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Targeting Neural-Restrictive Silencer Factor Sensitizes Tumor Cells to Antibody-Based Cancer Immunotherapy In Vitro via Multiple Mechanisms

Martin V. Kolev,* Marieta M. Ruseva,* B. Paul Morgan,* and Rossen M. Donev*†

Tumor cells escape clearance by complement by abundantly expressing CD59 and other membrane complement regulators. Recently, we designed a peptide derived from the neural-restrictive silencer factor (REST), REST68, which we showed to inhibit expression of CD59 in tumors lacking the full-length REST and proposed a detailed model for regulation of CD59 expression via interplay between REST and nucleolin (NCL) transcription factors. In this paper, we study in detail the mechanisms for sensitization of malignant cells to Ab-based cancer immunotherapy by the REST68 peptide and the implications of the REST/NCL model for the design of treatment resulting in higher tumor susceptibility. REST68 inhibited CD59 expression in malignant cells expressing either truncated or full-length REST, but not in nonmalignant cells. However, activation of protein kinase C (PKC) in nonmalignant cells, a process that contributes to cellular transformation, phosphorylated NCL and enabled suppression of CD59 expression by the REST68. Combined treatment of different tumor types with REST68 and PKC inhibitor synergized to further suppress CD59 expression and reduce resistance to complement lysis. The combined treatment also increased susceptibility of tumors expressing either of the REST isoforms to PBMC-mediated killing, which, at least in part, accounted for the strong promotion of apoptosis by the REST68/PKC inhibitor. These data demonstrate that REST68 sensitizes tumors to Ab-based cancer immunotherapy via multiple mechanisms. Furthermore, the REST/NCL interplay model for regulation of expression of cd59 and other genes involved in cell survival enables the design of treatments for different tumor types to achieve more efficient tumor clearance. The Journal of Immunology, 2010, 184: 000–000.
model for regulation of CD59 expression in tumors expressing ei-
ther of the REST isoforms (12). In this study, we comprehensively
address the multiple mechanisms by which REST68 peptide sen-
tizes tumor cells to Ab-based cancer immunotherapy and, based
on the REST/NCL model for regulation of CD59 expression, we
propose a strategy for achieving more efficient killing of tumors.

Materials and Methods

Cell lines and treatments

Human neuroblastoma cell line Kelly, the human colon carcinoma cell line Caco2, and the malignant melanoma cell line G361 (European Collection of Animal Cell Cultures, Salisbury, U.K.) were maintained in RPMI 1640 with
10% heat-inactivated FCS and supplemented with glutamine, penicillin, and streptomycin (Invitrogen, Paisley, U.K.). Normal human dermal fibroblasts (HDFs [Millipore, Watford, Hertfordshire, U.K.]) were maintained in basal HDF medium with supplement as recommended by the supplier. Cells were
transfected with the REST68-expressing construct or empty vector as a control as previously described (11). In some studies, cells were treated
for 24 h with 10 μM GF109203X (GF) inhibitor of PKC kinases, 4 μM
PDBu activator of PKC (Merck, Nottingham, U.K.), or 100 μM BI-667195
activator of apoptosis (Sigma-Aldrich, Gillingham, Dorset, U.K.).

Prior to each analysis, cells were harvested, centrifuged at low speed
(100 × g), and the percentage of dead cells determined by trypan blue exclusion.

Preparation of nuclear extracts and Western blotting

Nuclear protein extracts were prepared from all cell lines as described previously (16). Expression of REST was detected in the extracts by Western blotting (17) with rabbit polyclonal anti-REST Ab (H-290) raised
against aa 1–290 of the protein (Santa Cruz Biotechnology, Santa Cruz,
CA). This Ab recognizes both the full-length and truncated REST.

REST68, NCL, and phosphorylated NCL were detected with mouse
monoclonal anti-His (Millipore), anti-NCL (Millipore), and anti–NCL-
phosphorylated (Thr76/Thr84) (Biologend, San Diego, CA), respectively.

Flow cytometry

The effect of different expression constructs and treatments on expression of
CD59, CD55, and CD46 at the protein level was assessed by staining the
cells (3 × 106) with mouse anti-CD59 mAb (BRIC229), mouse anti-
CD55 (BRIC216), and rabbit polyclonal anti-CD46 (generated in-house), respectively, for 30 min on ice. The unbound Ab was removed
by three washes with flow cytometry buffer (PBS containing 10 mmol/l
EDTA, 1% BSA [pH 7.4]). The cells were then incubated for another 30
min with a 1:100 dilution of FITC-conjugated anti-mouse or anti-rabbit Igs
(Sigma-Aldrich, Gillingham, Dorset, U.K.).

Prior to each analysis, cells were harvested, centrifuged at low speed
(100 × g), and the percentage of dead cells determined by trypan blue exclusion.

Complement-dependent cytotoxicity

Normal human serum (NHS) was the source of complement in all
experiments (18). Cells were suspended in RPMI 1640 culture medium
without FCS and transfected into 96-well plates (104 cells per well) with
anti-GD2 mAb (for Kelly and G361 cells) clone 14.2Ga (Millipore) at
a concentration of 10 μg/ml, which was previously shown to yield max-
imum lysis at these conditions (19). In Caco2 and HDF cells, complement
was activated by sensitizing with 10 μg/ml mouse anti-human EpCAM
(clone B29.1; Perbio Science, Cramlington, Northumberland, U.K.) or
mouse anti-fibroblast Ab (clone 5B5; Acris Antibodies, Herford, Ger-
many), respectively. In experiments with mCReg blocking, excess of Fab
fragments (100 μg/ml each determined by flow cytometry to block com-
pletely the mCReg; data not shown) generated from rabbit polyclonal Abs
against CD59, CD55, and CD46 raised in-house against the whole solubile
mCReg molecules (ImmunoPure Fab preparation kit, Perbio Science) were
preincubated with the cells for 30 min at 37˚C. NHS was diluted as ap-
propriate in RPMI 1640 and added to the cells. The lysis assay was carried out
using Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, South-
ampton, U.K.) that measures the release of lactate dehydrogenase (LDH)
by the cells. Spontaneous release was assessed by incubation without mAb
and with heat-inactivated NHS (15 min at 56˚C). All experiments were
performed in triplicate for each condition. The percentage of lysed cells
was calculated using the following formula: % Lysis = [(test release –
specific release)/(total release – spontaneous release)] × 100.

Dead cells were also identified by staining with propidium iodide (50 μg/
ml; Sigma-Aldrich) and analysis on an FACS Calibur (BD Biosciences)
instrument to confirm the data obtained by measuring LDH release. The
experiment was replicated twice, and data were analyzed by Student t test.

PBMC-mediated killing

Cells were incubated first with either anti-GD2 (10 μg/ml, Kelly and G361
cells), anti-EpCAM (10 μg/ml, Caco2 cells), or anti-fibroblast (10 μg/mL
HDF cells) Abs for 30 min at 4˚C and, when required, incubated for an-
other 30 min with 100 μg/ml Fab fragments against mCReg. PBMCs
were isolated from human blood by gradient centrifugation on lymphocyte
separation medium (LSM 1077; PAA Laboratories, Pasching, Austria).

PBMCs were maintained in RPMI 1640 supplemented with 10% FCS,
glutamine (2 mmol/l), and streptomycin (100 μg/ml). PBMCs as effector
cells were mixed with target cells at 20:1 and 40:1 E:T cell ratio and in-
cubated for 4 h at 37˚C in 5% CO2. The cells were then centrifuged and the supernatant
assayed for LDH release with the CytoTox 96 Non-Radioac-
tive Cytotoxicity kit (Promega). The percentage of specific lysis was
determined using the same formula as for the complement-dependent
cytotoxicity (CDC). Cells were stained with propidium iodide and ana-
lyzed on an FACS Calibur as described above to confirm LDH data.

Some of the PBMC killing experiments were carried out in the presence
of 10% C8-depleted human serum, generated using a monoclonal affinity
column as described (20). The absence of C8 in the serum excludes the
CDC effect of the serum while allowing early complement activation, thus
testing effects of target opsonization on PBMC killing. For simplicity in
this complex assay, target cell lysis was measured by preloading with
CFSE and determining percentage of lysed cells using the Guava Cell
Toxicity Assay (Millipore). Results were corrected for background lysis
using the CytoTox 96-depleted serum alone.

Apopotosis, cell proliferation, and soft agar assays

Cells transfected either with REST68-expressing or a control plasmid were
 treated with GF inhibitor or the same volume of DMSO as described above.
The percentage of apoptotic cells was determined by staining with FITC-
Annexin V (Apoptosis Detection Kit; BD Pharmingen, San Diego, CA)
following the manufacturer’s recommendations and analysis on a flow

Statistical analysis

The data are expressed as mean ± SD and were analyzed for statistical
significance by the two-tailed Student t test to compare two paired groups
of data. A value of p < 0.05 was considered to be statistically significant.

Results

REST68 inhibits expression of CD59 in cells with high
phosphorylation level of NCL

Recently, we proposed a model for activation of genes controlled by
REST, which have overlapping binding sites for REST and NCL
transcription factors. This REST/NCL model implies that protein
kinase inhibitors, which have become increasingly popular for the
treatment of different malignancies, will be inefficient suppressors
of genes regulated by REST/NCL interplay, particularly in tumors
that lack full-length REST. This group of genes includes immu-
nomodulatory, tumor growth, and survival genes, for which in-
hibition is essential for the success of such a treatment. Even in
tumors expressing the full-length REST, levels of this transcrip-
tional suppressor are likely to be low due to the high activity of
protein kinases (22). Therefore, treatment of cancer cells with
REST68 might synergize with kinase inhibitors, resulting in
improved killing of tumors via multiple mechanisms. We addressed
this hypothesis by studying three tumor cell lines that expressed

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either the truncated REST (Kelly and Caco2) or the full-length REST (G361) and nonmalignant HDFs expressing the full-length REST (Fig. 1A). These cells, transfected with either empty expression plasmid or with REST68-expressing plasmid, were treated with GF inhibitor. Nonmalignant HDFs were also incubated in the presence of PDBu, an activator of PKC. Expression of REST68 (anti-His) and endogenous REST and the phosphorylation status of NCL were confirmed by Western blots (Fig. 1B). Phosphorylation of NCL was efficiently inhibited by GF treatment in all cell types examined, whereas the overall expression of total NCL was not affected by the treatment. In cells expressing the full-length REST (G361 and HDF), GF caused an increase in the expression of endogenous REST at the protein level, particularly in G361 cells; however, in cells expressing the truncated REST, levels were not altered by GF treatment. PDBu, an activator of PKC, yielded an efficient phosphorylation of NCL in HDF cells, which originally have low levels of phosphorylated NCL. This treatment also reduced the amount of endogenous REST in the HDF cells (Fig. 1B). Furthermore, PDBu treatment resulted in the formation of HDF colonies in soft agar (Fig. 1C), an indicator of cellular transformation.

GF treatment of Caco2 (Fig. 2A) and Kelly (Fig. 2B) cells did not alter expression of CD59. However, this PKC inhibitor reduced the expression of CD59 by 50% in G361 cells (Fig. 2C) and by 25% in HDF cells (Fig. 2D). REST68 peptide decreased CD59 expression in Caco2 and Kelly cells by around 50% (Fig. 2A, 2B). Even in G361 cells expressing the full-length REST, we observed a significant decrease of 20% in CD59 expression (Fig. 2C). However, in nonmalignant HDF cells, which have low phosphorylation levels of NCL, REST68 did not alter significantly expression of CD59 (Fig. 2D). Interestingly, PDBu treatment, which phosphorylated NCL and promoted cellular transformation, augmented expression of CD59 in HDF cells by 40%, and REST68 treatment considerably inhibited this increase. Combined treatment with REST68 and GF further suppressed expression of CD59 in Caco2, Kelly, and G361 cells, showing a cooperative effect of these two reagents in tumor cells. In HDF cells, this combined treatment resulted in an effect similar to that of GF alone. REST68 did not alter expression of CD55 or CD46 in either of the cell lines (Fig. 2). However, GF suppressed the expression of these two mCRegs by at least 50%, although this effect was more prominent in tumor cells than in nonmalignant HDF.

Cooperative effect of REST68 and GF treatment on tumor susceptibility to complement lysis

We next tested the effect of the REST68 and GF treatment on complement-mediated cytolysis triggered by anti-GD2 (Kelly and G361 cells) and anti-EpCAM (Caco2), mAbs used in cancer immunotherapy, or by anti-fibroblast mAb for the HDF (Fig. 3). Lysis assays were performed using different concentrations of human serum as a source of complement. Maximum lysis achieved for Caco2 cells with no pretreatment was ∼40% (Fig. 3A). Cells treated with GF were slightly more susceptible to complement-dependent killing at all serum doses, and ∼50% were lysed at maximum. Caco2 cells transfected with the REST68 expression plasmid were even more sensitive to complement lysis, which reached ∼65% at maximum. Combined treatment with REST68 and GF resulted in ∼80% lysis of cells by complement at 30% serum concentration. To confirm that reduced expression of the mCReg was responsible for the observed sensitization to complement, lytic susceptibility was assessed in Caco2 cells transfected either with REST68-expressing plasmid and treated with GF or empty plasmid controls, in which all three mCReg were preblocked with Fab fragments prepared from polyclonal Abs against the whole soluble mCReg molecules (Fig. 3A). After blocking mCReg, lytic susceptibility was increased and was similar

![FIGURE 1](http://www.jimmunol.org/) Expression pattern of NCL, phosphorylated NCL, REST, and REST68 as a result of REST68 and GF treatments. A, Western blot analysis of expression of REST isoforms in Caco2, Kelly, and G361 tumor cells, nonmalignant HDF cells, and normal colon tissue. Colon tissue, HDF, and G361 malignant melanoma express the full-length REST. Caco2 cells express predominantly the truncated REST, whereas Kelly cells express only the truncated REST. B, Effect of REST68, GF, or combined treatment on expression of NCL (anti-NCL), phosphorylated NCL (anti-phosphoNCL), endogenous REST (anti-REST), and REST68 (anti-His) peptide in Caco2, Kelly, G361, and HDF cells as determined by Western blots. HDF cells, transfected with either REST68-expressing construct or an empty plasmid as a control, were also incubated for 24 h with an activator of PKC, PDBu, to increase phosphorylation of NCL. Equal amounts of nuclear protein lysates were loaded in each lane. C, Soft agar assay for HDF, untreated, and PDBu-treated. Photographs (left panels) were taken after 3 wk of incubation. The numbers of foci per 6-cm dish (right panel) were automatically counted for each condition and are presented as average from three independent experiments. Bars indicate SD. **p < 0.001.
for both control and REST68/GF-treated cells, confirming that increased susceptibility of REST68- and/or GF-treated cells to complement lysis was a direct result of decreased mCReg expression.

Similar lysis assays were carried out for Kelly, G361, and HDF cells (Fig. 3B–D) for which we established stable populations of transfected cells by selecting with hygromycin B. The pattern of increased complement killing for Kelly, another line expressing truncated REST, following REST68 and/or GF treatment, was similar to that obtained for Caco2. GF treatment of G361 cells had a greater effect on susceptibility of the tumor cells to complement lysis than the REST68 treatment. Again, we observed a cooperative effect of both treatments on resistance of G361 cells to complement killing, resulting in ∼80% lysed cells at maximum E:T ratio (Fig. 3C). Blocking experiments with mCReg-specific Fab fragments confirmed that this cooperative effect was a result of decreased expression of mCReg only. In nonmalignant HDF, REST68 peptide had no significant effect on susceptibility to CDC (Fig. 3D). GF treatment increased the number of lysed HDF cells by 15%, but the combined REST68 and GF treatment did not increase further this percentage. mCReg-blocking experiments showed that the increased sensitization to complement attack by the GF was entirely a consequence of the inhibited expression of the mCReg.

**REST68 and GF treatments sensitize tumor cells to killing by PBMCs**

Recently, we showed that the interplay between REST and NCL plays a key role in the regulation of proapoptotic genes (12) and, because the major mechanism by which PBMCs kill cells is by instigating apoptosis, we reasoned that treatment of cells with REST68 and GF would alter tumor resistance to PBMC-mediated lysis, a key mechanism in cancer immunotherapy. For recruitment of cytolytic cells to the target cells, we used the same mAbs as in the CDC assays (see above). GF treatment of Caco2 (Fig. 4A) and Kelly (Fig. 4B) cells increased their sensitivity to PBMC killing by ∼15% at a 40:1 E:T ratio. REST68 caused a greater increase in PBMC-mediated killing (up to 40%) in these lines, although this peptide suppressed expression of CD59, but not CD46 or CD55 (Fig. 2). Combined REST68/GF treatment of these two cell lines further increased PBMC-mediated killing, up to 80% at maximum E:T ratio.

G361 tumor cell line, expressing full-length REST, was also sensitized by both GF and REST68 treatment to lysis by PBMCs (Fig. 4C). However, the sensitizing effect of these two treatments was comparable, unlike in the assays performed with Caco2 and Kelly cells expressing the truncated REST isoform. Again, combined REST68/GF treatment had a cooperative effect, further increasing G361 cell killing by PBMCs (up to 70% lysis at a 40:1 E:T ratio). In nonmalignant HDF cells, only the GF treatment had a sensitizing effect on PBMC-mediated lysis (Fig. 4D); REST68 had no effect.

**REST68- and GF-mediated tumor sensitization to PBMC killing is independent of expression of mCReg in a complement-free environment**

It was recently shown that blocking of CD59 and CD55 with miniantibodies, which do not contain Fc fragment, increases ADCC (4). Therefore, we addressed whether inhibition of mCReg expression by REST68 peptide and GF contributed to the sensitization of tumor cells to PBMC-mediated killing. Kelly (Fig. 5A) and G361 cells (Fig. 5B) expressing the truncated and full-length REST, respectively, were transfected with REST68-expressing construct or empty plasmid. Preblocking of either CD59 alone or CD46 and CD55 together with Fab fragments against these proteins did not alter the sensitivity of cells to PBMC killing. When all three mCRegs were blocked prior to the ADCC assay, there was a trend toward an increase in percentage of lysed cells, but this was not significant and could not explain the marked

![FIGURE 2. Inhibition of expression of mCReg by the REST68 and GF treatments. The effect of REST68, GF, or combined treatment on expression of CD59, CD55, and CD46 on Caco2 (A), Kelly (B), G361 (C), and nonmalignant HDF cells (D) was quantified by flow cytometry. Expression of the mCReg in PDBu-treated HDF cells transfected with either REST68-expressing construct or empty plasmid was also measured (D). Columns show results from three independent measurements, each performed in triplicate; bars indicate SD. *p < 0.01; **p < 0.001.](http://www.jimmunol.org/.../abs/doi-10.4049/jimmunol.1700313.html)
increase of ~50% in PBMC-mediated lysis caused by the REST68 peptide.

**Inhibition of expression of CD46/CD55 by GF contributes to sensitization of tumor to PBMC killing in the presence of complement**

We next tested whether altered mCReg expression induced by REST68 or GF treatment may have a role in sensitizing cancer cells to PBMC killing in the presence of complement. We performed the PBMC killing in 10% C8-depleted human serum to eliminate lysis to PBMC killing in the presence of complement. We performed the lysis assay with pre-incubation of control cells (-) and those with combined treatment (+) with blocking Fab fragments against all three mCReg was carried out as a control. Columns show results from three independent measurements, each performed in triplicate; bars indicate SD. *p < 0.01; **p < 0.001.

**FIGURE 4.** REST68 peptide sensitizes tumors to ADCC and has a cooperative effect with GF treatment. Caco2 (A), Kelly (B), G361 (C), and nonmalignant HDF cells (D) transfected either with REST68-expressing construct (-Δ-) or empty vector as a control (-○-) were tested for their sensitivity to PBMC-mediated ADCC triggered by anti-GD2 (Kelly and G361 cells), anti-EpCAM (Caco2 cells), or anti-fibroblast (HDF cells) mAbs. Tumor killing effect of PBMCs was studied at E:T ratios of 20:1 and 40:1. Combined treatment of the above cell lines with GF for 24 h (-○-, empty plasmid+GF treatment; -Δ-, REST68+GF), and the combined effect of both agents on sensitization of cell to CDC was determined. Lysis assay with pre-incubation of control cells (-) and those with combined treatment (+) with blocking Fab fragments against all three mCReg was carried out as a control. Columns show results from three independent measurements, each performed in triplicate; bars indicate SD. *p < 0.01; **p < 0.001.

**REST68 sensitizes tumors to PBMC killing by promoting apoptosis**

Considering our recent finding that REST and NCL are involved in regulation of antiapoptotic genes (12), we hypothesized that the difference in ADCC might be due to the effect of REST68 and GF treatments on apoptosis. We assessed the percentage of apoptotic cells in each of the different treatments (Fig. 7A). REST68 increased the number of Annexin V-positive Kelly and Caco2 cells by 3.5-fold, whereas for G361 cells expressing the full-length REST, this increase was 2-fold. Importantly, nonmalignant HDF cells were not affected by the transfection with REST68-expressing plasmid. GF treatment resulted in a 2-fold increase in percentage of apoptotic cells in all four cell lines investigated. We observed a cooperative effect of the combined treatment, with both reagents causing a 4-fold increase in the number of apoptotic Kelly and Caco2 cells compared with nontreated cells, whereas for G361, this combined treatment resulted in a 3-fold increase. In HDF cells, REST68 plus GF treatment did not alter significantly the percentage of Annexin V-positive cells compared with the GF treatment alone. To test whether the proapoptotic effect of REST68 is responsible for the sensitization of tumors to ADCC, Kelly and G361 cells transfected with either the REST68 expression vector or an empty control plasmid were treated with the BI-6C9 inhibitor of apoptosis (Fig. 7B). The BI-6C9 treatment reduced the percentage of apoptosis in the REST68-transfected cells to values very close to those determined for the corresponding noncontrol cells. The inhibitor also decreased the PBMC-mediated lysis for both Kelly and G361 cells expressing the full-length REST, to levels very similar to the non-malignant HDF cells.

**FIGURE 3.** Cooperative effect of REST68 peptide and GF treatment on sensitization of tumor cells to CDC. Caco2 (A), Kelly (B), G361 (C), and nonmalignant HDF cells (D) transfected either with REST68-expressing construct (-Δ-) or empty vector as a control (-○-) were tested for their resistance to CDC triggered by anti-GD2 (Kelly and G361 cells), anti-EpCAM (Caco2 cells), or anti-fibroblast (HDF cells) mAbs. The transfected cells were also treated with GF for 24 h (-○-, empty plasmid+GF treatment; -Δ-, REST68+GF), and the combined effect of both agents on sensitization of cell to CDC was determined. Lysis assay with pre-incubation of control cells (-) and those with combined treatment (+) with blocking Fab fragments against all three mCReg was carried out as a control. Columns show results from three independent measurements, each performed in triplicate; bars indicate SD. *p < 0.01; **p < 0.001.
The application of humanized antitumor mAbs as a targeted therapy holds great clinical promise and has become more widely used in clinical practice (23). The mechanisms by which antitumor Abs inhibit or kill tumor cells are diverse and include inhibition of growth factor receptors (for example, trastuzumab and cetuximab), ADCC, and CDC. However, mCRegs overexpressed on tumor cells diminish the contribution of CDC and ADCC mechanisms triggered by therapeutic mAbs in vitro and in vivo (1, 3, 5, 10, 24, 25). The role of CD59, the only mCReg inhibitor of the cytolytic membrane attack complex of complement, in enhancing tumor survival and growth in vivo was clearly demonstrated (7), prompting the search for an efficient strategy for CD59 suppression in tumors. Recently, we proposed a new strategy for inhibition of expression of CD59 by targeting transcription factors responsible for its overexpression on tumors (11, 20). We identified a peptide, named REST68, which can inhibit expression of CD59 in tumors that lack full-length REST (11). Treatment of IMR32 neuroblastoma cells with REST68 did not affect expression of CD59, provoking the suggestion that this peptide will not affect CD59 expression on cells expressing the full-length REST isoform. Our recently proposed model for regulation of expression of cd59 and other genes involved in cell survival by interplay between REST and NCL demonstrated that increased phosphorylation of NCL also plays a major role in overexpression of these genes (12). Low NCL phosphorylation is typical for the nonmalignant cells (Fig. 1); however, IMR32 cells also show a similar low phosphorylation pattern (12) that may explain the lack of effect of REST68 in this cell type.

The REST/NCL model for regulation of CD59 expression implies that REST68 peptide will not significantly alter gene expression in nonmalignant cells that have low activity of protein kinases, resulting in a longer $t_{1/2}$ of REST (22) and a lower number of phosphorylated NCL molecules. However, abundant phosphorylation in tumors will enable REST68 to suppress genes regulated by the REST/NCL interplay, replacing the degraded endogenous REST protein. This was confirmed by the inhibitory effect of the REST68 peptide on CD59 expression in G361 malignant melanoma cells expressing the full-length REST, whereas no significant alteration was found in nonmalignant HDF (Fig. 2). The key role of protein kinase activity in enabling REST68 to modulate CD59 expression was further confirmed by activation of PKC in HDF cells that recovered expression of CD59 to a level similar to that in nontreated cells (Fig. 2), Treatment of cancer cell lines expressing both full-length REST and truncated REST, with a combination of REST68 and an inhibitor of PKC kinases, inhibited NCL phosphorylation and led to additional suppression of CD59 (Figs. 1, 2). We previously showed that inhibition of NCL phosphorylation could also be achieved by using LY294002 (12), an inhibitor of PI3K. However, this inhibitor may be less useful in combination with REST68 peptide because inhibition of PI3K results in overexpression of CD55 (26), which will have an impact on tumor resistance to CDC (Fig. 3) and ADCC in the presence of complement (Fig. 6). Notably, GF treatment did not affect expression of CD59 in Caco2 and Kelly cells expressing the truncated REST. This can be explained by the REST/NCL model because of a lack of competitor for the NCL binding to DNA. However, in the presence of REST68 in these two cell lines, GF treatment further increased the inhibitory effect of this peptide on expression of CD59 (Fig. 2).

Our data on the complement-mediated killing of tumors (Fig. 3) confirmed CD59 as the major target for increasing CDC of tumors, contributing to ADCC by PBMCs in complement-free environment. PMBC-mediated killing of Kelly (A) and G361 cell lines (B) transfected with REST68-expressing plasmid and/or treated with GF. To elucidate the involvement of mCReg in ADCC, preblocking of CD59 alone, CD46 and CD55 together, or all three mCRegs with Fab fragments against these proteins was carried out. E:T ratio of 40:1 was used in all experiments. Columns show results from two independent experiments, each performed in triplicate; bars indicate SD.

**FIGURE 5.** Downmodulation of mCReg on tumors by REST68 peptide or GF does not contribute to ADCC by PMBCs in complement-free environment. PMBC-mediated killing of Kelly (A) and G361 cell lines (B) transfected with REST68-expressing plasmid and/or treated with GF. To elucidate the involvement of mCReg in ADCC, preblocking of CD59 alone, CD46 and CD55 together, or all three mCRegs with Fab fragments against these proteins was carried out. E:T ratio of 40:1 was used in all experiments. Columns show results from two independent experiments, each performed in triplicate; bars indicate SD.

**FIGURE 6.** Inhibition of expression of mCReg by GF but not REST68 contributes to ADCC by PMBCs in presence of serum. Kelly cells transfected with REST68-expressing plasmid and/or treated with GF were incubated with PBMCs (40:1 E:T ratio) in presence of 10% C8-depleted serum to eliminate the effect of CDC. Columns show results from two independent experiments each performed in quadruplet; bars indicate SD. *$p < 0.01; **p < 0.001.
which is not surprising considering that it is the only mCReg of the terminal pathway of complement cascade. Importantly, the resistance of HDF cells to CDC was not affected by the REST68 peptide, explicable by the low activity of protein kinases in nonmalignant cells. The combined REST68 and GF treatment yielded better CDC in all types of tumor cells, suggesting that such a combination of drugs would be beneficial for enhancing immunoclearance of tumors by the complement system. Furthermore, we observed a marked increase in the PBMC-mediated clearance of tumors by the complement system. Further in vivo studies will be necessary to confirm that the observations made in this study do not contribute to it. Published literature supports a role for opsonization in enhancing killing by PBMCs (reviewed in Ref. 1).

Recently shown that blocking of CD59 and CD55 on tumor cells with miniantibodies, which do not contain Fc fragment, increased PBMC-mediated killing (4). Thus, we addressed whether inhibition of mCReg expression by REST68 and GF contributes to the sensitization of tumor cells to ADCC. In our in vitro assays, which lack complement, we did not detect any significant contribution of the mCReg to the REST68-enhanced ADCC (Fig. 5). Considering that the majority of PBMC cell types kill tumors by instigating apoptosis and that the REST/NCL interplay is a key mechanism in expression of antiapoptotic genes (12), we reasoned that this difference might be due to the effect of REST68 and GF treatments on cell survival and/or apoptosis. Indeed, we found that REST68 is a strong promoter of apoptosis (Fig. 7), which we demonstrated to be a major mechanism for the marked sensitization of tumors to ADCC in a complement-free environment. Our conclusion is strongly supported by a recent finding that pharmacologic modulation of antiapoptotic proteins (i.e., Bcl-2, Mcl-1, or their upstream regulators) sensitizes B cell lymphoma to rituximab therapy (27). Notably, mcl-1 and bcl-2 genes are regulated by the REST/NCL interplay (12), and a treatment with REST68 would inhibit its expression. In the presence of complement opsonins, we showed that inhibition of CD55 and CD46 expression (which will enhance opsonization), but not CD59 (which will not), also contributes to PBMC-mediated lysis of cancer cells (Fig. 6). We did not investigate the exact mechanism for this enhancement because REST68 peptide, the major focus of this study, does not contribute to it. Published literature supports a role for opsonization in enhancing killing by PBMCs (reviewed in Ref. 1).

Our results demonstrate the complex nature of sensitization of tumors to Ab-based immunotherapy by the REST68 peptide and PKC inhibitors. Sensitization by the REST68 peptide may have advantages over RNA interference or mCReg-blocking mAb against a specific single target (i.e., CD59), because REST68 simultaneously inhibits expression of a number of genes responsible for tumor resistance to apoptosis and lysis mediated by cytolytic cells and complement system. Further in vivo studies will be necessary to confirm that the observations made in this study in vitro are valid in animal models; however, this study provides the fundamental tools essential for future in vivo investigations. Our data in this study demonstrate that the REST/NCL interplay model for regulation of expression of cd59 and other genes involved in cell survival and apoptosis enables

**FIGURE 7.** REST68 peptide sensitizes tumors to PBMC-mediated killing by promoting apoptosis. A, The ability of REST68, GF, or combined treatment to induce apoptosis was assessed by Annexin V staining of Kelly, Caco2, G361, and nonmalignant HDF cells transfected with REST68-expressing construct and/or treated with GF. The percentage of stained cells was determined by flow cytometry. Columns show results from two independent experiments, each performed in triplicate; bars indicate SD. B, Annexin V staining of Kelly and G361 cells transfected with empty vector as controls or with REST68-expressing construct and treated with 100 μM BI-6C9 inhibitor of apoptosis. Percentage of stained cells was determined by flow cytometry. Columns show results from two independent experiments, each performed in triplicate; bars indicate SD. C, PBMC-mediated killing at E:T ratio 40:1 was carried out with cells treated as in B. Columns show results from two independent experiments, each performed in triplicate; bars indicate SD. *p < 0.01; **p < 0.001.
Disclosures

The authors have no financial conflicts of interest.

References

Letter of Retraction

We wish to retract the article titled “Targeting Neural-Restrictive Silencer Factor Sensitizes Tumor Cells to Antibody-Based Cancer Immunotherapy In Vitro via Multiple Mechanisms” by Martin V. Kolev, Marieta M. Ruseva, B. Paul Morgan, and Rossen M. Donev, The Journal of Immunology, 2010, 184: 6035–6042.

Bands in Fig. 1A and 1B were pasted from multiple gels without indicating that this had been done. The last author, Rossen M. Donev, takes full responsibility for this action; the other authors were unaware of and had no part in the manipulation of the images. The findings and conclusions of the above article have been independently verified. However, due to the inappropriate manipulation of the data, we wish to retract the article.

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