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SIGNIFICANCE OF THE INTERACTION BETWEEN CALRETICULIN AND COMPLEMENT PROTEIN C1Q IN BREAST CANCER CELLS TREATED WITH ANTHRACYCLIN

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ABSTRACT

The initiation and maintenance of immunogenic cancer cell death is currently seen as one of the major aims of cancer chemotherapy and radiotherapy. Some chemotherapeutic agents such as anthracyclins mediate a rapid transfer of calreticulin from the lumen of endoplasmic reticulum to the cell surface. Presence of calreticulin on the cell surface can potentially lead to binding of soluble complement proteins such as C1q and thrombospondin, triggering an immune-mediated killing of cancer cells. The calreticulin-C1q interaction might also alter C1q-mediated immune response, since the N- and P-domains of calreticulin interacts with the globular head region of C1q to inhibit C1q-mediated haemolysis of IgM sensitized erythrocytes. However, details concerning the effect of these interactions on C1q binding to chemotherapy-induced cancer cells remain unknown. Using pMal-c2 protein fusion and purification system and solid-phase assay methods, we show here that C1q binds more to the N-domain of calreticulin at high concentrations and more to the P-domain at moderate and low concentrations, with very minimal interaction with the C-domain of calreticulin at all concentrations. We further demonstrate that the binding of C1q to the N- and P- domains of calreticulin increases C1q binding to anthracyclin-treated breast cancer cells, while binding of C1q to the C-domain of calreticulin inhibits C1q binding to anthracyclin-induced breast cancer cells. These findings suggest that the N- and P- domains of calreticulin might bind to a site on C1q (globular head) that differs from that to which the C-domain of calreticulin binds and that the binding site for the C-domain of calreticulin might also be the site via which C1q interacts with drug-induced breast cancer cells. The effect of this on C1q-mediated anti-cancer immune response however, remains unknown.

KEYWORDS: Calreticulin, cancer cells, complement proteins, immune system

INTRODUCTION

The immune system functions principally to protect its host against environmental agents such as toxic chemicals and microorganisms, in order to maintain the body's integrity [1]. Individuals with compromised immune responses are therefore highly susceptible to serious and sometimes life-threatening infections caused by these agents [2]. The role of chronic infections, especially viral infections, is well established in the pathogenesis of various human neoplasms, including prostatic

carcinoma, colorectal cancers[3]; cervical cancers, B-cell lymphoma, hepatic carcinomas and Kaposi sarcoma [4].

Chemical carcinogens also play important roles in the aetio-pathogenesis of cancers and there is massive evidence to suggest that the immune system does respond to cancer cells. The immune system functions by recognizing and responding to antigens present on the surfaces of cells and microorganisms as well as toxic chemical substances which can also serve as antigens. Toxic chemical substances and cells bearing such antigens are often recognized and destroyed by a competent immune system, thus protecting the body from harm.

Immune Surveillance of Cancer Cells

The first proponents of the immune surveillance theory by Erlich in 1909 proposed the "magic bullet" strategy, whereby soluble factors which we now refer to as antibodies, deliver toxins directly to malignant cells, leading to their recognition, killing and consequent control of cancer growth [5]. Immune surveillance suggests that both precancerous and malignant cells are capable of stimulating immune responses that result in the destruction of malignant cells.

Three basic mechanisms used by the immune system to prevent the development of cancers have been identified. In the first instance, the immune system protects its host from virus-induced cancers such as colorectal cancer, cervical cancer, B-cell lymphoma, liver cancers and Kaposi sarcoma by suppressing or eliminating such viral infections. Secondly, the timely intervention of the immune system ensures that pathogens are promptly eliminated and inflammation resolved in order to prevent the establishment of a chronic inflammatory environment which is conducive for tumour growth. The immune system can also recognize and kill tumour cells expressing some tumour-specific antigens or molecules that are induced by cellular stress such as starvation, cytotoxic agents, reactive oxygen species and DNA damage. This third mechanism constitutes the concept called 'immune surveillance' [6].

The original immune surveillance theory as envisioned by Erlich was later supported by several findings, including the discovery of lymphocytes as mediators of immune response. In 1959, Lederberg proposed the self-non-self-discrimination theory, in which it was suggested that lymphocytes are the mediators of tumour surveillance which protects immune competent hosts against tumours [7]. Lederberg's theory suggested that since most cancers occur after maturation of the immune system, any tumour-specific antigen expressed on cancer cells is easily recognized as non-self. The immune system therefore monitors the body for the development of tumours, which are promptly eliminated as they arise.

Further evidence in support of the immune surveillance theory was provided by [8], who proposed the 'danger' model [9]. Under this model, healthy cells do not send danger signals, whereas, cells that are distressed, damaged and those dying abnormally (by necrosis) usually activate local APCs by sending danger signals also referred to as 'alarm signals' or 'signal zero', resulting in immune responses. Some of the molecules involved in sending Matzinger's danger signals have been identified to include the nuclear protein HBGM-1, uric acid, Interferon-α (IFN- α), heat shock proteins, alternatively spliced domains of fibronectin and some endogenous nucleic acids. These molecules are collectively referred to as 'alarmins', some of which utilize the same Toll-like receptors seen in microbial recognition [9].

Calreticulin in Cancer Immunity

Calreticulin is a 46 kDa protein composed of at least three domains, the N-terminal, P- and C-terminal domains [10] that have been shown to enhance lymphocyte infiltration of cancer cells by promoting the expression of Intracellular Adhesion Molecule-1 (ICAM-1) on tumour endothelial cells [11]. Several studies have suggested that translocation and surface expression of calreticulin on dying cancer cells determines the immunogenicity of cancer cell death. Obeid *et al*. (2007) demonstrated that chemotherapy-induced surface expression of calreticulin resulted in DC-mediated phagocytosis of cancer cells even before the appearance of other morphological changes of apoptosis. Blockage of surface-expressed calreticulin by use of antibodies was also shown to inhibit DC-mediated phagocytosis of these cancer cells [12]. Also investigated the relationship between surface expression of calreticulin on malignant cells and immune responses, before and after induction chemotherapy. Results revealed that surface expression of calreticulin occurred in some acute myeloid leukaemia (AML) patients prior to induction chemotherapy and this correlated with enhanced secretion of IFN-Ɣ by T-cells, following stimulation by Dendritic Cells (DCs) [13]. This suggests that expression of calreticulin on the surfaces of AML cells promotes natural cellular anti-cancer immune responses.

Effects of calreticulin-C1q interaction on C1q-dependent immune responses

Several studies carried out in patients suffering from autoimmune diseases such as Systemic Lupus Erythematosus (SLE) have shown that surface calreticulin binds to and modulates the activities of various molecules of innate immunity, including C1q, the first subcomponent of complement and mannose binding lectin (MBL). Surface calreticulin also binds strongly to CD40 ligand, Tumour Necrosis Factor- Related Apoptosis Inducing Ligand (TRAIL) and Fas Ligand (FasL) [14]; while its peptide-binding site interacts with the collagen-like domain of immunoglobulin-bound C1q to facilitate clearance of immune complexes and apoptotic cells [15], suggesting that binding of surface calreticulin to components of innate immunity might enhance the immunogenicity and clearance of dying tumour cells.

Detailed study of the calreticulin-C1q interaction has also revealed that the N- and P- domains of calreticulin bind strongly to the globular head region of C1q. The N-domain of calreticulin was observed to be the most auto-antigenic, since anti-calreticulin autoantibody reactions occurred against this domain in most SLE patients [16]. Studies have also shown that phosphatidylserine (PS) and calreticulin – both ‘eat me’ signals – could interact to modulate C1q binding to apoptotic cells, including dying cancer cells. Using Surface Plasmon Resonance (SPR) analysis and co-sedimentation techniques, [17]. demonstrated that PS was actively involved in the externalization of calreticulin during apoptotic stress and that the polar head of PS binds directly to calreticulin with an affinity that is 10-fold higher than PS-C1q interaction, suggesting that calreticulin might interfere with PS-C1q interaction by competing with C1q for binding to PS, a key ‘eat me’ signal of apoptotic cells. Further experiments with Small interfering Ribonucleic Acid (Si RNA) against calreticulin also revealed an increase in the binding of apoptotic cells to C1q and consequent increase in their uptake by phagocytes [17]. This observation suggests that a particular domain of calreticulin might bind more to PS than C1q to disrupt the PS-C1q interaction on apoptotic cells and inhibit C1q dependent clearance of dying cancer cells by macrophages. These findings also suggest that the effects of the calreticulin-C1q interaction on immune responses is quite complex and may vary widely, depending on other immune molecules involved, activating cells and some other yet undefined factors.

For instance, results of [18] and [19] all suggest that while the globular head region of C1q might interact with the N- and P- domains of calreticulin to inhibit immune responses of the classical complement pathway, the collagen tail region of C1q is more likely to interact with calreticulin to activate components of innate immunity. The specific conditions under which these varying observations might occur, remains unknown. Detailed effects of the interactions between the N-, P- and C- domains of calreticulin and the globular head region of C1q on the binding of C1q to anthracycline-treated cancer cells is also not known. In the present study, we sought to characterize the interaction between the N-, P- and C- domains of calreticulin and C1q. We also aimed to examine the effect of these interactions on the binding of C1q to anthracycline-induced breast cancer cells.

MATERIALS AND METHODS

Making of competent BL21 E. coli cells

Single colonies of BL21 *E. coli* cells were inoculated in 5 ml of Luria Broth (LB) in sterile falcon tubes overnight at 37^{o}C and 200 rmp in an incubator shaker. Next day, 500 µl of inoculated cells was inoculated in 2.5 ml of LB for one and half hours at 37^{o}C and 200 rmp, until the optical density (OD) reached 0.34. Culture was then placed in ice for 5 minutes, after which it was centrifuged at 4000 revolutions per minute (rpm) for 10 minutes at 4^{o}C. After discarding the supernatant, cells were initially suspended in 1 ml of 0.1 M ice-cold $CaCl_2$, which was then made up to 12.5 ml and placed in ice for one hour. This was followed by centrifuging at 4000 rpm for 10 minutes at 4^{o}C, after which supernatant was discarded and pellets re-suspended in 2 ml of 0.1 M ice-cold $CaCl_2$ and stored at 4^{o}C until when needed.

Transformation of competent cells with pMal-c2 plasmids

1 µl of pMAL-c2 plasmids was added to 200 µl of competent E. coli cells and placed in ice for one hour. Transformation efficiency was optimized by heat-shocking at 42^{o}C for 20 seconds in a water bath, after which cells were returned to ice for 5 minutes and 800 µl of LB added, followed by incubation at 37^{o}C for 45 minutes. Two agar culture plates containing ampicillin were then spread with 50 µl and 100 µl cells and incubated at 37^{o}C overnight. Several colonies of transformed BL21 *E. coli* cells were observed the next day.

Protein expression

Following confirmation of expression of the three domains of calreticulin from results of a pilot expression, up-scaling was done, in order to obtain adequate amounts of proteins. 2 ml each of the primary cultures of the N-, P- and C- domains of calreticulin were incubated in 100 ml of LB in an incubator shaker at 37^{o}C and OD checked at intervals until it reached 0.73, 0.69 and 0.72 respectively.

1 ml of induced and un-induced samples were collected for all three domains of calreticulin as previously described, while the remaining samples were dispensed into sterile 50 ml falcon tubes and centrifuged at 4,500 rpm for 10 minutes. Supernatants were then discarded and pellets stored at - 4^{o}C until needed. Next day, pellets were placed in ice for one hour to thaw the cells, after which the three domains of calreticulin were each suspended separately in 10 ml of lysis buffer and 5 µl of Phenyl Methyl Sulfonyl Fluoride (PMSF) and stirred on a magnetic stirrer. After several rounds of sonication, samples were centrifuged at 13,000 rpm for 10 minutes and 500 µl of supernatant and

pellets was collected for the N-, P- and C- domains of calreticulin. 100 µl of 2 x treatment buffer was then added to all samples collected, including the induced, un-induced, pellets and supernatants for all three domains of calreticulin and heated at 100^{o}C for 10 minutes, using a heat block. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was then run to track the expression of the three protein domains of calreticulin.

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

Glass plates were properly washed, dried and SDS-PAGE apparatus assembled. Using 1 ml pipette and tips, distilled water was introduced between glass plates to test for leakages. In the absence of any leakages, 5 ml of 10% resolving gel was prepared by adding the following amounts of reagents to a small bottle labelled 'R': 1.9 ml of distilled water, 1.7 ml of 30% acrylamide, 1.3 ml of 1.5 M Tris HCL (pH 8.8) and 0.05 ml of 10% SDS. 10% ammonium persulfate (APS) was also freshly prepared by dissolving 0.1 g in 1 ml of distilled water. This was then added to the resolving gel, followed by 10 µl of Tetramethyl ethylene diamine (TEMED), after which the gel was quickly loaded (before commencement of polymerization) and incubated at 37^{o}C to allow polymerization. 5 ml of 10% stacking gel was also prepared by adding 3.4 ml of distilled water, 0.83 ml of 30% acrylamide mix, 0.63 ml of 1.5 M Tris HCL (pH 6.8), 0.05 ml of 10% APS (freshly prepared) and 9 µl of TEMED into a second bottle labelled 'S' and this was immediately loaded onto the set resolving gel.

The comb was quickly applied and gel incubated at 37^{o}C to allow complete polymerization. 10 mls of SDS was then added to 10x running buffer and the volume made up to 1 L with 890 ml of distilled water. After setting up the apparatus, the tank was flooded with the running buffer and comb gently removed, to expose the wells. 5 µl of a 14.4 – 116 kDa pegGold protein marker was then loaded into the first well, while 10 µl of un-induced and induced samples of N-, P- and C-domains of calreticulin were loaded into the second and third; fifth and sixth; and eight and ninth wells respectively. Gel was set to run at 110 V for 90 minutes, after which the apparatus was de-assembled and gel placed in a petri dish flooded with stain and left overnight on a shaker. Next day, stain was discarded, gel cleared with water, flooded with de-stain and returned to the shaker for one hour. De-stain was then changed at intervals until gel was clear to read the bands.

Protein purification

Lysis buffer was prepared from the following volumes of stock solutions: 1 ml of 20 mM Tris HCL (pH 8.0), 5 ml of 500 mM $NaCl_2$, 0.1 ml of 1 mM EDTA (pH 8.0), 20 µl of 0.02% Tween 20, Sodium azide, Lysozyme (final concentration of 100 µg/ml), and 0.1 mM PMSF. 200 ml of column buffer was also prepared, using 4 ml of 20 mM Tris HCL (pH 8.0), 4 ml of 100 mM $NaCl_2$, 2 ml of 1 mM EDTA (pH 8.0), and 10 ml of 5% glycerol. This was divided into two 100 ml bottles and 20 µl of Tween 20 added to one of the 100 ml bottles (column buffer with Tween 20). Elution buffer was then made, containing 4 ml of 20 mM Tris HCL (pH 8.0), 4 ml of 100 mM $NaCl_2$, 0.8 ml of 2 mM $CaCl_2$, 10 mls of 5% glycerol and 10 mM of maltose.

Different columns containing 3 ml (bed volume) of amylose resin were set up for the different domains of calreticulin and washed with 5 x bed volume (15 ml) of distilled water. 5 x bed volumes of 0.1% SDS was then passed through the columns and washed again with 5 x bed volumes of distilled water. This was followed by 5 x bed volumes of column buffer with Tween 20, after which protein samples (supernatants) were diluted with 5 x bed volumes of buffer with Tween 20 and passed through the columns. After collecting all flow through, the columns were washed with 2 x

bed volumes of buffer with Tween 20 and wash collected. 5 x bed volumes of column buffer without Tween 20 was then passed and each column eluted with 1 ml fractions of elution buffer. Eluted protein samples for the N-, P- and C- domains of calreticulin were collected in seven eppendorf tubes for each domain and labelled 1-7, for O.D reading at an absorbance of A_{280} and running of SDS-PAGE. (Fig. 4).

Prior to purification of whole C1q from human serum, non-immune IgG was coupled to CnBr-activated sepharose using the technique described by GE healthcare, UK. 200 ml of human plasma was then made in 5mM EDTA by addition of 200 mM of sodium EDTA (pH 7.4). Next, IgG-sepharose was added to the plasma and incubated on ice for 45 minutes, with occasional gentle stirring. Rapid washing of IgG-sepharose on a sintered glass funnel containing 5 x 50 ml of ice-cold 10 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 140 mM NaCl and 0.5 mM Ethylene diamine tetra acetic acid (EDTA) (pH 7.4) was also done, until the OD_{280} was observed to be less than 0.04. After rapidly packing the IgG-sepharose into the column, bound C1q was eluted using a high pH (11.2) salt buffer containing 50 mM CAPS, 1 M NaCl and 5 mM EDTA. pH of eluted fractions was then lowered by addition of 0.3 volume of 1 M sodium phosphate (pH 7) and analysed by SDS-PAGE (picture not shown).

Calreticulin-C1q interaction using solid-phase assays.

50 µl of purified C1q containing 15 µg (0.3 µg/µl) of protein was diluted with 1.2 ml of carbonate buffer and mixed thoroughly, after which 200 µl was loaded into the top six wells of an ELISA plate. 100 µl of carbonate buffer (not containing C1q) was also added into the next two wells running below the top six wells. 100 µl of buffer-treated C1q was then pipetted from the top six wells and added to the next well below. This was repeated down the columns for the two wells running below the top six wells, with the last 100 µl being discarded for each column thus producing protein concentrations of 1.25 µg and 0.63 µg for the two wells down the column below the top six wells. The enzyme-linked immunosorbent assay (ELISA) plate was then incubated at 4^oC overnight.

Next day, the wells were emptied thoroughly, after which each well was blocked with 100 µl of 2 % Bovine serum albumin (BSA) in Phosphate buffered saline (PBS) with tween and incubated at 37^oC for two hours. After two hours, wells were emptied and washed thoroughly with 100 µl of PBS with tween each. 2.5 µg of purified N-domain of calreticulin was then diluted with 100 µl of $CaCl_2$ buffer and added into the first two columns and labelled appropriately. This was repeated for the P- and C- domains of calreticulin, which were added into third and fourth; and the fifth and sixth columns, respectively. The ELISA plate was then incubated at 37^oC for one hour, followed by incubation at 4^oC for another one hour. After washing with 0.05 % of Tween 20 in PBS for three times, 1:5000 dilution of anti-MBP and PBS was added to each well and incubated at 37^oC for one hour. Wells were then washed with PBS with Tween, after which 1:5000 dilution of Immunoglobulin G-Horseradish Peroxidase (IgG-HRP) was added into each well and plate incubated at 37^oC for one hour. ELISA plate was again washed with PBS with Tween, followed by addition of 100 µl of OPD substrate into each well, after which O.D was read at A_{450}

Effect of C1q interaction with the N-, P- and C- domains of calreticulin on C1q-mediated anti-cancer immunity

15 µl of breast cancer cells and 185 µl of carbonate buffer were loaded into the top four wells of an ELISA plate. 100 µl of carbonate buffer was then added into the next two wells running down the

first three top wells. From the first well, 100 µl was pipetted and added into the next well below. This was repeated for the third well and the last 100 µl was discarded. The procedure was repeated for the second and third columns, while the single well on the fourth column served as control.
Following incubation at 4^{o}C overnight, the wells were thoroughly washed with PBS and 2.5 µg of C1q in 20 mM Tris HCL, 100 mM $NaCl_2$ and 5 mM $CaCl_2$ was added to all the wells. 5 µg of N-domain of calreticulin was then added to the wells of the first column, while 5 µg of P- and C-domains of calreticulin were also added into the wells of the second and third columns respectively. The ELISA plate was then incubated at 37^{o}C for one hour and 4^{o}C for another one hour. After thorough washing, 1:5000 dilution of anti-C1q antibody in PBS was added to all the wells and plate incubated at 37^{o}C for one hour. 1:5000 dilution of protein A in PBS was then added after washing and plate incubated at 37^{o}C for one hour. After washing, OPD was added to all wells and A_{405} units read.

RESULTS AND DISCUSSION

Expression and purification of recombinant N-, P- and C- domains of calreticulin

Chemically competent *E. coli* cells are suitable for transformation and protein expression, with a transformation efficiency of about 1-5 x 10^7 cfu/µg pUC 19 DNA. An important advantage of the BL21 and BL21 (DE3) strains of *E. coli* is that they are both deficient in Lon and Outer Membrane Protein T (OmPT) proteases, with reduced risk of protein degradation. The pMAL^{tm} protein fusion and purification system is also a reliable expression system, with substantial protein yields of up to 100 mg/L in most cases. Fusion to MBP (Maltose Binding Protein) enhances the solubility of proteins expressed in *E. coli* and allows gentle elution with maltose, without the use of detergents or risk of denaturation.
pMAL-c2 plasmids encoding *E. coli* Maltose Binding Protein (MBP)-fused recombinant N-, P- and C- domains of calreticulin were obtained for this study. Recombinant forms of the N-, P-, and C-domains of calreticulin were expressed as fusion proteins containing *E. coli* MBP, first on a pilot scale of 5 ml culture, which was then up-scaled to 100 ml of culture. The N-, P- and C- domains of calreticulin has predicted molecular masses of 20, 16 and 16 kDa respectively. When fused to MBP (42 kDa), these domains migrated as 62, 58 and 58 kDa proteins on a 12% SDS-PAGE gel (Fig. 1-3). Following purification and prior to solid-phase assays, anti-MBP antibody was used to block out binding of C1q to MBP.

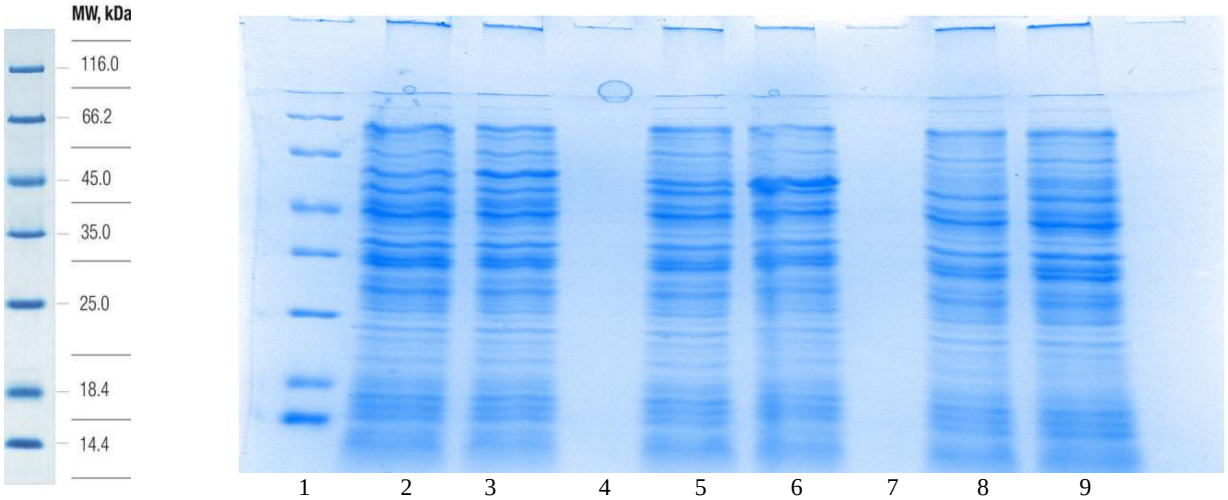

Figure 1: Expression of recombinant proteins showing MBP-fused N-, P- and C- domains of calreticulin which migrated as 62, 58 and 58 kDa proteins respectively on 12% SDS-PAGE gels.

Lane 1: 14.4-116kDa protein marker
Lane 2: Un-induced N-domain of calreticulin
Lane 3: Induced N-domain of calreticulin
Lane 5: Un-induced P-domain of calreticulin
Lane 6: Induced P-domain of calreticulin
Lane 8: Un-induced C-domain of calreticulin
Lane 9: Induced C-domain of calreticulin

The figure above shows the N-, P- and C- domains of calreticulin expressed as proteins fused to MBP in the induced samples, corresponding to the 62kDa, 58kDa and 58kDa bands respectively, on the standard protein marker. The molecular weight of MBP is 42kDa, while those of the N-, P- and C- domains of calreticulin are 20, 16 and 16 kDa respectively.

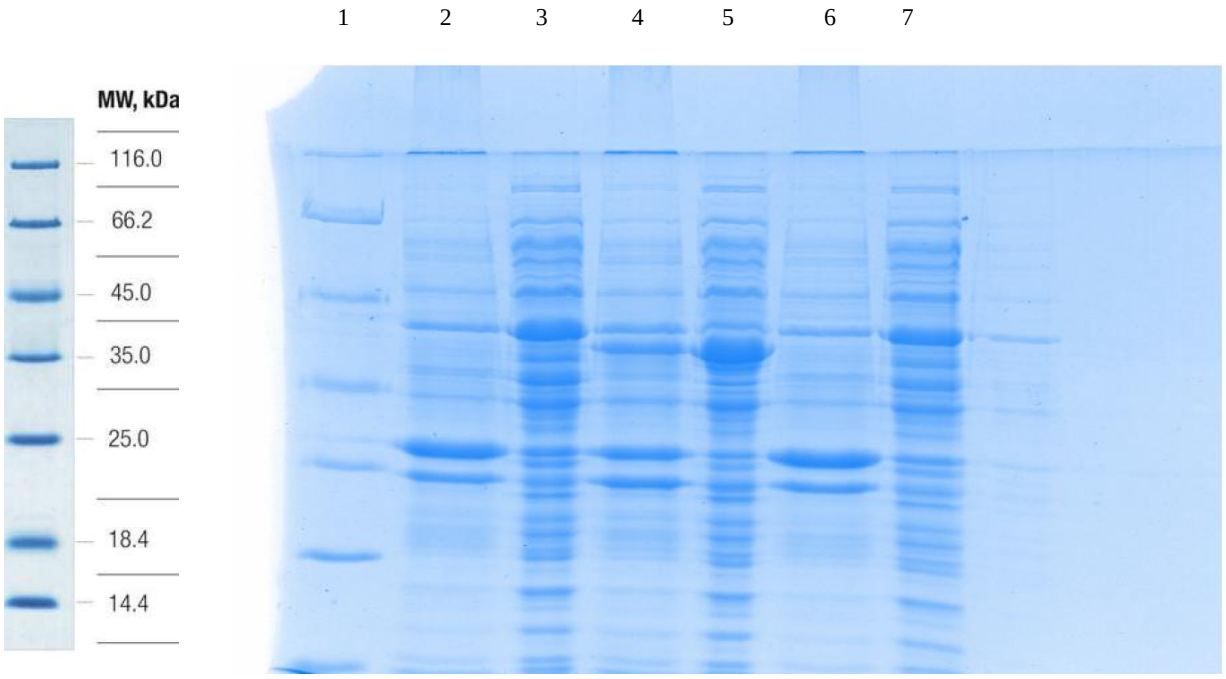

Figure 2: Sonicated pellets and supernatants of MBP-fused N-, P- and C- domains of calreticulin migrate as 62, 58 and 58 kDa proteins respectively on SDS-PAGE

Lane 1**:** 14.4-116 kDa protein marker
Lane 2: Sonicated pellets containing the N-domain of calreticulin
Lane 3: Sonicated supernatant containing the N-domain of calreticulin
Lane 4: Sonicated pellets containing the P-domain of calreticulin
Lane 5: Sonicated supernatant containing the P-domain of calreticulin
Lane 6: Sonicated pellets containing the C-domain of calreticulin
Lane 7: Sonicated supernatant containing the C-domain of calreticulin

Following sonication, samples were run again on a 12% SDS-PAGE. The figure above shows the N-, P- and C- domains of calreticulin all fused to MBP, being expressed more in the supernatant than in the pellets. The lower most bands of the standard protein marker and all protein samples are missing because gel was not allowed to run completely due to time constraint. The N-, P- and C-domains of calreticulin remained expressed at the 62, 58 and 58 kDa band levels on the standard

protein marker. The C-domain was however observed to be expressed at a slightly higher level, probably due to the presence of impurities.

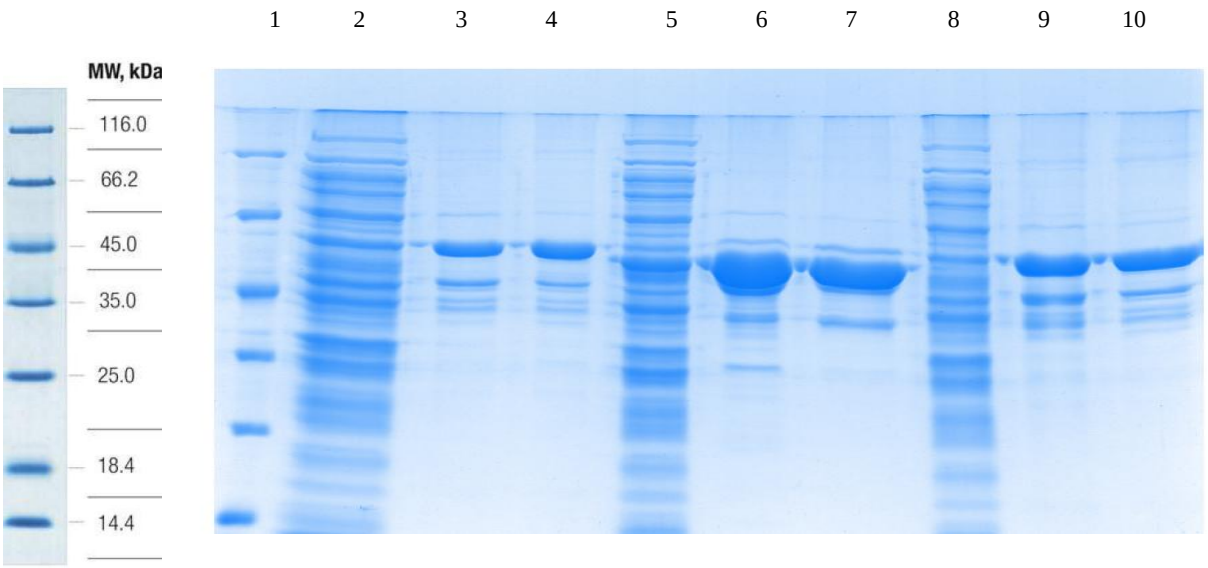

Figure 3: Concentrations of purified proteins obtained from a NANO drop 2000C (thermo scientific) spectrophotometer correlated with SDS-PAGE results.

Lane 1: 14.4-116 kDa protein marker
Lane 2: Flow through collected for the N-domain of calreticulin
Lanes 3 and 4: Purified N-domain of calreticulin
Lane 5: Flow through collected for the P-domain of calreticulin
Lanes 6 and 7: Purified P-domain of calreticulin
Lane 8: Flow through collected for the C-domain of calreticulin
Lanes 9 and 10: Purified C-domain of calreticulin

In figure 3, the SDS-PAGE result correlated well with the protein concentrations obtained from a NANO drop 2000C spectrophotometer. N-calreticulin (62kDa) is clearly seen to be expressed at a higher level than P- and C- calreticulin (both 58kDa). The presence of small amounts of impurities can be observed just below the bands, which could be eliminated by further purification by ion exchange. However, over 90% of the impurities present can be seen in the flow through (lanes 2, 5 and 8) collected for all three domains.

Calreticulin-C1q interaction using solid-phase assays

To characterize the binding of the different domains of calreticulin to C1q, different concentrations of whole human C1q were coated on different wells of an ELISA plate and non-specific bindings blocked with 2% BSA/PBS. Anti-MBP was also used to block possible binding of C1q to MBP. Following addition of O-phenylenediamine Dihydrochloride (OPD) to the wells of the ELISA plate and observation of colour changes, whole human C1q was observed to bind more to the N-domain of calreticulin at the highest C1q concentration of 2.5 µg at all times (between 5-30 minutes), while stronger bindings of C1q to the P-domain of calreticulin were observed at moderate and low concentrations of 1.25 µg and 0.63 µg. (Figs. 4-6). Minimal binding was observed between C1q and the C-domain of calreticulin at all concentrations between 5-30 minutess, in agreement with previous findings by [20]. All bindings occurred in a dose-dependent manner.

C1q binds more to the N-domain of calreticulin at high concentrations but more to the P-domain at moderate and low concentrations, with minimal binding to the C-domain of calreticulin at all concentrations of C1q.

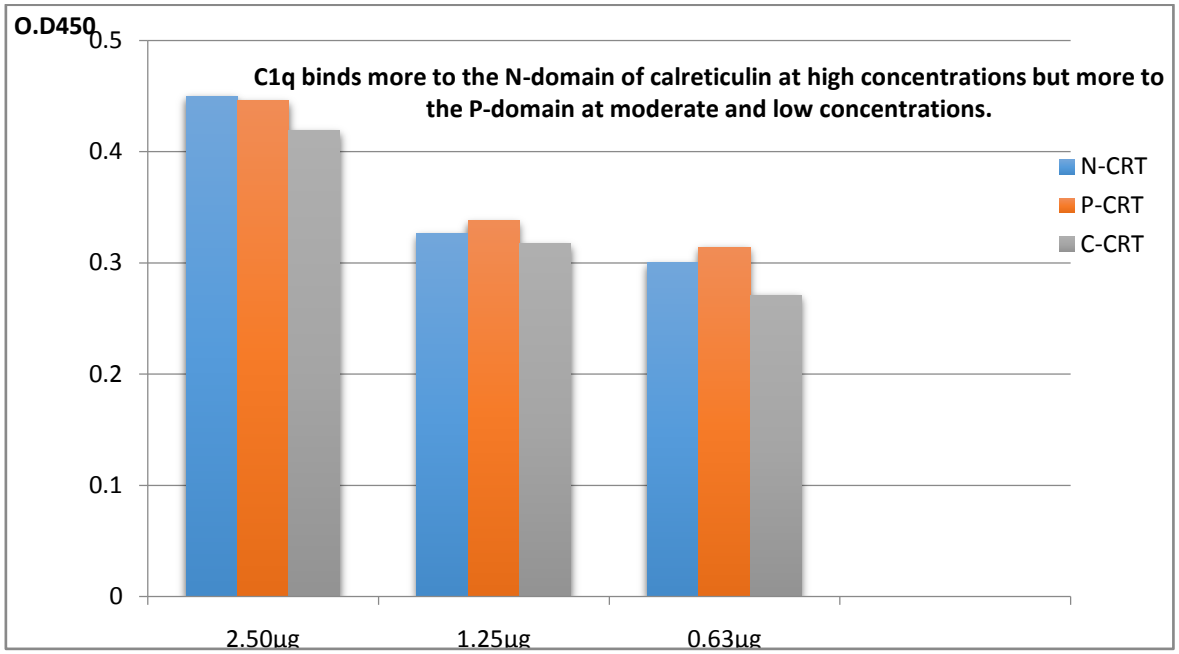

Fig. 4 Binding (A_{450}) of the N-, P- and C- domains of calreticulin to C1q, 5 minutes after addition of 100µl of OPD substrate into the respective wells. Average values were used for chart.

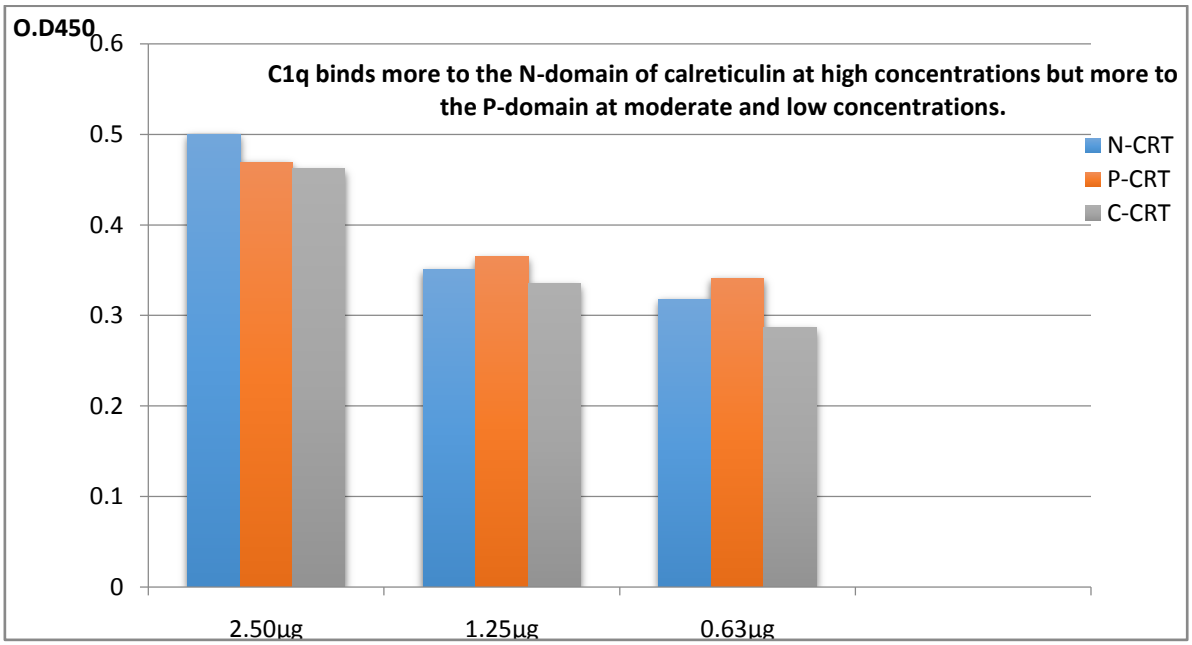

Fig. 5. Binding (A_{450}) of the N-, P- and C- domains of calreticulin to C1q, 15 minutes after addition of 100µl of OPD substrate into the respective wells. Average values were used for chart.

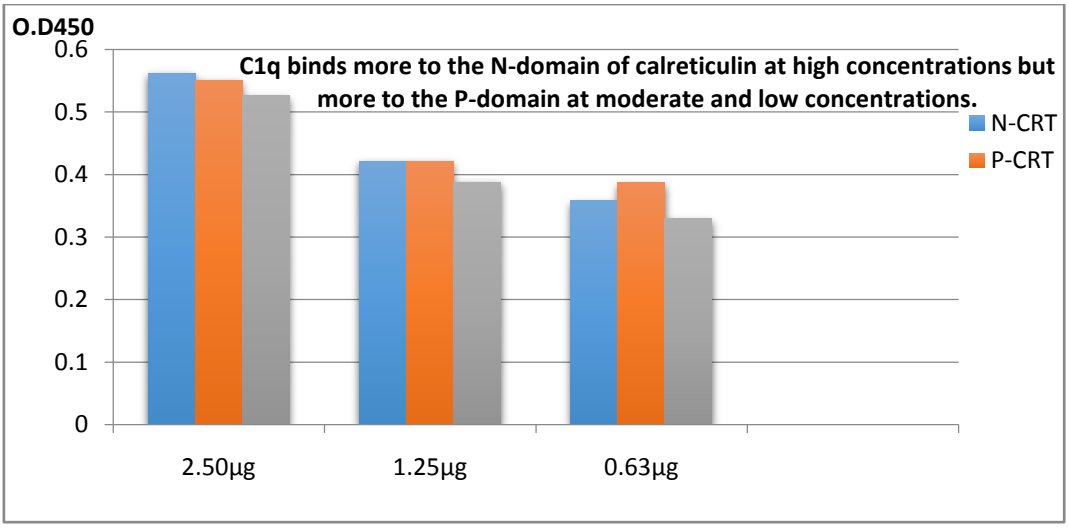

Fig. 6. Binding (A_{450}) of the N-, P- and C- domains of calreticulin to C1q, <u>30 minutes</u> after addition of 100µl of OPD substrate into the respective wells. Average values were used for chart.

The N- and P- domains of calreticulin increase the binding of C1q to anthracyclin-treated breast cancer cells, while the C-domain of calreticulin inhibits C1q binding to anthracyclin-treated breast cancer cells.

In fig. 7, binding of C1q to anthracyclin-induced breast cancer cells was first examined in the absence of any domain of calreticulin and an A_{405} value of about 0.16 was observed. In the presence of the N-domain of calreticulin, C1q binding to anthracyclin-induced breast cancer cells was observed to increase from 0.16 to a value slightly above 0.18 A_{405}, indicating an increase in binding of about 12%. The P-domain of calreticulin was also observed to increase C1q binding to anthracylin-induced breast cancer cells but to a much lower extent of about 2%. The interaction between C1q and the C-domain of calreticulin was however, observed to have an inhibitory effect on the binding of C1q to anthracyclin-induced breast cancer cells. As shown in figures 8 and 9, this interaction reduced the binding of C1q to anthracyclin-induced breast cancer cells from an A_{405} of about 0.16 to somewhere around 0.1 and 0.6 respectively, indicating a 37.5% reduction in binding.

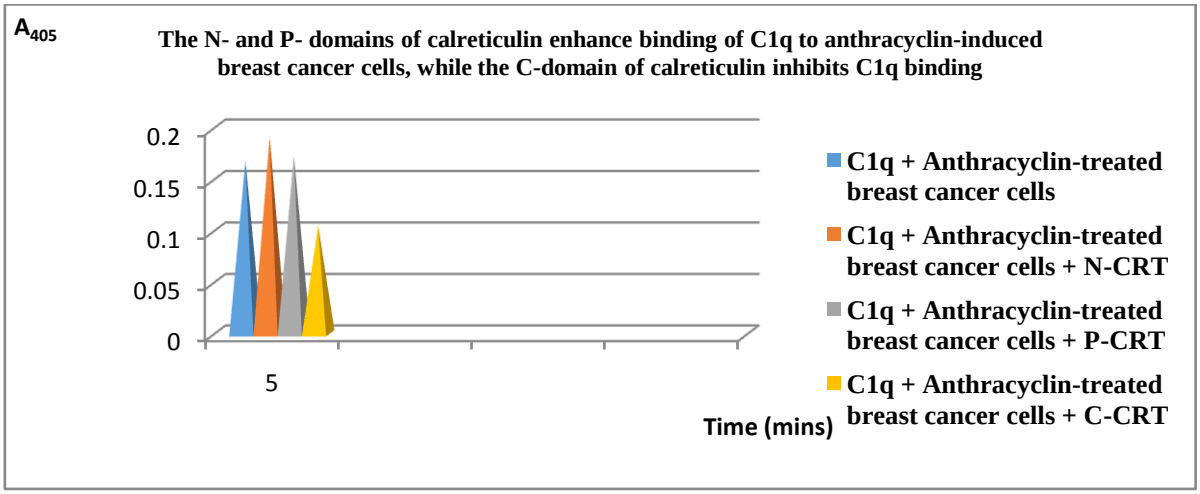

Fig. 7. Effect of C1q interaction with the N-, P- and C- domains of calreticulin on C1q binding to Anthracyclin-induced breast cancer cells, <u>5 minutes</u> after addition of 100µl of OPD substrate into the ELISA wells.

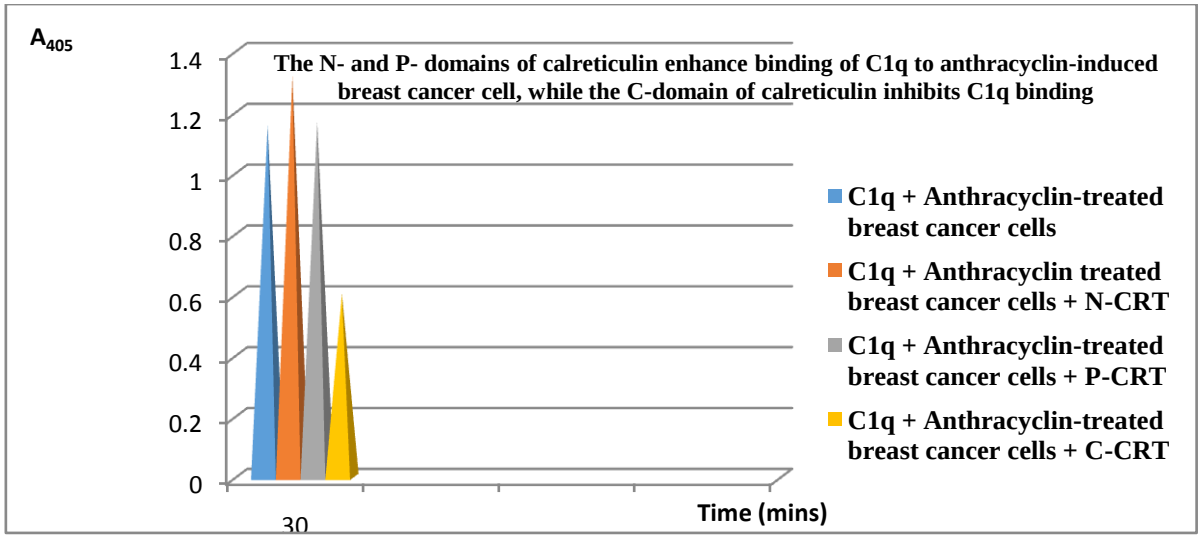

Fig. 8. Effect of C1q interaction with the N-, P- and C- domains of calreticulin on C1q binding to Anthracyclin-induced breast cancer cells, 30 minutes after addition of 100µl of OPD substrate into the ELISA wells.

DISCUSSION

Standard treatment protocol for cancer usually involves surgical resection, chemotherapy and radiotherapy. While surgical removal eliminates the primary tumour and reduces tumour load, radiotherapy and chemotherapy target metastatic tumour cells around the primary tumour and at distant parts of the body. Even with standard treatment, initial positive clinical responses are often followed by recurrence of malignancy several months or years later. Current efforts are therefore aimed at eliminating residual cancer cells by stimulating the body's immune response to fight against metastasized cells that are mainly responsible for cancer relapse.

Stimulation of an immune-mediated killing of cancer cells could therefore be one of the major aims of anticancer chemotherapy [12]. It is hoped that such anticancer immune responses will in principle, function as 'surveillance' to eliminate metastatic and chemotherapy-resistant cancer cells.

Calreticulin is located primarily within the lumen of the endoplasmic reticulum, where it is involved in calcium homeostasis. Varying amounts of the protein have also been detected in many other intracellular and extracellular locations including the nuclear membrane, cytoplasm, plasma, cytolytic T-lymphocytes and cell surfaces, where it is involved in a variety of other functions [21]. Studies have also shown that the amino acid sequence of calreticulin is identical to that of the N-terminal of a particular form of C1q receptor called cC1qR and that the N- and P- domains of calreticulin also bind to the globular head region (gC1q) of C1q [22]. Certain chemotherapeutic agents such as anthracyclins induce the translocation of calreticulin from the lumen of the endoplasmic reticulum to cell surfaces [12] and surface calreticulin is known to interact with a variety of immune molecules such as CD40 ligand, TRAIL, FasL, mannose binding lectin (MBL) and C1q [14]. Interaction between the N- and P- domains of calreticulin and C1q has also been shown to inhibit haemolysis of IgM sensitized erythrocytes [16].

Specifically, surface expression of calreticulin on apoptotic cells stimulates phagocytosis through binding and activation of CD91 on phagocytic cells, thereby enhancing phagocytic activity [18].

Furthermore, the binding of C1q and mannose binding lectin (MBL) to apoptotic cells is known to facilitate clearance of C1q-bound apoptotic cells by both macrophages and immature dendritic cells [23]. C1q has also been shown to prevent the development of autoimmunity through opsonisation and consequent recognition and clearance of apoptotic cells [20]. Binding of C1q to dying anthracyclin-treated cancer cells could therefore enhance their uptake by dendritic cells and macrophages. Although the interaction between C1q and the N- and P- domains of calreticulin has been shown to inhibit C1q-mediated haemolysis of IgM sensitized eruthrocytes, the direct effect of these interactions on chemotherapy-treated cancer cells is unknown.

In the present study, we therefore sought to dissect the effect of C1q interaction with the different domains of calreticulin on C1q binding and consequent killing of anthracyclin-induced cancer cells. We expressed and purified the N-, P- and C- domains of calreticulin as fusion proteins which migrated as 62, 58 and 58 kDa proteins respectively, on a 12% SDS-PAGE gel. Using solid-phase assay methods, C1q was observed to bind more to the N-domain of calreticulin at a high C1q concentration of 2.5µg, while a higher binding to the P-domain of calreticulin was observed at moderate and low concentrations of C1q. Minimal interaction occurred with the C-domain of calreticulin at all concentrations between 5 to 30 minutes. C1q interaction with N-, P- and C-domains of calreticulin all occurred in a dose-dependent manner.

We also observed that the N- and P- domains of calreticulin interacted with C1q to increase C1q binding to anthracycline-induced breast cancer cells by about 12% and 2% respectively. Previous studies have suggested that calreticulin play a central role in the immunogenicity of chemotherapy-induced cancer cells, as knockdown of calreticulin was observed to impair anti-cancer immune responses. Coating of cell surfaces with recombinant calreticulin has also been observed to promote immune responses against cancer cells treated with anti-cancer drugs [12].

Studies by [24] had suggested that presence of whole calreticulin on cell surfaces could promote activation of complement cascade, immunogenicity of cancer cell and clearance of apoptotic cells. In contrast to whole calreticulin, our results suggest that the C-domain of calreticulin inhibited the binding of C1q to anthracyclin-treated breast cancer cells by about 38% after 5 minutes of interaction with C1q and by as much as 48% after 30 minutes of C-calreticulin binding to C1q. This observation is significant, considering the minimal interaction that was observed between C-calreticulin and C1q and suggests a very strong affinity between C-calreticulin and its binding site on C1q.

Based on these findings, we further suggest that the site (globular head region) on C1q to which the N- and P- domains of calreticulin bind, might differ from an unknown site to which the C-domain of calreticulin binds. We also suggest that C1q interacts with anthracyclin-induced breast cancer cells through this undefined site to which C-calreticulin binds, thereby competitively inhibiting binding of C-calreticulin to both C1q and anthracycline-treated breast cancer cells. This again is in tandem with observations by [17], which suggested that the C1q receptor (calreticulin) could function as a bridging molecule between phagocytic cells and other ‘eat me’ signals on the surfaces of apoptotic cells. Occupation of this site by C-calreticulin and consequent interaction between C-calreticulin and other ‘eat me’ signals on the surfaces of anthracyclin-treated breast cancer cells would therefore effectively inhibit binding of C1q to anthracyclin-treated dying cancer cells. [20] have

previously observed that calreticulin binds predominantly to whole C1q and its globular head region, with negligible binding to the collagen tails of C1q. It is however, not known whether the collagen tail of C1q contains the yet undefined site to which the C-domain of calreticulin binds minimally to inhibit C1q binding to cancer cells. Our suggestion of a possible undefined binding site for the C-domain of calreticulin on the globular head or collagen-like tail regions of C1q is further strengthened by previous observations by [25], who suggested that these regions of C1q binds to a large number of ligands, resulting in its involvement in a wide range of biological functions such as complement activation, migration of neutrophils and eosinophils, chemotaxis of fibroblasts, activation of microglial cells of the CNS and macrophage-mediated clearance of apoptotic cells [25]. Similarly, [20] also observed that new discoveries concerning the structure-function relationship of the gC1q domain of C1q opened up more issues for discussion. Our findings here is also in complete agreement with this observation by suggesting that some unidentified binding sites might still exist within the head and tail regions of C1q.

While the 33kDa receptor for the globular head region of C1q known as gC1qR binds strongly to the globular head region (gC1q) of C1q, the calreticulin molecule previously known as cC1qR, binds strongly to the collagen-like tail region of C1q [25]. Binding between the collagen-like tail region of C1q and calreticulin has also been identified by other studies including those by [18]. These studies however, did not specify the domains of calreticulin involved in such interactions. Our search through the literature did not reveal any results specifying the sites on the collagen-like tail region of C1q where these bindings might occur. It is also possible that this site might differ from any previously defined site via which C1q is known to interact with other cancer cells or surface antigens.

Furthermore, there is little information concerning the level of similarity between the amino acid sequence of the different domains of calreticulin and that of the N-terminal of cC1qR or gC1qR. However, our results suggest that the N- and P- domains of calreticulin might carry amino acid sequences that are more identical to that of the N-terminal of gC1qR, when compared with C-calreticulin, resulting in their higher binding at this site, as previously identified by [20]. In line with previous observations, an increase in the binding of C1q to the N- and P- domains of calreticulin that is based on such sequence similarity would enhance C1q-dependent macrophage-mediated clearance of anthracyclin-induced breast cancer cells, hence immunogenicity of these cells.
In the absence of its C-domain, the N-, and P- domains of calreticulin will therefore bind to the globular head region of C1q to enhance C1q interaction with drug-induced breast cancer cells at the undefined site. Although surface exposure of full length calreticulin is known to facilitate immunogenicity of dying cancer cells, the effect of surface exposed full length calreticulin interaction with C1q on C1q binding to chemotherapy-treated cancer cells remain unknown. However, we suggest that developing antibodies against the C-domain of a full length calreticulin might be a first step towards enhancing the binding of C1q to dying cancer cells and this will have implications in the immunogenicity of anthracyclin-treated cancer cells.

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