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

J Immunol 2010; 184:6035-6042; Prepublished online 26 April 2010; doi: 10.4049/jimmunol.1000045
http://www.jimmunol.org/content/184/11/6035

References

This article cites 26 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/184/11/6035.full#ref-list-1

Why The JI? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Errata  An erratum has been published regarding this article. Please see
next page or:
/content/187/12/6581.full.pdf
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 inhibitors. 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: 6035–6042.
CD59 expression in tumors expressing either of the REST isoforms (12). In this study, we comprehensively address the multiple mechanisms by which REST68 peptide sensitizes 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-6672038 inhibitor 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. Only preparations with <5% dead cells were used in the subsequent assays.

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 (H4-90) raised against aa 1–290 of the protein (Santa Cruz Biotechnology, Santa Cruz, CA). This Ab recognizes both the full-length and truncated REST68, NCL, and phosphorylated NCL were detected with mouse monoclonal anti-His (Millipore), anti-NCL (Millipore), and anti-NCL phosphorylated (Thr76/Thr84) (Biolegend, 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 × 10^5) with mouse anti-CD59 mAb (BRIC229), mouse monoclonal anti-CD55 (BRIC226), 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% fetal EDTA, 1% BSA [pH 7.4]). The cells were then incubated for another 30 min with a 1:100 dilution of FITC-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) washed three times with flow cytometry buffer and analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA). Dead cells were excluded from the analyses by gating them out. All measurements were made in triplicate, and each experiment was replicated at least twice.

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 transferred into 96-well plates (10^5 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 maximum 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-ribonucleotidase Ab (clone 5B5; Acris Antibodies, Herford, Germany), respectively. In experiments with mCReg blocking, excess of Fab fragments (100 μg/ml each determined by flow cytometry to block completely the mCReg; data not shown) generated from rabbit polyclonal Abs against CD59, CD55, and CD46 raised in-house against the whole soluble mCReg molecules (ImmunoPure Fab preparation kit, Perbio Science) were preincubated with the cells for 30 min at 37˚C. NHS was diluted as appropriate in RPMI 1640 and added to the cells. The lysis assay was carried out using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Southamp ton, 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 – spontaneous 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-ribonucleotidase (10 μg/ml, HDF cells) Abs for 30 min at 4˚C and, when required, incubated for another 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 incubated 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-Radioactive 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 analyzed on an FACS Calibur as described above to confirm LDH data. Statistical analysis

The expression of PPI antibodies was determined by staining with FITC- Anti-Mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) or biotinylated goat anti-mouse IgG (Life Technologies, Carlsbad, CA). Cells were then fixed and permeabilized using CyQUANTDirect Cell Proliferation Assay Kit (Invitrogen).

To test colony-forming capacity of HDF, soft agar plates were prepared (21), and the HDF cells, split 1:2 a day earlier to ensure cells were growing exponentially, were seeded on the agar at 1 × 10^4 cells/6-cm dish. Fresh medium with or without PDBu (4 μM) was added twice a week and the number of foci present after 3 wk imaged and quantified using Quantity One 4.3.0 software (Bio-Rad, Hemel Hempstead, U.K.).

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 immunomodulatory, tumor growth, and survival genes, for which inhibition is essential for the success of such a treatment. Even in tumors expressing the full-length REST, levels of this transcriptional 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...
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.

### FIGURE 1

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.

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 cytolsis 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-expressing 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 lysis, susceptibility was assessed in Caco2 cells transfected either with REST68-expressing plasmid and treated with GF or with 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...
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 the 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 plasmid and/or treated with GF; 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.
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 caused by the terminal pathway of complement. In the presence of PBMC killing in the presence of complement, increased lysis of Kelly cells occurred with or without REST68 and/or GF treatment compared with the corresponding cells in a complement-free assay (Fig. 6). However, the difference in lysis between REST68-treated and nontreated cells was similar (~30%) in the presence of absence of complement, suggesting that inhibition of CD59 expression by REST68 does not contribute significantly to the PBMC-mediated killing of opsonized or nonopsonized cells. In contrast, following GF treatment, which inhibits expression of CD46 and CD55 in Kelly cells and will thus increase opsonization, killing was markedly enhanced by treatment with C8-depleted serum as a source of complement (~40% with complement versus ~20% without), implicating opsonization.

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 REST68 to levels very similar to the noncontrol cells, clearly demonstrating the dependence of the enhanced PBMC-mediated tumor killing on the proapoptotic effect of the REST68 peptide. Furthermore, this proapoptotic effect correlated well with a slower proliferation rate of tumor cell lines (Fig. 8). REST68 markedly suppressed proliferation of Kelly, Caco2, and G361 cells by ~50% and, in combination with GF, further slowed proliferation. In contrast, REST68 did not have a significant effect on proliferation of the nonmalignant HDF cells.
Discussion

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 (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 half-life 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 untransfected 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 CD55 in Caco2 and Kelly cells expressing the truncated REST.

Our data on the complement-mediated killing of tumors (Fig. 3) confirmed CD59 as the major target for increasing CDC of tumors, 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 half-life 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 untransfected 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 CD55 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,
performed in triplicate; bars indicate SD.

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 the sensitization of tumor cells to ADCC 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 the sensitization of tumors to ADCC.

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.

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...
Sensitization of tumors to immunotherapy by targeting REST

The authors have no financial conflicts of interest.

References

1. Yan, J., D. J. Allendorf, B. Li, R. Yan, R. Hansen, and R. Donev. 2008. The role of membrane complement regulatory proteins in cancer immunotherapy. Adv. Exp. Med. Biol. 632: 159–174.

2. Walport, M. J. 2001. Complement. First of two parts. N. Engl. J. Med. 344: 1058–1066.

3. Zilber, F., P. Macor, R. Bulla, D. Shlattero, R. Marzari, and F. Tedesco. 2005. Controlling complement expression in cancer by using human monoclonal antibodies that neutralize complement-regulatory proteins CD55 and CD59. Eur. J. Immunol. 35: 2175–2183.

4. Macor, P., C. Tripodo, S. Zorzetti, E. Pivov, F. Bossi, R. Marzari, A. Amadori, and F. Tedesco. 2007. In vivo targeting of human neutralizing antibodies against CD55 and CD59 to lymphoma cells increases the antitumor activity of rituximab. Cancer Res. 67: 10556–10563.

5. Varella, J. C., M. Imair, C. Atkinson, R. Ohla, M. Rapisardo, and S. Tomlinson. 2008. Modulation of protective T cell immunity by complement inhibitor expression on tumor cells. Cancer Res. 68: 6734–6742.

6. Golay, J., M. Lazzari, V. Facchinietti, S. Berrnasco, G. Borleri, T. Barbui, A. Rambaldi, and M. Intron. 2001. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. Blood 98: 3383–3389.

7. Chen, S. H., J. Hakulinen, M. Hagenaars, P. J. Kuppen, S. Meri, and A. Gorter. 2003. Membrane-bound complement regulatory proteins inhibit complement activation by an immunotherapeutic mAb in a syngeneic rat colorectal cancer model. Mol. Immunol. 40: 13–25.

8. Gelderman, K. A., P. J. Kuppen, W. Bruin, G. J. Fleuren, and A. Gorter. 2002. Enhancement of the complement activating capacity of 17-1A mAb to overcome the effect of membrane-bound complement regulatory proteins on colorectal carcinoma. Eur. J. Immunol. 32: 128–135.

9. Gelderman, K. A., J. Hakulinen, M. Hagenaars, P. J. Kuppen, S. Meri, and A. Gorter. 2003. Membrane-bound complement regulatory proteins inhibit complement activation by an immunotherapeutic mAb in a syngeneic rat colorectal cancer model. Mol. Immunol. 40: 13–25.

10. Zell, S., N. Geis, R. Barz, S. Schulz, T. Giese, and M. Kirschfink. 2002. Downregulation of CD55 and CD46 expression by anti-angiogenic phosphatidyl ethanolamine (S-ODNs) sensitizes tumour cells to complement attack. Eur. Exp. Immunol. 150: 576–584.

11. Donev, R. M., J. C. Gray, M. Introna, T. R. Hughes, C. W. van den Berg, and B. P. Morgan. 2008. Modulation of CD59 expression by restrictive silencer factor-derived peptides in cancer immunotherapy for neuroblastoma. Exp. Biol. Chem. 272: 681–690.

12. Tediose, T., M. Kolev, B. Sivasankar, P. Brennan, B. P. Morgan, and R. Donev. 2010. Upregulating CD59: a new strategy for protection of neurons from complement-mediated degeneration. Pharmacoimmunogenomics J. 10: 12–19.

13. Donev, R., R. Horton, S. Beck, T. Doneva, R. Vatcheva, W. R. Bowden, and D. Sheer. 2003. Recruitment of heterogeneous nuclear ribonucleoprotein A1 in vivo to the LMP/TAP region of the major histocompatibility complex. J. Biol. Chem. 278: 5214–5226.

14. Zhou, G., M. L. Seibenhener, and M. W. Wooten. 1997. Nucleolin is a protein kinase C-zeta substrate. Connection between cell surface signaling and nucleus in PC12 cells. J. Biol. Chem. 272: 31130–31137.

15. Le Good, J. A., and D. N. Brindle. 2004. Molecular mechanisms regulating protein kinase Czeta turnover and cellular transformation. Biochem. J. 378: 83–92.

16. Donev, R., R. Horton, S. Beck, T. Doneva, R. Vatcheva, W. R. Bowden, and D. Sheer. 2003. Recruitment of heterogeneous nuclear ribonucleoprotein A1 in vivo to the LMP/TAP region of the major histocompatibility complex. J. Biol. Chem. 278: 5214–5226.

17. Donev, R., A. Newall, J. Thome, and D. Sheer. 2007. A role for SC35 and hnRNP A1 in the determination of amyloid precursor protein isoforms. Mol. Psychiatry 12: 681–690.

18. Kolev, M. V., T. Tediose, B. Sivasankar, C. L. Harris, J. Thome, B. P. Morgan, and R. M. Donev. 2010. Upregulating CD59: a new strategy for protection of neurons from complement-mediated degeneration. Pharmacoimmunogenomics J. 10: 12–19.

19. Mugoo, K., D. A. Cheresh, H. M. Yang, and R. A. Reisfeld. 1987. Disialoganglioside GD2 on human neuroblastoma cells: target antigen for monoclonal antibody-mediated cytolyis and suppression of tumor growth. Cancer Res. 47: 1098–1104.

20. Donev, R. M., D. S. Cole, B. Sivasankar, T. R. Hughes, and B. P. Morgan. 2006. p53 regulates cellular resistance to complement lysis through enhanced expression of CD59. Cancer Res. 66: 2451–2456.

21. Kolev, M. V., T. Tediose, B. Sivasankar, C. L. Harris, J. Thome, B. P. Morgan, and R. M. Donev. 2010. Upregulating CD59: a new strategy for protection of neurons from complement-mediated degeneration. Pharmacoimmunogenomics J. 10: 12–19.

22. Tediose, T., M. Kolev, B. Sivasankar, P. Brennan, B. P. Morgan, and R. Donev. 2010. Interplay between REST and nucleolin transcription factors: a key mechanism in the overexpression of genes upon increased phosphorylation. Nucleic Acids Res. doi:10.1093/nar/gkq013.
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.

Martin V. Kolev  
B. Paul Morgan  
School of Medicine  
Cardiff University  
Cardiff, United Kingdom  

Marieta M. Ruseva  
Faculty of Medicine  
Imperial College  
London, United Kingdom  

Rossen M. Donev  
College of Medicine  
Swansea University  
Swansea, United Kingdom