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NKG2D- and T-cell receptor-dependent lysis of malignant glioma cell lines by human γδ T cells: Modulation by temozolomide and A disintegrin and metalloproteases 10 and 17 inhibitors

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ABSTRACT

The interaction of the MHC class I-related chain molecules A and B (MICA and MICB) and UL-16 binding protein (ULBP) family members expressed on tumor cells with the corresponding NKG2D receptor triggers cytotoxic effector functions in NK cells and γδ T cells. However, as a mechanism of tumor immune escape, NKG2D ligands (NKG2DLs) can be released from the cell surface. In this study, we investigated the NKG2DL system in different human glioblastoma (GBM) cell lines, the most lethal brain tumor in adults. Flow cytometric analysis and ELISA revealed that despite the expression of various NKG2DLs only ULBP2 is released as a soluble protein *via* the proteolytic activity of "a disintegrin and metalloproteases" (ADAM) 10 and 17. Moreover, we report that temozolomide (TMZ), a chemotherapeutic agent in clinical use for the treatment of GBM, increases the cell surface expression of NKG2DLs and sensitizes GBM cells to γδ T cell-mediated lysis. Both NKG2D and the T-cell receptor (TCR) are involved. The cytotoxic activity of γδ T cells toward GBM cells is strongly enhanced in a TCR-dependent manner by stimulation with pyrophosphate antigens. These data clearly demonstrate the complexity of mechanisms regulating NKG2DL expression in GBM cells and further show that treatment with TMZ can increase the immunogenicity of GBM. Thus, TMZ might enhance the potential of the adoptive transfer of *ex vivo* expanded γδ T cells for the treatment of malignant glioblastoma.

Abbreviations: ADAM, A disintegrin and metalloprotease(s); BrHPP, bromohydrin pyrophosphate; CFSE, carboxyfluoresceinsuccinimidyl ester; GBM, glioblastoma multiforme; GI, GI254023X; GW, GW280264X; MICA/B, MHC class I-related chain molecule A/B; MP, metalloprotease(s); NKG2D, Natural Killer Group 2, member D; NKG2DL, NKG2D Ligand; sMICA/B, soluble MICA/B; sULBP1/2, soluble ULBP1/2; TCR, T-cell receptor; TMZ, temozolomide; ULBP 1/2, UL-16 binding protein 1/2

Introduction

Glioblastoma multiforme, termed as glioblastoma for short (GBM) is the most lethal primary brain tumor in adults. Despite the aggressive standard therapeutic strategy including surgery and radio-chemotherapy, the median survival of GBM patients (14.6 mo) remains extremely low pointing to the urgent need for alternative treatment strategies such as immunotherapy.[1] Numerous studies demonstrated that *ex vivo* expanded immune cells could be a promising tool for the treatment of GBM.[2] Malignant GBM cells express several stress-inducible molecules which are sensed by the activating receptor NKG2D.[3] This interaction triggers cytotoxic activity in NKG2D-expressing killer cells and hence, the NKG2DL system is considered a promising target for the improvement of cell-based immunotherapies.[4] NKG2D is a C-type lectin-like receptor expressed on NK cells, NKT cells, γδ T cells, CD8+ T cells and a minor subset of immunoregulatory CD4+ T cells. The ligation of NKG2D triggers cytotoxicity in NK cells and co-stimulation in T-cell subsets.[5] Ligands for human NKG2D comprise two groups of MHC class I-related molecules, the MHC class I chain-related proteins A and B (MICA/B) and six members of the UL16-binding protein family (ULBP1-6).[6] Members of the ULBP family carry 2 MHC class I-like domains (α1, α2) and are either transmembrane proteins (ULBP4,5) or bound to the membrane with GPI-linkage (ULBP1,3,6). ULBP2 has the unique feature that it can be expressed at the cell surface either as a transmembrane protein or with a GPI anchor.[7] NKG2DLs are normally not expressed on healthy cells but expression can be induced by various types of cellular stress including viral infection, genotoxic stress or malignant

transformation.[8] As an example, in contrast to tissues isolated from meningioma patients, 10 out of 11 GBM specimens were stained positive for NKG2DLs.[9] NKG2DLs are excellent targets for NKG2D-mediated cytotoxicity and higher expression levels of these ligands are associated with increased cytotoxic activity of effector cells.[10] However, as a mechanism of immune escape, many tumor cells release soluble NKG2DLs (sNKG2D). NKG2DLs are frequently shed by metalloproteases (MP), specifically by distinct members of the ADAM family.[11] ADAM10 and ADAM17 have been implicated in the shedding of MICA/B and ULBP2 in various model systems,[12,13] whereas GPI-anchored ULBPs (ULBP1,2,3) are known to be processed bypho sphoinositide phospholipase C or are released in association with exosomes (reviewed in Chitadze et al.[14]). However, the exact mechanisms regulating NKG2DL in GBM cells are still elusive and require further investigation.

Current standard GBM treatment strategies include the administration of the chemotherapeutic agent TMZ in combination with radiotherapy after surgical removal of tumors. Temozolomide (TMZ) is an alkylating agent that induces apoptosis via methylation of guanine residues.[15] Since genotoxic stress is linked to the induction of NKG2DL expression, TMZ treatment transiently increased the expression of various NKG2DLs in TMZ-resistant GBM cell lines.[16]

NKG2D is expressed on human $\gamma\delta$ T cells, a minor population of peripheral blood lymphocytes (1–5%). The majority of these cells expresses a Vγ9Vδ2 TCR and recognizes microbial and eukaryotic pyrophosphate antigens (phosphoantigens) in a butyrophilin 3A1 (CD277)-dependent manner.[17-19] Since endogenous phosphoantigens are overproduced in transformed cells, $\gamma\delta$ T cells can distinguish transformed cells from healthy tissues.[20] Furthermore, recognition of pyrophosphate antigens by $\gamma\delta$ T cells is not restricted by MHC molecules which is advantageous in the setting of allogeneic adoptive cell transfer.[21] Vγ9Vδ2 T cells can be easily expanded in vitro with synthetic phosphoantigens or with nitrogen-containing bisphophonates such as zoledronate, combined with low doses of IL-2. Of note, $\gamma\delta$ T cells elicit potent antitumor activity against a broad range of malignant cells including GBM.[22,23]

In this study, we investigated the effects of MP inhibitors and TMZ on NKG2DL expression and shedding in several human GBM cell lines. We report that GBM cells express several NKG2DLs, but preferentially release ULBP2 into culture supernatants in an ADAM10/17-dependent manner. Moreover, we show that TMZ treatment increases the cell surface expression of NKG2DLs and also sensitizes GBM cells to $\gamma\delta$ T cell-mediated killing involving both NKG2D- and TCR-dependent mechanisms.

Results

GBM cell lines differentially express and release NKG2D ligands

Initially we screened the GBM cell lines A-172, T-98G, U-87MG and U-251MG for cell surface expression of the endogenous NKG2DLs MICA, MICB, ULBP1 and ULBP2 by flow cytometry (Fig. 1A). The expression levels of MICA, MICB, ULBP1 and ULBP2 varied considerably among GBM cell lines. Expression of MICA and ULBP2 was remarkably high when compared to MICB and ULBP1. Moreover, U-

87MG and U-251MG cells completely lacked MICB cell surface expression (Fig. 1A). In addition, U-251MG cells were deficient in cell surface expression of ULBP1. In parallel, levels of sNKG2DLs in cell culture supernatants were determined by ELISA to quantify the extent of shedding of MICA, MICB, ULBP1 and ULBP2 from the tested cell lines (Fig. 1B). Interestingly, despite considerable cell surface expression of the tested NKG2DLs, only ULBP2 was detected in cell culture supernatants as a soluble molecule. Some MICA alleles (specifically MICA*008) are known to be secreted in exosomes rather than being shed by MPs.[24] Biochemical and sequencing analysis revealed that both A-172 and U-87MG cells indeed expressed the truncated MICA*008:01:01 allele, whereas T-98G (MICA*001) and U-251MG (MICA*002:01) expressed full length MICA (not shown). In line, MICA was detected in exosome preparations of A-172 and U-87MG but not T-89G and U-251MG cells (not shown). It has been reported that ULBP2 can be either released in association with exosomes or by the activity of MPs.[25] To address this issue, we immunoprecipitated ULBP2 from cellular lysates, culture supernatants and isolated exosomes of GBM cell lines. As expected from ELISA results presented in Fig. 1B, western blot analysis revealed that ULBP2 was predominantly detected in the culture supernatants and not in the exosome preparations. However, a very weak signal at high exposure time was observed in T-98G-derived exosomal preparations. (Fig. S1).

Metalloproteases, particularly ADAM10 and ADAM17, mediate the release of soluble ULBP2

To address the role of MPs in ULBP2 release, we used a combination of the broad range MP inhibitors GM6001 and TAPI-1. Cells were cultured for 24 h in the presence or absence of inhibitors at non-toxic concentrations and cell surface expression of ULBP2 was analyzed by flow cytometry. As shown in Fig. 2, broad range inhibition of MPs slightly increased cell surface expression of ULBP2 (Fig. 2A) and simultaneously decreased the amount of sULBP2 in cell culture supernatants (Fig. 2B). In parallel, we inhibited the ADAM family members ADAM10 and ADAM17 with GI254023X (GI, ADAM10-specific) and GW280264X (GW, ADAM10/17-specific) compounds.[26] These studies revealed that ADAM10 and ADAM17 have a complementary effect on ULBP2 shedding. Inhibition of ADAM10 almost completely abrogated the shedding of ULBP2 in U-87MG cells but only partially in A-172, T-98G and U-251MG cells. However, in the latter cell lines ULBP2 shedding was effectively prevented upon additional inhibition of ADAM17 (Fig. 2B). In line with these results, flow cytometric analysis showed that inhibition of shedding by GI and GW increased the cell surface expression of ULBP2 with cell line-dependent variability (representative histograms are shown in Fig. 2A and a quantification of 3–4 experiments in Fig. S2A) but had no effect on surface expression of other NKG2DLs (not shown). Of note, there was no effect of the relevant DMSO control. Together these data indicate that ADAM10 and ADAM17 mediate the proteolytic release of ULBP2 in GBM cells. However, the contribution of ADAM10 and ADAM17 to ULBP2 shedding differs among cell lines. Flow cytometric analysis of ADAM10 and ADAM17 surface expression and Western blot
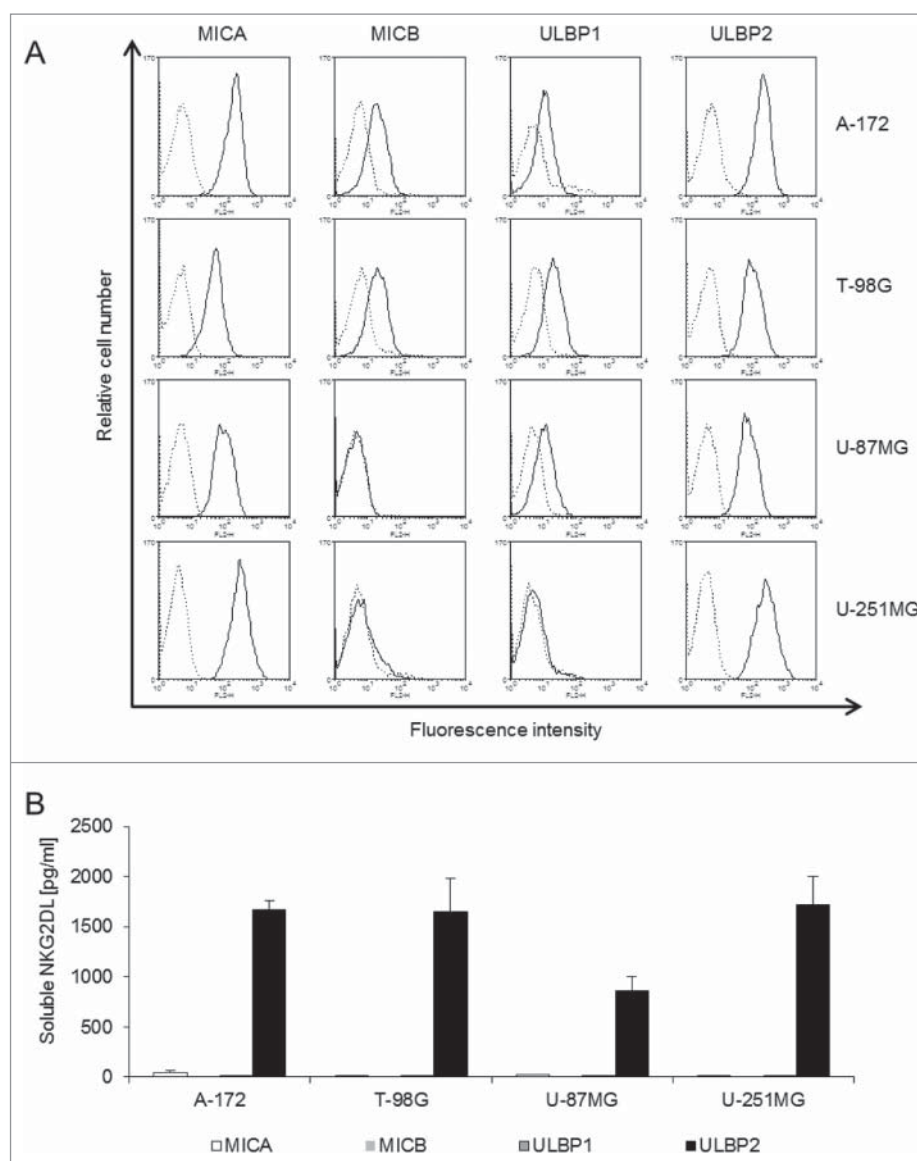
Figure 1. GBM cell lines express various NKG2DLs and release soluble ULBP2 into the supernatant. (A) Cell surface expression of MICA, MICB, ULBP1 and ULBP2 (solid lines) was determined by flow cytometry in A-172, T-98G, U-87MG and U-251MG cells. Dotted lines represent the respective isotype controls. (B) The amount of sMICA (white), sMICB (light gray), sULBP1 (dark gray) and sULBP2 (black) in cell culture supernatants was determined by ELISA. Data are presented as mean values of three independent experiments +/− SEM.

analysis of the active and the proform of ADAM10/17 in GBM cell lysates revealed no major differences among the four cell lines. (not shown).

TMZ treatment increases the cell surface expression of ULBP2

Since TMZ has already been shown to enhance NKG2DL expression,[16] we investigated the effect of TMZ on cell surface expression and shedding of ULBP2 in the various GBM cell lines. Cells were cultured for 24 h with various concentrations of TMZ or with DMSO as a solvent control. TMZ enhanced the cell surface expression of ULBP2 (Fig. 3A) and other NKG2DLs (not shown) in A-172, T-98G and U-87MG but only moderately in U-251MG cells (representative histograms are shown in Fig. 3A and a quantification of three experiments in Fig. S2B). Shedding

of ULBP2, however, was only weakly enhanced in two of the cell lines (A-172, U-87MG; Fig. 3B). Of note, even the highest tested concentration of TMZ (400 μg/mL) had only minor effects on cell viability of T-98G and U-251MG cells as revealed by PI staining. (Fig. S3A).

Phosphoantigen-activated $\gamma\delta$ T cells efficiently kill GBM cells

$\gamma\delta$ T cells have been shown previously to exert cytotoxic activity against GBM cells.[23,27] Before investigating the effect of TMZ, we analyzed the capacity of short-term expanded $\gamma\delta$ T cells to kill GBM cell lines in a Calcein AM-based cytotoxicity assay in the absence or presence of the phosphoantigen bromohydrin pyrophosphate (BrHPP).[28] In the absence of BrHPP, GBM cell lines were largely resistant to Vγ9Vδ2 T cell-mediated cytotoxicity with the exception of U-87MG
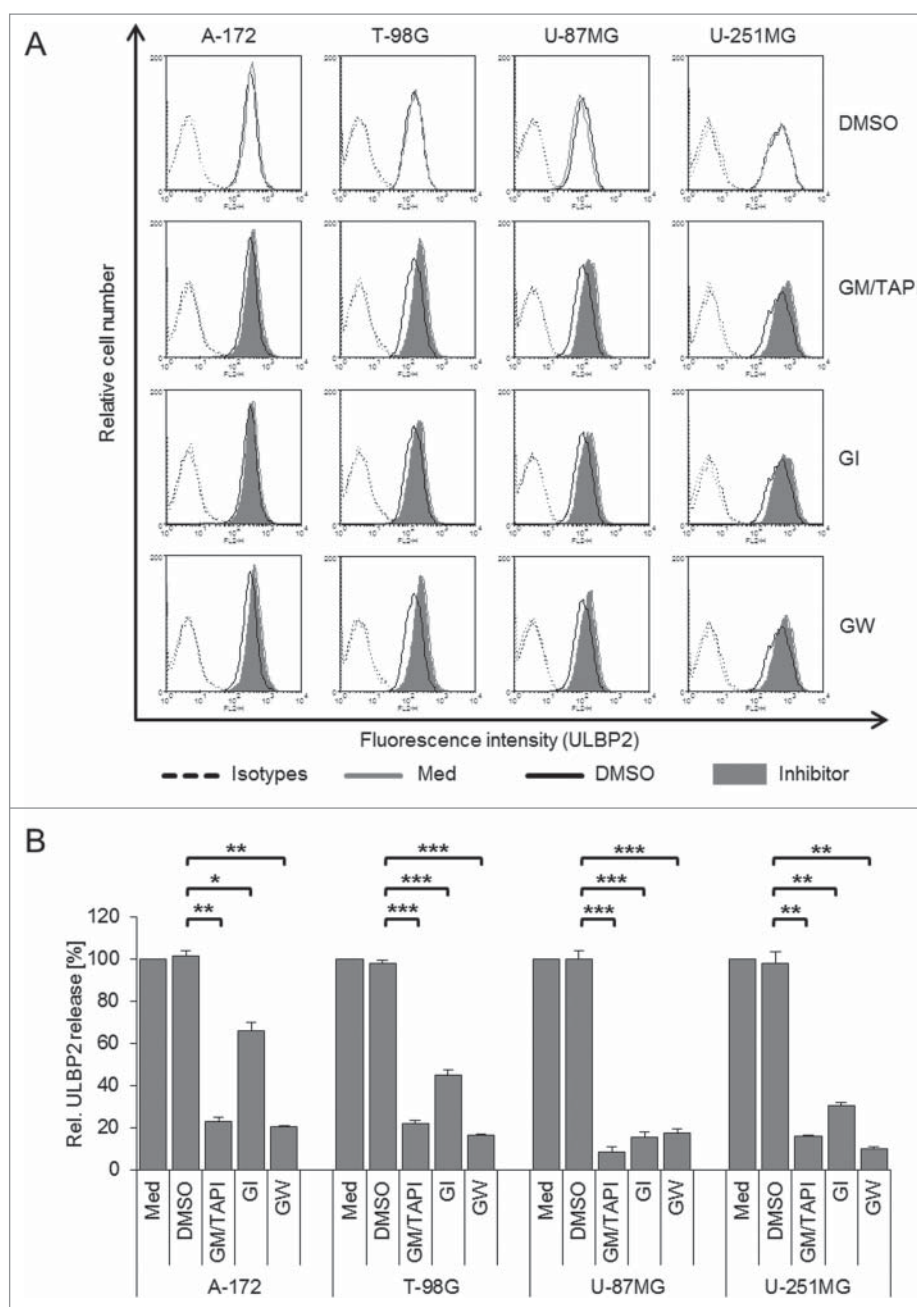
Figure 2. Metalloprotease inhibition modulates ULBP2 cell surface expression and reduces ULBP2 shedding. Cells were treated for 24 h with the combination of the broad range MP inhibitors GM6001 (5 μM) and TAPI-1 (5 μM), or with GI (3 μM) or GW (3 μM) to inhibit ADAM10 and ADAM10/17, respectively. DMSO was used as a solvent control. (A) Cell surface expression of ULBP2 was determined by flow cytometry after treatment with MP inhibitors (filled histograms), with DMSO solvent control (open black histograms), or without treatment (medium control, open gray histograms). Dotted lines represent the isotype controls. (B) The concentration of sULBP2 in supernatants of medium control, DMSO- and inhibitor-treated GBM cells was determined by ELISA. The medium control was set to 100%. Data are presented as mean values of three to four independent experiments +/− SEM. Statistical significance is displayed as *** for $p < 0.001$; ** for $p < 0.01$ and * for $p < 0.05$.

(Fig. 4A). Lysis of all four GBM cells was strongly increased when BrHPP was present during the 4 h cytotoxicity assay (Fig. 4A) suggesting that TCR-dependent killing plays a major role. In order to investigate the relative contribution of TCR- and NKG2D-dependent killing of GBM cells by Vγ9Vδ2 T cells, we performed the cytotoxicity assay in the absence or presence of monoclonal antibodies directed against the Vγ9 TCR (clone 7A5[29]) or NKG2D receptor (clone 149810) with U-87MG target cells.[22] Vγ9Vδ2 T cells were pre-incubated for 1 h with 10 μg/mL of blocking antibodies and then added to

Calcein AM-labeled U-87MG cells, again in the absence (Fig. 4B, left-hand panel) or presence of BrHPP (Fig. 4B, right-hand panel) to trigger TCR activation. The low level of spontaneous cytotoxic activity of $\gamma\delta$ T cells against U-87MG cells was reduced to almost the same extent by blocking of NKG2D or $\gamma\delta$ TCR (Fig. 4B, left-hand panel). In the presence of BrHPP, however, the contribution of NKG2D receptor to the cytotoxic activity of BrHPP-stimulated Vγ9Vδ2 T cells was negligible, whereas blockade of the $\gamma\delta$ TCR almost completely abrogated cytotoxic activity. (Fig. 4B, right-hand panel).
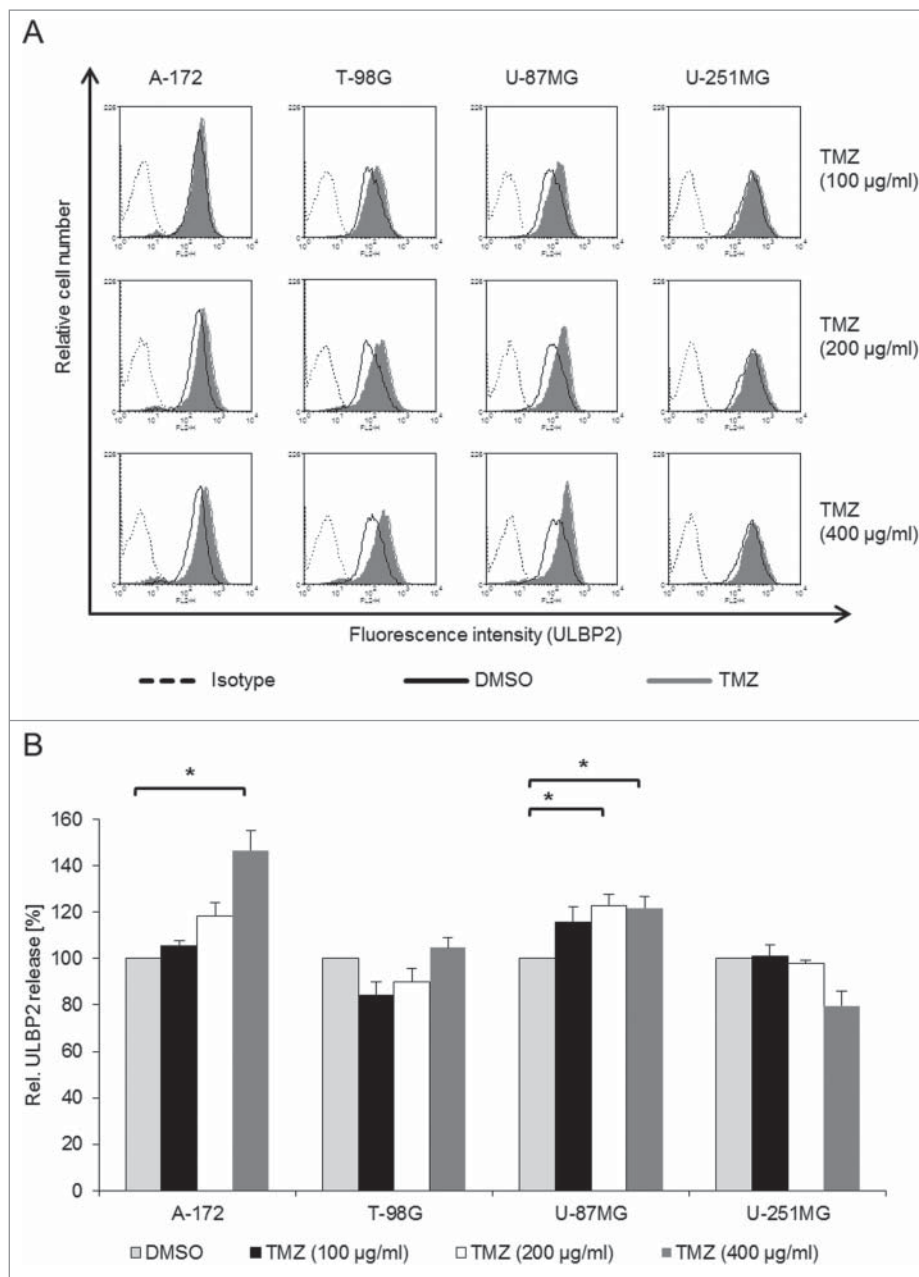
Figure 3. TMZ increases the cell surface expression and moderately the release of ULBP2. GBM cell lines were treated for 24 h with increasing concentrations of TMZ (100–400 μg/mL) and with DMSO as a control. (A) Cell surface expression of ULBP2 was determined by flow cytometry (filled histograms, TMZ; black open histograms, DMSO controls). Dotted lines represent the matched isotype controls. (B) The concentration of sULBP2 in supernatants of DMSO- and TMZ-treated GBM cells was determined by ELISA. The DMSO control was set to 100%. Data are presented as mean values of three to four independent experiments +/− SEM. Statistical significance is displayed as * for $p < 0.05$.

TMZ sensitizes GBM cells toward $\gamma\delta$ T-cell cytotoxicity

To judge functional implications of the TMZ-induced upregulation of NKG2DLs, we initially used TMZ-treated A-172 as target cells in a CFSE-based cytotoxicity assay. CFSE-labeled A-172 cells were treated with TMZ or with DMSO and incubated for 4 h at various effector/target (E/T) ratios with short-term Vγ9Vδ2 T-cell lines in the presence of BrHPP (TMZ was present in the co-culture at concentration of 100 μg/mL). Thereafter, co-cultures were stained with PI and analyzed for the percentage of CFSE-labeled dead target cells. Non-toxic concentrations of TMZ (Fig. 5A, right-hand panel) stabilized the cell surface expression of NKG2DLs (Fig. 5A, left-hand panel) and

sensitized A-172 cells to increased $\gamma\delta$ T cell-mediated cytotoxicity (Fig. 5B). This result was further validated in the Calcein AM-based cytotoxicity assay, where DMSO- and TMZ-treated A-172 cells were labeled with Calcein AM and used as targets for Vγ9Vδ2 effector T cells at an E/T ratio of 10:1, again in the absence or presence of BrHPP. As shown in Fig. 5C (right-hand panel), TMZ-pretreated A-172 cells displayed enhanced susceptibility to BrHPP activated Vγ9Vδ2 T cell-mediated killing. However, in the absence of BrHPP, there was only minimal lysis of A-172 cells (Fig. 5C, left-hand panel). Next, we investigated if TMZ could sensitize other GBM cell lines toward killing by Vγ9Vδ2 T cells in the absence of an additional stimulus. Short-
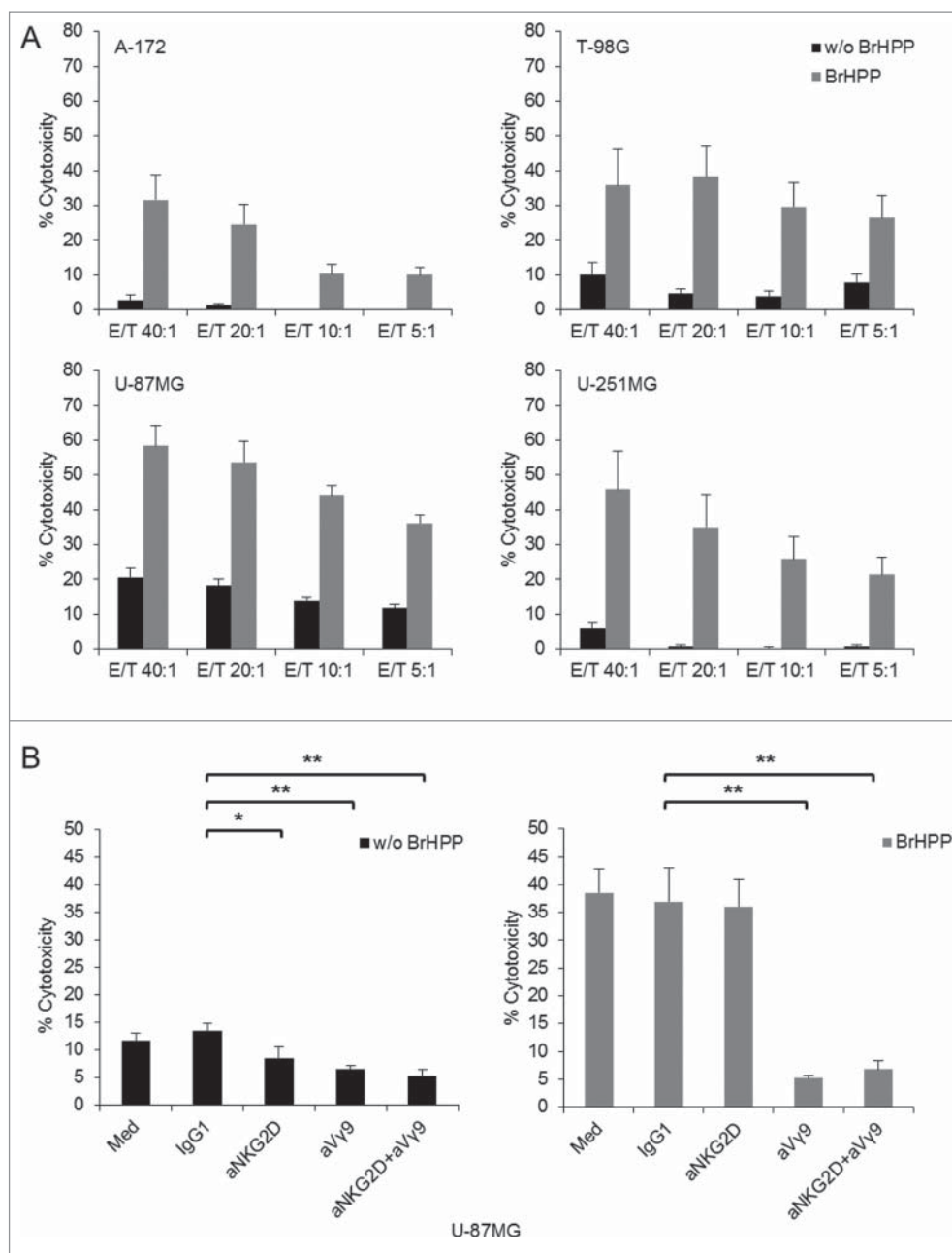
Figure 4. Lysis of GBM cell lines by short-term $\gamma\delta$ T cell lines. (A) Calcein-AM labeled A-172, T-98G, U-87MG and U-251MG target cells were co-cultured for 4 h at indicated E/T ratios with $\gamma\delta$ T cells expanded from four to five healthy donors. Cytotoxicity assays were performed in the absence (black bars) or presence (gray bars) of BrHPP. (B) Involvement of NKG2D and TCR in $\gamma\delta$ T cell-mediated killing. Calcein AM-labeled U-87MG cells were co-cultured for 4 h with $\gamma\delta$ T cells from five different healthy donors at an E/T ratio of 20:1 with (right-hand panel, gray bars) or without BrHPP (left-hand panel, black bars). Prior to the co-culture, $\gamma\delta$ T cells were pre-incubated for 1 h with 10 μg/mL anti-NKG2D and/or anti-Vγ9 TCR anti bodies or with IgG1 as an isotype control. Statistical significance is displayed as ** for $p < 0.01$ and * for $p < 0.05$.

term $\gamma\delta$ T cell lines from several different donors were analyzed in the Calcein AM cytotoxicity assay. The cytotoxic potential of $\gamma\delta$ T cells varied considerably between donors. In the absence of BrHPP, GBM cell lines were largely resistant to Vγ9Vδ2 T cell-mediated cytotoxicity with the exception of U-87MG cells (Fig. S4). Interestingly, TMZ pre-treatment sensitized A-172, T-98G ($p = 0.055$) U-87MG ($p < 0.05$) but not U-251G cells to $\gamma\delta$ T cell-mediated lysis (Fig. 6A for U-87MG, Fig. S4A for other cell lines). Comparable results were obtained with CFSE-based cytotoxicity assay (not shown). In extension of the results presented in Fig. 4B with untreated target cells, we performed

the cytotoxicity assay with TMZ pre-treated U-87MG target cells in the absence or presence of anti-Vγ9 TCR or anti-NKG2D receptor antibodies as above. As presented in Fig. 6A, the spontaneous cytotoxic activity of $\gamma\delta$ T cells was reduced to the same level by anti-NKG2D and anti-TCR antibodies. The effects of NKG2D- and Vγ9 TCR-blocking antibodies on killing of TMZ-sensitized U-87MG target cells varied considerably among the nine donors displayed in Fig. 6A. While blocking of the NKG2D receptor abrogated lysis of TMZ-pre-treated target cells by $\gamma\delta$ T cells from some donors, the $\gamma\delta$ TCR appeared to be more involved in other cases. In the presence of BrHPP, the
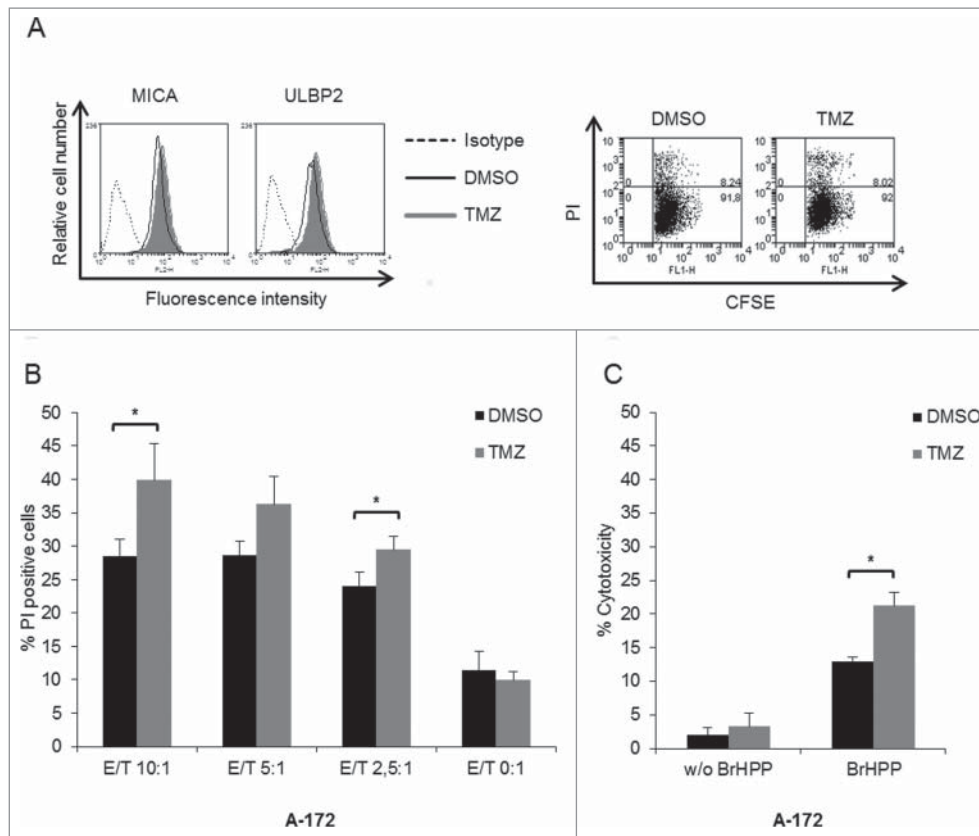
Figure 5. TMZ treatment sensitizes A-172 cells to Vγ9Vδ2 T cell-mediated killing. (A) A-172 cells were incubated overnight with 200 μg/mL TMZ or DMSO solvent control. This concentration of TMZ increased cell surface expression of MICA and ULBP2 (left-hand panel) but did not induce cell death as judged by PI staining (right-hand panel). (B) TMZ (gray bars)- or DMSO (black bars)-pre-incubated and CFSE-labeled A-172 cells were co-cultured in duplicates for 4 h with Vγ9Vδ2 T cells at various E/T ratios and stained with PI. BrHPP was present in the co-culture. PI/CFSE double-positive cells were considered as killed A-172 target cells. (C) TMZ (gray bars)- and DMSO (black bars)-pre-incubated A-172 cells were labeled with Calcein AM and co-cultured for 4 h with γδ T cells at E/T ratio 10:1 with (right-hand panel) or without BrHPP (left-hand panel). Data are presented as mean values of experiments with short-term γδ T-cell lines from 5 (B) and 4 (C) donors +/− SEM. Statistical significance is displayed as * for $p < 0.05$.

contribution of NKG2D receptor to the cytotoxic activity of BrHPP-stimulated Vγ9Vδ2 T cells against TMZ-pretreated U-87MG target cells was moderate, whereas the blockade of the γδ TCR almost completely abrogated the cytotoxic activity (Fig. 6B). Interestingly, the moderate sensitizing effect of TMZ pre-treatment on Vγ9Vδ2 T cell-mediated cytotoxicity was still detected even in the presence of both anti-NKG2D and anti-TCR antibodies (Fig. 6) suggesting that additional receptor-ligand interactions might be involved. Finally, we also investigated the susceptibility of U-87MG GBM cells pre-treated with TMZ and the MP inhibitor GW. GI and GW, either alone or in combination, did not have a major impact on GBM metabolic activity during the 48 h observation period as revealed by the XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), cell viability assay (Fig. S3B). Combined treatment with GW and TMZ synergistically increased the cell surface expression of ULBP2 on U-87MG (Fig. 7A, lower panel) and other GBM cells (Fig. S5A) but had little effect on MICA expression (Fig. 7A, upper panel). TMZ slightly increased shedding of ULBP2 from U-87MG cells in comparison to the relevant DMSO control which *per se* also increased ULBP2 shedding by 20% (Fig. 7B). ULBP2 shedding was less or not at all affected in the other GBM lines (Fig. S5B). Moreover, while both GW and TMZ alone sensitized U-87MG cells for killing by γδ T cells in the absence of BrHPP, in

combination they further increased the susceptibility. The solvent control DMSO had no effect. (Fig. 7C).

Discussion

To obtain more insight into the plasticity of the NKG2DL system in GBM, we analyzed the expression and shedding of NKG2DLs in four different GBM cell lines and studied the effects of MP inhibitors and TMZ on their expression and release. We observed high levels of MICA and ULBP2 expression on GBM cell lines and low to moderate levels of MICB and ULBP1. In line with our data, Wolpert *et al.* recently also reported high expression of MICA/ULBP2 on GBM initiating stem cells.[13] Although the precise reasons for the differential expression of MICA/ULBP2 *versus* MICB/ULBP1 are unclear, distinct NKG2DLs can be differentially regulated on the post-transcriptional and/or post-translational level by miRNAs, clathrin mediated-endocytosis, intracellular retention, MP/ADAM-mediated proteolytic cleavage or exosomal release.[30,31] However, MP/ADAM-mediated shedding cannot explain the low density of MICB or ULBP1 on GBM cells, since we did not detect any soluble MICB or ULBP1 by ELISA in culture supernatants. In fact, only ULBP2 was released as a soluble protein.
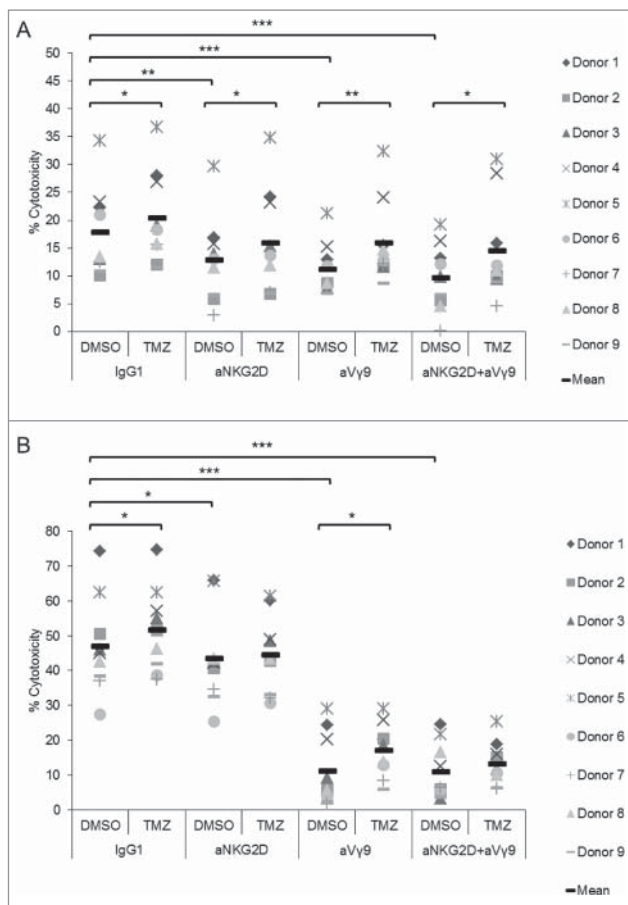
Figure 6. Involvement of NKG2D and TCR in $\gamma\delta$ T cell-mediated lysis of GBM cells. U-87MG cells were pre-treated for 20 h with TMZ or DMSO solvent control. (A) Calcein AM-labeled U-87MG target cells were co-cultured for 4 h with $\gamma\delta$ T cells at an E/T ratio of 20:1 in the absence of BrHPP. Prior to the co-culture, $\gamma\delta$ T cells were pre-incubated for 1 h with 10 μg/mL with blocking antibodies against NKG2D and/or Vγ9 TCR or with IgG1 as an isotype control. $\gamma\delta$ T cells were expanded from nine different healthy donors. (B) Similar set-up as in (A) but cytotoxicity assay performed in the presence of BrHPP. Statistical significance is displayed as *** for $p < 0.001$; ** for $p < 0.01$ and * for $p < 0.05$.

The lack of MICA shedding from A-172 and U-87MG cells can be partially explained by the exosomal release of the truncated MICA allele *008.[24,32] On the other hand, ULBP2 which can be attached to the cell surface *via* a GPI-anchor or expressed as a transmembrane protein, is released in association with exosomes or can be shed *via* the activity of MPs.[7,25] In the analyzed GBM cell lines, inhibition of MPs and specifically ADAM10/17 clearly abrogated ULBP2 shedding and increased its cell surface expression. Interestingly, both ADAM10 and ADAM17 contributed to ULBP2 shedding in GBM cells with the exception of U-87MG where the ADAM10-specific inhibitor GI alone was able to block the release of ULBP2 to the same extent as the ADAM10/17 inhibitor GW. Although the exact mechanism of the regulation of ADAM activity is not precisely known, trafficking, glycosylation, phosphorylation and interactions with accessory molecules might play important roles.[33,34] ADAMs cleave cell surface proteins in the juxta-membrane stalk region and substrate specificity is not dependent on the distinct amino acid sequence, but rather on the secondary structure of the protein.[34] ULBP2 harbors several single nucleotide polymorphisms

resulting in amino acid substitutions (see http://www.ncbi.nlm.nih.gov/SNP) that may cause alterations of the secondary structure of the protein and thus affect the substrate recognition by ADAM proteases. This particular aspect might also explain the lack of MICA shedding from T-98G and U-251MG in the presence of proteolytically active MP, altogether indicating that the regulation of shedding of distinct NKG2DLs is highly complex and divergent in different cell lines and primary tumor cells.

Shedding of NKG2DLs is considered as a tumor immune escape mechanism as it may either reduce the immunogenicity of tumors or even directly influence the expression of NKG2D and the functional activity of NK cells and T-cell subsets.[35,36] Proposed therapeutic strategies including the removal of immunosuppressive exosomes by affinity plasmapheresis or the removal of sMICA by neutralizing antibodies aim at controlling the serum levels of sNKG2DLs.[37,38] However, direct targeting of distinct ADAM proteases is of particular interest, as it may abrogate the shedding of NKG2DLs and simultaneously stabilize their cell surface expression.[10,13,39,40] Moreover, ADAM proteases, especially ADAM10 and ADAM17 are frequently over-expressed in cancer cells.[41] By the cleavage of various growth factors and their receptors, adhesion molecules or danger signals such as NKG2DLs, ADAM proteases are considered to be promising targets for tumor therapy.[42] Multiple inhibitors targeting ADAMs and other MPs were developed for the treatment of various malignancies. In this scenario, MP inhibitors were remarkably successful in experimental model systems.[43] However, although we show that inhibition of MP stabilizes ULBP2 surface expression, the clinical applicability of MP inhibitors is hampered by the lack of selectivity and specificity. For instance, in a phase II clinical trial conducted with GBM patients, the combined administration of the broad range MP inhibitor marimastat together with TMZ had minimal beneficial effect compared to TMZ single therapy but was rather associated with additional toxicity.[44,45] Therefore, the diverse physiological activities of ADAM proteases seem to preclude blocking strategies as a generally applicable principle in the context of cancer treatment.[46] Moreover, Deng *et al.* recently observed that sNKG2D can actually cause the activation of murine NK cells and thereby facilitate tumor elimination, which completely contradicts the dogma of the tumor-promoting properties of sNKG2D.[47] These findings definitely add more complexity to the NKG2D system and may also partially explain the failure of MP inhibitors in clinical trials for the treatment of various malignancies.

Thus, strategies aiming at enhancing cell surface expression rather than preventing shedding of NKG2DLs might be more promising. Drugs inducing cellular stress including histon deacetylase inhibitors or aldosteron antagonists have been used in different model systems to increase the immunogenicity of tumors by stabilizing the cell surface expression of NKG2DLs.[10,13,39] We found that TMZ, a standard chemotherapeutic drug for GBM treatment, increased the expression of NKG2DLs while moderately affecting ULBP2 shedding, and facilitated Vγ9Vδ2 T cell-mediated GBM tumor cell killing. Pre-treatment of A-172 cells and U-87MG cells with TMZ increased the susceptibility to BrHPP-activated $\gamma\delta$ T cells. As expected, cytotoxicity exerted by BrHPP-stimulated $\gamma\delta$ T cells was mainly mediated through
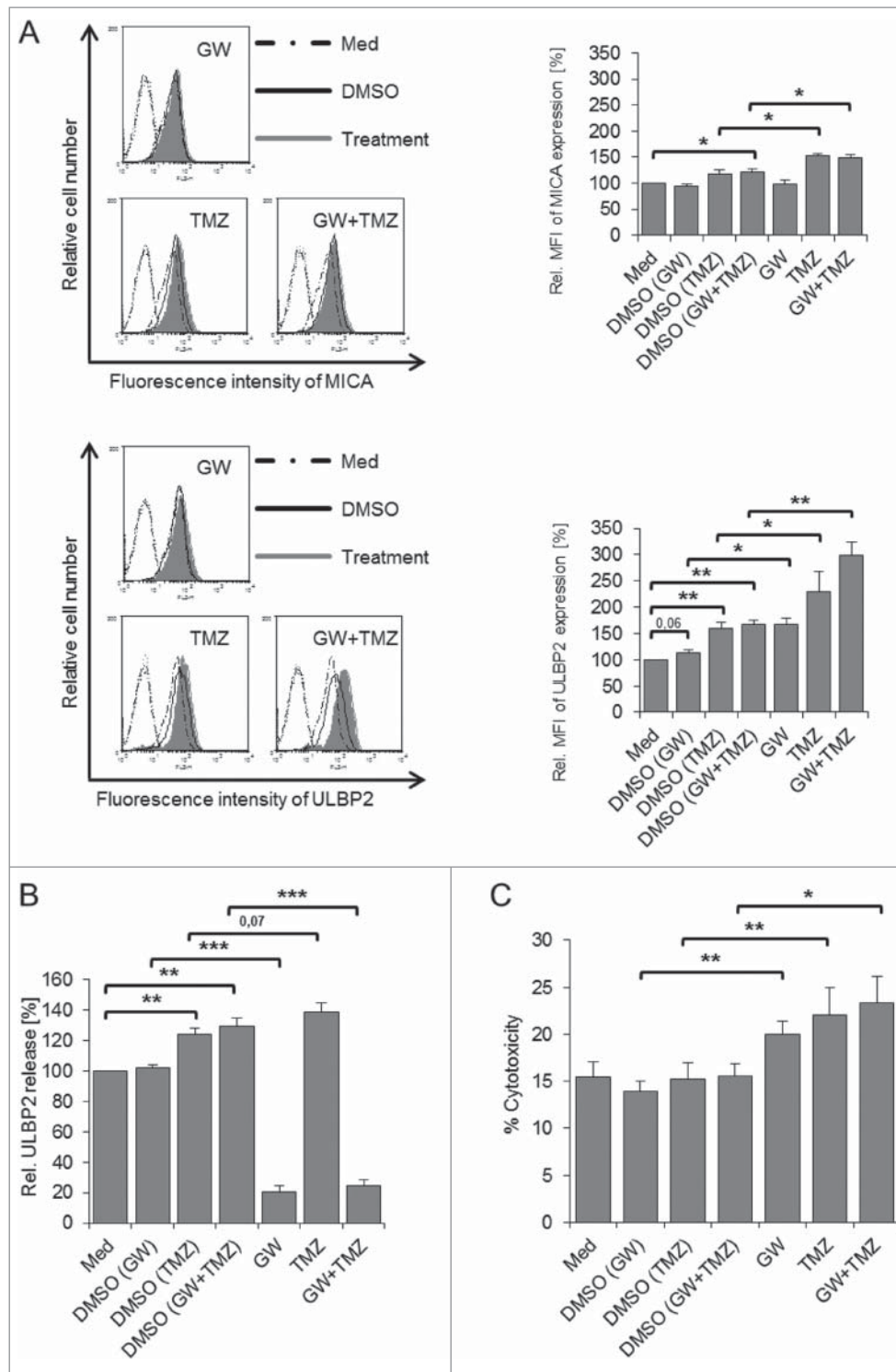
Figure 7. Combination of TMZ and GW treatment synergistically increases expression and shedding of ULBP2 and sensitizes U-87MG cells to Vγ9Vδ2 T cell-mediated killing. (A) U-87MG cells were incubated for 24 h with GW (3 μM), TMZ (200 μg/mL) or a combination of both. Cell surface expression of MICA and ULBP2 was determined by flow cytometry. Representative histograms are depicted in the left-hand panels (dotted histograms: medium control; black open histograms: DMSO solvent control; filled histograms: TMZ/GW treatment). A summary of results of four experiments is shown in the right-hand panels. Median fluorescence intensity (MFI) of MICA (upper right-hand panel) and ULBP2 (lower right-hand panel) expression was set to 100% in the medium-only treated cells. (B) ULBP2 shedding from GW and TMZ-treated U-87MG cells. U-87MG cells were pre-treated as in (A), and the concentration of sULBP2 was determined by ELISA in supernatants of medium-, DMSO solvent control-, GW-, TMZ- and GW/TMZ-treated samples. The amount of sULBP2 in medium control sample was set to 100%. (C) Combined treatment with GW and TMZ sensitizes U-87MG cells to Vγ9Vδ2 T cell-mediated killing. Medium-, DMSO-, GW-, TMZ- and GW/TMZ-treated U-87MG cells were labeled with Calcein AM and co-cultured in triplicates for 4 h with γδ T cells at an E/T ratio of 20:1 in the absence of BrHPP. Data are presented as mean values of five independent experiments +/− SEM. Statistical significance is displayed as[***] for $p < 0.001$, [**] for $p < 0.01$ and [*] for $p < 0.05$.

the $\gamma\delta$ TCR as shown by antibody blocking, with minimal contribution of the NKG2D receptor. Interestingly, Vγ9Vδ2 T-cells still exerted some cytotoxic activity even if both, NKG2D and $\gamma\delta$ TCR, were blocked indicating the possible involvement of additional cytotoxic trigger mechanisms. In the absence of BrHPP, antibody blocking of NKG2D also reduced the low levels of spontaneous $\gamma\delta$ T-cell cytotoxicity against GBM target cells. Due to the high donor-dependent variability and marginal effects of TMZ pre-treatment on $\gamma\delta$ T-cell killing potential, it is difficult to draw any definitive conclusions. Nevertheless, it seems that in line with investigations in other tumor systems,[22] both NKG2D and the TCR contribute to $\gamma\delta$ T cell-mediated lysis of GBM target cells. More importantly, even though A-172 cells and T-98G cells were completely resistant to spontaneous $\gamma\delta$ T-cell killing, these GBM cells were rendered susceptible upon TMZ pre-treatment. However, the same effect was not observed in U-251MG cells, indicating cell type-dependent variability. Together, these data indicate that TMZ pre-treatment increases the immunogenicity of tumors and augments anti-tumor activity of Vγ9Vδ2 T cells primarily in a TCR- and NKG2D-dependent manner. However, our results also illustrate the considerable heterogeneity between different GBM cell lines which makes it difficult to predict the $\gamma\delta$ T cell-sensitizing effect of TMZ in a clinical setting. The interaction between tumor cells and killer cells (NK cells, $\gamma\delta$ T cells) is not only regulated by activating receptors such as NKG2D but is also influenced by inhibitory receptors (e.g., NKG2A) and their ligands which we did not specifically investigate in the current study.[48] In addition to NKG2DLs or ligands for inhibitory receptors, TMZ might of course influence multiple other molecules important for the execution of $\gamma\delta$ T-cell cytotoxic activity, such as cell adhesion molecules[49] or BTN3A1/CD277,[17] an issue which we did not address in the current study but indeed requires further attention. For instance, cell adhesion molecule Nectin-like-molecule-5 binds to DNAX accessory molecule-1 (DNAM-1). DNAM1 is another NK cell activating receptor expressed on $\gamma\delta$ T cells playing a significant role in Vγ9Vδ2 T-cell cytotoxicity.[50] All four GBM lines expressed Nectin-like-molecule-5 which was increased by previous TMZ exposure (preliminary results, not shown). Therefore, DNAM-1 might indeed be involved in lysis of TMZ-treated GBM cells by $\gamma\delta$ T cells.

The application of TMZ in GBM patients can negatively influence the proliferation and cytotoxic activity of immune effector cells including $\gamma\delta$ T cells.[27,51] However, combination treatment with TMZ and adoptively transferred human cyto-kin-induced killer cells showed synergistic therapeutic efficacy in a xenograft mouse model with transplanted U-87MG cells.[52] Such a synergistic therapeutic effect might be also envisaged with $\gamma\delta$ T cells.

In conclusion, our results indicate that TMZ increases the immunogenicity of GBM cells and their susceptibility to Vγ9Vδ2 T-cell-mediated killing, suggesting that the combination of TMZ with the adoptive transfer of *in vitro* expanded Vγ9Vδ2 T cells might be beneficial for treatment of at least some GBM patients. In contrast to MP inhibitors, TMZ is a well-established component of clinical GBM treatment. Upon surgery of the primary tumor, the sensitizing effect of TMZ on

$\gamma\delta$ T cell killing could be determined in the individual patient. Large-scale in vitro expansion protocols for Vγ9Vδ2 T cells have already been established and several clinical trials documented the overall safety of Vγ9Vδ2 T-cell immunotherapy of various tumor entities[53-55] and the benefit in the treatment of metastatic solid tumors when used in combination with other therapeutic strategies.[56]

Materials and methods

Cells lines and reagents

The GBM cell lines A-172 (ECACC 880624218), T-98G (ECACC 92090213), U-87MG (ECACC 89081402) and U-251MG (formerly known as U-373MG; ECACC 89081403) were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Vγ9Vδ2 T-cell lines were established from healthy adult donors as previously described.[57] These studies have been approved by the appropriate institutional review board (D 405/10). Briefly, Ficoll-Hypaque-separated peripheral blood mononuclear cells were cultured in complete medium supplemented with 50 U/rIL-2 and were stimulated with 2,5 μM zoledronate (kindly provided by Novartis). Vγ9Vδ2 T cell lines were used as effector cells in cytotoxic assays between day 11 and day 15, referred to as short term $\gamma\delta$ T-cell lines. Cytotoxicity assays were performed in the absence or presence of 300 nMBr HPP (kindly provided by Innate Pharma, Marseille, France).[28] The purity of Vγ9Vδ2 T-cell lines used in the experiments was always >90%. Complete medium was RPMI-1640 (#52400-25, Life Technologies GmbH) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. All cell lines were kept at 37°C in a humidified atmosphere with 5% CO_2.

Where indicated, tumor cells were cultured for 24 h in the presence of the broad range MP inhibitors GM6001 (5 μM, Merck Millipore, #364206) and TAPI-1 (5 μM, Merck Millipore #579053) or with DMSO as a solvent control. For the selective inhibition of ADAM10 and ADAM10/17 GI254023X (GI, 3 μM) and GW280264X (GW, 3 μM)[26] were used (kindly provided by Prof. Paul Saftig, Institute of Biochemistry, Christian-Albrechts University, Kiel). Where indicated, tumor cells were cultured for 24 h in the presence of various concentrations of TMZ (#T2577, Sigma-Aldrich Chemie GmbH) or with DMSO as a solvent control.

Flow cytometry

The following mAb were used for cell surface staining: PE-conjugated anti-ADAM17 mAb (#FAB9301P, clone 111633), anti-MICB mAb (# FAB1599P, clone 236511) and anti-ULBP1 mAb (#FAB1380P, clone 170818) were purchased from R&D Systems. Unconjugated anti-MICA (clone AMO1, kindly provided by Prof. Alexander Steinle, Institute of Molecular Medicine, University of Frankfurt) and anti-ULBP2 mAb (#ab89930, clone MM0593-7F33) were obtained from BAMO-MAB and Abcam, respectively. Anti-ADAM10 mAb (#857.800.000, clone 11G2) was obtained from Gen-Probe. Tumor cells were detached from culture plates with trypsin/EDTA (0.05%/0.02 w/v; #L2153, Biochrom), washed and

blocked with Fc-receptor blocking reagent (#130-059-901, MiltenyiBiotec GmbH) according to the manufacturer's instructions. After an additional washing step, the cells were incubated for 30 min with specific antibodies and matched isotype controls. In case of unconjugated antibodies cells were further incubated with a polyclonal PE-conjugated goat-anti-mouse Ab (Life Technologies). After a washing step cells were fixed in 1% paraformaldehyde. 5,000 live cells were analyzed on a FACS Calibur flow cytometer using CellQuestPro Software (BD Biosciences, Heidelberg, Germany).

ELISA

GBM cells were cultured for 24 h and the cell-free culture supernatants were subjected to ELISA. The amounts of sMICB (#DY1599), sULBP1 (#DY1380) and sULBP2 (#DY1298) were determined with the respective sandwich ELISA kits (R&D Systems) following the manufacturer's instructions. sMICA was detected by sandwich ELISA as described by Salih *et al.* with slight modifications.[58] Briefly, a microtiter plate was coated overnight with anti-MICA mAb (clone AMO1, 2 μg/mL). Anti-MICA/B (#BAMO3-500, clone BAMO3, 5 μg/mL, IgG2a) was used as a detection Ab. After a washing step anti mouse-IgG2a-HRP was added to the plate and developed by using tetramethylbenzidine peroxidase substrate system (#DY999, R&D Systems). The absorbance was measured on a microplate reader. (Tecan Group Ltd, Männedorf, Switzerland) at 450 nm.

CFSE- and Calcein AM-based cytotoxicity assays

GBM cells were incubated in the presence of 10 μM CFSE (#21888, Fluka) at 37°C for 10 min. 10 mL of ice-cold RPMI medium containing 10% FCS were added to the cells for 5 min. After three washing steps, CFSE-labeled cells were incubated overnight with TMZ (200 μg/mL) or with DMSO as solvent control. Vγ9Vδ2 T cells were added to the plate at various E/T ratios in duplicates with or without BrHPP in the presence of 12.5 U/mL IL-2. After 4 h, cells were stained with PI and 5,000 CFSE-labeled target cells were analyzed by flow cytometry to quantify PI fluorescence. In Calcein AM-based cytotoxicity assays,[59] TMZ- and DMSO-treated cells were incubated with 5 μM Calcein AM (#C3099, Life Technologies) for 30 min at 37°C. Following two washing steps cells were co-cultured in triplicates in the presence of 12,5 U/mL rIL-2 with Vγ9Vδ2 T cells at various E:T ratios with or without BrHPP. Where indicated, effector cells were pre-incubated for 1 h with 10 μg/mL anti TCR-Vγ9 mAb 7A5,[29] anti-NKG2D mAb (#MAB139, clone 149810, R&D systems) or mouse isotype control IgG1 (#18443, clone MOPC-21, Abcam) before the addition of Calcein AM-labeled tumor target cells. 100 μL of supernatant were transferred to non-binding black μCLEAR® 96 well microplates (#147827, Greiner bio-one) and fluorescence intensities were measured using a Tecan microplate reader at 490 nm with reference filter at 530 nm. Fluorescence intensity correlates with the amount of calcein released into the supernatant.[59] Percentage cytotoxicity was calculated as follows: (extinction$_\text{experimental}$ − extinction$_\text{spontaneous}$/extinction$_\text{maximum}$ − extinction$_\text{spontaneous}$,

where the maximal calcein release was determined in wells containing target cells and 1% triton X-100.

Statistical analysis

The paired, two-tailed Student's t-Test was performed. Statistical significance was set at $p < 0.05$ and displayed as *** for $p < 0.001$; ** for $p < 0.01$ and * for $p < 0.05$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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