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C1q and C1q receptors (gC1qR and cC1qR) as potential novel targets for therapy against breast cancer

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ABSTRACT

The complement protein C1q and its receptors, gC1qR and cC1qR, are expressed on various normal as well as malignant cells and can play both pro- and anti-proliferative roles. These opposing roles, in turn, depend on the localization of these molecules i.e. surface versus secreted. The present studies were designed to examine the functions of the soluble (secreted) and membrane forms of both C1qRs and C1q using the SkBr3 Her2+ cancer cell line as a model for breast cancer. Our results show that SkBr3 cells express not only gC1qR and cC1qR, but also C1q. Importantly, co-culture of SkBr3 cells with either purified C1q or the C1q globular head (gh) modules, ghA, ghB and ghC, resulted in a significant inhibition of cell growth with ghA and ghC showing stronger effects than ghB. Conversely, co-culture of SkBr3 cells with either anti-C1q or an antibody recognizing the gC1qR site on ghA, resulted in complete inhibition of cell growth indicating that like gC1qR, membrane-bound C1q is also pro-proliferative. Co-culture of SkbR3 with anti-cC1qR was also found to inhibit cell proliferation. In contrast, addition of purified recombinant gC1qR to SkBr3 cells enhanced cell growth and reduced cell death even under nutrient-depleted conditions suggesting that it is a pro-proliferative autocrine signal. Although SkBr3 cells do not secrete C1q during normal cell proliferation, they release gC1qR. Interestingly, the anti-proliferative effect of exogenously added C1q was inhibited when added to SkBr3 cells in gC1qR-rich medium lending credence to the postulate that secreted gC1qR not only provides an autocrine signal for proliferation but also serves as a molecular checkpoint in the tumor cell microenvironment by denying C1q access to the cell surface. These observations demonstrate that cell surface-expressed C1q and C1qRs are involved in breast cancer cell proliferation, and that soluble gC1qR appears to serve as an autocrine growth signal. Thus, the C1q receptor–C1q axis may provide potential novel targets for therapeutic intervention in breast cancer.

KEYWORDS: breast cancer, complement, C1q, ghA, gC1qR, C1qBP, CR, cC1qR.

ABBREVIATIONS

gC1q, the globular heads of C1q; cC1q, the collagen domain of C1q; gC1qR, receptor for gC1q; cC1qR, receptor for cC1q; CR calreticulin,

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(another name for cC1qR); ghA, ghB, and ghC, globular heads (gh) of the A, B, and C chains of C1q; B1R, bradykinin receptor 1; B2R, bradykinin receptor 2; pAb, polyclonal antibody; mAb, monoclonal antibody; NIRG, non-immune IgG.

INTRODUCTION

There is an emerging trend, which supports the notion that locally expressed complement proteins participate in various ways to induce either cancer cell apoptosis or enhance cell proliferation. Prominent among these proteins are C1q and its receptors gC1qR (also known as: HABP-1, C1qBP, p32), and cC1qR (also known as calreticulin or CR), which are multi-compartmental and multifunctional molecules, expressed on a wide range of proliferating and non-proliferating cells [1-8]. However, the cell surface expression of these molecules is enhanced in various malignant cells–probably through a process of enzymatic modification and translocation from intracellular pools to the cell surface–as a sign of cell stress or malignancy. The seemingly opposing role of each molecule, and in particular of gC1qR and C1q, in turn depends on whether it is anchored on the cell membrane or released into the cell microenvironment [9, 10]. Earlier studies from our laboratory have shown that co-culturing of various cell lineages representing B cell (Raji, Daudi and Wil_2WT), T cell (Molt-4) and monocyte (U937), with endotoxin-free C1q showed a consistent and dose-dependent inhibition of cell proliferation [11, 12]. The effect of C1q in these studies was not cytotoxic but rather cytostatic since the number of dead cells in the C1q-treated cultures was not significantly different than in the untreated control [12]. This anti-proliferative response in turn was clearly shown to be due to the ability of C1q, but not heat-inactivated C1q, to bind to one or both of its receptors and inhibit the expression of the major immunoregulatory cytokine IL-1, which is produced by macrophages and B lymphoblastoid cell lines [12]. More importantly, additional studies also showed that when mitogen-induced peripheral T lymphocytes were cultured for 48 h in the presence of C1q and then pulsed with 1μCi [3H]-thymidine, proliferation was strongly inhibited as assessed by reduced [3H]-thymidine incorporation, indicating that the anti-proliferative effect of exogenously added C1q is universal and was not unique to immortalized cultured cell lines alone [13]. Although the presence and co-localization of both cC1qR and gC1qR have been documented in all types of cells, gC1qR, by virtue of its trimolecular structure and potential multivalency and high affinity (13.5 nM) for the globular heads of C1q [14], may play a critical role in modulating cell proliferation.

Evidence for the role of C1q and its receptors in cell proliferation has also been obtained from studies of non-hematologic malignancies. Hong and colleagues [15] have shown that addition of exogenous C1q to human DU145 prostate cancer cells induces apoptosis by activating the tumor suppressor molecule called WW-domain containing oxydoreductase 1 (WWOX1) thereby disrupting cell adhesion. Conversely, downregulation of C1q enhances prostate hyperplasia and cancerous formation due to failure of WWOX1 activation. Similarly, treatment of normal bronchial epithelial BEAS-2B and NHBE cells with C1q was found to induce apoptosis through activation of p38 and caspase-3, and cell death with autophagy through accumulation of LC3-II and autophagosomes, respectively [16]. Interestingly, this C1q-induced apoptosis was mitigated by treatment of the cells with either anti-gC1qR/p33 or anti-cC1qR/calreticulin indicating that both receptors may be involved. Since both cell lines are known to express ADAM28 (disintegrin and metalloproteinase 28) –a molecule involved in cell proliferation and progression–and treatment of C1q with recombinant ADAM28 prior to addition to culture media reduced C1q-induced cell death, these investigators hypothesized that ADAM28 plays a role in cell survival by suppression of C1q-induced cytotoxicity in bronchial epithelial cells. More recently Bulla and colleagues [8] showed that C1q is expressed in the stroma and vascular endothelium of several human malignant tumors. Using a C1q-deficient mouse model bearing syngeneic B16 melanoma the authors further showed slower tumor growth and prolonged survival compared to their C1q-sufficient counterparts, suggesting that locally synthesized C1q promotes tumor growth. Furthermore, another group [7] also found that the C1q globular head domain induces anti-proliferative responses in ovarian cancer cells presumably *via* tumor necrosis factor α (TNF-α) -induced apoptosis

and involving upregulation of the apoptosis-related proteins Bax and Fas. Finally, recent studies from our own laboratory have shown [17] overexpression of gC1qR in tumor tissues taken from pancreatic adenocarcinoma patients when compared to surrounding non-malignant pancreatic ones and that soluble gC1qR was detected in peripheral blood taken from patients with metastatic disease. Blood levels of soluble gC1qR rose with disease progression, and paralleled changes in traditional tumor biomarkers, carcino-embryonic antigen (CEA) and CA19.9 (carbohydrate antigen sialyl Lewis) [17].

Although the underlying mechanism(s) by which cancer cells utilize locally produced factors to enhance survival and disease progression is a complex and multifaceted process, the aforementioned examples provide strong evidence for a role for C1q and its receptors: cC1qR and gC1qR. The aim of the present study is to extend these observations to breast cancer by examining the role of C1q and its receptors in the proliferation of the HER2+ breast cancer cell line, SkBr3, as a model. The results demonstrate cell surface expression not only of cC1qR and gC1qR but also of C1q, as well as the release of soluble gC1qR into the culture medium. The data suggest a model in which breast cancer cell proliferation is affected by the interplay between C1qRs, cell surface C1q, and soluble gC1qR, functioning as an autocrine growth factor.

MATERIALS AND METHODS

Chemicals and general reagents

The following reagents and chemicals were purchased or obtained from the sources indicated: Dulbecco's phosphate buffered saline (D-PBS) without calcium and magnesium (Mediatech Inc, Manassas, VA); RPMI 1640, 100x Penicillin/ Streptomycin, and trypsin/ethylenediaminetetraacetic acid (EDTA) (GIBCO-Invitrogen, Grand Island, NY); heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT); *p*-nitrophenyl phosphate (pNPP) (Pierce, Rockford, IL); Immu-Mount (Thermo Fisher, Waltham, MA). Alexa 488- or Alexa 594-Streptavidin, Alexa 488- or Alexa 594-$F(ab')_2$, goat anti mouse or anti rabbit; fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG $F(ab')_2$ or sheep anti-rabbit IgG $F(ab')_2$ (Invitrogen, Carlsbad, CA); alkaline phosphatase (AP)–conjugated goat anti-rabbit IgG and non-immune rabbit or goat IgG and bovine serum albumin (Pierce), Mini-PROTEAN precast TGX gels (Biorad CA), Pierce Cell Surface Protein isolation kit (Thermo Fisher, Waltham MA).

Expression of various versions of recombinant gC1qR proteins

The strategy for the construction of plasmids containing the full-length gC1qR was described in detail in our earlier publications [18-20]. Similarly, the recombinant globular head proteins, ghA, ghB, ghC, were expressed as a fusion with maltose-binding protein (MBP) in *E. coli* BL21 strain, and the recombinant proteins purified as described extensively in earlier publications [21, 22].

Proteins and antibodies

The purified proteins used in these studies were obtained from the following sources. Monoclonal as well as polyclonal antibodies to recombinant human gC1qR, and to cC1qR have been described in our previous publications and represent part of the antibody databank in our laboratory [19, 23]. In addition, immunoaffinity-purified antibodies were made to selected synthetic peptides from gC1qR. Rabbit anti-C1q was made and purified in our laboratory; goat anti-C1q and monoclonal anti-C1q as well as purified C1q were purchased from Quidel (San Diego, CA). Rabbit anti C1q-A chain was purchased from Life Technologies Corp (Carlsbad, CA).

Cultured cells

The SkBr3 cell line was purchased from ATCC and grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO-Invitrogen, Grand Island NY) and maintained in humidified air consisting of 5% CO_2 and 95% air. Prior to each experiment, the viability of cells was verified by Trypan blue exclusion and only cultures with ≥95% viability were used for experiments. The SkBr3 cell line was originally derived from the pleural effusion of a breast adenocarcinoma patient and is known to over-expresses HER-2 (human epidermal growth factor receptor) gene product. Overexpression of this

oncogene has been shown to play an important role in the development and progression of certain aggressive types of breast cancer [24]. In addition, we have also used other breast cancer cell lines including MDA-MB-231 and MDA-MB-468 which like the SkBr3, were derived from the pleural effusion of breast adenocarcinoma patients. However, the MCF10a cell line, was derived from the breast of a patient with fibrocystic disease and was used for comparison.

SDS-PAGE and Western blot analysis of membrane and intracellular C1q

Both whole cell and membrane lysates were made using standard procedures. To identify membrane proteins SkBr3 cells were cultured to confluency as described above and were surface labeled with NHS-LC biotinylation agent lysed, and prepared according to the protocol provided by the manufacturer. Briefly, cells were washed 2x with TBS, and re-suspended in 1 ml of lysis buffer. Cells were centrifuged again at 10,000 g at 4 ºC after which 100 µl of Neutravidin resin were added to the supernatant and incubated for 1 hr at room temperature (RT). The lysate-resin mixture was flowed through a column and washed 3x with wash buffer. The bound membrane proteins were then eluted from the resin using dithiothreitol (DTT) and Laemmli buffer. Analysis on SDS-PAGE was performed on mini-PROTEAN precast TGX 10% acrylamide gels with samples being run reduced and alkylated by boiling for 5 min in the presence of 10% 2-β-Mercaptoethanol. After electrophoresis, the protein was electrotransferred to polyvinyl difluoride (PVDF) nitrocellulose membranes, and blocked with 2% bovine serum albumin (BSA) in TBST (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20). The bound proteins were then probed with an appropriate dilution of target-specific antibodies, and then visualized by chemiluminescence horseradish peroxidase-conjugated species-specific antibody followed by reaction with 4-chloro-1-naphthol substrate.

Detection of secreted gC1qR (sgC1qR) by Ag-capture ELISA assay

SkBr3 cells were seeded in RPMI containing 10% FBS overnight, after which medium was replaced with serum-free RPMI. Cell supernatant was collected after 24, 48, and 72 hr incubations and centrifuged to remove remaining cells. Microtiter wells were coated with 100 µl of 10 µg/ml rabbit anti-gC1qR in carbonate buffer, pH 9.6 (15 mM Na_2CO_3 and 35 mM $NaHCO_3$) overnight at 4 °C after which the unbound proteins were discarded, and the unreacted sites of the well blocked with 1% heat inactivated BSA (37 °C, 1 hr). Next, the wells were washed three times with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween-20), and 100 µl of SkBr3 supernatant was added to each well in duplicates and incubated overnight at 4 °C. After removal of the unbound proteins, the wells were washed three times with TBST, and bound proteins were detected using biotinylated mAb 60.11 against human gC1qR. The bound gC1qR was then visualized by sequential incubation (37 °C, 1 hr each) with alkaline phosphatase conjugated streptavidin followed by pNPP solution. The absorbance of the resulting color development was then measured at 405 nm using a V_{Max} Kinetic plate reader (Molecular Devices, Menlo Park, CA, USA). The experiments were done at least three times in duplicates.

Immunofluorescence microscopy

Immunofluorescence studies were performed on non-permeabilized SkBr3 cells grown on glass cover slips to 70% confluency. The attached monolayer of cells was then incubated first with PBS containing 0.1% BSA and 1% heat-inactivated human serum or 1 µg/ml Fc fragments to block Fc receptors, followed by incubation with rabbit anti-gC1qR-recognizing peptides 50-63, or peptides 144-155, goat anti-C1q or mAb anti-C1q at concentrations of 2.5 µg/ml. Cells incubated with non-immune rabbit, goat IgG or MOPC-21 served as negative controls. After fixing for 10 min with 10% (v/v) formalin, the cover slips were air-dried, placed face down onto microscope slides, sealed using mounting solution (Immu-Mount), and examined by three-dimensional imaging using deconvolution microscopy.

Cell proliferation assays

SkBr3 cells were seeded in tissue culture-treated 6-well plates at a concentration of 1.25×10^5 cells/ml in 2 ml of RPMI supplemented with 10% FBS for 96 hrs. The cells were then treated with

or without 10 or 5 μg/ml of purified protein or specific antibodies in duplicates. Untreated or mock-treated cells were used as control. At 96 hr, the supernatant was removed, and the cells were trypsinized, re-suspended in 1 ml of medium and 100 μl of trypan blue was added as an indicator of viability and the cells examined and counted using a hemocytometer. Proliferation studies were conducted separately on plates that were incubated for 48, 72, and 96 hrs.

Alternatively, proliferation studies were conducted in which untreated 12-well plates were first coated with 20 μg/ml of either, C1q, gC1qR, or various antibodies to C1q in endotoxin-free carbonate buffer, pH 9.6 (15 mM Na_2CO_3 and 35 mM $NaHCO_3$). Control wells were coated with BSA in carbonate buffer or culture medium alone. All wells were coated overnight at room temperature under sterile conditions and subsequently washed with PBS to remove excess buffer. Cells were then seeded at a concentration of 1.25 x 10^5 cells/ml in a total volume of 1 ml/well. At the end of incubation, images were then taken using a light microscope under 10X, every 12 hours for 96 hours at which point all bound and unbound cells were collected and counted using a trypan blue exclusion method as described above.

Statistical analysis

Student t-tests were performed using statistical software (Excel; Microsoft, Redmond, WA, USA). A value of $p = 0.05$ was considered to be a significant difference (n = separate experiments performed in duplicates).

RESULTS

Expression of gC1qR, cC1qR and C1q on SkBr3 cells

Figure 1 demonstrates the expression of both gC1qR and cC1qR on non-permeabilized SkBr3 cells. Moreover, co-localization of gC1qR and cC1qR on the cell surface is observed. Both gC1qR and cC1qR have been reported to interact with distinct functional domains of C1q. Whereas gC1qR interacts predominantly with the globular domain of C1q [23], cC1qR (calreticulin or CR) binds to the collagen tail of C1q [25-29], although interaction with the globular domain, particularly under low ionic strength conditions has also been described [27]. Importantly, cC1qR/CR has been shown to form a bi-molecular complex with gC1qR *in vitro* [30], and *in vivo*, where the cytoplasmic association has been shown to prevent cell death [31].

In addition to C1q receptors, cell membrane C1q expression has also been documented on select cell types including monocytes, dendritic cells, and fibroblasts, as well as on intestinal and liver epithelial cells [1]. Since SkBr3 cells are epithelial cell-derived cancer cells, we set out to investigate whether they also express C1q in addition to gC1qR, which is expressed in moderate amount in all types of cells. In the present study, the presence of C1q and gC1qR on SkBr3 cells was first analyzed using flow cytometry immunofluorescence microscopy (Figure 2A and B). Then the presence of C1q on SkBr3 cell lysates (Figure 3A) and cell membranes (Figure 3B) was analyzed by Western blotting. Interestingly, comparison of cellular C1q with plasma C1q by Western blotting (Figure 3A) reveals that the breast cancer cell lysate-derived C1q runs at a slightly higher molecular weight than plasma C1q, which likely occurs because the cellular protein is of a higher molecular weight probably due to the presence of an extra membrane-anchoring domain, which is not found in plasma C1q [32]. This fact was further demonstrated when cell membranes of various types of breast cancer–malignant (MDA231 and MDA 468) and non-malignant (MCF10a)–were compared to that of SkBr3 cell line (Figure 3B). In addition to the higher molecular weight A-chain, there appears to be an additional ~20 kDa band that is detected by an immunoaffinity-purified anti-C1q A-chain (Figure 3A). This suggests that either there are two species of the A-chain–a higher and a lower molecular weight–or that the higher molecular weight A-chain is enzymatically cleaved to generate the low mol. wt. ~20 kDa protein, which is detected by an antibody to the A-chain.

As expected, the membrane expression of gC1qR is not unique to SkBr3 cells since most breast cancer cell lines (MDA231, MDA-468) as well as a cell line representing fibrocystic disease (MCF10a) tested so far express the molecule in a manner similar to the SkBr3 cells (Figure 4).

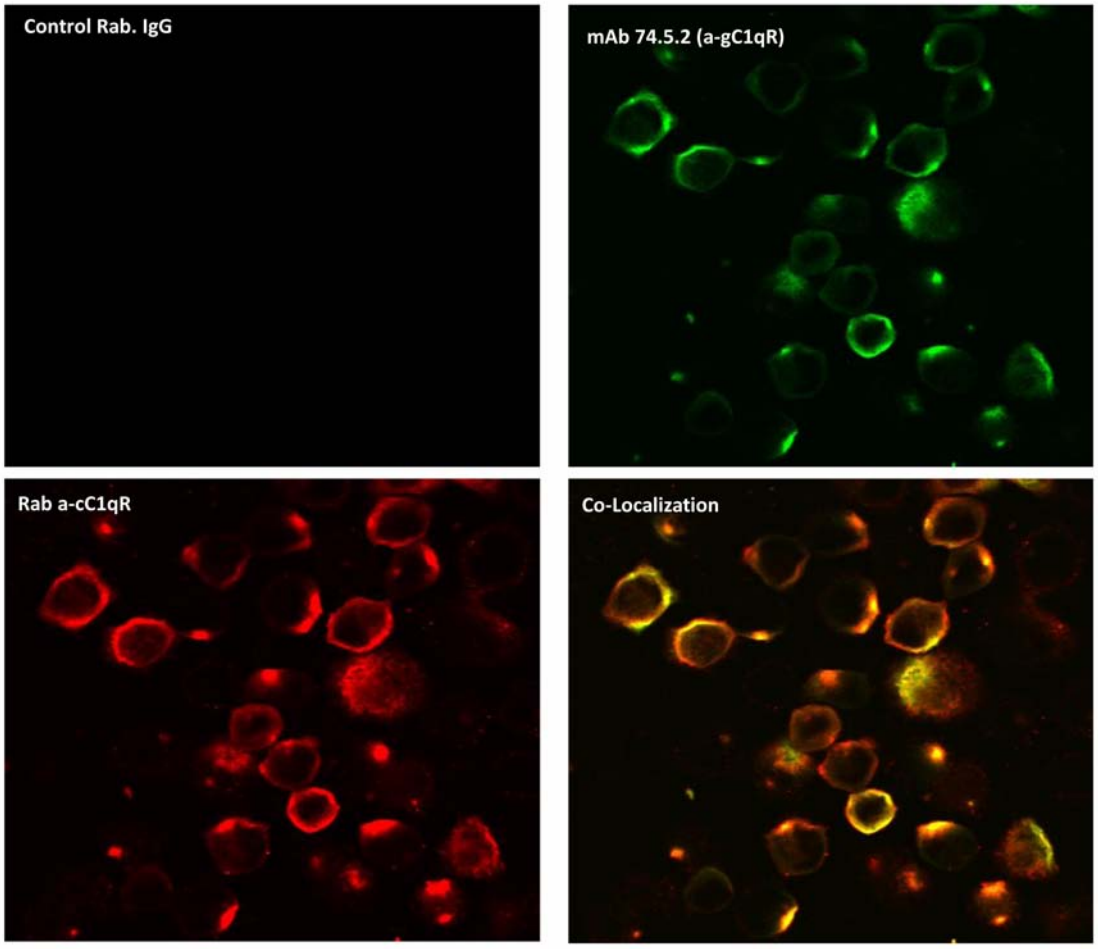

Figure 1. gC1qR expressed on SkBr 3 cells co-stains with cC1qR. SkBr3 cells, grown on glass cover slips, were incubated with PBS containing 0.1% BSA and 1 mg/ml Fc fragments to block FcRs. The cells were then probed with either control antibodies (MOPC-21 (not shown), and non-immune rabbit IgG (NIRG)), mAb 74.5.2 anti-gC1qR, or affinity-purified rabbit pAb anti-cC1qR. Bound Abs were visualized with Alexa Fluor 488-anti-mouse Abs (green) or Alexa Fluor 594 anti-rabbit Abs (red). The first panel shows control staining with NIRG. A similar lack of fluorescence staining was observed with MOPC 21 (not shown). gC1qR (green), cC1qR (red) staining and co-localization of gC1qR with cC1qR (yellow) are shown. Original magnification = x68.

Curiously however, the MDA-468, which is the only breast cancer line originally derived from an African American female patient did not show expression of gC1qR (Figure 4). Whether this is a single anomaly or a predictor of a significant observation will depend on the results from the comparison of several Caucasian and African American-derived cell lines.

Inhibition of SkBr3 cell proliferation by anti cC1qR and gC1qR antibodies

Previous studies from our laboratory have shown that blockade of gC1qR with mAb 60.11, which recognizes the major globular head C1q-binding site on gC1qR, results in inhibition of proliferation of a wide range of cultured cell lines [3]. Figure 5 shows that blockade of gC1qR with mAb 60.11 results in moderate but significant inhibition of SkBr3 cell proliferation. However, blockade of cC1qR with a pAb directed against the putative binding domain on the collagen tail of C1q resulted in almost complete inhibition of SkBr3 cell proliferation (*** $p < 0.005$). The difference in the inhibitory potency of the antibodies could be attributed to the fact that a multivalent pAb was used to block the cC1qR, whereas mAb was

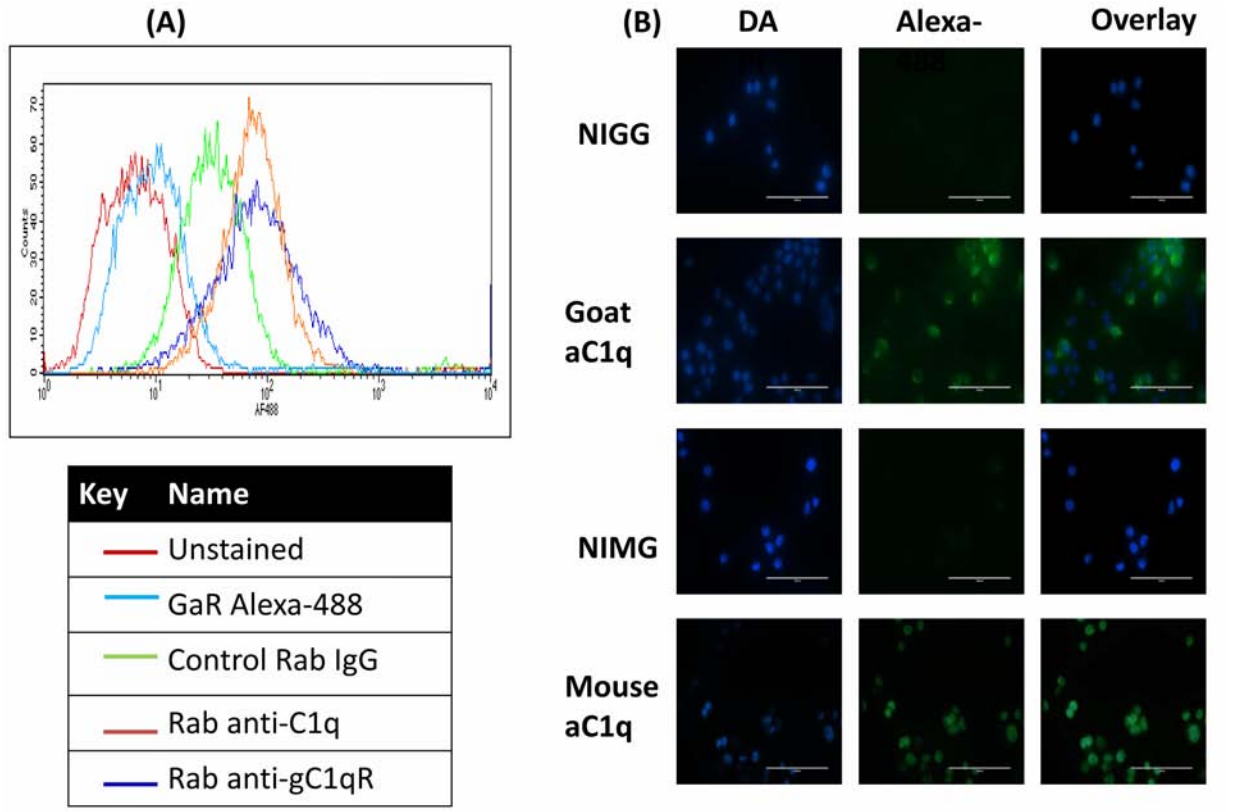

Figure 2. Cell surface expression of C1q by SkBr3 cells. (A) Flow cytometry analysis was performed on SkBr3 cells grown to confluency and detached using 10 mM EDTA solution. Cells were then blocked with human Fc fragments for 30 min on ice and subsequently probed with either 10 µg of non-immune rabbit IgG, rabbit anti-C1q, or rabbit anti-gC1qR for 30 min on ice. Then, Alexa Fluor 488-conjugated mouse anti-rabbit Ab was added and incubated for 30 min on ice. The cells were stained with propidium iodide to assess viability. (B) Immunofluorescence studies on SkBr3 cells grown in chamber slides to 90% confluency were done to determine C1q expression. Monoclonal anti-C1q and goat anti-C1q as well as the isotype matched non-immune IgG controls were incubated for 30 min at room temp. After incubation with Alexa Fluor 488-conjugated donkey anti-mouse or rabbit anti-goat antibodies (30 min), the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear visualization. The cells were imaged to detect membrane staining.

used to block gC1qR. It is also possible that the inhibitory effect of the cC1qR antibody could be due to steric inhibition of the cC1qR-gC1qR complex, given the co-localization of the two C1q receptors on the SkBr3 cell surface.

The role of cellular C1q in SkBr3 cell proliferation

Figure 6 demonstrates that blockade of SkBr3 cell surface C1q with a polyclonal anti-C1q antibody results in significant inhibition of cell proliferation. However, blockade of the A-chain with a specific anti-C1q-A antibody and more specifically, an antibody directed against the gC1qR-binding domain on the C1q A chain (ghA) also demonstrated marked inhibition of cell proliferation. Although direct comparison of inhibition using anti-ghB and anti-ghC has not been done in these experiments, the fact that an antibody directed against a well-recognized gC1qR site on the A-chain of C1q blocks cell proliferation [33] suggests that the C1q A-chain is indeed a critical player in tumor cell growth.

The effect of extracellular C1q on SkBr3 cell proliferation

The antiproliferative effect of C1q on a number of lymphocyte-derived tumor cell lines in culture [11, 12] and several epithelial cell-derived carcinomas

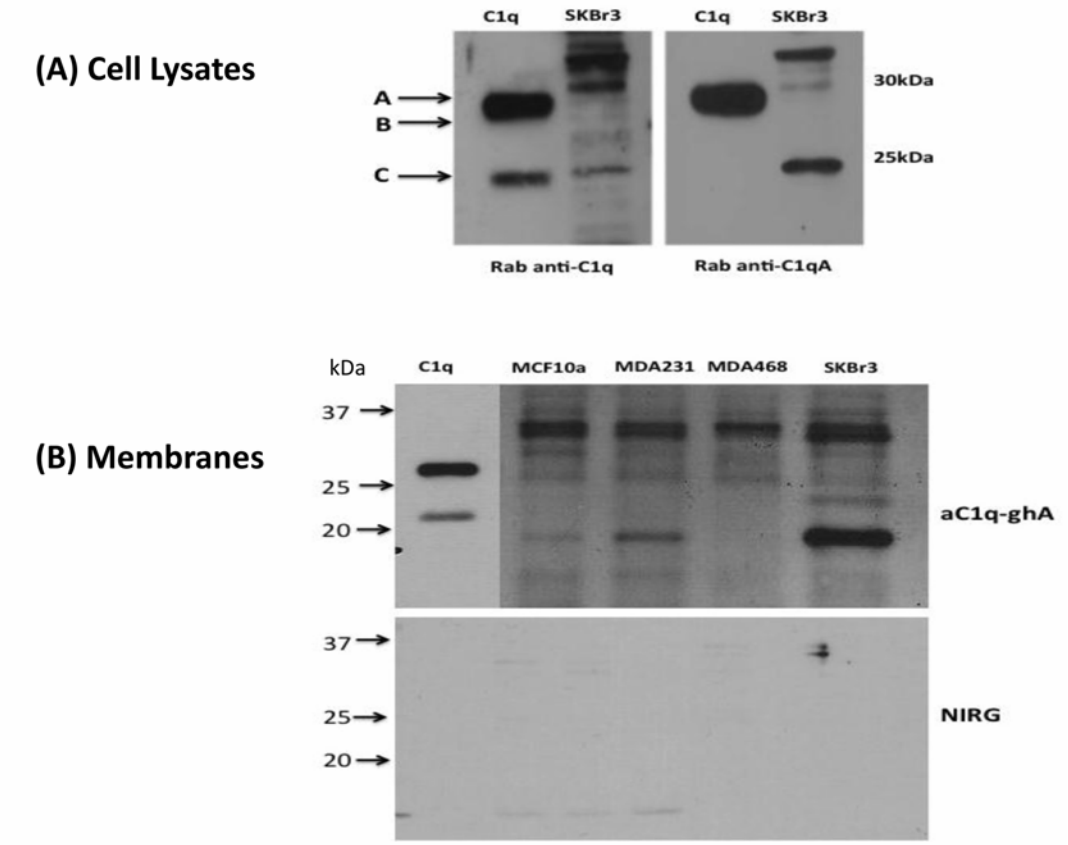

Figure 3. Western blot analysis of C1q expressed by SkBr 3 cells. Cell lysates (Figure 3A) as well as purified C1q (20 ng) were reduced and electrophoresed on a 10% polyacrylamide gel, transferred onto nitrocellulose membranes, blocked with 2% BSA in TBST and then incubated overnight at 4 ºC with either rabbit non-immune IgG (NIRG), or rabbit antibodies recognizing either the intact C1q, or the A chain of C1q. Bound antibodies were then detected by chemiluminescence using horse radish peroxidase (HRP)-conjugated goat anti-rabbit antibody and a chemiluminescence substrate and then exposed on film. Figure 3B compares the membrane-expressed C1qA on several breast cancer cell lines detected with a specific rabbit antibody which recognizes the ghA region of C1q. Staining of the same cells with non-immune rabbit IgG is also shown for comparison (Figure 3B). The figures are representative of 3 such experiments (n = 3).

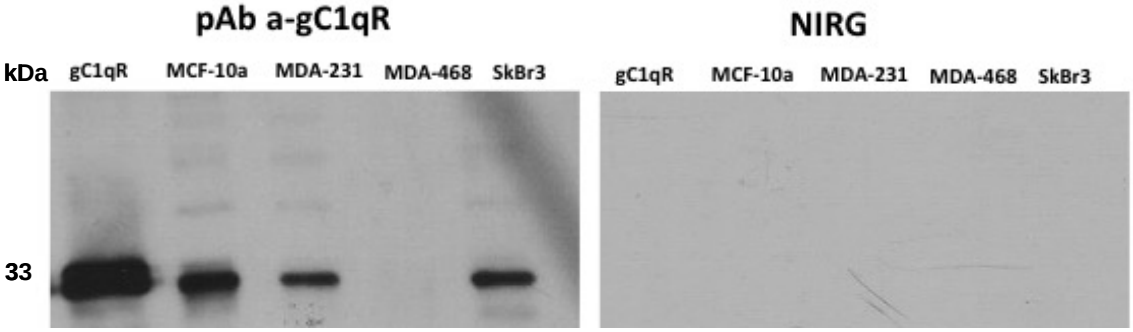

Figure 4. Detection of gC1qR on purified membrane proteins. Western blot analysis was performed on purified membrane proteins from several breast cancer cell lines and the presence of gC1qR was detected using an affinity-purified anti-gC1qR peptide.

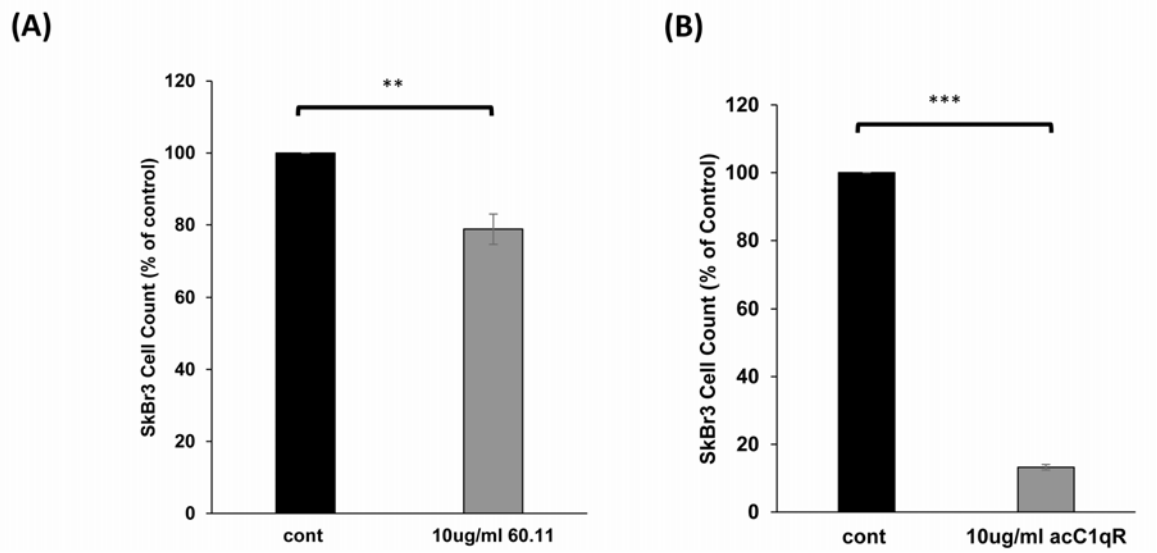

Figure 5. Effect of antibody-induced C1q receptor blockade on SkBr cell proliferation. SkBr3 cells were seeded in 6-well plates at 1 x 10^5 cells/ml in the presence or absence of either (A) 10 µg/ml mAb 60.11 recognizing the C1q site on gC1qR or (B) 10 µg/ml affinity-purified rabbit anti-cC1qR recognizing the putative C1q binding domain on cC1qR. After 96 hr, viable cells were counted in a hemocytometer in the presence of 10 µl/ml trypan blue. Results show cell count represented as % of control treated, with n = 4. Significance is represented by *** and represents $p < 0.005$, using the student's t-test.

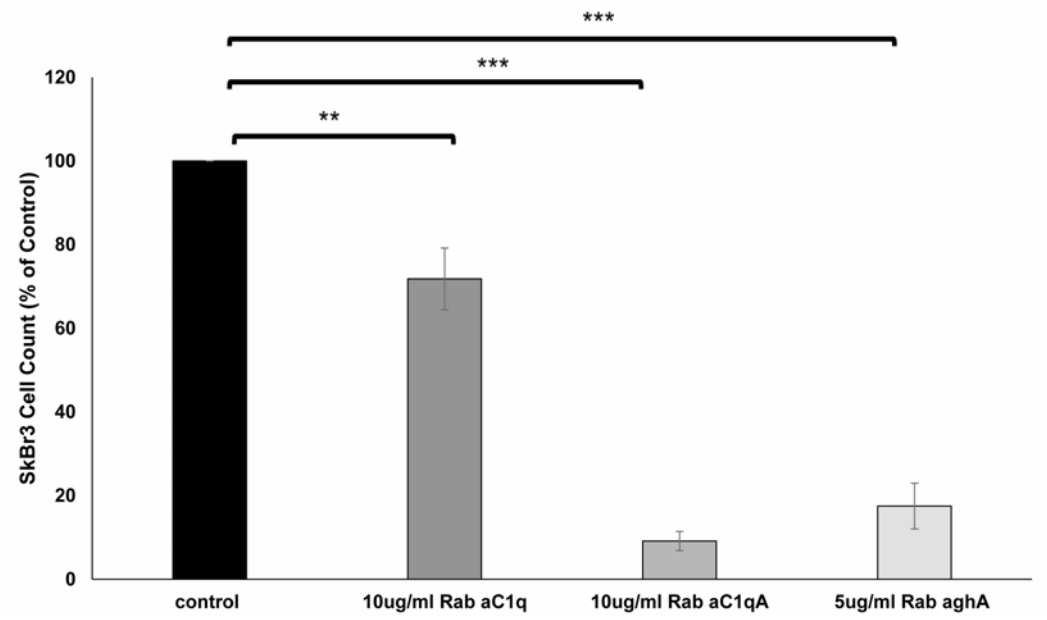

Figure 6. The A-chain of C1q is vital for cell survival. Proliferation assays were conducted using SkBr3 cells seeded at 1 x 10^5 cells/ml in the presence or absence of rabbit anti-C1q (10 µg/ml), anti C1qA (10 µg/ml), or rabbit anti-ghA. Cells were then incubated for 96 hr, after which they were counted in a hemocytometer in the presence of trypan blue. Control wells were either untreated or supplemented with isotype-matched non-immune rabbit IgG (NIRG). Results for NIRG-supplemented cells were no different from untreated cells (control) and are not included here. Results are representative of four different experiments (n = 4) run in duplicates. Significance is represented by ** ($p < 0.01$) and *** ($p < 0.005$) when compared to control using student's t-test.

has been described before [7, 15, 16], but has not been investigated in breast cancer. To evaluate the effect of extracellular C1q in SkBr3 cell proliferation, cells were seeded at 1 x 10^5 cells/ml in the presence or absence of 10 µg/ml of C1q or its globular head modules, ghA, ghB, or ghC. As shown in Figure 7A, a significant decrease in cell number was observed in cells treated with exogenous C1q, as well as cell cultures treated with purified recombinant globular head domains of C1q (Figure 7B). These observations strongly support the interaction of the globular domain of C1q with cell surface gC1qR.

Soluble gC1qR is released from SkBr3 cells and modulates cell proliferation

Since the presence of soluble gC1qR has been described in blood and body fluids of patients [17], we examined whether Skbr3 cells release gC1qR into their culture medium, using an antigen-capture ELISA. For these studies, SkBr3 cells were grown in serum-free medium and supernatants were collected at 24, 48, and 72 hr and tested for the presence of gC1qR using an antigen capture ELISA. Indeed, significant and time-dependent release of soluble gC1qR into SkBr3 culture supernatants was observed (Figure 8). Moreover, to understand the role of secreted gC1qR better, we cultured SkBr3 cells in the presence of soluble recombinant gC1qR (10 µg/ml). This resulted in an approximately 30% increase in cell number after 96 hr, indicating that secreted gC1qR enhances cell proliferation (Figure 9). Finally, we showed that the presence of soluble gC1qR in culture supernatant of SkBr3 cells could overcome the antiproliferative effect of exogenously added C1q (Figure 10). For these studies, SkBr3 cells were cultured for 48 h to allow measurable secretion of gC1qR (as shown in Figure 8), then the medium was removed from the test wells, which were washed to remove residual secreted gC1qR before addition of fresh medium. The remaining control wells were left in the original "gC1qR-rich" medium. Subsequently, C1q (10 µg/ml) was added to both test and control cells, and SkBr3 cell proliferation was examined after additional 96 hr incubation. As shown in Figure 10, SkBr3 cells that were maintained in the

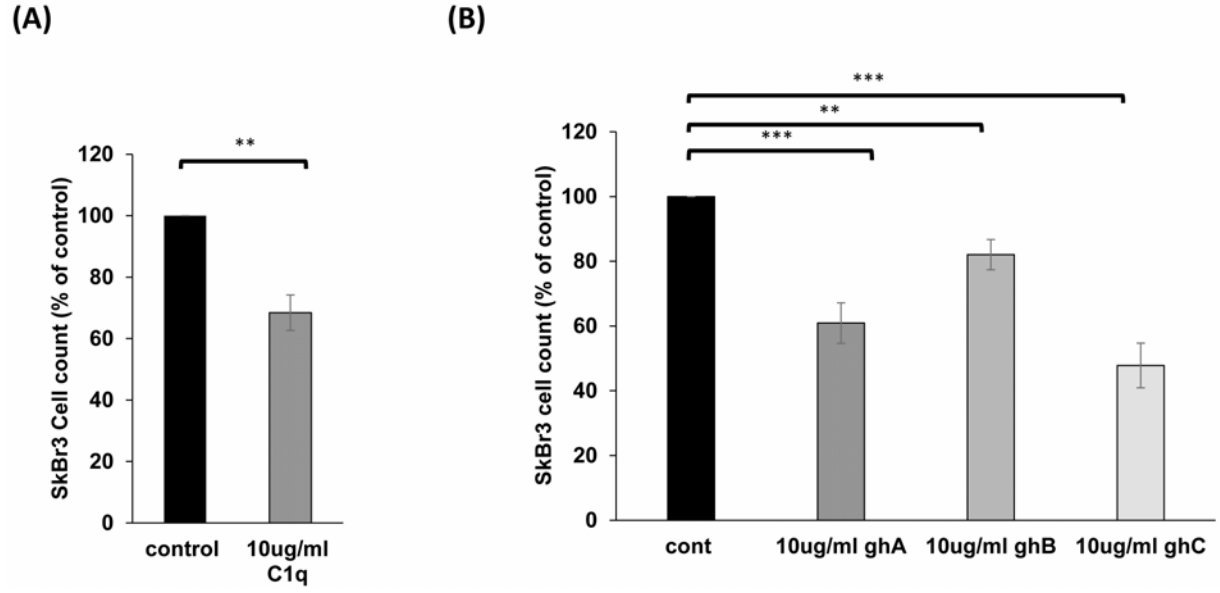

Figure 7. The anti-proliferative effect of C1q and its globular head modules on SkBr3 cells. SkBr3 cells were seeded in 6-well plates at 1 x 10^5 cells/ml in the presence or absence of either 10 µg/ml C1q (7A) or ghA, ghB, or ghC (7B). Each plate was counted after 96 hr using a hemocytometer in the presence of trypan blue. Results show cell proliferation levels represented as the mean of four assays run in duplicates. Values represent mean of cell count as percent of control values of 4 samples $\pm$ SD with significance ** ($p < 0.01$) and *** ($p < 0.005$) when compared to control using student's t-test.

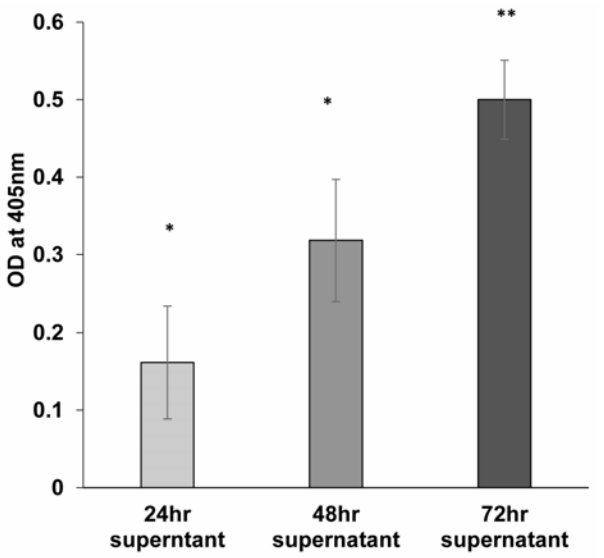

Figure 8. SkBr3 cells secrete gC1qR into the culture medium. Antigen-capture-ELISA on SkBr3 cell supernatants grown in serum-free RPMI for 24, 48, or 72 hrs (n = 4) was performed using microtiter plate wells coated with or without 100 µl of immunoaffinity-purified (10 µg/ml) rabbit anti-gC1qR peptide (1 h, 37 ºC). After blocking with 1% BSA (1 h, 37 ºC), 100 µl of either control medium or culture supernatants was added and incubated (1 h, 37 ºC). The captured gC1qR was detected by sequential incubation (1 h, 37 ºC) with biotinylated mAb 60.11 (2 µg/ml), alkaline phosphatase-conjugated Neutravidin and PNPP substrate as described in the 'Materials and Methods' section.

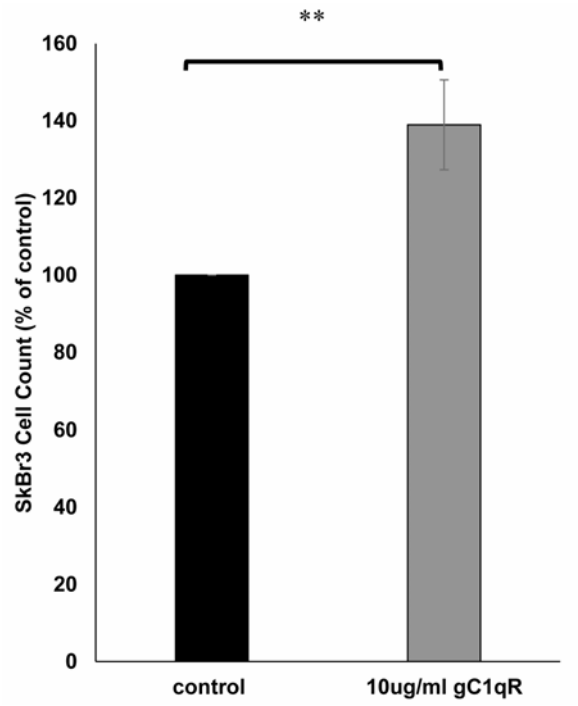

Figure 9. Soluble gC1qR is an autocrine signal of cell proliferation. Proliferation assay was done in which SkBr3 cells (10^5/ml) were co-cultured with or without 10 ug/ml gC1qR. After 96 hrs, cells were trypsinized and counted to determine cell number and viability. Results show cell count as a percent of control cell number. Data shown are representative of mean of n = 3 $\pm$ SD; significance denoted by ** ($p < 0.01$).

gC1qR-rich medium were "protected" from the anti-proliferative effects of C1q.

Differential response to growth by SkBr3 cells on C1q and gC1qR-coated surface

We have shown previously that C1q-coated plates can support and facilitate human umbilical vein-derived endothelial cell (HUVEC) adhesion and spreading in a manner that was similar to collagen-coated plates. The C1q-mediated endothelial cell adhesion and spreading in turn, was mediated in part by the cooperation of C1q receptors and β1 and α5 integrins [32]. We therefore wanted to see if C1q-coated plates would also support SkBr3 cell growth in a manner similar to that seen with HUVECS.

To further examine the interaction between SkBr3 cells and extracellular C1q or gC1qR, we performed adhesion studies using microplate wells coated with or without 20 µg/ml of purified C1q or gC1qR in endotoxin-free carbonate buffer. Wells were then blocked with 10% BSA and the SkBr3 cells were grown for 96 hrs and microscopic images (4X) were taken every 24 hr with a light microscope. As shown in Figure 11A, SkBr3 cells grew at a faster rate on gC1qR-coated wells compared to cells grown on control BSA-coated plates.

In contrast, cells did not attach on the C1q-coated wells and instead, they formed large clusters that got progressively bigger as the exposure time progressed. After 96 hr, cells were trypsinized, resuspended in fresh medium and analyzed for cell viability using the trypan blue exclusion assay. As shown in Figure 11B, while cell proliferation was enhanced in gC1qR-coated wells, the opposite response was observed when grown on a C1q-coated surface. Importantly,

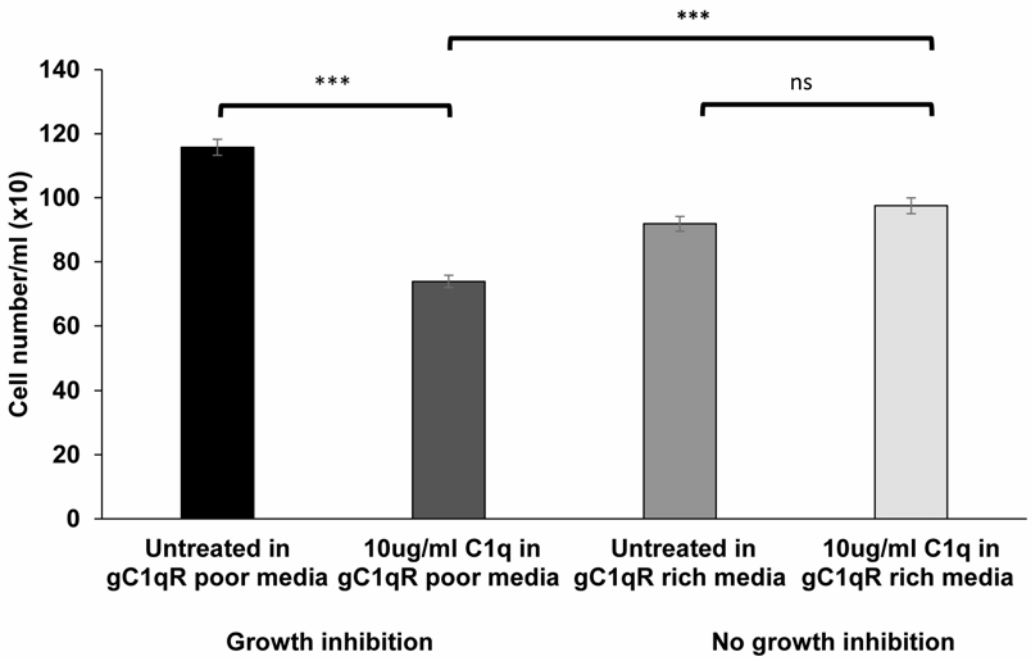

Figure 10. Soluble gC1qR secreted into the cell culture media reduces the inhibition of cell proliferation by exogenous C1q. SkBr3 cells were seeded in duplicate wells at 1 x 10^5 cells/ml and grown for 48 hr to allow for gC1qR secretion (n = 3). Then, the culture medium was removed from half of the wells and replaced with fresh medium, while the other half of the wells remained in the "gC1qR-rich" medium. Next, exogenous C1q or control heat-inactivated BSA was added and viable cells were counted after 96 h. Significance is represented by ** ($p < 0.01$) and *** ($p < 0.005$) when compared to control.

the gC1qR-coated surface showed about a 40% increase in cell number even when compared to those grown in buffer- or culture medium-coated plates indicating that gC1qR provides a strong growth signal.

DISCUSSION

The expression of C1q, gC1qR and cC1qR by many tumor types [34-42] and the poor prognosis associated with elevated gC1qR levels in breast and ovarian cancer in particular [38, 42, 43], raise interesting questions with regard to their collective functions in tumor biology. The present study makes several novel observations using SkBr3 cells as an *in vitro* model of breast cancer. First, cell surface gC1qR and cC1qR co-localize on the cell surface. Second, C1q is expressed on the surface of SkBr3 cells, but is not secreted. Although a wide range of cell types including cancer cells are known to express C1q [8, 42], this is the first time the presence of C1q has been documented on breast cancer cells, represented here by SkBr3 cells and other breast cancer cells derived from the pleural effusion of patients with breast adenocarcinomas. Third, gC1qR is released into the culture medium by proliferating SkBr3 cells. Although the mechanism for gC1qR release from breast cancer cells is as yet unknown, it is conceivable that membrane-associated gC1qR may be cleaved by the membrane type 1 MMP (MT1-MMP), which has been shown to cleave gC1qR at position Gly^{79}–Gln^{80} [44, 45]. We further present data that demonstrate a complex interplay between soluble and membrane-associated C1q and C1q receptors that contributes to cell proliferation. A model based on our experimental data is proposed in Figure 12. This model proposes that the interaction between membrane-associated C1q and either membrane-associated C1q receptors or soluble gC1qR supports cell proliferation. When this interaction is blocked by either antibodies to gC1qR, C1q, or by the addition of exogenous C1q, or its gC1qR-binding globular head modules, cell proliferation is diminished. In this regard, the observations by

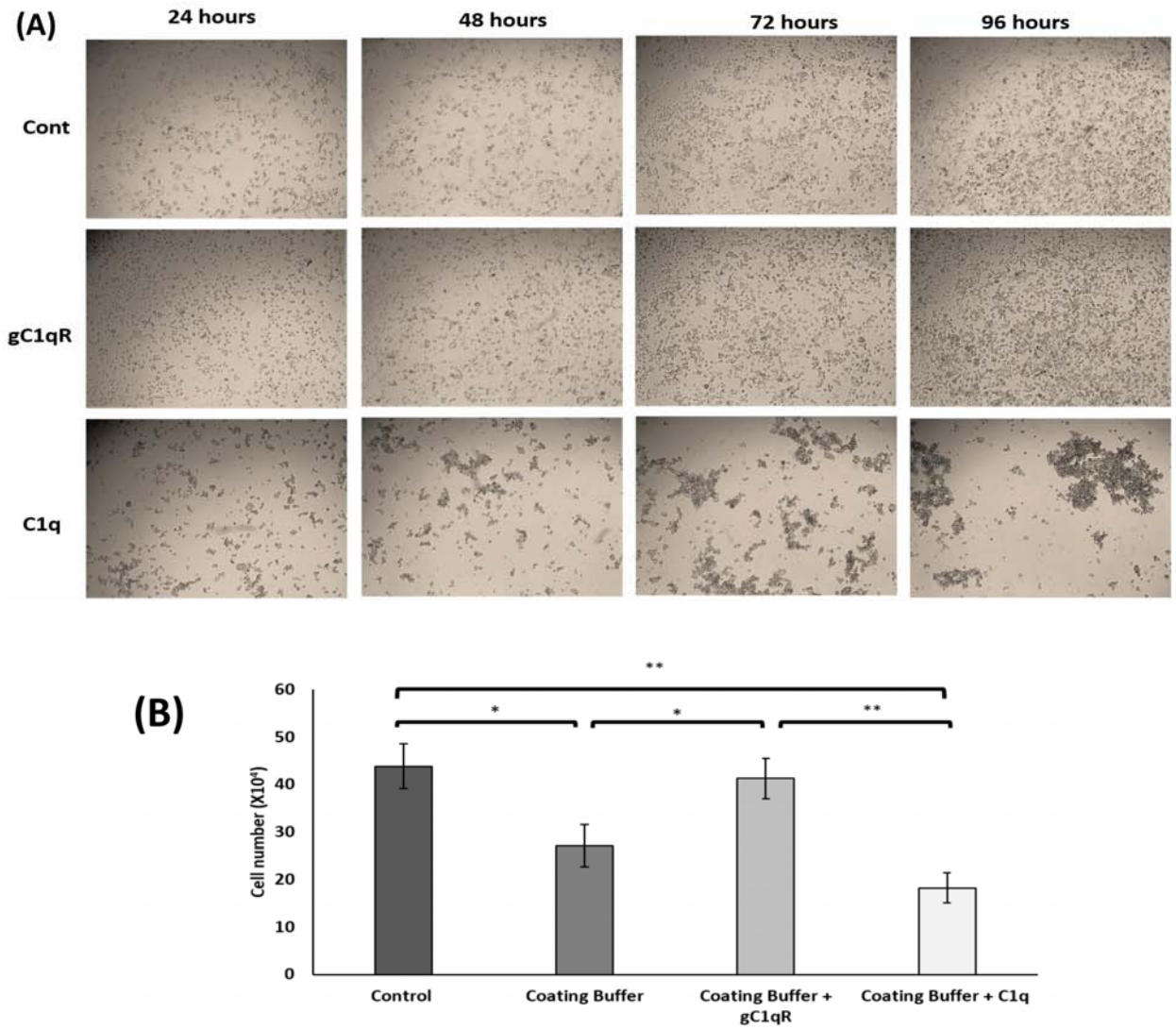

Figure 11. Adhesion assay on C1q- and gC1qR-coated plates. Microtiter plates (12-well) were coated (overnight at 20 ºC under sterile conditions) with either 20 µg/ml of C1q or gC1qR in carbonate buffer, pH 9.6 (15 mM Na_2CO_3 and 35 mM $NaHCO_3$). Excess buffer was removed and the cells were washed with sterile PBS before the addition of 1 x 10^5 cells/well in 1 ml of RPMI medium. (A) A light microscope was used to take 4X images of the wells every 24 hours for 96 hours. Buffer-coated wells were used as control and did not show any significant difference to cells in culture medium-coated wells. The images are representative of 3 experiments run in triplicates. (B) Cell supernatants were collected after 96 hr, and the remaining adherent cells were trypsinized after which supernatants and trypsinized cells were combined, stained with trypan blue and viability established. Students t-tests were applied to determine significance (* $p < 0.05$, ** $p < 0.01$).

Bulla and colleagues [8] demonstrating slower tumor growth and prolonged survival in C1q-deficient mice bearing syngeneic B16 melanoma compared to their C1q-sufficient counterparts suggest additional and potentially cancer cell-specific roles for C1q and C1q receptors in cancer cell biology.

Although both cC1qR and gC1qR are multicompartmental cellular proteins, they appear to be abundantly expressed on the cell surface [46, 47] of many malignant cells, particularly epithelial cell-derived malignancies. However, since neither cC1qR nor gC1qR possess a "conventional" motif for a transmembrane domain,

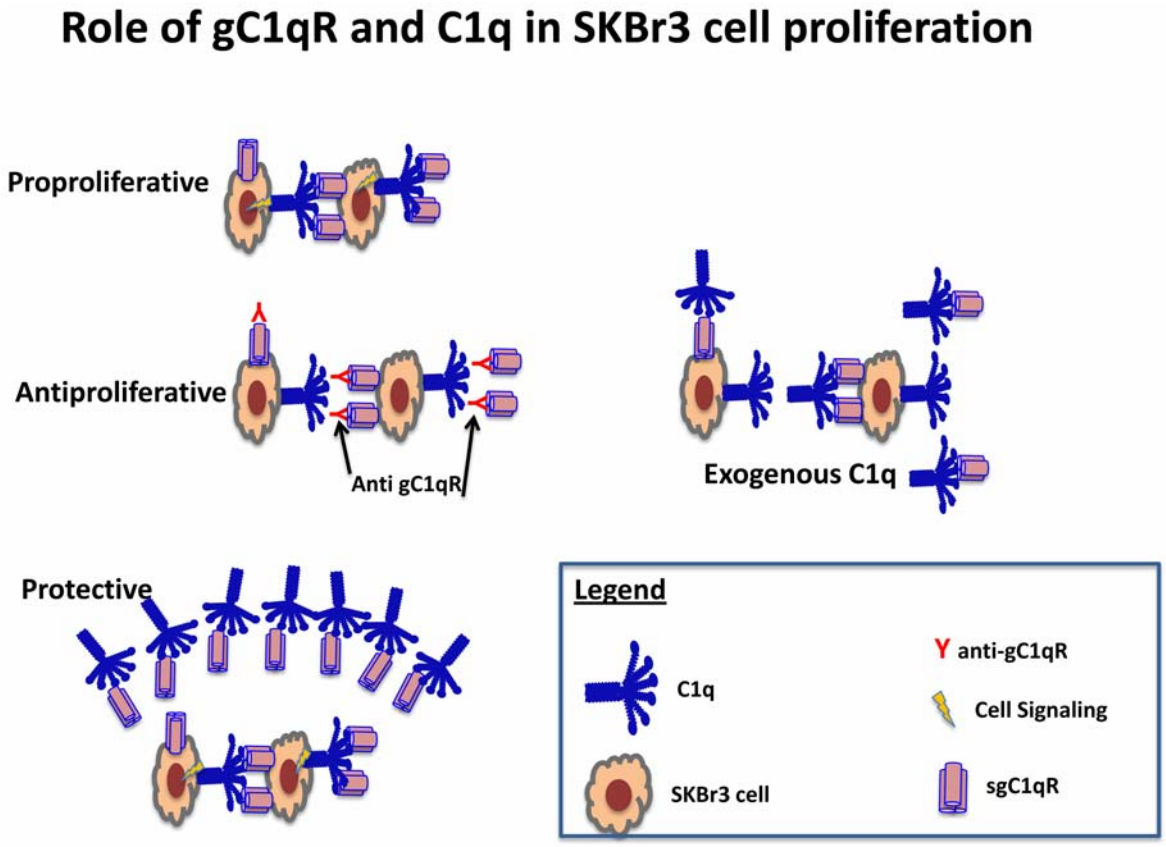

Figure 12. Schematic representation of the hypothesized roles of membrane-bound and soluble form(s) of C1q and gC1qR in breast cancer. The interaction of surface-expressed C1q on breast cancer cells may generate a pro-proliferative signal when engaged with membrane-associated or soluble gC1qR. This interaction can be blocked with antibodies to gC1qR, C1q and excess exogenous C1q. Soluble gC1qR released by breast cancer cells may represent an autocrine growth signal and further serve to ward off complement-mediated tumor recognition *via* engagement of C1q that may be present in the tumor microenvironment.

they are proposed to signal by "proxy" i.e by partnering with various cell-specific transmembrane receptors, which, in the case of gC1qR include CD44, DC-SIGN and β1-integrins and α1B-adrenergic receptor [34, 48, 49], and in the case of cC1qR, CD59 [50]. In the present study, we envision signaling *via* membrane-expressed C1q, which, based on observations made in macrophages and dendritic cells [51], is anchored in the cell membrane *via* a leader domain in the A-chain that serves as a type II membrane receptor. The gC1qR binding site on C1q may also be key for C1q-mediated signaling. This hypothesis is supported by the observed inhibition of SkBr3 cell proliferation not only by C1q but also by its globular head domains. These data are consistent with and mirror the preferential binding of the globular head domains to gC1qR seen using solid phase binding assays [33, 52].

Although the mechanism by which exogenous C1q exerts its anti-proliferative effect is yet to be determined, multiple pathways may be involved. The simplest explanation based on our studies, is that exogenous C1q binds soluble gC1qR and inhibits its pro-proliferative effect, by preventing its binding to cell surface C1q. In addition, exogenous C1q has previously been shown to induce cell apoptosis through activation of p38 and caspase-3 and cell death with autophagy through accumulation of LC3-II and autophagosomes [16]. This is particularly true when one considers the fact that C1q serves as a potent autocrine regulator of a plethora of cellular functions [10]. For example, C1q can induce-through either

cC1qR or gC1qR–the expression of IL-6, IL-8 and TNF-αR [53, 54], which in turn can activate the extrinsic apoptotic pathway and induce cell death through the TNF-α-TNFR-1-initiated apoptotic cell death pathway [55]. Alternatively, as an ancestral molecule of the TNF family of proteins which has retained some of its TNF-like functions [56, 57], exogenous C1q itself may interact with both gC1qR and TNFR-1 to activate the extrinsic apoptotic cell death pathway and induce downstream events leading to caspase-dependent cell death. However, other pathways involving mTOR survival factors may also play a role in down-regulating mTOR expression thus inhibiting cell proliferation [7].

In cancer, the C1q, as a self-molecule expressed on the tumor cell surface may serve not only to support proliferation when bound to gC1qR–either membrane associated or soluble–but also to evade recognition and destruction by invading reactive T cells. With multiple globular heads available for maximal contact, it may even inhibit the function of reactive T cells in the tumor microenvironment in a manner akin to that of PDL-1 and PD-1 interaction, with the membrane-expressed C1q playing the role of the PDL-1 and the T cell-expressed gC1qR serving as PD-1. This hypothesis is strongly supported by our previous observations that exogenously added C1q can suppress CD4+ T cells–ostensibly *via* gC1qR [13]– which is an immunosuppressive function that is indeed mimicked by several gC1qR-recognizing "danger" or "pathogen-associated" antigens such as those on hepatitis C virus, [58, 59], hantavirus [60]**,** HIV-1 gp41 [61] and plasmodium falciparum [62]. Although the tumor cell microenvironment is likely to contain numerous C1q- as well as gC1qR- and cC1qR-binding extracellular matrix proteins secreted by the cell, including heparan sulfate, chondroitin sulfate and hyaluronic acid that can play a role in regulating C1q functions, the secreted gC1qR in the tumor cell microenvironment nonetheless, is predicted to be a major player in the protection, sustenance, growth and metastasis of tumor cells by virtue of its ability to recruit and activate two of the most powerful proinflammatory cascades: the complement and the kinin systems [63-67].

In the present studies, we did not find any evidence that the surface-expressed C1q is secreted into the pericellular milieu. In contrast, SkBr3 cells, like most proliferating cells including malignant cells, are able to release or secrete a soluble and functional form of gC1qR [68]. While the secretion of gC1qR in itself is a very useful diagnostic molecular marker of cell proliferation and malignancy–as has been suggested earlier [3, 17]– the major function of the secreted gC1qR may be to orchestrate diverse activities in the tumor cell microenvironment, that collectively sustain cell growth as well as to promote metastasis through activation of the kinin system leading to generation of bradykinin. Therefore, on the basis of the present findings, we propose that both surface-expressed gC1qR as well as soluble gC1qR are pro-proliferative (Figure 12). This conclusion is supported by the fact that blockade of surface gC1qR with either anti-gC1qR antibodies or the gC1qR ligands–C1q or its gh domains–results in significantly reduced cell proliferation. Conversely, addition of purified gC1qR to cultured cells enhances their proliferation in an autocrine manner presumably by binding to surface-expressed C1q or other surface molecules including cC1qR. Also, gC1qR secreted into the pericellular microenvironment constitutes a "protective" molecular shield that prevents circulating "free" C1q from inducing anti-proliferative response leading to cell death. This postulate in turn is supported by the fact that the anti-proliferative effect of exogenously added C1q is inhibited by "gC1qR-enriched" medium (Figure 9).

CONCLUSION

In summary, our findings reveal that the SkBr3 cell line, which was used in these studies as a model for breast cancer, not only expresses cell surface gC1qR, cC1qR and C1q, but also releases soluble gC1qR into the extracellular milieu. This constellation of ligand and receptor expression appears to support cell proliferation *in vitro*. These studies, while not complete, have nonetheless laid a foundation for future investigations that seek to unravel more precisely the C1q- and gC1qR- and/or cC1qR-specific signaling mechanisms. Moreover, our studies support the notion that C1q and its receptors represent novel molecular targets for the development of therapeutic modalities, not only against breast cancer but also other cancer types that are known to express these molecules.

CONFLICT OF INTEREST STATEMENT

The authors have no financial or personal interests to disclose.

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