Erroneous expression of NKG2D on granulocytes detected by phycoerythrin-conjugated clone 149810 antibody

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Abstract
Background: The activating Natural killer group 2 member D (NKG2D) receptor is typically expressed on NK cells, CD8 T lymphocytes, γδ T cells and small subsets of CD4 T lymphocytes. During the course of an extensive flow cytometry phenotyping of immune cells in the peripheral blood of patients with glioblastoma multiforme (GBM) we noticed an unexpected expression of NKG2D receptor on granulocytes using the phycoerythrin (PE)-conjugated clone 149810 antibody.

Methods: Peripheral blood samples from 35 patients with GBM and 22 age-matched healthy control (HC) donors were analyzed using flow cytometry, imaging cytometry and real-time quantitative reverse transcription PCR to validate the observed expression of NKG2D receptor on myeloid cells.

Results: Reactivity with PE-149810 was mostly observed on granulocytes from GBM patients on dexamethasone treatment where it correlated with inferior survival rates. Surprisingly, such NKG2D expression on granulocytes was not observed using the allophycocyanin (APC)-conjugate of the same clone 149810 antibody or an indirect staining procedure with unconjugated clone 149810 antibody. Moreover, the PE-conjugate of a different anti-NKG2D clone (1D11) also did not stain granulocytes. Imaging cytometry indicated cell surface and intracellular localization of PE-149810 but not of PE-1D11 in granulocytes.

Conclusion: Our results uncover an erroneous and false positive reactivity of PE-labeled (but not of APC-labeled or unconjugated) anti-NKG2D antibody 149810 on granulocytes from dexamethasone-treated GBM patients and raise a note of caution for studies of NKG2D expression on non-lymphoid cells.

KEYWORDS
cross-reactivity, false positive, flow cytometry, granulocytes, glioblastoma multiforme, natural killer group 2 member
1 | INTRODUCTION

Natural killer group 2 member D (NKG2D) is a C-type lectin receptor expressed on NK cells, CD8 T cells, γδ T cells and certain subsets of CD4 T cells (Wensveen et al., 2018). Upon binding of stress-inducible, Major Histocompatibility Complex class I Chain related gene A/B (MICA/B) ligands or members of the UL16-binding proteins (ULBP1-6), the human NKG2D receptor initiates a signaling cascade via the transmembrane adaptor protein DAP10. Upon phosphorylation, DAP10 recruits the p85 subunit of phosphoinositide-3-kinase (PI3-K) and Grb2-Vav1, which stimulates survival and cytotoxicity of NK cells and provides costimulatory signals to T cells (Lopez-Soto et al., 2015). As many solid tumor cells and leukemias/lymphomas express one or several of those NKG2D ligands, it is generally assumed that the NKG2D/NKG2D ligand axis constitutes an important immune surveillance mechanism, a concept which is supported by increased rates of spontaneous malignancies observed in NKG2D-deficient mice (Guerra et al., 2008).

NKG2D (also termed CD314) is a widely investigated molecule where a number of specific antibodies have been studied and characterized over the years. Clone 149810 is one of the well characterized antibodies which has originally contributed to identify the CD314 cluster of differentiation (Warren, 2005). We have previously performed a detailed immunophenotyping study in patients with glioblastoma multiforme (GBM), the most malignant brain tumor in adults. While the treatment with dexamethasone (Dex) further reduced the number of immune cells in the peripheral blood of GBM patients, our studies revealed a compromised immune cell distribution with or without Dex treatment (Chitadze et al., 2017). The numbers of NKG2D-positive lymphocytes were significantly reduced in the Dex-treated group, due to the depleting effect of Dex mainly on NK cells and CD8 T cells. Conversely, the numbers of neutrophils were significantly increased in Dex-treated GBM patients (Chitadze et al., 2017). Unexpectedly, during the course of these studies, we noticed that granulocytes from some Dex-treated GBM patients and some healthy control donors appeared to express the NKG2D receptor as detected by a PE-conjugated anti-NKG2D antibody. However, the thorough analysis presented here revealed that this positive staining was erroneously due to the use of the PE-conjugate of this particular antibody clone.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

We analyzed peripheral blood samples from 35 patients with GBM and 22 age-matched healthy control (HC) donors. Clinical details of GBM patients have been previously reported (Chitadze et al., 2017). EDTA blood was collected for immediate flow cytometric analysis and further storage in liquid nitrogen until RNA extraction for real-time quantitative reverse transcription PCR (qRT-PCR). Peripheral blood mononuclear cells (PBMC) and granulocytes of healthy donors (n = 17) and GBM patients (n = 8) were isolated by Ficoll–Hypaque density gradient centrifugation (see below). The study was approved by the Ethics committee of the Medical Faculty of University of Kiel (D485/13 and D405/14), and informed consent was obtained from all GBM patients and HC. Guidelines of the Helsinki Declaration of 1975 were followed.

2.2 | Flow cytometry (FCM)

To study the NKG2D expression on lymphocytes, monocytes and granulocytes in peripheral blood, whole blood was stained with mAb directed against CD14 and CD66b: CD14-APC (clone MFE2, #555399, BD Biosciences), CD66b (CD66b-FITC, clone 04, #11729-MM04-F, Sino Biologicals) together with anti-NKG2D antibodies: anti-NKG2D-PE (clone 149810, #FAB139P, R&D systems), or anti-NKG2D-PE (clone 1D11, #12-5878-41 eBioscience), and incubated for 20 min at room temperature (RT). After red blood cell lysis with BD Lysing-solution (#349202, BD Biosciences), cells were spun down, washed with FACS washing buffer (WB) (PBS containing 1% BSA and 0.1% sodium azide) and resuspended in 1% BSA and 0.1% sodium azide. To analyze the expression of NKG2D on isolated leukocytes we lysed the red blood cells using RBC Lysis buffer (#420301, Biolegend) and blocked the cells with Fc-receptor blocking reagent (#130-059-901, Miltenyi Biotec GmbH) for 20 min on ice. To analyze the expression of NKG2D on isolated granulocytes, the cell pellet after Ficoll–Hypaque density gradient centrifugation consisting of erythrocytes and granulocytes was resuspended in RBC lysis buffer. Following the erythrocyte lysis step, remaining granulocytes were subjected to further analysis. Neutrophils were isolated using the MACS® Whole Blood Neutrophil Isolation Kit (#130-104-434 Miltenyi Biotec GmbH) according to the manufacturer’s instructions. Briefly, whole blood cells were incubated with the antibody mix for 5 min and afterwards placed in the magnetic field for another 15 min. The flow-through containing the unlabeled neutrophils was carefully collected and subjected to further analysis. Isolated granulocytes and neutrophils were blocked with Fc-receptor blocking reagent. After the blocking step, cells were stained with anti-NKG2D-PE (clone 1D11 or clone 149810) or with anti-NKG2D-AF700 (clone 149810, #FAB139N, R&D systems), anti-NKG2D-APC (clone 149810, #FAB139A, R&D systems), or with unconjugated NKG2D (clone 149810, #MAB139, R&D systems; or clone 1D11, #14-5878-82, eBioscience) at 4°C. In case of unconjugated antibodies, cells were further incubated with a PE-conjugated goat-anti-mouse (GAM) second-step Ab (#A10543, Invitrogen). After a washing step, cells were fixed in 1% paraformaldehyde (PFA). The samples were measured on a Fortessa LRS II analyzer (BD Biosciences) using FACS Diva software or on a FACS Calibur using FACS CellQuest Pro software (BD Biosciences). Data collected on FACS Calibur and Fortessa LRS was analyzed with FlowJo software (FlowJo v10).

2.3 | Quantitative RT-PCR

PBMC and granulocytes were isolated using Ficoll–Hypaque density gradient centrifugation. After careful aspiration of the PBMC
interphase layer and removal of the remaining Ficoll solution, RBC were lysed and the remaining cells (granulocytes) were collected. After washing, pellets of isolated granulocytes and PBMC were directly resuspended in PeqGold Trifast (#30-2010, Peqlab) and stored at −80°C until RNA extraction. RNA extraction, cDNA synthesis and qRT-PCR were performed as described previously (Kalyan et al., 2014). All PCR reactions were run in duplicates using 2.5 µL of the cDNA (transcribed from 10 ng total RNA). qRT-PCR was performed using a Rotorgene 3000 (Corbett, LT, Wasserburg, Germany) with SYBR green-based qPCR mix (#95072-05K, Quanto Biosciences). Primers for NKG2D receptors were designed using the Web-based primer3 software (http://primer3.wi.mit.edu/) and purchased from TIB MOLBIOL (Berlin, Germany), together with primers for housekeeping genes β-actin (ACTB), β-2-microglobulin (B2M) and RNA (18S) as described in ref (Chitadze et al., 2017). Threshold levels for Ct-determination were chosen manually. Data analysis was performed according to the ΔCt method (Nolan et al., 2006). Briefly, the mean Ct value of three housekeeping genes was subtracted from the Ct value of the gene of interest (ΔCt) and transformed with equation $X = 2^{-\Delta Ct}$.

2.6 | Statistical analysis

We used Graph Pad Prism 6, SPSS 24 (IBM). Two-tailed Wilcoxon’s rank-sum test was used to compare the data without adjusting for multiple testing between GBM patients and HC and between groups expressing NKG2D on granulocytes at low and at high levels. GBM-associated overall survival was analyzed with Kaplan–Meier approach. Relative median fluorescence intensity values (rel. MFI) were compared between groups (MFI of the NKG2D signal on the population of interest is divided by the MFI from NKG2D-negative lymphocytes). Relative MFI of anti-NKG2D-PE obtained on lymphocytes, monocytes and granulocytes was dichotomized based on the respective median value as a cut off and a log rank test was run to determine differences in the survival distribution for the two groups. A two-tailed $p$-value of 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Expression of NKG2D receptor on granulocytes, monocytes and lymphocytes in the peripheral blood of GBM patients and healthy control donors

Blood samples of GBM patients collected before tumor resection ($n = 35$) and of age-matched healthy control donors (HC, $n = 22$) were analyzed by 3-color FCM for NKG2D-expression on lymphocytes, granulocytes and monocytes using cell scatter-properties and expression of CD66b and CD14 for granulocytes and monocytes, respectively. The NKG2D expression was analyzed by using anti-NKG2D-PE lgG1 antibody clones 149810 or 1D11, both at 25 µg/mL concentration. PE-1D11 detected the same amount of NKG2D-positive lymphocytes as PE-149810; however, the results were not comparable regarding the NKG2D expression on granulocytes. Representative results with blood from a GBM patient with Dexamethasone (Dex) treatment (left panel) and a GBM patient without Dex treatment (right panel) are shown in Figure 1. Clone 1D11 (Figure 1a, right dot plots in both panels), in contrast to clone 149810, (Figure 1a, left dot plots in both panels) did not detect any positive signal on the cell surface of granulocytes. Both antibodies revealed rather unspecific staining on the monocytes in patients and HC.

22 patients received Dex treatment before the surgery. To address the effects of steroids on the intensity of the NKG2D signal using PE-149810 mAb on lymphocytes and granulocytes, we separated the patients in two subgroups, GBM patients who did not receive steroid treatment (GBM, n = 13) and GBM patients treated with Dex (GBM-Dex, n = 22). Relative MFI values of NKG2D-PE signal in GBM patients was compared to sex- and age-matched HC. For comparison of results between the steroid-untreated patients and HC, 14 sex- and age-matched HC were selected (HC-I) out of the total group of 22 HC. The results obtained from patients in the GBM-Dex group were compared to the data of all 22 HC included in the study. Thus, HC-I represents a subgroup of HC (Figure 1b).
FIGURE 1  NKG2D expression on lymphocytes, monocytes and granulocytes. (a) Whole blood leukocytes of two GBM patients being treated with dexamethasone (Dex; left) or not (right) were analyzed for NKG2D expression by FCM. NKG2D expression was detected using PE-conjugated antibody clones 149810 and 1D11, depicted as red dots. The isotype control (blue dots) was IgG1-PE. (b) Granulocytes (CD66b+ cells), monocytes (CD14+ cells) and lymphocytes defined as cells with low side scatter properties without the expression of CD66b and CD14 were analyzed for NKG2D expression using PE-149810 in GBM patients treated with steroids (GBM-Dex, n = 22) or not (GBM, n = 13) and in age-matched HC-I (subgroup of HC; n = 13) and HC (n = 22) (upper left panel). Relative MFI values of NKG2D expression on granulocytes (upper right panel), monocytes (lower right panel) and on NKG2D-positive lymphocytes (lower left panel) were compared between groups using Wilcoxon’s rank-sum test. Statistical significance is displayed as *** and * for \( p < 0.001 \) and 0.05, respectively [Color figure can be viewed at wileyonlinelibrary.com]
This comparison showed that there was no difference in the intensity of staining with PE-149810 between HC and GBM patients on monocytes and lymphocytes (Figure 1b, lower left and right panels). In contrast, there was a significant difference in the relative MFI of PE-149810 staining on granulocytes between steroid-treated GBM patients and steroid-naïve GBM patients and HC (Figure 1b, upper right panel). For staining of RBC-lysed blood samples we did not use the FC-blocked reagent, as serum contains Immunoglobulins at a concentration of approximately 10 mg/mL (Gonzalez-Quintela et al., 2008). Moreover, the blocking of negatively isolated neutrophils or granulocytes with a FC-blocking reagent did not affect the overall PE-149810 staining on granulocytes, but only minimally reduced the MFI (Figure S1). Non-specific binding of PE-149810 was also not due to an excess use of antibody as shown in titration experiments (Figure S2).

CD14 is expressed at low levels on granulocytes (Antal-Szalmas et al., 1997) and might be upregulated in the course of activation (Zarco et al., 1998). In order to evaluate a possible link between CD14 expression and NKG2D-positivity of granulocytes, we assessed the relative MFI values of CD14 on granulocytes, in patients and healthy controls and we did not find any significant differences between relative CD14 MFI values in defined cohorts nor a correlation between history of steroid intake and PE-NKG2D signal on granulocytes (not shown).

3.2 High levels of reactivity with anti-NKG2D mAb PE-149810 on granulocytes is associated with inferior survival

Survival data of 32 GBM patients were available. To understand the impact of the granulocytic NKG2D expression as revealed by PE-149810 mAb on the overall survival we performed Kaplan-Meier’s analysis. Relative MFI values of PE-149810 expression on granulocytes, monocytes and NKG2D-positive lymphocytes were dichotomized at the median (6.9, 15.2, and 19.5 median relative MFI values, respectively) and were compared between two groups using the log-rank test. 240 days median Overall Survival (OS) (95% confidence interval, 207–273 days) was estimated as the time elapsed

![Figure 2](image-url)

**Figure 2** Kaplan-Meier survival analysis of GBM patients. Relative MFI of NKG2D expression as revealed by PE-149810 staining on granulocytes (a), on monocytes (b) and on NKG2D-positive lymphocytes (c) was dichotomized (median as a cut-off) and used to generate Kaplan-Meier curves in the GBM patient cohort \(n = 32\), 6 patients censored at the date of the last follow up. The levels of variables above (high) and below (low) median were compared by log-rank test. Statistical significance is displayed as ** for \(p < 0.01\).
between including the GBM patients into the study and death (total \( n = 26 \): GBM-dex 17, GBM 9), or the date of the last follow up (6 patients censored, all primary GBM; GBM-Dex 3, GBM 3). Survival analysis revealed that high PE-149810 relative MFI on granulocytes and on monocytes (\( p \leq 0.054 \)) were associated with inferior survival (Figure 2a: granulocytes: 180 vs 308 days; Figure 2b: monocytes: 183 vs 252 days). NKG2D expression levels on lymphocytes did not correlate with the overall survival (217 vs 240 days, Figure 2c).

3.3 Quantitative real-time PCR analysis of NKG2D in isolated PBMC and granulocytes

To correlate the results of FCM analyses with mRNA expression, we analyzed the expression of mRNA for full-length NKG2D and a truncated isoform by qRT-PCR in isolated granulocytes (Gr) and PBMC. The recently described truncated NKG2D-isoform lacks the cell surface domain and negatively regulates NKG2D-functions (Karimi

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**FIGURE 3** Expression of NKG2D mRNA in GBM patients and HC. (a) Primer design for full-length FL-NKG2D (dark blue arrows) and truncated TR-NKG2D (orange arrows) isoforms. (b) mRNA level of the full-length (NKG2D-FL, upper panel) and truncated NKG2D (NKG2D-TR) was analyzed in isolated granulocytes and PBMC of GBM patients (\( n = 8 \)) and HC (\( n = 17 \)). The data was divided in two groups based on relative MFI of NKG2D expression (as detected by PE-149810 staining) (median as a cut-off) on granulocytes as NKG2D-high (\( n = 3 \) GBM, \( n = 9 \) HC) and NKG2D-low group (\( n = 5 \) GBM, \( n = 8 \) HC) and compared between these groups using Wilcoxon’s rank-sum test. CY-cytosolic; EC, extracellular; LBD, ligand-binding domain; ST, stalk; TR, transmembrane [Color figure can be viewed at wileyonlinelibrary.com]
et al., 2014). For this, cells from 8 GBM patients and 17 HC were available. The primers were designed to detect the truncated (TR-NKG2D) or the full-length (FL-NKG2D) isoforms of NKG2D as shown in Figure 3a.

The relative MFI values of NKG2D expression as detected by PE-149810 mAb on granulocytes of all analyzed samples were pooled together to quantify the median (6.9 median relative MFI value of NKG2D) as a cut-off in order to separate samples in two groups based on the NKG2D expression intensity on granulocytes. The relative MFI of NKG2D expression on the granulocytes in the NKG2D-high group (NKG2D-hi) was 20 for GBM patients and 15.3 for HC, in the NKG2D-low group (NKG2D-lo) 3.9 and 4.9 for GBM patients and for HC, respectively (Figure 3b, left panels). Surprisingly, even in samples with high intensity of PE-149810 staining in granulocytes, mRNA levels of both NKG2D isoforms were low in granulocytes (Figure 3b, left panels) compared to PBMC from the same individuals (Figure 3b, left panels). The relative MFI of NKG2D expression on the NKG2D-positive lymphocytes in the NKG2D-hi group was 19.3 for GBM patients and 16.3 for HC, in the NKG2D-lo group 19.5 and 17.8 for GBM patients and for HC, respectively (Figure 3b, right panel). Interestingly, there was a tendency that mRNA levels of the truncated NKG2D isoform in granulocytes were expressed at lower levels in the NKG2D-hi subgroup in HC controls (Figure 3b, lower left panel), possibly pointing towards a low negative regulatory effect on full length NKG2D expression. This was also observed in PBMC of GBM patients but not HC (Figure 3b, lower right panel) although the low sample size

**FIGURE 4**  NKG2D expression by FCM and imaging cytometry. (a) Whole blood samples were stained and analyzed by FCM. Red dots: PE-conjugated clone 149810. Dark blue dots: PE-conjugated clone 1D11. Light blue dots: IgG1-PE isotype control. (b) 2-step staining procedure was performed using the unconjugated anti-NKG2D (clone 149810, red) and anti-NKG2D (clone 1D11, blue) antibody and a second-step GAM-PE antibody. (c) Cells were stained with CD66b-FITC (green) and PE-NKG2D-antibodies (clone 149810, left panel, clone 1D11, right panel, yellow) to study expression of NKG2D on granulocytes (upper panel) and on lymphocytes (lower panel), and visualized using imaging cytometry [Color figure can be viewed at wileyonlinelibrary.com]
impedes a definite interpretation. Overall, we could not verify NKG2D mRNA expression in granulocytes using qRT-PCR.

3.4 | Antibody clone- and fluorochrome-based variability in NKG2D expression on granulocytes

To reconcile the obvious discrepancy between the very low or absent mRNA expression (Figure 3) and the positive NKG2D expression with one (PE-149810) but not another anti-NKG2D clone (PE-1D11) in granulocytes (Figure 1a), we extended the FCM analysis to other fluorochrome conjugates of clone 149810 (AF700, APC, Figure 4a) as well as unconjugated 149810 clone (Figure 4b) with a second-step Ab. The PE-conjugated clone 1D11 did not stain granulocytes and monocytes but clearly stained lymphocytes (Figure 4a, left panel, dark blue dots), in striking contrast to PE-conjugated clone 149810 which clearly stained granulocytes and monocytes, in addition to lymphocytes (Figure 4a, left panel, red dots). Surprisingly, different fluorochrome conjugates of clone 149810 showed either an intermediate staining pattern on granulocytes and monocytes (AF700-conjugate; Figure 4a, middle panel, red dots), or no reactivity at all (APC-conjugate; Figure 4a, right panel, red dots). Furthermore, an indirect staining procedure with unconjugated clone 149810 followed by PE-labeled GAM Ig also did not stain granulocytes or monocytes (Figure 4b). Note that in all instances lymphocytes were clearly stained by the anti-NKG2D mAb, irrespective of the clone and the fluorochrome.

Although all antibody stainings were performed on ice, we reasoned that the PE-conjugated clone 149810 mAb might be non-specifically taken up by granulocytes, resulting in the observed positive staining in FCM. To address this, we used imaging cytometry which allows to capture single cells, and analyzed the localization and intensity of the fluorescence signal (Figure 4c). Granulocytes were identified with FITC-conjugated anti-CD66b mAb. The PE-conjugated anti-NKG2D clone 149810 detected a positive signal with high intensity localized inside the granulocytes as well as on the cell surface, as evidenced by the yellow overlay depicted in Figure 4c (upper left picture). In contrast, such a co-localization with CD66b-FITC was not observed employing the PE-conjugated anti-NKG2D clone 1D11 (Figure 4c, upper right picture). As expected, PE-conjugated antibody clone 149810 as well as the clone 1D11 gave a positive signal on lymphocytes (identified as CD66b-negative cells), mainly localized at the cell membrane (Figure 4c, lower picture).

To further validate the hypothesis that uptake of PE-conjugated clone 149810 might result in the observed positive staining of granulocytes in FCM, we performed antibody-blocking assays on ice to avoid the internalization and uptake by granulocytes or monocytes.

![Figure 5](https://wileyonlinelibrary.com) Impact of blocking on NKG2D expression on granulocytes and lymphocytes. (a) RBC-lysed whole blood cells were preincubated for 30 min on ice with 20x excess of IgG1 isotype control, unconjugated clone 149810 or unconjugated clone 1D11 anti-NKG2D antibodies and subsequently stained with PE-conjugated anti-NKG2D clone 149810 or 1D11 antibodies as indicated. (b) Control stainings with PE-IgG1, PE-149810 and PE-1D11 without prior pre-incubation [Color figure can be viewed at wileyonlinelibrary.com]
To this end, RBC-lysed blood cells were incubated with 20x excess of unconjugated anti-NKG2D antibodies clone 149810 or clone 1D11 (or isotype control) for 30 min on ice. Thereafter, cells were stained with PE-conjugated anti-NKG2D mAb clone 149810, assuming that cell surface NKG2D expression would have been blocked with the excess of antibodies during pre-incubation and thus result in the reduction of positive signal on granulocytes (Figure 5a). In control stainings, cells were not preincubated with any antibodies (Figure 5b). As shown in Figure 5a, binding of PE-149810 was successfully blocked on lymphocytes by pre-incubation with either clone 149810 (Figure 5a, middle panel) or clone 1D11 (Figure 5a, right panel) but not with IgG1 isotype control (Figure 5a, left panel). However, following pre-incubation with either antibody clone, granulocytes still remained positive for staining with PE-149810 (Figure 5a, middle and right panels). These results clearly demonstrate that the PE-conjugated anti-NKG2D mAb clone 149810 non-specifically binds to the cell surface of granulocytes and therefore reflects a false positive signal. In addition, using two different lots of PE-149810 antibody clone (LCP0713021, lot used during the initial study (Chitadze et al., 2017) and LCP0918011, recent lot, used in experiments shown in Figures S2 snd S3) the positive signal detected on granulocytes differed between two lots even in the same blood donor (Figure S2) whereas the similar NKG2D expression pattern on lymphocytes was detected, hinting towards the potential false positive staining on granulocytes also when using a recent lot (Figure S2, Donor 3).

4 | DISCUSSION

Here we report our findings of an erroneous expression of NKG2D on granulocytes using PE-labeled clone 149810, mainly occurring in steroid-treated-patients diagnosed with GBM and even linked to poor outcome. APC-conjugates of the same 149810 clone or PE-conjugated NKG2D antibody clone 1D11 did not detect the false positive signal on granulocytes. Different labeling approaches (amines (−NH₂), thiol groups (−SH) and carbohydrate residues of an antibody or the glycosylation status of monoclonal antibodies produced in different hybridoma cell lines might differently affect the tertiary structure and thus influence antigen/Fc-receptor binding affinities. This might possibly lead to differences between distinct fluorochrome conjugates of the same antibody clone or of PE-conjugates of distinct clones as described here (Adamczyk et al., 1999; Shrestha et al., 2012). The conjugation efficiency can also vary between distinct lots of the same antibody. Moreover, human Fc-receptors for IgG (FcγRI) can cross-react with murine IgGs (Bruhns, 2012; Lubeck et al., 1985; Temming et al., 2020). Here used mouse IgG1 does not normally bind to FcγRI/FcγRII but interacts efficiently with FcγRIIa, with particularly high affinity to FcγRIIa-131 polymorphic variant (Temming et al., 2020). If the expression of FcγRIIa-131, a frequent allelic variant in Caucasian population (van Schie & Wilson, 2000) was associated with here described cross-reactivity it was not addressed in this study. We used whole blood samples containing serum immunoglobulins, known to prevent unspecific binding (Andersen et al., 2016; Gonzalez-Quintela et al., 2008). However, Dex and corticosteroids in general are known to reduce serum immunoglobulin levels (Butler & Rossen, 1973; Nunley Jr. et al., 1982), for instance by down-regulating genes associated with immunoglobulin production (Lee et al., 2016). Considering this, granulocytes of patients receiving steroids might unspecifically bind to PE-149810 due to reduced levels of immunoglobulins and impaired Fc-blocking. However, the lack of PE-1D11 (IgG1) non-specific binding to granulocytes of steroid-treated patients does not support this hypothesis. In addition, we used Fc receptor blocking reagents in control experiments. These approaches did not abolish the erroneous signal, making an insufficient blocking rather an improbable reason for the observed false-positivity.

While non-specific binding of antibodies is an often reported phenomenon, it is a rare observation that a selected fluorophore-conjugated antibody clone unspecifically binds a distinct immune cell subset. For instance, PE-conjugated antibodies, but not FITC- or APC-conjugates of antibodies selectively stained murine plasma cells due to a direct interaction between PE and specific molecules related to plasma cell differentiation, irrespective of the antibody epitope or isotype (Kim & Kim, 2013). Moreover, human neutrophils “express” program death receptor ligand 1 (PD-L1) only when co-stained with AlexaFluor™700-conjugated anti-CD16 antibodies, due to an interaction between selected anti-PD-L1 clone and the AlexaFluor™700 fluorophore (Hughes et al., 2020). If the described cross-reactivity occurred selectively in the context of neutrophils was not addressed in the study.

The erroneous NKG2D-positivity of granulocytes described here was associated with poor outcome in GBM patients. A possible link between NKG2D cross-reactivity and inferior survival might be steroid intake, recently linked with poor outcomes in GBM patients in the context of radio-chemotherapy (Petrelli et al., 2021). Steroids might induce changes in plasma composition and/or in the binding/uptake capacities of granulocyte, thus possibly resulting in the cross-reactivity of the PE-conjugated 149810 clone of NKG2D antibody that we observed. For instance, Bukowska-Strakova et al. (2006) described an interesting phenomenon where the cross-linking of two antibodies directed against CD4 and CD8, caused an erroneous CD8-positivity on CD4 T-cells in the presence of so far undefined plasma factor(s) in only some patients. By incubating lymphocytes obtained from a healthy donor with patient plasma, these factors were transferred to the donor lymphocytes, resulting in false CD8 positivity on CD4 cells also in healthy individuals. Even after plasma removal cells remained “double positive” hinting towards membrane-attachment of these factors. Of note, this occurred only when the clone SK1 and not the clone RPA-T8 of the anti-CD8 mAb was used (Bukowska-Strakova et al., 2006). In our case, non-specific staining was irrespective of a second antibody and possibly involved factors/immune complexes present in some individuals in soluble form or attached to the membrane. Additional experiments addressing the effects of soluble factors obtained from NKG2D-“positive” individuals or more elaborate tests comparing conjugated antibody structures (in SDS gel) and conjugation efficiency of distinct NKG2D clones are needed to further delineate the reasons for here described erroneous expression of NKG2D.
In summary, our study has uncovered an unexpected reactivity of the PE-conjugate of a well-characterized anti-NKG2D antibody clone 149810 (Warren, 2005) with granulocytes from GBM patients and in some healthy donors. This false positive staining pattern even associated with poor outcome and seems to be a specific feature of the PE-conjugate of this antibody as we observed an expected (i.e., negative) staining pattern with unconjugated or APC-conjugated clone 149810 and the PE-conjugate of another anti-NKG2D mAb. Our results raise a note of caution for the final interpretation of data and for the selection of appropriate antibodies/fluorochrome-conjugates in studies on NKG2D expression on non-lymphoid cells.

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Additional supporting information may be found online in the Supporting Information section at the end of this article.

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