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The CCL2 chemokine is a negative regulator of autophagy and necrosis in luminal B breast cancer cells

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Abstract

Luminal A and B breast cancers are the most prevalent forms of breast cancer diagnosed in women. Compared to luminal A breast cancer patients, patients with luminal B breast cancers experience increased disease recurrence and lower overall survival. The mechanisms that regulate the luminal B subtype remain poorly understood. The chemokine CCL2 is overexpressed in breast

cancer, correlating with poor patient prognosis. The purpose of this study was to determine the role of CCL2 expression in luminal B breast cancer cells. Breast tissues, MMTV-PyVmT and MMTV-Neu transgenic mammary tumors forming luminal B-like lesions, were immunostained for CCL2 expression. To determine the role of CCL2 in breast cancer cells, CCL2 gene expression was silenced in mammary tumor tissues and cells using TAT cell-penetrating peptides non-covalently cross linked to siRNAs (Ca-TAT/siRNA). CCL2 expression was examined by ELISA and flow cytometry. Cell growth and survival were analyzed by flow cytometry, immunocytochemistry, and fluorescence microscopy. CCL2 expression was significantly increased in luminal B breast tumors, MMTV-PyVmT and MMTV-Neu mammary tumors, compared or normal breast tissue or luminal A breast tumors. Ca-TAT delivery of CCL2 siRNAs significantly reduced CCL2 expression in PyVmT mammary tumors, and decreased cell proliferation and survival. CCL2 gene silencing in PyVmT carcinoma cells or BT474 luminal B breast cancer cells decreased cell growth and viability associated with increased necrosis and autophagy. CCL2 expression is overexpressed in luminal B breast cancer cells and is important for regulating cell growth and survival by inhibiting necrosis and autophagy.

Keywords

CCL2; siRNA; TAT cell-penetrating peptide; Luminal breast cancer; Necrosis; Autophagy

Introduction

With approximately 174,000 cases per year, luminal breast cancers are the most common forms of breast cancer diagnosed in the US and Canada [1, 2]. In contrast to Her2-overexpressing breast cancers, or triple negative breast cancers, which lack expression of estrogen receptor (ER), Progesterone receptor, and Her2, luminal breast cancers frequently express hormone and Her2 receptors, rendering these luminal breast cancers responsive to anti-hormonal and Her2-targeted therapies [2–4]. Gene profiling studies have revealed that a subset of luminal breast cancers show high expression of cell proliferation genes. This poorly understood subset, classified as luminal B, is significantly associated with lower patient survival, and is less responsive to anti-hormonal therapy compared to luminal A breast cancers [1, 3, 5]. Understanding the mechanisms that drive luminal B breast cancers would contribute to improved treatments tailored specifically to this disease.

CCL2 (MCP-1) is a chemotactic cytokine (8 kDa), which binds to CCR2 G-protein-coupled receptors to regulate macrophage recruitment during normal processes such as wound healing [6–13], and during inflammatory diseases such as rheumatoid arthritis [14, 15]. CCL2 is overexpressed in breast cancer tumors [16–18]. CCL2 expression in the stroma correlates with macrophage recruitment and poor patient prognosis [18, 19]. Antibody neutralization of CCL2 in tumor-bearing mice inhibits breast tumor growth and metastasis, and reduces the number of M2 polarized macrophages in primary tumors [19, 20]. Macrophage depletion through clodronate treatment or knockout of the CSF-1 Receptor in tumor-bearing mice inhibits tumor growth and metastasis, associated with a reduction in angiogenesis, cell proliferation, and survival [19, 21]. These studies demonstrate an important role for CCL2-mediated macrophage recruitment in breast cancer progression.

Recent studies showed that CCL2 was highly expressed in tumor epithelium, and signaled directly to breast cancer cells, to regulate cell survival, migration, and invasion through CCR2-dependent mechanisms [22, 23]. With few analytical tools, the role of epithelial CCL2 expression has not been well studied. Neutralizing antibodies block CCL2 activity non-specifically in tissues [19, 20, 24]. Small interfering RNAs (siRNAs) could selectively inactivate critical oncogenes if coupled to a carrier that targeted carcinoma cells [25, 26]. The HIV-1 derived trans-activating transcriptor peptide (TAT$_{49-57}$: RKKRRQRRR) exhibits unusual properties by efficiently penetrating cell membranes, independent of temperature or cell surface receptor expression [27]. In recent studies, TAT peptides calcium cross linked to siRNAs or plasmids (Ca-TAT/siRNA or Ca-TAT/plasmid) formed stable nanoparticles that transfected basal and lung carcinoma cells, with lower toxicity and higher efficiency than conventional polymeric carriers or TAT peptides alone [28, 29]. Thus, Ca-TAT peptides may be used to block CCL2 expression and activity in breast carcinoma cells.

The relevance of CCL2 expression in specific subtypes has remained unclear. We show for the first time that CCL2 expression is overexpressed in luminal B breast cancer. Using Ca-TAT/siRNA complexes to knockdown CCL2 expression in *ex vivo* tissue models and in breast cancer cell lines, we demonstrate that epithelial-specific CCL2 positively regulates carcinoma cell growth, and negatively regulates necrosis and autophagy. These studies reveal novel mechanisms for the regulation of luminal B breast cancers, and identify a potentially important therapeutic target for this breast cancer subtype.

Methods

Tissue microarrays

Paraffin-embedded breast cancer tissue microarrays were obtained from the National Cancer Institute Diagnostics Program. The arrays were composed of 24 cores of normal breast tissue 1 mm in diameter (6 cores/slide), and 400 cores of invasive breast ductal carcinoma 0.6 mm in diameter (100 cores/slide). Using clinical criteria previously validated [2, 30, 31], luminal A breast cancers were identified as ER+ and/or PR+, Her2−, with a mitotic score of 1. Luminal B breast cancers were identified as ER and/or PR+, Her2+ or Her2−, with a mitotic score of 2+.

Tissue harvesting

MMTV-PyVmT positive animals were generated as described [32], and were maintained in accordance with AAALAC and University of Kansas Medical Center guidelines. Late-stage carcinomas were harvested from PyVmT transgenic females 14–16 weeks of age for *ex vivo* cultures, or were fixed in 10 % neutral formalin buffer (10 % NBF) for immunohistochemistry, as described previously [33]. MMTV-Neu tumors derived from FVB mice 8–10 weeks of age were kindly provided by Harold L. Moses, M.D (Vanderbilt University Medical Center). Normal mammary glands were harvested from age-matched wild type FVB females.

Immunohistochemistry

Five micron sections were stained for CCL2 expression as described [22]. Briefly, sections were heated in 1 M urea, blocked in PBS containing 5 % rabbit serum, and incubated with antibodies (1:100) to murine or human CCL2 (Santa Cruz Biotechnology), overnight at 4 °C. CCL2 expression was detected using secondary goat biotinylated antibodies (Vector Laboratories, 1:1000 dilution), conjugated to streptavidin peroxidase (Vector Laboratories). Reactions were catalyzed with 3,3′-Diaminobenzidine (DAB) substrate (Dako).

Cell culture

Sum225 cells originated from Asterand. Raw 264.7, BT474, MCF10A, and MCF-7 cell lines were obtained from the American tissue culture collection. PyVmT carcinoma cells were isolated from transgenic mice (Jackson Laboratories), as described [23, 34]. All cell lines were cultured as commercially specified. Normal fibroblasts were isolated from mouse mammary tissue, validated, and cultured as described [33].

siRNA reagents

Sense and anti-sense oligonucleotides were synthesized and annealed by Dharmacon Fisher. The following siRNA targeting sequences were designed: enhanced green fluorescent protein (eGFP) [35] as a negative control: 5′-GCUGACCCUGAAGUUCAUC-3′, mCCL2si: 5′-AAACCUGGAUCGGAACCAA-3′, huCCL2si1: 5′-ACCUGCUGUUAUAACUUCA-3′, huCCL2si2: 5′-CAGCAAGUGUCCCAAAGAA-3′.

Preparation of Ca-TAT complexes

The following formula was used to determine the amount of TAT peptide needed for a specific N/P ratio per μg of DNA or siRNA: μg of TAT $= 0.446 \times (N/P$ ratio$) + 0.116$. TAT peptides were mixed with siRNA or plasmid DNA in 45 μl sterile deionized water containing: 37.5, 75, or 112.5 mM $CaCl_2$. The solution was pipetted 20 times and incubated on ice for 20 min. For in vitro studies, these complexes were added directly to cells. For *ex vivo* cultures, 25 μl of 10 % glucose was added to Ca-TAT complexes. Ca-TAT complexes were diluted in PBS to a total volume of 100 μl, before injection.

Luciferase assay

40,000 cells/well were plated in duplicate onto 24-well plates, and transfected with Ca-TAT peptides complexed to 2 μg MSCV-luciferase plasmid(Addgene) for 48 h. Cells were analyzed for luciferase activity using the Promega Luciferase Assay System, and a Veritas Microplate Luminometer.

ELISA

40,000 cells/well were seeded in duplicate onto 24-well plates, and incubated in 400 μl/well serum-free DMEM for 24 h. Conditioned media were analyzed by murine or human CCL2 ELISA kit (Peprotech). Reactions were catalyzed using tetramethylbenzidine substrate (RnD systems). Absorbance was read at OD 450 nM using a BioTek Microplate Reader.

Ex vivo culture

Using a scalpel, tumors were partitioned into 0.125 cm^3 sizes, placed in mesh filters (70 micron pores) fitted to 6-well plates, and cultured in DMEM/10 % FBS with antibiotics for 24 h. Ca-TAT peptides were complexed to 10 μg siRNA, and injected into four different areas of the tumor at 25 μl increments using a 27-gage needle. Tissues were incubated for 48 h, before analysis.

Flow cytometry

PyVmT mammary tumors were digested into single-cell suspensions, as described [36], and then fixed in 10 % NBF overnight at 4 °C. Cells were permeabilized with 0.1 % Triton-X-100 at 37 °C for 15 min, washed in PBS, incubated with the following antibodies overnight at a 1:50 dilution in PBS/2 % BSA: murine CCL2 (Santa Cruz Biotechnology), Ki67 (Santa Cruz Biotechnology), HMGB1 (Cell Signaling Technology) or LC3B (Invitrogen), CD24 (Santa Cruz Biotechnology), Fsp1 (Abcam) or Cd11b-FITC (BD Biosciences). CCL2 or CD24 proteins were detected by anti-goat-Alexa-488 (Life Technologies, 1: 500 dilution), on ice for 1 h. Ki67, HMGB1, LC3B, or Fsp1 proteins were detected by anti-rabbit-Alexa-647 (Life Technologies, 1: 500 dilution). Cells were washed with PBS three times, before analysis on an LSRII Flow cytometer.

Propidium iodide assay

500,000 cells/well were seeded in 6-cm dishes for 24 h. Cells were transfected with Ca-TAT peptides complexed to 6 μg siRNA for 48 h. Cells were detached by 5 mM EDTA treatment and stained with 1 μg/ml of PI for 15 min at room temperature. Cells were assayed using an LSRII Flow cytometer.

Immunocytochemistry

10,000 cells/well were seeded in triplicate onto 96-well plates for 24 h. Cells were transfected with Ca-TAT peptides complexed to 500 ng siRNA for 48 h. Cells were fixed in 10 %NBF, methanol permeabilized, blocked with PBS/3 % FBS, and immunostained overnight with antibodies to Ki67 (Santa Cruz Technology, 1:50 dilution), HMGB1 (Cell Signaling Technology, 1:100 dilution), LC3B expression (Cell Signaling Technology, 1:100 dilution), and conjugated to rabbit-biotinylated antibodies (1:1000 dilution). Expression was detected by incubation with streptavidin-peroxidases and DAB substrate. Three fields per sample were captured using a Motic AE31 inverted microscope. Protein expression was quantified by Image J [23], and normalized to hematoxylin counterstain.

Viability/cell count growth assay

8000 cells/well were seeded in triplicate onto 96-well plates for 24 h. Cells were transfected with Ca-TAT complexed to 500 ng siRNA for up to 48 h, washed with PBS, detached by trypsin and stained with 0.1 % trypan blue. The number of trypan blue-positive cells was counted by hemocytometer and normalized to the total number of cells per field. Brightfield images were captured at ×10 magnification using a Motic microscope. Growth was measured by the total number of viable cells at 24 and 48 h.

Autophagy assay

100,000 cells were seeded onto coverslips coated with 0.1 % gelatin. Cells were transfected with Ca-TAT complexed to 5 μg siRNA for 48 h. Cells were incubated with Cyto-ID® green dye uptake as described (Enzo Life Sciences). Images were captured at ×20 magnification using a Motic microscope. Uptake was quantified by Image J [23], and normalized to DAPI counterstain.

Statistical analysis

Experiments were performed in a minimum of triplicate. Data are expressed as mean ± SE of the mean (SEM). Statistical analysis was performed using Mann–Whitney two-sample test or ANOVA with Bonferroni post-hoc comparisons using Graphpad Software. Statistical significance was determined by $p < 0.05$. *$p < 0.001$, **$p < 0.01$, ***$p < 0.05$, ****$p > 0.05$.

Results

Expression patterns of CCL2 in luminal breast cancer

To determine the relevance of CCL2 expression in luminal breast cancer, we analyzed patient samples for CCL2 expression by immunohistochemistry. CCL2 expression was lowly expressed in normal breast epithelium, and in some stromal cells including fibroblasts and macrophages. Luminal B breast tumors showed the highest levels of CCL2 expression, compared to luminal A breast tumors and normal breast tissues (Fig. 1a). Animal models are vital for the testing of new molecular therapies [37, 38]. Therefore, we analyzed CCL2 expression in mouse mammary tumors induced by polyoma virus middle T antigen (PyVmT) or Neu overexpression, under the control of the mouse mammary tumor virus promoter (MMTV). PyVmT and Neu transgenic mice form invasive mammary tumors histologically similar to breast ductal carcinomas [34, 35], and are of luminal B classification [39, 40]. Similar to patient samples, CCL2 was more highly expressed in MMTV-PyVmT and MMTV-Neu mammary tumors compared to normal tissues (Fig. 1b, c). These studies demonstrate increased CCL2 expression in luminal B breast cancer.

Calcium chloride and charge ratios modulate transfection efficiency to mammary carcinoma cells

To study the functional role of CCL2 in luminal B breast cancer, we designed a strategy to silence CCL2 expression in mammary carcinoma cells using Ca-TAT peptides. Studies have indicated that transfection efficiency of Ca-TAT/siRNA or Ca-TAT/plasmid complexes depends on cell type, $CaCl_2$ concentration, and charge ratio (N/P) ratio of amino groups to phosphate groups [28, 29]. To optimize transfection conditions for mammary carcinoma cells, PyVmT cells were transfected with Ca-TAT/luciferase plasmid complexes, formulated at varying $CaCl_2$ concentrations and N/P ratios. Ca-TAT/plasmid complexes formulated at $N/P = 5$ in 75 mM $CaCl_2$ induced the highest level of luciferase activity in PyVmT mammary carcinoma cells (Fig. 2a). Mesenchymal cells such as mammary fibroblasts and macrophages, showed minimal luciferase activity at this formulation. These data indicate that Ca-TAT/plasmid complexes preferentially transfected mammary carcinoma cells.

Using this formulation, PyVmT cells were transfected with Ca-TAT peptides complexed to control or CCL2 siRNAs (mCCL2si) complexes. Ca-TAT/mCCL2si complexes significantly knocked down CCL2 expression in PyVmT cells, but not in cultured macrophages or fibroblasts (Fig. 2b). While cultured PyVmT cells showed lower levels of CCL2 compared to stromal cells, flow cytometry analysis revealed that carcinoma cells comprised the bulk (75%) of PyVmT tumor tissues (Supplemental Fig. 1). These studies indicate that Ca-TAT/ plasmid or Ca-TAT/siRNA complexes formulated at $N/P = 5$ and 75 mM $CaCl_2$ optimally transfect mammary carcinoma cells, a major source for CCL2 expression in tumor tissues.

CCL2 gene silencing inhibits cell growth and survival in PyVmT mammary tumor tissues

To determine the functional relevance of CCL2 expression in the context of tumor tissues, we used a modified *ex vivo* tissue culture system [41]. This efficient and cost-effective system allowed us to control for the size of tissue samples and dosage of Ca-TAT/siRNA delivery, minimizing potential variations in treatment. PyVmT mammary tumors were cultured in suspension (Fig. 3a), injected with Ca-TAT/siRNA complexes, and analyzed by flow cytometry. Injecting tumor samples with Ca-TAT/mCCL2si complexes significantly reduced CCL2 protein expression by 40 % (Fig. 3b). CCL2 expression was not affected in normal mammary tissue (Fig. 3b), indicating a preference for Ca-TAT/siRNA complexes in targeting mammary tumor tissues.

We analyzed the effects of CCL2 knockdown on expression of markers associated with cell proliferation or cell death. Compared to control siRNA-treated tumors, tumors injected with Ca-TAT/mCCL2si complexes showed a 30 % decrease in Ki67 expression (Fig. 4a), indicating decreased cell proliferation [2]. CCL2 knockdown did not significantly affect expression of cleaved caspase-3 (Fig. 4b), a marker for apoptosis [41]. CCL2 knockdown significantly decreased intracellular HMGB1 expression by 33 % (Fig. 4c), indicating increased necrosis [42, 43]. CCL2 knockdown increased expression of LC3B by 30 % (Fig. 4d), demonstrating increased autophagy [44]. These studies indicate that CCL2 knockdown inhibited PyVmT tumor cell proliferation and survival in *ex vivo* cultures.

CCL2 gene silencing enhances necrosis and autophagy in cultured PyVmT mammary carcinoma cells

We next determined which cell types were most affected by CCL2 knockdown, using cultured cells. Ca-TAT/mCCL2si complexes significantly decreased cell proliferation in PyVmT cells, as determined by Ki67 expression (Fig. 5a), and cell count assay (Supplemental Fig. 2). Moreover, CCL2 knockdown significantly increased trypan blue staining in PyVmT cells (Fig. 5b). Ca-TAT/siRNA complexes did not affect cell proliferation or viability of macrophages or fibroblasts (Supplemental Fig. 3). These studies indicate that Ca-TAT/mCCL2si complexes selectively inhibited PyVmT carcinoma cell proliferation and survival.

We further examined the effects of CCL2 silencing on necrosis and autophagy in cultured cells. PyVmT mammary carcinoma cells were examined for changes in propidium iodide (PI) staining, which labels cells undergoing cellular necrosis [45]. Consistent with decreased HMGB1 expression in *ex vivo* cultures, CCL2 knockdown significantly increased the

number of PI+ cells, indicating increased cellular necrosis (Fig. 6a). To examine the role of CCL2 expression on cellular autophagy, PyVmT cells were transfected with Ca-TAT/siRNA complexes, and examined for live cell uptake of Cyto-ID® substrate, which labels lysosomal/autophagic vacuoles. CCL2 knockdown significantly increased the uptake of Cyto-ID® (Fig. 6b), which corresponded to increased trypan blue staining (Fig. 5b). In summary, CCL2 knockdown in PyVmT mammary carcinoma cells enhances necrosis and cellular autophagy, ultimately decreasing cell viability.

CCL2 gene silencing enhances necrosis and autophagy of luminal B breast cancer cells

To determine the relevance of CCL2 expression in human breast cancer cells, we examined: BT474 and Zr-75-1, luminal B breast cancer cell lines, MCF-7 and Sum225, luminal A cell lines and MCF10A, a benign breast epithelial cell line [46]. The molecular subtypes of the breast cancer cell lines were characterized in studies on gene expression profiling, and ER, PR, and Her2 expression patterns of breast cancer cell lines [30, 47–49]. By ELISA, BT474 and Zr-75-1 conditioned medium showed the highest levels of CCL2 expression (Fig. 7a). Next, we examined the effects of CCL2 knockdown on BT474 cell growth and survival using human-specific siRNA sequences (huCCL2si1 and huCCL2si2), which targeted two non-overlapping regions of CCL2 (Supplemental Fig. 4). Ca-TAT delivery of huCCL2si1 or huCCL2si2 to BT474 cells significantly reduced CCL2 expression, compared to control siRNA delivery (Fig. 7b). CCL2 knockdown using either siRNA decreased cell proliferation as indicated by decreased Ki67 expression (Fig. 7c), and cell count assay (Supplemental Fig. 5). Moreover, CCL2 knockdown increased expression of HMGB1 and LC3B proteins, corresponding to increased trypan blue staining (Fig. 7d–f). These studies indicate that CCL2 positively regulates cell growth and negatively regulates necrosis and autophagy in BT474 luminal B breast cancer cells.

Discussion

The role of CCL2 in regulating macrophage recruitment during breast cancer progression is well recognized [14, 15, 19, 20]. Here, we demonstrate that CCL2 is important for promoting luminal B breast cancer cell proliferation and negatively regulating necrosis and autophagy independent of macrophage recruitment. We previously demonstrated an important role for paracrine CCL2 signaling in regulating migration and apoptosis, but not autophagy, necrosis, or proliferation of breast cancer cells [22]. These results suggest a unique role for autocrine CCL2 signaling in breast cancer cells in modulating breast cancer cell growth and survival.

Previous studies demonstrated that paracrine CCL2 signaling positively regulating cell migration and negatively regulated apoptosis through Smad3- and MAPK-dependent mechanisms. However, it is not likely that these same pathways are involved in autocrine CCL2-mediated proliferation, autophagy and necrosis, breast cancer cells alone expressed low to undetectable levels of these phosphorylated proteins [22]. In prostate cancer cells, recombinant CCL2 treatment inhibited autophagic cell death and enhanced cell proliferation and migration through PI3-K-, AKT-, and AMPK-dependent mechanisms [50–52]. It is possible that autocrine CCL2 signaling modulates breast cancer cell proliferation and

autophagy through similar mechanisms. In addition, necrosis is modulated by a number of pathways, including RIPK1/3 and poly(ADP-ribose) polymerase to inhibit glycolysis and deplete ATP levels [53, 54]. Studies are currently being conducted to understand how autocrine CCL2 signaling integrates cell proliferation with survival in breast cancer cells.

We show that CCL2 knockdown in luminal B breast cancer cells enhanced necrosis. Breast tumors frequently exhibit central necrosis, a factor associated with poor prognosis [55, 56]. Functional studies indicate that aggressive tumor growth contributes to necrosis, as proliferative cells consume the existing pool of nutrients and promote a hypoxic environment [57–59]. Hypoxia induces angiogenesis and macrophage recruitment through HIF-1-dependent mechanisms [60, 61]. Necrosis itself benefits tumor progression by releasing HMGB1 from cells into the extracellular space. HMGB1 acts as a pro-inflammatory cytokine to enhance the recruitment of M2 polarized macrophages [43]. Anti-cancer drug treatment also enhances necrosis in solid tumors. However, these drugs also inhibit cell proliferation, suppressing tumor growth [54]. These studies indicate that cellular necrosis may be tumor suppressive when accompanied by decreased tumor cell proliferation. As CCL2 knockdown enhances necrosis and decreases breast cancer cell proliferation, CCL2 functions as a tumor promoter in luminal B breast cancer.

We showed that CCL2 knockdown enhanced autophagy of breast cancer cells. While autophagy enhances cancer cell survival to conserve energy during metabolic stress, cytotoxic drugs such as the mTOR inhibitor RAD001 and sorafenib induce and sustain autophagy, leading to cell death in cancers including: glioma, hepatocellular carcinoma, and ovarian cancer [62]. In prostate cancer, CCL2 protects against autophagic cell death induced by serum deprivation and rapamycin [52]. Here, CCL2 silencing in breast cancer cells enhanced LC3B expression correlating with decreased survival. These results indicate that CCL2 expression in breast cancer cells may protect against autophagic cell death. Alternatively, CCL2 may enhance autophagy as an initial protective response to necrosis signals. To distinguish these possibilities, it would be necessary to conduct further biochemical studies on CCL2-mediated autophagy and necrosis in breast cancer cells.

Tissue specificity and penetration are major challenges in nanomedicine [63, 64]. Here, we demonstrated that Ca-TAT/mCCL2si complexes penetrated PyVmT mammary tumors preferentially over normal tissues to significantly reduce CCL2 expression and inhibit mammary tumor cell proliferation and survival. Net ionic charge and nanoparticle size are two factors modulating specificity of Ca-TAT. Complexing TAT peptides with siRNAs with $CaCl_2$ results in a net positive charge of Ca-TAT complexes [28]. $CaCl_2$ also condenses TAT/siRNA complexes to nanoparticle sizes. These positively charged nanocomplexes may interact with negatively charged phospholipids to form micelle structures, which facilitate entry into the cell [28, 65, 66]. Furthermore, differences in cell membrane organization could render tumor cells more easily penetrable to Ca-TAT complexes, compared to normal cells [67].

Future studies using in vivo models will further determine the contribution of CCL2 signaling in epithelial cells versus macrophages in breast cancer progression. Here, we show that CCL2 expression is associated with luminal B breast cancers, and plays an important

role in regulating growth and survival of this breast cancer subtype. Inducing necrosis and autophagic cell death provides a potential therapeutic advantage where tumors acquire resistance to drug-induced apoptosis [68]. Moreover, Ca-TAT/siRNA complexes may be used as a therapeutic targeting agent to control expression and activity of molecules in selected cell types [25]. By understanding the role of CCL2 signaling in luminal B breast cancer progression, we could design alternative therapies to more effectively treat this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

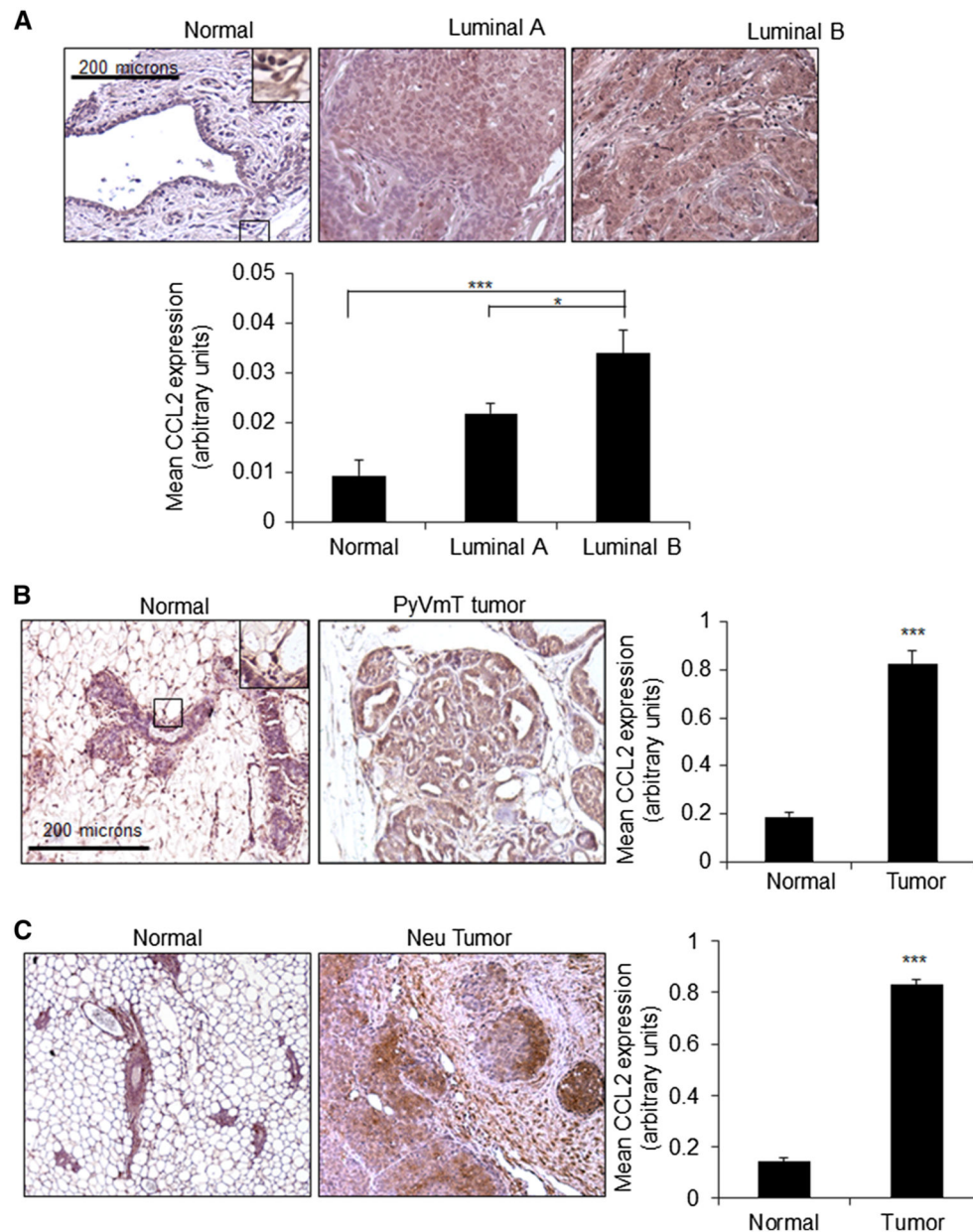|	
Ca-TAT	Calcium crosslinked TAT
CCL2	Chemokine (C–C motif) ligand 2
siRNA	Small interfering RNA
MMTV	Mouse mammary tumor virus
PyVmT	Polyoma virus middle T
HMGB1	High mobility group box 1
LC3B	Light chain 3B
TNF-a	Tumor necrosis alpha
TRAIL	TNF-related apoptosis inducing ligand
RIPK	Receptor interacting protein kinase
FBS	Fetal bovine serum
BSA	Bovine serum albumin
NBF	Neutral formalin buffer
SEM	Standard error of the mean
ANOVA	Analysis of variance

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Fig. 1.

CCL2 expression is increased in luminal B breast cancer.

a Immunohistochemistry staining was performed for CCL2 expression in patient samples of normal breast tissue ($n = 24$), luminal A ($n = 173$) or luminal B ($n = 81$) breast tumor tissues.

b Immunohistochemistry staining was performed for CCL2 expression in normal mouse mammary gland ($n = 11$) or PyVmT mammary tumor tissues ($n = 17$).

c Immunohistochemistry staining was performed for CCL2 expression in normal mammary gland ($n = 7$) or Neu-overexpressing mammary tumor tissues ($n = 7$). Magnified inset shows low-level expression in normal epithelium and stromal cells. Expression was quantified by Image J. Statistical analysis was performed by One Way ANOVA with Bonferroni post hoc

comparison (A) or Mann–Whitney two-sample test (B). Statistical significance was determined by p value < 0.05. $*p < 0.05$, $***p < 0.001$. *Values* are shown as Mean ± SEM
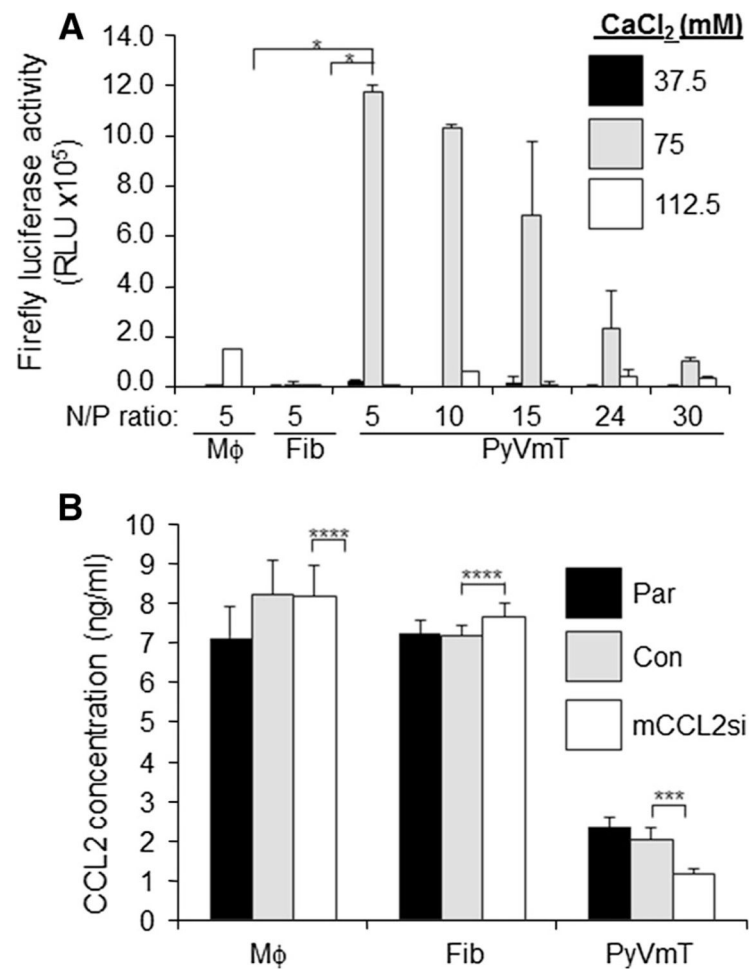
Fig. 2.

Calcium concentrations and *N/P* ratios modulate transfection efficiency of Ca-TAT complexes to PyVmT mammary carcinoma cells. **a** Mammary fibroblasts (Fib), Raw 264.7 macrophages (*Mφ*) or PyVmT mammary carcinoma cells, were transfected with Ca-TAT peptides complexed to MSCV-luciferase plasmids at the indicated $CaCl_2$ concentrations and *N/P* ratios. Luciferase activity was measured 48 h post-transfection. *RLU* relative light units. **b** Ca-TAT peptides were complexed to control siRNA (Con) or CCL2 siRNAs (mCCL2si), and transfected into the indicated cell lines. CCL2 expression was measured in parental (Par) or transfected cells by ELISA 48 h later. Statistical analysis was performed by One-Way ANOVA, followed by Bonferroni post-hoc comparisons. Statistical significance was determined by *p* value < 0.05. *$p < 0.001$,***$p < 0.05$, ****$p > 0.05$. *Values* are shown as Mean ± SEM
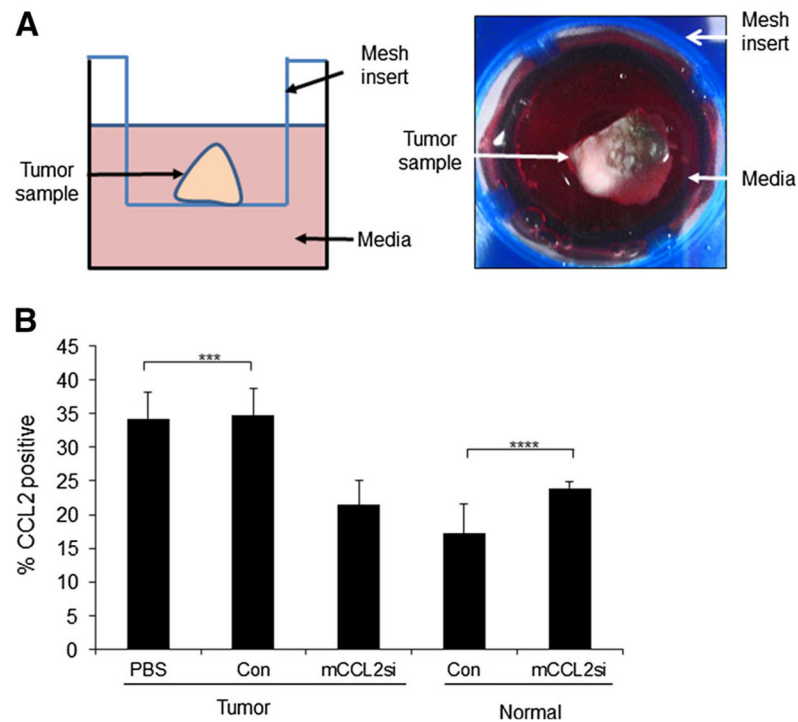
Fig. 3.
Ca-TAT/siRNA complexes selectively target CCL2 gene expression in mammary tumor tissues cultured *ex vivo*. **a** *Left panel* Diagram of PyVmT mammary tumors cultured in suspension *ex vivo*. Ca-TAT complexes are injected directly into the tumor tissue. *Right panel* representative image of a tumor sample shown in an overhead view. **b** Flow cytometry analysis was performed to measure overall CCL2 expression in normal mammary tissues or PyVmT tumors injected with phosphate buffered saline (PBS vehicle control), or Ca-TAT peptides complexed to control siRNA (Con) or CCL2 siRNA (mCCL2si). CCL2 expression levels were normalized to secondary antibody only control, and are relative to whole cell population in either the tumor or normal group. $N = 6$ per group. Statistical analysis was performed using One-Way ANOVA with Bonferroni post-hoc comparison. Statistical significance was determined by p value < 0.05. ***$p < 0.05$, ****$p > 0.05$. *Values* are shown as Mean $\pm$ SEM
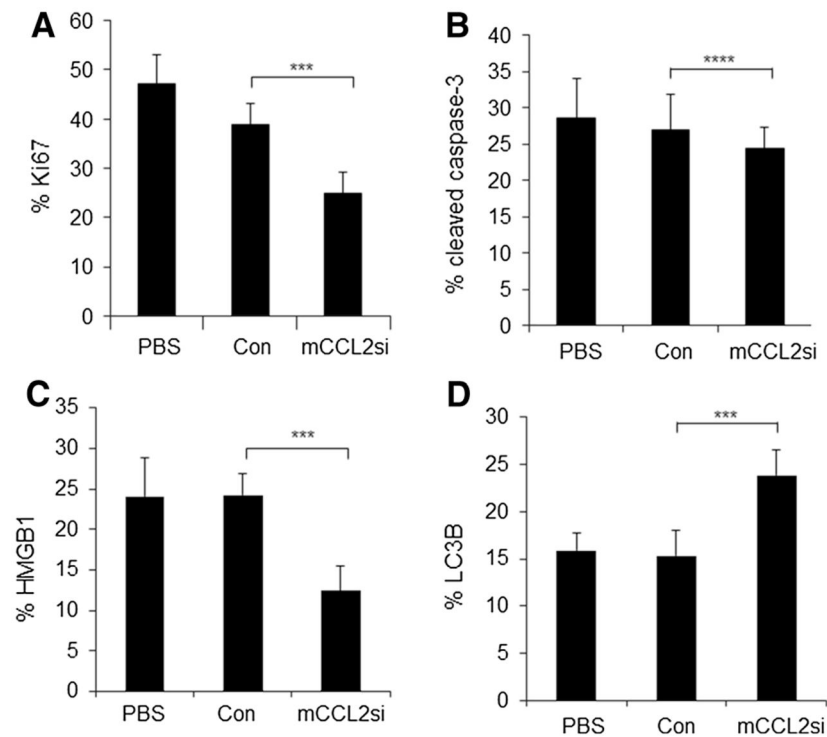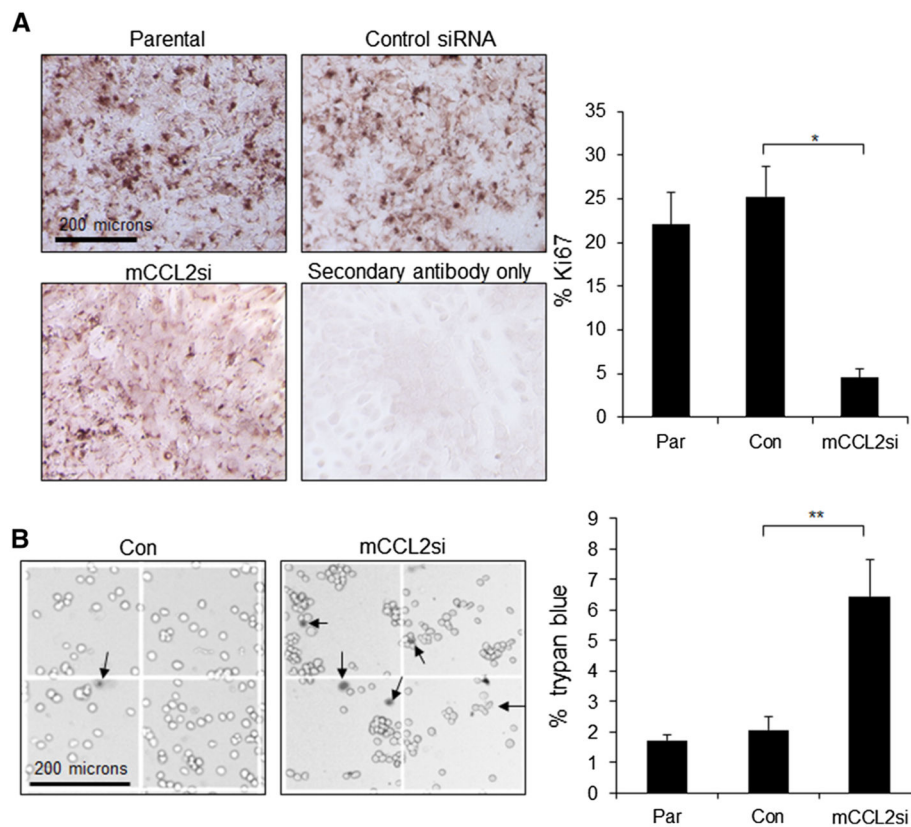
Fig. 4.
Ca-TAT-mediated delivery of CCL2 siRNAs to PyVmT *ex vivo* cultures lead to decreased cell proliferation and increased necrosis and autophagy. PyVmT tumor tissues were cultured for 24 h before injection with: PBS vehicle control, or Ca-TAT complexed to control siRNA (Con) or CCL2 siRNA (mCCL2si). Samples were analyzed by flow cytometry 48 h post-injection, for expression of the following: **a** Ki67, **b** cleaved caspase-3, **c** HMGB1, or **d** LC3B. Expression levels were normalized to samples stained with secondary antibody only. Statistical analysis was performed using One-Way ANOVA followed by Bonferroni post-hoc comparisons. Statistical significance was determined by *p* value < 0.05, ***p < 0.05, ****p > 0.05. *Values* are expressed as Mean ± SEM. *N* = 6 per group

Fig. 5.
CCL2 gene silencing in PyVmT mammary carcinoma cells leads to decreased cell proliferation and viability. PyVmT mammary carcinoma cells were transfected with Ca-TAT peptides complexed to control (Con) or CCL2 siRNAs (mCCL2si). Parental cells (Par) or transfected cells were analyzed for changes in the following: **a** Ki67 expression by immunocytochemistry staining, followed by hematoxylin counterstain. Expression was quantified by Image J, and normalized to hematoxylin staining. **b** Cell viability by trypan *blue staining. Arrows* in magnified sample images point to positive staining. Statistical analysis was performed using One-Way ANOVA with Bonferroni post-hoc comparisons. Statistical significance was determined by *p* value < 0.05.
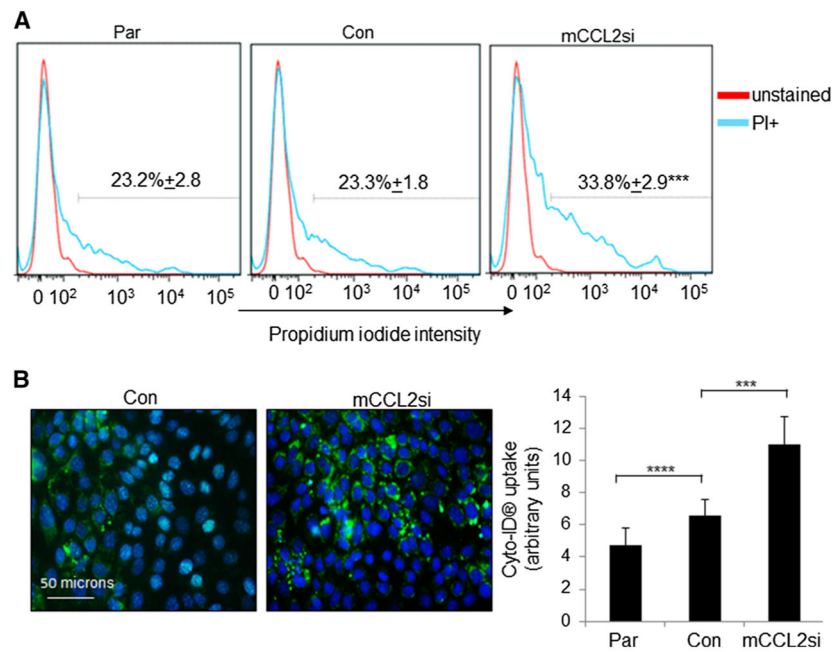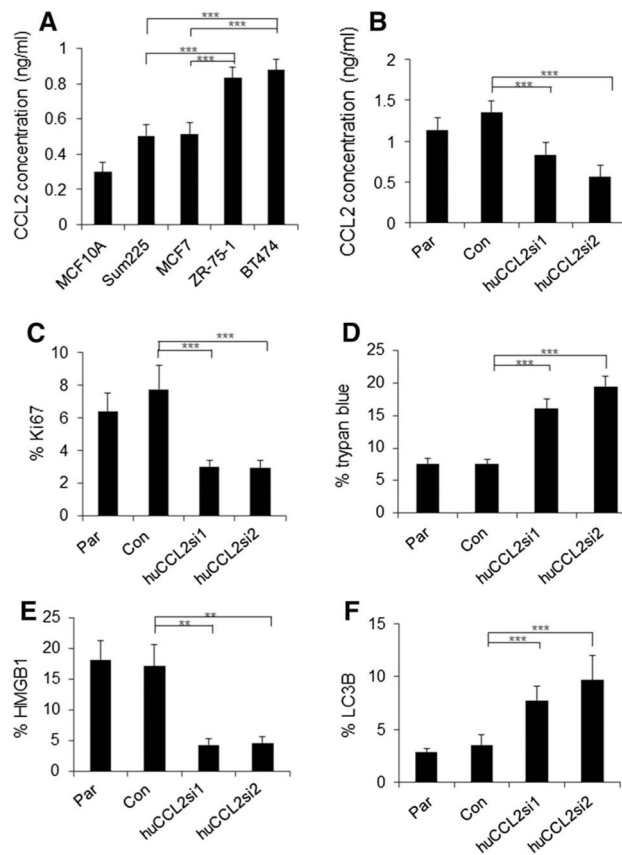*$p < 0.001$,**$p < 0.01$. *Values* are shown as Mean ± SEM

Fig. 6.
CCL2 gene silencing in PyVmT mammary carcinoma cells leads to increased necrosis and autophagy. PyVmT cells were transfected with Ca-TAT peptides complexed to control (Con) or CCL2 siRNAs (mCCL2si). 48 h later, transfected cells or parental cells (Par) were analyzed for the following: **a** Propidium iodide (PI) staining by flow cytometry. **b** Autophagosome formation by uptake of Cyto-ID® green dye. LC3B expression was quantified by Image J. Levels were normalized to DAPI stain. Statistical analysis was performed using One-Way ANOVA with Bonferroni post-hoc comparisons. Significance was determined by $p < 0.05$. ***$p < 0.05$, ****$p > 0.05$. For (A), ***$p < 0.05$ versus Control siRNA group. *Values* are shown as Mean ± SEM

Fig. 7.
Ca-TAT delivery of CCL2 siRNAs inhibits CCL2 expression, cell growth, and survival in BT474 breast cancer cells. **a** The indicated breast epithelial cell lines were analyzed for CCL2 expression by ELISA. **b–f** BT474 breast cancer cells were transfected with Ca-TAT peptides complexed to control (Con) or CCL2 siRNAs (huCCL2si1, huCCL2si2). Parental (Par) or transfected cells were analyzed for CCL2 expression by ELISA (**b**), Ki67 expression (**c**), trypan blue staining (**d**), HMGB1 expression (**e**), and LC3B expression (**f**). Statistical analysis performed by One-Way ANOVA followed by Bonferroni post hoc comparisons. Statistical significance determined by p value < 0.05. **$p < 0.01$, ***$p < 0.05$. *Values* are shown as Mean ± SEM