The multidrug-resistance transporter Abcc3 protects NK cells from chemotherapy in a murine model of malignant glioma

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ABSTRACT
Abcc3, a member of the ATP-binding cassette transporter superfamily, plays a role in multidrug resistance. Here, we found that Abcc3 is highly expressed in blood-derived NK cells but not in CD8+ T cells. In GL261 glioblastoma-bearing mice treated with the alkylating agent temozolomide (TMZ) for 5 d, an early increased frequency of NK cells was observed. We also found that Abcc3 is strongly upregulated and functionally active in NK cells from mice treated with TMZ compared to controls. We demonstrate that Abcc3 is critical for NK cell survival during TMZ administration; more importantly, Akt, involved in lymphocyte survival, is phosphorylated only in NK cells expressing Abcc3. The resistance of NK cells to chemotherapy was accompanied by increased migration and homing in the brain at early time points. Cytotoxicity, evaluated by IFNγ production and specific lytic activity against GL261 cells, increased peripherally in the later phases, after conclusion of TMZ treatment. Intra-tumor increase of the NK effector subset as well as in IFNγ, granzymes and perforin-1 expression, were found early and persisted over time, correlating with a profound modulation on glioma microenvironment induced by TMZ. Our findings reveal an important involvement of Abcc3 in NK cell resistance to chemotherapy and have important clinical implications for patients treated with chemoinmunotherapy.

Introduction
Current therapeutic options for glioblastoma (GBM) patients include chemotherapy with the alkylating agent TMZ.1,2 Recent data have suggested that some chemotherapeutic agents, previously viewed as immunosuppressive, possess immune-modulatory effects3,4 and influence the vaccine-induced immune response affecting the quality and efficacy of the T cell response5 or enhancing the immunogenicity of dying tumor cells.3,4 TMZ leads to transient lymphodepletion6 and may interfere with regulatory T cell (Treg) trafficking to the tumor,7 thereby creating a “time-window” for improved efficacy of vaccinations; moreover, dendritic cell (DC) immunotherapy may increase TMZ sensitivity.8 Preclinical evidence has implicated the inhibition of glioma growth by NK cells9,10 and recently, we reported a significant, positive correlation of NK cell response and survival of patients affected by recurrent GBM treated with DCs loaded with autologous tumor lysates.11 Treg depletion by TMZ could relieve the suppression of NK cells restoring the innate antitumor response.12 Previous attempts have been made to decipher the mechanisms through which NK cells are more radio- and chemotherapy resistant than other lymphoid cells. It has been observed that NK cells express high levels of P-glycoprotein 1 (P-gp1), a transmembrane transporter encoded by the multidrug-resistance 1 (MDR1) gene, as well as MRPI (Abcc1) and MRP2 (Abcc2).13 Discrepancies were found in terms of the expression and function in T cells of multidrug-resistance proteins, specifically P-gp1 and Abcc114,15. In a clinical trial currently active at our institution (DENDR1 - EUDRACT No. 2008-005035-15), 24 patients with first diagnosis of GBM have been treated with DCs loaded with autologous tumor lysate together with standard radiotherapy and chemotherapy with TMZ. Peripheral blood lymphocytes (PBLs) from patients were analyzed by flow cytometry for immunotherapy follow-up. Their ratio of vaccine/baseline frequencies (V/B ratio) was correlated with the progression-free survival (PFS) of each patient. The increased V/B ratio of NK cells but not CD8+ T cells was significantly associated with prolonged PFS (Pellegatta et al., manuscript in preparation).

To investigate the specific contribution of TMZ-based chemotherapy to differential responses of NK and T cells, we used the GL261 pre-clinical model of glioma.

We found that blood-derived NK cells (but not CD8+ T cells) are resistant to and activated by TMZ. Multidrug resistance is primarily associated with Abcc3 expression (a member of the MRP family), which was upregulated and functionally active in NK cells during TMZ treatment. Furthermore, NK cells displayed migratory and cytotoxic activities that were positively influenced by TMZ.
Results

Local and systemic NK cell frequency is positively influenced by TMZ

Nine days after intracranial implantation of GL261 gliomas, immune competent glioma-bearing mice were treated with intraperitoneal injections (i.p.) of 5 mg/kg TMZ or DMSO for 5 d (Fig. 1A). To characterize the effect of TMZ on the immune system, PBLs and tumor-infiltrating lymphocytes (TILs) were harvested at different time points, and immune cell populations quantified using flow cytometry. TMZ induced rapid and reversible lymphopenia: CD8\(^+\) T cells decreased significantly at 48 h, after two administrations of chemotherapy \((p < 0.0001\) vs. controls) and quickly increased at 72 h \((p < 0.01\) vs. 48h; Fig. 1B). On the contrary, peripheral blood NK cells increased significantly at early time point, doubled 72 h after the first TMZ administration and remained higher than controls throughout the entire treatment (Fig. 1C). To assess a possible delayed effect of TMZ on immune cells, we performed similar evaluations at day 19, 5 d after ending chemotherapy. We did not observe a significant difference between CD8\(^+\) T cells in the blood of TMZ-treated mice compared to controls (Fig. 1B) while NK cells were still increased in blood of TMZ-compared to vehicle-treated mice (Fig. 1C). In non-glioma-bearing mice, TMZ induced a modulation of CD8\(^+\) T lymphocytes and NK cells similar to TMZ-treated tumor bearing mice (Fig. S1).

Tumor-infiltrating immune cells were isolated from fresh gliomas by Percoll gradient and quantified by flow cytometry as NK1.1\(^+\)CD3\(^-\) and CD8\(^+\) CD3\(^-\); at 48 h CD8\(^+\) T cells decreased in TMZ-treated mice compared with controls (Fig. 1D); at 240 h no differences were found between TMZ-treated mice and controls \((p = 0.3\); Fig. 1D). NK cells in the tumor showed a similar pattern of systemic NK cells, increasing significantly at 72 h after the first TMZ administration and continued to be significantly elevated at 240 h \((p < 0.005\) at both time points, Fig. 1E).

Evidence of a vigorous immune cell infiltration in response to TMZ compared with the vehicle at 72 h was confirmed by hematoxilin and eosin staining. In situ immunofluorescence...
confirmed the predominant infiltration of NK cells located into the tumor mass (Fig. 1F). Quantitative determination of TILs from the same groups of treatment was obtained by flow cytometry (Fig. 1G).

In another set of experiments, immune cells from spleen, cervical lymph nodes and bone marrow were analyzed: the results did not indicate a significant influence of TMZ on lymphocytes in these organs (not shown).

These results show that trafficking and NK cell homing to the tumor are positively influenced by TMZ administration.

**Expression of genes involved in drug resistance and chemotaxis is upregulated in NK cells from TMZ-treated mice**

To further characterize the molecular effects of TMZ, we compared gene expression profiles of NK cells obtained by magnetic sorting of PBLs from TMZ- and vehicle-treated glioma-bearing mice (n = 50/group) 72 h after treatment onset. We used the GeneChip Mouse Gene 2.0 ST Array and identified differentially expressed genes (DEGs) using a ≥2-fold-change (FC) threshold for transcript comparisons. A robust difference was observed between the transcriptome levels of the two NK cell groups (Table S1) and 211 DEGs passed the FC cut-off.

Based on Gene Ontology annotations, the transcripts were grouped because of their involvement in multidrug resistance, anti-apoptosis and migration. We focused the validation experiments on genes indicating the relationship of NK cells with drug-resistance. In particular, three upregulated genes were related to ABC drug transporters: Abcc3, Abca9 and Abca6. Other genes were related to the inhibition of apoptosis (CDSL and Naip1) and cell survival (Ednrh, Gatad6 and Fgfr1), an indication of the predisposition of NK cells to resist the cytotoxic effect of TMZ.

In addition, data from genes regulating cytoskeleton organization, microtubule-based movement, actin polymerization and chemotaxis (Ccr1, Efnb2, Alox15, Lbp and Lrp1) supported the idea that NK cells from TMZ-treated mice migrated more than NK cells from controls. Notably, at this time point, downregulated genes were related to NK cell-mediated cytotoxicity (GzmD, GzmE, GzmG and GmzC) and to secretory pathway or inflammatory response (Sggb1a1 and Elane).

Gene expression profiling was also performed on CD8\(^+\) T cells purified from the same mice. No significant differences could be observed between CD8\(^+\) T cells sorted from TMZ-treated mice and those of controls (Table S1).

Overall, these findings suggest that TMZ influences the activity of NK cells by activating pathways relevant in the acquisition of chemo-resistance.

**NK cells respond to chemotherapy by over-expressing Abcc3**

The validation of ABC transporter over-expression was performed by real time PCR. The analysis revealed that two of the three ABC transporters, Abcc3 and Abca6, were significantly upregulated in peripheral NK cells from TMZ-treated mice compared to controls (3.46 ± 0.01-fold; p < 0.0001, and 2.75 ± 0.045-fold; p < 0.001, respectively; Fig. 2A–B).

To investigate *in vitro* the expression of Abca6 and Abcc3 during TMZ administration, we treated PBLs from naïve mice with 1 \(\mu\)M TMZ or DMSO at different time points. The dosage was determined according to TMZ concentrations measured in the plasma of patients treated with “standard” schedule. The upregulation of Abca6 expression was observed only after 6 and 8 h of TMZ treatment (2.0 ± 0.1-fold and 3.3 ± 0.1-fold, respectively vs. DMSO-treated PBLs; p = 0.01, p < 0.005; Fig. 2C). On the contrary, the upregulation of Abcc3 expression was detectable after 4 h (p < 0.05) and increased over the time during TMZ treatment, suggesting a direct effect of chemotherapeutic and its expression (Fig. 2D).

The remarkable increase of Abcc3 expression on the surface of NK cells was confirmed *in vivo*. At flow cytometry, NK cells (but not CD8\(^+\) T cells) displayed a high basal expression of Abcc3 (31.2 ± 0.8% Abcc3\(^+\)NK cells vs. 2.0 ± 0.6% Abcc3\(^+\)CD8\(^+\) T cells; p < 0.00001). Moreover, NK cells from TMZ-treated mice exhibited a significant upregulation of Abcc3 compared to controls during chemotherapy (31.2 ± 0.8% vehicle NK cells vs. 59.8 ± 1.1% TMZ-treated NK cells, p < 0.0001; Fig. 2E, left). No significant difference in Abcc3 expression was found in CD8\(^+\) T cells from TMZ-treated mice compared to controls. (2.0 ± 0.6% vehicles vs. 1.5 ± 0.5% TMZ-treated mice, Fig. 2E, right).

These results show that Abcc3 is differentially expressed in NK cells compared to CD8\(^+\) T cells and is increased in NK cells from TMZ-treated glioma-bearing mice compared with controls.

**Abcc3 expressed in NK cells is functionally active**

To investigate whether Abcc3 expression is related to a greater ABC transporter activity and a drug-resistant phenotype, we used a flow cytometry assay to measure the efflux activity of the three clinically most important ABC transporter families involved in cancer multidrug resistance. The assay is based on determining the fluorescence intensities of cells after a short incubation with a fluorescent substrate in the presence or absence (control) of specific ABC transporter inhibitors. Inhibition of active ABC transporters results in increased fluorescence intensity due to the accumulation of the substrate. PBLs from naïve mice were treated *in vitro* with 1 \(\mu\)M TMZ or DMSO for 4 h.

NK and CD8\(^+\) T cells were gated on PBLs and a multidrug-resistance activity factor (MAF) was calculated. Cells exhibiting drug resistance have increased fluorescence and a MAF greater than 25%. TMZ-NK cells showed greater fluorescence in the presence of a multidrug-resistance protein inhibitor for MRP, of which Abcc3 is a key member resulting in MAF = 73.4% (Fig. 2G).

TMZ-treated NK cells were also tested for the MDR and BCRP inhibitors included in the assay, confirming a high efflux activity. MRP exhibited the strongest efflux activity (p < 0.001; Fig. 2G). Results obtained with a lower dose of TMZ suggested that the efflux activity was dose-dependent in NK cells (Fig. S2). No evidence of a resistant phenotype was found in TMZ-treated CD8\(^+\) T cells, showing a MAF < 25% for all ABC transporter families (Fig. 2H).
These findings highlight the rapid activation of ABC multidrug-resistance transporters in NK cells but not in CD8$^+$ T lymphocytes during TMZ treatment, supporting the NK cell ability to react to the cytotoxic effects of chemotherapy.

**Abcc3 is critical for survival and expansion of NK cells during TMZ administration**

To determine whether Abcc3 is required for NK cell survival, we treated PBLs from naïve mice with 1 μM TMZ or DMSO for 2, 6 and 15 h. Apoptotic cells were measured by flow cytometry on gated NK and CD8$^+$ T cell populations using Annexin V and Propidium Iodide (PI) staining (Fig. 3A–C).

Cells in early apoptosis (EA) were Annexin V positive and PI negative, cells in late apoptosis (LA) or dead were Annexin V and PI positive. NK cells showed a low percentage of early apoptotic cells that slightly increased after 6 and 15 h ($p < 0.01$; Fig. 3A). Abcc3 negative NK cells showed a higher percentage of apoptotic cells compared to Abcc3 positive NK cells in response to TMZ (Fig. 3B). CD8$^+$ T cells showed a remarkable increase of early and LA after 2 h and later ($p < 0.005$, $p < 0.001$; Fig. 3C).

Because the pathways involving Akt activation could promote lymphocyte survival, 25,26 we investigated Akt activation by analyzing phosphorylation in PBLs in response to chemotherapy. NK cells and CD8$^+$ T cells were purified from naïve mice and treated with 1 μM TMZ or DMSO in vitro at two different time points. Western blots showed a
basal phosphorylation of Akt in NK cells with a time-dependent increase of pAkt with TMZ treatment (Fig. 3D). On the contrary, in CD8+ T cells, no significant difference in Akt activation was detected in TMZ-treated cells (Fig. 3E). We confirmed these results by analyzing Akt activation in NK and CD8+ T cells from glioma-bearing mice treated with TMZ or vehicle. We sacrificed mice after the third TMZ (or DMSO) administration every 5 min for 30 min, and Akt activation in PBLs was measured by a flow cytometry phospho-specific staining (Miltenyi Biotec). Akt phosphorylation was only detected in NK cells from TMZ-treated mice expressing Abcc3 (p < 0.01 vs. vehicle-treated mice; Fig. 3E). Abcc3+CD8+ T cells from TMZ-treated and control mice did not exhibit Akt phosphorylation (2.9 ± 1.2% TMZ-treated vs. 3.3 ± 0.9% control mice; Fig. 3E).

Inactivation of Abcc3 function by its specific inhibitor MK571 induced a significant increase of NK cell apoptosis in PBLs treated with 1 μM TMZ for 4h (Fig. 3F).

Together, these results confirm the important role of Abcc3 in survival and response to cytotoxic effects of chemotherapy.

**NK cell migration and maturation are positively influenced by TMZ**

To validate the signature related to migration and chemotaxis in NK cells from TMZ-treated glioma-bearing mice, we measured NK cell migration using the transwell system. NK cells purified from PBLs of TMZ-treated glioma-bearing mice and controls were evaluated for their ability to migrate toward conditioned medium derived from GL261 cells treated with 150 μM TMZ or DMSO for 24 h. We observed a 1.8-fold increase in the migration of NK cells from TMZ-treated mice toward the supernatant from TMZ-stimulated GL261 compared to the vehicle supernatant (p < 0.005). Migration of NK cells from treated mice increased by 2.3- and 30-fold compared with NK cells from control mice (p < 0.0001, Fig. 4A).

We then analyzed the expression of CD49b and CD49d integrin subunits involved in cellular adhesion and leukocyte tissue infiltration. CD49b+CD49d+ double positive NK cells increased in blood of TMZ-treated mice compared to controls (57.1 ± 3.4% vs. 39.7 ± 2.2%, respectively, p <0.01), supporting their greater homing ability into tumors (Fig. 4B).
We also characterized the effect of TMZ on the maturation status of NK cells by evaluating the surface density of CD11b and CD27, two markers associated with the four-stage developmental program in mice and humans. In blood, immature CD11blowCD27low NK cells significantly decreased after 3 d of chemotherapy (p < 0.001 vs. controls). Interestingly, TMZ led to a significant enrichment of the CD11b lowCD27high and CD11b highCD27high NK cell subsets with migratory potential and a simultaneous decrease of the CD11b highCD27low cytotoxic subset (p < 0.001 vs. controls; Fig. 4C). The increase of the CD11b highCD27low cytotoxic subset was observed 5 d after the end of TMZ administration (p = 0.001; Fig. 4D).

On the contrary, in gliomas of TMZ-treated mice there was a strong accumulation of the CD11b highCD27 lowNK effector subset 72 h after the first TMZ administration (p < 0.005, Fig. 4E). This increase persisted at later time points (p < 0.005, Fig. 4F).

These data showed a direct effect of TMZ on the progressive maturation of NK cells, with consequent influence on their migratory and cytotoxic phenotype in blood and gliomas.

Local and systemic NK cell cytotoxicity is triggered by TMZ

We further aimed to verify the cytotoxic ability of NK cells during and after completion of TMZ treatment.

The significant accumulation of the effector subset in blood of TMZ-treated mice suggested an activation of systemic cytotoxicity against tumors. In parallel, IFNγ production in blood-derived NK cells from TMZ-treated mice increased compared to controls (7.5 ± 1.0% vs. 16.9 ± 1.2%; p < 0.005; Fig. 5A).

To test the cytotoxic specificity of NK cells, PBLs from naive mice, and TMZ- or vehicle-treated glioma-bearing mice were stimulated with autologous irradiated tumor cells. NK cells from TMZ-treated mice, purified by magnetic sorting, exhibited a greater lytic activity against GL261 cells than NK from vehicle or naïve mice (p < 0.0001, Fig. 5B).

The accumulation of the NK cytotoxic subset in gliomas was associated to increased expression of Perforin 1 (Prf1), Granzyme B (GzmB) and IFNγ (by 9.3 ± 0.04-fold, 8.1 ± 0.02-fold and 4.1 ± 0.03-fold, respectively; p < 0.0001) in TMZ-treated mice (Fig. 5C). This upregulation persisted on day 19, 5 d after the end of TMZ treatment (Prf1: 3.97 ± 0.07-fold, GzmB: 2.2 ± 0.06-fold and IFNγ: 2.4 ± 0.02-fold, p < 0.0001 vs. controls; Fig. 5D).

Thus, TMZ is able to modulate NK cell function increasing their effector activity.

Glioma microenvironment is converted by TMZ into a site permissive for an efficient effector immune response

To investigate whether the glioma microenvironment could be modulated by TMZ, we looked for expression levels of galectin-1, −3 and −9, that suppress NK immune surveillance.
found that they were expressed at high levels in tumors of vehicle-treated mice and significantly less in those of TMZ-treated mice (Fig. 6A).

A decrease in the expression of H2-Q1 (Hla-e), a non-classical Major Histocompatibility Complex class I (Mhc I) molecule normally implicated in immune escape mechanism and inhibition of NK cell-mediated lysis, was also found in gliomas from TMZ-treated mice (Fig. 6B). We also investigated the effects of TMZ administration on intratumor expression of chemokines and cytokines involved in modulating the infiltration of immune cells: CCL3 (whose expression is related to NK cell accumulation\(^\text{10}\)), TNF-\(\alpha\) (which is not only involved in antitumor immune response but also responsible for decreased GL261 proliferation\(^\text{38}\)), IL-7 (which plays a role in promoting NK cell survival and inhibiting apoptosis\(^\text{34}\)), and IL-27 (which is an important stimulator of NK cell effector function\(^\text{35}\)), all increased in gliomas from TMZ-treated mice compared to controls (Fig. 6C). In contrast CXCL10, which role in glioma progression is contradictory,\(^\text{36-38}\) and CCL5, which expression was recently most related with CD8A levels,\(^\text{39}\) and previously described as immunosuppressor by us and others,\(^\text{10,40}\) significantly decreased.

Finally, we investigated in vitro the effect of TMZ on glioma immunogenicity. GL261 cells were treated with 50 and 150 \(\mu\)M TMZ or DMSO at different time points. TGF-\(\beta1\) and TGF-\(\beta2\) concentrations in the supernatant from TMZ-treated GL261 cells significantly decreased, as evaluated by ELISA (Fig. 6D). Nkg2d ligand (Nkg2dl), involved in Nkg2d-mediated NK cell recognition of tumor cells and weakly expressed in GL261 cells, was upregulated in a time- and dose-dependent manner after TMZ treatment (Fig. S2). Similarly, we found a time- and dose-dependent increase of the Rae-1 -\(\beta\), -\(\epsilon\) and -\(\delta\), ligands for the Nkg2d receptor (Fig. 6E); while B7-h3, a NK cell inhibitory molecule highly expressed in GL261 cells, was significantly decreased (Fig. S3).

These results indicate that TMZ modulates glioma microenvironment into a site favoring NK cell infiltration and antitumor cytotoxicity.

Discussion

Our results show, for the first time, that NK cells in peripheral blood are resistant to chemotherapy due to expression of Abcc3, which was slightly or not expressed by CD8\(^+\) T cells. Abcc3 was upregulated and active in NK cells from glioma-bearing mice during TMZ treatment, whereas CD8\(^+\) T cells did not exhibit a drug-resistant phenotype. We also confirmed the ability of NK, but not CD8\(^+\) T cells, to react to cytotoxic effects of chemotherapy by measuring their apoptosis in vitro, which was low or almost absent in NK cells during TMZ treatment.

ABC transporters promote cell survival independently of cytotoxic drug efflux, as shown in a study where the inhibition of endogenous expression of ABC transporters resulted in reduced expression of Bcl2 protein levels and activation of the apoptotic cascade.\(^\text{41}\) Based on these observations, ABC transporters can be responsible for the drug-resistance phenotype through direct drug efflux and by other intrinsic pathways, including the phosphorylation of Akt, a key regulatory molecule involved in cell survival, that is activated in response to TMZ contributing to chemo-resistance.\(^\text{42}\) This is supported by data presented here showing that Abcc3-expressing NK cells from TMZ-treated glioma-bearing mice exhibit significant Akt phosphorylation, a protective mechanism against cell death.\(^\text{43}\)

Notably, Akt activation has been correlated with increased expression and activity of some ABC transporters.\(^\text{44-46}\) and there is evidence that Akt and PI3K/Akt pathway are responsible for cytotoxic and killing ability of NK cells.\(^\text{47-50}\) Our data showing that Akt is phosphorylated only in Abcc3\(^+\) NK cells support the involvement of Abcc3 in the cytotoxicity of NK cells.

We also found that TMZ led to an enrichment of NK cells with migratory function, as observed by investigating the four-stage model of NK cell maturation.\(^\text{28}\) While different studies have shown that ABC transporters play a role in the migration of cancer and normal cells, including immune cells,\(^\text{51,52}\) a direct action of TMZ on NK cell maturation/migration has not been previously reported. The increase of migratory subsets is
concomitant with a consistent modulation of the glioma microenvironment of TMZ-treated mice, where we found a significant increase of chemokines that are important for NK cell recruitment.53,54

Intriguingly, the expression of galectin-1 and other galectins, that are potent suppressors of antitumor immune surveillance, decreased in glioma-bearing mice treated with TMZ. Recently, galectin-1 was found responsible for the inhibition of NK cell function and viability: galectin-1 deficient gliomas could be eradicated by infiltrating NK cells with cytotoxic function.30

The evidence that the "cytotoxicity signature" after three chemotherapy treatments resulted down-modulated in the periphery and upregulated in the gliomas of TMZ-treated mice, supports the contention that TMZ can modulate glioma microenvironment facilitating NK cell infiltration and cytotoxic function.

It has long been known that NK cells can control and reinforce antitumor immune responses mediated by DC.57 NK cells and DCs work in synergy, taking advantage of one another.58,59 The reciprocal interaction of NK cells and DCs and the ability of NK cells to recognize and kill tumor cells represent an important rationale for their monitoring during immunotherapeutic approaches with DCs in correlation with clinical outcomes. Our results obtained on a group of recurrent GBM patients treated with DCs loaded with autologous tumor lysate showed a significant increase of NK cells producing IFNγ in patients with prolonged survival.11 We are now evaluating the combination of DC immunotherapy with TMZ in patients with first diagnosis GBM. The NK cell response significantly correlates with survival, whereas the CD8+/ T cell response does not appear to influence clinical outcomes. Patients with evidence of an NK cell response showed a significant upregulation of ABCC3 in PBLs in comparison with non-responders (manuscript in preparation).

In agreement with these results, we observed that trafficking and NK cell homing increased in glioma-bearing mice during TMZ treatment. Notably, we also found that murine NK cells can efficiently overcome the drug-mediated toxicity of chemotherapy by expressing multidrug-resistance genes. It has been previously described that NK cells are resistant to chemotherapy.56,60 After chemotherapy treatment, NK cells are the first lymphoid cells to recover and may represent the principal lymphocytes for the initial months after treatment, suggesting a more rapid reaction to cytotoxic effect of drugs than other immune cells.60 Murine and human NK cells were found to express high levels of multidrug transporters, which could confer protection against chemotherapeutic agents.51 TMZ administration, similar to other chemotherapeutics, can cause the
development of moderate to severe lymphopenia and myelo-suppression,\textsuperscript{52,63} indicating that immune inhibitory effects take place through selective toxicity on proliferating lymphocytes and inhibition of immune effector differentiation.\textsuperscript{63,64} However, there is evidence that following a transient chemotherapy-induced lymphopenia, lymphocytes undergo homeostatic proliferation that enhances antitumor, vaccine-induced immune responses\textsuperscript{65,66} with positive clinical outcome correlation.\textsuperscript{67}

Discrepancies have been reported about the effect of TMZ on NK cells. It has been shown that TMZ significantly decrease the absolute number of CD3\textsuperscript{-} CD56\textsuperscript{+} effector cells in blood of GBM patients.\textsuperscript{68} However, other studies have reported that circulating NK cells are relatively resistant to chemotherapy, with their frequency and absolute number, as well as their effector functions, unaffected by TMZ.\textsuperscript{55,56}

In conclusion, our data indicate that chemotherapy is able to modulate tumor microenvironment and reinforce tumor infiltration of NK effector cells and that can contribute to the adjuvant effect of chemotherapy. The different sensitivity of NK and T cells to TMZ, however, may disrupt their interactions, like that of 2B4 (CD244) and CD48,\textsuperscript{69,70} relevant to generate a T cell memory and possibly amplify antitumor responses. Confirmation of this hypothesis, driven by the results we reported here, may imply a careful re-evaluation of chemotherapy/immunotherapy schedules.

**Materials & methods**

**Cell culture**

GL261 cells were cultured as neurospheres in a stem cell growth medium containing DMEM-F12 Glutamax, B-27 (Life Technologies), penicillin/streptomycin, human recombinant epidermal growth factor (EGF; 20 ng/mL) and human recombinant fibroblast growth factor-2 (FGF-2; 20 ng/mL; Peprotech). PBls were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), \(\beta\)-mercaptoethanol, sodium pyruvate, nonessential amino acids, L-glutamine, and penicillin/streptomycin (all from EuroClone). Human recombinant interleukin-2 (hIL-2; 10 U/ml; Roche) was added to the medium.

**In vivo experiments**

C57BL/6N 6-week-old female mice (Charles River Laboratories) received intracranial injections of \(10^5\) GL261 cells using specific stereotactic coordinates into the nucleus caudatum (0.7 mm posterior, 3 mm left lateral and 3.5 mm deep, with respect to the bregma). Mice were divided in two groups, treated 9 d after glioma implantation with intraperitoneal injections (i.p.) of 5 mg/kg TMZ or vehicle (DMSO) on days 1–5 and sacrificed at different time points. The animals were monitored every day and euthanized when suffering, in accordance with the current directives of the Campus animal IFOM-IEO house facility and the Minister of Health. Animal experiments were performed in accordance to the Italian Principle of Laboratory Animal Care (D.Lgs. 26/2014) and European Communities Council Directives (86/609/EEC and 2010/63/UE).

**Isolation of local and systemic lymphocytes**

PBls were isolated using Lympholyte-M (Cedarlane Labs) according to the manufacturer’s instructions. An indirect magnetic labeling system was used to immune-isolate CD8\textsuperscript{+} T and NK cells (NK and CD8\textsuperscript{+} T Cell Isolation Kits, Miltenyi Biotec) resulting in a 97 ± 1.5% and 93.2 ± 2.9% pure CD8\textsuperscript{+} T and NK cell population, respectively, as evaluated by flow cytometry. TILs were isolated with the Tumor Dissociation Kit and GentleMACS (Miltenyi Biotec) according to the manufacturer’s instruction. A Percoll-density gradient centrifugation (30%–40%–80%–100% isotonic Percoll, 400xg, 15 min at 20°C) was used to separate lymphocytes from the tumor single cell suspension. Immune cells were recovered from the 40–80 gradient interphase.

**Flow cytometry**

Cells were stained in a cold buffering at 4°C in the dark. The following antibodies were used: CD45, CD8\textsuperscript{+}, CD4\textsuperscript{+}, CD3, CD11b, CD27, NK1.1, NKP46, CD49b, CD49d, IFN\(\gamma\) and pAkt (Miltenyi Biotec), Abcc3 (Abcam), B7-H3 (Biolegend) and NKG2D-L (eBioscience). DAPI was added to exclude dead cells. Flow cytometry acquisition was performed on a MACS-Quant instrument, and data were analyzed with MACSQuantify Software (Miltenyi Biotec).

**Microarray analyses and Real Time-PCR**

Total RNA from purified NK and CD8\textsuperscript{+} T cells was extracted with TRIzol reagent (Life Technologies) using RNase-Free DNase Set (Qiagen). Microarray analyses were performed after three TMZ or DMSO administrations. Mouse Gene 2.0 ST Array GeneChip (Affymetrix), which includes 35,240 mouse transcripts, was used following standard procedures. Differentially expressed genes were identified using a fold-change threshold \(\geq 2\) for all transcript comparisons. The functional annotation of genes that passed the FC and expression signal cut-offs was performed using the Gene Ontology (GO) Biological Process category. Fast SYBR Green chemistry (Life Technologies) was used for real-time PCR expression analyses. Relative mRNA levels were measured using a ViiA7 Real Time-PCR System (Life Technologies) and calculated using the \(\Delta\Delta Ct\) method normalizing to the housekeeping Gapdh, Actin and \(\beta2M\) levels. The primer sequences (Primm S. r.l.) are reported in Supplemental Materials.

**Migration assay**

Migration was assessed in vitro using 8 \(\mu\)m Transwell migration chambers. Purified NK cells (\(4 \times 10^5\)transwell) were placed in the upper chamber and evaluated for their ability to transmigrate toward the lower chamber. Chemotactant in the lower chamber was represented by medium from GL261 cells previously treated for 24 h with DMSO or 150 \(\mu\)g/mL TMZ. After 12 h, migrating cells were stained with crystal violet solubilized in 10% acetic acid.
**Western blot analysis and proteome profiler array**

Briefly, cells were washed with cold PBS and lysed in a buffer supplemented with protease and phosphatase inhibitors. Membranes with transferred proteins were incubated with the primary antibody anti-p-AKT (Ser473) (1:1000, Cell Signaling) or anti-vinculin (1:10000). The primary antibody incubation was followed by incubation with peroxidase conjugated to the secondary antibody (anti-rat, 1:10000). A chemiluminescence reaction using the ECL Plus kit (GE Healthcare) was detected using G:BOX iChemi system (Syngene). Tumor relative levels of cytokines and chemokines were measured using the Mouse Cytokine Array Panel A kit (R&D Systems) following the manufacturer’s instructions. Images of the blots were acquired with G:BOX Chemi system (Syngene) and quantitative analyses were performed using ImageJ software. The 40 cytokines and chemokines of interest were normalized to the corresponding positive controls.

**Apoptosis assay**

Resistance of NK and CD8⁺ T cells to the cytotoxic effects of TMZ was evaluated with Annexin V (Biolegend) and propidium (PI). Early and later apoptosis were distinguished with Annexin V positivity and Annexin V-PI double positivity, respectively. The selective MRP inhibitor MK571 at a concentration of 25 μM was used to test the Abcc3 role in chemoresistance. Briefly, naïve lymphocytes were treated with 1 μM TMZ or DMSO for 4 h in vitro. MK571 was added to the medium 30 min before or after the pharmacological treatment and apoptosis was evaluated.

**ABC transporter activity**

The eFluxx-ID® Green Multidrug-Resistance Assay (Enzo Life Sciences) was used to detect the multidrug-resistant phenotype of NK and CD8⁺ T cells by monitoring the efflux activity of the three major multidrug-resistance proteins: MDR1, MRP and BCRP. Following the manufacturer's instructions, specific inhibitors (Verapamil, MK571 and Novobiocin) were used to define the resistance activity factor (MAF) in PBLs from naïve mice treated with 1 μM TMZ or DMSO for 4 h in vitro. MAF values >25 are indicative of multidrug-resistance positive phenotype.

**IFNγ production and LDH assay**

PBLs were cultured in a 24-well tissue culture plate in 1 mL of completed RPMI supplemented with 10 U/mL IL-2. Unspecific stimulation was performed with 50 ng/mL phorbol myristate acetate (PMA), 1 μg/mL Ionomycin and 10 μg/mL Brefeldin A for a total of 4 h. Lymphocytes were stained for IFNγ (BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit, BD Bioscience) according to the manufacturer's instructions. A non-radioactive cytotoxic assay (Cytotoxicity detection kitplus LDH, Roche) was performed to test PBL capacity of TMZ-treated, control and naïve mice to recognize and lyse GL261 cells, according to manufacturers' instructions. PBLs were pre-stimulated for 5 d in the presence of 20 Gy-irradiated GL261 cells and tested 5 d later for GL261 cell specific cytotoxicity.

**Histology and immunofluorescence**

Double immunofluorescence was performed on 5 μm paraffin-embedded sections. Paraffin was removed with xylene and the sections were rehydrated in graded alcohol. Tumor sections were incubated with anti-CD8⁺ (1:10, BD Bioscience) and anti-NK1.1 (1:10, Miltenyi) antibodies overnight at 4°C. After a counterstained with DAPI, sections were examined using a Nikon confocal microscope and analyses were performed on 3 to 5 independent fields per tumor using the 40X objective. Tumor sections were also stained with hematoxylin and eosin to assess the volume of tumor growth and acquired using the Aperio ScanScope slide scanner (Leica).

**Statistical analysis**

Statistical comparison was performed using two-tailed Student’s t-test. Results were considered significant at p < 0.05.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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