Targeting Suppressive Myeloid Cells Potentiates Checkpoint Inhibitors to Control Spontaneous Neuroblastoma

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Abstract

Purpose: Neuroblastoma is the most common extracranial solid cancer type in childhood, and high-risk patients have poor prognosis despite aggressive multimodal treatment. Neuroblastoma-driven inflammation contributes to the induction of suppressive myeloid cells that hamper efficient antitumor immune responses. Therefore, we sought to enhance antitumor immunity by removing immunosuppression mediated by myeloid cells.

Experimental Design: The prognostic values of myeloid cells are demonstrated by analyzing genomic datasets of neuroblastoma patients. The impact of tumor-derived factors on myelopoiesis and local induction of suppressive myeloid cells is dissected by in vitro culture models using freshly isolated human CD34+ hematopoietic stem cells, primary human monocytes, and murine bone marrow cells. To test the therapeutic efficacy of BLZ945 as a monotherapy or in combination with checkpoint inhibitors, we used a transgenic murine model (TH-MYCN) that develops aggressive spontaneous neuroblastoma.

Results: We report that infiltrating CSF-1R+ myeloid cells predict poor clinical outcome in patients with neuroblastoma. In vitro, neuroblastoma-derived factors interfere with early development of myeloid cells and enable suppressive functions on human monocytes through M-CSF/CSF-1R interaction. In a transgenic mouse model (TH-MYCN) resembling high-risk human neuroblastoma, antagonizing CSF-1R with a selective inhibitor (BLZ945) modulates the induction of human and murine suppressive myeloid cells and efficiently limit tumor progression. While checkpoint inhibitors are insufficient in controlling tumor growth, combining BLZ945 with PD-1/PD-L1 blocking antibodies results in superior tumor control.

Conclusions: Our results demonstrate the essential role of CSF-1R signaling during the induction of suppressive myeloid cells and emphasize its clinical potential as an immunotherapy for human cancers.

Introduction

Neuroblastoma is a solid pediatric tumor of the developing sympathetic nervous system (1). High-risk metastatic tumors frequently show amplified MYCN proto-oncogene, and overall patient survival is less than 50% (1, 2). Progression of neuroblastoma is associated with expression of inflammatory factors (3), and intratumoral myeloid cells predict poor clinical outcome (4, 5). A transgenic murine model resembling the aggressive growth pattern of high-risk human neuroblastoma has been characterized, where the human MYCN oncogene (TH-MYCN) drives the establishment of spontaneous tumors (6, 7). We and others have reported prolonged survival of these animals using novel treatments (8–10). However, once the tumors are established, it is difficult to achieve tolerable therapeutic effects, due to the known short treatment window and rapid disease progression (7, 11, 12).

Recently, eliciting antitumor immunity has been proposed to be a promising option for treating high-risk childhood neuroblastoma (13, 14). However, tumor-induced inflammation limits efficient antitumor immune responses through recruitment of various suppressive immune cell types (15), including myeloid-derived suppressor cells (MDSC) and "M2-biased" tumor-associated macrophages (TAM) (16–18). These cells demonstrate overlapping phenotypic markers as well as inhibitory mechanisms (19, 20) and predict poor clinical outcome in patients with cancer (21–24).

Macrophage colony-stimulating factor (M-CSF or CSF-1) is known to be essential for the differentiation and survival of myeloid cells (25). In malignant conditions, tumor-derived M-CSF is associated with poor survival in various human cancers (26). Thus, targeting strategies against its receptor (CSF-1R) have been extensively explored (27–29). When included in combination approaches, blockade of CSF-1R signaling exhibits synergistic effects of various anticancer therapies (30–34).

Here, we show that infiltration of CSF-1R+ myeloid cells predicts poor survival in patients with neuroblastoma. Further,
Flow cytometry (penicillin and streptomycin), and 2 mM glutamine (Life Technologies). Tumor-conditioned medium (TCM) was harvested and stored in aliquots at −80 °C when cells reached 90% confluence.

Flow cytometry
Detailed information of fluorochrome-conjugated antibodies utilized in this study was summarized in Supplementary Table S3. Briefly, 1 × 10^6 to 2 × 10^6 cells were placed in a 96-v-bottom plate and washed twice in 200 μL FACS buffer (PBS with 1% human serum albumin, HSA). Next, cells were resuspended in 20 μL PBS containing the appropriate antibody cocktails for extracellular antigens and incubated at 4°C for 25 minutes. For samples requiring intracellular staining, cells were fixed and permeabilized in BD cytofix/cytoperm buffer for 15 minutes and stained for 40 minutes at 4°C. All cells were washed and acquired in a BD LSRII instrument, and data were further analyzed with FlowJo software (Treestar Inc.).

TH-MYCN murine neuroblastoma model
TH-MYCN animals were obtained from the Mouse Model of Human Cancer Consortium Repository as an N16 backcross to the 129 × 1/SvJ background and have been kept as a continuous inbreeding. All experimental protocols were reviewed and approved by the regional ethical committee (ethical permit N42/14). Heterozygous mice received abdominal palpations 3 times weekly to follow tumor development and were monitored closely for signs of discomfort. At the time tumors were palpable (day 0), mice were treated with BLZ945 by daily oral gavage as described above for 10 consecutive days. Alternatively, blocking antibodies for PD-1 (clone: RMP1-14) and PD-L1 (clone: 10F.9G2; both purchased from BioXcell) were injected intraperitoneally at 12.5 mg/kg on days 0, 3, and 6 or concurrently with BLZ945. The combination was well tolerated, and neither weight loss nor toxicity of the internal organs was observed.

Isolation of primary human cells
To obtain human primary monocytes, peripheral blood mononuclear cells (PBMC) were first isolated from buffy coats by ficoll gradient centrifugation (GE Healthcare) and washed three times in PBS. Next, monocytes were labeled with CD14^+ selection microbeads and purified using an LS column (Miltenyi Biotech). CD34^+ hematopoietic progenitor cells were purified from umbilical cord blood provided by the Karolinska University Hospital. In brief, T cells were first removed during gradient centrifugation using a RosetteSep CD3^+ depletion kit (StemCells), followed by CD34^+ positive selection using microbeads and MS columns (Miltenyi Biotech).

CSF-1R inhibitors
BLZ945, a highly selective small-molecule inhibitor for tyrosine kinase of CSF-1R (>3,200-fold more than other tyrosine kinases; ref. 27), was kindly provided by Novartis. For in vitro–blocking experiments, stock solutions were prepared by dissolving BLZ945 or GW2580 (Selleck Chemicals) in DMSO at 10 mM and 1 mM, respectively. For in vivo treatment, BLZ945 was dissolved in 20% Captisol at 16 mg/mL and delivered by daily oral gavage at the dose of 200 mg/kg, according to a previous study (29).

Differentiation of CD34^+ hematopoietic progenitor cells
Maturation of CD34^+ cells was performed using 900 μL culture medium containing 50 ng/mL GM-CSF and 5 ng/mL TNFα (both were from PeproTech) in a 24-well plate. Alternatively, supernatants harvested from the three above-mentioned human neuroblastoma cell lines were added in addition to the cytokines at a 2:1 ratio to the progenitor cells. CD34^+ cells maintained in culture medium were used as controls. To block CSF-1R signaling, BLZ945 (500 nmol/L) was added to cells matured with cytokines or the combination of cytokines and SK-N-BE(2) supernatant.
After 7 days, all cells were harvested by washing and gently scraping, and phenotypes and functions of the cells were evaluated by flow cytometry or in CFSE-based T-cell proliferation assays.

Human monocyte–tumor coculture

Primary human monocytes were cocultured with human neuroblastoma cell lines according to our published protocol (35). In brief, monocytes were cocultured with 4 × 10^5 SK-N-BE (2), SK-N-AS, or 6 × 10^5 SK-N-FI neuroblastoma cells in 3-ml culture medium in a 6-well plate. Monocytes cultured without tumor cells were used as controls. After 64 hours, cells were harvested by vigorously washing, followed by gently scraping of the plates. Phenotypic changes of the cells were evaluated by flow cytometry and HLA-DR^+ cells were sorted with microbeads and MS columns (Miltenyi Biotech). To investigate the role of CSF-1R, 500 nmol/L BLZ945 (Novartis) or 1 μmol/L GW2580 (Selleck Chemicals) was added to monocytes or the cocultures.

Differentiation of murine bone marrow cells

Suppressive myeloid cells were induced from bone marrow cells harvested from negatively genotyped TH-MYC N mice, in accordance with a previously described protocol (36). In brief, 1 × 10^6 isolated bone marrow cells were cultured in 6-well plates in the presence of NHO2A tumor-conditioned medium (TCM, 1:1 dilution to fresh medium). As controls, cells were cultured in fresh medium or with M-CSF (20 ng/mL, Biologend). To block CSF-1R signaling, BLZ945 or GW2580 were added at 1 μmol/L to the cultures and DMSO was included as control. After 4 days, cells were harvested by collecting floating cells and carefully scraping the adherent cells of the wells, and flow cytometric analysis or T-cell suppression assays were conducted subsequently.

T-cell suppression assays

Primary human T cells were isolated from PBMCs by CD3^+ positive selection and MS columns (Miltenyi Biotech) and labeled with 1.5 μmol/L 5(6)-CFDA/CFSE tracing dye (Invitrogen). For the tumor-educated monocytes, 1 × 10^6 T cells were added together at 2:1 or 4:1 with HLA-DR–sorted cells and activated by microbeads coated with anti-CD3/CD28 mAb (Invitrogen). After 4 days, cells were harvested and proliferations of T cells were evaluated by flow cytometry. In mixed lymphocyte reactions, CFSE-labeled T cells from PBMCs were mixed at 4:1, 8:1, or 16:1 with differentiated CD34^+ cells, and proliferation of T cells was analyzed after 6 days. In some experiments, IFNγ concentrations in the culture medium at the end of proliferation assays were also measured by ELISA (Mabtech).

In order to evaluate murine T-cell functions, 1 × 10^6 CFSE-labeled splenocytes from naive mice were preincubated for 24 hours at different ratios with Gr1^+ cells isolated from spleens of tumor-bearing mice or bone marrow–derived myeloid cells differentiated in vitro in a 96-well plate. Next, T cells were activated with 1 μl microbeads coated with anti-CD3/CD28 mAb (Invitrogen) and proliferation and expression of CD69 on T cells were measured by flow cytometry after 4 days. To investigate the suppressive mechanisms, pharmacologic inhibitors for IDO (1-DL-MT, 250 μmol/L, Sigma-Aldrich), JAK/STATs (AG490, 10 μmol/L, Sigma-Aldrich), arginase (nor-NOHA, 200 IU/mL, Calbiochem), or iNOS (1400W, 200 μmol/L, Sigma-Aldrich) were added to the wells during the T-cell activation.

Cytokine analysis

Cytokine contents in culture medium or supernatants harvested from SK-N-BE(2), SK-N-AS, or SK-N-FI neuroblastoma tumor cell lines were analyzed by a 27-parameter Luminex multiplex assay (R&D Systems) in the core facility at Karolinska University Hospital. Concentrations of human or murine M-CSF (CSF-1) in the TCM were determined using ELISA (R&D Systems).

R2 database

Multiple parameters were searched and correlated in patient datasets available in the public "R2: microarray analysis and visualization platform" (http://r2.amc.nl). For comparisons between benign and malignant tumors, three datasets, including Versteeg (cohort 1, n = 88), Hiyama (cohort 2, n = 51), and Lastowska (cohort 3, n = 30), were compared with the Miller neuroblastoma dataset (n = 86) using one-way ANOVA. For survival of patients, Kaplan–Meier analysis was performed on two previously reported datasets of patients with untreated neuroblastoma tumor biopsies, including Versteeg (cohort 1, n = 88; ref. 37) and Seeger (cohort 4, n = 102; ref. 5), and the cutoff expression levels were calculated within the R2 database. In addition, a larger dataset, Kocak (cohort 5, n = 649; ref. 38), was included to study the correlations between expression of CSF-1R and other markers.

Statistical analysis

Unless otherwise stated, all results were collected from multiple experiments, and figures were prepared in the Prism software (GraphPad). Normal distribution and equal variant of the datasets were first tested, thereafter appropriate Student t tests or nonparametric, Mann–Whitney U tests were applied (as stated in the figure legends). All results were presented as means ± SD, and representative histograms or pictures were selected based on the average values.

Results

Infiltrating CSF-1R^+ myeloid cells predicted poor survival in patients with neuroblastoma

To investigate the immune signatures in human neuroblastoma, we analyzed the expression patterns in different patient datasets (‘R2: microarray analysis and visualization platform’, http://r2.amc.nl).

In comparison to benign neuroblastoma, neuroblastoma from three patient cohorts (refs. 37, 39; P values are summarized in Supplementary Table S1) all showed significantly increased expression of CD14 and CD68 monocytic/macrophage markers (Fig. 1A). In contrast, granulocytes (CD66b), regulatory T cells (FoxP3) or dendritic cell (DC) markers CD11c and CD83 remained unchanged (Supplementary Fig. S1A). Moreover, CSF-1R showed enhanced expression in tumor tissues (Fig. 1B) and correlated strongly with expression levels of CD14 and CD68 (R = 0.801 and 0.752, respectively; Fig. 1C). Accordingly, the myeloid differentiation factor M-CSF (Fig. 1D), but not GM-CSF, G-CSF, or another CSF-1R ligand, IL34 (Supplementary Fig. S1B), was expressed at significantly higher levels in human neuroblastoma.

Furthermore, we validated the prognostic significance of M-CSF and CSF-1R in two previously published datasets. We restricted these analyses to data collected from biopsies of untreated patients (5, 37), because myeloid cells are sensitive to treatment,
CD56 survival in patients with neuroblastoma. Moreover, expression of CSF-1R (Fig. 1E), for example, by chemotherapy (40). Expression levels of M-CSF (Fig. 1F), as well as CD14 or CD68 (data not shown), strongly associated with poor overall and relapse-free survival in patients with neuroblastoma. Moreover, expression of NK cell marker CD56 (Supplementary Fig. S1C) predicted better survival in high-risk patients with neuroblastoma (age >18 months), but the presence of CD4 T cells was associated with poor prognosis (Supplementary Fig. S1D).

Myelopoiesis of human CD34⁺ progenitor cells was modulated by tumor-derived factors

To evaluate how inflammatory factors released from neuroblastoma cells could influence the early development of myeloid cells in patients with cancer, we established an in vitro model where human CD34⁺ hematopoietic progenitor cells were differentiated in the presence of tumor-derived factors. In the absence of cytokines, progenitor cells failed to differentiate (Fig. 2A) and were not capable of stimulating allogeneic T cells (Supplementary Fig. S2A). In contrast, addition of GM-CSF and TNFα promoted rapid proliferation and myelopoiesis of CD34⁺ progenitor cells, resulting in clusters of nonadherent cells and subsets of adherent cells, which contained DCs (CD14⁺CD11c⁺CD1a⁺CD86⁺), macrophages (CD11b⁺CD68⁺) and monocytes/macrophages (CD14⁺c-kit⁺) (Fig. 2A and gating strategy in Supplementary Fig. S2B). As a result, frequencies of c-kit⁺CD14⁺ immature cells diminished (Fig. 2B), and these cells enabled T cells to proliferate and produce IFNγ (Fig. 2C). However, when progenitor cells were exposed to neuroblastoma-derived factors, their maturation was significantly hampered. Frequencies of DCs, monocytes, and macrophages (Fig. 2A), and the overall activation of myeloid cells (Supplementary Fig. S2C) were inhibited. Functionally, these cells demonstrated impaired ability to stimulate T cells (Fig. 2C and Supplementary Fig. S2D).

To uncover the essential factors contributing to the alterations in myelopoiesis, we analyzed 28 soluble factors in tumor-conditioned media and identified M-CSF (Supplementary Fig. S3A and S3B) as the only factor that was absent in the culture medium but produced at high levels by all three tumor cell lines. Indeed, antagonizing CSF-1R during differentiation of

Figure 1.
Infiltrating CSF-1R⁺ myeloid cells predicted poor survival in patients with neuroblastoma. Genomic information was analyzed by searching the R2 database, and intratumoral expression of CD14 or CD68 myeloid markers (A), CSF-1R (B) or M-CSF (D) were compared between 3 neuroblastoma patient cohorts (cohort 1, Versteeg, n = 88; cohort 2, Hiyama, n = 51; and cohort 3, Lastowska, n = 50) and benign neurofibroma patients (Miller, n = 86) using one-way ANOVA (see statistical summary in Supplementary Table S2). C, correlations between CSF-1R and myeloid markers CD14 (r = 0.801) or CD68 (r = 0.752) were analyzed using a dataset containing 649 neuroblastoma tumor samples (cohort 5, Kocak). For evaluating the prognostic values of intratumoral expression of M-CSF (E) and CSF-1R (F), two patient datasets containing untreated tumor biopsies (cohorts 1 and 4) were analyzed by Kaplan-Meier estimates.
CD34⁺ cells by a highly selective inhibitor (BLZ945) specifically limited the differentiation of macrophages and monocytes (Fig. 2D and Supplementary Fig. S3C) that had high expression of CSF-1R (Fig. 2E). However, it had no impact on DCs (Fig. 2D), because of their lower expression of CSF-1R (Supplementary Fig. S3D and S3E). Notably, BLZ945 decreased monocytes and macrophages in cultures with only cytokines (Fig. 2D), but this did not lead to consistent changes of their T cell-activating capacities (Fig. 2F).

CSF-1R blockade reverted suppressive functions on tumor-educated human monocytes

Next, we cocultured primary monocytes freshly isolated from healthy individuals with three human neuroblastoma cell lines (SK-N-BE(2), SK-N-AS, and SK-N-FI). The presence of neuroblastoma cells upregulated the expression of CD14 (Supplementary Fig. S3A) and CD206 (Fig. 3A), which is consistent with the microenvironment of tumor cells. The expression of CSF-1R was also significantly increased in tumor-educated monocytes (Fig. 3B). When CSF-1R was blocked, expression of CD14 and CD206 were significantly decreased (Supplementary Fig. S3A and S3B).

**Figure 2.**

Myelopoiesis of human CD34⁺ progenitor cells was modulated by tumor-derived factors. The impact of tumor-derived factors on differentiation of human CD34⁺ hematopoietic stem cells was measured in the presence of TCM harvested from 3 human neuroblastoma cell lines (SK-N-BE(2), SK-N-AS, and SK-N-FI). A, frequencies of DCs (CD14⁺CD11c⁺ CD14⁻CD11c⁻), monocytes/macrophages (CD14⁻CD16⁻), and monocytes (CD14⁻CD16⁺) were evaluated by flow cytometry. B, the ability of myeloid cells matured in different conditions to stimulate proliferation and IFNγ release from allogeneic T cells was tested in mixed lymphocyte reactions. The effects of the CSF-1R inhibitor BLZ945 on the maturation of CD34⁺ progenitor cells to different myeloid subsets (D) and expression of CSF-1R on monocytes or macrophages (E) were tested by flow cytometry. F, stimulatory functions of myeloid cells on T-cell proliferation after CSF-1R inhibition were demonstrated. G, representative histograms of the T-cell proliferation were chosen based on average values. *P < 0.05; **P < 0.01; nonparametric Mann-Whitney U test. Each dot represented an individual experiment.
Notably, direct CSF-1R inhibition led to phenotypic changes on primary monocytes (Fig. 3D) and resulted in a threefold increase of CSF-1R expression (Fig. 3D). Even though reported in a previous study (33), CSF-1R inhibitors did not modulate expression of PD-L1 on tumor cells in our coculture system (Supplementary Fig. S4D).

Functionally, SK-N-BE(2) or SK-N-AS tumor-educated monocytes acquired strong suppressive capacity against autologous T cells, and CSF-1R inhibition was sufficient to revert their suppressive functions on T cells \( (P < 0.05; \text{Fig. 3E and representative histograms based on average values of the seven independent experiments in Fig. 3F; Supplementary Fig. S4E and S4F).} \}

Figure 3.
CSF-1R blockade reverted suppressive functions on tumor-educated human monocytes. Using the monocyte-neuroblastoma coculture model, we investigated the changes of phenotypic markers (A) and expression of CSF-1R on primary human monocytes (B) induced by different neuroblastoma cell lines. C, next, we evaluated the effects of CSF-1R inhibitor BLZ945 (500 nmol/L) or GW2580 (1 \( \mu \)mol/L) on the phenotypic changes of tumor-educated monocytes. D, the direct effects of BLZ945 on phenotypes of human primary monocytes were demonstrated. E, we demonstrated the suppressive functions of tumor-educated monocytes against T cells with or without CSF-1R inhibition using primary monocytes from 7 donors. F, representative histograms of 7 independent proliferation assays were shown. \( *, P < 0.05; \), \( **, P < 0.01; \) nonparametric Mann-Whitney U test. Each dot represented an individual experiment.
contrast, treatment of healthy monocytes with BLZ945 did not show changes in their functions toward T-cell proliferation (Supplementary Fig. S4G).

**BLZ945 elicited therapeutic effects through modulating suppressive myeloid cells**

To confirm that the M-CSF/CSF-1R interaction in mice is involved in the development of suppressive myeloid cells, we established an in vitro model based on a previous study (36). Naive bone marrow cells were differentiated in the presence of recombinant murine M-CSF. Similar to the results using primary human myeloid cells, TCM and M-CSF induced expansion of MDSCs and TAMs (Supplementary Fig. S5A), and the resulted cells inhibited proliferation of autologous T cells (Supplementary Fig. S5B). Blocking CSF-1R signaling efficiently abolished the induction of CSF-1R–expressing suppressive myeloid cells (Supplementary Fig. S5C and S5D) and recovered functions of T cells (Supplementary Fig. S5E).

Motivated by our in vitro findings, we sought to evaluate the therapeutic potential of BLZ945 in vivo in a transgenic murine model (TH-MYCN) that resembles the aggressive growth pattern of high-risk human neuroblastoma (6, 7). As shown in Supplementary Fig. S7A, a 10-day daily oral gavage of BLZ945 was initiated when the abdomen-located spontaneous tumors were palpable.

In the control group, mice developed tumors at the average size of 1.5 g at 10 days after tumor detection (Fig. 4A). Tumor-bearing animals demonstrated significantly higher levels of MDSCs of the granulocytic (P < 0.01, grMDSCs, Ly6G+Ly6C−) and monocytic (P < 0.0001, moMDSCs, Ly6G−Ly6Chigh) lineages, as well as F4/80+ macrophages (P < 0.01), in comparison to tumor-free mice (Fig. 4B) and these myeloid cells demonstrated high expression of CSF-1R (Supplementary Fig. S6A). Functionally, splenic Gr1+ cells sorted from tumor-bearing mice potently inhibited proliferation of T cells in a ratio-dependent manner (Supplementary Fig. S6B and S6C). This suppression could be rescued by blocking enzymatic activity of iNOS and IDO (Supplementary Fig. S6D). However, frequencies of regulatory T cells did not change significantly (Supplementary Fig. S6E), although lower CD4+ T-cell frequencies were observed (Supplementary Fig. S6F).

As a monotherapy, BLZ945 treatment significantly controlled progression of established tumors (P < 0.01; Fig. 4A). This could

![Figure 4](image-url)

**Figure 4.**

BLZ945 elicited therapeutic effects through modulating suppressive myeloid cells. All mice received 3 abdominal palpations per week and immune composition in tumors and spleens were evaluated 10 days after tumors were palpable. A, comparison of tumor weights between control and BLZ945-treated mice (200 mg/kg in 20% Captisol) was demonstrated. B, frequencies of moMDSCs, grMDSCs, or macrophages and CSF-1R or PD-L1 (C) expression of macrophages in the spleens of naive, control or BLZ945-treated mice were compared. D, percentages of CD4+ and CD8+ T cells and E, activation in the spleens of naive, control or BLZ945-treated mice were shown. F, frequencies of tumor-infiltrating T cells and G, expression of PD-1 and K6D9 on CD8 T cells in tumors of control or BLZ945-treated mice were evaluated. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; nonparametric Mann-Whitney U test. Each dot represented an individual mouse.
be explained by the selective effects on CSF-1R–expressing myeloid cells, because numbers of CD11b+ cells (P < 0.01; Supplementary Fig. S7B), including grMDSCs (P < 0.01), moMDSCs (P < 0.001), and macrophages (P < 0.0001), were significantly reduced in spleens of the treated mice (Fig. 4B). Further, BLZ945 treatment strongly limited the expression of CSF-1R (P < 0.01) and PD-L1 (P < 0.01) on macrophages and MDSCs (Fig. 4C and Supplementary Fig. S7C).

In addition, treatment with BLZ945 led to a significant recovery of CD4+ (P < 0.05) and CD8+ (P < 0.01) T cells in spleens (Fig. 4D). Activation of CD4+ and CD8+ T cells were improved (Fig. 4E), demonstrated by significantly higher expression of CD25 (P < 0.05) and CD69 (P < 0.01). However, the CD4/CD8 ratio remained unchanged in the treated mice when compared with the controls (Supplementary Fig. S7D).

In tumors of BLZ945-treated mice, neither did we observe changes in CD11b+ cell numbers (Supplementary Fig. S7E), nor the frequencies of grMDSCs, moMDSCs, and macrophages (Supplementary Fig. S7F). However, lower numbers of infiltrating CD4+ T cells but increased CD8+ T cells were observed (Fig. 4F), leading to decreased CD4/CD8 ratios (Supplementary Fig. S7G). Moreover, tumor-infiltrating CD8+ (Fig. 4G) and CD4+ T cells (Supplementary Fig. S7H) were more activated in BLZ945-treated mice.

Combining CSF-1R inhibition with checkpoint blockade enabled superior tumor control

Given that suppressive myeloid cells expressed PD-L1 at high levels and T cells (data not shown) did not delay progression of established spontaneous tumors in the TH-MYCN mice. In contrast, the addition of BLZ945 to the checkpoint-blocking agents resulted in potent synergistic antitumor effects (P < 0.05; Fig. 5A). In 6 of 8 (75%) treated mice (Fig. 5B), we observed complete tumor regression or small tumors (<0.5 g). Strikingly, the combination prevented the progressive growth of large tumors (>1.0 g; Fig. 5B).

In comparison to checkpoint blockade, the combination efficiently normalized the numbers of CD11b+ cells (P < 0.05; Supplementary Fig. S8C), including macrophages and grMDSCs (P < 0.05; Fig. 5C), and all treatments could diminish moMDSCs (Fig. 5C) in the spleens. Strikingly, the combined treatment reactivated macrophages in spleens, demonstrated by decreased numbers of CSF-1R+ (P < 0.01) or PD-L1+ (P < 0.01) macrophages, but increased expression of MHC class II (P < 0.05) on macrophages (Fig. 5D). Although intratumoral frequencies of CD11b+ cells (Supplementary Fig. S8D) or macrophages (Supplementary Fig. S8E) were not significantly modulated, we observed similar effects on macrophages in tumor tissues (Fig. 5E). Despite the low total numbers, there was also a marked reduction of grMDSCs (P < 0.05) and moMDSCs in the tumors of mice received combination therapy (Supplementary Fig. S8E).

When checkpoint-blocking agents were administered, we observed enhanced infiltration (Supplementary Fig. S8G) and activation (Fig. 5F) of T cells in tumor tissues. It was more pronounced on CD4+ T cells, leading to increased CD4/CD8 ratios (Supplementary Fig. S8F and S8G). Removal of suppressive myeloid cells by CSF-1R inhibition did not further elevate T-cell numbers in spleens (Supplementary Fig. S8F) or tumors (Supplementary Fig. S8G), but boosted activation of T cells (Fig. 5F).

Discussion

Stimulated by the finding that CSF-1R+ myeloid cells in tumors of patients with neuroblastoma predict poor overall and disease-free survival, we investigated the functional and therapeutic significance of targeting CSF-1R signaling in this study. The essential role of the tumor-driven M-CSF/CSF-1R axis on the differentiation and activation of suppressive myeloid cells is dissected by in vitro models, reflecting myelopoiesis in the bone marrow or activation of myeloid cells in the tumor microenvironment. As therapeutic approaches, the highly selective CSF-1R inhibitor BLZ945 elicits strong antitumor effects against established aggressive spontaneous tumors in vivo and could exert superior tumor control when combined with PD-1/PD-L1-blocking antibodies.

Emerging evidence has demonstrated that tumor-derived inflammatory mediators support circulating hematopoietic progenitor cells to engraft in the spleen, leading to splenic production of immature suppressive myeloid cells (43, 44). Previous studies have confirmed the importance of GM-CSF, G-CSF, and IL6 (36, 45) in driving the differentiation of suppressive myeloid cells from murine bone marrow. However, the detailed mechanisms of this effect in humans remain elusive. In a recent study, Wu and colleagues confirmed the role of GM-CSF and IL6 during the expansion of MDSCs from human CD34+ hematopoietic progenitor cells (46).

Utilizing murine bone marrow cells, human CD34+ hematopoietic progenitor cells and a human monocyte-neuroblastoma coculture model, we provided insightful results emphasizing the key contribution of the M-CSF/CSF-1R pathway during the induction of MDSCs and TAMs. Notably, CSF-1R inhibition did not rescue frequencies of DCs after in vitro differentiation of CD34+ progenitor cells. This indicated that other tumor-derived factors, such as IL10 or VEGF, might be responsible for the suppression. Given that long-term in vitro culture could result in growth of selected tumor cell subsets, it is important to validate these findings with primary or short-passage human neuroblastoma cells (47).

It is well documented that MDSCs and TAMs are associated with poor clinical outcome in patients with solid tumors (22–24). In human neuroblastoma, tumor-infiltrating inflammatory myeloid cells were analyzed by immunohistochemistry stainings in 133 metastatic tumors and demonstrated independent prognostic values (4). However, due to the extremely heterogeneous nature of suppressive myeloid cells, it remains challenging to specifically target these cells. Our analyses revealed that human neuroblastoma produced M-CSF at high levels and recruited substantial numbers of CSF-1R+ myeloid cells. These findings were validated in vitro utilizing human neuroblastoma cell lines and primary monocytes. Indeed, M-CSF levels as well as numbers of CSF-1R+ myeloid cells in human tumors predicted worse patient outcome. Collectively, we proposed that the M-CSF/CSF-1R axis is clinically
Combining CSF-1R inhibition with checkpoint blockade enabled superior tumor control. Once spontaneous tumors were palpable (day 0), mice received checkpoint-blocking antibodies for PD-1 and PD-L1 intraperitoneally at 12.5 mg/kg on days 0, 3, and 6. Alternatively, antibodies were injected concurrently with BLZ945, or the combination with BLZ945 were grouped and compared according to the tumor weight distribution. Activation of T cells in spleens and tumors was compared among control, checkpoint blockade, and the combinational treatment groups.

In the clinic, antibodies blocking PD-1/PD-L1 interactions have achieved remarkable therapeutic benefits in patients with solid cancers (49, 50). We showed that checkpoint-blocking agents were potent in enhancing T-cell functions in vivo, but had minor impacts on the myeloid compartment. Thus, it could be one of the resistance mechanisms underlying the insufficient in vivo potency. Indeed, inhibition of CSF-1R synergized with checkpoint-blocking antibodies and elicited striking antitumor effects that were superior to either treatment. Recently, three independent early-stage clinical studies have been initiated, where a small-molecule inhibitor (NCT02452424) or monoclonal antibodies (NCT02526017 and NCT02323191) targeting CSF-1R signaling are investigated in combination with anti-PD-1 or PD-L1 therapeutic antibodies in various types of human solid cancers.

According to published findings using the TH-MYCN murine model of high-risk human neuroblastoma, it is extremely difficult to achieve meaningful therapeutic effects due to the known short

Figure 5.
Combining CSF-1R inhibition with checkpoint blockade enabled superior tumor control. Once spontaneous tumors were palpable (day 0), mice received checkpoint-blocking antibodies for PD-1 and PD-L1 intraperitoneally at 12.5 mg/kg on days 0, 3, and 6. Alternatively, antibodies were injected concurrently with BLZ945, or the combination with BLZ945 were grouped and compared according to the tumor weight distribution. Activation of T cells in spleens and tumors was compared among control, checkpoint blockade, and the combinational treatment groups.

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According to published findings using the TH-MYCN murine model of high-risk human neuroblastoma, it is extremely difficult to achieve meaningful therapeutic effects due to the known short

valuable for targeting neuroblastoma-induced suppressive myeloid cells.

The detailed in vivo effects of CSF-1R inhibition are still unclear (48). In a number of murine tumor models, CSF-1R inhibition resulted in only moderate tumor control, despite potent depletion of suppressive myeloid cells in vivo (29, 31, 32, 34). In contrast, others have demonstrated remarkable therapeutic benefits from interrupting CSF-1R signaling, predominantly through repolarizing intratumoral myeloid cells (27–29, 33). In this study, treatment with the highly selective CSF-1R inhibitor BLZ945 demonstrated growth control of established spontaneous tumors in the TH-MYCN neuroblastoma murine model. In spleens of the treated mice, frequencies of suppressive myeloid cells were completely normalized when compared with naïve mice. However, intratumoral numbers of myeloid cells were not altered. This is in line with a previous study (27), where tumor-derived GM-CSF sustained myeloid cell survival in the tumor microenvironment.

In the clinic, antibodies blocking PD-1/PD-L1 interactions have achieved remarkable therapeutic benefits in patients with solid cancers (49, 50). We showed that checkpoint-blocking agents were potent in enhancing T-cell functions in vivo, but had minor impacts on the myeloid compartment. Thus, it could be one of the resistance mechanisms underlying the insufficient in vivo potency. Indeed, inhibition of CSF-1R synergized with checkpoint-blocking antibodies and elicited striking antitumor effects that were superior to either treatment. Recently, three independent early-stage clinical studies have been initiated, where a small-molecule inhibitor (NCT02452424) or monoclonal antibodies (NCT02526017 and NCT02323191) targeting CSF-1R signaling are investigated in combination with anti-PD-1 or PD-L1 therapeutic antibodies in various types of human solid cancers.

According to published findings using the TH-MYCN murine model of high-risk human neuroblastoma, it is extremely difficult to achieve meaningful therapeutic effects due to the known short
treatment window and rapid tumor progression once the tumors are established and palpable (7, 12). Novel therapeutic modalities of these animals developed by us and others (9, 10) resulted in prolonged survival, but in contrast to current findings, not a single animal was cured or even showed shrinkage of tumors. However, due to abdominally located tumors in the TH-MYCNC mice, it is challenging to determine an objective endpoint to monitor the longitudinal effects of the treatment. Facilitated by imaging tools, several well-established murine models of neuroblastoma should be used, in order to further explore the mechanistic insights and longitudinal impact of combination immunotherapy (51–54).

Taken together, we conclude that tumor-driven CSF-1R signalling regulates the induction of suppressive myeloid cells, which hampers antitumor effects of checkpoint inhibitors. Given that the therapeutic efficacy of PD-1 blockade and prognostic values of suppressive myeloid cells are being validated in clinical trials, our results support CSF-1R inhibition as a novel treatment option for immunotherapy in human cancers, including high-risk childhood neuroblastoma.

Disclosure of Potential Conflicts of Interest

P. Kogner is a consultant/advisory board member for Roche/Genentech. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: Y. Mao, N. Eissler, J.I. Johnsen, P. Kogner, R. Kiersling
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Mao, N. Eissler, P. Kogner, R. Kiersling
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Mao, N. Eissler, P. Kogner

References


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Clinical Cancer Research

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Yumeng Mao, Nina Eissler, Katarina Le Blanc, et al.


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