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

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Chapter 1

General introduction

Glioblastoma, the deadly tumor

Glioblastoma (GBM), a grade IV astrocytoma, is the highest-grade and the most common form of malignant brain tumor. It is one of the most lethal form of human cancer: without treatment, the median survival is approximately 3 months with a rapid development of clinical symptoms [1]. Clinically, patients with GBM may present with headaches, focal neurologic deficits, confusion, memory loss, personality changes or seizures depend on the location and size of tumor [2].

Each year in western world 3-4 new cases of glioblastomas are diagnosed every 100,000 inhabitants. Although brain tumors account for <2% of all primary neoplasms, high mortality rates mean they rank high as leading cause of cancer-related death [3]. Most patients die within one year from the diagnosis, and only 5% survive more than 5 years despite aggressive therapies (CBTRUS, 2011). Despite several progresses, the current standard treatment for GBM patients still consists of maximal surgical resection followed by radiotherapy plus concomitant and adjuvant temozolomide (TMZ) [4,5]. Many features help to explain the refractory nature of GBM to treatments including capacity for infiltration, and presence of the blood–brain barrier, a significant obstacle to chemotherapy. Recurrences are almost inevitable remaining an incurable disease with a survival range of 12–15 months [4].

GBM is usually described into primary or secondary form, indistinguishable histologically but differ in genetic and epigenetic profiles [6] (figure 1).

Primary GBM is the most common form (about 95%) and arises *de novo* from glial cells as a combination of genetic and epigenetic alterations. Typically has a clinical history < 6 months and is most common in older patients. It displays deletion in cell cycle-related genes $p16^{INK4A}$ and $p19/p14^{ARF}$, mutations in the promoter of the telomerase reverse transcriptase (TERT), loss of chromosome 10 or PTEN mutation and gene amplification of the epidermal growth factor receptor (EGFR), usually occurring in conjunction with the variant III (EGFRvIII) activating mutation.

Secondary GBM, less frequent and studied, develops over months or years from preexisting low-grade astrocytomas and predominantly affects younger patients [7]. It is characterized by mutations in TP53, amplifications of cyclin-dependent kinase 4 (CDK4) or loss of retinoblastoma (Rb) and overexpression of platelet-derived growth factor (PDGF). IDH1 mutations were found in less than 5% of primary GBM and in greater than 80% of secondary GBM, characteristically associated with improved survival.

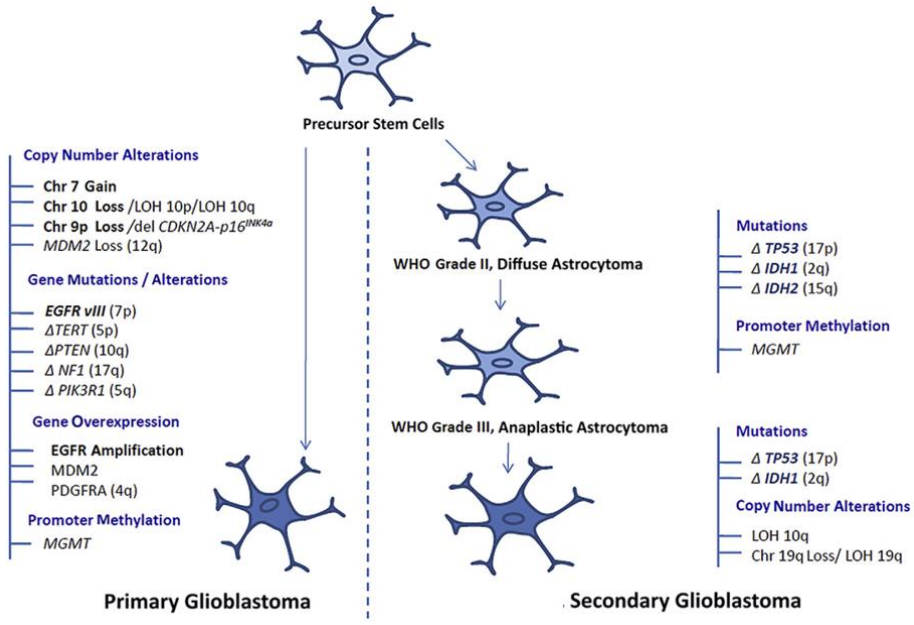

Figure 1. Primary and secondary GBM development. The most frequent and relevant molecular abnormalities of primary versus secondary GBM [8].

The recent characterization of the genome [9,10] and transcriptome [11–13] of GBM results in the generation of molecularly defined subtypes (i.e., proneural, neural, classical and mesenchymal). Molecular subclasses are typically associated with different genetic alterations and expression profiles that can better predict survival and treatment response. Thus, GBM subtypes suggest the possibility to appropriately stratify patients and to treat them with more innovative and personalized cares.

It is reported, for example, that the proneural (PN) signature can be considered the less aggressive form with a more favorable prognosis [12]. On the contrary, the mesenchymal (MES) subtype is considered the most aggressive profile carrying not only an angiogenic but also an

inflammatory signature that makes MES GBM more responsive to immune-based therapies [14,15].

Due to the infiltrative nature of GBM, with diffuse dissemination of tumor cells beyond the tumor mass and general resistance to therapy, there have been intensified research efforts studying new targeted therapies.

Novel therapeutic approaches created expectations for increased survival, including anti-angiogenic therapy and immunotherapy, however heterogeneity favors escape strategies.

In the last years, research focused on targets as EGFR, vascular endothelium growth factor (VEGF) and platelet-derived growth factor (PDGF) receptors to inhibit mechanisms of proliferation and angiogenesis. Bevacizumab is a recombinant humanized monoclonal antibody that neutralizes the activity of VEGF and is normally used as treatment for recurrent GBM. Recently, results of a phase III study of bevacizumab added to standard treatments in newly diagnosed GBM showed a significantly increased in progression free survival [16,17].

A growing body of evidence indicate encouraging results with immunotherapeutic approaches to fight GBM [18,19]. Immunotherapy with dendritic cells (DCs), loaded with whole tumor lysate or glioma-associated antigen peptides, revealed tolerable and safe with promising increased survival [20–22]. Mitchell et al. suggested in a recent clinical study that pre-conditioning the vaccine site with a potent recall antigen as tetanus toxoid can significantly improve the lymph node homing and the efficacy of DC immunotherapy [23].

EGFRvIII is a constitutively activated and immunogenic mutation not expressed in normal tissues but widely expressed in GBM and for that the most important target for different immunotherapeutic approaches. A phase II peptide-based immunotherapy trial was conducted and demonstrated safety with evidence of immune response and increased patient survival [24]. A phase III randomized study of Rindopepimut/GM-CSF (ACT IV) in patients affected by newly diagnosed GBM is ongoing but the recruitment is concluded. The EGFRvIII epitope is also proposed as target for chimeric antigen receptor (CAR) T cell therapy in murine models demonstrating a robust antitumor activity [25,26]. Different clinical trials exploring the safety and effectiveness of CARs against HER2, IL-13α2 and EGFRvIII just started [27].

Finally, immunotherapy with immune checkpoint inhibitors for cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) have provided substantial improvements in melanoma and lung cancer, and there is evidence of a potential benefit for GBM patients [28]. Tumor progression involves multiple immunosuppressive mechanisms, making combination of immunotherapeutic agents that target different pathways could be a promising approach.

Glioblastoma & Immune System

The central nervous system (CNS) has long been considered an immunological privileged site not affected by apparent immune responses. Initially, CNS immune privilege was supported by (i) restrictions imposed by the blood–brain barrier (BBB), structured to restrict the transport of molecules and cells from the circulation to the

parenchyma, (ii) the lack of draining lymphatics and (iii) the dearth of antigen-presenting cells (APCs).

Over the last 20 years, accumulating data re-evaluated this idea suggesting that the CSN is neither isolated nor passive in its interactions with the immune system [29]. Indeed, peripheral immune cells can cross the intact BBB and infiltrate the parenchyma to perform routine immune surveillance functions against infections and cancer [30]. BBB represents only a relative barrier to lymphocyte trafficking, especially in pathological states where it is disrupted, often in the case of GBM. Microglia are the resident immunocompetent cells with the role of APCs participating in the regulation of innate and adaptive immune responses. Moreover, under a variety of inflammatory conditions, peripheral DCs are quickly recruited to the site of the brain lesion [31]. Although conventional lymphatics are missing in the CNS, antigen-loaded DCs have access to secondary lymphoid organs, where T-cell priming takes place, through cerebrospinal fluid and perivascular spaces (figure 2). Furthermore, subcutaneous vaccination with genetically modified cytokine-secreting tumor cells or systemic immunotherapy with DCs induce an efficient immune response against intracranial tumors, confirming that the CNS is not truly immunologically privileged but a site for immunological responses and a candidate for immunotherapeutic approaches [32–34].

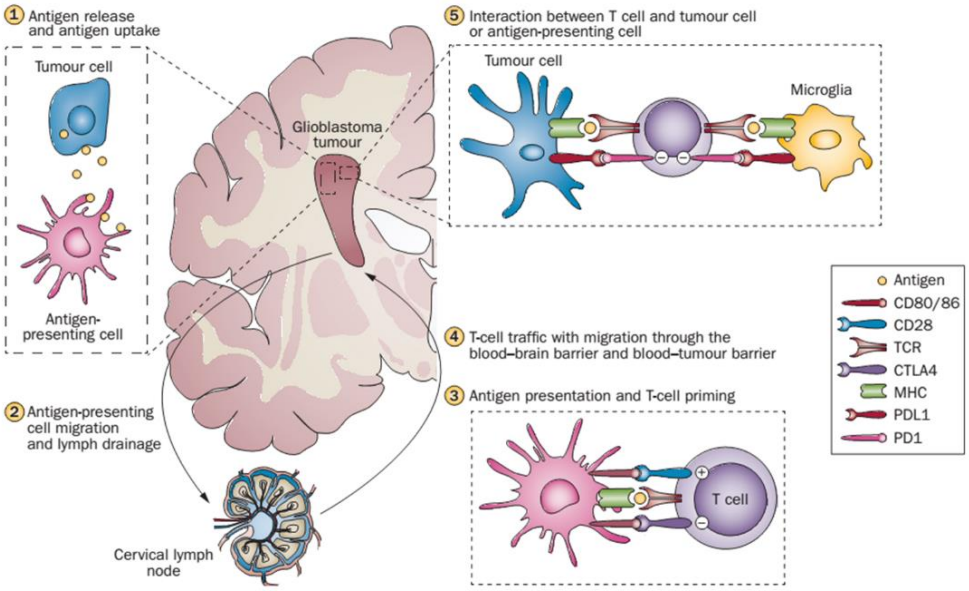

Figure 2. Immune response cycle of GBM. Antigens released from dying tumor cells are up-taken by APCs and presented to T cells that are then primed and activated in lymph nodes. The cytotoxic T cells migrate to tumor site where they infiltrate and specifically recognized tumor cells. During this cycle, various ligand-receptor interactions between APCs and T cells and between tumor cells and T cells provide signals to stimulate or inhibit the immune response [28].

Very recently Louveau and colleagues discovered the presence of functional lymphatic vessel in mouse CNS [35]. In searching for some immune cell gateway, the scientists observed that meninges, that cover the brain and contain blood vessels and cerebrospinal fluid are associated with structures showing a vessel-like patterns and expressing markers of the lymphatic system. These vessels carried fluids and immune cells from the cerebrospinal fluid into cervical lymph nodes. They also identified a potentially similar structure in human dura, however additional studies are necessary to demonstrate and

characterize the existence and the location and organization of meningeal lymphatic vessels in the human CNS.

Clinical observations suggests that high densities of tumor-infiltrating lymphocytes (TILs) correlate with improved clinical outcome in melanoma, ovarian, colon, breast, lung, and other types of solid tumors. The majority of these studies observed a robust infiltration of $CD3^+$ T cells, cytotoxic T lymphocytes (CTLs) and $CD45RO^+$ memory T cells that are associated with an improved survival [36].

The characterization of TILs needs to be properly extended in malignant glioma. Indeed, contradictory reports exist regarding the presence and the prognostic factor of immune infiltration in GBM patients [37]. Donson et al. showed as gene expression microarray profiles of high-grade astrocytomas, including GBM, from long-term survivors have increased expression of immune-related genes. Histology validated results revealing an increased immune cell infiltration significantly associated with longer survival and a better Karnofsky performance status [38]. Similarly, a recent study described a significant correlation between increased $CD3^+$ and $CD8^+$ T cell infiltration in tumor bed and improved patient survival, even in presence of $CD4^+CD25^+FOXP3^+$ regulatory T (Treg) cells and surface expression of inhibitory molecules [39]. Yang et al. reported natural killer (NK) cell infiltration in GBM suggesting their important role in antitumor immune responses [40].

On the contrary, some reports indicated a predominant and variable infiltration of $CD3^+$ T cells, often represented by Treg cells, in GBM specimens with a negative or not significant correlation with patient prognosis [41–43]. The difficulty to sustain an anti-tumor T cell mediated

cytotoxic response is principally due to the strong immunosuppressive microenvironment of GBM. Indeed, Fecci et al. found TILs with poor effector functions and an up-regulation of inhibitory receptors, such as CD95, PD-1, PD-L1, CTLA-4, LAG3 and TIM3, indicating immune exhaustion [44].

Tumors cells responds with numerous strategies to counteract the immune system. It has largely reported that GBM cells secrete high levels of transforming growth factor beta (TGF-β), responsible for decreased expression of the activating receptor NKG2D on effector cells [45], IL-10, prostaglandin E2 (PGE_2), VEGF and galectins [15]. Moreover, glioma cells promote trafficking of suppressive Tregs, myeloid-derived suppressor cells (MDSCs) and M2 macrophages by production of chemoattractants as indoleamine 2,3- dioxygenase (IDO), CCL22 and CCL2 [46,47]. Down-regulation of major histocompatibility complex (MHC) and B7 family proteins [48,49], increase of PD-L1 and HLA-E expression [50,51] and secretion of NKG2D ligands MICA/B [52] contribute to the immunosuppressive feature of GBM environment.

These mechanisms of immune suppression and the dynamic nature of interactions between T lymphocytes and tumor cells develop a mechanism of immune escape as a negative consequence of an anti-tumor immune response (figure 3).

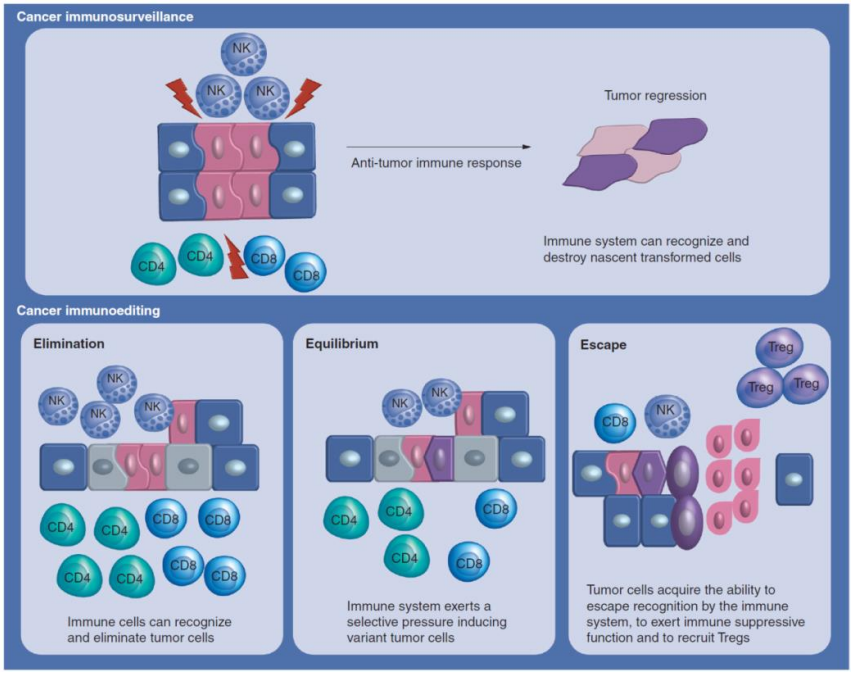

Figure 3. The three Es of cancer immunoediting: elimination, equilibrium, and escape. Immunosurveillance hypothesis describes the ability of the immune system to recognize and eliminate tumor cells. Immunoediting is a process triggered after the encounter between immune components and tumor cells. Transformed tumors cells may be recognized and eliminated by different cell types of the immune system. If elimination is unsuccessful, the immune system and the cancer can reach an equilibrium phase. Equilibrium involves the continuous elimination of tumor cells and the production of resistant tumor variants by immune selection pressure. This continuous sculpting can lead to escape, in which mutated cancer cells become able to inhibit the immune system and cancer can growth uncontrolled [53].

Several different immunotherapeutic approaches are proposed to potentiate immune system against glioma cells with encouraging clinical results [18,27]. However, the mechanism of cancer immunoediting

used by GBM to evade immune clearance [54] during immunotherapy suggests that this cancer requires multimodal therapeutic strategies for an effective treatment. Additionally, given the heterogeneity and the complexity of mechanisms involved in GBM pathogenesis, a more detailed characterization of tumor immune microenvironment is necessary.

Chemotherapy: a secret ally

Conventional anticancer drugs were firstly selected for their ability to arrest cell cycle progression leading to cell death. A growing body of evidence suggests that chemotherapeutic agents, when used at clinically useful doses, have an impact on therapeutic outcome beyond their cytotoxic properties on neoplastic cells. Indeed, the majority of these agents, initially considered immunologically silent or immunosuppressive, are able to stimulate the innate and adaptive immune system and evoke long-term protective memory T cell responses facilitating tumor eradication [55]. It has been shown that tumor cells, pretreated with chemotherapy, can evoke a durable adaptive immune response upon transfer into immunocompetent mice and an efficient rejection of tumor rechallenge [56].

It is reported that chemotherapy is able to enhance antitumor immune response by (i) directly inducing tumor cell death with consequent release of tumor-derived antigens and immunostimulatory danger signals, (ii) by modifying tumor microenvironment and (iii) by modulating functions of immune cells, including DCs, MDSCs, tumor-associated macrophages (TAMs), CTLs and Tregs.

Certain chemotherapeutic agents confer an immunogenic cell death, which stimulates an immune response against dead-cell antigens, in particular when they derive from cancer cells. The immunogenic cell death involves changes occurring in a defined temporal sequence, in the composition of the cell surface as well as the release of soluble mediators called damage-associated molecular patterns (DAMPs). DAMPs stimulate inflammasome-dependent response, recruit specific cells of the innate and adaptive immune system to the tumor bed and trigger a therapeutically relevant immune response against malignant cells (figure 4).

Pre-apoptotic exposure of calreticulin (CRT) on dying tumor cell surfaces promote phagocytosis by DCs and the up-regulation of their costimulatory molecules and antigen-processing machinery components [57]. It is reported that the inhibition of CRT exposure in tumors negatively affects the efficacy of chemotherapy in immunocompetent mice, suggesting that this stress–dependent immunogenic signal is indispensable for the elicitation of anticancer immune responses [58,59]. The exposure of CRT is accompanied by changes in the endoplasmic reticulum (ER) ultrastructure and by signs of an ER stress response [60]. Moreover, the transcriptional up-regulation of heat-shock proteins (HSPs) is another important mechanism induced in response to cellular stress. HSPs are considered potently immunoadjuvants able to determine a strong immunogenicity of stressed tumor cells, facilitating cross-presentation of tumor antigens, DC maturation and also activating NK cell immune responses [61]. Thus, a second line of immunogenic factors that stimulate antigen processing is required in addition to these signals. In late apoptosis the high-

mobility group box 1 (HMGB1), a non-histone chromatin-binding nuclear protein, is released and acts as a pro-inflammatory signaling factor. The release of HMGB1 and its interaction with the toll-like receptor 4 (TLR4) on the DC surface are mandatory for the optimal presentation of tumor antigens to T lymphocytes [62] and stimulate the synthesis of pro-inflammatory cytokines leading to NK and T cell activation [63]. Furthermore, many chemotherapeutic agents induce production and release of other alarmin molecules as uric acid, adenosine triphosphate (ATP) and uridine triphosphate (UTP) that bind the purinergic receptor P2X7 at the surface of DCs, resulting in the activation of the NLRP3 inflammasome and therefore in the secretion of interleukin-1β (IL-1β). Several reports indicated as IL-1β is required for the efficient priming and expansion of $CD4^+$ and $CD8^+$ T lymphocytes and elicitation of antitumor immune response [64].

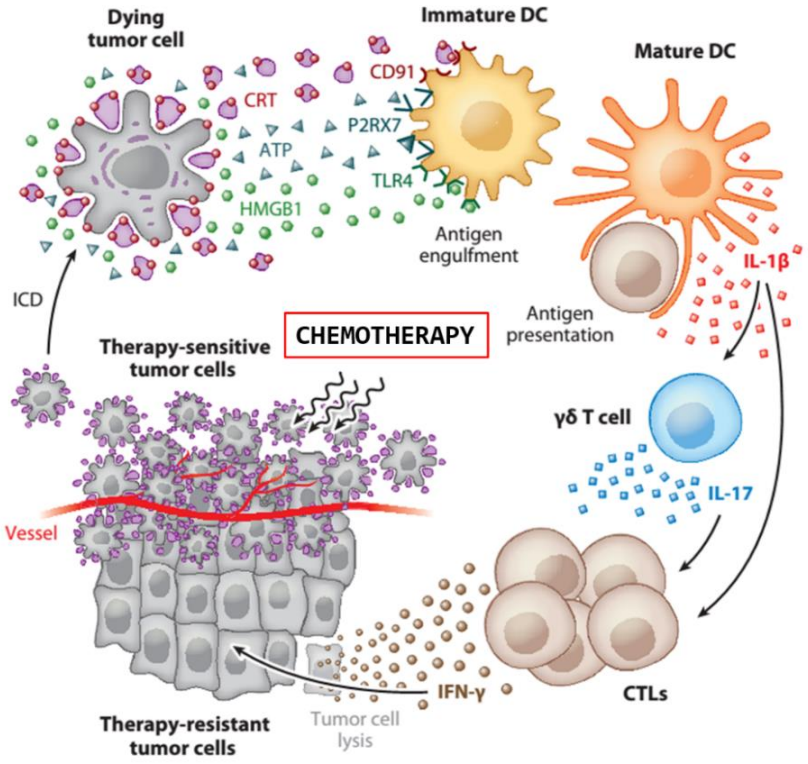

Figure 4. Immunogenic cell death (ICD) induced by chemotherapy. Stressed-cancer cells undergo to ICD releasing DAMPs that facilitate the recruitment and activation of DCs into the tumor bed and the conditions for optimal antigen presentation to T cells. These processes result in a potent IFN-γ mediated immune response involving both γδT cells and CTLs, which eventually can lead to the eradication of chemotherapy-resistant tumor cells. Adapted from [65].

In addition, chemotherapy can also render cancer cells more susceptible to CTL and NK cell killing inducing changes in their immunogenic properties. It is reported the capacity of some anti-neoplastic agents to increase the permeability of tumor cells to granzymes facilitating the CTL-mediated lysis even if they do not express the antigen recognized by CTLs [66]. Similarly, van der Most et al. showed as the alkylating

agent cyclophosphamide can sensitize tumor cells to TRAIL-dependent $CD8^+$ T cell anti-tumor responses [67]. Anti-cancer agents inducing DNA damage promote functional down-regulation of inhibitory molecules and up-regulation of stimulatory DNAM-1 and NKG2D ligands on the surface of cancer cells, resulting in augmented NK and $CD8^+$ T cell cytotoxicity and IFN-γ production [68–70].

Tumor microenvironment contains immune-suppressive cytokines, immune checkpoints and regulatory subsets of cells, such as TAM, MDSCs and Tregs, that can contribute to a failure of immune-based approaches in clinics. Chemotherapy has the ability to modify tumor microenvironment converting it into a permissive state for the activation of a local and systemic antitumor immunity [71]. Indeed, current data have indicated as chemotherapeutic drugs induce an alteration of cytokine and chemokine profile in the tumor microenvironment decreasing amounts of immunosuppressive IL-4, IL-10 and TGF-β, and promoting production of pro-inflammatory mediators such as IL-8, TNF-α, IL-2, IL-7, IL-15, IL-21 and IFNs [72].

In response to anti-neoplastic agents, the composition of tumor immune infiltrate changes with a crucial effect on therapy outcomes. Thus, an increased number of IFN-γ producing effector cells over immunosuppressive cells within the tumor microenvironment after chemotherapy predict favorable therapeutic responses in different types of tumors [73]. Indeed, many conventional chemotherapeutic compounds can lead to a transient suppression of functions or direct depletion of intra-tumoral and peripheral inhibitory cells restoring a potent and durable cytotoxic antitumor immune response and improving patient

survival [74,75]. Accumulating evidence suggest that chemotherapy treatment, most of all a metronomic administration, results in an enhanced serum levels of IFN-γ and IL-2 with consequent $CD8^+$ T cell response in both murine models and patients with different solid tumors [76,77]. Similarly, some types of anticancer drugs elicit NK cell recruitment to tumors and regulate their cytotoxic activity in several models of cancer [68,74,78].

These evidences suggest that many anticancer agents have the ability to evoke an anti-tumor immune response and may cooperate with immunostimulatory approaches, as immunotherapy, to develop combined protocols. The rationale for this dual approach is that chemotherapy has the potential to induce sufficient tumor cell death and counteract the immunosuppressive factors in tumor microenvironment synergizing immune-based therapy and harnessing the maximum antitumor immune response.

Temozolomide (TMZ)

TMZ is the most widely used and effective chemotherapeutic drug for GBM patients. A major benefit of TMZ is its ability to cross the blood-brain barrier with almost 100% bioavailability. TMZ is a prodrug that spontaneously hydrolyzes at physiological pH to the active metabolites 5-(3-methyltriazen-1-yl)-imidazo-4-carboximide (MTIC) and methylhydrazine. TMZ exerts its antitumor effects by methylating guanine at the N^7 and O^6 positions and adenine at the N^3 position. These adducts create a DNA damage in tumor cells that results in apoptosis and cytotoxicity [79].

TMZ was first evaluated as a single treatm
ent for recurrent malignant glioma, where it showed a good antitumor activity and acceptable side effects. A successive pilot phase 2 trial demonstrated the feasibility of the concomitant administration of TMZ with fractionated radiotherapy, followed by six cycles of adjuvant TMZ for newly diagnosed GBM patients with a median survival benefit of 2.5 months compared to radiations alone [4].

The most important prognostic factor for survival and TMZ response is the methylation status of O^6-methylguanine-DNA methyltransferase (MGMT), an alkyl guanine transferase that repairs lesions created by TMZ and other alkylating agents. It has been well documented that the level of MGMT activity is correlated with tumor cell resistance to chemotherapeutic drugs [80].

Although TMZ is well-tolerated and has an overall beneficial impact on patient survival, it has also been well-described to induce immunosuppression, most often described as various forms of lymphopenia and abnormal levels of white blood cells [79]. Although bone marrow cells are particularly vulnerable to TMZ due to their high proliferation rate, severe myelosuppression and bone marrow failure are rare adverse effects. It has been suggested that TMZ selectively targets monocytes but not dendritic cells that derived from them [81].

Several studies pointed out that alkylating agents have immune inhibitory effects via selective toxicity to proliferating lymphocytes and inhibition of differentiation of immune effectors [82,83] highlighting the drug-mediated toxicity of chemotherapy to immune effector cells. However, lymphopenia caused by TMZ is preferential associated to depletion of highly potent immunosuppressive cells as Tregs and

MDSCs in blood and within the tumor microenvironment [84]. This lymphopenic state has been positively correlated with clinical outcome due to relieve suppression of antitumor effector cells [85,86]. Treg reduction was associated with the possibility to exploit a "time-window" of lymphopenia in order to amplify immune responses. Recently, concomitant use of immunotherapy and TMZ regimen has been proposed: a lymphopenic state can induce homeostatic lymphocyte proliferation and enhance antitumor immune responses [87,88]. Thus, the efficacy of antitumor vaccines can be enhance by vaccination immediately after lymphodepleting chemotherapy [89].

Growing amount of evidence show as systemic TMZ does not inhibit antitumor immunity and has a robust efficacy when used in combination with recombinant IFN-β or DC vaccination in different animal models [90–92]. Clinically, TMZ appears to be beneficial when combined with peptide vaccines in GBM and metastatic melanoma patients [93,94]. Moreover, prolonged survival has been observed in patients with GBM who receive chemotherapy after DC vaccination, compared with patients who receive vaccination only [95]. Significant clinical benefits were observed also in a group of GBM patients treated with DC vaccines and subsequent chemotherapy suggesting a synergism between two approaches

TMZ contribution to tumor microenvironment is mediated by down-regulation of the expression of TGF-β, the most immunosuppressive factor in GBM, [96] and by reduced CCL2 secretion, an important chemokine involved in Treg migration, by glioma cells [46]. TMZ has also demonstrated significant anti-angiogenic effects in experimental gliomas [97]. It has been recently described the ability of TMZ to modify

the chemokine network, in particular CXC chemokines with a role in glioma cell biology [98]. Furthermore, Hong et al. showed that TMZ can induce the intratumoral expression of different chemokines resulting in the recruitment of immune cells with antitumor activity [99]. Finally, it has been reported also the ability of TMZ to induce on tumor cells the expression of stress-associated NKG2D ligands on TMZ-resistant glioma cells, potentially rendering them vulnerable to lymphocyte recognition and lysis [100].

ABC transporters

ATP-binding cassette (ABC) transporters are one of the largest protein families evolutionarily conserved present in all living organism from prokaryotes to mammals.

ABC transporters are large, membrane-bound proteins that utilize the energy of ATP hydrolysis to carry out the translocation of various substrates across membrane [101].

All ABC transporters consist of two distinct domains: the transmembrane domain (TMD) and the nucleotide-binding domain (NBD). The sequence and architecture of TMDs is variable, reflecting the chemical diversity of substrates that can be translocated. The NBD or ABC-domain is located in the cytoplasm and has a highly conserved sequence. ABC proteins are organized either as full transporters containing two TMDs and two NBDs, or as half transporters. The latter must form either homodimers or heterodimers to constitute a functional transporter.

It has been established that these pumps recognize a very wide range of substrates and their major physiological function is to transport

exogenous and endogenous hydrophobic compounds through the membrane [102].

The ABC transporters, expressed in numerous cell types under physiological conditions, are also targets of therapeutic interventions in medicine, including cancer drug-resistance, lipid and other metabolic disorders.

Multidrug-resistance

Multidrug-resistance (MDR) is defined as the ability to resist to several drugs that may have different structures and mechanism of action. MDR is one of the major reason for the failure in the treatment of cancer. Chemotherapeutic drugs commonly enter cells by passive diffusion throughout the membrane. The MDR phenotype in tumors is usually associated with the overexpression of certain ABC transporters, resulting in increased drug efflux and reduced intracellular drug accumulation [103].

It is known that exist at least 48 members of ABC transporters associated to MDR belonging to 7 different subfamilies that are divided on the basis of sequence and structural homology in humans. The best clinically characterized MDR transporters belong to three major groups: ABCB, ABCC and ABCG families [104].

ABCB and ABCG preferentially extrude large hydrophobic, positively charged molecules, while the members of the ABCC family can extrude both hydrophobic uncharged molecules and water-soluble anionic compounds in physiological conditions.

The most extensively studied gene in ABCB family is ABCB1, also known as MDR1 or P-glycoprotein (Pgp1). Pgp1 was the first described

membrane transporter characterized by its ability to confer multidrug-resistance in cancer cells to cytotoxic drugs such as methotrexate, anthracyclines, vinca alkaloids and taxanes [105].

The ABCC family, also known as multidrug resistance-associated protein family (MRPs), contains the greatest number of known drug transporters that confer resistance to a similar, but not identical, spectrum of drugs of Pgp1 [106]. It has been established that there are 13 genes belonging to the ABCC family and only three of these genes encode proteins are not involved in drug resistance. Thus, ABCC7 transporter is the cystic fibrosis transmembrane conductance regulator (CFTR), the only member that functions as a channel for chloride ion efflux. ABCC8 and ABCC9 are cell surface receptors that regulate potassium channels modulating insulin secretion [107].

The most characterized member in ABCG family is ABCG2, an half transporter with a single NBD and TMD, also known as the breast cancer resistance protein (BCRP) found for the first time in cell lines resistant to anthracycline and mitoxantrone. It is expressed in many types of solid tumors, conferring resistance to most of topoisomerase I or II inhibitors such as topotecan, irinotecan and doxorubicin [108].

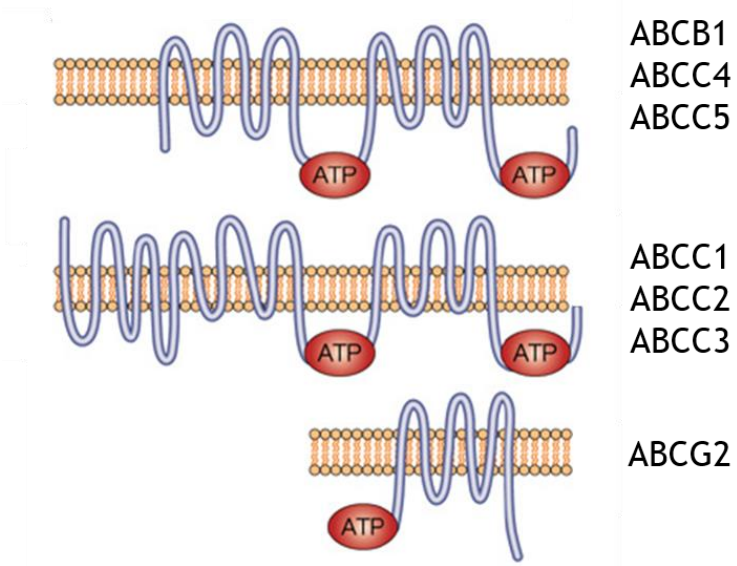

Figure 5. Structure of the best clinically characterized MDR transporters. ABCB1 and ABCC4/5 transporters have 12 transmembrane domains and two ATP-binding sites. The structures of ABCC1/2/3 are similar but contain also an additional domain that is composed of five transmembrane segments at the amino-terminal end, giving them a total of 17 transmembrane domains. ABCG2 contains 6 transmembrane domains and one ATP-binding region [109].

Other Functions

Since ABC transporters have an important role in tumor biology, they are large studied and associated to some of the cancer hallmarks as apoptosis, proliferation and migration or invasion.

Several reports provide evidences that ABC proteins might promote cell survival independently of cytotoxic drug efflux [110,111]. For example, the increased expression of Pgp1 has been reported to delay apoptosis in response to a wide range of harmful stimuli in normal and cancer cells. Indeed, apoptotic cascade needs intracellular acidification and the over-expression of multidrug-resistance proteins, associated with

intracellular alkalinization, inhibits the progression of death [112,113]. The downregulation of ABC transporter expression or the inhibition of their activity may result in cell cycle arrest and alteration of the apoptotic cascade with reduced expression of anti-apoptotic proteins [114,115].

Moreover, ABC transporters seem to be involved in proliferative mechanisms. Kuss et al. described as a down-regulation of ABCC1 expression not only increases tumor cell death but also reduces the mitotic index indicating a lower cell turnover in vitro and in vivo [116]. Similarly, other ABC pumps, normally associated to MDR, have a role in contributing directly to the proliferation of tumor and normal cells [117,118].

Lastly, several ABC transporters play some roles in normal and tumor cell migration. The physiological expression of ABC proteins promotes migration in different type of cells, such as DCs or endothelial cells [119,120]. It is reported that the reduction of ABC protein levels reduced migration in vitro and in vivo in different cancer cell lines promoting their invasive phenotype [121,122].

ABC transporters and Immune system

In the last years, the expression of ABC proteins has been reported in numerous cell types under physiological conditions. Peripheral blood cells have also been found to express several ABC transporters with an important role in the resistance to anti-inflammatory, anti-viral, and anti-cancer drugs [123–125]. Although the relative expression of this type of proteins in lymphocytes are controversial [126,127], it is accepted that ABC transporters can be protagonists of immune processes influencing

the differentiation and maturation of immune cells and promoting the migration of immune effectors to sites of inflammation [123,128].

Indeed, ABC proteins can transport inflammatory mediators such as platelet activating factor, leukotrienes and prostaglandins [129–131] exerting a pro-inflammatory effect and facilitating the migration of immune cells to lymph nodes [132].

It is well known the role of ABC transporters, in particular Pgp1, ABCC1 and ABCC4, in DC migration and differentiation [119,133]. In addition, several member of ABCB family are responsible for intracellular peptide transport, including a role in major histocompatibility complex (MHC) class I antigen presentation [134].

A controversial point remains whether ABC transporters are involved in the efflux of inflammatory cytokines and chemokines. Several studies proposed an active role of ABC transporters in extrude molecules as TNF-α, IFN-γ, perforins and interleukins [135–137]. However, the molecular weight of some of these molecules exceeds size of the most common substrates of these transporters. Cytokine secretion may result as a secondary and indirect effect in response to efflux of other substrates.

Several studies reported that Pgp1, ABCC1 and ABCC2 transporter activity is related to cytotoxicity in NK cells and its inhibition was accompanied by suppression of IFN-γ secretion [123,135]. On the contrary, there are evident discrepancies about the expression and function of ABCC1 and Pgp1 in T cells: some studies suggested an increased expression upon T cell activation while others indicated a decrease [138,139].

Since ABC transporters have an important role during chemotherapy treatment due to MDR acquisition, their function should be investigated intensively in immune cells to assess their potential roles in basal drug-resistance. For now, the greatest interest for ABC transporters is for drug therapy of leukemias: Pgp1, BCRP and several MRPs have already been found to confer resistance against anti-leukaemic drugs and indicated as prognostic factors [140,141].

A substantial variability in ABC expression levels of each transporter is reported in different lymphocyte subsets, [126]. Expression of ABC transporter in $CD8^+$ and $CD4^+$ T cells are controversial and related to activation and differentiation status [142]. In Tregs a low expression of ABC transporters and an extreme sensitivity to chemotherapy [123,143] support the absence of a specific drug efflux. Conversely, a number of studied revealed that NK cells appear more chemo-resistant than other lymphocytes principally due to their relatively high levels of Pgp1 and ABCC1 [128,144,145]. Moreover, it has been suggested that the highly aggressive clinical course and strong refractory to chemotherapy of patients with NK lymphomas could be related to activity of Pgp1 and other ABC transporters [146].

ABCC3, a marker of drug-resistance

ABCC3, also known as MRP3, is a member of the large C family involved in active extrusion of substrates out of the cells. ABCC3 is expressed in many polarized cells and localized in the basolateral membrane. It was first localized in human and rat hepatocytes where it can mediate the efflux of organic anions into sinusoidal blood [147,148]. It

is expressed in the adrenal glands and the intra-hepatic bile ducts, colon, kidney, pancreas, spleen and some lymphoid cells [123,149].

ABCC3 is also an organic anion transporter and prefers glucuronate conjugates as transported substrates. Like other MRPs, it was largely characterized for its role in detoxification through export of numerous toxic molecules. This detoxification is reported also in numerous tumors where it contributes to drug-resistance actively extruding a broad spectrum of anticancer drugs [107,150]. Overexpression and activation of ABCC3 leads to the reduction of intracellular drug levels and consequent drug insensitivity. Indeed, ABCC3 is proposed as a marker of MDR and predictor for clinical outcome and response to chemotherapy in several cancer types [151–154]. Moreover, it has been shown to be upregulated and related to tumor grading in pancreatic cancer and malignant gliomas [155,156].

Its expression correlated with decreased sensitivity to different anticancer drugs such as vincristine, etoposide, and cisplatin [157,158], and also to methotrexate and doxorubicin [148,159,160].

Giannoudis et al. suggested that ABCC3 efflux activity could have a role in imatinib treatment failure in patients with chronic myeloid leukemia. ABCC3 pharmacological inhibition showed a better cellular retention of imatinib demonstrating as this drug is actively efflux by ABCC3 transporter. Moreover, patients with high ABCC3 expression at diagnosis were less responsive to imatinib treatment supporting this hypothesis [161].

Although ABCC3 expression and efflux activity is not broadly studied in lymphocytes and other immune cells, increasing evidences supported its role in lymphoid leukemias [162]. Mature leukocytes display basal

constitutive MRP-related transport activity regardless of cell lineage suggesting a potential role of ABCC3 in chemo-protection of immune cells.

Unraveling the specific role of ABCC3 and other transporters in MDR phenotype of the immune system may therefore reveal new links for future immune-chemotherapeutic approaches.

Scope of the thesis

Glioblastoma (GBM) is one of the most lethal human cancer. Standard treatment of GBM includes three steps: surgical resection of the tumor mass, radiotherapy and chemotherapy with temozolomide (TMZ). Despite this multimodal and aggressive strategy, GBM continues to have a dismal prognosis with almost inevitable recurrences. There is the need to find long-term strategies to target residual tumor cells infiltrated in the adjacent areas of brain and responsible for tumor perpetuation.

Cancer immunotherapy is now considered a potent weapon aims to harness the body's own immune system to fight tumor cells. Recently, the number of immunotherapy approaches to fight GBM is growing with encouraging results[18,19]. Moreover, data show that several anticancer agents, traditionally viewed as immunosuppressive, are now proposed as immune-adjuvants able to enforce anti-tumor immune response when combined with immunotherapy[163].

In our institute, we are currently carrying out a clinical trial based on immunotherapy with dendritic cells (DCs) loaded with whole tumor lysate. Patients with first diagnosis of GBM after surgery and leukapheresis receive standard treatment with radio-chemotherapy (RT-TMZ) and TMZ as adjuvant during DC vaccines. Flow cytometry analyses on peripheral blood lymphocytes indicated a significant activation of an NK, but not $CD8^+$ T, cell response correlated with a prolonged survival of patients. In light of these data, we moved from clinical observations to a murine malignant glioma model to evaluate a potential direct effect of TMZ on immune system.

We have found that NK cells, but not $CD8^+$ T lymphocytes, are able to up-regulate Abcc3, a multidrug resistant ATP transporter, as we extensively demonstrated in "**The multidrug-resistance transporter Abcc3 protects NK cells from chemotherapy in a murine model of malignant glioma**" OncoImmunology, Oct 29 2015 (DOI 10.1080/2162402X.2015.1108513).

In this study, our principal goal was to analyze the potential ability of TMZ to modulate local and systemic immune cells involved in anti-tumor response and influence their activity in GL261 glioma-bearing mice.

The first step was to characterize the direct impact of TMZ on frequency, cytotoxic ability, migration and consequent homing of NK and $CD8^+$ T cells.
Nine days after GL261 intracranial injection, mice were treated with TMZ for five days. Trafficking of $NK1.1^+$ $CD3^-$ NK and of $CD8^+$ T cells was investigated by flow cytometry in blood, their homing ability into the brain was evaluated as tumor infiltration by flow cytometry and immunofluorescence.
We also investigated the role of TMZ in modulating tumor microenvironment and providing conditions that can favor NK cell homing and activation. We observed in fact that TMZ treatment is able to exert a local modulation of the immunogenicity and to modify the chemokine-cytokine pattern increasing infiltration of NK cells with a stronger anti-tumor effector activity.

The second step was to evaluate the direct molecular mechanisms induced by TMZ on NK cells, important protagonists in anti-tumor

immune response of our patients, focusing on their ability to react to the cytotoxic effects of chemotherapy. By microarray analysis we identified three important gene signature related to migration, inhibition of apoptosis and multidrug resistance. Notably a high expression of Abcc3 was found physiologically expressed in NK cells and up-regulated during TMZ treatment.

The third goal was to specifically characterize the functional role of Abcc3 expressed by NK cells and its involvement in resistance to TMZ-induced apoptosis. NK cells expressing Abcc3 do not undergo apoptosis and show an activation of Akt, a key protein for immune cell survival. Pharmacological inhibition of Abcc3 in NK cells caused an increase of apoptosis demonstrating the crucial role of Abcc3 in resistance of NK cells to TMZ.

Results obtained will guide us to implement the knowledge of combined therapy modalities taking into account our clinical trials where GBM patients received chemo-immunotherapy.

Based on these pre-clinical observations we plan to verify the expression of Abcc3 in NK cells of patients before, during and after chemo-immunotherapy in order to define its potential role in the immune response correlated with a greater survival.

References

1. Bartek J, Ng K, Fischer W, Carter B, Chen CC. Key concepts in glioblastoma therapy. J Neurol Neurosurg Psychiatry 2012; 83:753–60.
2. Reardon DA, Rich JN, Friedman HS, Bigner DD. Recent advances in the treatment of malignant astrocytoma. J Clin Oncol 2006; 24:1253–65.
3. Guilfoyle MR, Weerakkody RA, Oswal A, Oberg I, Jeffery C, Haynes K, Kullar PJ, Greenberg D, Jefferies SJ, Harris F, et al. Implementation of neuro-oncology service reconfiguration in accordance with NICE guidance provides enhanced clinical care for patients with glioblastoma multiforme. Br J Cancer 2011; 104:1810–5.
4. Stupp R, Mason W, van den Bent MJ, Weller M, Fisher BM, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U, et al. Radiotherapy plus Concomitant and Adjuvant Temozolomide for Glioblastoma. N Engl J Med 2005; 352:987–96.
5. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJB, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009; 10:459–66.
6. Ohgaki H, Kleihues P. Genetic pathways to primary and secondary glioblastoma. Am J Pathol 2007; 170:1445–53.
7. Ohgaki H, Kleihues P. The definition of primary and secondary glioblastoma. Clin cancer Res 2013; 19:764–72.
8. Crespo I, Vital AL, Gonzalez-tablas M, Otero A, Lopes C, Oliveira C De, Domingues P, Orfao A, Dolores M, Tabernero MD. Molecular and Genomic Alterations in Glioblastoma Multiforme. Am J Pathol 2015; 185:1820–33.
9. Parsons DW, Jones S, Zhang X, Lin JC-H, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu I-M, Gallia GL, et al. An integrated genomic analysis of human glioblastoma multiforme. Science 2008; 321:1807–12.
10. The Cancer Genome Atlas. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008; 455:1061–8.
11. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Wilkerson MD, Miller CR, Ding L, Golub T, Jill P, Alexe G, et al. An integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by

abnormalities in PDGFRA, IDH1, EGFR and NF1. Cancer Cell 2011; 17:1–25.

12. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro JM, Colman H, Soroceanu L, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006; 9:157–73.

13. De Bacco F, Casanova E, Medico E, Pellegatta S, Orzan F, Albano R, Luraghi P, Reato G, D'Ambrosio A, Porrati P, et al. The MET oncogene is a functional marker of a glioblastoma stem cell subtype. Cancer Res 2012; 72:4537–50.

14. Prins RM, Soto H, Konkankit V, Odesa SK, Eskin A, Yong WH, Nelson SF, Liau LM. Gene Expression Profile Correlates with T-Cell Infiltration and Relative Survival in Glioblastoma Patients Vaccinated with Dendritic Cell Immunotherapy. Clin Cancer Res 2010; 17:1603–15.

15. Tiffany Doucette, Ganesh Rao, Arvind Rao, Li Shen, Kenneth Aldape JW, Kristine Dziurzynski, Mark Gilbert and ABH. Immune Heterogeneity of Glioblastoma Subtypes: Extrapolation from the Cancer Genome Atlas. Cancer Immunol Res 2013; 29:997–1003.

16. Norden AD, Young GS, Setayesh K, Muzikansky A, Klufas R, Ross GL, Ciampa AS, Ebbeling LG, Levy B, Drappatz J, et al. Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence. Neurology 2008; 70:779–87.

17. Gil-Gil MJ, Mesia C, Rey M, Bruna J. Bevacizumab for the treatment of glioblastoma. Clin Med Insights Oncol 2013; 7:123–35.

18. Finocchiaro G, Pellegatta S. Perspectives for immunotherapy in glioblastoma treatment. Curr Opin Oncol 2014; 26:608–14.

19. Weathers S-P, Gilbert MR. Current challenges in designing GBM trials for immunotherapy. J Neurooncol 2015; 123:331–7.

20. Pellegatta S, Eoli M, Frigerio S, Antozzi C, Bruzzone MG, Cantini G, Nava S, Anghileri E, Cuppini L, Cuccarini V, et al. The natural killer cell response and tumor debulking are associated with prolonged survival in recurrent glioblastoma patients receiving dendritic cells loaded with autologous tumor lysates. Oncoimmunology 2013; 2:e23401.

21. Bregy A, Wong TM, Shah AH, Goldberg JM, Komotar RJ. Active immunotherapy using dendritic cells in the treatment of glioblastoma multiforme. Cancer Treat Rev 2013; 39:891–907.

22. Prins RM, Wang X, Soto H, Young E, Lisiero DN, Fong B, Everson R, Yong WH, Lai A, Li G, et al. Comparison of glioma-associated antigen

peptide-loaded versus autologous tumor lysate-loaded dendritic cell vaccination in malignant glioma patients. J Immunother 2013; 36:152–7.

23. Mitchell DA, Batich KA, Gunn MD, Huang M, Sanchez-perez L, Nair SK, Congdon KL, Reap EA, Archer GE, Desjardins A, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. Nature 2015;

24. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, Friedman HS, Gilbert MR, Herndon JE, McLendon RE, Mitchell DA, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. J Clin Oncol 2010; 28:4722–9.

25. Johnson LA, Scholler J, Ohkuri T, Kosaka A, Patel PR, Mcgettigan SE, Nace AK, Dentchev T, Thekkat P, Loew A, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. Sci Transl Med 2015; 7.

26. Sampson JH, Choi BD, Sanchez-Perez L, Suryadevara CM, Snyder DJ, Flores CT, Schmittling RJ, Nair SK, Reap EA, Norberg PK, et al. EGFRvIII mCAR-modified T-cell therapy cures mice with established intracerebral glioma and generates host immunity against tumor-antigen loss. Clin Cancer Res 2014; 20:972–84.

27. Ampie L, Woolf EC, Dardis C. Immunotherapeutic advancements for glioblastoma. Front Oncol 2015; 5:1–8.

28. Preusser M, Lim M, Hafler DA, Reardon DA, Sampson JH. Prospects of immune checkpoint modulators in the treatment of glioblastoma. Nat Rev Neurol 2015; 11:504–14.

29. Carson MJ, Doose JM, Melchior B, Schmid CD, Ploix CC. CNS immune privilege: hiding in plain sight. Immunol Rev 2006; 213:48–65.

30. Tran Thang NN, Derouazi M, Philippin G, Arcidiaco S, Di Berardino-Besson W, Masson F, Hoepner S, Riccadonna C, Burkhardt K, Guha A, et al. Immune infiltration of spontaneous mouse astrocytomas is dominated by immunosuppressive cells from early stages of tumor development. Cancer Res 2010; 70:4829–39.

31. Grauer OM, Wesseling P, Adema GJ. Immunotherapy of Diffuse Gliomas: Biological Background, Current Status and Future Developments. Brain Pathol 2009; 19:674–93.

32. Heimberger AB, Crotty LE, Archer GE, McLendon RE, Friedman A, Dranoff G, Bigner DD, Sampson JH. Bone marrow-derived dendritic cells pulsed with tumor homogenate induce immunity against syngeneic intracerebral glioma. J Neuroimmunol 2000; 103:16–25.

33. Sampson JH, Archer GE, Ashley DM, Fuchs HE, Hale LP, Dranoff G, Bigner DD. Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8+ cell-mediated immunity against tumors located in the "immunologically privileged" central nervous system. Proc Natl Acad Sci U S A 1996; 93:10399–404.

34. Pellegatta S, Poliani PL, Corno D, Menghi F, Ghielmetti F, Suarez-Merino B, Caldera V, Nava S, Ravanini M, Facchetti F, et al. Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas. Cancer Res 2006; 66:10247–52.

35. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, Derecki NC, Castle D, Mandell JW, Lee KS, et al. Structural and functional features of central nervous system lymphatic vessels. Nature 2015; 523:337–41.

36. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity 2013; 39:11–26.

37. Dunn GP, Dunn IF, Curry WT. Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human glioma. Cancer Immun 2007; 7:12.

38. Donson AM, Birks DK, Schittone S a, Kleinschmidt-DeMasters BK, Sun DY, Hemenway MF, Handler MH, Waziri AE, Wang M, Foreman NK. Increased immune gene expression and immune cell infiltration in high-grade astrocytoma distinguish long-term from short-term survivors. J Immunol 2012; 189:1920–7.

39. Kmiecik J, Poli A, Brons NHC, Waha A, Eide GE, Enger PØ, Zimmer J, Chekenya M. Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level. J Neuroimmunol 2013; 264:71–83.

40. Yang I, Han SJ, Sughrue ME, Tihan T, Parsa AT. Immune cell infiltrate differences in pilocytic astrocytoma and glioblastoma: evidence of distinct immunological microenvironments that reflect tumor biology. J Neurosurg 2011; 115:505–11.

41. Kuppner MC, Hamou MF, de Tribolet N. Immunohistological and functional analyses of lymphoid infiltrates in human glioblastomas. Cancer Res 1988; 48:6926–32.

42. Caleb Rutledge W, Kong J, Gao J, Gutman D a., Cooper L a D, Appin C, Park Y, Scarpace L, Mikkelsen T, Cohen ML, et al. Tumor-infiltrating

lymphocytes in glioblastoma are associated with specific genomic alterations and related to transcriptional class. Clin Cancer Res 2013; 19:4951–60.

43. El Andaloussi A, Lesniak MS. CD4+ CD25+ FoxP3+ T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas. J Neurooncol 2007; 83:145–52.

44. Fecci PE, Heimberger AB, Sampson JH. Immunotherapy for primary brain tumors: no longer a matter of privilege. Clin cancer Res 2014; 20:5620–9.

45. Crane C a, Han SJ, Barry JJ, Ahn BJ, Lanier LL, Parsa AT. TGF-beta downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients. Neuro Oncol 2010; 12:7–13.

46. Jordan JT, Sun W, Hussain SF, DeAngulo G, Prabhu SS, Heimberger AB. Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy. Cancer Immunol Immunother 2008; 57:123–31.

47. Wainwright DA, Balyasnikova I V, Chang AL, Ahmed AU, Moon K-S, Auffinger B, Tobias AL, Han Y, Lesniak MS. IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival. Clin cancer Res 2012; 18:6110–21.

48. Facoetti A, Nano R, Zelini P, Morbini P, Benericetti E, Ceroni M, Campoli M, Ferrone S. Human leukocyte antigen and antigen processing machinery component defects in astrocytic tumors. Clin cancer Res 2005; 11:8304–11.

49. Anderson RCE, Anderson DE, Elder JB, Brown MD, Mandigo CE, Parsa AT, Goodman RR, McKhann GM, Sisti MB, Bruce JN. Lack of B7 expression, not human leukocyte antigen expression, facilitates immune evasion by human malignant gliomas. Neurosurgery 2007; 60:1129–36.

50. Wintterle S, Schreiner B, Mitsdoerffer M, Schneider D, Chen L, Meyermann R, Weller M, Wiendl H. Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis. Cancer Res 2003; 63:7462–7.

51. Wolpert F, Roth P, Lamszus K, Tabatabai G, Weller M, Eisele G. HLA-E contributes to an immune-inhibitory phenotype of glioblastoma stem-like cells. J Neuroimmunol 2012; 250:27–34.

52. Eisele G, Wischhusen J, Mittelbronn M, Meyermann R, Waldhauer I, Steinle A, Weller M, Friese M a. TGF-beta and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. Brain 2006; 129:2416–25.

53. Pellegatta S, Cuppini L, Finocchiaro G. Brain cancer immunoediting: novel examples provided by immunotherapy of malignant gliomas. Expert Rev Anticancer Ther 2011; 11:1759–74.

54. Ehtesham M, Black KL, Yu JS. Recent progress in immunotherapy for malignant glioma: treatment strategies and results from clinical trials. Cancer Control 2004; 11:192–207.

55. Galluzzi L, Senovilla L, Zitvogel L, Kroemer G. The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov 2012; 11:215–33.

56. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008; 8:59–73.

57. Shurin G V, Tourkova IL, Kaneno R, Shurin MR. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. J Immunol 2009; 183:137–44.

58. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini J-L, Castedo M, Mignot G, Panaretakis T, Casares N, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 2007; 13:54–61.

59. Panaretakis T, Joza N, Modjtahedi N, Tesniere A, Vitale I, Durchschlag M, Fimia GM, Kepp O, Piacentini M, Froehlich K-U, et al. The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death. Cell Death Differ 2008; 15:1499–509.

60. Panaretakis T, Kepp O, Brockmeier U, Tesniere A, Bjorklund A-C, Chapman DC, Durchschlag M, Joza N, Pierron G, van Endert P, et al. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. EMBO J 2009; 28:578–90.

61. Ma Y, Conforti R, Aymeric L, Locher C, Kepp O, Kroemer G, Zitvogel L. How to improve the immunogenicity of chemotherapy and radiotherapy. Cancer Metastasis Rev 2011; 30:71–82.

62. Kepp O, Galluzzi L, Martins I, Schlemmer F, Adjemian S, Michaud M, Sukkurwala AQ, Menger L, Zitvogel L, Kroemer G. Molecular determinants of immunogenic cell death elicited by anticancer chemotherapy. Cancer Metastasis Rev 2011; 30:61–9.

63. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med 2000; 192:565–70.

64. Ben-Sasson SZ, Wang K, Cohen J, Paul WE. IL-1β strikingly enhances

antigen-driven CD4 and CD8 T-cell responses. Cold Spring Harb Symp Quant Biol 2013; 78:117–24.

65. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annu Rev Immunol 2013; 31:51–72.

66. Ramakrishnan R, Assudani D, Nagaraj S, Hunter T, Cho H-I, Antonia S, Altiok S, Celis E, Gabrilovich DI. Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. J Clin Invest 2010; 120:1111–24.

67. van der Most RG, Currie AJ, Cleaver AL, Salmons J, Nowak AK, Mahendran S, Larma I, Prosser A, Robinson BWS, Smyth MJ, et al. Cyclophosphamide chemotherapy sensitizes tumor cells to TRAIL-dependent CD8 T cell-mediated immune attack resulting in suppression of tumor growth. PLoS One 2009; 4:e6982.

68. Hervieu A, Rébé C, Végran F, Chalmin F, Bruchard M, Vabres P, Apetoh L, Ghiringhelli F, Mignot G. Dacarbazine-Mediated Upregulation of NKG2D Ligands on Tumor Cells Activates NK and CD8 T Cells and Restrains Melanoma Growth. J Invest Dermatol 2012; :1–10.

69. Soriani A, Zingoni A, Cerboni C, Iannitto ML, Ricciardi MR, Di Gialleonardo V, Cippitelli M, Fionda C, Petrucci MT, Guarini A, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. Blood 2009; 113:3503–11.

70. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature 2005; 436:1186–90.

71. Kang TH, Mao C-P, Lee SY, Chen A, Lee J-H, Kim TW, Alvarez R, Roden RBS, Pardoll DM, Hung C-F, et al. Chemotherapy acts as an adjuvant to convert the tumor microenvironment into a highly permissive state for vaccination-induced antitumor immunity. Cancer Res 2013;73(8):2493-504.

72. Bracci L, Schiavoni G, Sistigu a, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. Cell Death Differ 2014; 21:15–25.

73. Slaney CY, Kershaw MH, Darcy PK. Trafficking of T Cells into Tumors. Cancer Res 2014; :1–8.

74. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, Solary E, Le Cesne A, Zitvogel L, Chauffert B. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores

T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007; 56:641–8.

75. Medler TR, Cotechini T, Coussens LM. Immune Response to Cancer Therapy: Mounting an Effective Antitumor Response and Mechanisms of Resistance. Trends in Cancer 2015; 1:66–75.

76. Chang C-L, Hsu Y-T, Wu C-C, Lai Y-Z, Wang C, Yang Y-C, Wu T-C, Hung C-F. Dose-Dense Chemotherapy Improves Mechanisms of Antitumor Immune Response. Cancer Res 2013; 73:119–27.

77. Mattarollo SR, Loi S, Duret H, Ma Y, Zitvogel L, Smyth MJ. Pivotal Role of Innate and Adaptive Immunity in Anthracycline Chemotherapy of Established Tumors. Cancer Res 2011; 71:4809–20.

78. Liu P, Jaffar J. Administration of cyclophosphamide changes the immune profile of tumor-bearing mice. J Immunother 2010; 33:53–9.

79. Sengupta S, Marrinan J, Frishman C, Sampath P. Impact of temozolomide on immune response during malignant glioma chemotherapy. Clin Dev Immunol 2012; 2012.

80. Jiang G, Wei Z-P, Pei D-S, Xin Y, Liu Y-Q, Zheng J-N. A novel approach to overcome temozolomide resistance in glioma and melanoma: Inactivation of MGMT by gene therapy. Biochem Biophys Res Commun 2011; 406:311–4.

81. Briegert M, Kaina B. Human monocytes, but not dendritic cells derived from them, are defective in base excision repair and hypersensitive to methylating agents. Cancer Res 2007; 67:26–31.

82. Litterman AJ, Zellmer DM, Grinnen KL, Hunt M a, Dudek AZ, Salazar AM, Ohlfest JR. Profound Impairment of Adaptive Immune Responses by Alkylating Chemotherapy. J Immunol 2013; 190(12):6259-68.

83. Roos W, Baumgartner M, Kaina B. Apoptosis triggered by DNA damage O6-methylguanine in human lymphocytes requires DNA replication and is mediated by p53 and Fas/CD95/Apo-1. Oncogene 2004; 23:359–67.

84. Banissi C, Ghiringhelli F, Chen L, Carpentier AF. Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model. Cancer Immunol Immunother 2009; 58:1627–34.

85. Iversen TZ, Brimnes MK, Nikolajsen K, Andersen RS, Hadrup SR, Andersen MH, Bastholt L, Svane IM. Depletion of T lymphocytes is correlated with response to temozolomide in melanoma patients. Oncoimmunology 2013; 2:e23288.

86. Ghiringhelli F, Ménard C, Martin F, Zitvogel L. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. Immunol Rev 2006; 214:229–38.

87. Sampson JH, Aldape KD, Archer GE, Coan A, Desjardins A, Friedman AH, Friedman HS, Gilbert MR, Herndon JE, McLendon RE, et al. Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. Neuro Oncol 2011; 13:324–33.

88. Asavaroengchai W, Kotera Y, Mulé JJ. Tumor lysate-pulsed dendritic cells can elicit an effective antitumor immune response during early lymphoid recovery. Proc Natl Acad Sci U S A 2002; 99:931–6.

89. Neyns B, Tosoni A, Hwu W-J, Reardon DA. Dose-dense temozolomide regimens: antitumor activity, toxicity, and immunomodulatory effects. Cancer 2010; 116:2868–77.

90. Park J-A, Joe Y-A, Kim T-G, Hong Y-K. Potentiation of antiglioma effect with combined temozolomide and interferon-beta. Oncol Rep 2006; 16:1253–60.

91. Kim T-G, Kim CKC-H, Park J-S, Park S-D, Kim CKC-H, Chung D-S, Hong Y-K. Immunological factors relating to the antitumor effect of temozolomide chemoimmunotherapy in a murine glioma model. Clin vaccine Immunol 2010; 17:143–53.

92. Candolfi M, Yagiz K, Wibowo M, Ahlzadeh GE, Puntel M, Ghiasi H, Kamran N, Paran C, Lowenstein PR, Castro MG. Temozolomide does not impair gene therapy-mediated antitumor immunity in syngeneic brain tumor models. Clin cancer Res 2014; 20:1555–65.

93. Heimberger AB, Sun W, Hussain SF, Dey M, Crutcher L, Aldape K, Gilbert M, Hassenbusch SJ, Sawaya R, Schmittling B, et al. Immunological responses in a patient with glioblastoma multiforme treated with sequential courses of temozolomide and immunotherapy: case study. Neuro Oncol 2008; 10:98–103.

94. Kyte JA, Gaudernack G, Dueland S, Trachsel S, Julsrud L, Aamdal S. Telomerase peptide vaccination combined with temozolomide: a clinical trial in stage IV melanoma patients. Clin cancer Res 2011; 17:4568–80.

95. Liau LM, Prins RM, Kiertscher SM, Odesa SK, Kremen TJ, Giovannone AJ, Lin J-W, Chute DJ, Mischel PS, Cloughesy TF, et al. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. Clin cancer Res 2005; 11:5515–25.

96. Zhang D, Jing Z, Qiu B, Wu A, Wang Y. Temozolomide decreases invasion of glioma stem cells by down-regulating TGF-β2. Oncol Rep 2011; 26:901–8.

97. Kim JT, Kim J-S, Ko KW, Kong D-S, Kang C-M, Kim MH, Son MJ, Song

HS, Shin H-J, Lee D-S, et al. Metronomic treatment of temozolomide inhibits tumor cell growth through reduction of angiogenesis and augmentation of apoptosis in orthotopic models of gliomas. Oncol Rep 2006; 16:33–9.

98. Bruyère C, Mijatovic T, Lonez C, Spiegl-Kreinecker S, Berger W, Kast RE, Ruysschaert J-M, Kiss R, Lefranc F. Temozolomide-induced modification of the CXC chemokine network in experimental gliomas. Int J Oncol 2011; 38:1453–64.

99. Hong M, Puaux A-L, Huang C, Loumagne L, Tow C, Mackay C, Kato M, Prévost-Blondel A, Avril M-F, Nardin A, et al. Chemotherapy induces intratumoral expression of chemokines in cutaneous melanoma, favoring T-cell infiltration and tumor control. Cancer Res 2011; 71:6997–7009.

100. Lamb LS, Bowersock J, Dasgupta A, Gillespie GY, Su Y, Johnson A, Spencer HT. Engineered Drug Resistant γδ T Cells Kill Glioblastoma Cell Lines during a Chemotherapy Challenge: A Strategy for Combining Chemo- and Immunotherapy. PLoS One 2013; 8:e51805.

101. Chang G. Multidrug resistance ABC transporters. FEBS Lett 2003; 555:102–5.

102. Szakács G, Váradi A, Ozvegy-Laczka C, Sarkadi B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov Today 2008; 13:379–93.

103. Ozben T. Mechanisms and strategies to overcome multiple drug resistance in cancer. FEBS Lett 2006; 580:2903–9.

104. Gillet J, Efferth T, Remacle J. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. Biochim Biophys Acta - Rev Cancer 2007; 1775:237–62.

105. Fletcher JI, Haber M, Henderson MJ, Norris MD. ABC transporters in cancer: more than just drug efflux pumps. Nat Rev Cancer 2010; 10:147–56.

106. Haimeur a, Conseil G, Deeley RG, Cole SPC. The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Metab 2004; 5:21–53.

107. Deeley RG. Transmembrane Transport of Endo- and Xenobiotics by Mammalian ATP-Binding Cassette Multidrug Resistance Proteins. Physiol Rev 2006; 86:849–99.

108. Szakács G, Hall MD, Gottesman MM, Boumendjel A, Kachadourian R, Day BJ, Baubichon-Cortay H, Di Pietro A. Targeting the Achilles heel of multidrug-resistant cancer by exploiting the fitness cost of resistance. Chem Rev 2014; 114:5753–74.

109. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002; 2:48–58.

110. Pallis M, Russell N. P-glycoprotein plays a drug-efflux-independent role in augmenting cell survival in acute myeloblastic leukemia and is associated with modulation of a sphingomyelin-ceramide apoptotic pathway. Blood 2000; 95:2897–904.

111. Tainton KM, Smyth MJ, Jackson JT, Tanner JE, Cerruti L, Jane SM, Darcy PK, Johnstone RW. Mutational analysis of P-glycoprotein: suppression of caspase activation in the absence of ATP-dependent drug efflux. Cell Death Differ 2004; 11:1028–37.

112. Robinson LJ, Roberts WK, Ling TT, Lamming D, Sternberg SS, Roepe PD. Human MDR 1 Protein Overexpression Delays the Apoptotic Cascade in Chinese Hamster Ovary Fibroblasts. Biochemistry 1997; 36:11169–78.

113. Johnstone RW, Cretney E, Smyth MJ. P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. Blood 1999; 93:1075–85.

114. Lehne G, Sørensen DR, Tjønnfjord GE, Beiske C, Hagve T-A, Rugstad HE, Clausen OPF. The cyclosporin PSC 833 increases survival and delays engraftment of human multidrug-resistant leukemia cells in xenotransplanted NOD-SCID mice. Leukemia 2002; 16:2388–94.

115. Peaston AE, Gardaneh M, Franco A V, Hocker JE, Murphy KM, Farnsworth ML, Catchpoole DR, Haber M, Norris MD, Lock RB, et al. MRP1 gene expression level regulates the death and differentiation response of neuroblastoma cells. Br J Cancer 2001; 85:1564–71.

116. Kuss BJ, Corbo M, Lau WM, Fennell DA, Dean NM, Cotter FE. In vitro and in vivo downregulation of MRP1 by antisense oligonucleotides: a potential role in neuroblastoma therapy. Int J cancer 2002; 98:128–33.

117. Sassi Y, Lipskaia L, Vandecasteele G, Nikolaev VO, Hatem SN, Cohen Aubart F, Russel FG, Mougenot N, Vrignaud C, Lechat P, et al. Multidrug resistance-associated protein 4 regulates cAMP-dependent signaling pathways and controls human and rat SMC proliferation. J Clin Invest 2008; 118:2747–57.

118. Katoh S-Y, Ueno M, Takakura N. Involvement of MDR1 function in proliferation of tumour cells. J Biochem 2008; 143:517–24.

119. van de Ven R, Scheffer GL, Reurs AW, Lindenberg JJ, Oerlemans R, Jansen G, Gillet J-P, Glasgow JN, Pereboev A, Curiel DT, et al. A role for multidrug resistance protein 4 (MRP4; ABCC4) in human dendritic cell migration. Blood 2008; 112:2353–9.

120. Barakat S, Turcotte S, Demeule M, Lachambre M-P, Régina A, Baggetto

LG, Béliveau R. Regulation of brain endothelial cells migration and angiogenesis by P-glycoprotein/caveolin-1 interaction. Biochem Biophys Res Commun 2008; 372:440–6.

121. Miletti-González KE, Chen S, Muthukumaran N, Saglimbeni GN, Wu X, Yang J, Apolito K, Shih WJ, Hait WN, Rodriguez-Rodriguez L. The CD44 receptor interacts with P-glycoprotein to promote cell migration and invasion in cancer. Cancer Res 2005; 65:6660–7.

122. Colone M, Calcabrini A, Toccacieli L, Bozzuto G, Stringaro A, Gentile M, Cianfriglia M, Ciervo A, Caraglia M, Budillon A, et al. The multidrug transporter P-glycoprotein: a mediator of melanoma invasion. J Invest Dermatol 2008; 128:957–71.

123. van de Ven R, Oerlemans R, van der Heijden JW, Scheffer GL, de Gruijl TD, Jansen G, Scheper RJ. ABC drug transporters and immunity: novel therapeutic targets in autoimmunity and cancer. J Leukoc Biol 2009; 86:1075–87.

124. Giraud C, Manceau S, Declèves X, Goffinet F, Morini J-P, Chappuy H, Batteux F, Chouzenoux S, Yousif S, Scherrmann J-M, et al. Influence of development, HIV infection, and antiretroviral therapies on the gene expression profiles of ABC transporters in human lymphocytes. J Clin Pharmacol 2010; 50:226–30.

125. Janneh O, Jones E, Chandler B, Owen A, Khoo SH. Inhibition of P-glycoprotein and multidrug resistance-associated proteins modulates the intracellular concentration of lopinavir in cultured CD4 T cells and primary human lymphocytes. J Antimicrob Chemother 2007; 60:987–93.

126. Elliott JI, Raguz S, Higgins CF. Multidrug transporter activity in lymphocytes. Br J Pharmacol 2004; 143:899–907.

127. Albermann N, Schmitz-Winnenthal FH, Z'graggen K, Volk C, Hoffmann MM, Haefeli WE, Weiss J. Expression of the drug transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, BCRP/ABCG2, and PXR in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver. Biochem Pharmacol 2005; 70:949–58.

128. Köck K, Grube M, Jedlitschky G, Oevermann L, Siegmund W, Ritter C a, Kroemer HK. Expression of adenosine triphosphate-binding cassette (ABC) drug transporters in peripheral blood cells: relevance for physiology and pharmacotherapy. Clin Pharmacokinet 2007; 46:449–70.

129. Raggers RJ, Vogels I, van Meer G. Multidrug-resistance P-glycoprotein (MDR1) secretes platelet-activating factor. Biochem J 2001; 357:859–65.

130. Leier I, Jedlitschky G, Buchholz U, Cole SP, Deeley RG, Keppler D. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. J Biol Chem 1994; 269:27807–10.

131. Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J, Borst P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 2003; 100:9244–9.

132. Honig SM, Fu S, Mao X, Yopp A, Gunn MD, Randolph GJ, Bromberg JS. FTY720 stimulates multidrug transporter- and cysteinyl leukotriene-dependent T cell chemotaxis to lymph nodes. J Clin Invest 2003; 111:627–37.

133. Randolph GJ, Beaulieu S, Pope M, Sugawara I, Hoffman L, Steinman RM, Muller WA. A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. Proc Natl Acad Sci U S A 1998; 95:6924–9.

134. Herget M, Tampé R. Intracellular peptide transporters in human--compartmentalization of the "peptidome". Eur J Physiol 2007; 453:591–600.

135. Ludescher C, Pall G, Irschick EU, Gastl G. Differential activity of P-glycoprotein in normal blood lymphocyte subsets. Br J Haematol 1998; 101:722–7.

136. Drach J, Gsur A, Hamilton G, Zhao S, Angerler J, Fiegl M, Zojer N, Raderer M, Haberl I, Andreeff M, et al. Involvement of P-glycoprotein in the transmembrane transport of interleukin-2 (IL-2), IL-4, and interferon-gamma in normal human T lymphocytes. Blood 1996; 88:1747–54.

137. Frank MH, Denton MD, Alexander SI, Khoury SJ, Sayegh MH, Briscoe DM. Specific MDR1 P-glycoprotein blockade inhibits human alloimmune T cell activation in vitro. J Immunol 2001; 166:2451–9.

138. Zhang J, Alston MA, Huang H, Rabin RL. Human T cell cytokine responses are dependent on multidrug resistance protein-1. Int Immunol 2006; 18:485–93.

139. Pilarski LM, Paine D, McElhaney JE, Cass CE, Belch AR. Multidrug transporter P-glycoprotein 170 as a differentiation antigen on normal human lymphocytes and thymocytes: modulation with differentiation stage and during aging. Am J Hematol 1995; 49:323–35.

140. Takeuchi K, Shibata M, Kashiyama E, Umehara KEN. Expression levels of multidrug resistance-associated protein 4 (MRP4) in human leukemia and lymphoma cell lines , and the inhibitory effects of the MRP-specific inhibitor MK-571 on methotrexate distribution in rats. Exp Ther Med 2012; 4:524–32.

141. Sarkadi B, Homolya L, Szakács G, Váradi A. Human multidrug

resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. Physiol Rev 2006; 86:1179–236.

142. Giraud C, Manceau S, Treluyer J-M. ABC transporters in human lymphocytes: expression, activity and role, modulating factors and consequences for antiretroviral therapies. Expert Opin Drug Metab Toxicol 2010; 6:571–89.

143. Dimeloe S, Frick C, Fischer M, Gubser PM, Razik L, Bantug GR, Ravon M, Langenkamp A, Hess C. Human regulatory T cells lack the cyclophosphamide-extruding transporter ABCB1 and are more susceptible to cyclophosphamide-induced apoptosis. Eur J Immunol 2014; 44:3614–20.

144. Lowdell MW. Natural killer cells in haematopoietic stem cell transplantation. Transfus Med 2003; 13:399–404.

145. Lamy T, Drenou B, Fardel O, Amiot L, Grulois I, Le Prise PY, Loughran TP, Fauchet R. Multidrug resistance analysis in lymphoproliferative disease of large granular lymphocytes. Br J Haematol 1998; 100:509–15.

146. Egashira M, Kawamata N, Sugimoto K, Kaneko T, Oshimi K. P-glycoprotein expression on normal and abnormally expanded natural killer cells and inhibition of P-glycoprotein function by cyclosporin A and its analogue, PSC833. Blood 1999; 93:599–606.

147. König J, Nies A, Cui Y, Leier I, Keppler D. Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochim Biophys Acta 1999; 1461.

148. Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A 1999; 96:6914–9.

149. Keppler D. Drug Transporters. Handb Exp Pharmacol 2011; 201.

150. Borst P, Zelcer N, van de Wetering K. MRP2 and 3 in health and disease. Cancer Lett 2006; 234:51–61.

151. Zhao Y, Lu H, Yan A, Yang Y, Meng Q, Sun L, Pang H, Li C, Dong X, Cai L. ABCC3 as a marker for multidrug resistance in non-small cell lung cancer. Sci Rep 2013; 3:3120.

152. Bruhn O, Cascorbi I. Polymorphisms of the drug transporters ABCB1, ABCG2, ABCC2 and ABCC3 and their impact on drug bioavailability and clinical relevance. Expert Opin Drug Metab Toxicol 2014; 10:1337–54.

153. O'Brien C, Cavet G, Pandita A, Hu X, Haydu L, Mohan S, Toy K, Rivers CS, Modrusan Z, Amler LC, et al. Functional genomics identifies ABCC3

as a mediator of taxane resistance in HER2-amplified breast cancer. Cancer Res 2008; 68:5380–9.

154. Yu Z, Zhang C, Wang H, Xing J, Gong H, Yu E, Zhang W, Zhang X, Cao G, Fu C. Multidrug resistance-associated protein 3 confers resistance to chemoradiotherapy for rectal cancer by regulating reactive oxygen species and caspase-3-dependent apoptotic pathway. Cancer Lett 2014; 353:182–93.

155. König J, Hartel M, Nies AT, Martignoni ME, Guo J, Büchler MW, Friess H, Keppler D. Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. Int J cancer 2005; 115:359–67.

156. Kuan C-T, Wakiya K, Herndon JE, Lipp ES, Pegram CN, Riggins GJ, Rasheed A, Szafranski SE, McLendon RE, Wikstrand CJ, et al. MRP3: a molecular target for human glioblastoma multiforme immunotherapy. BMC Cancer 2010; 10:468.

157. Young LC, Campling BG, Cole SP, Deeley RG, Gerlach JH. Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. Clin cancer Res 2001; 7:1798–804.

158. Kruh GD, Belinsky MG. The MRP family of drug efflux pumps. Oncogene 2003; 22:7537–52.

159. Zelcer N, Saeki T, Reid G, Beijnen JH, Borst P. Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3). J Biol Chem 2001; 276:46400–7.

160. Lockhart AC, Tirona RG, Kim RB. Pharmacogenetics of ATP-binding cassette transporters in cancer and chemotherapy. Mol Cancer Ther 2003; 2:685–98.

161. Giannoudis A, Davies A, Harris RJ, Lucas CM, Pirmohamed M, Clark RE. The clinical significance of ABCC3 as an imatinib transporter in chronic myeloid leukaemia. Leukemia 2014; 28:1360–3.

162. Plasschaert SLA, de Bont ESJM, Boezen M, vander Kolk DM, Daenen SMJG, Faber KN, Kamps WA, de Vries EGE, Vellenga E. Expression of MRPs predicts prognosis in childhood and adult acute lymphoblastic leukemia. Clin cancer Res 2005; 11:8661–8.

163. Emens L a, Middleton G. The Interplay of Immunotherapy and Chemotherapy: Harnessing Potential Synergies. Cancer Immunol Res 2015; 3:436–43.

Chapter 2

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 glioma-bearing mice treated with the alkylating agent temozolomide (TMZ) for five days, an early increased frequency of NK cells was observed. We also found that Abcc3 is strongly up-regulated 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 IFN-γ 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 IFN-γ, 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 chemo-immunotherapy.

Introduction

Current therapeutic options for glioblastoma (GBM) patients include chemotherapy with the alkylating agent temozolomide (TMZ).[1,2] Recent data have suggested that some chemotherapeutic agents, previously viewed as immunosuppressive, possess immune-modulatory effects[3,4] and influence the vaccine-induced immune response affecting the quality and efficacy of the T cell response[5] or enhancing the immunogenicity of dying tumor cells.[3,4] TMZ leads to transient lymphodepletion[6] 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 cells[9,10] 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 anti-tumor 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 MRP1 (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 ABCC1.[14,15]

In a clinical trial currently active at our institution (DENDR1 - EUDRACT N° 2008-005035-15), 24 patients with first diagnosis of GBM have been treated with DC 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 up-regulated 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 days (Figure 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 hours (hrs), after two administrations of chemotherapy ($p < 0.0001$ vs controls) and quickly increased at 72 hrs ($p < 0.01$ vs 48hrs; Figure 1B). On the contrary, peripheral blood NK cells increased significantly at early time point, doubled 72 hrs after the first TMZ administration and remained higher than controls throughout the entire treatment (Figure 1C). To assess a possible delayed effect of TMZ on immune cells, we performed similar evaluations at day 19, 5 days after ending chemotherapy. We did not observe a significant difference between $CD8^+$ T cells in blood of TMZ-treated mice compared to controls (Figure 1B) while NK cells were still increased in blood of TMZ- compared to vehicle-treated mice (Figure 1C). In non-glioma-bearing mice TMZ induced a modulation of $CD8^+$ T lymphocytes and NK cells similar to TMZ-treated tumor bearing mice (Figure 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 hrs $CD8^+$ T cells decreased in TMZ-treated mice compared with controls (Figure 1D); at 240 hrs, no differences were found between TMZ-treated mice and controls ($p = 0.3$; Figure 1D). NK cells in the tumor showed a similar pattern of systemic NK cells, increasing significantly at 72 hrs after the first TMZ administration and continued to be significantly elevated at 240 hrs ($p < 0.005$ at both time points, Figure 1E).

Hematoxilin & eosin staining confirmed the evidence of a vigorous immune cell infiltration in response to TMZ compared with the vehicle at 72 hrs. In situ immunofluorescence established the predominant infiltration of NK cells located into the tumor mass (Figure 1F). Quantitative determination of TILs from the same groups of treatment was obtained by flow cytometry (Figure 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 up-regulated 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 hrs 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 up-regulated genes were related to ABC drug transporters: *Abcc3*, *Abca9* and *Abca6*[16]. Other genes were related to the inhibition of apoptosis (*CD5L* and *Naip1*) and cell survival (*Ednrb*, *Gata6* and *Fgfr1*), an indication of the predisposition of NK cells to resist the cytotoxic effect of TMZ[17–20].

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[21,22].

Notably, at this time point, down-regulated genes were related to NK cell-mediated cytotoxicity (*GzmD*, *GzmE*, *GzmG* and *GmzC*) and to secretory pathway or inflammatory response (*Scgb1a1* and *Elane*)[23].

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 up-regulated 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; Figure 2A and B).

To investigate *in vitro* the expression of Abca6 and Abcc3 during TMZ administration, we treated PBLs from naïve mice with 1 µM TMZ or DMSO at different time points. The dosage was determined according to TMZ concentrations measured in the plasma of patients treated with the "standard" schedule.[24] The up-regulation of Abca6 expression was observed only after 6 and 8 hrs of TMZ treatment (2.0 ± 0.1–fold and 3.3 ± 0.1–fold, respectively vs DMSO-treated PBLs; $p = 0.01$, $p < 0.005$; Figure 2C). On the contrary, the up-regulation of Abcc3 expression was detectable after 4 hrs ($p < 0.05$) and increased over the time during TMZ treatment, suggesting a direct effect of chemotherapy on its expression (Figure 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 up-regulation of Abcc3 compared to controls during chemotherapy (31.2 ± 0.8% vehicle NK cells vs 59.8 ± 1.1% TMZ-treated NK cells, $p < 0.0001$; Figure 2E, left). No significant difference in Abcc3 expression was

found in $CD8^+$ T cells from TMZ-treated mice compared to controls ($2.0 \pm 0.6\%$ vehicles vs $1.5 \pm 0.5\%$ TMZ-treated mice, Figure 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 µM TMZ or DMSO for 4 hrs. NK and $CD8^+$ T cells were gated on PBLs, and the 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% (Figure 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 ($p<0.001$; Figure 2G). Results obtained with a lower dose of TMZ suggested that the efflux activity

was dose-dependent in NK cells (Figure S2). No evidence of a resistant phenotype was found in TMZ-treated $CD8^+$ T cells, showing a MAF < 25% for all ABC transporter families (Figure 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 hrs. Apoptotic cells were measured by flow cytometry on gated NK and $CD8^+$ T cell populations using Annexin V and Propidium Iodide (PI) staining (Figure 3 A-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 hrs ($p < 0.01$; Figure 3A). Abcc3 negative NK cells showed a higher percentage of apoptotic cells compared to Abcc3 positive NK cells in response to TMZ (Figure 3B). $CD8^+$ T cells showed a remarkable increase of early and late apoptosis after 2 hrs and later ($p < 0.005$, $p < 0.001$; Figure 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 (Figure 3D). On the contrary, in $CD8^+$ T cells, no significant difference in Akt activation was detected in TMZ-treated cells (Figure 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 minutes for 30 minutes, 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; Figure 3E). $Abcc3^+CD8^+$ T cells from TMZ-treated and control mice did not exhibit Akt phosphorylation ($2.9 \pm 1.2\%$ TMZ-treated vs $3.3 \pm 0.9\%$ control mice; Figure 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 4hrs (Figure 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 hrs. 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 ($p<0.0001$, Figure 4A).

We then analyzed the expression of CD49b and CD49d integrin subunits involved in cellular adhesion and leukocyte tissue infiltration.[27] $CD49b^+$ $CD49d^+$ 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 (Figure 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.[28,29] In blood, immature $CD11b^{low}CD27^{low}$ NK cells significantly decreased after three days of chemotherapy ($p < 0.001$ vs controls). Interestingly, TMZ led to a significant enrichment of the $CD11b^{low}CD27^{high}$ and $CD11b^{high}CD27^{high}$ NK cell subsets with migratory potential and a simultaneous decrease of the $CD11b^{high}$ $CD27^{low}$ cytotoxic subset ($p < 0.001$ vs controls; Figure 4C). The increase of the $CD11b^{high}$ $CD27^{low}$ cytotoxic subset was observed five days after the end of TMZ administration ($p=0.001$; Figure 4D).

On the contrary, in gliomas of TMZ-treated mice there was a strong accumulation of the $CD11b^{high}CD27^{low}$NK effector subset 72 hrs after the first TMZ administration ($p < 0.005$, Figure 4E). This increase persisted at later time points ($p < 0.005$, Figure 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, IFN-γ production in blood-derived NK cells from TMZ-treated mice increased compared to controls (7.5 ± 1.0% vs 16.9 ± 1.2%; $p < 0.005$; Figure 5A).

To test the cytotoxic specificity of NK cells, PBL from naïve 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 ($p < 0.0001$, Figure 5B).

The accumulation of the NK cytotoxic subset in gliomas was associated to increased expression of *Perforin 1* (*Prf1*), *Granzyme B* (*GzmB*) and *Ifn-γ* (by 9.3± 0.04-fold, 8.1± 0.02-fold and 4.10 ± 0.03-fold, respectively; $p<0.0001$) in TMZ-treated mice (Figure 5C). This up-regulation persisted on day 19, five days after the end of TMZ treatment (*Prf1*: 3.97± 0.07-fold, *GzmB*: 2.2 ± 0.06-fold and *Ifn-γ:* 2.4 ± 0.02-fold, $p<0.0001$ vs controls; Figure 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[30–32] and found that they were expressed at high levels in tumors of vehicle-treated mice and significantly less in those of TMZ-treated mice (Figure 6A).

A decreased 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 (Figure 6B). We also investigated the effects of TMZ administration on intra-tumor expression of chemokines and cytokines involved in modulating the infiltration of immune cells. CCL3 (which expression is related to NK cell accumulation[10]), TNF−α (which is not only involved in anti-tumor immune response but also responsible for decreased GL261 proliferation[33]), IL-7 (which plays a role in promoting NK cell survival and inhibiting apoptosis[34]), and IL-27 (which is an important stimulator of NK cell effector function[35]), all increased in gliomas from TMZ-treated mice compared to controls (Figure 6C). In contrast, CXCL10, which role in glioma progression is contradictory[36–38], and CCL5, which expression was recently most related with CD8A levels[39], and previously described as immunosuppressor by us and others[10,40], significantly decreased.

Finally, we investigated *in vitro* the effect of TMZ on glioma immunogenicity. GL261 cells were treated with 50 and 150 μM TMZ or DMSO at different time points. TGF-β1 and TGF-β2 concentrations

in the supernatant from TMZ-treated GL261 cells significantly decreased, as evaluated by ELISA (Figure 6D). Nkg2d ligand (*Nkg2dl*), involved in Nkg2d-mediated NK cell recognition of tumor cells and weakly expressed in GL261 cells, was up-regulated in a time- and dose-dependent manner after TMZ treatment (Figure S3). Similarly, we found a time- and dose-dependent increase of the Rae-1 -β, -ε and –δ, ligands for the Nkg2d receptor (Figure 6E); while B7-h3, a NK cell inhibitory molecule highly expressed in GL261 cells, was significantly decreased (Figure S3).

These results indicate that TMZ modulate glioma microenvironment into a site favoring NK cell infiltration and anti-tumor 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 up-regulated 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 Bcl-2 protein levels and activation of the apoptotic cascade.[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.[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.[36] Notably, Akt activation has been correlated with increased expression and activity of some ABC transporters[44–46] and there is evidence that Akt and PI3K/Akt pathway are responsible for cytotoxic and killing ability of NK cells.[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 4-stage model of NK cell maturation.[28] While different studies have shown that ABC transporters play a role in the migration of cancer and normal cells, including immune cells[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 up-regulated 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 anti-tumor immune responses mediated by dendritic cells.[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 IFN-□ 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 up-regulation 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.[49] Murine and human NK cells were found to express high levels of multidrug transporters, which could confer protection against chemotherapeutic agents.[61] TMZ administration, similar to other chemotherapeutics, can cause the development of moderate to severe lymphopenia and myelosuppression[62,63], indicating that immune inhibitory effects take place through selective toxicity on proliferating lymphocytes and inhibition of immune effector differentiation.[63,64] However, there is evidence that following a transient chemotherapy-

induced lymphopenia, lymphocytes undergo homeostatic proliferation that enhances anti-tumor, vaccine-induced immune responses[6,65,66] with positive clinical outcome correlation.[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 $CD3^-$ $CD56^+$ effector cells in blood of GBM patients.[57] 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.[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[6970], relevant to generate a T cell memory and possibly amplify anti-tumor 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), β-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 2 groups, treated 9 days 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 $CD8^+$ T and NK cells (NK and $CD8^+$ T Cell Isolation Kits, Miltenyi Biotec) resulting in a 97±1.5% and 93.2±2.9% pure $CD8^+$ 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 staining buffer at 4°C in the dark. The following antibodies were used: CD45, CD8, CD4, CD3, CD11b, CD27, NK1.1, NKp46, CD49b, CD49d, IFN-γ and pAkt (Miltenyi Biotec), Abcc3 (Abcam), B7-h3 (Biolegend) and Nkg2dl (eBioscience). DAPI was added to exclude dead cells. Flow cytometry acquisition was performed on a MACSQuant instrument, and data were analyzed with MACSQuantify Software (Miltenyi Biotec).

Microarray analyses and Real Time-PCR

Total RNA from purified NK and $CD8^+$ T cells was extracted with TRIzol reagent (Life Technologies) using RNeasy Mini Kit and 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 ≥ 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 ΔΔCt method normalizing to the housekeeping Gapdh, Actin and β2M levels. The primer sequences (Primm S.r.l.) are reported in Table S2.

Migration assay

Migration was assessed in vitro using 8μm Transwell migration chambers. Purified NK cells ($4x10^5$/transwell) were placed in the upper chamber and evaluated for their ability to transmigrate toward the lower chamber. Chemoattractant in the lower chamber was represented by medium from GL261 cells previously treated for 24 hours with DMSO or 150μM TMZ. After 12h, 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-pAKT (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 chemo-resistance. Briefly, naïve lymphocytes were treated with 1 μM TMZ or DMSO for 4 h in vitro. MK571 was added to the medium 30 minutes 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 manufactures' 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.

IFN-γ 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 hrs. Lymphocytes were stained for IFN-γ (BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit, BD Bioscience) according to the manufacturer's instructions.

A non-radioactive cytotoxic assay (Cytotoxicity detection kit^{plus} LDH, Roche) was performed to test PBL capacity of TMZ-treated, control and naïve mice to recognize and lyse GL261 cells, according to manufactures' instructions. PBLs were pre-stimulated for 5 days in the presence of 20 Gy-irradiated GL261 cells and tested 5 days later for GL261 cell specific cytotoxicity.

Histology and immunofluorescence

Double immunofluorescence was performed on 5μm 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 three to five 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 were performed using two-tailed Student's T-test. Results were considered significant at $p < 0.05$.

References

1. Stupp R, Mason W, van den Bent MJ, Weller M, Fisher BM, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U, et al. Radiotherapy plus Concomitant and Adjuvant Temozolomide for Glioblastoma. N Engl J Med 2005; 352:987–96.
2. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJB, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009; 10:459–66.
3. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008; 8:59–73.
4. Galluzzi L, Senovilla L, Zitvogel L, Kroemer G. The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov 2012; 11:215–33.
5. Emens LA, Jaffee EM. Leveraging the activity of tumor vaccines with cytotoxic chemotherapy. Cancer Res 2005; 65:8059–64.
6. Sampson JH, Aldape KD, Archer GE, Coan A, Desjardins A, Friedman AH, Friedman HS, Gilbert MR, Herndon JE, McLendon RE, et al. Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. Neuro Oncol 2011; 13:324–33.
7. Jordan JT, Sun W, Hussain SF, DeAngulo G, Prabhu SS, Heimberger AB. Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy. Cancer Immunol Immunother 2008; 57:123–31.
8. Kim T-G, Kim CKC-H, Park J-S, Park S-D, Kim CKC-H, Chung D-S, Hong Y-K. Immunological factors relating to the antitumor effect of temozolomide chemoimmunotherapy in a murine glioma model. Clin Vaccine Immunol 2010; 17:143–53.
9. Alizadeh D, Zhang L, Brown CE, Farrukh O, Jensen MC, Badie B. Induction of anti-glioma natural killer cell response following multiple low-dose intracerebral CpG therapy. Clin Cancer Res 2010; 16:3399–408.
10. Cantini G, Pisati F, Pessina S, Finocchiaro G, Pellegatta S. Immunotherapy against the radial glia marker GLAST effectively triggers specific antitumor effectors without autoimmunity. Oncoimmunology 2012; 1:884–93.

11. Pellegatta S, Eoli M, Frigerio S, Antozzi C, Bruzzone MG, Cantini G, Nava S, Anghileri E, Cuppini L, Cuccarini V, et al. The natural killer cell response and tumor debulking are associated with prolonged survival in recurrent glioblastoma patients receiving dendritic cells loaded with autologous tumor lysates. Oncoimmunology 2013; 2:e23401.

12. Banissi C, Ghiringhelli F, Chen L, Carpentier AF. Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model. Cancer Immunol Immunother 2009; 58:1627–34.

13. Oselin K, Mrozikiewicz PM, Pähkla R, Roots I. Quantitative determination of the human MRP1 and MRP2 mRNA expression in FACS-sorted peripheral blood CD4+, CD8+, CD19+, and CD56+ cells. Eur J Haematol 2003; 71:119–23.

14. Donnenberg VS, Burckart GJ, Zeevi A, Griffith BP, Iacono A, McCurry KR, Wilson JW, Donnenberg AD. P-glycoprotein activity is decreased in CD4+ but not CD8+ lung allograft-infiltrating T cells during acute cellular rejection. Transplantation 2004; 77:1699–706.

15. Van de Ven R, Oerlemans R, van der Heijden JW, Scheffer GL, de Gruijl TD, Jansen G, Scheper RJ. ABC drug transporters and immunity: novel therapeutic targets in autoimmunity and cancer. J Leukoc Biol 2009; 86:1075–87.

16. Glavinas H, Krajcsi P, Cserepes J, Sarkadi B. The role of ABC transporters in drug resistance, metabolism and toxicity. Curr Drug Deliv 2004; 1:27–42.

17. Liston P, Fong WG, Korneluk RG. The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene 2003; 22:8568–80.

18. Kuwata K, Watanabe H, Jiang S-Y, Yamamoto T, Tomiyama-Miyaji C, Abo T, Miyazaki T, Naito M. AIM inhibits apoptosis of T cells and NKT cells in Corynebacterium-induced granuloma formation in mice. Am J Pathol 2003; 162:837–47.

19. Cai W, Shen F, Li J, Feng Z, Wang Y, Xiao H, Xu B. Activated protease receptor-2 induces GATA6 expression to promote survival in irradiated colon cancer cells. Arch Biochem Biophys 2014; 555-556:28–32.

20. Wan X, Zhang L, Jiang B. Role of endothelin B receptor in oligodendroglioma proliferation and survival: In vitro and in vivo evidence. Mol Med Rep 2014; 9:229–34.

21. Zamora DO, Babra B, Pan Y, Planck SR, Rosenbaum JT. Human leukocytes express ephrinB2 which activates microvascular endothelial cells. Cell Immunol 2006; 242:99–109.

22. Bergström S-E, Bergdahl E, Sundqvist K-G. A cytokine-controlled mechanism for integrated regulation of T-lymphocyte motility, adhesion and activation. Immunology 2013; 140:441–55.

23. Ewen CL, Kane KP, Bleackley RC. A quarter century of granzymes. Cell Death Differ 2012; 19:28–35.

24. Ostermann S, Csajka C, Buclin T, Leyvraz S, Lejeune F, Decosterd LA, Stupp R. Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients. Clin Cancer Res 2004; 10:3728–36.

25. Bommhardt U, Chang KC, Swanson PE, Wagner TH, Tinsley KW, Karl IE, Hotchkiss RS. Akt Decreases Lymphocyte Apoptosis and Improves Survival in Sepsis. J Immunol 2004; 172:7583–91.

26. Plas DR, Rathmell JC, Thompson CB. Homeostatic control of lymphocyte survival: potential origins and implications. Nat Immunol 2002; 3:515–21.

27. Gan Y, Liu R, Wu W, Bomprezzi R, Shi FD. Antibody to α4 integrin suppresses natural killer cells infiltration in central nervous system in experimental autoimmune encephalomyelitis. J Neuroimmunol 2012; 247:9–15.

28. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. Blood 2009; 113:5488–96.

29. Fu B, Wang F, Sun R, Ling B, Tian Z, Wei H. CD11b and CD27 reflect distinct population and functional specialization in human natural killer cells. Immunology 2011; 133:350–9.

30. Baker GJ, Chockley P, Yadav VN, Doherty R, Ritt M, Sivaramakrishnan S, Castro MG, Lowenstein PR. Natural killer cells eradicate galectin-1-deficient glioma in the absence of adaptive immunity. Cancer Res 2014; 74:5079–90.

31. Wang W, Guo H, Geng J, Zheng X, Wei H, Sun R, Tian Z. Tumor-released Galectin-3, a soluble inhibitory ligand of human NKp30, plays an important role in tumor escape from NK cell attack. J Biol Chem 2014; 289:33311–9.

32. Golden-Mason L, McMahan RH, Strong M, Reisdorph R, Mahaffey S, Palmer BE, Cheng L, Kulesza C, Hirashima M, Niki T, et al. Galectin-9 functionally impairs natural killer cells in humans and mice. J Virol 2013; 87:4835–45.

33. Pellegatta S, Poliani PL, Stucchi E, Corno D, Colombo CA, Orzan F, Ravanini M, Finocchiaro G. Intra-tumoral dendritic cells increase efficacy

of peripheral vaccination by modulation of glioma microenvironment. Neuro Oncol 2010; 12:377–88.

34. Michaud A, Dardari R, Charrier E, Cordeiro P, Herblot S, Duval M. IL-7 enhances survival of human CD56bright NK cells. J Immunother 2010; 33:382–90.

35. Ziblat A, Domaica CI, Spallanzani RG, Iraolagoitia XLR, Rossi LE, Avila DE, Torres NI, Fuertes MB, Zwirner NW. IL-27 stimulates human NK-cell effector functions and primes NK cells for IL-18 responsiveness. Eur J Immunol 2015; 45:192–202.

36. Cai J, Zhang W, Yang P, Wang Y, Li M, Zhang C, Wang Z, Hu H, Liu Y, Li Q, et al. Identification of a 6-cytokine prognostic signature in patients with primary glioblastoma harboring M2 microglia/macrophage phenotype relevance. PLoS One 2015; 10:e0126022.

37. Huang B, Lei Z, Zhao J, Gong W, Liu J, Chen Z, Liu Y, Li D, Yuan Y, Zhang G-M, et al. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. Cancer Lett 2007; 252:86–92.

38. Maru S V, Holloway KA, Flynn G, Lancashire CL, Loughlin AJ, Male DK, Romero IA. Chemokine production and chemokine receptor expression by human glioma cells: role of CXCL10 in tumour cell proliferation. J Neuroimmunol 2008; 199:35–45.

39. Triozzi PL, SchoenfieldL, Plesec T, Sauntharajah Y, Tubbs RR and Singh AD. Molecular profiling of primary uveal melanomas with tumor-infiltrating lymphocytes. OncoImmunology 2014. DOI:10.4161/21624011.2014.947169.

40. Conforti R, Ma Y, Morel Y, Paturel C, Terme M, Viaud S, Ryffel B, Ferrantini M, Uppaluri R, Schreiber R, et al. Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. Cancer Res 2010; 70:490–500.

41. Peaston AE, Gardaneh M, Franco A V, Hocker JE, Murphy KM, Farnsworth ML, Catchpoole DR, Haber M, Norris MD, Lock RB, et al. MRP1 gene expression level regulates the death and differentiation response of neuroblastoma cells. Br J Cancer 2001; 85:1564–71.

42. Caporali S, Levati L, Graziani G, Muzi A, Atzori MG, Bonmassar E, Palmieri G, Ascierto PA, D'Atri S. NF-κB is activated in response to temozolomide in an AKT-dependent manner and confers protection against the growth suppressive effect of the drug. J Transl Med 2012; 10:252.

43. Bellacosa A, Kumar CC, Cristofano A Di, Testa JR. Activation of AKT kinases in cancer: Implications for therapeutic targeting. Adv. Cancer Res.2005; 94:29–86.

44. Chiarini F, Del Sole M, Mongiorgi S, Gaboardi GC, Cappellini A, Mantovani I, Follo MY, McCubrey JA, Martelli AM. The novel Akt inhibitor, perifosine, induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrug-resistant human T-acute leukemia cells by a JNK-dependent mechanism. Leukemia 2008; 22:1106–16.

45. Tazzari PL, Cappellini A, Ricci F, Evangelisti C, Papa V, Grafone T, Martinelli G, Conte R, Cocco L, McCubrey JA, et al. Multidrug resistance-associated protein 1 expression is under the control of the phosphoinositide 3 kinase/Akt signal transduction network in human acute myelogenous leukemia blasts. Leukemia2007; 21:427–38.

46. Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW, Holland EC. PTEN/PI3K/Akt Pathway Regulates the Side Population Phenotype and ABCG2 Activity in Glioma Tumor Stem-like Cells. Cell Stem Cell 2009; 4:226–35.

47. Su Y-C, Li S-C, Hsu C-K, Yu C-C, Lin T-J, Lee C-Y, Liao H-F. G-CSF downregulates natural killer cell-mediated cytotoxicity in donors for hematopoietic SCT. Bone Marrow Transplant 2012; 47:73–81.

48. Segovis CM, Schoon RA, Dick CJ, Nacusi LP, Leibson PJ, Billadeau DD. PI3K links NKG2D signaling to a CrkL pathway involved in natural killer cell adhesion, polarity, and granule secretion. J Immunol 2009; 182:6933–42.

49. Kim N, Saudemont A, Webb L, Camps M, Ruckle T, Hirsch E, Turner M, Colucci F. The p110delta catalytic isoform of PI3K is a key player in NK-cell development and cytokine secretion. Blood 2007; 110:3202–8.

50. Wai L-E, Fujiki M, Takeda S, Martinez OM, Krams SM. Rapamycin, But Not Cyclosporine or FK506, Alters Natural Killer Cell Function. Transplantation 2008; 85:145–9.

51. Miletti-González KE, Chen S, Muthukumaran N, Saglimbeni GN, Wu X, Yang J, Apolito K, Shih WJ, Hait WN, Rodriguez-Rodriguez L. The CD44 receptor interacts with P-glycoprotein to promote cell migration and invasion in cancer. Cancer Res 2005; 65:6660–7.

52. Honig SM, Fu S, Mao X, Yopp A, Gunn MD, Randolph GJ, Bromberg JS. FTY720 stimulates multidrug transporter- and cysteinyl leukotriene-dependent T cell chemotaxis to lymph nodes. J Clin Invest 2003; 111:627–37.

53. Robertson MJ. Role of chemokines in the biology of natural killer cells. J Leukoc Biol 2002; 71:173–83.

54. Franciszkiewicz K, Boissonnas A, Boutet M, Combadière C, Mami-Chouaib F. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. Cancer Res 2012; 72:6325–32.

55. Alvino E, Pepponi R, Pagani E, Lacal PM, Nunziata C, Bonmassar E, D'Atri S. O(6)-benzylguanine enhances the in vitro immunotoxic activity of temozolomide on natural or antigen-dependent immunity. J Pharmacol Exp Ther 1999; 291:1292–300.

56. Ellsworth S, Balmanoukian A, Kos F, Nirschl CJ, Nirschl TR, Grossman S a, Luznik L, Drake CG. Sustained CD4(+) T cell-driven lymphopenia without a compensatory IL-7/IL-15 response among high-grade glioma patients treated with radiation and temozolomide. Oncoimmunology 2014; 3:e27357.

57. Fernandez NC, Lozier a, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 1999; 5:405–11.

58. Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat Rev Immunol 2002; 2:957–64.

59. Van Elssen CHMJ, Oth T, Germeraad WT V, Bos GMJ, Vanderlocht J. Natural killer cells: the secret weapon in dendritic cell vaccination strategies. Clin Cancer Res 2014; 20:1095–103.

60. Lowdell MW. Natural killer cells in haematopoietic stem cell transplantation. Transfus Med 2003; 13:399–404.

61. Köck K, Grube M, Jedlitschky G, Oevermann L, Siegmund W, Ritter C a, Kroemer HK. Expression of adenosine triphosphate-binding cassette (ABC) drug transporters in peripheral blood cells: relevance for physiology and pharmacotherapy. Clin Pharmacokinet 2007; 46:449–70.

62. Sengupta S, Marrinan J, Frishman C, Sampath P. Impact of temozolomide on immune response during malignant glioma chemotherapy. Clin Dev Immunol 2012; 2012.

63. Litterman AJ, Zellmer DM, Grinnen KL, Hunt M a, Dudek AZ, Salazar AM, Ohlfest JR. Profound Impairment of Adaptive Immune Responses by Alkylating Chemotherapy. J Immunol 2013; 190(12):6259-68.

64. Roos W, Baumgartner M, Kaina B. Apoptosis triggered by DNA damage O6-methylguanine in human lymphocytes requires DNA replication and is mediated by p53 and Fas/CD95/Apo-1. Oncogene 2004; 23:359–67.

65. Asavaroengchai W, Kotera Y, Mulé JJ. Tumor lysate-pulsed dendritic cells can elicit an effective antitumor immune response during early lymphoid recovery. Proc Natl Acad Sci U S A 2002; 99:931–6.

66. Sanchez-Perez L a, Choi BD, Archer GE, Cui X, Flores C, Johnson L a, Schmittling RJ, Snyder D, Herndon JE, Bigner DD, et al. Myeloablative temozolomide enhances $CD8^+$ T-cell responses to vaccine and is required for efficacy against brain tumors in mice. PLoS One 2013; 8:e59082.

67. Iversen TZ, Brimnes MK, Nikolajsen K, Andersen RS, Hadrup SR, Andersen MH, Bastholt L, Svane IM. Depletion of T lymphocytes is correlated with response to temozolomide in melanoma patients. Oncoimmunology 2013; 2:e23288.

68. Fadul CE, Fisher JL, Gui J, Hampton TH, Côté AL, Ernstoff MS. Immune modulation effects of concomitant temozolomide and radiation therapy on peripheral blood mononuclear cells in patients with glioblastoma multiforme. Neuro Oncol 2011; 13:393–400.

69. Lee K-M, Bhawan S, Majima T, Wei H, Nishimura MI, Yagita H, Kumar V. Cutting edge: the NK cell receptor 2B4 augments antigen-specific T cell cytotoxicity through CD48 ligation on neighboring T cells. J Immunol 2003; 170:4881–5.

70. Assarsson E, Kambayashi T, Schatzle JD, Cramer SO, von Bonin A, Jensen PE, Ljunggren H-G, Chambers BJ. NK Cells Stimulate Proliferation of T and NK Cells through 2B4/CD48 Interactions. J Immunol 2004; 173:174–80.

Figures

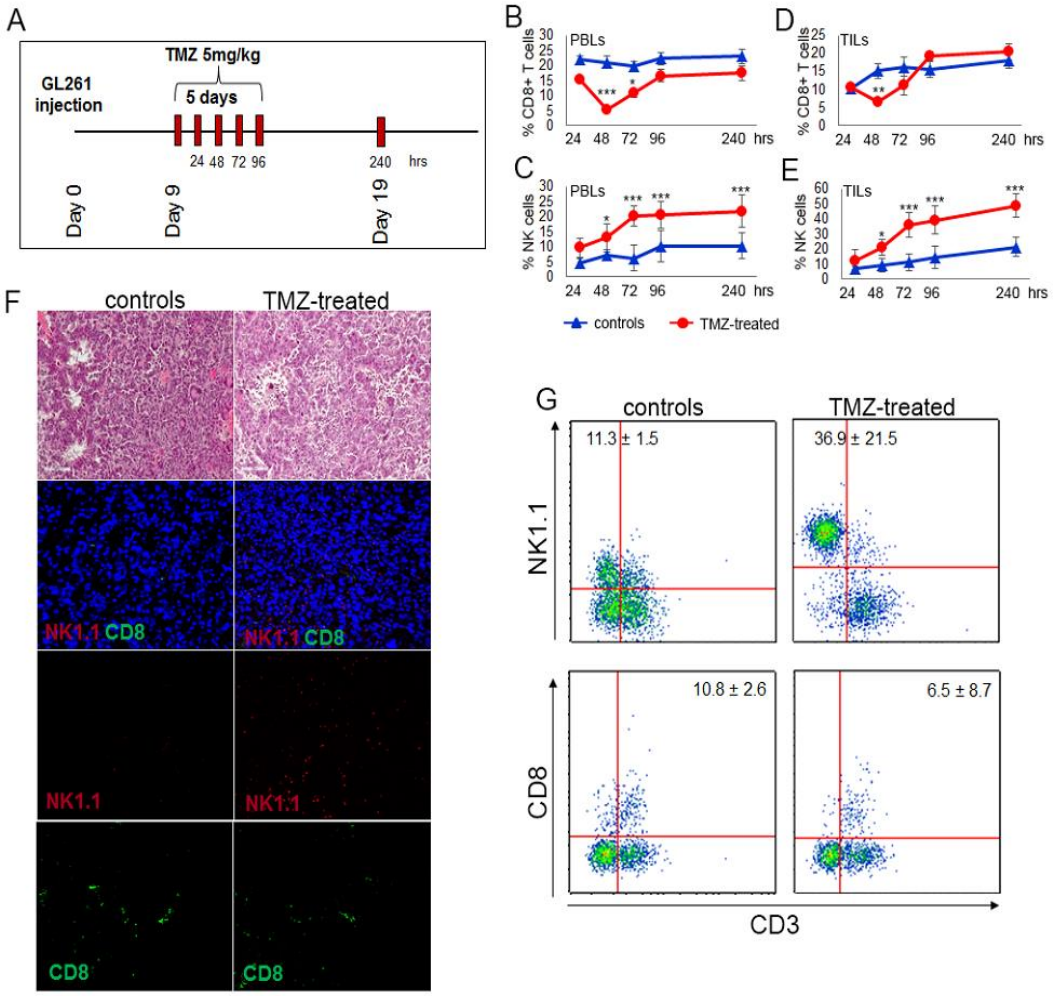

Figure 1. TMZ treatment influences local and peripheral immune cell frequency.

(**A**) Experimental schema of in vivo treatment. C57BL6 were i.c. injected with GL261 cells and treated for five days with i.p. injection of 5mg/kg TMZ or vehicle (DMSO) 9 days after tumor implantation. On days 9-13 and 19 after tumor implantation (24-96 and 240 hrs after TMZ treatment), n = 5 mice per group/each time point were sacrificed for immune

monitoring. (**B**) Peripheral percentages of $CD8^+$ T cells ($CD8^+$ $CD3^+$): 22.2 ± 1.2% vs 15.5 ± 0.2% at 24 hrs; 21.1 ± 2.0% vs 5.5 ± 1.0% at 48hrs; 19.8 ± 1.4% vs 10.8 ± 1.3% at 72hrs; 22.4 ± 2.1 vs 16.6 ± 2.1% at 96 hrs; 22.6 ± 0.4% vs 17.3± 1.4% at 240 hrs, controls vs TMZ-treated mice, respectively; *p < 0.01; ***p<0.0001. (**C**) Percentages of blood NK cells ($NK1.1^+$ $CD3^-$): 4.8 ± 1.2% vs 9.8 ± 3.2% at 24 hrs; 7.2 ± 1.2% vs 13.3 ± 1.2% at 48 hrs; 6.3 ± 1.6% vs 20.2 ± 1.9% at 72 hrs; 10.2 ± 2.1% vs 20.6 ± 2.3% at 96 hrs, 7.2 ± 2.1% vs 21.8 ± 2.3% at 240 hrs, controls vs TMZ-treated mice respectively; *p < 0.01; ***p<0.0001. (**D**) Tumor-infiltrating $CD8^+$ T cells: 10.3 ± 1.2% vs 10.5 ± 0.2% at 24 hrs; 15.2 ± 2.1% vs 6.5 ±1.0% at 48 hrs; 16.3 ± 2.7% vs 11.2 ± 2.6% at 72 hrs; 15.7 ± 2.2% vs 19.3 ± 1.1% at 96 hrs, 18.2 ± 2.2% vs 20.4 ± 2.3% at 240 hrs, controls vs TMZ-treated mice respectively; **p < 0.001. (**E**) Tumor-infiltrating NK cells during and after TMZ administration: 7.2 ± 12.5% vs 12.5 ± 7.4% at 24 hrs; 9.1 ± 4.5% vs 21.1 ± 5.3% at 48 hrs; 11.3 ± 5.6% vs 36.2 ± 8.1% at 72 hrs; 14.5 ± 7.6% vs 39.2 ± 9.2% at 96 hrs; 21.4 ± 6.5% vs 48.9 ± 7.7% at 240 hrs, controls vs treated mice respectively; *p < 0.01; ***p<0.0001. (**F**) Characterization of immune infiltration in gliomas by IF at 72 hrs after beginning of TMZ treatment. (**G**) Quantitative determination of TILs by flow cytometry obtained from the same groups of gliomas used for IF studies.

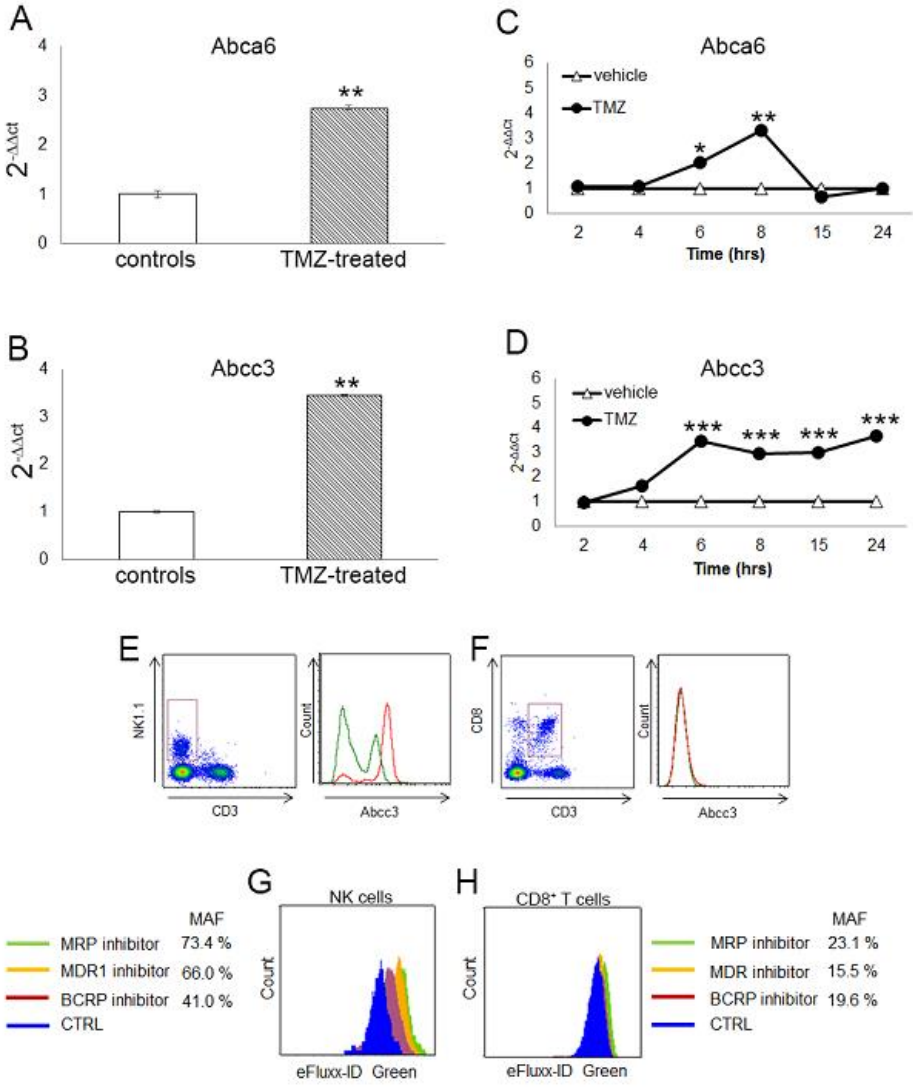

Figure 2. Abcc3 is responsible for NK cell chemo-resistance.

(**A and B**) Relative expression of Abca6 and Abcc3 transporters in blood NK cells at 72 hrs, **p < 0.001. (**C and D**) Time course of Abca6 and Abcc3 expression in PBLs from naïve mice treated in vitro with 1µM TMZ

or DMSO; *p = 0.01, **p < 0.005 and ***p < 0.0001. (**E and F**) Abcc3 levels in NK and $CD8^+$ T cells from glioma-bearing mice after 3 treatments of TMZ (red line) or DMSO (green line). (**G and H**) MDR activity assay in NK and $CD8^+$ T cells from PBLs of naïve mice treated in vitro with 1 μM TMZ or DMSO for 4hrs. MAF values > 25 are indicative of multidrug-resistant phenotype.

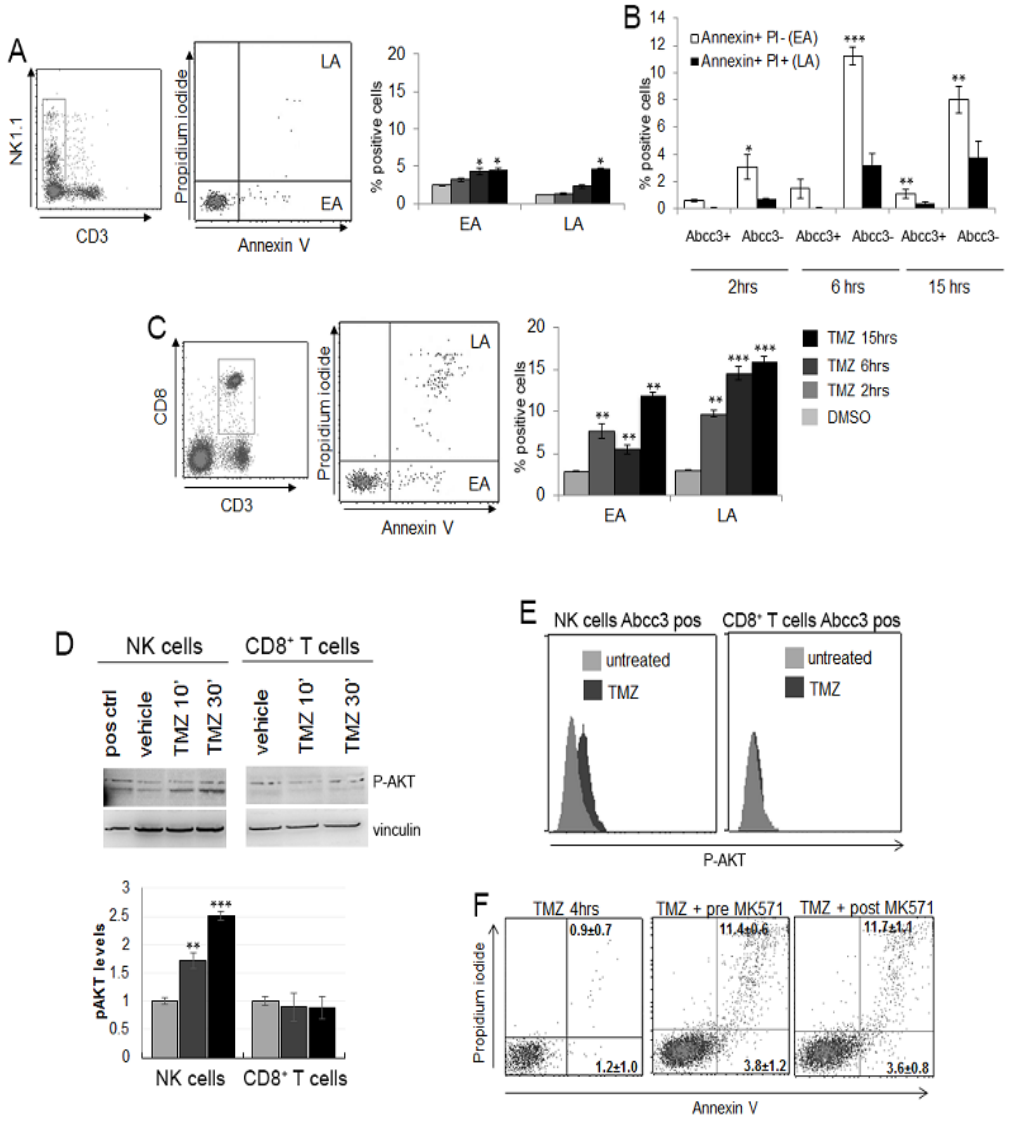

Figure 3. NK cells exhibit resistance to TMZ-induced apoptosis.

(**A and C**) Early and late apoptosis (EA and LA, respectively) induced by TMZ treatment in NK and $CD8^+$ T cells from PBLs of naïve mice. (**B**) EA and LA of $Abcc3^+$ and $Abcc3^-$ NK cells treated in vitro with 1 μM TMZ.

(**D**) WB analysis and densitometric quantification of pAkt in blood immune-separated NK and $CD8^+$ T cells of naïve mice treated with 1 μM TMZ or DMSO. (**E**) Intracellular staining of pAkt in blood-derived $Abcc3^+$ NK and $CD8^+$ T cells from glioma-bearing mice at 72hrs. (**F**) Representative dot plots showing apoptosis in NK cells treated for 4 hrs in vitro with 1 μM TMZ and 25 μM Abcc3 inhibitor added to the medium 30 minutes before (pre MK571) or after (post MK571) the pharmacological treatment. *$p < 0.01$, **$p < 0.001$ and ***$p < 0.0001$.

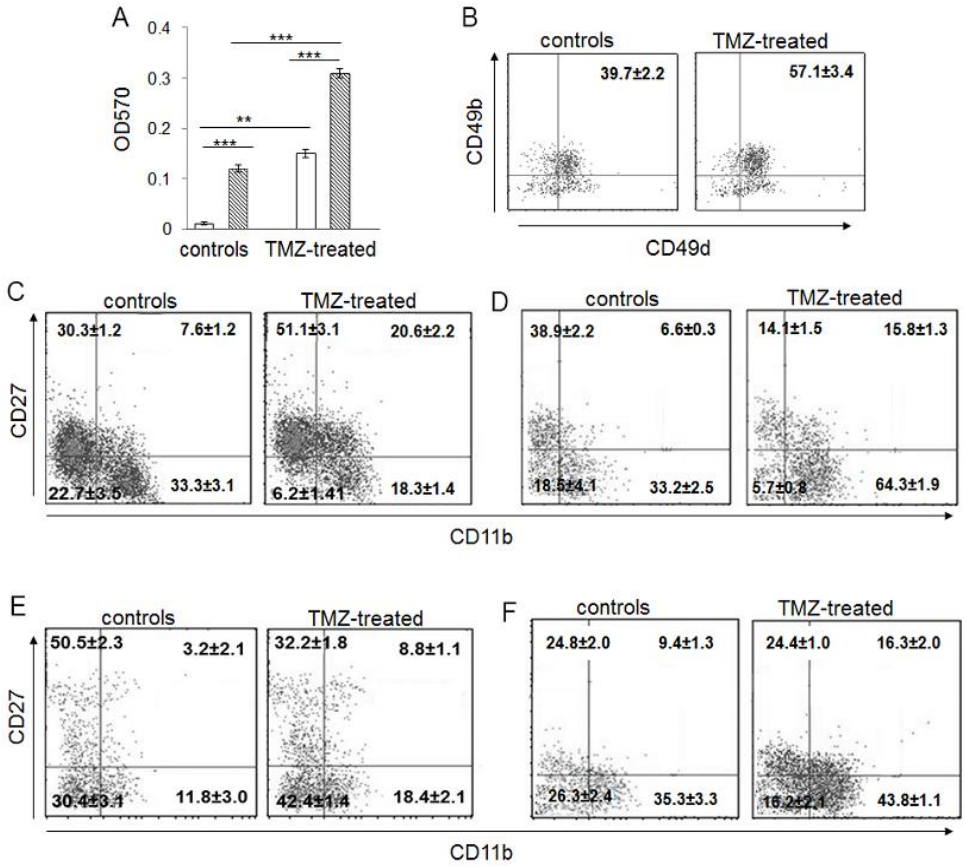

Figure 4. The developmental stage of NK cells from TMZ-treated mice was influenced by TMZ. (**A**) Migration of NK cells from PBLs of TMZ-treated and control mice at 72hrs toward medium from DMSO-treated (white bars) and TMZ-treated (striped bars) GL261 cells. **p < 0.005; ***p < 0.0001. (**B**) CD49b and CD49d integrin surface expression in blood-derived NK cells from TMZ-treated and control mice isolated at 72 hrs; p < 0.001. (**C and D**) Representative dot plots of the NK cell four-developmental stages basing on surface expression of CD27 and CD11b in blood of TMZ- and vehicle-treated mice on days 12 (C) and 19 (D); p < 0.01. (**E and F**) Representative dot plots showing CD27 and CD11b expression in tumor-infiltrating NK cells isolated on day 12 (E) and 19 (F) from gliomas, p < 0.005.

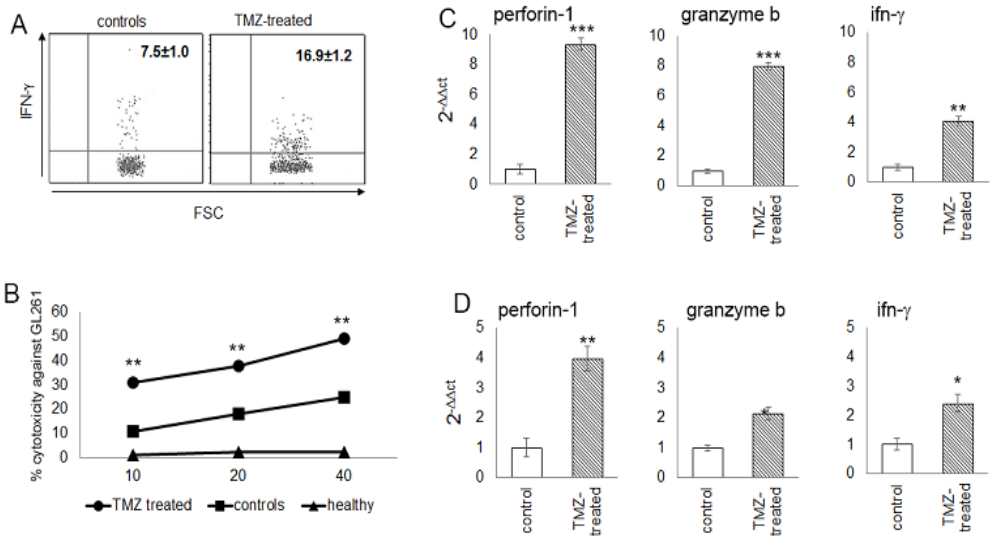

Figure 5. Cytotoxicity of glioma-infiltrating NK cells is promoted by TMZ treatment.

(**A**) IFN-γ production in blood NK cells (n = 4/group); $p < 0.005$. (**B**) Cytotoxic specificity of peripheral NK cells from naïve mice, TMZ- and vehicle-treated glioma-bearing mice (n = 10/group) against GL261 cells in vitro. Effector : target ratio (E:T) 10:1, 20:1, 40:1. $p < 0.001$. (**C and D**) Relative expression of *Prf1*, *GzmB* and *Ifn-γ* in TILs at early (C) and later time point (D). *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$.

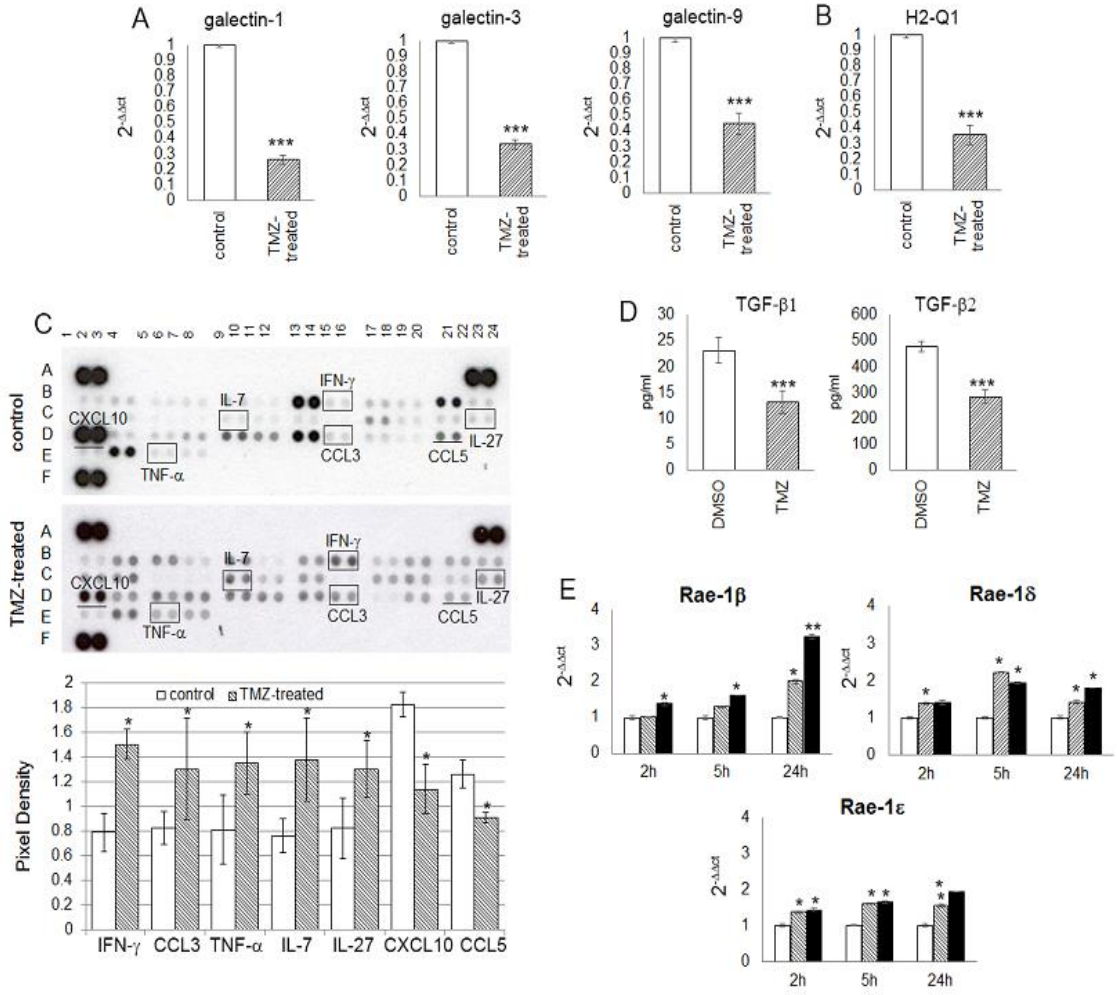

Figure 6. **TMZ modifies glioma microenvironment favoring an NK cell antitumor activity.**

(**A and B**) Relative expression of the most immunosuppressive molecules normally expressed in glioma cells: *galectin-1*, *-3*, *-9* (3.85 ± 0.03-, 2.94 ± 0.02- and 2.22 ± 0.03-fold, respectively vs controls) and *H2-q1 (*2.81 ± 0.08-fold vs controls). (**C**) Cytokine and chemokine protein array blots with pixel density quantification of lysates from gliomas of control and TMZ-treated mice. Blots are representative of experiments performed on

a pool of gliomas from controls or TMZ-treated mice. Cytokines and chemokines which expression changed in both experiments were considered: IFN-γ, CCL3, TNF-α, IL-7 and IL-27 were 1.9 ± 0.6-, 1.6 ± 0.4-, 1.7 ± 0.2-, 1.8 ± 0.4-, 1.6 ± 0.2-fold higher, CXCL10 and CCL5 were 0.6 ± 0.7- and 0.7 ± 0.4-fold lower in TMZ-treated compared to controls; * $p \leq 0.01$. (**D**) TGF-β1 and -β2 levels in medium of GL261 cells treated in vitro with DMSO or TMZ: from 23.2 ± 2.5 pg/ml and 478.4 ± 19.3 pg/ml in vehicle to 13.2 ± 2.2 pg/ml and 283.8 ± 25.9 pg/ml in TMZ-treated cells. (**E**) Relative expression of Nkg2d ligands in GL261 cells treated in vitro with 50 and 150 μM TMZ (striped and black bars, respectively) and DMSO used as vehicle (white bars) at different time points. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$.

Supplementary

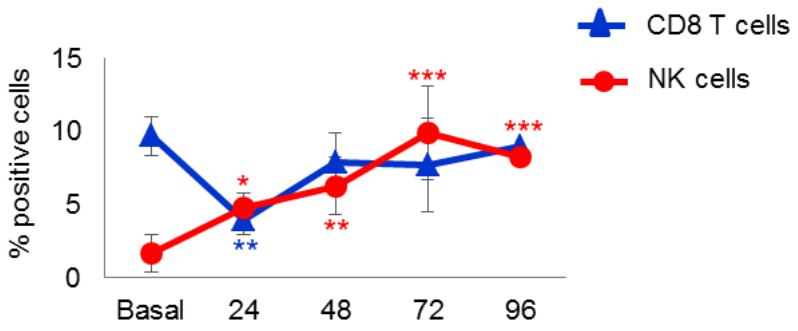

Figure S1. TMZ enhances the NK cell frequency in non-glioma-bearing mice.

A group of non-glioma-bearing mice were treated with TMZ. PBLs were harvested 24, 48, 72 and 96 hrs after treatment. TMZ induced a modulation of $CD8^+$ T cells and NK cells similar to TMZ-treated tumor bearing mice (Figure 1B and D). Specifically, NK cell frequency increased significantly at early time points (* $p < 0.01$; ** $p < 0.005$; p*** $p<0.001$), $CD8^+$ T cells decrease was rapid but reversible (** $p < 0.005$).

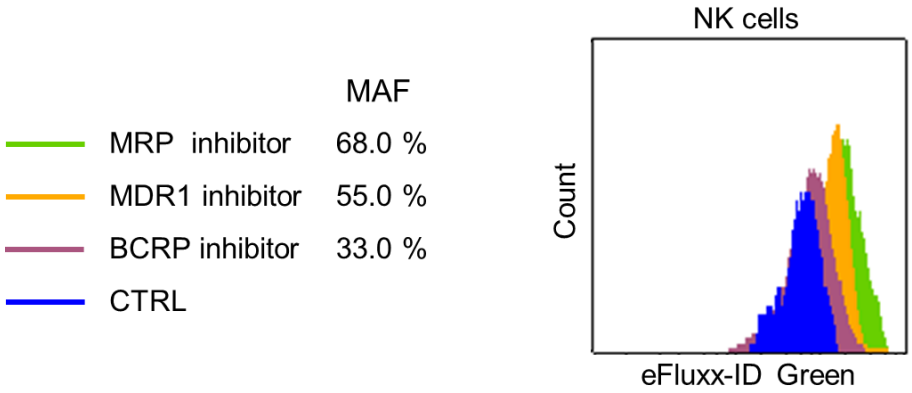

Figure S2. Peripheral blood NK cells show a dose-dependent drug-resistance activity.

Efflux activity of ABC transporters assessed by flow cytometry with a fluorescent dye in presence or absence of specific ABC inhibitors to calculate the multidrug-resistance activity factor values (MAF). NK cells from naïve mice were treated in vitro with 0,5 μM TMZ or DMSO for 4 hrs. MAF values > 25 are indicative of multidrug-resistant phenotype.

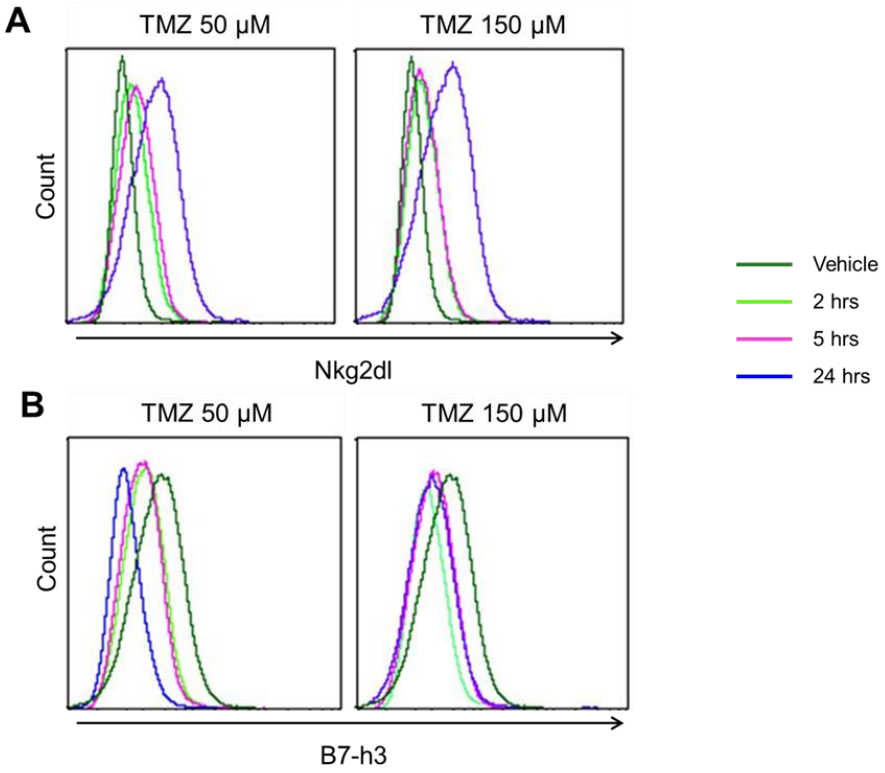

Figure S3. TMZ influences the GL261 cell immunogenicity.

Representative dot plots of GL261 cells treated in vitro with 50 and 150 µM TMZ or DMSO (vehicle) at different time points. (**A**) Nkg2dl rapidly increased (4.1± 0.2% vs 9.4 ± 1.1% and 10.6 ± 0.9% at 2 hrs; 3.2 ± 1.0% vs 13.6 ± 1.0%and 17.6 ± 1.3%at 5 hrs;3.9 ± 0.6% vs 20.2 ± 2.3% and 27.7 ± 1.3% at 24hrs, controls vs. 50 and 150 µM TMZ-treatedmice respectively; *p < 0.01). (**B**) B7-H3 significantly decreased (23.9± 0.2% vs 18.4 ± 1.5% and 6.1 ± 1.6% at 2 hrs; 23.2 ± 1.0% vs 10.7 ± 1.0%and 6.0 ± 1.5%at 5 hrs;23.9 ± 0.6% vs 4.8 ± 0.3% and 6.4 ± 0.9% at 24hrs, controls vs. 50 and 150 µM TMZ-treated mice respectively; *p < 0.01). Flow cytometry analyses were obtained from 3 different experiments.

Table S1.

Probe Set ID	NK CTRL	NK TMZ	CD8 CTRL	CD8 TMZ	logFC NK	logFC CD8	Gene Symbol	Gene Bank
17362539	7,72202	3,242859	2,866335	2,828359	-4,479161	-0,037976	Scgb1a1	NM_011681
17306991	5,994791	2,021417	2,445533	2,329827	-3,973374	-0,115706	Gzmd	NM_010372
17306983	6,139857	2,709189	2,941385	3,022386	-3,430668	0,081001	Gzme	NM_010373
17489363	6,428623	3,300454	2,478789	2,344999	-3,128169	-0,13379	Hamp	NM_032541
17466530	8,309629	5,47775	3,184305	3,266585	-2,831879	0,08228	Rny1	NR_004419
17306999	5,211462	2,416042	2,940237	2,979622	-2,79542	0,039385	Gzmg	NM_010375
17235011	7,567307	4,952219	3,304193	3,500477	-2,615088	0,196284	Elane	NM_015779
17283634	6,251194	3,755193	3,030057	3,156768	-2,496001	0,126711	Serpina1c	NM_009245
17438886	9,429761	7,098767	4,373413	3,481985	-2,330994	-0,891428	Alb	NM_009654
17307025	4,977774	2,666936	2,911402	3,095509	-2,310838	0,184107	Gzmc	NM_010371
17351831	6,627881	4,440394	7,413254	6,670288	-2,187487	-0,742966	Snord58b	NR_028552
17305520	7,387096	5,22775	3,074114	2,902435	-2,159346	-0,171679	Ear1	NM_007894
17477832	8,77969	6,654347	4,169685	5,017468	-2,125343	0,847783	Ftl1	NM_010240
17312032	7,696645	5,612951	3,031745	2,946713	-2,083694	-0,085032	Ndrg1	NM_008681
17285706	10,43502	8,39452	5,278312	5,203849	-2,0405	-0,074463	Hist1h4m	NM_001195421
17264036	9,386904	7,403713	7,042769	6,920182	-1,983191	-0,122587	Ubb	ENSMUST00000019649
17250742	8,66349	6,7166	7,953413	7,965178	-1,94689	0,011765	Snord49a	NR_028550
17547723	7,702611	5,854451	5,840998	6,089122	-1,84816	0,248124	Ywhaq	NM_011739
17548348	9,131264	7,313562	6,380653	6,421731	-1,817702	0,041078	Actg1	NM_009609
17218680	9,861839	8,075171	8,931215	8,56097	-1,786668	-0,370245	Snord47	NR_028543
17314679	7,01271	5,22708	5,930602	6,067333	-1,78563	0,136731	Tuba1b	NM_011654
17236800	7,39912	5,62707	4,930883	4,31751	-1,77205	-0,613373	Dcn	NM_007833
17232731	7,586909	5,842309	6,427805	6,052614	-1,7446	-0,375191	Rnu3a	NR_002842
17339392	8,181211	6,505793	4,807532	4,453646	-1,675418	-0,353886	Myl12a	ENSMUST00000024846
17547608	8,508842	6,837495	5,176863	5,225361	-1,671347	0,048498	Actg1	NM_009609
17471778	7,76548	6,138906	2,566724	2,469116	-1,626574	-0,097608	Klra3	NM_010648
17246936	7,113566	5,522482	3,727224	3,828931	-1,591084	0,101707	Rasl10a	NM_145216
17225173	10,7736	9,208171	10,10508	9,887005	-1,565429	-0,218075	Snord82	NR_002851
17254877	7,119788	5,560782	3,753373	3,839166	-1,559006	0,085793	Mpo	ENSMUST00000020779
17324398	7,262779	5,705933	7,378842	7,178941	-1,556846	-0,199901	Snora81	NR_034048
17357659	8,12729	6,61431	7,682272	8,015916	-1,51298	0,333644	Ms4a4b	NM_021718
17547620	8,859197	7,348626	6,197084	6,349689	-1,510571	0,152605	Ftl1	NM_010240
17471757	10,1179	8,609843	2,713829	3,135477	-1,508057	0,421648	Klra9	NM_010651
17295032	7,459177	5,975341	3,641421	3,915324	-1,483836	0,273903	Arsb	NM_009712
17356556	8,938833	7,455145	8,78065	8,328193	-1,483688	-0,452457	Malat1	NR_002847
17305986	10,50259	9,055317	10,01262	10,27451	-1,447273	0,26189	Rpph1	NR_002142
17329023	8,605267	7,160343	10,82889	10,93757	-1,444924	0,10868	Iglv1	M94350
17471768	9,013568	7,593667	3,737961	3,716724	-1,419901	-0,021237	Klra10	NM_008459
17349552	8,164172	6,808004	7,231226	7,424065	-1,356168	0,192839	Snora74a	NR_002905
17306975	6,341461	4,988468	2,87951	2,807576	-1,352993	-0,071934	Ctsg	NM_007800
17547869	9,344457	7,997941	7,318279	7,238977	-1,346516	-0,079302	Ywhaz	AK139259
17306937	7,291237	5,946251	2,916593	2,985589	-1,344986	0,068996	Cma1	NM_010780
17228102	8,13068	6,802405	5,947146	5,78095	-1,328275	-0,166196	Arpc5	NM_026369
17304510	7,301034	5,990347	7,204192	6,906984	-1,310687	-0,297208	Snord69	NR_028531
17308731	9,176225	7,885982	8,533373	8,526903	-1,290243	-0,00647	Tpt1	NM_009429
17318020	7,034488	5,746267	9,09833	9,27974	-1,288221	0,18141	Ly6d	NM_010742

17221375	8,816829	7,561432	6,22869	6,461329	-1,255397	0,232639	Eya1	NM_010164
17265126	8,920765	7,678303	7,033771	7,224906	-1,242462	0,191135	Gabarap	NM_019749
17228864	8,899477	7,670825	5,102848	5,35863	-1,228652	0,255782	Fasl	NM_010177
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17254283	9,131736	7,990671	4,455153	4,639244	-1,141065	0,184091	Ccl4	NM_013652
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17301932	7,296147	6,183538	3,109515	3,151845	-1,112609	0,04233	Esd	NM_016903
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17302054	7,903318	6,810967	7,705496	7,324223	-1,092351	-0,381273	Snora31	NR_028481
17321241	6,85377	5,770359	6,693675	6,396474	-1,083411	-0,297201	Snora34	NR_034051
17353673	6,903749	5,834694	5,866147	6,026212	-1,069055	0,160065	Ube2d2a	NM_019912
17250740	10,88953	9,834885	10,23115	10,32846	-1,054645	0,09731	Snord49b	NR_028526
17211416	7,314589	6,268205	2,887611	2,848359	-1,046384	-0,039252	Khdc1a	NM_183322
17306968	6,89051	5,851244	3,465873	3,27207	-1,039266	-0,193803	Mcpt8	NM_008572
17229187	10,6378	9,605013	5,639183	5,516995	-1,032787	-0,122188	Xcl1	NM_008510
17471707	7,418639	6,389576	4,594179	4,386136	-1,029063	-0,208043	Klra6	NM_008464
17307033	10,94476	9,924092	5,745749	6,068019	-1,020668	0,32227	Gzmb	NM_013542
17215097	6,185431	5,16647	5,207719	5,148394	-1,018961	-0,059325	B3gnt7	NM_145222
17312679	8,37664	7,36377	7,314449	7,190969	-1,01287	-0,12348	Apol7e	NM_001134802
17357148	7,941168	6,930844	7,214105	7,279716	-1,010324	0,065611	Snord22	NR_004445
17231116	6,516153	5,521481	7,197991	7,293609	-0,994672	0,095618	Gstp2	BC094623
17313641	8,558916	7,580473	9,498824	8,990781	-0,978443	-0,508043	Rnu12	NR_004432
17288059	8,441198	7,463277	9,840925	9,418514	-0,977921	-0,422411	Taf1d	BC056964
17292800	6,939976	5,96291	7,151183	7,100213	-0,977066	-0,05097	Uimc1	NM_011307
17282008	7,659172	6,68407	7,483053	7,466557	-0,975102	-0,016496	Zbtb25	NM_001172104
17268645	9,996293	9,022996	9,792955	9,387423	-0,973297	-0,405532	Snora21	NR_028078
17222465	7,83616	6,864261	9,597407	9,461366	-0,971899	-0,136041	Aff3	NM_010678
17250755	7,402809	6,434532	6,246735	6,127377	-0,968277	-0,119358	Mmgt2	ENSMUST00000129162
17548027	7,054536	6,088927	7,006764	6,951341	-0,965609	-0,055423	Ddx39b	NM_019693
17331863	3,057429	2,10425	2,291425	2,211405	-0,953179	-0,08002	Krtap16-2	NM_130876
17293513	7,909463	6,972289	9,569397	9,064346	-0,937174	-0,505051	Taf1d	BC056964
17302606	8,958868	8,023295	8,954804	8,681014	-0,935573	-0,27379	Tpm3	NM_001253740
17254743	9,743053	8,807734	9,719027	9,24151	-0,935319	-0,477517	Rnu3b1	NR_004415
17219171	8,616061	7,688538	4,392973	4,540123	-0,927523	0,14715	Sh2d1b1	ENSMUST00000027984
17309099	7,113311	6,186494	5,557748	5,740354	-0,926817	0,182606	Klf12	NM_010636
17296281	8,508589	7,58205	3,986543	3,808605	-0,926539	-0,177938	Gpx8	NM_027127
17237589	8,164701	7,247658	4,654826	4,7737	-0,917043	0,118874	Ifng	NM_008337
17231203	7,788134	6,875824	8,378237	8,513258	-0,91231	0,135021	Traf3ip3	NM_153137
17262046	7,178063	6,275447	5,161349	5,508403	-0,902616	0,347054	Sft2d1	ENSMUST00000093169
17332932	8,602118	7,707323	6,117021	5,754463	-0,894795	-0,362558	Sytl3	NM_183368
17232316	8,438663	7,563082	4,45795	4,482922	-0,875581	0,024972	Samd3	NM_001115154
17285859	8,466693	7,598425	8,32604	8,242228	-0,868268	-0,083812	Hist1h1c	NM_015786
17324787	8,537018	7,671464	3,347514	3,465678	-0,865554	0,118164	Tm4sf19	NM_001160402
17238705	8,10652	7,24109	8,774448	8,302185	-0,86543	-0,472263	Tespa1	NM_183264
17301718	6,967107	6,108297	5,804009	6,174373	-0,85881	0,370364	Egr3	NM_018781
17233794	6,566071	5,707977	5,309245	5,007273	-0,858094	-0,301972	Ddx21	NM_019553
17260150	7,204992	6,362369	7,194204	6,995586	-0,842623	-0,198618	Znrf3	NM_001080924
17284354	9,497367	8,655708	11,34842	11,48989	-0,841659	0,14147	Igh-VJ558	BC003495
17286354	9,035644	8,197952	5,388192	5,486094	-0,837692	0,097902	Serpinb6b	ENSMUST00000110293
17340532	10,36806	9,544233	8,212882	7,7073	-0,823827	-0,505582	Sytl3	NM_031395
17231477	9,1174	8,305917	3,192828	3,214253	-0,811483	0,021425	Myct1	NM_026793
17330751	8,75443	7,944432	9,089464	8,598955	-0,809998	-0,490509	Cd96	NM_032465
17219435	6,612782	5,807183	9,426852	9,5171	-0,805599	0,090248	Slamf6	NM_030710
17332317	6,368155	5,564988	6,241686	6,450589	-0,803167	0,208903	Pigp	NM_001159619

17327613	8,441427	7,639778	7,565238	7,518968	-0,801649	-0,04627	Nlrc3	NM_001081280
17343426	9,015028	8,213956	8,712572	8,520144	-0,801072	-0,192428	Rasal3	NM_178785
17262600	8,569902	7,772296	10,95838	10,53023	-0,797606	-0,42815	Tcf7	ENSMUST00000072425
17229391	8,958097	8,160543	9,765763	9,347804	-0,797554	-0,417959	Uck2	NM_030724
17296286	10,53042	9,736224	5,335327	5,123867	-0,794196	-0,21146	Gzma	NM_010370
17212252	9,681068	8,889685	5,093229	5,293192	-0,791383	0,199963	Il18rap	ENSMUST00000027237
17308800	7,871169	7,083346	7,71402	7,596732	-0,787823	-0,117288	Dnajc15	NM_025384
17425276	9,582831	8,797203	6,357544	6,544458	-0,785628	0,186914	Amd1	NM_009665
17216976	7,15169	6,366389	8,953716	9,296986	-0,785301	0,34327	Faim3	ENSMUST00000038829
17342027	8,325926	7,540999	7,258554	7,163834	-0,784927	-0,09472	Snora78	NR_028515
17236531	9,51541	8,738447	8,522184	8,313869	-0,776963	-0,208315	Lta4h	NM_008517
17471744	9,6378	8,862185	4,052271	4,167145	-0,775615	0,114874	Klra14	NM_008461
17243458	9,718661	8,944473	9,732668	9,571295	-0,774188	-0,161373	Rpl23a	NM_207523
17219011	6,447505	5,677557	7,381426	6,774175	-0,769948	-0,607251	Pou2f1	NM_011137
17268820	9,263456	8,497277	9,60421	9,592226	-0,766179	-0,011984	Ikzf3	NM_011771
17281419	8,160708	7,396818	8,305159	8,238655	-0,76389	-0,066504	Fkbp3	NM_013902
17289986	7,534341	6,774198	4,048173	4,140341	-0,760143	0,092168	Cdc20b	XM_138861
17241181	10,00853	9,252814	4,06883	4,06882	-0,755716	0	Adamts14	NM_001081127
17227025	8,63718	7,888536	7,874898	7,820453	-0,748644	-0,054445	Lax1	NM_001159649
17340629	9,74552	8,99758	8,46234	8,285722	-0,74794	-0,176618	Mir692-1	NR_030465
17351384	9,285126	8,537655	8,262767	8,408219	-0,747471	0,145452	Sec11c	NM_025468
17356173	8,65758	7,910756	8,37185	8,335217	-0,746824	-0,036633	Ptprcap	ENSMUST00000061086
17335330	9,559889	8,816904	9,749522	9,945054	-0,742985	0,195532	Rpl10a	NM_011287
17268445	10,23153	9,492514	5,510755	5,70501	-0,739016	0,194255	Tbx21	NM_019507
17226622	7,168702	6,434578	8,563266	8,657395	-0,734124	0,094129	Cd55	NM_010016
17290682	11,02663	10,29295	10,26991	10,21478	-0,73368	-0,05513	Ywhaq	NM_011739
17217566	8,743455	8,016308	7,829902	7,54802	-0,727147	-0,281882	Ptpn7	NM_177081
17308280	9,241116	8,521441	8,624108	8,770431	-0,719675	0,146323	Ppp3cc	NM_008915
17329345	3,740905	3,025362	2,788297	2,784504	-0,715543	-0,003793	Crygs	NM_009967
17348833	5,346639	4,632923	3,660481	3,628634	-0,713716	-0,031847	Ttr	NM_013697
17231229	7,278599	6,566498	6,328564	6,240336	-0,712101	-0,088228	Hsd11b1	ENSMUST00000016338
17229178	9,667757	8,957817	8,188601	7,813097	-0,70994	-0,375504	Atp1b1	NM_009721
17232394	5,073312	4,363488	9,812919	8,944581	-0,709824	-0,868338	Themis	ENSMUST00000056097
17323828	8,027083	7,319416	9,449989	9,728542	-0,707667	0,278553	B3gnt5	NM_001159407
17383127	7,6682	6,968411	6,989335	7,271997	-0,699789	0,282662	Snora43	NR_028572
17284247	7,976331	7,276897	8,597324	8,410034	-0,699434	-0,18729	Gpr132	NM_019925
17309644	8,015487	7,327188	8,120798	8,666903	-0,688299	0,546105	Gpr18	NM_182806
17211867	8,066287	7,378091	8,145213	7,481831	-0,688196	-0,663382	Zap70	NM_009539
17262102	8,574433	7,886278	9,694916	9,317152	-0,688155	-0,377764	Itk	NM_010583
17337110	9,637356	8,949302	8,988371	9,101721	-0,688054	0,11335	H2-Q5	NR_051981
17507462	7,133271	6,446615	7,377557	7,209774	-0,686656	-0,167783	Ankrd10	NR_030779
17335628	6,7878	6,101765	7,864737	7,407816	-0,686035	-0,456921	Dnahc8	NM_013811
17345926	7,694689	7,010238	9,67535	9,05047	-0,684451	-0,62488	Satb1	ENSMUST00000144331
17330544	7,453719	6,769765	8,367424	8,487717	-0,683954	0,120293	Sidt1	NM_001159419
17256823	7,597329	6,916411	4,491824	4,06953	-0,680918	-0,422294	Arl4d	NM_025404
17217247	6,422698	5,747318	7,675901	7,838312	-0,67538	0,162411	Pik3c2b	NM_001099276
17266362	9,867433	9,195583	9,996347	10,00752	-0,67185	0,011173	Rpl23a	NM_207523
17335550	8,462671	7,791561	8,043688	7,703098	-0,67111	-0,34059	Tbc1d22b	NM_198647
17336476	7,195491	6,525324	9,606166	9,856952	-0,670167	0,250786	H2-Ob	NM_010389
17292117	7,507554	6,837662	5,226664	5,06265	-0,669892	-0,164014	Gfod1	NM_001033399
17219397	9,409743	8,747982	8,108435	8,425161	-0,661761	0,316726	Cd48	ENSMUST00000068584
17319401	6,992645	6,332792	6,944668	6,644951	-0,659853	-0,299717	Snord83b	NR_028282
17318100	10,88581	10,22758	9,696198	9,64756	-0,65823	-0,048638	Ly6c2	NM_001099217
17248064	6,053524	5,395494	6,173096	6,231428	-0,65803	0,058332	Mtif2	NM_133767

17248894	7,56391	6,91022	7,977855	7,748202	-0,65369	-0,229653	Snord95	NR_028564
17212724	10,62824	9,975967	9,806267	9,843521	-0,652273	0,037254	Stat4	NM_011487
17215820	8,188369	7,538746	5,001016	4,968524	-0,649623	-0,032492	Gpc1	NM_016696
17249980	10,05675	9,408862	7,838535	7,730333	-0,647888	-0,108202	Igtp	NM_018738
17283871	6,381108	5,733329	10,0225	9,14764	-0,647779	-0,87486	Bcl11b	ENSMUST00000066060
17331173	7,873653	7,2305	6,054777	6,184859	-0,643153	0,130082	St3gal6	ENSMUST00000137035
17258118	8,615083	7,972767	6,738274	6,69655	-0,642316	-0,041724	Rab37	NM_001163753
17306785	8,420643	7,778876	7,792899	7,73721	-0,641767	-0,055689	Nedd8	NM_008683
17313050	9,564374	8,924964	9,949554	10,26131	-0,63941	0,311756	Apobec3	NM_001160415
17220475	7,509834	6,87098	9,259194	8,817899	-0,638854	-0,441295	Dusp10	ENSMUST00000048655
17309580	6,074012	5,43732	6,551866	6,678033	-0,636692	0,126167	Dock9	NM_001081039
17548405	8,764709	8,131117	7,932292	7,820493	-0,633592	-0,111799	Myl12a	ENSMUST00000123686
17278268	6,947299	6,318162	6,245343	6,50313	-0,629137	0,257787	Serpina3f	NM_001168294
17229767	8,692522	8,06673	8,438124	8,665705	-0,625792	0,227581	Ly9	NM_008534
17214053	6,552958	5,928838	7,361716	7,348809	-0,62412	-0,012907	Xrcc5	NM_009533
17323473	8,297984	7,674548	7,720813	7,557796	-0,623436	-0,163017	Crkl	NM_007764
17286375	9,415531	8,792261	4,403741	4,416109	-0,62327	0,012368	Serpinb9b	ENSMUST00000006392
17251518	7,858993	7,238272	8,915357	8,997569	-0,620721	0,082212	Vamp2	NM_009497
17298791	10,30448	9,686543	10,31118	10,28728	-0,617937	-0,0239	Rpl23a	NM_207523
17336649	5,579643	4,964034	7,517755	6,389677	-0,615609	-1,128078	Prrt1	ENSMUST00000015620
17226043	9,665359	9,053116	9,322617	9,368759	-0,612243	0,046142	Bcl2	NM_009741
17232853	8,603196	7,991085	7,621121	7,669891	-0,612111	0,04877	Mical1	NM_138315
17223574	8,005469	7,396096	8,322408	8,05392	-0,609373	-0,268488	Sumo1	NM_009460
17214983	8,228064	7,622594	7,870612	7,599275	-0,60547	-0,271337	Cab39	NM_133781
17294974	8,579552	7,977632	8,450578	8,327712	-0,60192	-0,122866	Papd4	NM_133905
17266967	11,24631	10,64597	5,718506	7,141956	-0,60034	1,42345	Ccl3	NM_011337
17306690	6,931663	6,333368	5,60846	5,38979	-0,598295	-0,21867	Dcaf11	ENSMUST00000133256
17237937	9,804963	9,208323	10,07581	10,09739	-0,59664	0,02158	B4galnt1	NM_008080
17366278	8,679462	8,084273	9,162449	9,135664	-0,595189	-0,026785	Sp100	ENSMUST00000141998
17334147	8,354542	7,760951	9,794441	9,650993	-0,593591	-0,143448	Mir5125	NR_039586
17303472	9,876981	9,284418	9,61978	9,204252	-0,592563	-0,415528	Ptma	NM_008972
17348054	7,463392	6,870926	7,23273	7,429677	-0,592466	0,196947	Mettl4	NM_176917
17219542	7,336897	6,746457	7,964671	7,715325	-0,59044	-0,249346	Pigm	NM_026234
17285851	11,86335	11,27338	8,265327	8,410759	-0,58997	0,145432	Hist1h2bc	NM_023422
17217619	7,122962	6,533694	4,994402	5,072389	-0,589268	0,077987	Phlda3	NM_013750
17229277	5,564948	4,976171	7,482156	6,778565	-0,588777	-0,703591	Rcsd1	AK030934
17257085	8,504443	7,920609	7,905697	7,050145	-0,583834	-0,855552	Hexim1	NM_138753
17342182	7,245903	6,664486	5,909534	5,763075	-0,581417	-0,146459	Baiap3	NM_001163270
17228788	5,607321	5,026563	7,424744	7,525654	-0,580758	0,10091	Mir1927	NR_035448
17231287	5,103554	4,522939	6,738204	6,651904	-0,580615	-0,0863	Cd46	ENSMUST00000162650
17324394	7,261841	6,685762	8,435038	8,076769	-0,576079	-0,358269	Snord2	NR_030705
17308556	9,055775	8,483037	5,86439	5,770777	-0,572738	-0,093613	Cysltr2	NM_133720
17293362	10,86562	10,29385	7,145607	6,9282	-0,57177	-0,217407	Ctla2a	NM_007796
17357306	7,347357	6,775671	6,608177	6,832348	-0,571686	0,224171	B3gat3	NM_024256
17224620	6,768267	6,198813	5,639653	5,926612	-0,569454	0,286959	Chpf	NM_001001565
17347210	7,166876	6,601146	6,018254	5,717389	-0,56573	-0,300865	Memo1	NM_133771
17218653	7,267068	6,70248	7,448176	7,384833	-0,564588	-0,063343	Gas5	NR_002840
17294222	8,51128	7,946997	9,256173	8,571746	-0,564283	-0,684427	Rpl9	ENSMUST00000080545
17345740	7,342778	6,778806	7,456551	7,398767	-0,563972	-0,057784	Foxp4	NM_001110824
17288896	10,56974	10,00764	9,081757	9,02475	-0,5621	-0,057007	Actg1	NM_009609
17229782	9,601611	9,040549	8,03731	8,42053	-0,561062	0,38322	Slamf7	NM_144539
17286365	9,762955	9,204165	6,711953	6,944495	-0,55879	0,232542	Serpinb9	ENSMUST00000063191
17312829	10,91237	10,35781	9,37781	9,346898	-0,55456	-0,030912	Lgals1	NM_008495
17284360	10,12793	9,574241	11,49501	11,71039	-0,553689	0,21538	Ighm	AB067787

17265030	8,777009	8,223427	8,799217	8,777853	-0,553582	-0,021364	Acap1	NM_153788
17222913	8,523819	7,976355	6,471075	6,595484	-0,547464	0,124409	Nab1	ENSMUST00000069792
17336190	6,969216	6,421898	6,079786	6,078209	-0,547318	-0,001577	Ndufa7	NM_023202
17278056	8,655529	8,108963	6,976704	7,079153	-0,546566	0,102449	Rin3	NM_177620
17289181	9,885352	9,340539	6,678686	7,168914	-0,544813	0,490228	Arsb	NM_009712
17225360	7,866826	7,323525	7,354815	6,904544	-0,543301	-0,450271	Arl4c	NM_177305
17329636	10,5496	10,00633	4,519377	4,065839	-0,54327	-0,453538	Gp5	NM_008148
17233694	10,6716	10,12976	6,68435	6,681859	-0,54184	-0,002491	Prf1	NM_011073
17353143	6,841847	6,302286	6,779686	6,590427	-0,539561	-0,189259	Rprd1a	NM_144861
17306272	7,103533	6,56557	7,005251	7,037005	-0,537963	0,031754	Snord8	NR_028542
17234339	8,580913	8,044294	7,66598	7,83583	-0,536619	0,16985	Adora2a	NM_009630
17275524	7,111043	6,574971	7,746321	7,52486	-0,536072	-0,221461	Srp54c	NM_001100110
17356194	7,388518	6,8529	7,512085	7,639639	-0,535618	0,127554	Clcf1	ENSMUST00000046506
17328958	7,394947	6,859927	4,261745	4,061539	-0,53502	-0,200206	Gp1bb	NM_001001999
17276714	7,189328	6,655676	5,506394	5,218909	-0,533652	-0,287485	Mpp5	NM_019579
17260364	8,331414	7,800178	7,768806	7,593963	-0,531236	-0,174843	Purb	NM_011221
17216563	7,337948	6,807835	7,32009	7,193344	-0,530113	-0,126746	Mki67ip	NM_026472
17303916	8,440359	7,912599	7,858069	7,637767	-0,52776	-0,220302	Camk2g	NM_178597
17223793	6,712744	6,185215	4,588263	4,669118	-0,527529	0,080855	Fzd5	NM_022721
17297678	8,587286	8,059834	8,513708	7,980078	-0,527452	-0,53363	Cnot1	NM_178078
17315312	7,677176	7,150307	4,044717	4,359251	-0,526869	0,314534	Soat2	ENSMUST00000023806
17337065	6,816663	6,29005	6,570727	6,380967	-0,526613	-0,18976	H2-Q2	ENSMUST00000074806
17319009	10,84138	10,31536	8,721974	8,647979	-0,52602	-0,073995	Il2rb	NM_008368
17320705	9,633808	9,107987	6,715457	6,907989	-0,525821	0,192532	Yaf2	NR_028315
17243051	10,27437	9,7493	8,798029	8,924009	-0,52507	0,12598	Mob3a	NM_172457
17242931	7,670016	7,145276	8,482405	8,466669	-0,52474	-0,015736	Tcf3	NM_001164147
17261417	7,354632	6,830637	4,281227	4,151361	-0,523995	-0,129866	Chac2	NM_026527
17212229	9,042225	8,518259	6,757498	7,141586	-0,523966	0,384088	Il18r1	NM_008365
17237915	7,683266	7,160321	7,512674	7,463905	-0,522945	-0,048769	Agap2	NM_001033263
17284795	7,671269	7,149072	7,534875	7,488251	-0,522197	-0,046624	Sp4	NM_009239
17255626	7,164841	6,643003	8,287263	7,724408	-0,521838	-0,562855	Skap1	NM_001033186
17280184	7,900993	7,379378	7,173523	7,285791	-0,521615	0,112268	Zfp125	AJ005350
17274658	7,954896	7,43544	6,310131	6,29651	-0,519456	-0,013621	Tssc1	NM_201357
17292941	7,599355	7,083307	7,799572	7,684948	-0,516048	-0,114624	Fam193b	NM_145382
17236339	8,570982	8,056081	7,569371	7,686084	-0,514901	0,116713	Gnptab	NM_001004164
17348850	7,117806	6,603748	7,42284	7,270076	-0,514058	-0,152764	Rnf138	NM_207623
17284037	9,858269	9,345045	9,022526	8,830379	-0,513224	-0,192147	Brp44l	NM_018819
17217145	8,085062	7,573195	8,398483	8,361729	-0,511867	-0,036754	Elk4	NM_007923
17239702	8,274086	7,762582	7,35342	7,107204	-0,511504	-0,246216	Tbpl1	NM_011603
17280842	7,299767	6,788553	6,336206	6,370578	-0,511214	0,034372	Ifrd1	ENSMUST00000001672
17337122	10,86945	10,35939	10,6478	10,65594	-0,51006	0,00814	H2-Q6	NM_207648
17289094	5,65484	5,144958	5,18576	5,323533	-0,509882	0,137773	Ankrd34b	ENSMUST00000168871
17343449	7,703459	7,193591	7,304299	7,122027	-0,509868	-0,182272	Pglyrp2	NM_021319
17293273	8,442746	7,934557	6,665953	6,375139	-0,508189	-0,290814	Isca1	NM_026921
17309340	7,865636	7,359308	5,659228	5,526604	-0,506328	-0,132624	Spry2	NM_011897
17230304	5,966102	5,461136	7,050429	6,985984	-0,504966	-0,064445	Smyd3	ENSMUST00000128302
17231859	10,70357	10,1993	10,53372	10,34626	-0,50427	-0,18746	Ifngr1	NM_010511
17302251	6,655107	6,151074	6,592021	6,633879	-0,504033	0,041858	Sugt1	NM_026474
17284327	5,988863	5,487208	6,405217	5,919301	-0,501655	-0,485916	Ighg	AB097847
17240645	7,610886	7,109783	6,143065	6,523561	-0,501103	0,380496	Prdm1	NM_007548
17354212	6,555723	6,096356	6,955244	6,410246	-0,459367	-0,544998	Sumo2	NM_133354
17483301	6,555723	6,096356	6,955244	6,410246	-0,459367	-0,544998	Sumo2	NM_133354
17300173	5,167911	4,723652	9,845593	9,202727	-0,444259	-0,642866	Trav12-2	X04330
17349514	7,9982	7,5809	6,231769	7,173673	-0,4173	0,941904	Egr1	NM_007913

17217846	3,599054	3,190356	8,247457	6,893147	-0,408698	-1,35431	Mir181b-1	NR_029820
17330998	5,543056	5,150113	7,887892	7,242175	-0,392943	-0,645717	Cep97	NM_028815
17219407	10,61739	10,24315	7,882639	7,112572	-0,37424	-0,770067	Slamf1	NM_013730
17547965	5,862079	5,548983	6,617781	6,070514	-0,313096	-0,547267	Cbr1	NM_007620
17246803	6,90621	6,607695	6,20713	7,316542	-0,298515	1,109412	Osm	ENSMUST00000075221
17225169	8,387702	8,09376	9,488194	8,766941	-0,293942	-0,721253	Snora75	NR_028478
17215697	4,98603	4,710763	6,885127	5,980136	-0,275267	-0,904991	Ramp1	NM_001168392
17228353	7,584553	7,312157	8,530529	7,851785	-0,272396	-0,678744	Mr1	NM_008209
17216753	7,310708	7,048003	7,472921	6,965268	-0,262705	-0,507653	Mgat5	NM_145128
17330805	4,371064	4,13599	8,840948	7,944216	-0,235074	-0,896732	Trat1	NM_198297
17229658	11,33652	11,10233	7,533551	8,608191	-0,23419	1,07464	Fcer1g	NM_010185
17230111	8,455375	8,257897	4,345897	5,736881	-0,197478	1,390984	Ifi205	NM_172648
17222825	9,240271	9,04487	8,963826	8,402598	-0,195401	-0,561228	Obfc2a	NM_028696
17354219	5,162158	4,989333	3,493811	3,857747	-0,172825	0,363936	Trim36	NM_178872
17227797	11,51649	11,3647	6,526243	7,409307	-0,15179	0,883064	Rgs18	ENSMUST00000027603
17227780	6,86869	6,727451	7,286811	7,835445	-0,141239	0,548634	Rgs1	ENSMUST00000172388
17213462	6,473576	6,347395	8,528661	7,98141	-0,126181	-0,547251	Cd28	NM_007642
17360842	8,921074	8,80429	8,246848	8,340968	-0,116784	0,09412	Rps12	NM_011295
17289037	7,305953	7,201729	9,245312	8,311029	-0,104224	-0,934283	Ssbp2	NM_024272
17356583	6,609526	6,528538	7,458825	7,658788	-0,080988	0,199963	Syvn1	NM_028769
17295038	8,448845	8,378065	4,964139	5,573284	-0,07078	0,609145	Scamp1	ENSMUST00000022197
17313433	5,233052	5,168207	7,865443	7,261367	-0,064845	-0,604076	Xrcc6	ENSMUST00000100399
17300360	7,971485	7,914164	9,144283	8,977753	-0,057321	-0,16653	Pabpn1	NM_019402
17214685	4,339372	4,296576	6,619501	5,902818	-0,042796	-0,716683	Acsl3	NM_028817
17289792	3,060398	3,020478	8,447563	7,356251	-0,03992	-1,091312	Mir1904	NR_035442
17321263	6,800642	6,81018	7,478119	6,448468	0,009538	-1,029651	Adcy6	NM_007405
17260137	6,715715	6,747581	9,148178	8,215382	0,031866	-0,932796	Kremen1	NM_032396
17290695	5,639749	5,682642	7,909864	7,998283	0,042893	0,088419	Gpr137b	NM_031999
17253912	7,120241	7,164826	7,733023	7,594581	0,044585	-0,138442	Rhot1	NM_001163354
17228497	2,782127	2,828932	7,964214	6,536144	0,046805	-1,42807	Tdrd5	NM_001134741
17353676	4,489139	4,544283	4,165155	3,689264	0,055144	-0,475891	Nrg2	ENSMUST00000115713
17297374	5,642543	5,705226	7,109665	6,36235	0,062683	-0,747315	Nkiras1	NM_023526
17213548	4,067666	4,134457	6,286251	6,871441	0,066791	0,58519	Nrp2	NM_001077406
17374488	11,25486	11,32608	8,187112	10,18821	0,07122	2,001098	Thbs1	NM_011580
17312700	7,76849	7,842728	7,889555	8,438079	0,074238	0,548524	Ncf4	NM_008677
17288145	5,896847	5,982243	5,876764	6,039476	0,085396	0,162712	Habp4	NM_019986
17332084	6,933	7,036024	7,462723	7,457321	0,103024	-0,005402	Gcfc1	ENSMUST00000118522
17291953	5,153798	5,282709	5,598669	5,742994	0,128911	0,144325	Muted	NM_139063
17218861	11,14262	11,3079	6,46808	9,011907	0,16528	2,543827	F5	NM_007976
17250178	6,567119	6,739287	7,416512	6,655526	0,172168	-0,760986	Hist3h2a	NM_178218
17212355	7,147372	7,342451	8,072539	7,059476	0,195079	-1,013063	Nck2	NM_010879
17300251	7,847435	8,072048	6,149077	6,75549	0,224613	0,606413	Abhd4	NM_134076
17357947	5,34164	5,585937	5,893805	7,185633	0,244297	1,291828	Olfr1502	NM_146797
17280125	8,232164	8,524548	6,924429	7,566913	0,292384	0,642484	Pqlc3	NM_001161111
17300189	1,933763	2,267333	7,829066	7,166917	0,33357	-0,662149	Trav3-4	Z86035
17314460	7,833005	8,176063	7,187557	7,708617	0,343058	0,52106	Irak4	NM_029926
17262178	6,269192	6,631094	6,452964	6,440458	0,361902	-0,012506	Trim41	NM_145377
17225472	6,986139	7,358756	7,614451	8,147987	0,372617	0,533536	Zfp330	AK139907
17329026	1,681696	2,062349	2,484488	2,368513	0,380653	-0,115975	Olfr164	ENSMUST00000056727
17344387	5,255955	5,647359	7,687472	7,07621	0,391404	-0,611262	Gtf2h4	ENSMUST00000001565
17343897	6,468628	6,881639	7,309086	6,618672	0,413011	-0,690414	Ppt2	ENSMUST00000169067
17292634	6,804196	7,224471	6,585979	7,177952	0,420275	0,591973	Nfil3	ENSMUST00000071065
17218366	7,210876	7,640054	8,214086	7,580537	0,429178	-0,633549	Stx6	NM_021433
17319607	7,622211	8,063448	6,758389	7,300317	0,441237	0,541928	Naga	NM_008669
17286283	7,940247	8,382278	6,931294	7,486552	0,442031	0,555258	Dusp22	NM_134068
17339729	9,442807	9,884975	5,408422	7,34491	0,442168	1,936488	Ltbp1	NM_019919

17289103	6,449024	6,902662	8,602953	7,944226	0,453638	-0,658727	Serinc5	NM_172588
17231066	5,945387	6,42391	7,232356	6,687385	0,478523	-0,544971	Dtl	NM_029766
17326510	9,258803	9,746803	4,678825	6,492658	0,488	1,813833	Pros1	NM_011173
17296128	6,049269	6,54127	6,30602	7,071895	0,492001	0,765875	Gapt	NM_177713
17329433	7,165043	7,663781	7,661284	6,973663	0,498738	-0,687621	Bcl6	ENSMUST00000023151
17334419	8,05252	8,553817	6,752113	7,047427	0,501297	0,295314	Sepx1	NM_013759
17253431	6,716819	7,218339	7,404024	7,217378	0,50152	-0,186646	Flot2	NM_008028
17270888	7,797262	8,300146	6,875265	7,076592	0,502884	0,201327	Ern1	ENSMUST00000001059
17327038	9,130974	9,640001	8,559582	8,8566	0,509027	0,297018	Ifnar2	NM_010509
17298805	8,802176	9,312473	8,938039	9,226872	0,510297	0,288833	Glud1	NM_008133
17232634	4,345088	4,856625	8,669348	7,572428	0,511537	-1,09692	Tube1	NM_028006
17214998	8,030888	8,542461	7,467901	7,64795	0,511573	0,180049	Itm2c	ENSMUST00000027425
17232951	7,109286	7,621343	7,799578	7,739091	0,512057	-0,060487	Sec63	NM_153055
17216727	6,243887	6,759185	4,036674	4,605269	0,515298	0,568595	Slc35f5	NM_028787
17327331	6,228119	6,74617	7,073561	6,538659	0,518051	-0,534902	Chaf1b	NM_028083
17249057	7,148994	7,669847	8,091006	7,397471	0,520853	-0,693535	Mapk9	NM_016961
17356563	7,0764	7,597256	6,425904	6,6693	0,520856	0,243396	Slc25a45	ENSMUST00000025732
17301757	7,214467	7,735385	8,018515	7,871659	0,520918	-0,146856	Reep4	NM_180588
17290301	6,029445	6,552946	5,68573	5,702523	0,523501	0,016793	Akr1e1	NM_018859
17351232	6,764033	7,294382	7,520123	7,478109	0,530349	-0,042014	Wdr7	NM_001014981
17241162	7,14415	7,674551	7,284641	7,561146	0,530401	0,276505	Sgpl1	NM_009163
17219096	8,216847	8,749981	8,274993	8,30149	0,533134	0,026497	Aldh9a1	NM_019993
17258714	6,818596	7,352375	6,455593	6,443652	0,533779	-0,011941	Sec14l1	NM_001166506
17233629	10,51824	11,0534	10,74282	10,89393	0,53516	0,15111	Psap	NM_001146120
17272785	9,435004	9,972488	7,731281	8,14407	0,537484	0,412789	Lgals3bp	ENSMUST00000043722
17222179	5,384631	5,922555	7,069615	6,700094	0,537924	-0,369521	Lman2l	ENSMUST00000125304
17298312	6,117821	6,657413	6,170248	6,119926	0,539592	-0,050322	Glt8d1	NM_029626
17297227	6,853437	7,393343	7,702649	7,707954	0,539906	0,005305	Slc4a7	NM_001033270
17347475	6,579448	7,120022	6,853564	6,190247	0,540574	-0,663317	Hnrpll	NM_144802
17307588	6,86782	7,412256	7,040932	7,089822	0,544436	0,04889	Fdft1	NM_010191
17219546	5,077194	5,623463	6,268832	6,785285	0,546269	0,516453	Slamf9	ENSMUST00000027830
17350517	6,285724	6,832091	6,848124	6,692298	0,546367	-0,155826	Hsd17b4	NM_008292
17247533	9,079891	9,628637	9,443911	9,41308	0,548746	-0,030831	Rab1	NM_008996
17308132	7,169549	7,718704	8,83729	8,725042	0,549155	-0,112248	Slc25a37	NM_026331
17255402	7,48515	8,034584	7,738726	7,710865	0,549434	-0,027861	Spop	NM_025287
17347571	6,20408	6,754478	6,795957	6,560254	0,550398	-0,235703	Map4k3	NM_001081357
17317296	6,656888	7,20871	6,531343	6,694017	0,551822	0,162674	Tmem65	ENSMUST00000072113
17244439	7,007654	7,560632	7,696054	7,965966	0,552978	0,269912	Plxnc1	NM_018797
17256345	7,387208	7,941107	8,280304	8,081751	0,553899	-0,198553	Eif1	NM_011508
17293757	7,636679	8,190578	7,367367	7,502349	0,553899	0,134982	Hiatl1	ENSMUST00000155487
17253235	8,582083	9,139152	9,168846	9,389271	0,557069	0,220425	Ssh2	NM_177710
17230256	8,095563	8,652717	8,205182	8,29938	0,557154	0,094198	Adss	ENSMUST00000016105
17308714	7,069029	7,627498	7,17178	7,386947	0,558469	0,215167	Slc25a30	NM_026232
17301722	8,124253	8,683105	8,155062	8,36409	0,558852	0,209028	Bin3	NM_021328
17340332	7,602308	8,161225	7,995773	8,164858	0,558917	0,169085	Klraq1	NM_028658
17228860	8,883883	9,444352	9,288135	9,294095	0,560469	0,00596	Aph1a	NM_146104
17350869	8,287262	8,852432	8,620236	8,517699	0,56517	-0,102537	Isoc1	NM_025478
17282723	9,014027	9,57972	9,130428	9,300289	0,565693	0,169861	Tmed10	NM_026775
17256414	6,971656	7,537413	6,230683	6,543866	0,565757	0,313183	Dnajc7	NM_019795
17283422	6,958607	7,524775	7,494435	7,526799	0,566168	0,032364	Atxn3	NM_029705
17332050	7,328505	7,89607	6,70852	6,967167	0,567565	0,258647	Synj1	NM_001164483
17288467	5,701721	6,269762	7,441571	7,693471	0,568041	0,2519	Lpcat1	ENSMUST00000022099
17282420	6,673059	7,244739	7,17173	7,075949	0,57168	-0,095781	Zfyve1	NM_183154
17222650	6,169672	6,743066	6,060338	6,180779	0,573394	0,120441	Uxs1	ENSMUST00000126008
17274875	6,971498	7,548201	7,482179	7,602814	0,576703	0,120635	Nampt	NM_021524
17334304	6,424127	7,003391	7,312895	7,337049	0,579264	0,024154	Pkd1	NM_013630

17322559	7,589106	8,170588	8,151337	8,246286	0,581482	0,094949	Hmox2	ENSMUST00000004172
17229607	8,324808	8,906755	8,643161	9,13483	0,581947	0,491669	Fcgr2b	NM_001077189
17331564	6,93132	7,513807	7,777517	7,399642	0,582487	-0,377875	Nrip1	ENSMUST00000121927
17215968	8,010756	8,597423	8,612072	8,691059	0,586667	0,078987	Ppp1r7	ENSMUST00000027494
17233226	9,697678	10,29039	7,937216	9,196314	0,592712	1,259098	Lilrb4	NM_013532
17320307	4,24427	4,838446	6,409072	6,939296	0,594176	0,530224	Mapk12	NM_013871
17236836	9,096325	9,690732	9,711331	9,98899	0,594407	0,277659	Atp2b1	NM_026482
17354995	6,832865	7,431355	7,303247	7,224578	0,59849	-0,078669	Fech	NM_007998
17272913	6,895566	7,496176	6,706542	7,210859	0,60061	0,504317	Sgsh	NM_018822
17240053	6,671791	7,273341	6,979804	7,120914	0,60155	0,14111	Ncoa7	NM_172495
17255026	6,949783	7,563349	8,69096	8,800943	0,613566	0,109983	Trim25	NM_009546
17221756	6,687934	7,302514	4,588058	5,139194	0,61458	0,551136	Ogfrl1	NM_001081079
17334803	7,194066	7,810008	8,133793	8,183661	0,615942	0,049868	Jmjd8	ENSMUST00000133595
17500275	6,681298	7,298223	6,380334	6,72434	0,616925	0,344006	Erlin2	NM_153592
17269911	7,105235	7,722378	6,891325	7,549478	0,617143	0,658153	Vat1	NM_012037
17238594	6,633016	7,261329	4,334667	4,435435	0,628313	0,100768	Cd63	NM_001042580
17211774	6,76707	7,395988	7,526197	7,704848	0,628918	0,178651	Plekhb2	NM_145516
17254166	7,885752	8,515647	7,827438	7,978152	0,629895	0,150714	Slfn2	NM_011408
17282563	9,275988	9,909496	9,244076	9,457718	0,633508	0,213642	Npc2	NM_023409
17353337	6,619605	7,255728	5,833216	5,505816	0,636123	-0,3274	Gypc	NM_001048207
17238482	7,058269	7,695981	7,205977	7,405742	0,637712	0,199765	Rnf41	NM_001164237
17305685	7,825674	8,46529	8,024742	8,034334	0,639616	0,009592	Ddhd1	NM_001042719
17272437	6,803088	7,444184	6,783096	7,200078	0,641096	0,416982	Rhbdf2	NM_172572
17304906	7,347307	7,990122	9,332798	9,596867	0,642815	0,264069	Wdfy4	NM_001146022
17350740	6,228748	6,871938	7,455481	7,048126	0,64319	-0,407355	Lmnb1	NM_010721
17243022	6,709941	7,353891	6,884367	6,797133	0,64395	-0,087234	Btbd2	NM_145361
17324553	8,52689	9,178108	8,052605	8,686935	0,651218	0,63433	Ccdc50	ENSMUST00000100026
17342101	6,474099	7,125337	7,112027	7,171763	0,651238	0,059736	Hn1l	ENSMUST00000024981
17286254	6,041173	6,695262	6,192604	6,299879	0,654089	0,107275	Mboat1	NM_153546
17383467	6,406147	7,060637	5,634828	6,380713	0,65449	0,745885	Ntng2	NM_133501
17281971	7,931169	8,585807	8,325535	8,447086	0,654638	0,121551	Sgpp1	NM_030750
17213428	7,147408	7,802353	6,492986	7,086036	0,654945	0,59305	Cyp20a1	ENSMUST00000060608
17227483	6,560924	7,21599	7,155493	7,298692	0,655066	0,143199	Camsap2	NM_001081360
17343035	7,200163	7,857397	7,815231	7,948244	0,657234	0,133013	Mtch1	NM_019880
17283930	7,566288	8,236075	7,678843	7,862485	0,669787	0,183642	Wars	NM_001164314
17346349	6,036273	6,710959	7,346448	7,848524	0,674686	0,502076	Rfx2	NM_027787
17229620	7,694138	8,369534	5,215715	6,375921	0,675396	1,160206	Fcgr3	NM_010188
17277387	7,395317	8,075942	7,802212	8,297579	0,680625	0,495367	Fos	NM_010234
17268729	7,084195	7,767599	7,934349	7,90494	0,683404	-0,029409	Fbxl20	NM_028149
17335297	6,714341	7,400104	6,843205	7,077991	0,685763	0,234786	Ppard	NM_011145
17329079	6,393589	7,080763	6,808092	6,772245	0,687174	-0,035847	Parl	NM_001005767
17321597	7,093781	7,781806	7,982627	8,048676	0,688025	0,066049	Racgap1	NM_012025
17274249	6,888887	7,586748	6,835749	7,072966	0,697861	0,237217	Pdia6	NM_027959
17223799	6,701841	7,402831	7,965491	8,125345	0,70099	0,159854	Plekhm3	NM_001039493
17347163	8,48396	9,185109	5,920551	6,959862	0,701149	1,039311	Xdh	NM_011723
17244140	7,058547	7,760675	6,987411	6,805999	0,702128	-0,181412	Apaf1	NM_001042558
17262357	6,425956	7,133457	6,850734	6,953052	0,707501	0,102318	Rufy1	NM_172557
17281843	7,454978	8,168287	6,600838	7,186118	0,713309	0,58528	Dhrs7	NM_025522
17350611	8,248373	8,962262	9,42992	9,756411	0,713889	0,326491	Snx2	NM_026386
17272619	5,99809	6,712692	7,665372	7,954583	0,714602	0,289211	Socs3	NM_007707
17214142	7,242959	7,962944	4,939416	5,328135	0,719985	0,388719	Cxcr2	NM_009909
17326895	7,664179	8,386475	7,982733	7,482109	0,722296	-0,500624	Bach1	NM_007520
17317858	6,285686	7,018915	5,862745	6,228966	0,733229	0,366221	Ptk2	NM_007982
17268120	6,054599	6,788635	5,679688	5,405785	0,734036	-0,273903	Pdk2	NM_133667
17273165	8,549341	9,288026	7,720013	8,021994	0,738685	0,301981	P4hb	ENSMUST00000026122
17243576	6,435679	7,181304	5,923933	6,436069	0,745625	0,512136	Appl2	ENSMUST00000020500

17356743	7,550539	8,299877	7,470304	7,830759	0,749338	0,360455	Ehd1	ENSMUST00000025684
17330523	7,282652	8,031994	7,324233	7,411808	0,749342	0,087575	Atp6v1a	ENSMUST00000114666
17239856	6,563095	7,317572	5,219164	5,768072	0,754477	0,548908	Akap7	NM_018747
17284065	6,995052	7,752645	6,307638	6,753062	0,757593	0,445424	Cdc42bpb	NM_183016
17233022	8,649394	9,408406	10,18995	10,30242	0,759012	0,11247	Cd24a	NM_009846
17258140	7,088554	7,850896	7,233706	7,325498	0,762342	0,091792	Tmem104	NM_001033393
17353948	7,367426	8,132895	7,098341	7,399754	0,765469	0,301413	Gnpda1	NM_011937
17295588	7,378003	8,144991	6,284079	7,173254	0,766988	0,889175	Naip5	NM_010870
17226771	6,500519	7,274589	7,985357	8,278639	0,77407	0,293282	Ikbke	NM_019777
17224270	6,357535	7,132519	5,406294	5,685338	0,774984	0,279044	Tmbim1	ENSMUST00000113796
17329316	7,046887	7,823175	6,171291	5,734249	0,776288	-0,437042	Dgkg	NM_138650
17356002	8,611594	9,397997	9,788301	10,01997	0,786403	0,231669	Unc93b1	NM_019449
17327995	5,837494	6,632035	5,799664	5,709239	0,794541	-0,090425	Carhsp1	NM_025821
17217182	6,123728	6,919407	6,311366	6,757595	0,795679	0,446229	Nuak2	NM_001195025
17231003	6,039546	6,836799	6,965693	6,812791	0,797253	-0,152902	Mfsd7b	NM_001081259
17222527	6,725369	7,527747	5,307161	5,970162	0,802378	0,663001	Tbc1d8	NM_018775
17314190	5,157845	5,963087	3,793981	4,35394	0,805242	0,559959	Shank3	ENSMUST00000109309
17306856	8,234545	9,04537	8,680914	8,794653	0,810825	0,113739	Dhrs1	NM_026819
17234175	6,619415	7,435453	7,658133	7,635149	0,816038	-0,022984	Ipmk	NM_027184
17329723	8,176059	8,995285	8,857574	8,928021	0,819226	0,070447	Acap2	NM_030138
17223932	6,304934	7,131555	7,383703	7,247778	0,826621	-0,135925	Lancl1	NM_001190985
17329105	6,106488	6,943847	7,068325	6,671293	0,837359	-0,397032	Abcc5	NM_013790
17231891	7,221858	8,069007	7,877102	8,209234	0,847149	0,332132	Map3k5	NM_008580
17295569	6,106658	6,956167	5,05092	5,99629	0,849509	0,94537	Naip2	NM_010872
17310882	6,954261	7,810472	4,857493	5,534661	0,856211	0,677168	Pgcp	NM_018755
17241130	6,644825	7,502509	7,536669	7,574985	0,857684	0,038316	Slc29a3	NM_023596
17307518	5,183063	6,041934	5,804796	5,325121	0,858871	-0,479675	Mir15a	NR_029733
17316956	6,317077	7,179224	6,322385	6,346126	0,862147	0,023741	Trps1	NM_032000
17268227	8,052107	8,916366	8,515485	8,32166	0,864259	-0,193825	Abi3	NM_025659
17238424	7,118496	7,98763	7,802932	7,833811	0,869134	0,030879	Cnpy2	NM_019953
17289889	7,116374	7,995862	9,424937	9,405313	0,879488	-0,019624	Il6st	NM_010560
17293675	6,341447	7,221475	6,929873	7,138278	0,880028	0,208405	Cdc14b	NM_172587
17354434	5,892123	6,772179	7,430119	6,851239	0,880056	-0,57888	Aldh7a1	ENSMUST00000174518
17345038	7,846705	8,730124	9,022942	9,170952	0,883419	0,14801	Cd2ap	NM_009847
17259679	6,965156	7,860522	4,195534	5,027074	0,895366	0,83154	Metrnl	NM_144797
17314564	5,600322	6,506842	6,385886	6,640608	0,90652	0,254722	Tmem106c	NM_201359
17254188	5,412025	6,322153	5,455376	5,577021	0,910128	0,121645	Slfn3	NM_011409
17240330	6,607723	7,51919	5,977628	6,075196	0,911467	0,097568	Slc16a10	NM_001114332
17319192	7,224529	8,138163	8,049911	8,133665	0,913634	0,083754	Tmem184b	NM_172608
17322359	6,138439	7,054343	6,01297	6,382364	0,915904	0,369394	Zfp385a	NM_013866
17236882	7,854383	8,775178	7,749078	7,589499	0,920795	-0,159579	Dusp6	NM_026268
17342581	6,098496	7,021397	6,800559	7,020883	0,922901	0,220324	Decr2	ENSMUST00000040907
17328625	7,766894	8,697867	7,211559	7,803964	0,930973	0,592405	Sdf2l1	NM_022324
17304740	5,949855	6,893625	7,758348	8,148488	0,94377	0,39014	Sh3bp5	ENSMUST00000091903
17241692	6,390726	7,340114	7,735371	7,111695	0,949388	-0,623676	Cdk1	ENSMUST00000020099
17325979	6,764509	7,716749	3,795039	3,63881	0,95224	-0,156229	Gcet2	NM_001159297
17281025	7,576966	8,534466	6,64538	7,030034	0,9575	0,384654	Heatr5a	NM_177171
17301176	6,63165	7,589678	7,300785	7,309306	0,958028	0,008521	Wdfy2	NM_175546
17214368	6,435288	7,393861	5,127243	6,001647	0,958573	0,874404	Cyp27a1	NM_024264
17279404	8,648836	9,608633	9,588039	9,983295	0,959797	0,395256	Pld4	NM_178911
17315349	7,686694	8,657921	7,844229	8,241407	0,971227	0,397178	Mfsd5	NM_134100
17259235	6,641037	7,61781	6,244538	6,365293	0,976773	0,120755	Baiap2	ENSMUST00000026436
17334205	6,402025	7,389976	7,43251	7,628057	0,987951	0,195547	Abca3	NM_013855
17334545	6,172929	7,16865	5,96417	6,071802	0,995721	0,107632	Clcn7	NM_011930
17338472	6,216537	7,214079	6,482624	6,624117	0,997542	0,141493	Mocs1	NM_020042
17269521	8,394341	9,394493	8,395644	8,536424	1,000152	0,14078	Acly	NM_001199296

17245481	6,554946	7,564241	6,578812	7,174853	1,009295	0,596041	Rassf3	NM_138956
17226194	6,070665	7,089951	5,571286	5,8742	1,019286	0,302914	Ralb	NM_022327
17256473	6,311055	7,330361	6,429249	6,996138	1,019306	0,566889	Atp6v0a1	NM_016920
17326069	7,671528	8,691006	8,573864	9,122976	1,019478	0,549112	Retnla	NM_020509
17286587	9,106037	10,13084	9,893567	10,20862	1,024803	0,315053	Ly86	NM_010745
17355092	7,401188	8,427783	7,078792	7,462524	1,026595	0,383732	Lman1	ENSMUST00000120461
17228544	7,521658	8,549862	8,855238	8,532072	1,028204	-0,323166	Soat1	NM_009230
17327137	6,727843	7,756936	5,314826	6,305424	1,029093	0,990598	Itsn1	NM_010587
17268909	6,529805	7,561562	8,153514	7,592195	1,031757	-0,561319	Top2a	NM_011623
17222549	6,339431	7,371259	6,03305	6,253771	1,031828	0,220721	Rnf149	NM_001033135
17265247	6,164906	7,204969	6,547497	6,518496	1,040063	-0,029001	Pelp1	NM_029231
17357810	8,090467	9,148258	6,651259	7,503748	1,057791	0,852489	Mpeg1	NM_010821
17343263	7,407168	8,468616	10,74929	10,60341	1,061448	-0,14588	Sik1	NM_010831
17326567	7,278197	8,340281	5,883628	6,524311	1,062084	0,640683	Gbe1	ENSMUST00000163832
17339772	5,710759	6,774759	6,600175	7,158906	1,064	0,558731	Rasgrp3	NM_207246
17266952	8,274776	9,343382	7,103242	8,3866	1,068606	1,283358	Ccl9	NM_011338
17289249	6,82862	7,900139	7,44457	7,641573	1,071519	0,197003	Wdr41	NM_172590
17243617	6,006196	7,081518	6,506867	7,016105	1,075322	0,509238	Ckap4	NM_175451
17227278	6,988655	8,064938	6,762311	6,969554	1,076283	0,207243	Rnpep	NM_145417
17301558	6,743875	7,822746	6,363808	6,524606	1,078871	0,160798	Kctd9	NM_001111028
17266960	7,722705	8,802958	7,50527	8,615627	1,080253	1,110357	Ccl6	NM_009139
17274540	5,590309	6,672502	7,932548	7,402667	1,082193	-0,529881	Rrm2	NM_009104
17483577	9,616143	10,70433	7,455223	8,969418	1,088187	1,514195	Itgam	NM_001082960
17337269	6,68471	7,778625	7,515759	7,904148	1,093915	0,388389	Nrm	NM_134122
17338378	6,255622	7,349814	4,208319	4,381313	1,094192	0,172994	Treml4	NM_001033922
17324420	6,366271	7,462177	9,181595	9,36886	1,095906	0,187265	St6gal1	NM_145933
17332783	6,353034	7,453197	7,25614	7,651301	1,100163	0,395161	Snx9	ENSMUST00000002436
17337741	7,85463	8,959998	5,732572	6,887778	1,105368	1,155206	Tnfrsf21	NM_178589
17295607	7,041655	8,148333	6,025925	6,868568	1,106678	0,842643	Naip6	NM_010871
17346528	8,400613	9,507429	6,433522	7,582873	1,106816	1,149351	C3	NM_009778
17298364	5,621681	6,730614	8,257797	7,871689	1,108933	-0,386108	Nt5dc2	NM_027289
17352439	6,639665	7,75432	7,213013	7,18357	1,114655	-0,029443	Arhgap12	NM_001039692
17320538	5,56133	6,706966	5,428593	5,737524	1,145636	0,308931	Arsa	NM_009713
17243382	6,169161	7,323791	4,692461	5,629131	1,15463	0,93667	Gna15	NM_010304
17211713	5,217495	6,376225	5,350207	5,945888	1,15873	0,595681	Ptpn18	NM_011206
17345570	6,301345	7,461648	7,271828	7,444485	1,160303	0,172657	Cnpy3	NM_028065
17292915	7,216951	8,382987	8,474016	8,961411	1,166036	0,487395	Dok3	NM_013739
17326075	8,644369	9,815186	2,864657	3,653805	1,170817	0,789148	Retnlg	NM_181596
17252995	6,22764	7,399069	7,652436	7,768938	1,171429	0,116502	Slc43a2	NM_001199284
17217440	5,392055	6,574775	5,77127	6,165281	1,18272	0,394011	Cyb5r1	ENSMUST00000027726
17289527	7,148633	8,346066	8,875358	9,398293	1,197433	0,522935	Cd180	ENSMUST00000022124
17233536	6,521334	7,724533	6,937801	7,14242	1,203199	0,204619	P4ha1	NM_011030
17318713	6,414796	7,618502	6,516696	6,902083	1,203706	0,385387	Arhgap39	NM_001168288
17342642	8,622986	9,830132	9,820033	10,36827	1,207146	0,548237	Dusp1	ENSMUST00000025025
17218261	8,163346	9,377582	7,343568	8,148682	1,214236	0,805114	Ncf2	NM_010877
17493869	5,886518	7,102915	4,188938	4,768302	1,216397	0,579364	P2ry6	NM_183168
17347042	5,738036	6,97031	7,259377	7,023698	1,232274	-0,235679	Ndc80	NM_023294
17284114	6,446747	7,691731	6,741036	7,076285	1,244984	0,335249	Ckb	NM_021273
17251485	5,840549	7,089443	7,596467	7,207796	1,248894	-0,388671	Aurkb	NM_011496
17327084	7,157288	8,430135	8,590483	8,410227	1,272847	-0,180256	Ifngr2	ENSMUST00000023687
17314260	5,927593	7,203182	7,438414	7,803335	1,275589	0,364921	Lrrk2	NM_025730
17292215	5,337922	6,61362	5,065978	5,438004	1,275698	0,372026	Kif13a	NM_010617
17289794	6,033621	7,311998	6,687064	7,047769	1,278377	0,360705	Plk2	NM_152804
17327465	5,596242	6,878515	9,28458	8,627478	1,282273	-0,657102	Ets2	NM_011809
17507288	3,029332	4,312334	4,89523	4,306369	1,283002	-0,588861	Shcbp1	NM_011369
17211043	6,718893	8,007373	7,757994	7,954595	1,28848	0,196601	Sgk3	NM_133220

17307774	4,501289	5,799735	6,180698	5,702818	1,298446	-0,47788	Esco2	ENSMUST00000022613
17330918	6,676832	7,979583	6,008067	6,857502	1,302751	0,849435	Alcam	ENSMUST00000023312
17257444	7,074046	8,37866	4,131145	4,410233	1,304614	0,279088	Ace	NM_207624
17281084	5,923933	7,233448	7,229249	6,513737	1,309515	-0,715512	Egln3	NM_028133
17321761	5,846561	7,156965	5,773309	6,243011	1,310404	0,469702	Smagp	NM_174992
17338371	6,432153	7,771784	4,300385	4,798179	1,339631	0,497794	Trem3	NM_021407
17337228	7,08053	8,422618	5,938332	7,754211	1,342088	1,815879	Ier3	NM_133662
17231033	5,928178	7,270733	8,94608	9,293113	1,342555	0,347033	Atf3	NM_007498
17324446	5,672898	7,022024	5,948678	6,04128	1,349126	0,092602	Rtp4	NM_023386
17383216	6,999491	8,35165	6,253881	6,973873	1,352159	0,719992	Slc2a6	NM_172659
17319738	6,51567	7,870815	6,718919	7,367139	1,355145	0,64822	Nfam1	ENSMUST00000109503
17218349	8,267183	9,688818	8,028639	8,442446	1,421635	0,413807	Glul	NM_008131
17211286	7,80631	9,230669	7,81525	8,192135	1,424359	0,376885	Ly96	NM_016923
17497525	5,802816	7,24529	4,69452	5,212566	1,442474	0,518046	Adam8	ENSMUST00000106069
17310673	7,272774	8,723026	5,864444	7,024254	1,450252	1,15981	Ank	NM_020332
17306477	6,577716	8,028574	4,366122	5,948697	1,450858	1,582575	Slc7a8	NM_016972
17229502	6,038124	7,504622	6,597444	6,818183	1,466498	0,220739	Uap1	NM_133806
17217399	5,379539	6,853388	3,245401	3,312111	1,473849	0,06671	Chi3l1	NM_007695
17331918	5,833219	7,310307	5,329571	5,875249	1,477088	0,545678	Tiam1	ENSMUST00000114124
17357640	5,071126	6,553259	3,785595	3,955008	1,482133	0,169413	Ms4a4a	XM_986941
17279349	6,666887	8,149317	5,968636	6,547713	1,48243	0,579077	Adssl1	NM_007421
17315352	4,780274	6,275684	5,709484	5,763056	1,49541	0,053572	Espl1	NM_001014976
17495839	9,259654	10,76009	6,737537	8,84012	1,500436	2,102583	Igsf6	NM_030691
17242376	6,038552	7,549084	6,534376	6,793246	1,510532	0,25887	Pfkl	NM_008826
17291694	6,182758	7,694995	5,813475	6,515891	1,512237	0,702416	Serpinb1a	NM_025429
17254153	5,52689	7,062624	5,935768	6,12475	1,535734	0,188982	Slfn5	NM_183201
17241623	7,299825	8,846231	7,749156	8,083022	1,546406	0,333866	Reep3	NM_001204915
17493949	4,177262	5,726651	4,754477	5,378238	1,549389	0,623761	Folr2	NM_008035
17338416	5,163425	6,725471	3,648878	4,07131	1,562046	0,422432	Trem2	NM_031254
17256784	6,155197	7,719026	6,158118	6,359836	1,563829	0,201718	Tmem106a	ENSMUST00000100403
17333731	5,183623	6,759561	3,251913	3,167332	1,575938	-0,084581	Fpr2	NM_008039
17310044	5,666428	7,247518	7,503334	8,121386	1,58109	0,618052	Lifr	NM_013584
17256502	5,577532	7,166973	5,237371	5,780448	1,589441	0,543077	Naglu	NM_013792
17270877	6,13765	7,762251	6,442562	6,914905	1,624601	0,472343	Icam2	ENSMUST00000001055
17335467	6,474934	8,102732	6,59338	7,968711	1,627798	1,375331	Cdkn1a	NM_007669
17339108	7,99635	9,625868	8,59907	8,968794	1,629518	0,369724	Man2a1	ENSMUST00000086723
17211790	6,050535	7,68284	8,140153	7,975585	1,632305	-0,164568	Hs6st1	NM_015818
17258107	6,020436	7,67225	4,786313	5,079708	1,651814	0,293395	Cd300a	NM_170758
17354764	5,438623	7,092581	4,133324	4,284307	1,653958	0,150983	Arhgef37	NM_177828
17256959	8,210927	9,874049	7,967088	8,891502	1,663122	0,924414	Grn	NM_008175
17245700	6,863377	8,531673	8,376373	8,715645	1,668296	0,339272	Tspan31	NM_025982
17259907	7,993747	9,678206	7,242939	8,690989	1,684459	1,44805	Tcn2	ENSMUST00000109993
17216458	8,243145	9,931284	5,985657	8,825514	1,688139	2,839857	Serpinb2	NM_011111
17233394	7,953105	9,650748	8,129412	9,027002	1,697643	0,89759	Smpdl3a	ENSMUST00000020022
17506991	5,190357	6,888694	6,126982	5,972383	1,698337	-0,154599	Nrp1	NM_008737
17254395	5,580338	7,292683	5,96351	6,275317	1,712345	0,311807	Acaca	NM_133360
17264835	8,017208	9,732675	7,214764	8,459773	1,715467	1,245009	Cd68	NM_009853
17358214	6,490279	8,208713	6,423411	7,027411	1,718434	0,604	Klf9	NM_010638
17301213	8,71772	10,44006	9,27864	9,959899	1,72234	0,681259	Ctsb	NM_007798
17254171	6,384534	8,106915	7,432618	7,901112	1,722381	0,468494	Slfn1	NM_011407
17351465	6,21597	7,972548	7,30476	7,812508	1,756578	0,507748	Tubb6	NM_026473
17328937	6,019662	7,808821	6,604642	7,289473	1,789159	0,684831	Comt	ENSMUST00000115609
17306344	5,515233	7,305635	6,787159	7,292461	1,790402	0,505302	Slc7a7	NM_011405
17279131	5,345794	7,144465	4,219324	4,739029	1,798671	0,519705	Tnfaip2	ENSMUST00000102745
17224971	6,740494	8,547483	5,193279	6,51728	1,806989	1,324001	Pid1	NM_001003948
17230045	7,321849	9,135925	3,720185	5,111405	1,814076	1,39122	Ifi204	NM_008329

17230484	5,482994	7,297328	7,097932	7,433383	1,814334	0,335451	Ephx1	ENSMUST00000036928
17346749	7,109239	8,936438	7,586003	8,136797	1,827199	0,550794	Rab31	NM_133685
17218845	8,684654	10,54799	5,951889	8,218878	1,863336	2,266989	Selp	NM_011347
17328508	4,895505	6,779959	3,39824	4,194564	1,884454	0,796324	Fgd4	ENSMUST00000161861
17268884	5,382138	7,294327	5,402496	5,179055	1,912189	-0,223441	Nr1d1	ENSMUST00000064941
17272770	7,820322	9,740899	7,653145	8,406399	1,920577	0,753254	Timp2	ENSMUST00000017610
17337796	6,515199	8,448761	3,791042	5,289989	1,933562	1,498947	Pla2g7	ENSMUST00000024706
17254176	6,803511	8,744821	4,172538	5,549662	1,94131	1,377124	Slfn4	NM_011410
17308257	5,514946	7,508637	4,630305	5,77283	1,993691	1,142525	Sorbs3	NM_011366
17321823	6,024487	8,031663	5,396936	6,444937	2,007176	1,048001	Krt80	NM_028770
17335506	4,984896	6,998934	3,606941	4,711351	2,014038	1,10441	Pi16	ENSMUST00000114701
17484167	4,92322	6,94167	3,622232	4,609194	2,01845	0,986962	Dock1	NM_001033420
17504444	7,363705	9,384002	7,331205	8,30427	2,020297	0,973065	Cmtm3	NM_024217
17342625	6,138542	8,164219	6,193325	7,181438	2,025677	0,988113	Itfg3	ENSMUST00000114988
17223863	7,332762	9,363936	5,971759	7,213199	2,031174	1,24144	Idh1	NM_001111320
17341963	5,082928	7,122298	4,866551	5,847231	2,03937	0,98068	Slc9a3r2	NM_023055
17320337	6,498953	8,556587	5,765083	6,91497	2,057634	1,149887	Plxnb2	NM_138749
17360977	5,13719	7,201246	5,988608	5,752959	2,064056	-0,235649	Lrp5	NM_008513
17382920	5,425421	7,492276	5,788852	5,950367	2,066855	0,161515	Card9	ENSMUST00000100303
17532569	5,433601	7,501606	4,842346	5,687298	2,068005	0,844952	Ccr1	NM_009912
17265268	4,82003	6,891633	5,573634	6,423313	2,071603	0,849679	Cxcl16	ENSMUST00000019064
17331284	5,035634	7,118655	5,050541	5,839437	2,083021	0,788896	Crybg3	NM_174848
17245648	4,778471	6,872993	5,455342	6,217944	2,094522	0,762602	Slc16a7	ENSMUST00000063318
17287984	6,669252	8,801469	7,202742	7,396691	2,132217	0,193949	Dapk1	NM_029653
17325608	5,745573	7,883711	8,067148	8,45969	2,138138	0,392542	Cd80	NM_009855
17219199	4,591934	6,73474	3,82971	4,513111	2,142806	0,683401	Fcgr4	NM_144559
17267601	6,844967	9,005307	8,231976	8,786457	2,16034	0,554481	Scpep1	NM_029023
17239227	6,203192	8,364386	5,573036	6,849443	2,161194	1,276407	Rab32	NM_026405
17287827	8,064057	10,23659	6,699467	8,574282	2,172533	1,874815	Tgfbi	NM_009369
17281721	6,115036	8,306716	4,734724	5,437407	2,19168	0,702683	Pygl	NM_133198
17293706	6,27047	8,481782	7,921008	7,387086	2,211312	-0,533922	Ctsl	NM_009984
17243868	5,512076	7,731533	4,430312	5,457442	2,219457	1,02713	Dram1	NM_027878
17256870	6,160548	8,420387	5,475978	6,459056	2,259839	0,983078	Cd300lg	NM_001160711
17530243	8,057837	10,36986	6,63758	8,406939	2,312023	1,769359	Trf	ENSMUST00000035158
17267846	4,160737	6,493881	4,103273	4,544879	2,333144	0,441606	Abcc3	NM_029600
17351053	7,33147	9,683061	5,816728	7,594163	2,351591	1,777435	Csf1r	NM_001037859
17245231	7,095935	9,523712	7,83452	9,248249	2,427777	1,413729	Lyz1	NM_013590
17352036	5,825021	8,292607	6,430522	7,063242	2,467586	0,63272	Pstpip2	NM_013831
17216469	7,171445	9,643112	4,307654	7,325596	2,471667	3,017942	Serpinb10	NM_198028
17418519	4,526365	7,008114	4,384923	4,817927	2,481749	0,433004	Oscp1	NM_172701
17352985	5,241015	7,729548	6,069003	6,77262	2,488533	0,703617	B4galt6	NM_019737
17363470	7,012671	9,504123	6,018223	7,360303	2,491452	1,34208	Gda	NM_010266
17271443	3,522923	6,043066	3,723533	4,006015	2,520143	0,282482	Abca6	NM_147218
17344064	4,624222	7,168561	4,037507	4,788486	2,544339	0,750979	Cfb	NM_008198
17214197	6,67437	9,223215	5,558557	7,310296	2,548845	1,751739	Slc11a1	NM_013612
17271724	6,024632	8,583055	4,934464	6,156918	2,558423	1,222454	Cd300ld	ENSMUST00000045075
17394153	7,226619	9,802204	6,05878	8,637745	2,575585	2,578965	Slpi	NM_011414
17474974	5,801637	8,388187	8,296436	8,64188	2,58655	0,345444	Plaur	ENSMUST00000002284
17375997	4,300755	6,926266	3,954049	5,023597	2,625511	1,069548	Mertk	ENSMUST00000014505
17224071	9,361686	11,98952	9,819113	11,4637	2,627834	1,644587	Fn1	NM_010233
17245923	6,965324	9,654119	5,746463	7,690616	2,688795	1,944153	Lrp1	NM_008512
17338617	6,84933	9,546226	4,613992	6,609534	2,696896	1,995542	Emr4	NM_139138
17354101	4,581501	7,300237	4,509981	5,189752	2,718736	0,679771	Dpysl3	ENSMUST00000025379
17462796	5,921942	8,655614	4,523855	6,376474	2,733672	1,852619	Clec4d	NM_010819
17326801	3,329509	6,063628	3,6471	4,286726	2,734119	0,639626	Jam2	NM_023844
17258912	5,31407	8,090231	5,673363	6,435722	2,776161	0,762359	Engase	ENSMUST00000135383

17312219	4,749608	7,54222	3,160961	4,289943	2,792612	1,128982	Ly6g	XM_001475753
17437198	5,976456	8,78579	5,464659	6,862984	2,809334	1,398325	Bst1	NM_009763
17283445	6,1225	8,958448	6,456817	7,529007	2,835948	1,07219	Lgmn	ENSMUST00000021607
17470627	6,484612	9,331956	4,695291	6,978383	2,847344	2,283092	Clec4e	NM_019948
17346975	7,814077	10,67872	6,754671	8,967196	2,864643	2,212525	Emilin2	NM_145158
17394297	6,799634	9,664432	7,768081	9,093259	2,864798	1,325178	Pltp	NM_011125
17452705	4,974228	7,871373	3,449855	3,655536	2,897145	0,205681	Niacr1	NM_030701
17421033	3,180879	6,086344	3,582338	4,79048	2,905465	1,208142	Epha2	NM_010139
17309935	4,500522	7,419732	5,610706	6,881321	2,91921	1,270615	Dab2	ENSMUST00000080880
17537081	5,258751	8,185442	3,977983	5,680908	2,926691	1,702925	Tlr13	NM_205820
17320652	5,600131	8,528525	3,209014	5,221596	2,928394	2,012582	Abcd2	NM_011994
17489464	4,886589	7,835878	3,756119	5,557484	2,949289	1,801365	Scn1b	NM_011322
17528692	3,771353	6,72083	4,298397	5,512817	2,949477	1,21442	Cgnl1	NM_026599
17501633	5,008878	7,981215	4,4476	6,149157	2,972337	1,701557	Lpl	ENSMUST00000015712
17495610	4,500727	7,491356	5,18361	5,614125	2,990629	0,430515	Gprc5b	NM_022420
17432014	4,822825	7,81644	4,236468	5,888321	2,993615	1,651853	Padi4	ENSMUST00000026381
17433265	4,030719	7,082666	4,115662	4,548475	3,051947	0,432813	Car6	ENSMUST00000030817
17419277	7,101611	10,15654	6,737793	8,714136	3,054929	1,976343	Sdc3	NM_011520
17329093	3,908152	7,009271	3,974698	4,711331	3,101119	0,736633	Cyp2ab1	ENSMUST00000023513
17393868	5,326731	8,458218	5,588224	6,421997	3,131487	0,833773	Mafb	NM_010658
17346150	5,298428	8,488145	5,146091	6,499998	3,189717	1,353907	Lrg1	NM_029796
17540521	7,243188	10,48279	7,798534	9,109869	3,239602	1,311335	Cfp	ENSMUST00000001156
17453611	3,670662	7,043983	5,203271	6,483829	3,373321	1,280558	Ccl24	NM_019577
17348282	4,081113	7,489085	4,375387	5,452421	3,407972	1,077034	Colec12	NM_130449
17331669	6,665879	10,08151	6,294654	8,15694	3,415631	1,862286	App	NM_001198823
17384100	4,364265	7,820585	4,102789	5,519053	3,45632	1,416264	Garnl3	ENSMUST00000102810
17297010	6,441812	9,958832	7,053974	8,353849	3,51702	1,299875	Flnb	NM_134080
17307134	5,541005	9,061911	7,208986	7,926934	3,520906	0,717948	Cryl1	NM_030004
17512611	5,553152	9,090222	5,014908	7,112585	3,53707	2,097677	Dpep2	ENSMUST00000081998
17289547	2,738994	6,299932	3,008013	4,123499	3,560938	1,115486	Etv1	NM_007960
17378827	4,49807	8,070959	4,286355	6,114587	3,572889	1,828232	Lbp	NM_008489
17353747	4,345096	7,920608	5,378677	7,35069	3,575512	1,972013	Cd14	ENSMUST00000061829
17500108	5,774091	9,350451	6,08082	8,100088	3,57636	2,019268	Fgfr1	NM_010206
17271399	5,123266	8,70355	4,708789	6,508837	3,580284	1,800048	Abca9	ENSMUST00000044850
17382737	3,750983	7,336894	4,89595	5,993488	3,585911	1,097538	Fcna	ENSMUST00000028307
17262316	3,776561	7,455116	3,999305	5,740701	3,678555	1,741396	Ltc4s	NM_008521
17493875	4,209774	7,933028	3,677578	4,965712	3,723254	1,288134	P2ry2	NM_008773
17227696	5,992113	9,793705	5,389149	7,728874	3,801592	2,339725	Cfh	NM_009888
17339013	6,258738	10,14557	6,355885	8,713228	3,886832	2,357343	Emr1	NM_010130
17295627	3,240003	7,136444	4,639411	5,331629	3,896441	0,692218	Naip1	ENSMUST00000022142
17403255	4,512371	8,417868	4,408272	6,102687	3,905497	1,694415	Gbp1	NM_010259
17508850	5,667226	9,581429	5,931888	8,213323	3,914203	2,281435	Msr1	NM_001113326
17439464	4,76975	8,707665	3,835568	5,545413	3,937915	1,709845	Anxa3	NM_013470
17227892	6,655327	10,61322	8,47919	10,64473	3,957893	2,16554	Prg4	NM_021400
17507321	3,4493	7,459631	4,167992	6,074322	4,010331	1,90633	Efnb2	NM_010111
17439367	6,685216	10,72537	8,271397	9,868114	4,040154	1,596717	Cxcl13	NM_018866
17265229	7,235255	11,32977	8,009497	10,42574	4,094515	2,416243	Alox15	NM_009660
17431612	3,94887	8,179264	4,72221	6,658095	4,230394	1,935885	C1qc	ENSMUST00000046332
17462832	3,747929	8,005649	3,390403	4,830787	4,25772	1,440384	Vmn2r26	NM_019917
17431607	6,319914	10,66183	7,430313	9,350456	4,341916	1,920143	C1qb	NM_009777
17394679	3,58815	8,251455	4,372238	6,3088	4,663305	1,936562	Ptgis	ENSMUST00000018113
17348400	4,695361	9,367277	3,952312	6,803017	4,671916	2,850705	Gata6	NM_010258
17230830	4,154748	9,029202	5,322719	7,337743	4,874454	2,015024	Tgfb2	NM_009367
17309268	5,688822	10,60203	5,931916	8,575165	4,913208	2,643249	Ednrb	NM_007904
17343918	5,139817	10,07237	6,479048	8,46674	4,932553	1,987692	C4b	NM_009780
17233384	4,944606	9,913021	6,418943	8,491428	4,968415	2,072485	Fabp7	ENSMUST00000020024
17233384	4,944606	9,913021	6,418943	8,491428	4,968415	2,072485	Fabp7	ENSMUST00000020024
17438995	4,485271	9,603101	6,947411	8,889437	5,11783	1,942026	Cxcl2	ENSMUST00000075433
17404091	4,209189	9,333618	4,371784	6,457975	5,124429	2,086191	Fabp4	NM_024406
17248860	4,040284	9,207533	5,307802	7,554366	5,167249	2,246564	Timd4	NM_178759
17491193	5,357244	10,82792	6,483482	9,568161	5,470676	3,084679	Saa3	NM_011315
17543365	3,184662	8,985921	5,298122	7,64684	5,801259	2,348718	Vsig4	NM_177789
17398734	3,011252	8,858755	3,914339	6,889477	5,847503	2,975138	Cd5l	NM_009690

Table S2.

Gene	Forward	Reverse
Abca6	ATGATGACTGGTTTCCTGGTCA	GCAACAGAACAGCCATCAGG
Abcc3	GGACCATGAAGCCTTGCAAAAT	GGCTGGCTCATTGTCTGTCA
Galectin-1	CTCAAAGTTCGGGGAGAGGT	CATTGAAGCGAGGATTGAAGT
Galectin-3	GCCTACCCCAGTGCTCCT	GGTCATAGGGCACCGTCA
Galectin-9	GCATTGGTTCCCCTGAGAATA	TCCAGTAAAGGGGATGATCG
Gapdh	ATGGTGAAGGTCGGTGTGA	AATCTCCACTTTGCCACTGC
Gzm B	GCTGCTCACTGTGAAGGAAGT	TGGGGAATGCATTTTACCAT
Gzm C	TCCTGATTCTCCTGACCCTACT	GACGGGAATGTGGACTGATCT
Gzm D	CTGCAAAAACAGCTCTGTCCAA	TGATCTGCTGTGTCTCCTCCT
Gzm E	GGTAATAGGAGATACTGTGGAGG	TGTGACTGTCATTGTCCTGTTC
Gzm G	AGAAAGCATGGCACCAATGAC	ATTGGGTCTGGGCAACTTGA
H2-q1	CTACGGCTGGGAAATGAGAC	GCACCTCAGGGTGACTTCAT
Ifn-γ	ATCTGGAGGAACTGGCAAAA	TTCAAGACTTCAAAGAGTCTGAGGTA
Prf 1	TGAGAAGACCTATCAGGACCAG	GTCAAGGTGGAGTGGAGGTT
Rae-1β	CAGCAAATGCCACTGAAGTGAA	GGTCTTGTGAGTGTCCACTTTG
Rae-1δ	CTCCTACCCCAGCAGATGAAG	CCCTGGGTCACCTGAAGTC
Rae-1ε	GACCCACAGACCAAATGGCA	CTCTGTCCTTTGAGCTTCTTGC
β2M	GAATGGGAGCCGAACATAC	CCGTTCTTCAGCATTTGGAT
β-actin	GATGTGGATCAGCAAGCAGGA	AGCTCAGTAACAGTCCGCCTA

Chapter 3

Summary

The idea that some chemotherapeutic agents, including classical chemotherapeutic compounds, may exert immunostimulatory effects has generate considerable interest in their combination with immunotherapy. We are testing this strategy in a clinical trial (DENDR1 - EUDRACT N° 2008-005035-15) currently active at our Institute. Patients affected by newly diagnosis of glioblastoma (GBM), enrolled in DENDR1 clinical trial and treated with dendritic cells (DCs) in combination with temozolomide (TMZ), showed a significant increase of peripheral cytotoxic NK cell percentage but a failure of $CD8^+$ T lymphocyte response. From these clinical observations, we moved to evaluate the potential direct effect of TMZ on NK cell response by using our preclinical malignant glioma model.

We treated GL261 glioma-bearing mice with five intraperitoneal injections of a low-dose of TMZ 9 days after intracranial tumor implantation. Peripheral blood lymphocytes (PBLs) and tumor-infiltrating lymphocytes (TILs) were analyzed by flow cytometry to characterize a potential direct effect of TMZ on immune effectors. TMZ induced a rapid and reversible lymphopenia of $CD8^+$ T cells with a concomitant limited homing to the brain. Conversely, the frequency of peripheral blood NK cells from TMZ-treated mice increased significantly at early time point and remained higher than controls throughout the entire treatment.

Microarray analysis performed on blood-purified NK cells revealed that Abcc3 was one of the most up-regulated genes in TMZ-treated mice compared to controls during chemotherapy administration. Abcc3, a

member of the ATP-binding cassette transporter superfamily, is implicated in acquired multidrug-resistance in different types of tumors. In the present study, we provided the evidence that Abcc3 plays an important role in protecting blood-derived NK cells from the cytotoxic effects of the alkylating agent TMZ. Indeed, we found that Abcc3 was over-expressed and functionally active in response to TMZ in NK cells, and not in $CD8^+$ T lymphocytes, from naïve and glioma-bearing mice. Moreover, our results indicated that NK cells displayed a high expression of this transporter in physiological conditions suggesting a potential basal drug-resistant phenotype.

The major finding of this study was the demonstration of the critical role of Abcc3 in protecting NK cells from apoptosis. NK cells negative for Abcc3 were more prone to undergo apoptosis during chemotherapy treatment and the percentage of apoptotic cells was higher compared to NK cells Abcc3 positive. The inhibition of Abcc3 activity with the specific molecule MK571 significantly induced apoptosis in NK cells. Because the pathways involving Akt activation promotes lymphocyte survival, we investigated Akt phosphorylation in response to chemotherapy. Only NK cells expressing Abcc3 presented Akt activation during TMZ treatment.

We also found that TMZ is able to influence positively the maturation status and functions of NK cells. During TMZ treatment, there was a significant enrichment of the NK cell subsets with migratory potential resulting in a greater tumor-infiltration ability. NK cells infiltrate the tumor mass early leading to an increase and persistent expression of cytotoxic signals including Ifn-γ, granzymes and perforin. Additionally,

TMZ modified the glioma microenvironment reducing the strong immunosuppressive components and providing the optimal condition for NK cell recruitment and effector function.
Finally, we found that cytotoxicity, evaluated as IFN-γ production and specific lytic activity against GL261 cells by peripheral NK cells, significantly increased compared to control mice.

These findings demonstrated for the first time the direct involvement of Abcc3 in conferring NK cell resistance to chemotherapy and the influence of TMZ on immune effector functions and glioma microenvironment, observations to consider for trials where glioblastoma patients are treated with chemo-immunotherapy.

Conclusions

Glioblastoma (GBM) remains a particularly devastating solid tumor for which standard treatment options offer only modest efficacy. Cancer immunotherapy, the scientific breakthrough of the year 2013 by Science [1], represents the most promising cancer treatment approach with a potential to achieve a complete, long-lasting remission with few or no side effects. The advances in cancer immunotherapy are mostly related to the treatment of melanoma and other solid tumors [2,3]. However, a growing amount of studies indicates that immunotherapy is a well-suited opportunity to target glioma cells for destruction with high specificity and efficiency improving the outcome of patients [4,5]. Unfortunately, one of the major limitation of immune-related strategies in GBM is the highly immunosuppressive tumor microenvironment.

Despite conventional dogma that indicates chemotherapy as immunosuppressive, now it is proposed its ability to overcome or realistically to reduce the immune suppressive factors, responsible for T cell anergy and amplification of the regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) [6], produced by cancer cells. In fact, chemotherapy could be used to influence the immune system and tumor to create an environment where cancer vaccines have a better chance of success [7,8].

TMZ is proposed as a possible immune adjuvant and already administered with immune vaccines [9,10]. Although TMZ reaches its anti-tumor effects through direct killing of tumor cells, it demonstrated several direct and indirect effects also on immune cells by inducing lymphopenia and modulating tumor microenvironment [11–13].

In this scenario of co-administration with immune approaches, an important goal was represented by characterization of the potential direct effects of chemotherapy on immune system. Gaining better insights of the molecular mechanisms induced by TMZ on immune cells is essential to design efficient therapeutic strategies of chemo-immunotherapy.

Our experience with DC-based immunotherapy started in 2008 in close collaboration with the Unit of Cell Therapy Production located within our institute [14]. Currently, we have two clinical studies for DC immunotherapy (DENDR1, EUDRACT 2008-005035-15 and DENDR2, EUDRACT 2008-005038-62, respectively for the treatment of first diagnosis and recurrent GBM) coordinated by Dr. Finocchiaro and approved by the Italian Ministry of Health. In both studies, we are testing the effects of immunotherapy with DCs loaded with tumor lysate and simultaneous treatment with TMZ [15].

Analyses on blood lymphocytes before and after DC vaccines indicated an increased NK, but not $CD8^+$ T, cell cytotoxic response significantly associated with prolonged survival.

Consistent with these clinical studies, we found a great increase in peripheral trafficking and local homing of NK cells in GL261 glioma-bearing mice treated with TMZ. In fact, murine NK cells can efficiently overcome the drug-mediated toxicity of chemotherapy and infiltrate tumors producing an efficient anti-glioma immune response.

First, we found that the expression and function of Abcc3 transporter is a protective mechanism activated by NK cells to escape death during TMZ administration. Abcc3 expression is already associated in acquired MDR in different tumors [16,17]. Its relative expression in

lymphocytes is still controversial [18–20]. However, it is commonly accepted that in leukocytes its efflux function could be involved in protection against toxins and chemotherapy compounds. Indeed, Abcc3 expression is associated to the strong refractory to chemotherapy in patients with leukemia suggesting its active role in efflux of anticancer agents [21,22]. The expression and activity of MDR transporters could be one important point to take into account when the outcome of combination therapy regimens is considered.

Another important goal was to highlight the positive influence of TMZ on NK cell anti-tumor activity. The effects of different chemotherapeutic drugs on NK cell activity have been already observed suggesting negative or no modulation of NK cell cytotoxic functions [11,23]. While the direct effect of TMZ on NK cell effector activity has not been well investigated, another alkylating agent as cyclophosphamide has been proposed as a potent inductor of NK-dependent antitumor immunity [24–26]. Our results demonstrate a direct effect of TMZ on influencing NK cell activation, migration and specific anti-glioma cytotoxicity.

These important findings suggest a translational potential for the definition of novel chemo-immunotherapy protocols for GBM. Several groups, including ours, proposed the relevance of NK cells in inhibiting glioma growth [27–30]. Since NK cells have been shown to positively influence multiple aspects of immune response [31–34], their activation by chemotherapy could be exploited for enhancing the magnitude of both innate and adaptive immunity against tumors resulting in a better clinical outcome.

These findings highlighted two important aspects poorly examine in depth: the molecular mechanisms mediating chemo-resistance in immune cells during chemotherapy treatment and the role of NK cells in immune approaches to treat brain tumors.

Overall our observations may help to set up the best conditions for a clinical trial taking into account the strong different influence of TMZ on NK cells and $CD8^+$ T lymphocytes which are, normally, protagonists of effector response during a DC-based immunotherapy.

Future perspectives

During the last four years, we have observed one of the most important revolutions in all the history of the medicine. First of all, in 2011 Ralph Steinman received the Nobel Prize for his discovery of the dendritic cells. Only two years later, immunotherapy, considered for many years to be more a theory than therapeutic strategy, was defined the breakthrough of the year 2013 [1]. The main advances were preferentially related to the successful obtained from the use of checkpoints inhibitors in some solid tumors, especially metastatic melanoma [35].

Our immune system contains all the potential solutions we need to ward off the cancer, however it is not perfect. Immunotherapy can potentiate specific anti-tumor immune responses and the efficacy of this strategy may be increased by secret and unexpected allies [36]. Recent studies suggest that some chemotherapeutic agents possess immune stimulatory effects [37] and can effectively favor immune response against tumors. However, a detailed understanding of the cellular and molecular basis of relationship between chemotherapy and immune system is essential for planning the optimal strategy for combining immune approaches with standard therapies for cancer.

Our preclinical data showed that temozolomide (TMZ), the chemotherapeutic drug used as standard therapy for patients affected by GBM, influences the activity and the phenotype of NK cells. Most important, Abcc3 transporter with its efflux activity confers to NK cells chemo-resistance to TMZ protecting them from apoptosis.

These observations encourage us to investigate the potential involvement of ABCC3 in influencing the immune response, and

specifically the NK cell response of GBM patients enrolled in our clinical trial DENDR1. We assessed ABCC3 expression by real-time PCR on PBLs of 17 GBM patients and found a significant higher expression in responders (PFS $\geq$ 12 and high NK cell response) compared to non-responders ($p = 0.0006$). ABCC3 expression also increases significantly at the time of first vaccine after radio-chemotherapy ($p = 0.01$), and after concomitant vaccine and TMZ administration ($p = 0.01$) (Figure 1). In order to measure the ABCC3 expression by flow cytometry specifically on NK and $CD8^+$ T cells, we used an anti-human ABCC3 antibody. Preliminary results indicated that ABCC3 is differentially expressed in NK cells compared to $CD8^+$ T cells also in human PBLs before TMZ administration (68.6 ± 16.8 vs 1.2 ± 0.8, NK vs $CD8^+$ T cells respectively, $p = 0.0002$; Figure 2). Now we are going to investigate ABCC3 expression in PBLs before, after and during DC immunotherapy. Specific attention will be given to last four vaccines co-administrated with adjuvant TMZ in order to understand whether ABCC3 plays the same protection role against chemotherapy potentiating NK cell anti-glioma response.

Next, we plan to characterize widely the peripheral effector response in our DENDR1 patients. Flow cytometry analyses showed an increased cytotoxic NK cell frequency correlated with a prolonged PFS. Unexpectedly, we have a lack of an efficient antitumor $CD8^+$ T cell response. Although several studies suggest as NK cell activation promote CTL antitumor response [32,38], other data affirm that a strong NK cell response may be inhibitory [39,40]. Based on these data we aim to study and characterize some potential effect of the robust NK cell cytotoxicity on $CD8^+$ T activation and expansion.

Our pre-clinical observations indicated a more sensitivity of $CD8^+$ T cells to cytotoxic effects of TMZ in contrast with a potent NK cell activation. To further improve the survival of our patients and delay the unavoidable cancer immunoediting [41,42] and consequently the recurrence, we need to trigger an efficient CTL response. In addition, since a critical requirement of an efficient cancer immunotherapy is the generation of long-lasting and specific memory, we plan to investigate the $CD8^+$ T (effector) memory formation during DC vaccinations and concomitant TMZ administration.

Therefore, our main challenge is to figure out any issues related to the current treatment schedule evaluating the possibility to limit repeated exposure to TMZ during DC vaccines.

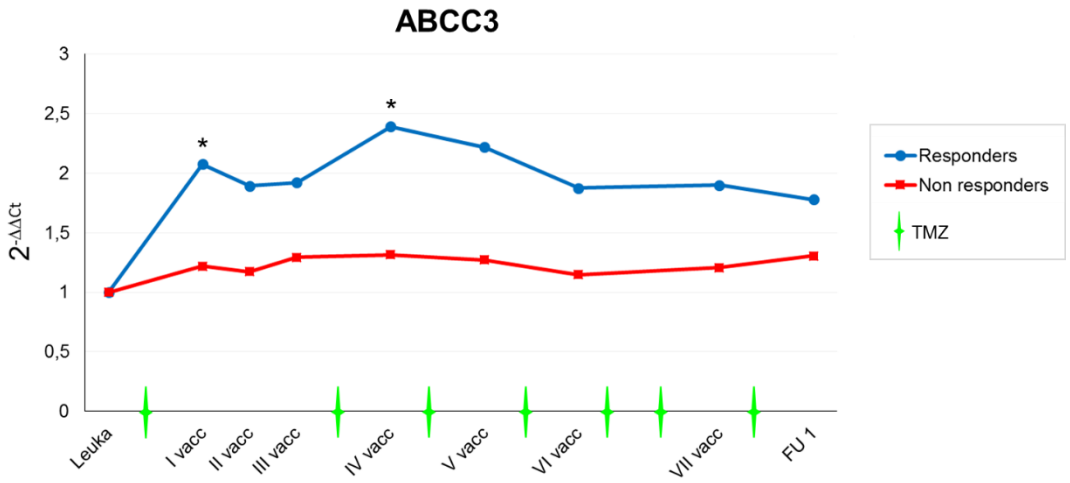

Figure 1. ABCC3 expression in PBLs of DENDR1 patients. ABCC3 transporter is differentially expressed in responders (patients with a PFS ≥ 12 and high NK cell response) and non-responders. ABCC3 is over-expressed at the time of first vaccine after radio-TMZ, and after the third vaccine in concomitance with adjuvant chemotherapy. * p = 0.01.

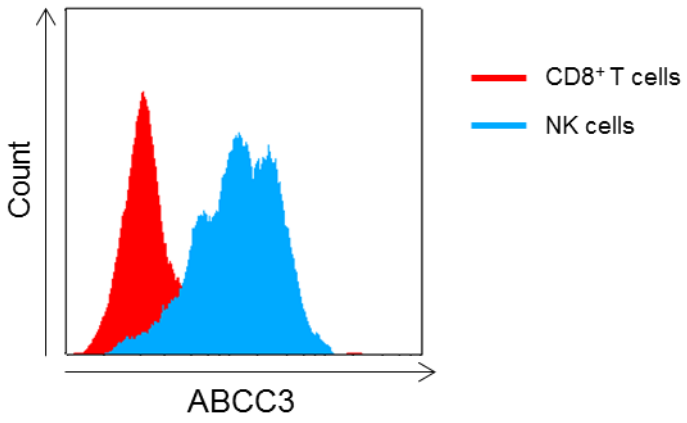

Figure 2. Differential expression of ABCC3 in PBLs of a GBM patient enrolled in DENDR1 study. Representative dot plot of basal ABCC3 positivity expression in NK cells versus $CD8^+$ T lymphocytes.

References

1. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. Science (80-) 2013; 342:1432–3.

2. Galluzzi L, Vacchelli E, Pedro JB, Buqué A, Senovilla L, Baracco EE, Bloy N, Castoldi F, Abastado J, Agostinis P, et al. Classification of current anticancer immunotherapies. Oncotarget 2014; 5.

3. Zavala VA, Kalergis AM. New clinical advances in immunotherapy for the treatment of solid tumours. Immunology 2015; 145:182–201.

4. Bregy A, Wong TM, Shah AH, Goldberg JM, Komotar RJ. Active immunotherapy using dendritic cells in the treatment of glioblastoma multiforme. Cancer Treat Rev 2013; 39:891–907.

5. Weathers S-P, Gilbert MR. Current challenges in designing GBM trials for immunotherapy. J Neurooncol 2015; 123:331–7.

6. Sengupta S, Marrinan J, Frishman C, Sampath P. Impact of temozolomide on immune response during malignant glioma chemotherapy. Clin Dev Immunol 2012; 2012.

7. Weir GM, Liwski RS, Mansour M. Immune Modulation by Chemotherapy or Immunotherapy to Enhance Cancer Vaccines. Cancers 2011; 3:3114–42.

8. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. Immunity 2013; 39:74–88.

9. Wheeler CJ, Das A, Liu G, Yu JS, Black KL. Clinical responsiveness of glioblastoma multiforme to chemotherapy after vaccination. Clin cancer Res 2004; 10:5316–26.

10. Heimberger AB, Sun W, Hussain SF, Dey M, Crutcher L, Aldape K, Gilbert M, Hassenbusch SJ, Sawaya R, Schmittling B, et al. Immunological responses in a patient with glioblastoma multiforme treated with sequential courses of temozolomide and immunotherapy: case study. Neuro Oncol 2008; 10:98–103.

11. Bracci L, Schiavoni G, Sistigu a, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. Cell Death Differ 2014; 21:15–25.

12. Kang TH, Mao C-P, Lee SY, Chen A, Lee J-H, Kim TW, Alvarez R, Roden RBS, Pardoll DM, Hung C-F, et al. Chemotherapy acts as an adjuvant to convert the tumor microenvironment into a highly permissive state for vaccination-induced antitumor immunity. Cancer Res 2013; Apr 15;73(8):2493-504.

13. Hong M, Puaux AL, Huang C, Loumagne L, Tow C, Mackay C, Kato M, Prévost-Blondel A, Avril MF, Nardin A, et al. Chemotherapy induces intratumoral expression of chemokines in cutaneous melanoma, favoring T-cell infiltration and tumor control. Cancer Res 2011; 71:6997–7009.

14. Nava S, Dossena M, Pogliani S, Pellegatta S, Antozzi C, Baggi F, Gellera C, Pollo B, Parati EA, Finocchiaro G, et al. An optimized method for manufacturing a clinical scale dendritic cell-based vaccine for the treatment of glioblastoma. PLoS One 2012; 7:e52301.

15. Nava S, Lisini D, Pogliani S, Dossena M, Bersano A, Pellegatta S, Parati E, Finocchiaro G, Frigerio S. Safe and Reproducible Preparation of Functional Dendritic Cells for Immunotherapy in Glioblastoma Patients. Stem Cells Transl Med 2015; 4:1164–72.

16. Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A 1999; 96:6914–9.

17. Hagmann W, Jesnowski R, Faissner R, Guo C, Löhr JM. ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. Upregulation in 5-fluorouracil-resistant cells. Pancreatology 2009; 9:136–44.

18. Giraud C, Manceau S, Declèves X, Goffinet F, Morini J-P, Chappuy H, Batteux F, Chouzenoux S, Yousif S, Scherrmann J-M, et al. Influence of development, HIV infection, and antiretroviral therapies on the gene expression profiles of ABC transporters in human lymphocytes. J Clin Pharmacol 2010; 50:226–30.

19. Elliott JI, Raguz S, Higgins CF. Multidrug transporter activity in lymphocytes. Br J Pharmacol 2004; 143:899–907.

20. Albermann N, Schmitz-Winnenthal FH, Z'graggen K, Volk C, Hoffmann MM, Haefeli WE, Weiss J. Expression of the drug transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, BCRP/ABCG2, and PXR in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver. Biochem Pharmacol2005; 70:949–58.

21. Yee SW, Mefford JA, Singh N, Percival M-E, Stecula A, Yang K, Witte JS, Takahashi A, Kubo M, Matsuda K, et al. Impact of polymorphisms in drug pathway genes on disease-free survival in adults with acute myeloid leukemia. J Hum Genet 2013; 58:353–61.

22. Steinbach D, Lengemann J, Voigt A, Hermann J, Zintl F, Sauerbrey A. Response to chemotherapy and expression of the genes encoding the multidrug resistance-associated proteins MRP2, MRP3, MRP4, MRP5, and SMRP in childhood acute myeloid leukemia. Clin cancer Res 2003; 9:1083–6.

23. Markasz L, Stuber G, Vanherberghen B, Flaberg E, Olah E, Carbone E, Eksborg S, Klein E, Skribek H, Szekely L. Effect of frequently used chemotherapeutic drugs on the cytotoxic activity of human natural killer cells. Mol Cancer Ther 2007; 6:644–54.

24. Banissi C, Ghiringhelli F, Chen L, Carpentier AF. Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model. Cancer Immunol Immunother 2009; 58:1627–34.

25. Doloff JC, Waxman DJ. VEGF receptor inhibitors block the ability of metronomically dosed cyclophosphamide to activate innate immunity-induced tumor regression. Cancer Res 2012; 72:1103–15.

26. Ghiringhelli F, Ménard C, Martin F, Zitvogel L. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. Immunol Rev 2006; 214:229–38.

27. Castriconi R, Daga A, Dondero A, Zona G, Poliani PL, Melotti A, Griffero F, Marubbi D, Spaziante R, Bellora F, et al. NK cells recognize and kill human glioblastoma cells with stem cell-like properties. J Immunol 2009; 182:3530–9.

28. Pellegatta S, Eoli M, Frigerio S, Antozzi C, Bruzzone MG, Cantini G, Nava S, Anghileri E, Cuppini L, Cuccarini V, et al. The natural killer cell response and tumor debulking are associated with prolonged survival in recurrent glioblastoma patients receiving dendritic cells loaded with autologous tumor lysates. Oncoimmunology 2013; 2:e23401.

29. Cantini G, Pisati F, Pessina S, Finocchiaro G, Pellegatta S. Immunotherapy against the radial glia marker GLAST effectively triggers specific antitumor effectors without autoimmunity. Oncoimmunology 2012; 1:884–93.

30. Ogbomo H, Cinatl J, Mody CH, Forsyth PA. Immunotherapy in gliomas: limitations and potential of natural killer (NK) cell therapy. Trends Mol Med 2011; 17:433–41.

31. Mocikat R, Braumüller H, Gumy A, Egeter O, Ziegler H, Reusch U, Bubeck A, Louis J, Mailhammer R, Riethmüller G, et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity 2003; 19:561–9.

32. Wong JL, Mailliard RB, Moschos SJ, Edington H, Lotze MT, Kirkwood JM, Kalinski P. Helper Activity of Natural Killer Cells During the Dendritic Cytotoxic T Cells. J Immunother 2011; 34:270–8.

33. Crome SQ, Lang P a, Lang KS, Ohashi PS. Natural killer cells regulate diverse T cell responses. Trends Immunol 2013; 34:342–9.

34. Van Elssen CHMJ, Oth T, Germeraad WT V, Bos GMJ, Vanderlocht J.

Natural killer cells: the secret weapon in dendritic cell vaccination strategies. Clin cancer Res 2014; 20:1095–103.

35. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, Schadendorf D, Dummer R, Smylie M, Rutkowski P, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med 2015; 373:23–34.

36. Galluzzi L, Senovilla L, Zitvogel L, Kroemer G. The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov 2012; 11:215–33.

37. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008; 8:59–73.

38. Adam C, King S, Allgeier T, Braumüller H, Lüking C, Mysliwietz J, Kriegeskorte A, Busch DH, Röcken M, Mocikat R. DC-NK cell cross talk as a novel CD4+ T-cell-independent pathway for antitumor CTL induction. Blood 2005; 106:338–44.

39. Barber MA, Zhang T, Gagne BA, Sentman CL. Tumor-Specific CTLs in a Syngeneic Lymphoma Model. J Immunol 2007;

40. Deniz G, Erten G, Kucuksezer UC, Kocacik D, Karagiannidis C, Aktas E, Akdis C a., Akdis M. Regulatory NK Cells Suppress Antigen-Specific T Cell Responses. J Immunol 2008; 180:850–7.

41. O'Sullivan T, Saddawi-Konefka R, Vermi W, Koebel CM, Arthur C, White JM, Uppaluri R, Andrews DM, Ngiow SF, Teng MWL, et al. Cancer immunoediting by the innate immune system in the absence of adaptive immunity. J Exp Med 2012; 209:1869–82.

42. Gross E, Sunwoo JB, Bui JD. Cancer immunosurveillance and immunoediting by natural killer cells. Cancer J 2013; 19:483–9.

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