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
Macrophage infiltration promotes regrowth in MYCN-amplified neuroblastoma after chemotherapy

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ABSTRACT

Despite aggressive treatment, the 5-year event-free survival rate for children with high-risk neuroblastoma is <50%. While most high-risk neuroblastoma patients initially respond to treatment, often with complete clinical remission, many eventually relapse with therapy-resistant tumors. Novel therapeutic alternatives that prevent the recurrence of therapy-resistant tumors are urgently needed. To understand the adaptation of neuroblastoma under therapy, we analyzed the transcriptomic landscape in 46 clinical tumor samples collected before (PRE) or after (POST) treatment from 22 neuroblastoma patients. RNA sequencing revealed that many of the top-upregulated biological processes in POST $MYCN$ amplified (MNA$^+$) tumors compared to PRE MNA$^+$ tumors were immune-related, and there was a significant increase in numerous genes associated with macrophages. The infiltration of macrophages was corroborated by immunohistochemistry and spatial digital protein profiling. Moreover, POST MNA$^+$ tumor cells were more immunogenic compared to PRE MNA$^+$ tumor cells. To find support for the macrophage-induced outgrowth of certain subpopulations of immunogenic tumor cells following treatment, we examined the genetic landscape in multiple clinical PRE and POST tumor samples from nine neuroblastoma patients revealing a significant correlation between an increased amount of copy number aberrations (CNA) and macrophage infiltration in POST MNA$^+$ tumor samples. Using an in $vivo$ neuroblastoma patient-derived xenograft (PDX) chemotherapy model, we further show that inhibition of macrophage recruitment with anti-CSF1R treatment prevents the regrowth of MNA$^+$ tumors following chemotherapy. Taken together, our work supports a therapeutic strategy for fighting the relapse of MNA$^+$ neuroblastoma by targeting the immune microenvironment.

Introduction

Neuroblastoma is a pediatric solid tumor that arises from the sympathetic nervous system and presents with a mass in the adrenal gland or along the sympathetic chain.[1] It is the most common extracranial solid tumor in children accounting for 6–10% of pediatric cancers.[2,3] Despite advances in risk stratification and therapy, neuroblastoma remains a therapeutic challenge, accounting for approximately 15% of all pediatric cancer deaths.[4–6] Neuroblastoma is stratified into low-, intermediate- or high-risk groups, according to the International Neuroblastoma Risk Group (INRG) consensus criteria, which include age at diagnosis, histological category, and genetic characteristics such as $MYCN$ amplification (MNA$^+$).[7] MNA$^+$ is found in approximately 20% of all neuroblastoma patients and accounts for 40% of all high-risk neuroblastoma cases.[8] Increased expression of MYCN is a tumor-initiating event responsible for the development of high-risk neuroblastoma.[9,10] Besides having a direct effect on neuroblastoma development, MNA$^+$ induces an immunosuppressive tumor microenvironment. MYCN negatively regulates ligands

for natural killer (NK) receptors, and MNA$^+$ is associated with the downregulation of MHC-I expression in neuroblastoma, rendering tumor cells less susceptible to recognition and killing by NK- and T-cells.[11–13] Moreover, MNA-positivity correlates with the infiltration of tumor-associated macrophages (TAMs) into neuroblastoma tumors.[13,14]

Most patients with MNA$^+$ neuroblastoma are treated with intensive chemotherapeutic induction regimens according to contemporary European protocols, typically Rapid COJEC. Rapid COJEC comprises cisplatin (C), vincristine (O), carboplatin (J), etoposide (E), and cyclophosphamide (C), administered in eight cycles, one every ten days and all within 70 days from the first to the last drug administered.[15] Induction chemotherapy is typically followed by surgery and later high-dose treatment with allogeneic stem cell transplantation and radiotherapy, followed by isotretinoin and anti-GD2 monoclonal antibodies to treat any residual disease. While most high-risk neuroblastoma patients initially respond to the treatment, often with complete clinical remission, many eventually relapse with therapy-resistant tumors. Therapy resistance could be due

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to either acquired drug resistance or due to the selection of therapy-resistant subclones in a genetic intratumor heterogeneity already present at diagnosis.[1,16,17] Chemotherapy resistance in neuroblastoma has been attributed to tumor cells with a mesenchymal gene profile.[17] There are two main differentiation states of neuroblastoma tumor cells; committed adrenergic cells and undifferentiated mesenchymal cells.[17–19] Importantly, intratumor genetic heterogeneity is common in neuroblastoma, where high-risk tumors are distinguished by a mosaic pattern of distinct clonal areas across the primary tumor space,[20,21] which in turn forms a substrate for tumor relapse with an increased mutational burden and activation of the RAS-MAPK pathway.[22–24] Emerging data from other tumor types further demonstrate that resistance to therapy can be promoted by the tumor microenvironment.[25] For example, in melanoma, MAPK pathway inhibitors increase the infiltration of macrophages, fostering tumor cell resistance to BRAF and MEK inhibition through macrophage-derived TNFα.[26] A therapeutic strategy for fighting therapy resilience could accordingly be to target the tumor microenvironment itself.

Here we demonstrate that the immune landscape and the genetic landscape change in chemotherapy-treated MNA$^+$ neuroblastoma tumors. Using neuroblastoma patient-derived xenograft (PDX) models, we show that immunogenic tumor cells expand following treatment and that inhibition of macrophage recruitment after chemotherapy can prevent the regrowth of MNA$^+$ neuroblastoma tumors.

Materials and methods

See Supplemental Information.

Results

Immune cells are recruited to MNA$^+$ neuroblastoma tumors following treatment

To understand the potential adaptations of neuroblastoma cells under therapy, we analyzed the transcriptomic landscape in 46 clinical tumor samples from 22 neuroblastoma patients (Supplementary Figure S1a, Supplementary Table S1). The tumor samples were collected before (PRE) or after (POST) treatment. RNA sequencing (RNA-seq) was performed, and differential gene expression analysis revealed major transcriptomic changes when comparing PRE MNA$^+$ tumors with POST MNA$^+$ tumors and PRE MNA$^-$ tumors with POST MNA$^-$ tumors (Figure 1a, Supplementary Figure S1b). Gene ontology (GO) analysis was performed to determine which biological processes were upregulated in POST tumor samples. Eight of the top 20 upregulated biological processes in POST MNA$^+$ tumors were immune-related pathways, such as the interferon-gamma-response and IL2-STAT5 signaling (Figure 1b). Similar correlations with immune-related pathways were not found in MNA$^-$ tumors (Supplementary Figure S1c).

To further elucidate how the immune landscape changed after treatment, we analyzed the RNA-seq data for selected immune cell markers. There was a significant increase in specific sets of genes, including those associated with macrophages and T-cells, in POST MNA$^+$ tumors compared to those in PRE MNA$^+$ tumors (Figure S1c, Supplementary Figure S2a). The transcriptomic results were further corroborated by IHC staining for various immune cell markers on consecutive tumor sections (Supplementary Fig. 1a), revealing a significant POST treatment shift in the immune infiltration of macrophages and T-cells only in MNA+ tumors (Figure 1d–f, Supplementary Figure 2b-f and Supplementary Figure 3). MNA+ patients with low infiltration of macrophages in their POST tumors had better survival (p-value = 0.059; Supplementary Figure S4a). A similar prognostic impact was not found for T-cells (p-value = 0.841; Supplementary Figure S4b). Caution should be taken when interpreting these prognostic data given our limited number of patients.

Spatial profiling reveals POST infiltration of pro-tumoral macrophages

Next, we investigated the immune landscape using a high-resolution spatial proteomics platform (Nanostring, GeoMx; Supplementary Table 2).[27,28] PRE and POST tumor samples from two MNA$^+$ neuroblastoma patients (selected based on having both PRE and POST tumor samples and having enough tissue available) were stained with a chromogranin A antibody, recognizing neuroblastoma tumor cells and with a CD45-antibody recognizing immune cells (Figure 2a). GeoMx analysis confirmed that the infiltration density of monocytes/macrophages (HLA-DR, CD11c, CD68, and CD14) increased in the POST MNA$^+$ samples (Figure 2b,c and Supplementary Table S3). Moreover, there was a significant increase in proteins associated with pro-tumor effects and immunosuppression such as Tim-3, B7-H3, and CD163.[29–31] CD163 is a scavenger receptor for hemoglobin and haptoglobin and a major marker for anti-inflammatory M2 macrophages and tumor-associated macrophages (TAMs).[31,32] To further validate the infiltration of TAMs in POST MNA$^+$ tumors we stained the neuroblastoma patient cohort with an anti-CD163 antibody (Figure 2d). The infiltration percentage of CD163$^+$ cells in tumors significantly correlated with that of CD68 (p-value <0.001; Spearman's rho, two-tailed p-value), supporting the notion that macrophages infiltrating POST MNA$^+$ tumors have TAM traits.

Expansion of CCL2-expressing tumor cells following treatment

To understand how macrophages are recruited, we re-analyzed patient RNA-seq data from both MNA$^-$ and MNA$^+$ tumors and specifically focused on the expression of chemokines/cytokines and their receptors (Supplementary Table 4). There were 37 differentially expressed genes for chemokines/cytokines and their receptors in POST MNA$^+$ compared to PRE MNA$^+$ tumors (Figure 3a). Among the top differentially expressed genes were two genes, namely colony-stimulating factor (CSF1) and chemokine (C-C motif) ligand 2 (CCL2), encoding potent monocyte/macrophage chemoattractant proteins, shown to be critical determinants of monocyte/macrophage recruitment and TAM accumulation in various cancers.[33–35]

To elucidate whether expression of CCL2 was induced during treatment or if subpopulations of tumor cells more abundant in
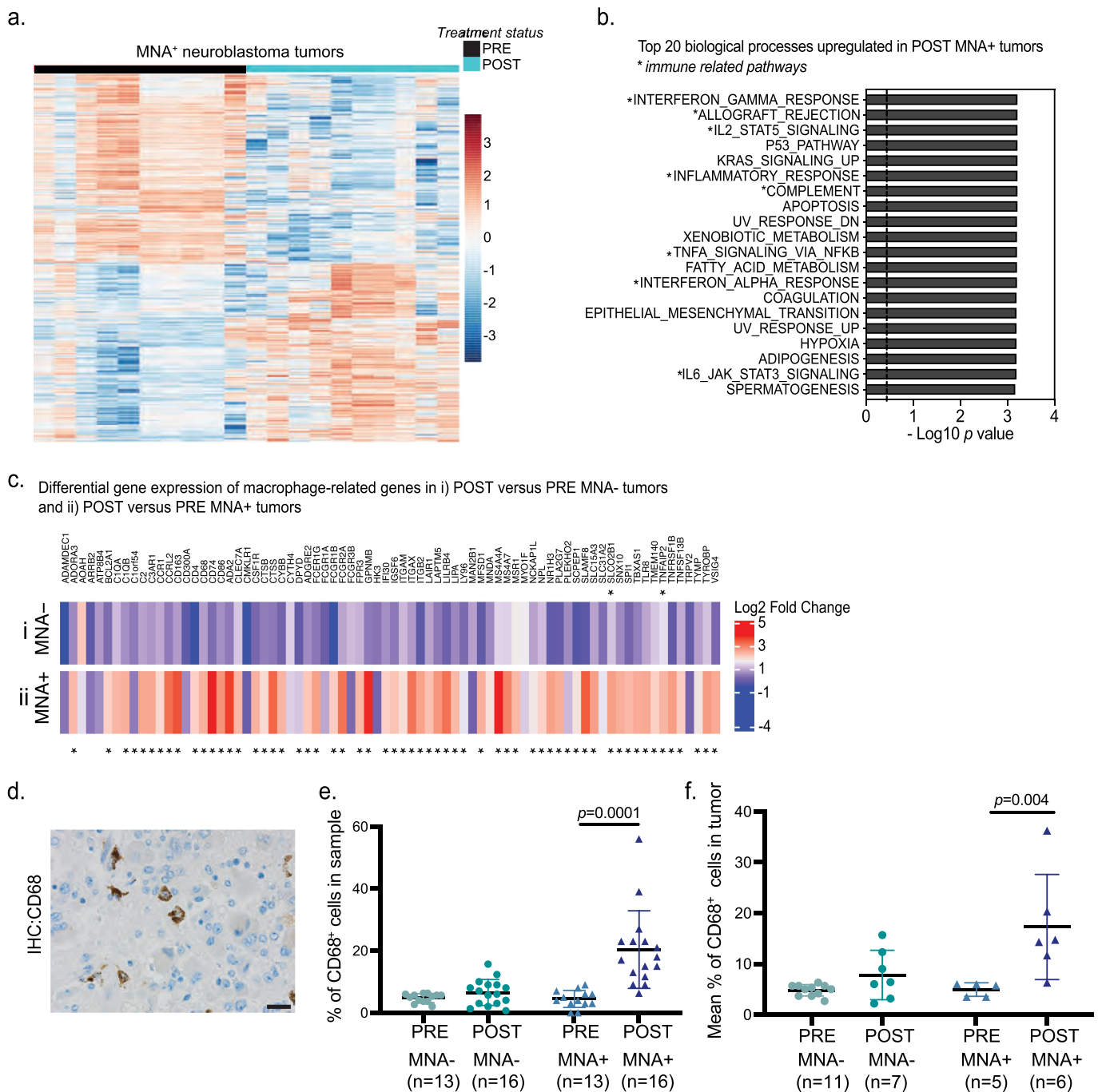
a.

b.

Top 20 biological processes upregulated in POST MNA+ tumors
* immune related pathways

c. Differential gene expression of macrophage-related genes in i) POST versus PRE MNA- tumors
and ii) POST versus PRE MNA+ tumors

d.

e.

f.

Figure 1. Immune cell infiltration in POST MNA+ neuroblastoma tumors a. Heatmap showing differentially expressed genes in PRE and POST MNA+ neuroblastoma tumors. The heatmap is based on (unsupervised) clustering of the genes that differed significantly in differential expression analyses. b. Top 20 biological processes upregulated in POST MNA+ neuroblastoma tumors. c. Expression of macrophage-related genes in POST MNA- and MNA+ neuroblastoma tumors compared to PRE MNA- and MNA+ neuroblastoma tumors, respectively. *Indicates significant differential expression, p-value. d. Representative IHC image for macrophages (CD68+ cells, here in a POST MNA+ neuroblastoma tumor). Scale bar = 100μm. e. Percentage of CD68+ cells in neuroblastoma tumors (1-7 tumor samples per patient) divided by treatment status and MYCN amplification. f. Mean percentage of CD68+ cells in patients' PRE and POST tumors. Mean ± SD, Mann-Whitney test.

CCL2 expanded after COJEC treatment, we analyzed RNA-seq data derived from a previously performed *in vivo* PDX#3-COJEC experiment.[36] We compared *CCL2* expression in untreated tumors, tumors collected during treatment, and tumors collected after COJEC treatment and regrowth (POST). Interestingly, *CCL2* gene expression was found to be increased in POST tumors (Figure 3b). This indicates that the increase of *CCL2* seen in POST tumors is not due to the treatment itself but rather due to an expansion of CCL2+ tumor cells following treatment. An

increase in CCL2 protein expression in POST tumors was further confirmed by IHC in clinical MNA+ tumor samples (Figure 3c,d).

CCL2 is mainly expressed by mesenchymal neuroblastoma tumor cells

Chemotherapy resistance in neuroblastoma has been attributed to tumor cells with a mesenchymal gene profile.[17] It was recently shown *in vitro* that adrenergic neuroblastoma
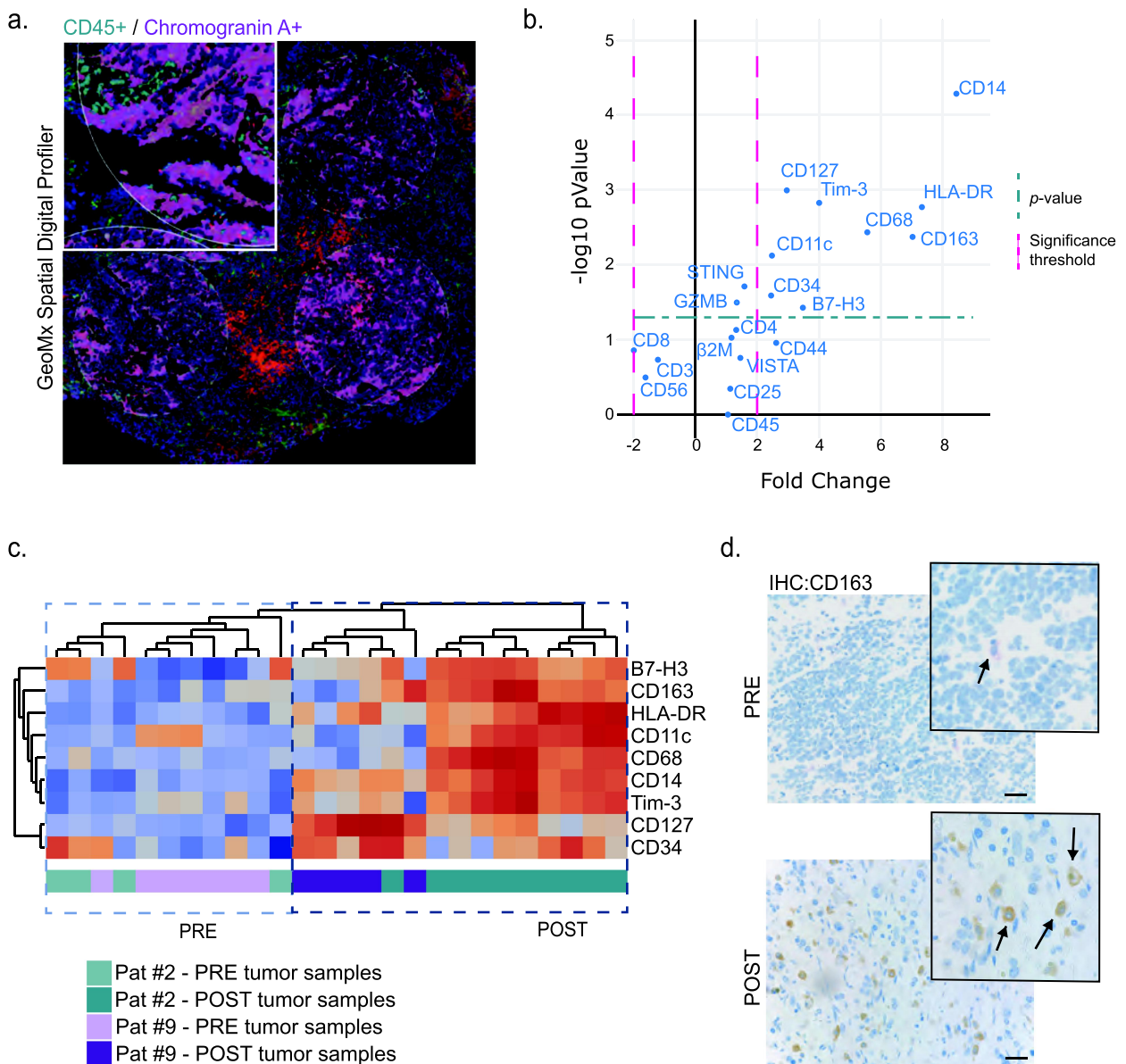
Figure 2. Infiltration of tumor associated macrophages in POST MNA+ neuroblastoma tumors a. Representative image of high-resolution spatial proteomics on neuroblastoma tumors stained for immune cells (CD45+, green) and tumor cells (chromogranin A+, purple). Circles indicate regions of interest (ROI). Red blood cell autofluorescence can be seen between ROI. b. Volcano plot of immune cell related proteins. Unpaired t-tests, p-value <-2 or > 2. c. Heatmap and dendrogram showing unsupervised hierarchical clustering of multiple PRE and POST tumor samples from two patients with MNA+ neuroblastoma, for the proteins that had significantly different expression comparing PRE and POST tumor samples. d. Representative IHC images for TAMs (CD163+ cells in PRE and POST MNA+ tumors from the same patient). Black arrow indicate CD163+ cells. Scale bar = 100 μm.

tumor cells that become therapy-resistant acquire a mesenchymal profile and, more importantly, have increased expression of immune response genes.[37] In line with that data, our clinical data revealed that, besides immune-related pathways, epithelial-mesenchymal transition (EMT) was among the top upregulated biological processes when we compared POST with PRE MNA$^+$ tumors (Figure 1b). To understand whether CCL2$^+$ tumor cells were of mesenchymal tumor cell lineage, we analyzed previously generated single-cell RNA sequencing (scRNA-seq) data from a treated MNA$^+$ neuroblastoma tumor (Figure 3e).[38] In line with previous work, the majority of CCL2-expressing tumor cells belonged to the mesenchymal population (Figure 3f).

Macrophage infiltration and changed genetic landscape in POST tumors

To find further support for a macrophage-induced outgrowth of subpopulations of tumor cells following treatment, we examined the genetic landscape in multiple clinical PRE and POST tumor samples from nine neuroblastoma patients. The number of copy number aberrations (CNA) in each sample correlated with macrophage infiltration. While macrophage infiltration and the number of CNAs in PRE tumor samples did not correlate, there was a significant correlation found in POST tumor samples between the number of CNA and macrophage infiltration ($p = .005$) (Figure 4a, Supplementary Table S5). Notably, only MNA$^+$ neuroblastoma tumors showed a significant increase in CNAs after treatment (Figure 4b).

a. Differential gene expression of genes related to cytokines/chemokines and their receptors in i) POST- versus PRE MNA- tumors and ii) POST- versus PRE MNA+ tumors

b.

c.

d.

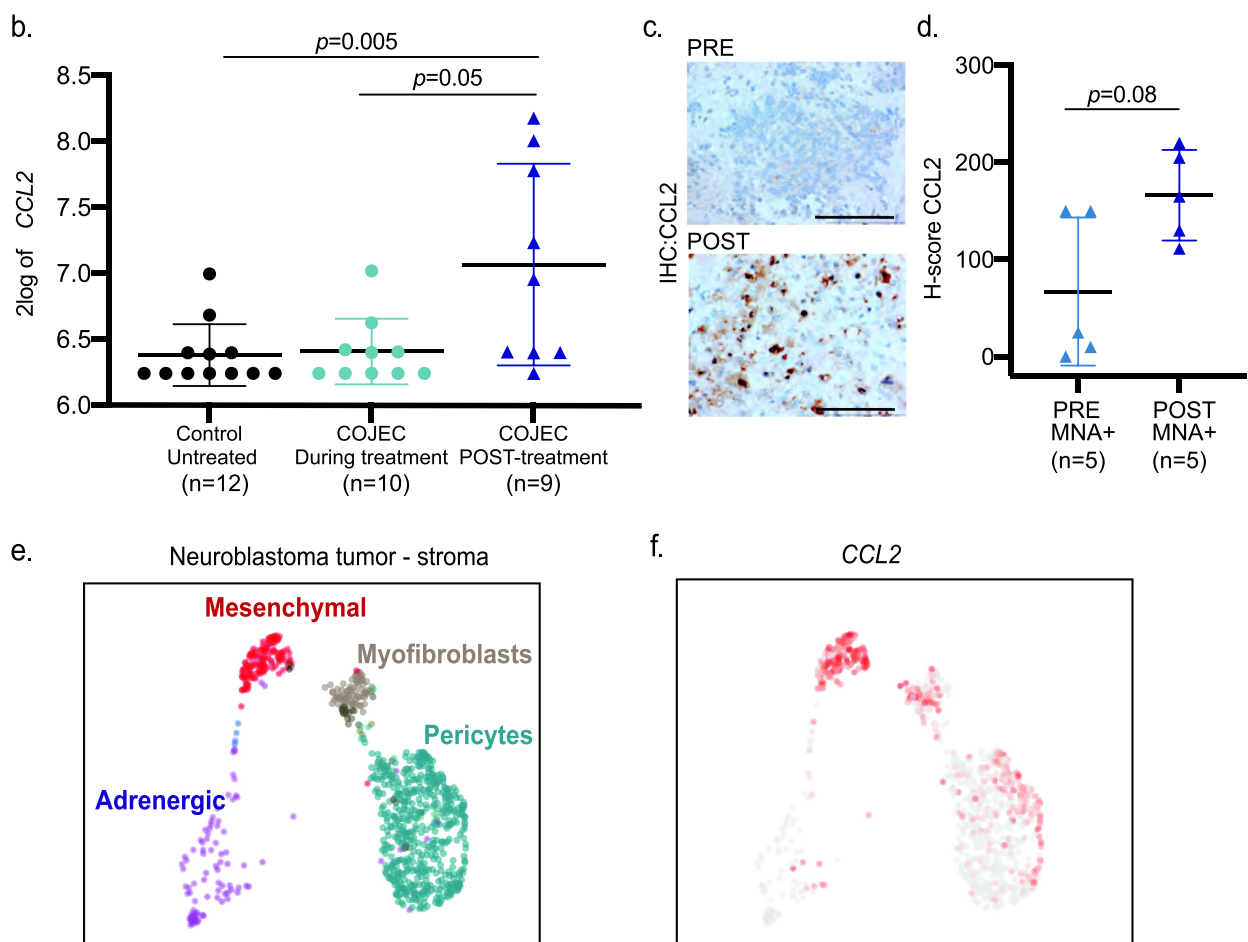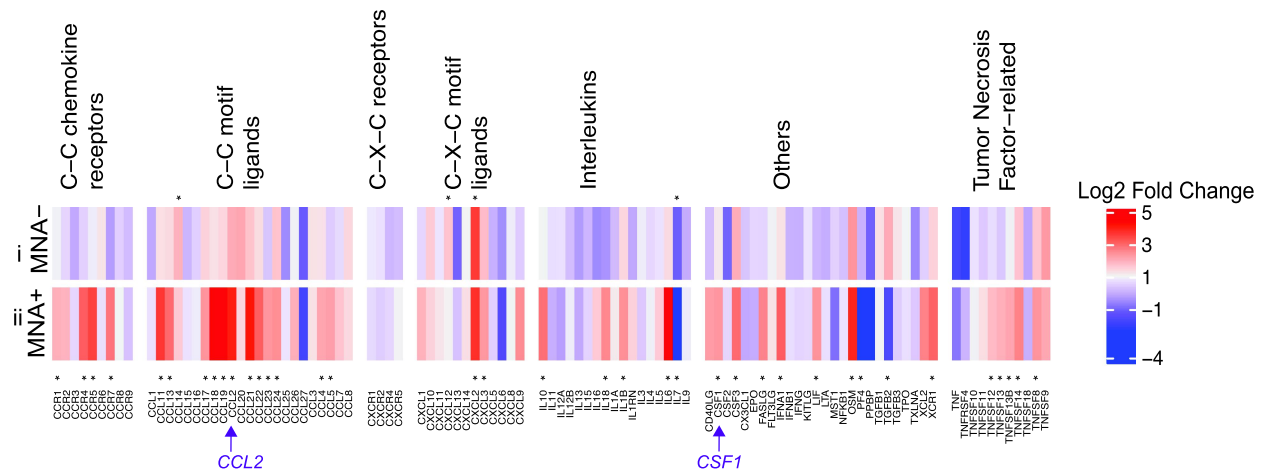e. Neuroblastoma tumor - stroma

f. CCL2

Figure 3. Monocyte/macrophage chemoattractant protein CCL2 increases in POST MNA+ tumors a. Genes related to cytokines and chemokines and their receptors in POST-treatment MNA- and MNA+ neuroblastoma tumors as compared to pre-treatment MNA- and MNA+ neuroblastoma tumors, respectively. *Indicates significant differential expression, p-value. b. Gene expression of CCL2 in PDX#3 tumors; untreated, collected during COJEC treatment or POST COJEC treatment. Mean ± SD, Mann-Whitney test. c. Representative IHC images for CCL2 in a PRE and POST MNA+ neuroblastoma tumor. Scale bar = 100µm. d. H-score of CCL2 protein levels in clinical MNA + neuroblastomas. Mean ± SD, Mann-Whitney test. e. Data analysis of scRNA-seq sample from one treated MNA+ neuroblastoma tumor, visualized using a common UMAP embedding is tumor and stroma compartment. f. gene expression shown in UMAP plot.

Inhibition of macrophage recruitment prevents tumor regrowth

Once high-risk neuroblastoma patients complete the rapid-COJEC therapy regimen, they undergo surgery to achieve complete removal of any remaining primary tumor. The time from completed treatment to surgery is typically a couple of weeks to allow recuperation. We hypothesized that, during the interval between chemotherapy and surgery, surviving tumor
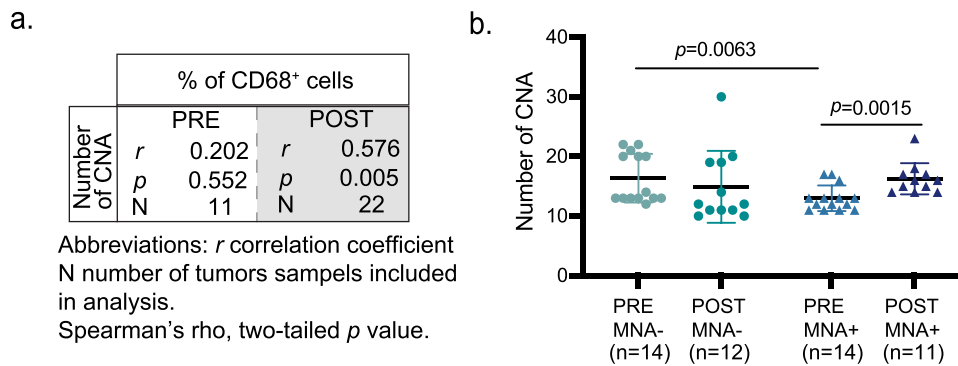
a.

	% of CD68+ cells			
	PRE		POST	
Number of CNA	r	0.202	r	0.576
	p	0.552	p	0.005
	N	11	N	22

Abbreviations: *r* correlation coefficient
N number of tumors sampels included
in analysis.
Spearman's rho, two-tailed *p* value.

b.

Figure 4. Macrophage infiltration and altered genetic landscape in POST-tumors a. Correlations between the percentage of CD68+ cells and the number of copy number aberrations (CNA) in clinical PRE and POST tumor samples. b. Number of CNA in clinical neuroblastoma tumor samples. Mean ± SD, Mann-Whitney test.

cells could proliferate in cooperation with macrophages. To explore whether inhibition of macrophage recruitment could prevent tumor regrowth we used an *in vivo* neuroblastoma PDX–COJEC model and anti-CSF1R, inhibiting the recruitment of macrophages.[36] Dissociated organoids from the PDX#3 model were subcutaneously (s.c.) injected into the flank of nude mice (Figure 5a).[39] COJEC treatment was administered for three weeks. After chemotherapy, the tumors were allowed to regrow for three weeks without or with intraperitoneal (i.p.) treatment with anti-CSF1R, inhibiting the recruitment of macrophages.[40,41] All PDX tumors treated with COJEC shrank during the treatment period and started to regrow once the treatment ended. Importantly, the anti-CSF1R treatment, which impaired macrophage infiltration (Figure 5d,e and Supplementary Fig 5a-c), prevented the regrowth of the tumors (Figure 5b,c). In line with the above-mentioned data, the POST tumors in this experiment had increased expression of CCL2 than the untreated tumors (Figure 5f).

Taken together, our data suggest that the regrowth of immunogenic tumor cells in MNA+ neuroblastoma depends on the infiltration of macrophages, and our work supports a therapeutic strategy for fighting the relapse of high-risk neuroblastoma by targeting macrophages (Figure 6).

Discussion

Despite aggressive treatment, the 5-year event-free survival rate of high-risk neuroblastoma patients is <50%. Here, we show that MNA+ tumors are infiltrated with pro-tumor macrophages following treatment, and inhibition of macrophage recruitment after chemotherapy prevents tumor regrowth, suggesting that a therapeutic strategy for fighting relapse of high-risk neuroblastoma could be to target the immune microenvironment.

The tumor microenvironment in neuroblastoma has been previously investigated, revealing a complex network of interactions between tumor cells and various immune cell populations.[38] However, to our knowledge, no previous study has compared the immune landscape in neuroblastoma tumors

before and after treatment. One study did, however, determine immune profiles in the peripheral blood of high-risk neuroblastoma patients before and over the course of treatment, revealing a high degree of interpatient immune variability and the existence of both immune-enhancing and regulatory responses during treatment.[42] While we did not find a significant difference in macrophage infiltration between clinical PRE MNA⁻ and MNA⁺ tumors, we did observe increased infiltration of macrophages in POST MNA⁺ tumors, compared to POST MNA⁻ tumors, PRE MNA⁻ and PRE MNA⁺ tumors. Other clinical studies focusing on the immune microenvironment in neuroblastoma tumors have compared MNA⁺ with MNA⁻ tumors and included primary untreated tumors or did not provide information about treatment status. This could explain the contradictory results in this field. RNA-seq was performed on 150 neuroblastomas revealing that MNA⁻ tumors had a significantly higher cytotoxic tumor-infiltrating lymphocyte (TIL) signature than MNA⁺ tumors. Importantly, all tumors included in that study were primary tumors collected before treatment, and the authors did not focus on myeloid signatures.[43] *In silico* immunological analysis of ~140 neuroblastomas (information about treatment was not provided) revealed significantly reduced transcripts related to major immune effector cells, including macrophages and T-cells, in MNA⁺ tumors compared to MNA⁻ tumors.[44] Another study examined 41 primary neuroblastomas (collected before therapy) using IHC and markers for macrophages (CD68 and CD163). They, however, found a correlation between macrophage infiltration and MNA⁺.[14]

Our neuroblastoma cohort had too few patients to perform comprehensive survival analyses. The patients with MNA⁺ tumors, which had high infiltration of macrophages following treatment, had a worse outcome than those with lower macrophage infiltration. Our prognostic data align with previous work showing that high gene expression of *CSF1R* in neuroblastoma predicts poor outcome,[45] but future studies investigating the importance of macrophages and other immune cell populations in a larger patient cohort are warranted to determine the prognostic impact of macrophage infiltration in PRE and POST neuroblastoma tumors. Interestingly, we also detected increased T-cell infiltration in POST MNA⁺ tumors. T-cell infiltration has been associated with improved clinical outcome for neuroblastoma patients.[46] A future goal will be to investigate T-cell
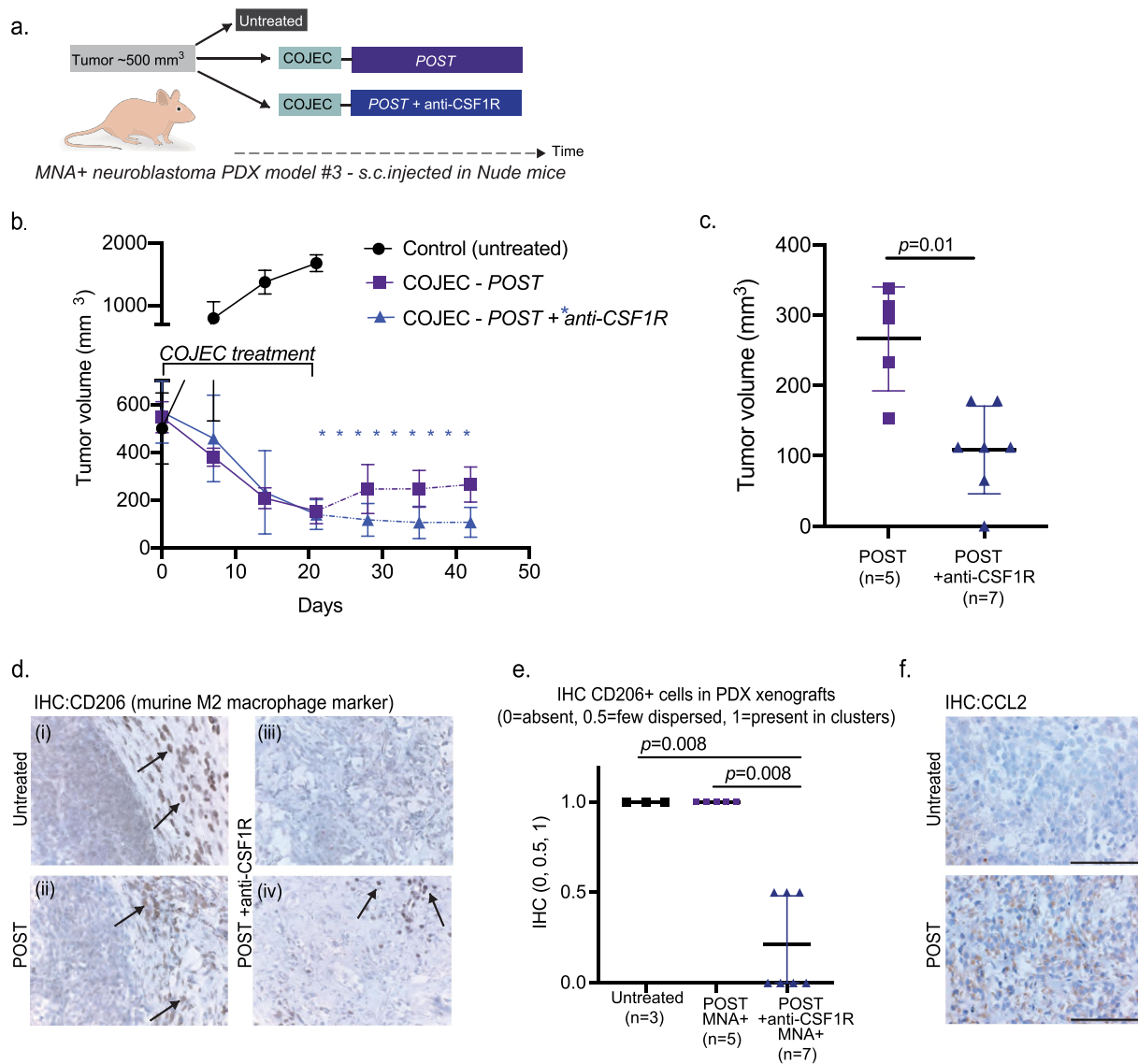
Figure 5. Inhibition of macrophage recruitment prevent POST tumor regrowth a. Schematic illustration of experimental approach. b. Tumor growth kinetics of untreated (n = 4) and COJEC treated PDX#3 tumors (n = 12). The treatment continued for three weeks after which the tumors were left to regrow for an additional three weeks. Seven of the COJEC-treated mice were administrated with anti-CSF1R during the last three weeks. Mean ± SD. c. Tumor volume of POST treatment (n = 5) and POST treatment + anti-CSF1R treated tumors (n = 7) last day of experiment (day 42). Mean ± SD, Mann-Whitney test. d. Representative IHC images for mouse CD206 in untreated (i), POST (ii) and POST+anti-CSF1R (iii- CD206+ cells absent; iv- few dispersed CD206+ cells present) PDX#3 tumors. Black arrows indicate CD206+ cells. e. Immunohistochemistry statistics of d. using Mann-Whitney test. Mean ± SD. f. Representative IHC images for human CCL2 in untreated and POST PDX#3 tumors. Scale bar = 100 μm.
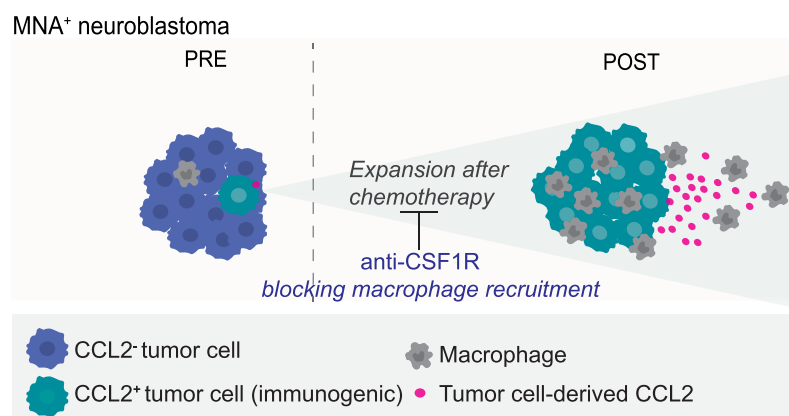

Figure 6. Immunogenic tumor cells have a growth advantage after treatment due to their ability to recruit pro-tumor macrophages. Blocking the recruitment of macrophages after chemotherapy prevents the outgrowth of immunogenic tumor cells.

subpopulations further and their role in PRE and POST neuroblastoma.

Besides potentially affecting tumor progression by supporting, e.g., angiogenesis and invasion, as shown in other cancers,[47] the unfavorable prognostic impact of macrophage infiltration in POST MNA+ neuroblastoma could be that macrophages are essential for the outgrowth of tumor cells that have survived therapy and are responsible for relapse. POST MNA[+] tumors did have enrichment for the EMT pathway. Importantly, therapy-resistant tumor cells in neuroblastoma have been shown to have a mesenchymal gene expression profile.[17,37]

MNA^{+} tumor cells surviving treatment were found to have an immunogenic gene expression profile compared to untreated MNA[+] tumors, with an increased expression of many cytokines/chemokines capable of recruiting monocytes/macrophages, e.g., *CSF1* and *CCL2*. A similar immunogenic shift was not observed for MNA[−] tumors. The reason for the increased production of CCL2, a potent chemoattractant for macrophages,[33] in POST MNA[+] tumor samples is unknown. It is noteworthy that *MYCN* has been shown to bind to the CCL2 promoter directly and negatively regulate the production of CCL2 in neuroblastoma.[48] Marrano *et al.* reported clinical cases of PRE MNA[+] neuroblastoma tumors that had *MYCN* amplified tumor cells throughout the tumor, but after treatment only had one or more foci with *MYCN* amplified tumor cells separated by foci of non-amplified tumor cells.[49] Hence, the increase in CCL2 following treatment might be explained by the intratumoral *MYCN* heterogeneity found in untreated primary MNA[+] neuroblastoma tumors and the expansion of therapy-resilient CCL2^{+}MNA^{−} tumor cells following treatment.

Using an *in vivo* neuroblastoma PDX-COJEC model, we furthermore showed that inhibition of macrophage recruitment prevented the short-term outgrowth of tumor cells that had survived COJEC-like therapy. These data are consistent with those of previous studies. Webb *et al.* showed that blocking the recruitment of macrophages with the macrophage inhibitor BLZ945 improved the efficiency of chemotherapy in various human neuroblastoma cell lines and in a PDX model, suggesting that subpopulations of neuroblastoma tumor cells are protected from chemotherapy through cooperation with macrophages.[50] Interestingly, BLZ945 treatment in a glioblastoma model did not deplete macrophages but impaired their tumor-promoting functions.[51] Alteration of macrophage polarization is an additional way that anti-macrophage treatment might prevent neuroblastoma regrowth after treatment. Moreover, metastatic MNA- neuroblastomas have been shown to have a higher infiltration of TAMs than locoregional tumors, emphasizing anti-macrophage therapy's potential in also preventing and targeting metastatic neuroblastoma.[52]

Taken together, we suggest that a therapeutic strategy for fighting relapse of high-risk neuroblastoma could be to inhibit macrophage infiltration following treatment, which would presumably prevent the outgrowth of therapy-resilient tumor cells, that have a mesenchymal and immunogenic profile and depend on macrophages for their expansion.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

CH and DG designed and supervised the research. All authors performed the research and discussed the results. AV, GC, and CH wrote the manuscript. All authors provided comments and feedback. All the authors have read and approved the final manuscript.

Data availability statement

Research data supporting this publication is available at DOI 10.17044/scilifelab.22188583.

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