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Characterization of G-CSF receptor expression in medulloblastoma

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Abstract

Background. Identifying mechanisms of medulloblastoma recurrence is a key to improving patient survival, and targeting treatment-resistant subpopulations within tumors could reduce disease recurrence. Expression of the granulocyte colony-stimulating factor receptor (G-CSF-R, CD114) is a potential marker of cancer stem cells, and therefore we hypothesized that a subpopulation of medulloblastoma cells would also express CD114 and would demonstrate chemoresistance and responsiveness to G-CSF.

Methods. Prevalence of CD114-positive (CD114+) cells in medulloblastoma cell lines, patient-derived xenograft (PDX) tumors, and primary patient tumor samples were assessed by flow cytometry. Growth rates, chemoresistance, and responses to G-CSF of CD114+ and CD114-negative (CD114–) cells were characterized in vitro using continuous live cell imaging and flow cytometry. Gene expression profiles were compared between CD114+ and CD114– medulloblastoma cells using quantitative RT-PCR.

Results. CD114+ cells were identifiable in medulloblastoma cell lines, PDX tumors, and primary patient tumors and have slower growth rates than CD114– or mixed populations. G-CSF accelerates the growth of CD114+ cells, and CD114+ cells are more chemoresistant. The CD114+ population is enriched when G-CSF treatment follows chemotherapy. The CD114+ population also has higher expression of the *CSF3R*, *NRP-1*, *TWIST1*, and *MYCN* genes.

Conclusions. Our data demonstrate that a subpopulation of CD114+ medulloblastoma cells exists in cell lines and tumors, which may evade traditional chemotherapy and respond to exogenous G-CSF. These properties invite further investigation into the role of G-CSF in medulloblastoma therapy and methods to specifically target these cells.

Key Points

- The expression of the G-CSF receptor defines a unique treatment-resistant subpopulation of medulloblastoma cells.
- CD114-positive medulloblastoma cells are capable of responding to exogenous G-CSF.

Medulloblastoma is a common childhood malignancy of the central nervous system, with poor outcomes for children with aggressive, metastatic, or relapsed disease. The 5-year overall survival rates for medulloblastoma range between 41.9% and 100%,[1] and relapsed disease is very difficult to cure.[2] Standard treatment for children with medulloblastoma is multimodal and

Importance of the Study

Our results represent the first demonstration of a potential role for CD114 as a marker of a chemoresistant medulloblastoma cell subpopulation and as a functional receptor for G-CSF in medulloblastoma cells. Our data show that CD114+ medulloblastoma cells demonstrate slower growth, chemoresistance, and responses to exogenous G-CSF. Our results suggest that exogenous G-CSF may promote the survival and proliferation of chemoresistant medulloblastoma cells and also may contribute to disease relapse. As G-CSF is used for supportive care in many patients with medulloblastoma, further study into the clinical relevance of this cell subpopulation is indicated.

includes surgical resection, craniospinal radiation, and cytotoxic chemotherapy. In younger patients, radiation is avoided or delayed by using myeloablative doses of chemotherapy followed by autologous stem cell rescue. Medulloblastoma treatment therefore has a high potential for both short-term and long-term morbidity, including late effects such as neurodevelopmental delays, endocrinopathies, hearing loss, and secondary malignancies.

Medulloblastoma is a heterogeneous disease and has been clinically divided into 4 subgroups, including the WNT, Sonic Hedgehog (SHH), G3, and G4 groups.[1] These subgroups have distinct molecular and genetic profiles as well as differences in outcomes, with the WNT and SHH groups named for the active signaling pathways in tumors of these groups, while tumors in Groups 3 and 4 are less well defined. Patients with tumors in the WNT subgroup have excellent overall survival rates, while Groups 3 and 4 tumors have much poorer outcomes.[1] Recognizing this, some clinical trials now risk-stratify medulloblastoma therapy in part by molecular subgroup (NCT01878617). While our understanding of the biology of medulloblastoma has advanced rapidly in recent years, there has been minimal progress in translating that information into improved survival rates and reduced treatment-related morbidity. Novel therapies are needed to improve patient outcomes, and therapies targeting medulloblastoma subpopulations that contribute to disease relapse are likely to reduce the risk of recurrences and improve survival rates.

Expression of the granulocyte colony-stimulating factor (G-CSF) receptor (CD114) has been implicated in the pathogenesis of multiple tumor types,[3–5] and recent investigations have identified CD114 as a marker of a cancer stem cell (CSC) population in neuroblastoma,[6,7] with CD114+ neuroblastoma cells demonstrating the ability to self-renew, generate differentiated progeny, and recapitulate a heterogeneous cancer cell population.[6] However, the significance of CD114 expression and activity in the pathogenesis of medulloblastoma is unknown. We hypothesized that CD114 expression would be found on a subpopulation of medulloblastoma cells and that these CD114+ cells would be resistant to chemotherapy and respond to G-CSF.

Materials and Methods

Cell Culture

Daoy medulloblastoma cells were obtained from the ATCC (www.atcc.org). Medulloblastoma cell lines D283 and D341 were provided by Dr Robert Wechsler-Reya (Sanford Burnham Prebys Medical Discovery Institute). Medulloblastoma cell lines HMB-5,[8] SL0024, SL00673, SL00668, SL00278, SL00362, SL00615, and SL00870[9] were derived directly from patient tumors and provided by Dr Donald Durden (University of California San Diego [UCSD]) and Dr Giselle L. Saulnier Sholler (Helen DeVos Children's Hospital). Daoy, D283, and D341 cell lines were validated by STR profiling, and patient tumor-derived cell lines were validated by flow cytometry for medulloblastoma cell surface markers and verified to match the patient of origin by DNA sequencing. Daoy, D283, and D341 cells were maintained in RPMI media with 20% fetal bovine serum (FBS), L-glutamine (0.3 g/L), streptomycin (100 IU/mL), and penicillin (100 IU/mL). HMB-5 cells were maintained in NeuroCult NS-A Proliferation Media (StemCell Technologies) with 20 ng/mL EGF, 10 ng/mL bFGF, streptomycin (100 IU/mL), and penicillin (100 IU/mL). SL cell lines were all maintained in DMEM with 20% FBS, 5% sodium pyruvate, L-glutamine (0.3 g/L), streptomycin (100 IU/mL), and penicillin (100 IU/mL).

For stable transfection, Daoy cells were seeded in a 6-well plate and allowed to grow to 70–90% confluence before transfection. About 2 µg of Human *CSF3R*/CD114/G-CSFR transcript variant 1 ORF mammalian expression plasmid (Sino Biological Inc., #HG10218-UT) was diluted in 200 µL of jetOPTIMUS buffer (PolyPlus Transfection, #117-01) and then incubated with 2 µL of jetOPTIMUS reagent (PolyPlus, cat#117-01). The transfection mixture was added to the cells and incubated for 24 h before medium change. The transfected cells were exposed to 50 µg/mL of Hygromycin B (Omega Scientific, #HG-80) for selection at 48 h post-transfection. The cells were selected for 9 days with medium containing selection antibiotic that was changed every 3 days, and cells were allowed to expand to reach high confluence. *CSF3R* expression was confirmed by quantitative PCR (qPCR) and CD114 expression was confirmed by flow cytometry. The stably transfected cells were maintained in complete medium supplemented with selection antibiotics until use.

Patient-Derived Xenograft Tumors

Medulloblastoma patient-derived xenograft (PDX) lines used for this study included Med-411-FH (Group 3) and Med-1712-FH (SHH) generated by the Olson laboratory,[10,11] CHOPMB-3933 (Group 4) obtained from Children's Hospital of Philadelphia, and RCMB18 (SHH) and RCMB24

(SHH) generated by the Wechsler-Reya laboratory.[12,13] PDX lines were generated by implanting patient cells directly into the cerebellum of immune-deficient NSG mice and propagating them from mouse to mouse without in vitro passaging[14]; the identity and subgroup of each line were validated by gene expression and/or methylation analysis. Mice were maintained in the animal facilities at the Sanford Consortium for Regenerative Medicine (La Jolla, CA). All experiments were performed in accordance with national guidelines and regulations, and all experiments were approved by the UCSD institutional animal care and use committee. For all experiments, tumor-bearing mice were euthanized and cells were prepared by dissecting the tumor tissue followed by papain enzymatic digestion (10 U/mL) (Worthington Biochemical Corporation) supplemented with 0.2 mg/mL L-cysteine (Sigma) and 25 U/mL DNase (Worthington Biochemical Corporation) for 30 min at 37°C. The papain reaction was stopped with 1× phosphate-buffered saline (PBS; Life Technologies) supplemented with 1% FBS (Seradigm) solution and 25 U/mL DNase (Worthington Biochemical Corporation), and single cells were strained through a 0.7 µm strainer, spun down at 300*g*, resuspended in 1× PBS supplemented with 5% FBS, and analyzed by flow cytometry.

Flow Cytometry

Flow cytometry for cell lines was conducted on a FACSCanto II 3-laser flow cytometer (BD Biosciences). FlowJo 10.0 (FlowJo) software was used to analyze flow cytometry data. All samples were gated using an unstained control as reference. PE-conjugated anti-CD114 antibodies (BioLegend, #346106), APC-conjugated anti-CD133 antibodies (BioLegend, #372805), and V450-conjugated anti-CD15 antibodies (BD Biosciences, #561584) were used for flow cytometry and fluorescence-activated cell sorting (FACS) as detailed below. Live cell discrimination was determined by the absence of DAPI staining. Flow cytometry of cells from tumor samples was performed on an LSFortessa X20 flow cytometer (BD Biosciences) as above with live cell discrimination determined by the absence of 7-AAD staining.

Fluorescence-Activated Cell Sorting

FACS was conducted on a FACSAria I or FACSAria II 3-laser cell sorter (BD Biosciences). BD FACSDiva 8.0.2 software was used to analyze FACS data. PE-conjugated anti-CD114 antibodies were used to label CD114 surface expression. Live cell discrimination was determined by the absence of DAPI staining. CD114+ and CD114– cells were harvested after sorting. Parental cells used in experiments were also sorted by isolating DAPI-negative live cells via FACS without further sorting for CD114 expression.

In Vitro Chemosensitivity and Growth Assay

Cells were counted manually (for experiments with unsorted cells) or by FACS (for experiments with sorted cells), seeded in equal numbers in wells of 96-well plates, and allowed to adhere at 37°C for 24 h. Chemotherapy agents (carboplatin, etoposide, and methotrexate) obtained from the UCSD Moores Cancer Center pharmacy were added after 24 h of incubation at 37°C. For experiments involving G-CSF, 1 or 10 ng/mL research-grade human G-CSF (Miltenyi, #130-096-346) was added alone or after removal of chemotherapy agents, when applicable. Cells were monitored using continuous live cell imaging with the IncuCyte Live-Cell Analysis System (Essen Bioscience) and confluence was calculated using the IncuCyte Analysis software. The change in percent confluence over time was normalized to the percent confluence at time zero. Change in confluence in treated cells was then normalized to the change in confluence in the untreated control cells of the same type. All experiments were performed in triplicate, and differences in normalized confluence were compared using Student's *t*-tests.

In Vitro Viability Assay

To determine cell viability, cells were treated as above and then 1× AlamarBlue viability reagent (ThermoFisher Scientific) was added. Cells were incubated for up to 12 h in the dark at 37°C, and fluorescence was then analyzed using a microplate reader with fluorescence excitation at 560 nm and emission at 590 nm (Tecan LifeSciences). Relative cell viability was calculated by normalizing the fluorescence of samples in the treated group to the fluorescence of samples in the untreated control group. All experiments were performed in triplicate, and differences in normalized fluorescence were compared using Student's *t*-tests. For the IncuCyte Caspase-3/7 Green Apoptosis Assay, cells were treated with 100 µL/well of Caspase-3/7 Reagent (Essen Bioscience) as well as IncuCyte NucLight Rapid Red Reagent 50 µL/well at the time of cell seeding. Red fluorescence and green fluorescence were photographed with the IncuCyte every 6 h. The numbers of live cells at each time point were calculated by subtracting green fluorescent events from red fluorescent events.

Quantitative PCR

Daoy and HMB-5 cells were sorted into CD114+, CD114–, and parental populations by flow cytometry as above and RNA was isolated from cell populations using a Qiagen RNeasy Kit. RNA was reverse transcribed to complementary DNA and qPCR was subsequently performed using primers for *CSF3R*, *PROM1*, *FUT4*, *SOX2*, *SOX9 NRP1*, *MSI1*, *MYCN*, and *TWIST1*, with primers for *GAPDH* used as a control (Supplementary Figure S1). Fold change in gene expression was calculated by comparing levels of the gene of interest against *GAPDH*.

Results

CD114 Expression in Medulloblastoma Cells and Tumors

To determine whether medulloblastoma cell lines and tumors contain a cell subpopulation with surface expression

of CD114, we analyzed medulloblastoma cell lines, PDX tumor samples, and primary patient tumor samples obtained from biopsies or resections at the time of initial diagnosis at Rady Children's Hospital in San Diego (except for RCMB66, which was from a recurrent tumor) by flow cytometry. CD114+ cells were identified in a subpopulation of all tested medulloblastoma cell lines and tumors (Table 1; Supplementary Figure S2). The CD114+ subpopulation ranged from 0.2% to 13.8% of the total live cell population and was generally higher in primary patient tumor samples and PDX tumor samples compared to cell lines.

The cell surface proteins CD133 and CD15 have been identified as CSC markers in medulloblastoma.[8,15–17] We aimed to explore whether CD114 was also expressed on these medulloblastoma CSC subpopulations. There was no significant coexpression of CD15 or CD133 in the population of CD114+ cells (Supplementary Figure S3). Furthermore, while *CSF3R* (CD114) expression was significantly higher in CD114+ cells compared to CD114– cells, gene expression of *PROM1* and *FUT4* (CD133 and CD15, respectively) was not significantly different in CD114+ and CD114– sorted cells (Supplementary Figure S3), indicating CD114 is expressed on a subpopulation of medulloblastoma cells independent of previously identified medulloblastoma CSCs.

Growth Rates of CD114+ Medulloblastoma Cells

To determine whether CD114+ medulloblastoma cells displayed altered growth, medulloblastoma cells were sorted into equal numbers of CD114+, CD114–, and unsorted parental cells and monitored by continuous live cell imaging. CD114+ cells demonstrated a slower rate of growth and took a longer time to achieve 100% confluence than the CD114– and parental populations (Figure 1). Cell morphology of CD114+ and CD114– cells appeared similar (Supplementary Figure S4), suggesting the difference in confluence is due to reduced cell number, rather than different cell size.

Chemoresistance of CD114+ Medulloblastoma Cells

To evaluate whether CD114+ medulloblastoma cells were resistant to chemotherapy agents used for medulloblastoma treatment, we exposed CD114+ and CD114– cells to carboplatin, etoposide, or methotrexate, chemotherapy agents used in standard treatment regimens for medulloblastoma, for 72 h. Relative chemotoxicity in sorted populations was evaluated using assays for confluence (Figure 2A) and viability (Figure 2B; Supplementary Figure S5), with CD114+ medulloblastoma cells demonstrating increased confluence and viability

Table 1. Identification of CD114 Surface Expression by Flow Cytometry in Medulloblastoma Cell Lines and Tumors

	%CD114+	Subtype (if known)
Established cell lines		
Daoy	1.6	SHH
D283	0.6	Group 3/4
D341	0.2	Group 3
Patient-derived cell lines		
HMB-5	5.9	SHH
SL00024-1-A	1.8	SHH
SL00673-1-A	1.7	SHH
SL00668-1-A	1.7	Group 3
SL00362-1-A	6.5	Group 3
SL00278-1-A	6.0	WNT
SL00870-2-A	0.9	Unknown
PDX tumors		
RCMB18	2.79	SHH
Med1712-FH	1.92	SHH
RCMB24	1.37	SHH
MED411-FH	0.8	Group 3
CHOP3933	0.03	Group 4
Primary tumors		
RCMB65	13.8	SHH
RCMB67	12.51	SHH
RCMB66	1.11	Group 3
RCMB70	0.45	WNT

The percentages of the total live cell population expressing CD114 in each cell line and tumor sample are listed.

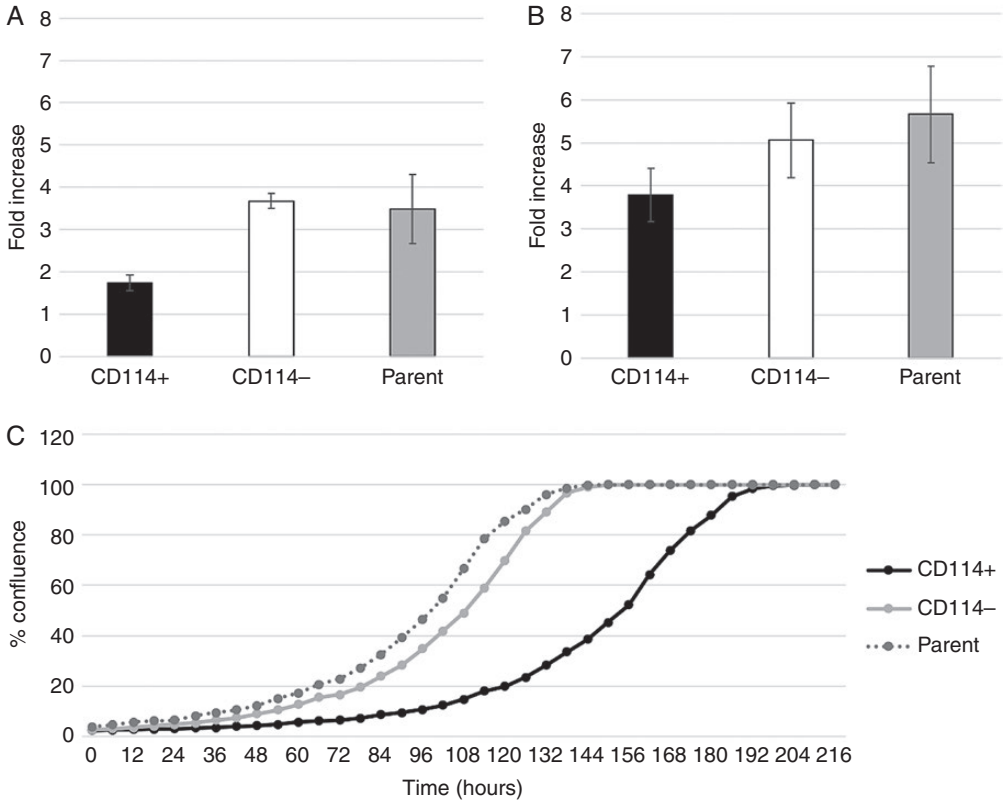

Figure 1. CD114-positive (CD114+) cells have slower growth than CD114-negative (CD114–) and unsorted populations. Equal numbers of CD114+, CD114–, and parental cells were plated in wells of 96-well plates and monitored with continuous live cell imaging. Cell confluence was calculated every 6 h and fold increase in confluence was calculated versus confluence at the time of cell seeding. (A and B) Fold increase in confluence after 120 h for D283 (A) and Daoy (B) cells. (C) Longitudinal change in confluence for Daoy cells over time.

after chemotherapy treatment compared to CD114– and parental cells. The percentage of CD114+ cells also was significantly increased after exposure to chemotherapy (Figure 2C), further suggesting increased chemoresistance of CD114+ medulloblastoma cells.

Responses of CD114+ Medulloblastoma Cells to G-CSF

To determine whether CD114+ medulloblastoma cells are capable of responding to exogenous G-CSF, CD114+ and CD114– medulloblastoma cells were treated with G-CSF and cell confluence and viability were measured over time. G-CSF exposure resulted in increased viability of CD114+ cells compared to untreated and to CD114– and parental cells (Figure 3A). G-CSF treatment also resulted in an increased confluence of CD114+ cells, while the CD114– and parental populations demonstrated no response to exogenous G-CSF (Figure 3B). The effect of G-CSF appears dose-dependent, with increased doses of G-CSF leading to increases in the percentage of CD114+ cells and in *CSF3R* gene expression (Supplementary Figure S6), further suggesting that medulloblastoma cells are capable of responding to exogenous G-CSF, likely via exogenous G-CSF directly binding to CD114.

To explore the effects of the sequential treatment of medulloblastoma cells with chemotherapy followed by G-CSF, we evaluated the percentage of CD114+ cells after chemotherapy alone for 48 h and after chemotherapy for 48 h followed by G-CSF. The percentage of CD114+ cells increased after the addition of chemotherapy, while subsequent exposure of cells to G-CSF after chemotherapy led to a significant increase in the percentage of CD114+ live cells (Figure 3C).

CD114+ Overexpression Results in Resistance to JAK–STAT Pathway Inhibition

To determine whether CD114 expression in medulloblastoma cells resulted in altered signaling through the JAK–STAT pathway, parental Daoy medulloblastoma cells and Daoy cells overexpressing *CSF3R* were treated with G-CSF. G-CSF treatment in *CSF3R*-overexpressing cells resulted in increases in phosphorylation of both JAK2 and STAT3, with minimal change in phosphorylation of JAK1 and STAT5 (Figure 4). Furthermore, Daoy-CSF3R cells demonstrated increased resistance to inhibition of JAK2 and STAT3 phosphorylation induced by the JAK inhibitor ruxolitinib (Figure 4).

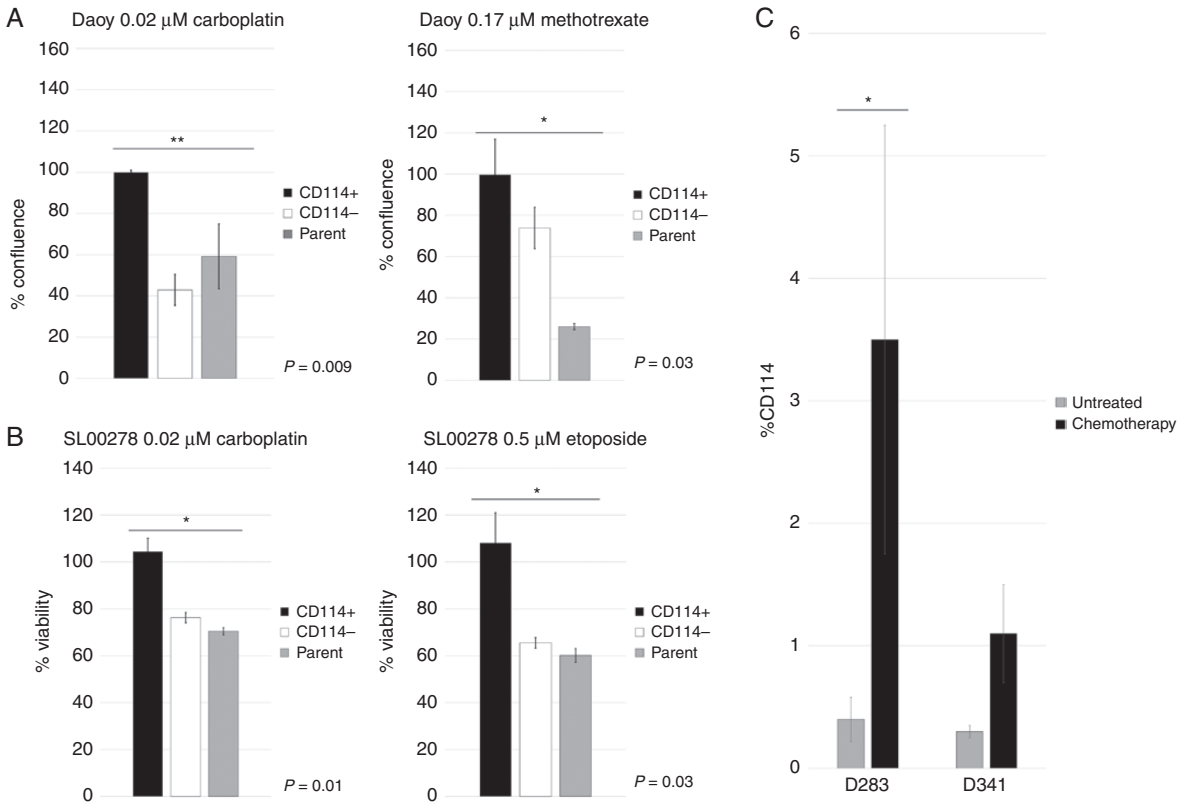

Figure 2. Efficacy of chemotherapy on sorted cell populations. Daoy cells (A) and SL00278 cells (B) were sorted by FACS into equal numbers of live CD114+, CD114–, and parental populations. Cells were then exposed to drug or vehicle (DMSO), and changes in confluence (A) and cell viability (B) were calculated over time (*$P < 0.05$, **$P < 0.01$). (C) Parental D283 cells were exposed to 0.17 µM methotrexate and parental D341 cells were exposed to 0.04 µM carboplatin for 48 h. Cells were harvested and analyzed by flow cytometry for CD114 expression, with differences between treated and untreated cells compared using Student's *t*-tests. *$P < .05$. $P = .02$ for D283 and $P = .12$ for D341.

Altered Gene Expression in CD114+ Medulloblastoma Cells

To determine whether CD114+ medulloblastoma cells demonstrated altered gene expression patterns, we evaluated the relative RNA expression of *NRP1*,[18] *MSI1*,[18,19] *TWIST1*,[20] *SOX2*,[21] *SOX9*,[22] and *MYCN*[23] in CD114+, CD114–, and parental populations. Isolated CD114+ cells demonstrated increased levels of *CSF3R* RNA as expected (Figure 5). CD114+ cells also demonstrated higher levels of *NRP1*, *MYCN*, *SOX9*, and *TWIST1* gene expression, compared to parental or sorted CD114– cells (Figure 5). Daoy CD114+ cells additionally had higher gene expression of *MSI1* and *SOX2*, while primary patient-derived HMB-5 cells did not demonstrate differences in *MSI1* and *SOX2* expression. These data suggest a unique RNA expression profile for CD114+ cells that is likely relevant for their unique cell behavior.

Discussion

Our results demonstrate that a subpopulation of medulloblastoma cells defined by surface expression of CD114 (the G-CSF receptor) demonstrates altered growth, chemoresistance, and responsiveness to G-CSF. CD114+ cells were identified in all tested medulloblastoma cell lines, PDX tumors, and primary patient tumor samples and have slower growth rates than CD114– and mixed parental cells. CD114+ cells also demonstrated resistance to chemotherapy agents used for standard medulloblastoma treatment, and the CD114+ population responded to G-CSF with increased growth and resistance to JAK/STAT inhibition. Furthermore, the treatment of medulloblastoma cells with chemotherapy followed by G-CSF resulted in a dramatic increase in the percentage of CD114+ cells. Our data therefore demonstrate that a CD114+ subpopulation of medulloblastoma cells exists in cell lines and tumors and may be capable of evading the effects of traditional chemotherapy and responding to exogenous G-CSF used for supportive care in treated patients. G-CSF is used after receipt of cytotoxic chemotherapy in nearly all patients undergoing therapy for medulloblastoma, in order to reduce the length of time of neutropenia and risk of severe infectious complications.

Molecular and cytogenetic analyses of medulloblastoma tumors have led to the identification of 4 major molecular medulloblastoma subtypes, including the WNT, SHH, G3,

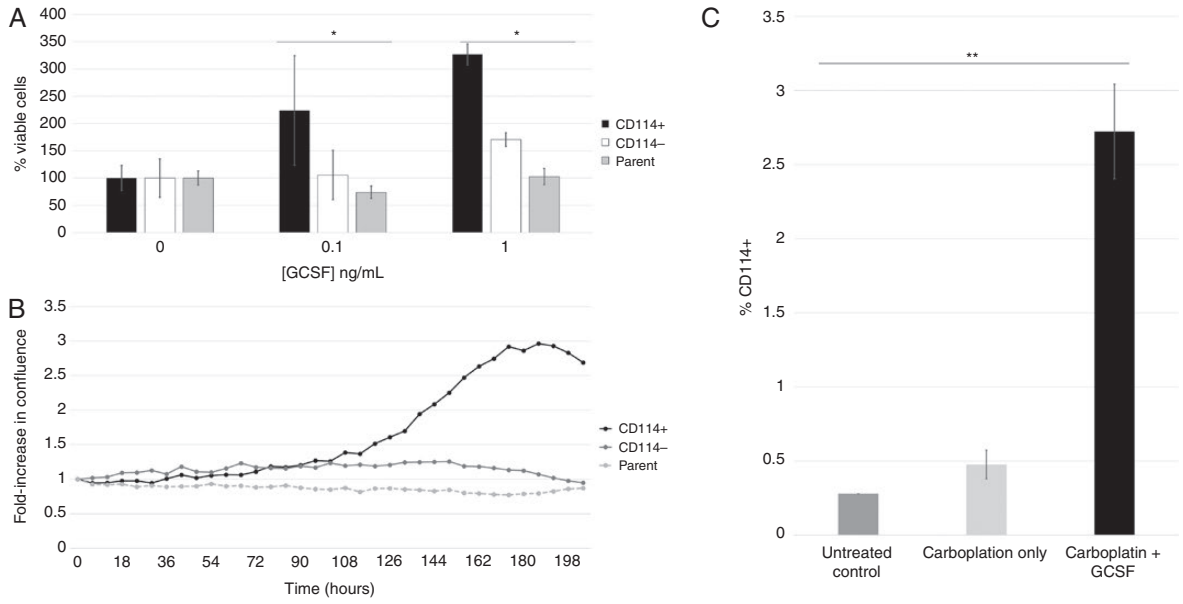

Figure 3. Changes in cell growth and CD114 surface expression after G-CSF exposure. (A) Daoy cells were sorted into CD114+, CD114–, and parental populations using FACS. Cells were exposed to vehicle (DMSO), 0.1 or 1 ng/mL G-CSF and at 200 h cell viability was determined by AlamarBlue assay. Differences in viability in CD114+ and parental cells were compared using Student's *t*-tests. $*P < .05$. (B) Longitudinal growth by confluence compared to vehicle control in sorted cells exposed to 1 ng/mL G-CSF for up to 198 h. Cells were plated in equal cell numbers immediately after sorting. Cell confluence was calculated via the IncuCyte live cell imaging system. (C) Parental D283 cells were plated and then treated with either 0.04 μM carboplatin or vehicle for 48 h. Media was then exchanged and cells were incubated in fresh media with or without 10 ng/mL G-CSF for 24 h. All cells were harvested and analyzed by flow cytometry for the percentage of live cells expressing CD114, and differences in CD114 expression were compared using Student's *t*-tests. $**P < .01$. *P*-value between untreated control and carboplatin + G-CSF is .006.

and G4 groups.[1] The WNT subgroup is defined by frequent mutations in the *CTNNB1* gene, leading to overactivity of the WNT signaling pathway, and patients with WNT tumors have very favorable outcomes. Tumors of the SHH subgroup have mutations in the *PTCH1*, *SMO*, and *SUFU* genes, along with *GLI2* and *MYCN* amplification, and can have favorable or unfavorable outcomes, depending on the presence of additional cytogenetic features, such as *P53* gene mutations. Group 3 tumors are aggressive tumors with the worst outcomes and primarily occur in infants and children, with a male predominance, and have frequent *MYC* amplification along with isochromosome 17q. Group 4 tumors are the most common subtype, but are also the most heterogeneous, with tumors having varying features such as isochromosome 17q, *KDM6A* mutations, and *MYCN* and *CDK6* amplification.[24] We have been able to identify a subpopulation of CD114+ cells in cell lines and tumors from each subtype. However, a larger sample size of cell lines and tumor samples is clearly needed to determine whether CD114 expression differs in tumors from different molecular subgroups and whether there is an association between CD114 expression and any of the known molecular or cytogenetic tumor features. We noted that the highest percentages of CD114+ cells were seen in tumor samples, while the smallest percentages were seen in the established cell lines, suggesting that the expression of CD114 may be downregulated over time in cells cultured in vitro.

Our results demonstrate that CD114+ medulloblastoma cells have slower rates of growth and increased viability after chemotherapy treatment when compared to CD114– and the parental cell populations, but the mechanisms underlying the altered growth rates and chemoresistance are unknown. Our results further demonstrate that medulloblastoma cells with CD114 expression have increased rates of growth in response to exogenous G-CSF compared to cells without CD114 expression and unsorted cells, suggesting that G-CSF binding to the G-CSF receptor is capable of inducing intracellular signaling leading to altered growth rates. Our results further demonstrate that the percentage of CD114+ cells is significantly increased after sequential exposure to chemotherapy followed by G-CSF, analogous to regimens used with patient treatment. This increase in the percentage of CD114+ cells suggests a potential mechanism for medulloblastoma disease relapse, with chemotherapy treatment followed by G-CSF leading to the selective growth of this chemoresistant subpopulation.

CSCs have been defined as a multipotent subpopulation of tumor cells with the ability to self-renew, generate differentiated progeny, and recapitulate a heterogeneous cancer cell population.[25] Recent studies have identified CD114 as a marker of a CSC subpopulation in neuroblastoma cells,[6] with G-CSF-induced intracellular signaling through the JAK–STAT pathway mediating the ability of this CSC subpopulation to self-renew and differentiate into all

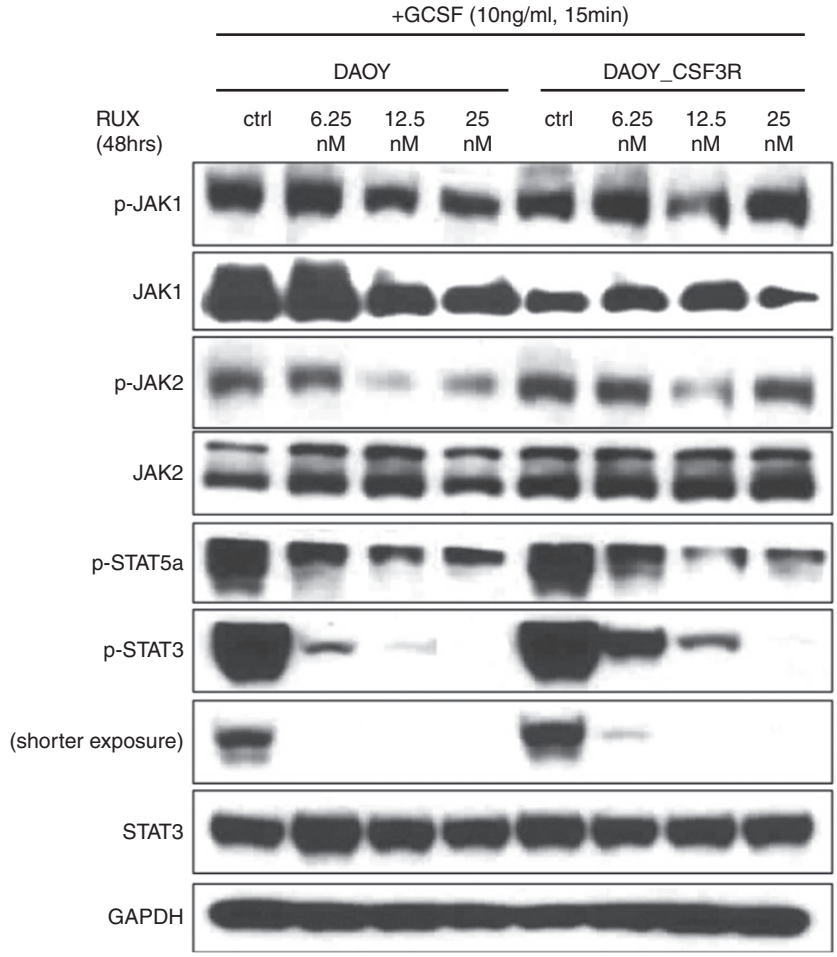

Figure 4. JAK–STAT pathway activity and response to ruxolitinib in medulloblastoma cells. Parental Daoy cells and Daoy cells transduced with *CSF3R* to overexpress CD114 (Daoy_CSF3R) were plated, treated with G-CSF (10 ng/mL for 15 min), and exposed to vehicle alone or increasing concentrations of ruxolitinib (RUX). Cells were harvested, separated by SDS-PAGE, and Western blots were performed for total and phosphorylated JAK1, JAK2, STAT3, and STAT5, with GAPDH used as a loading control.

tumor cell types.[26] CD114 expression has also been previously identified in established medulloblastoma cell lines and was associated with chemoresistance in both neuroblastoma and melanoma cell lines.[6,7] Expression of CD114 has also been implicated in the pathogenesis of multiple tumor types,[3–5] and CD114-mediated signaling induced by G-CSF promotes the survival and expansion of neural stem cells,[27] suggesting that the intracellular signaling pathways downstream of CD114 that are required for CSC maintenance and survival represent potentially effective therapeutic targets that could lead to the development of novel treatment strategies with minimal impact on G-CSF-stimulated myelopoiesis. However, the significance of CD114 expression and activity and the specific signaling pathways downstream of CD114 involved in the pathogenesis of medulloblastoma have not been delineated.

Recent studies have identified subpopulations of medulloblastoma cells that have CSC properties, including those defined by the expression of surface markers CD133[15,16] and CD15,[8,17] and the frequency of these CSC subpopulations correlates with more aggressive disease and poor prognosis,[15,28,29] suggesting that therapies directed against these CSCs are likely to improve patient outcomes. We have identified the expression of the CSC markers CD133 and CD15 on a subset of medulloblastoma cells that are largely distinct from CD114+ medulloblastoma cells. Therefore, the phenotype observed in CD114+ cells cannot be attributed to an effect of other CSC markers and is more likely due to the expression of CD114 and its downstream effects on the cell.

We also analyzed gene expression patterns of CD114+ medulloblastoma cells compared to CD114– and parental cells, and we identified higher expression of *NRP1*, *MYCN*, *SOX9*, and *TWIST1* in CD114+ cells. *NRP1* is found in higher concentration in medulloblastoma stem cells,[18] and *TWIST1* has been implicated in medulloblastoma metastasis and self-renewal.[20] *SOX9* expression and SOX9 protein function have been linked to increased malignancy in medulloblastoma tumors.[22] Musashi 1 (*MSI1*) has been proposed as a marker of CSCs and of worse prognosis

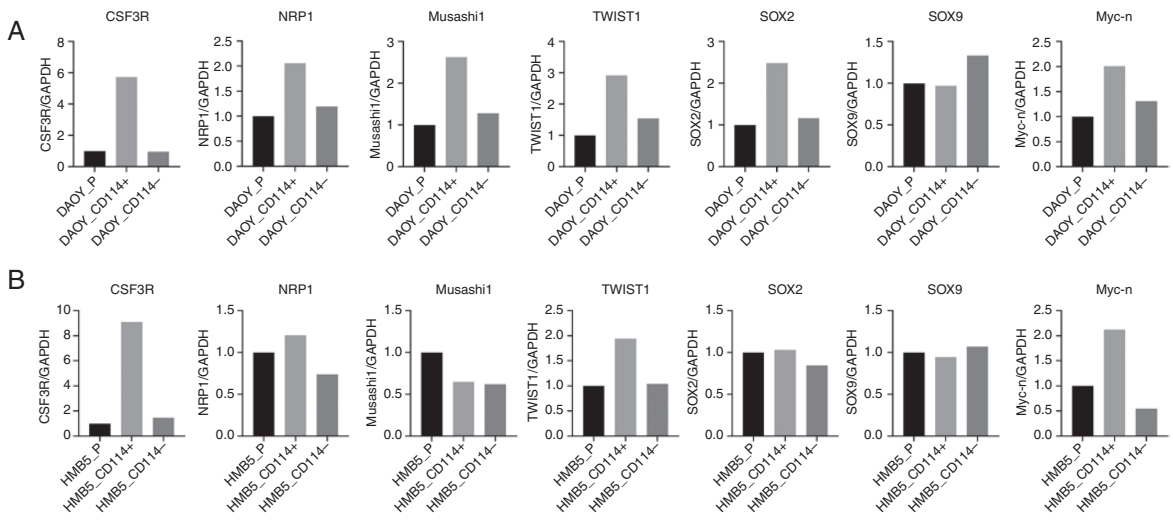

Figure 5. Changes in mRNA expression of select genes in CD114+, CD114- and parental cells. Daoy (A) and HMB-5 (B) cells were sorted by FACS and RNA was immediately isolated. Quantitative PCR was performed using primers for *CSF3R*, *NRP1*, *MSI1* (*Musashi1*), *Twist1*, *SOX2*, *SOX9*, and *MYCN* with *GAPDH* used as a control.

in gliomas[30] and medulloblastomas.[19] We found higher expression levels of *MSI1* in Daoy CD114+ cells, while in HMB-5 the levels were similar between CD114+ and CD114– cells, possibly suggesting cell type or molecular subtype differences. We conclude from this data that CD114 expression is associated with a unique cellular state as denoted by the differential expression of genes associated with more aggressive medulloblastoma tumors and worse patient outcomes, and further work is needed to determine the role of the altered expression of these and other genes in the behavior of CD114+ medulloblastoma cells.

Understanding medulloblastoma intratumoral cellular heterogeneity allows for identification and specific targeting of treatment-resistant cell subpopulations that likely to contribute to metastatic or recurrent disease. Our findings suggest a role for the G-CSF receptor in a unique subpopulation of medulloblastoma cells and tumors that could contribute to disease recurrence. Moreover, our data suggest that exogenous G-CSF may provide a survival benefit to this subpopulation of tumor cells. Alternative methods of supporting patient neutrophil recovery in children with medulloblastoma may be effective without stimulating the CD114+ medulloblastoma cell population; however, more studies are needed before recommending a change in clinical practice. Further work is required to clarify the role of this subpopulation in patients with medulloblastoma, and future work is needed to define the role of the CD114+ subpopulation in medulloblastoma pathogenesis and to evaluate the potential significance of CD114-mediated signaling as a therapeutic target.

Supplementary Data

Supplementary data are available at *Neuro-Oncology Advances* online.

Keywords

CD114 | chemoresistance | G-CSF | medulloblastoma

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