Block CSF-1R to improve checkpoint blockade in neuroblastoma

Targeting suppressive myeloid cells potentiates checkpoint inhibitors to control spontaneous neuroblastoma

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Translational Relevance

Tumor-driven induction of suppressive myeloid cells hampers sufficient anti-tumor immune responses. We show here that combining a highly selective CSF-1R inhibitor (BLZ945) with blocking antibodies against the PD-1/L1 axis provides striking tumor control in a transgenic murine model that resembles the aggressive growth pattern of high-risk human neuroblastoma. Further, we demonstrate the prognostic value of CSF-1R-expressing myeloid cells in neuroblastoma patients and reveal the mechanistic insight that the induction of suppressive myeloid cells is governed by CSF-1R signaling in humans and mice. Given that the therapeutic efficacy of PD-1 blockade and the prognostic values of suppressive myeloid cells are being validated in clinical trials of various human cancers, we believe that these findings have broad implications in designing novel combinational immunotherapies for cancer patients.

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 anti-tumor immune responses. Therefore, we sought to enhance anti-tumor 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 are 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
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monotherapy or in combination with checkpoint inhibitors, we employed 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 neuroblastoma patients. *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 limited tumor progression. While checkpoint inhibitors were 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.
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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 anti-tumor immunity has been proposed to be a promising option for treating high-risk childhood neuroblastoma (13, 14). However, tumor-induced inflammation limits efficient anti-tumor immune responses through recruitment of various suppressive immune cell types (15) including myeloid-derived suppressor cells (MDSCs) and ‘M2-biased’ tumor-associated macrophages (TAMs) (16-18). These cells demonstrate overlapping phenotypic markers as well as inhibitory mechanisms (19, 20) and predict poor clinical outcome in cancer patients (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 combinational approaches, blockade of CSF-1R signaling exhibits synergistic effects of various anti-cancer therapies (30-34).
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Here, we show that infiltration of CSF-1R+ myeloid cells predicts poor survival in neuroblastoma patients. Further, we reveal the mechanistic insight of the M-CSF/CSF-1R axis during the induction of suppressive MDSCs and TAMs in humans and mice. Finally, combining a highly selective CSF-1R inhibitor, BLZ945, with PD-1/PD-L1 blocking agents elicits robust anti-tumor effects against established aggressive tumors in the TH-MYCN murine neuroblastoma model.

Materials and Methods

Tumor cell lines

NHO2A murine neuroblastoma cell line was maintained in RPMI1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies), 1% antibiotics (penicillin, streptomycin) and 2 mmol/L L-glutamine (Life Technologies). Tumor-conditioned medium was harvested and used on the same day when the cells reached 90% confluence.

Human neuroblastoma cell lines, including SK-N-BE(2), SK-N-AS and SK-N-FI, were routinely maintained in IMDM medium (Life Technologies) supplemented with 10% heat-inactivated FBS. Cell lines were routinely tested for mycoplasma contamination (MycoAlert, Lonza) and authenticated by an STR identifier kit (Table S2, Applied Biosystems) For obtaining tumor-conditioned medium, cells were cultured in IMDM medium supplemented with 10% heat-inactivated, pooled human AB serum from 6 donors (referred as ‘culture medium’ below) and media were harvested and stored in aliquots at -80 ºC when cells reached 90% confluence.

Flow cytometry

Detailed information of fluorochrome-conjugated antibodies utilized in this study was summarized in Table S3. Briefly, 1-2×10^5 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 stainings, 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 was 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 129X1/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 day 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. Spleens and tumors were collected and homogenized, erythrocytes were lysed by BD Pharm Lyse™ Buffer (BD Biosciences) and immune cells were analyzed by flow cytometry. For tumors smaller than 0.5 grams, an additional staining for tumor antigen GD2 was performed in order to confirm the presence of neuroblastoma cells.

Isolation of primary human cells

To obtain human primary monocytes, peripheral blood mononuclear cells (PBMCs) 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
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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 (>3200 folds
more than other tyrosine kinases (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 at 2-to-1 ratios to the progenitor cells. CD34+ cells maintained in culture medium were
used as controls. To block CSF-1R signaling, BLZ945 (500 nM) 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 co-culture

Primary human monocytes were co-cultured with human neuroblastoma cell lines according to
our published protocol (35). In brief, monocytes were co-cultured with $4 \times 10^5$ SK-N-BE(2), SK-
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N-AS, or $6 \times 10^5$ 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 nM BLZ945 (Novartis) or 1 µM GW2580 (Selleck Chemicals) was added to monocytes or the co-cultures.

Differentiation of murine bone marrow cells

Suppressive myeloid cells were induced from bone marrow cells harvested from negatively genotyped TH-MYCN mice, in accordance to a previously described protocol (36). In brief, $1 \times 10^6$ isolated bone marrow cells were cultured in 6-well plates in presence of NHO2A tumor-conditioned medium (1:1 dilution to fresh medium). As controls, cells were cultured in fresh medium or with M-CSF (20 ng/ml, Biolegend). To block CSF-1R signaling, BLZ945 or GW2580 were added at 1 µM 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 µM 5(6)-CFDA/CFSE tracing dye (Invitrogen). For the tumor-educated monocytes, $1 \times 10^5$ 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 mix 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
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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, 1x10⁶ CFSE-labeled splenocytes from naive mice were pre-incubated 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, pharmacological inhibitors for IDO (1-DL-MT, 250 μM, Sigma-Aldrich), JAK/STATs (AG490, 10 μM, Sigma-Aldrich), arginase (nor-NOHA, 200 IU/ml, Calbiochem), or iNOS (1400W, 200 μM, 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 tumor-conditioned medium 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, 3 datasets including Versteeg (cohort-1, n=88), Hiyama (cohort-2, n=51) and Lastowska (cohort-3, n=30) were compared with the Miller neurofibroma dataset (n=86) using one-way ANOVA. For survival of patients, Kaplan-Meier analysis were performed.
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on 2 previously reported datasets of patients with untreated neuroblastoma tumor biopsies including Versteeg (cohort-1, n=88) (37) and Seeger (cohort-4, n=102) (5) and the cut-off expression levels were calculated within the R2 database. In addition, a larger dataset Kocak (cohort-5, n=649) (38) was included to study the correlations between expression of CSF-1R and other markers.

Statistics

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 non-parametric, 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 neuroblastoma patients

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 neurofibroma, neuroblastoma from 3 patient cohorts (37, 39) ($P$ values are summarized in Table S1) all showed significantly increased expression of $CD14$ and $CD68$ monocyte/macrophage markers (Figure 1A). In contrast, granulocytes ($CD66b$), regulatory T cells ($FoxP3$) or dendritic cell markers $CD11c$ and $CD83$ remained unchanged (Figure S1A). Moreover, CSF-1R showed enhanced expression in tumor tissues (Figure 1B) and correlated strongly with expression levels of $CD14$ and $CD68$ (R=0.801 and 0.752, respectively, Figure 1C). In accordance, myeloid-differentiation factor $M-CSF$ (Figure 1D), but not $GM-CSF$, $G-CSF$
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or another CSF-1R ligand IL-34 (Figure 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), since myeloid cells are sensitive to treatment e.g. by chemotherapy (40).

Expression levels of M-CSF (Figure 1E), CSF-1R (Figure 1F), as well as CD14 or CD68 (data not shown), strongly associated with poor overall and relapse-free survival in neuroblastoma patients. Moreover, expression of NK cell marker CD56 (Figure S1C) predicted better survival in high-risk neuroblastoma patients (age>18 months) but presence of CD4 T cells was associated with poor prognosis (Figure 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 cancer patients, 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 (Figure 2A) and were not capable of stimulating allogeneic T cells (Figure S2A). In contrast, addition of GM-CSF and TNFα promoted rapid proliferation and myelopoiesis of CD34+ progenitor cells, resulting in clusters of non-adherent cells and subsets of adherent cells, which contained DCs (CD14negCD11c+CD1a+CD86+), macrophages (CD11b+CD68+) and monocytes/macrophages (CD14+c-kitneg) (Figure 2A and gating strategy in Figure S2B). As a result, frequencies of c-kit+CD14neg immature cells diminished (Figure 2B) and these cells enabled T cells to proliferate and produce IFNγ (Figure 2C). However, when progenitor cells were exposed to neuroblastoma-derived factors, their maturation was significantly hampered. Frequencies of DCs, monocytes and
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macrophages (Figure 2A), and the overall activation of myeloid cells (Figure S2C) were inhibited. Functionally, these cells demonstrated impaired ability to stimulate T cells (Figure 2C and 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 (Figure S3A and B) 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 CD34+ cells by a highly selective inhibitor (BLZ945) specifically limited the differentiation of macrophages and monocytes (Figure 2D and Figure S3C) that had high expression of CSF-1R (Figure 2E). However, it had no impact on dendritic cells (Figure 2D), because of their lower expression of CSF-1R (Figure S2E). Functionally, BLZ945 significantly recovered the potential of myeloid cells to stimulate T cells (Figure 2F and G), with comparable effects on CD8+ and CD4+ subsets (Figure S3D and E). Notably, BLZ945 decreased monocytes and macrophages in cultures with only cytokines (Figure 2D), but this did not lead to consistent changes of their T cell activating capacities (Figure 2F).

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

Next, we co-cultured primary monocytes freshly isolated from healthy individuals with 3 human neuroblastoma cell lines, according to our previously published protocol (35). This co-culture model allowed us to dissect the impact of tumor-derived factors on myeloid cells that are recruited into the neuroblastoma tumor microenvironment.

Presence of neuroblastoma cells up-regulated the expression of CD14 (Figure 3A) and CSF-1R (Figure 3B), but impaired the expression of HLA-DR and CD86 on primary human monocytes (Figure 3A). However, different tumor cell lines demonstrated distinct abilities to regulate the expression of CD206 (Figure 3A) and PD-L1 (Figure S4A) on monocytes. Importantly, CSF-1R
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inhibition did not influence growth of neuroblastoma tumor cell lines due to their lack of CSF-1R expression (data not shown).

When CSF-1R was blocked, expression of CD14 and CD206 (Figure 3C) on tumor-educated monocytes were reduced, but expression of HLA-DR and CD86 (Figure 3C) was partially recovered. However, CSF-1R inhibition did not significantly alter the expression of PD-L1 (Figure S4B) or CSF-1R (Figure S4C) on tumor-educated monocytes.

Notably, direct CSF-1R inhibition led to phenotypic changes on primary monocytes (Figure 3D) and resulted in a 3-fold increase of CSF-1R expression (Figure 3D). Even though reported in a previous study (33), CSF-1R inhibitors did not modulate expression of PD-L1 on tumor cells in our co-culture system (Figure 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, Figure 3E and representative histograms based on average values of the 7 independent experiments in Figure 3F; Figure S4E and F). In contrast, treatment of healthy monocytes with BLZ945 did not show changes in their functions towards T cell proliferation (Figure S4G).

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

To confirm also in mice that M-CSF/CSF-1R interaction is involved in the development of suppressive myeloid cells, we established an *in vitro* model based on a previous study (36). Naïve bone marrow cells were differentiated in the presence of NHO2A neuroblastoma-conditioned medium (TCM) or recombinant murine M-CSF. Similar to the results using primary human myeloid cells, TCM and M-CSF induced expansion of MDSCs and TAMs (Figure S5A) and the resulted cells inhibited proliferation of autologous T cells (Figure S5B). Blocking CSF-1R
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signaling efficiently abolished the induction of CSF-1R-expressing suppressive myeloid cells (Figure S5C and D) and recovered functions of T cells (Figure 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 Figure 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 grams at 10 days after tumor detection (Figure 4A). Tumor-bearing animals demonstrated significantly higher levels of MDSCs of the granulocytic (p<0.01, grMDSCs, Ly6G+Ly6Clow) and monocytic (p<0.0001, moMDSCs, Ly6GnegLy6Chigh) lineages, as well as F4/80+ macrophages (p<0.01), in comparison to tumor-free mice (Figure 4B) and these myeloid cells demonstrated high expression of CSF-1R (Figure S6A). Functionally, splenic Gr1+ cells sorted from tumor-bearing mice potently inhibited proliferation of T cells in a ratio-dependent manner (Figure S6B and C). This suppression could be rescued by blocking enzymatic activity of iNOS and IDO (Figure S6D). However, frequencies of Tregs did not change significantly (Figure S5E), although lower CD4+ T cell frequencies were observed (Figure S6F).

As a monotherapy, BLZ945 treatment significantly controlled progression of established tumors (p<0.01, Figure 4A). This could be explained by the selective effects on CSF-1R-expressing myeloid cells, because numbers of CD11b+ cells (p<0.01, Figure S7B), including grMDSCs (p<0.01), moMDSCs (p<0.001) and macrophages (p<0.0001), were significantly reduced in spleens of the treated mice (Figure 4B). Further, BLZ945 treatment strongly limited the expression of CSF-1R (p<0.01) and PD-L1 (p<0.01) on macrophages and MDSCs (Figure 4C and S7C).
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In addition, treatment with BLZ945 led to a significant recovery of CD4+ (p<0.05) and CD8+ (p<0.01) T cells in spleens (Figure 4D). Activation of CD4+ and CD8+ T cells were improved (Figure 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 to the controls (Figure S7D).

In tumors of BLZ945-treated mice, neither did we observe changes in CD11b+ cell numbers (Figure S7E), nor the frequencies of grMDSCs, moMDSCs and macrophages (Figure S7F). However, lower numbers of infiltrating CD4+ T cells but increased CD8+ T cells were observed (Figure 4F), leading to decreased CD4/CD8 ratios (Figure S7G). Moreover, tumor-infiltrating CD8+ (Figure 5G) and CD4+ T cells (Figure 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 demonstrated elevated PD-1 expression after CSF-1R inhibition, we asked whether checkpoint blockade could further improve the therapeutic effects of BLZ945 treatment. Given that PD-1 has multiple ligands (41) and PD-L1 could inhibit functions of PD-1-deficient T cells (42), we treated tumor-bearing mice with blocking antibodies against both PD-1 and PD-L1 to maximize the blockade of this pathway, as monotherapy or combined with BLZ945 (Figure S8A). This therapeutic scheme was motivated by the observation that both PD-L1 and PD-L2 were positively correlated with CSF-1R expression in human neuroblastoma (R=0.542 and 0.512, respectively, Figure S8B).

To our surprise, antagonizing the PD-1/PD-L1 axis (Figure 5A) or the isotype controls (data not shown) did not delay progression of established spontaneous tumors in the TH-MYCN mice. In contrast, addition of BLZ945 to the checkpoint blocking agents resulted in potent synergistic anti-tumor effects (p<0.05, Figure 5A). In 6 out of 8 (75%) treated mice (Figure 5B), we
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observed complete tumor regression or small tumors (<0.5g). Strikingly, the combination prevented the progressive growth of large tumors (>1.0g) (Figure 5B).

In comparison to checkpoint blockade, the combination efficiently normalized the numbers of CD11b+ cells (p<0.05, Figure S8C), including macrophages and grMDSCs (p<0.05, Figure 5C) and all treatments could diminish moMDSCs (Figure 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 (Figure 5D). Although intratumoral frequencies of CD11b+ cells (Figure S8D) or macrophages (Figure S8E) were not significantly modulated, we observed similar effects on macrophages in the tumor tissues (Figure 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 (Figure S8E).

When checkpoint blocking agents were administered, we observed enhanced infiltration (Figure S7G) and activation (Figure 5F) of T cells in the tumor tissues. It was more pronounced on CD4+ T cells, leading to increased CD4/CD8 ratios (Figure S8F and G). Removal of suppressive myeloid cells by CSF-1R inhibition did not further elevate T cell numbers in spleens (Figure S8F) or tumors (Figure S8G), but boosted activation of T cells (Figure 5F).

Discussion

Stimulated by the finding that CSF-1R+ myeloid cells in tumors of neuroblastoma patients 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
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inhibitor BLZ945 elicits strong anti-tumor 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 IL-6 (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 IL-6 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 co-culture 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 dendritic cells after in vitro differentiation of CD34+ progenitor cells. This indicated that other tumor-derived factors, such as IL-10 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...
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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 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 to naive 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 anti-tumor 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...
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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 one single animal was cured or even showed shrinkage of tumors. However, due to the abdominally-located tumors in the TH-MYCN 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 employed, in order to further explore the mechanistic insights and longitudinal impact of the combination immunotherapy (51-54).

Taken together, we conclude that tumor-driven CSF-1R signaling regulates the induction of suppressive myeloid cells, which hampers anti-tumor effects of checkpoint inhibitors. Given that the therapeutic efficacy of PD-1 blockade and the 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.
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References

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**Figure Legends**

**Figure 1, Infiltrating CSF-1R+ myeloid cells predicted poor survival in neuroblastoma patients.** Genomic information was analyzed by searching the R2 database and intratumoral expression of A) CD14 or CD68 myeloid markers, B) CSF-1R or D) M-CSF were compared between 3 neuroblastoma patient cohorts (cohort-1, Versteeg, n=88, cohort-2, Hiyama, n=51 and cohort-3, Lastowska, n=30) and benign neurofibroma patients (Miller, n=86) using one-way ANOVA (see statistical summary in 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 E) intratumoral expression of M-CSF and F) CSF-1R, two patient datasets containing untreated tumor biopsies (cohort-1 and cohort-4) were analyzed by Kaplan-Meier estimates.

**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 tumor-conditioned medium harvested from 3 human neuroblastoma cell lines (SK-N-BE(2), SK-N-AS and SK-N-FI). A) Frequencies of dendritic cells (CD14negCD1a+CD11c+CD86+), monocytes/macrophages (CD14+c-kitneg), macrophages (CD11b+CD68+) or B) immature cells (c-kit+CD14neg) were evaluated by flow cytometry. C) The ability of myeloid cells matured in different conditions to stimulate proliferation and IFN-γ release from allogeneic T cells was tested in mixed lymphocyte reactions. The effects of CSF-1R inhibitor BLZ945 on the D) maturation of CD34+ progenitor cells to different myeloid subsets and E) expression of CSF-1R on monocytes or macrophages 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
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average values. *, p<0.05; **, p<0.01; non-parametric Mann-Whitney U test. Each dot represented an individual experiment.

**Figure 3**, CSF-1R blockade reverted suppressive functions on tumor-educated human monocytes. Using the monocyte-neuroblastoma co-culture model, we investigated the changes of A) phenotypic markers and B) expression of CSF-1R on primary human monocytes induced by different neuroblastoma cell lines. Next, we evaluated C) the effects of CSF-1R inhibitor BLZ945 (500 nM) or GW2580 (1 μM) 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; non-parametric Mann-Whitney U test. Each dot represented an individual experiment.

**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 C) CSF-1R or PD-L1 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 CD69 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; non-parametric Mann-Whitney U test. Each dot represented an individual mouse.
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 day 0, 3 and 6. Alternatively, antibodies were injected concurrently with the BLZ945 oral treatment. After 10 days, A) tumors were harvested and tumor weights were compared. B) Control mice, mice treated with checkpoint blockade or the combination with BLZ945 were grouped and compared according to the tumor weight distribution. C) Frequencies of macrophages, grMDSCs or moMDSCs, or D) the expression of CSF-1R or PD-L1 on macrophages in the spleens or E) in tumors were compared among different treatment groups. F) Activation of T cells in spleens and tumors was compared among control, checkpoint blockade and the combinational treatment groups. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not significant; non-parametric Mann-Whitney U test.

Each dot represented an individual mouse.
Figure 1. Infiltrating CSF-1R+ myeloid cells predicted poor survival in neuroblastoma patients.

A. CD14, CD68

B. CSF-1R

C. CSF-1R vs CD14 (cohort-5)

D. M-CSF

E. M-CSF (cohort-4)

F. CSF-1R (cohort-4)

M-CSF (cohort-1)

CSF-1R (cohort-1)

High (28)
Low (60)

P value 0.032

Overall survival

Time (months)

High (28)
Low (60)

P value 0.032

Overall survival

Time (months)

High (25)
Low (77)

P value 3.9e-04

Relapse-free survival

Time (months)

High (55)
Low (47)

P value 3.3e-03

Relapse-free survival

Time (months)

High (22)
Low (66)

P value 0.034

Relapse-free survival

Time (months)
Figure 2. Myelopoiesis of human CD34+ progenitor cells was modulated by tumor-derived factors.
Figure 3. CSF-1R blockade reverted suppressive functions on tumor-educated human monocytes

A

CD14

HLA-DR

CD86

CD206

B

CSF-1R

C

CD14

HLA-DR

CD86

CD206

D

Monocytes+BLZ945

E

CD3+ T cells

F

T alone

T+Beads

Control Mono

Tumor-educated

BLZ945 (500 nM)

Cell Counts

n = 7

% of proliferated cells

T cell only

T cell: Mono
Figure 4. BLZ945 elicited therapeutic effects against established tumors through modulating suppressive myeloid cells

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor Weights (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl (n=18)</td>
<td>2.5</td>
</tr>
<tr>
<td>BLZ945 (n=11)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Figure B**

- grMDSCs
- moMDSCs
- Macrophages

**Figure C**

- CSF-1R
- PD-L1

**Figure D**

T cell numbers (Spleen)

- CD4+ T cells
- CD8+ T cells

**Figure E**

T cell activations (Spleen)

- CD4+ T cells
- CD8+ T cells

**Figure F**

Tumor

- CD4+ T cells
- CD8+ T cells

**Figure G**

CD8 activation (Tumor)

- PD1+ T cells
- CD69+ T cells
Figure 5. Combining CSF-R inhibition with checkpoint blockade enabled superior tumor control.

A. Tumor Weights (g) (n = 18) (n = 8) (n = 8)

B. Control αPD-1+αPD-L1

BLZ945+αPD1+αPD-L1

Total=18

Total=8

C. Macrophages grMDSCs moMDSCs

D. CSF-1R PD-L1 MHC II (I-A/I-E)

E. CSF-1R on TAMs (Tumor)

F. Spleen

T cell activation (Spleen)

T cell activation (Tumor)
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Targeting suppressive myeloid cells potentiates checkpoint inhibitors to control spontaneous neuroblastoma

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