Modulation of C5a-C5aR interactions against murine mammary cancer cell line

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Abstract

Background: Complement C5a is a potent inflammatory chemoattractant and might be a beneficial therapeutic target for the induction of an effective anti-tumour response. C5a agonist and antagonist modulation have demonstrated to have either promotes or inhibits tumour development, EMT6 murine mammary cancer cells through both in vitro and in vivo studies.

Methods: For the in-vitro studies, Alamar Blue cell viability assay was used for cell viability determination and immunofluorescence assay was used to determine the location of C5aR expression on EMT6 cell line. For the in-vivo experiment, female Balb/c mice were subcutaneously injected with EMT6 tumour cells and subsequently treated with both C5aR agonist and antagonist peptides. At the end of in-vivo study period of 14 days, liver and tumour samples were obtained for an ELISA assay to quantify the levels of TNF-α, caspase-3, C5a and VEGF-A signals following the treatment with both C5aR agonist and antagonist. One-way ANOVA test was performed to evaluate the differences between the mean of treatments given in a group and that of the negative control

Results: The in-vitro experiment revealed an expression of C5aR was found on the cell membrane of the EMT6 cells and treatment with EP54; which is the C5aR agonist has shown low cell viability after 48 hours post-treatment. For the in-vivo experiment, the ELISA assay outcome have shown that EP54 significantly promote high numbers of circling of signaling proteins except for VEGF-A, suggesting that the C5aR agonist modulation might inhibits tumour development and also trigger the induction of apoptosis.

Conclusion: C5aR modulation through the influence of EP54 agonism may have beneficial effects in terms of reduction of the tumour size and attenuation of its development.

Background

The inflammatory response may promote tumour regression (1), which may be a useful therapeutic option in combating resistant tumours (2). Conversely, while acute inflammation is a protective response (3, 4), chronic inflammatory processes may contribute to the pathogenesis of specific diseases, including neoplasia (3, 5). Differences in the inflammatory mediators or pathways may be responsible for this contradictory outcome.

Complement is a key mediator of inflammation (6, 7) and activation of the complement system results in the formation of a pore-like membrane attack complex (MAC) (8) and the release of the potent C5a peptide (9). C5a induces inflammation following interaction with its specific receptor, C5aR (10, 11) and has been associated with various disease processes, such as rheumatoid arthritis (12), brain disorders (13, 14), inflammatory bowel diseases (15), cancers (16), sepsis (17) and ischemia reperfusion injuries (18).

The functional role of complement in cancer immunotherapy is poorly understood, since C5a has been reported to enhance lung cancer in mice by inhibiting myeloid-derived suppressor cells (19), while inducing regression of mammary tumours in mice (20). Furthermore, while complement components and MAC have been found within tumour cells (21, 22, 23), lysis of the tumour cell following complement activation may have failed due to the constitutive expression of membrane regulatory proteins, such as membrane co-factor protein (MCP, CD46), decay-accelerating factor (DAF, CD55) and CD59 (24). However, it has also been reported that the presence of the complement regulatory protein enhanced the immune-mediated clearance of tumour cells (25).

The aim of the current study was to further investigate the role of C5a in tumour cell growth. A preliminary study will identify the location of the C5aR in cells from an established tumour cell line (EMT6 mouse mammary tumour) and then administrationof both C5aR agonist and antagonist to these cells and to measure the concentration of inflammatory mediators released. In a second study, EMT6 cells were implanted into mice and tumour growth in vivo in response to the same agonist and antagonist was measured. At the end of this study, the liver from these mice were analysed for the concentrations of the same inflammatory mediators as the cell lines.

Methods

In vitro study

Cell culture condition

EMT6 cell lines from a transplantable murine mammary carcinoma were purchased from American Type Culture Collection (ATCC; Rockville, MD). Cells were maintained in Waymouth's medium supplemented with 2 mM L-glutamine (Gibco, Life Technologies), 1% of penicillin-streptomycin (10,000 U/mL) solution and 10% fetal bovine serum (FBS). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 ^{o}C.

In vitro drug preparation

The C5a agonist EP54 (YSFKPMPLaR) (2) was purchased from GL Biochem, Shanghai, China Ltd. The C5aR antagonist PMX205 (hydrocinnamate-[OPdChaWR]) (15) was obtained from collaborators at the School of Biomedical Sciences, University of Queensland, Australia. A positive control drug; Tamoxifen was purchased from Sigma-Aldrich, USA. All drugs/peptides were diluted with 5% glucose to the concentration of 0.1-mg/0.1 mL, while a 5% of glucose solution was used as a placebo control.

Immunofluorescence staining for C5aR

EMT6 cells were seeded on glass cover slips and were allowed to grow for 2–3 days before fixation for 10 minutes in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4). The cells were then incubated with primary rat anti-mouse C5aR/CD88 (Hycult Biotechnology, Netherlands) antibody or irrelevant immunoglobulin (negative control) followed by the appropriate secondary antibodies. Cover

slips were mounted with gelvatol mounting medium and were observed under a fluorescence microscope. A 20 µL of Hoescht dye was used at concentration of 1:500. This dye will stain the EMT6 cell's nucleus, which promotes the differentiation between the nucleus and membrane when the images were merged.

Alamar Blue assay for cell viability / proliferation

EMT6 cells were trypsinized from sub-confluent culture and suspended in culture medium containing 10% FBS. Cell suspensions were then seeded in duplicates of 5×10^3 cells per well in a 96-well plate. The media was discarded and washed using 20 µL of PBS after 24 h incubation, then 50 µL of fresh complete media without serum were added to the wells and incubated for 24 h. Five µL of glucose and each peptide/drug with concentration 0.1 mg/0.1 mL were added into the cells in the 96-well plate. The plates were incubated and the cells were collected at a different time points of 0, 24, 48 and 72 h, respectively. Following incubation, Alamar Blue solution was added 2 hours prior to the reading to indicate cell viability/proliferation, with absorbance measured using a microplate reader (Infinite® 200 Pro, Switzerland) at wavelength of 570 nm and 630 nm, respectively. The percentage (%) of AB reductions represents the number of viable cells after 72 H of incubation time.

Inflammatory mediator response of EMT6 cells in response to agonists and antagonists of C5aR

EMT6 cells were treated with 1.5 mL of each peptide/drug at a concentration of 0.1 mg/mL and 5% glucose (control group) in 75-cm3 flasks for 72 h. Two mL of PBS solution were added to flask and the cells were then scraped using a cell scraper. The solutions containing the cells were then centrifuged for 20 min at 510-x *g* in 4 ^{o}C. The supernatant was collected and stored for further ELISA assays.

In vivo study

Induction of -tumour in mice and respective treatments

Female Balb/c mice (6–8 weeks old) were purchased from Comparative Medicine Division (National University of Singapore) and housed at Laboratory Animal Facilities and Management (LAFAM), Universiti Teknologi Mara (UiTM), Puncak Alam. The mice were kept in micro-isolator cages in a pathogen-free environment. Twenty mice were divided into four groups of five mice and each mouse was subcutaneously injected with 1×10^5 viable cells/mL of EMT6 cells in suspension. The mice were then divided into four groups as follows: Group A - EP54 (agonist drug); Group B - PMX205 (antagonist drug); Group C – Tamoxifen (positive control) and Group D - untreated (negative control). The specific treatments for each group are outlined in Table 1. Treatment was given daily for 14 days and general observation was performed daily. The diameters of the tumours (width · length) were measured daily throughout the experiment using an electronic caliper. Throughout the study, mice with a tumour growth of 8 mm in diameter will be culled and excluded.

Table 1
Treatments administered to each group of rats.

Group	No. of mice	Treatment	Dose administered	Dosing frequency
A	5	EP54	0.1 mg/kg in 0.1 mL of 5% glucose	Single dose per day
B	5	PMX205	0.1 mg/kg in 0.1 mL of 5% glucose	Single dose per day
C	5	Tamoxifen	0.1 mg/kg in 0.1 mL of 5% glucose	Single dose per day
D	5	Control	0.1 mL of 5% glucose	Single dose per day

Liver collection

After 14 days, all surviving mice were euthanased by an overdose of pentobarbital followed by exsanguinations via cardic puncture. The liver was excised post-mortem and flushed with cold PBS to get off of excess blood. One g of each mouse liver was weighed, then cut into small pieces using scissors and solubilized in cold PBS to a final concentration of 1 mg tissue/mL to make up 10% homogenates. Tissues were completely homogenized by using a Tissue Ruptor (Qiagen, Netherlands) and samples were centrifuged at 510-x g, 4^{o}C for 10 min for post-nuclear supernatant. The supernatant was collected and divided into small portions and stored at -20 ^{o}C before further analysis. The entire procedure was carried out at 4 ^{o}C and protease inhibitor (Roche, Germany) was added to avoid any potential protease activity. Total protein concentration was estimated using Bicinchonic Acid (BCA) protein assay and the homogenates were utilized for ELISA analysis.

Measurement of inflammatory mediators

Cell lysates from EMT6 cells and liver homogenates from each treated individual mouse were processed for ELISA assay. The ELISA assay kits used were Mouse TNF-alpha Instant ELISA (eBioscience, San Diego, USA), Mouse VEGF-A Plantinum ELISA (eBioscience), Mouse Caspase 3 (Casp-3) ELISA Kit (Cusabio Biotech Co., Ltd, China) and ELISA Kit for Complement Component 5a (C5a) (Cloud-Clone Corp., Texas, USA). All the procedures and protocols were followed according to the manufacturer's instructions. The data obtained was normalized to the total protein obtained from the BCA protein assay, according to manufacture (PierceTM, Thermo Scientific).

Statistical Analysis

All statistical analyses were performed using INSTAT software (GraphPad software, San Diego, CA) to calculate mean and standard error (SE) of the ELISA concentration measurements. One-way ANOVA test was performed to evaluate the differences between the mean of treatments given in a group and that of the negative control. The level of significance was set at $p < 0.05$.

Results

C5aR expression on EMT6 mammary cancer cells line

A green color appeared in the EMT6 cells and indicated expression of C5aR on the plasma membrane only, which was differentiated from the cell nucleus by the blue fluorescent color (Fig. 1). In comparison, there was no C5aR expression in the control group.

Alamar Blue (AB) assay for EMT6 cell viability against incubation time

EMT6 cells treated with PMX205 showed a higher % of AB reduction, compared to cells treated with EP54 or tamoxifen (Table 2).

Table 2
Percentage of viability reduction of EMT6 cells after treated with different treatment (EP54, PMX205 and Tamoxifen) by Alamar blue. * indicates means that are statistical significance between time points of each treatments given.

Treatment	% AB viability reduction (hours)		
	24	48	72
EP54	15.57*	14.25	13.50*
PMX205	15.50*	15.10	15.90*
Tamoxifen	13.75*	13.15*	14.00*

Mice studies

Tumour growth in mice

Tumours became visible at day 5-post inoculation. Growth of the tumours was variable, in terms of size and also palpability, for the agonist-treated group, but more consistent in the antagonist-treated group, with several mice becoming moribund before the end of the 14-days study period (data not shown). The results of tumour growth rates following the various treatments are presented in Fig. 2.

Production of inflammatory mediators in liver tissue

The representative experimental group (Fig. 3 and Table 2) of the liver tissue homogenates showed significant concentration response to every tested ELISA assays.

Table 2
Expression of TNF- α, Caspase-3, C5a and VEGF-α detected in liver tissue homogenates in different treatment group of in-vivo studies. * indicate means that are statistical significance between treatments of each ELISA assay tested.

ELISA	Concentration (pg/mL)			
	EP54	PMX205	Tamoxifen	Control
TNF-α	0.55*	0.51*	0.78	0.854
Caspase-3	2.14*	1.81*	1.84*	1.308
C5a	1.70	1.29	1.74*	1.535
VEGF-α	0.34*	0.39*	0.44*	0.543

Production of inflammatory mediators in EMT6 cells

The results showed the concentration of TNF-α in EP54 treated group was higher compared to the other group (Fig. 4 and Table 3). However, Tamoxifen showed the highest concentration of caspase-3 and C5aR.

Table 3
Expression of TNF- α, Caspase-3 and C5a detected in EMT6 cell homogenates in different treatment group of in-vitro studies. * indicate means that are statistical significance between treatments of each ELISA assay tested.

ELISA	Concentration (pg/mL)			
	EP54	PMX205	Tamoxifen	Control
TNF-α	4.12×10^{-3}*	3.36×10^{-3}*	3.30×10^{-3}*	4.625×10^{-3}
Caspase-3	2.06×10^{-3}*	1.97×10^{-3}*	2.08×10^{-3}*	1.411×10^{-3}
C5a	1.08	1.01*	1.17	1.328

In vivo and in vitro study results showed that EP54 induced significantly higher activities of TNF-α, caspase-3 and C5aR, compared to other groups. In contrast, in vivo VEGF-α activity (ELISA assay) was higher following PMX205 treatment compared to EP54.

Discussion

There is limited understanding of the effects of the anaphylatoxin C5a in attenuating tumour growth in murine breast cancer. C5a exerts a pro-inflammatory role that induces regression of tumour growth (Gunn and others 2010) and this action is favourable against tumour immunity (Patel and others 1993). In the current study, C5aR were found to be express abundantly on the cell membrane of EMT6 murine mammary cancer cells (see Fig. 1). The expression of the C5aR was reported to be extensively on both immune and non-immune cells, leukocytes (e.g., neutrophils, basophils, monocytes, mast cells) (26, 27, 28) and several tissues (17), supporting similar reports of C5aR expression in lung (19) and cervical cancer cells from syngenic mice (16).

The current study has also shown, for the first time, that a C5aR agonist directly inhibited the growth and viability of mammary tumour cells. Conversely, treatment with a C5aR antagonist significantly enhanced tumour cell proliferation. This was an unexpected finding and contrasted to a study by Markiewski and others (2008) that showed that C5aR activation led to progression of cervical cancer in mice (28).

Several factors may account for the differences between the current study and that of Markiewski and others, 2008 (28). A synthetic C5a synthetic agonist was used in the current study since the C5a molecule itself is unstable (20, 30, 31). EP54 reduced the number and viability of the EMT6 mammary tumour cells, while PMX205 enhanced the growth of these cells. It could be speculated that autogenous production of C5a may limit but not inhibit the growth of EMT6 mammary tumour cells. A further factor to consider is that EP54 is a dual C5a/C3a receptor agonist (32) and activation of the C3a receptor (C3aR) may contribute to its anti-tumour activity, although PMX205 is a specific C5aR antagonist. Finally, the Markiewski and others, 2008 study investigated the effects of C5a on cervical tumour cells and there are reports to suggest that C5a may have varying effects in different cancer cell types (28).

The initial hypothesis for the current study was that C5a released during the inflammatory response, particularly chronic inflammation may initiate and propagate neoplastic changes in cells, based on the findings of Markiewski and others, 2008 (28). However, the current study actually supported other studies that proposed that C5a is a molecular adjuvant inducing anti-tumour immune responses in mouse models of mammary cancer and melanoma (20, 33). The immune properties of C5a potentially derived from the migration of myeloid cells into the mammary tumours with the subsequent release of inflammatory mediators contributing towards tumour cell death (20). However, the current study also showed that C5a might have a direct effect on tumour cell growth and viability, particularly the in vitro results, which removed the confounding effects of immune cells or their products. This study needs to be repeated to see if the effects are consistent with other neoplastic cell lines. Importantly, administration of EP54 also reduced the growth of the EMT6 tumours in vivo. The mechanism by which EP54 may inhibit growth in tumour cells is unclear, but certain peptides will kill cells and delay cell division (34) and EP54 induced significant increases in the signaling proteins for TNF-α, caspase-3 and C5a, compared to C5aR antagonist-treated group.

Further evidence to support the anti-tumour activity of C5a can be found in the response of some neoplastic cells able to express soluble or membrane-associated regulators of complement (e.g.: CD55) (35, 36) that protect against complement-dependent cytolysis (37, 38). Our initial observation indicates that C5a magnitude was higher in the C5a agonist treatment, which might leads to the activation of apoptosis. The increase in TNF-α following EP54 treatment was also interesting since TNF-α is used to treat local or metastatic melanomas and other unresectable tumours, particularly in conjunction with other cytostatic drugs (39). The response of TNF-α to EP54 treatment was lower in vitro than in vivo, which agrees with other reports (40, 41) and may reflect synergies with other mediators in vivo.

In addition to TNF-α, we found that EP54 increased the concentration of caspase-3, in vitro and in vivo. This contrasted with an earlier study in which a C5aR agonist had a protective effect on cultured rat granule neurons by inhibiting the activation of caspase-3 (42). In contrast, other studies have shown that an increased concentration of caspase-3 induced apoptosis in tumour cells (43), including in response to other chemotherapeutic drugs (26). However, it should also be noted that caspase can be present as an active and/or inactive form and apoptosis will depend on the relative ratio of the two forms (44) Furthermore, human breast cancer carcinoma cells (MCF-7) have been reported to resist the apoptotic stimuli by deletion of 47 base pairs in exon 3 of CASP3 gene, which is necessary for caspase-3 expression (42, 45).

Finally, antagonism of the C5aR by PMX205 induced a significant increase in the signaling protein for VEGF-α, with subsequent vascular growth known to be supportive of tumourigenesis (46). Increased expression of VEGF-α was also found to promote tumour growth and angiogenesis in vivo in a nude mouse model (47, 48). In the current study, measurement of VEGF-α activity could only be undertaken in vivo since active blood vessels are required. Similarly, expression of VEGF-α has been shown to increase tumour growth, angiogenesis and metastases in vivo in a nude mouse model, but had no effect on the growth of MCF cells in vitro (12, 47, 49).

Conclusions

In this preliminary study, abundant expression of C5aR was found on the membrane of EMT6 mouse mammary cancer cell line and activation of this receptor by an agonist (EP54) appeared to inhibit cell proliferation and viability. However, this effect appeared to be more pronounced in vivo and may reflect subsequent expression on other inflammatory mediators, including TNF-α and caspase-3. Furthermore, a reduction in the synthesis of VEGF-α if the C5aR is not inhibited adds support for a role of C5a to suppress the growth of tumour cells. However, further studies on the mechanism are required in order to maximize the range and ability of C5aR agonist and antagonist in cancer cells development.

Abbreviations

MAC : Membrane attack complex

C5A : Complement component 5A

MCP/CD46 : Membrane co-factor protein

DAF/CD55 : Decay-accelerating factor

AB : Alamar blue

ELISA : Enzyme-linked immunosorbent assay

TNF-α : Tumour necrosis factor alpha

VEGF-α : Vascular endothelial growth factor alpha

MCF-7 : Michigan Cancer Foundation-7 mammary cancer cell lines

CASP3 : Caspase 3

Declarations

Ethics approval and consent to participate

All experiments and care involving laboratory animal utilization were performed in accordance to the Universiti Teknologi MARA (UiTM) Committee on Animal Research and Ethics (CARE) guidelines and approval with the certificate number UITM 2013/2014. All experimental protocols in the laboratory settings were performed accordance to the guidelines by the Research and Postgraduate Studies Office, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Consent for publication

I, as the corresponding author, give my consent of identifiable details, which can include figure(s) and/or table(s) and/or details within the text to be published in the above Journal and Article.

Availability of data and materials

The data and materials from this article are made available from the Sultan Abdul Samad Library, University Putra Malaysia, Selangor, Malaysia under the repositories of postgraduate thesis collection.

Competiting Interests

The author(s) declare that this article have no conflicts of interest.

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funding body approved the design of the study; collection, analysis, and interpretation of data.

Author contributions

MNM Hezmee, HA Hassim, A Hafandi. and TRTA Rinalfi conceived and planned the experiments. NH Kamarudin and NF Bachek carried out the experiments. NH Kamarudin and NF Bachek contributed to sample preparation. MNM Hezmee, HA Hassim, A Hafandi, TRTA Rinalfi, NH Kamarudin and NF Bachek contributed to the interpretation of the results. MNM Hezmee took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript. All authors have read and approved the manuscript.

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Figures

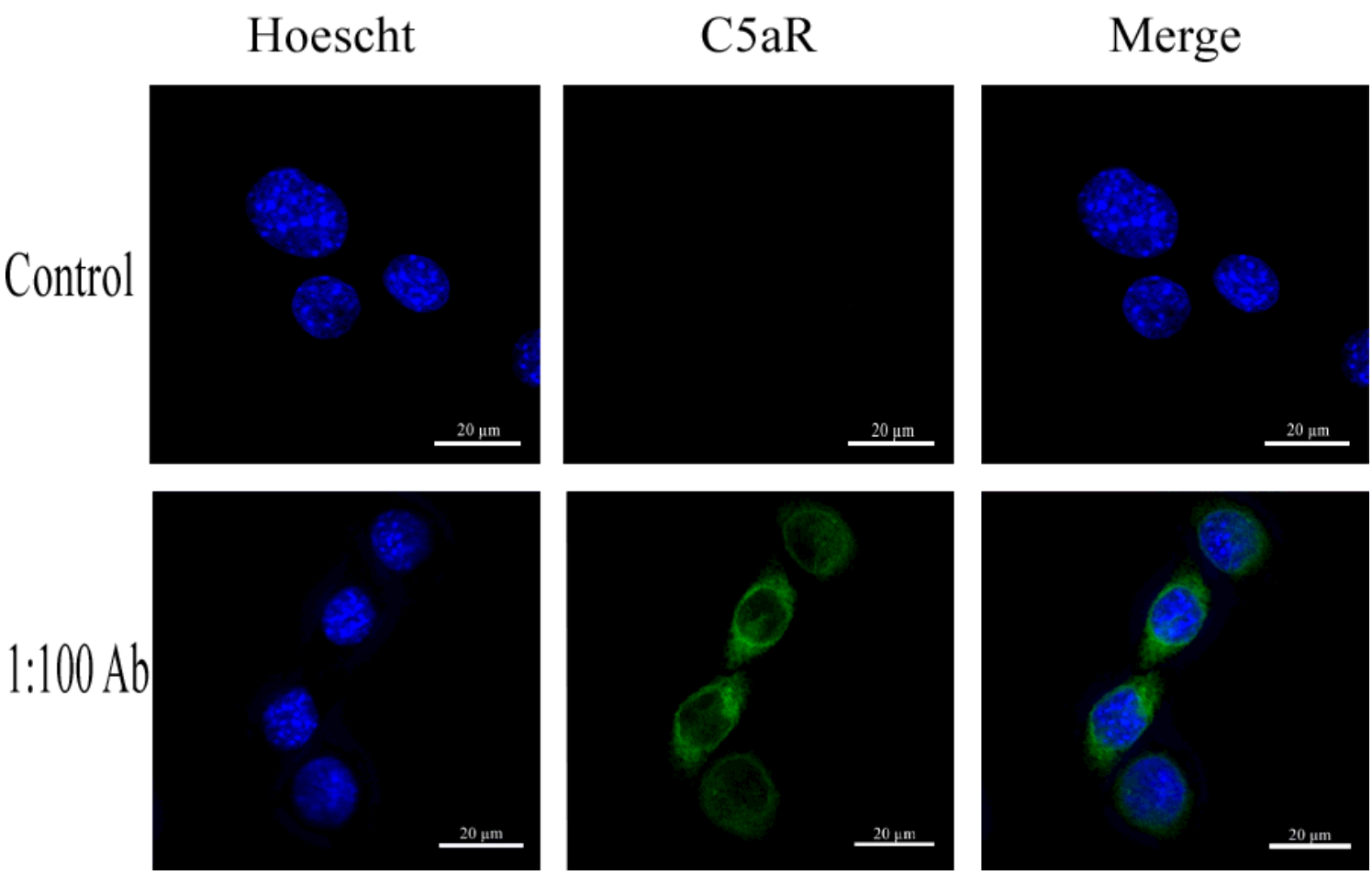

Figure 1

Confocal immunofluorecent micrograph of C5aR expression on EMT6 mammary cancer cells line. Left panel: Cell nucleus was stained with Hoescht dye. Middle panel: The cells stained with green showed the C5aR protein expression while no expression for unstained cells. Right panel: The merge image of EMT6 nuclear stained and C5aR. The C5aR protein expression was located at plasma membrane of EMT6 cells. Scale bar 20µm.

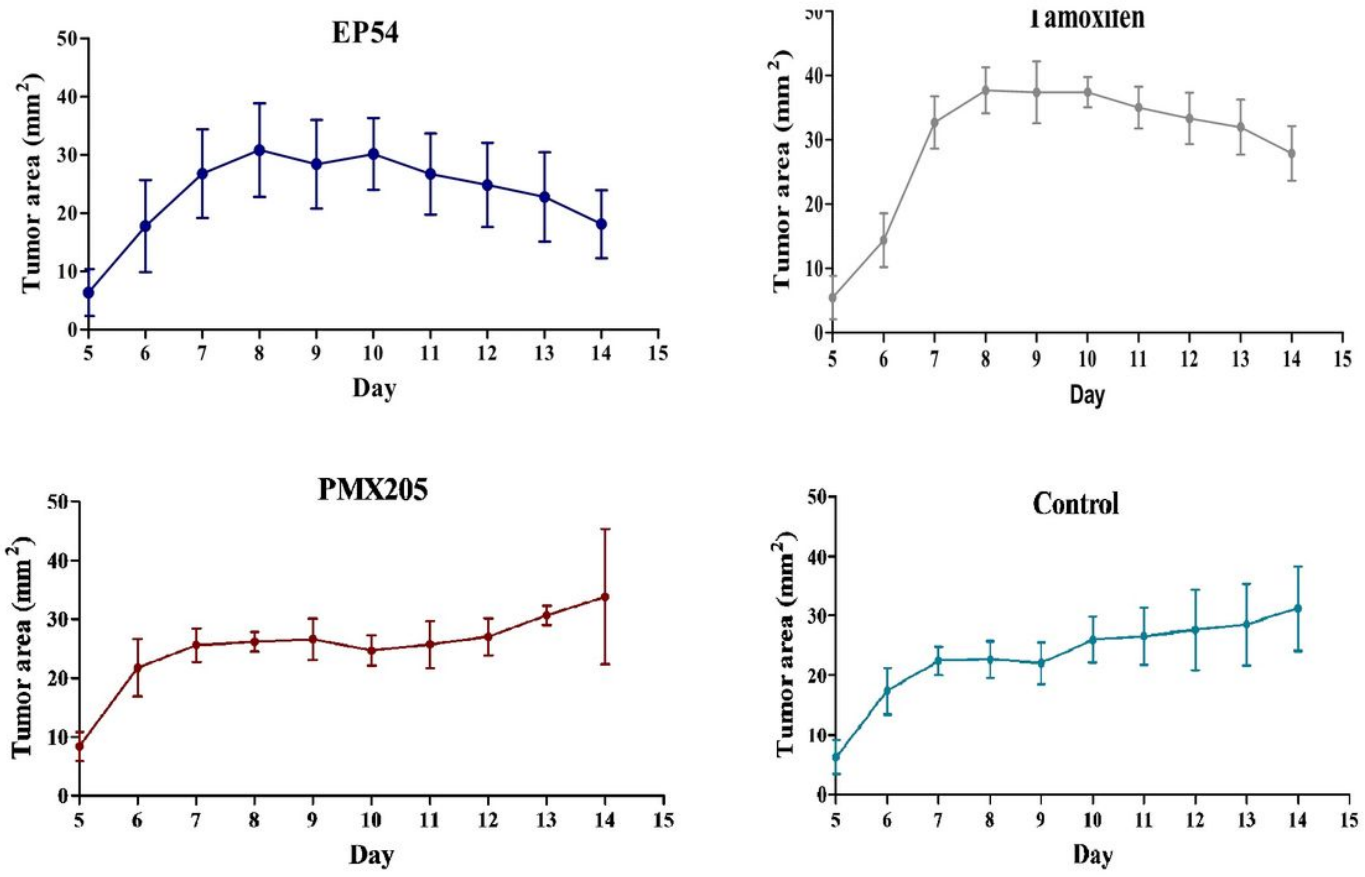

Figure 2

Tumour area (mm2) of all mice (n=20) within 14 days. A) Tumour area of mice (n=5) following EP54 treatment. B) Tumour area of mice (n=5) following PMX205 treatment. C) Tumour area of mice (n=5) following Tamoxifen. D) Tumour area of mice, which implanted with EMT6 cells only without any treatment given. The data represented as average (Mean ± SE).

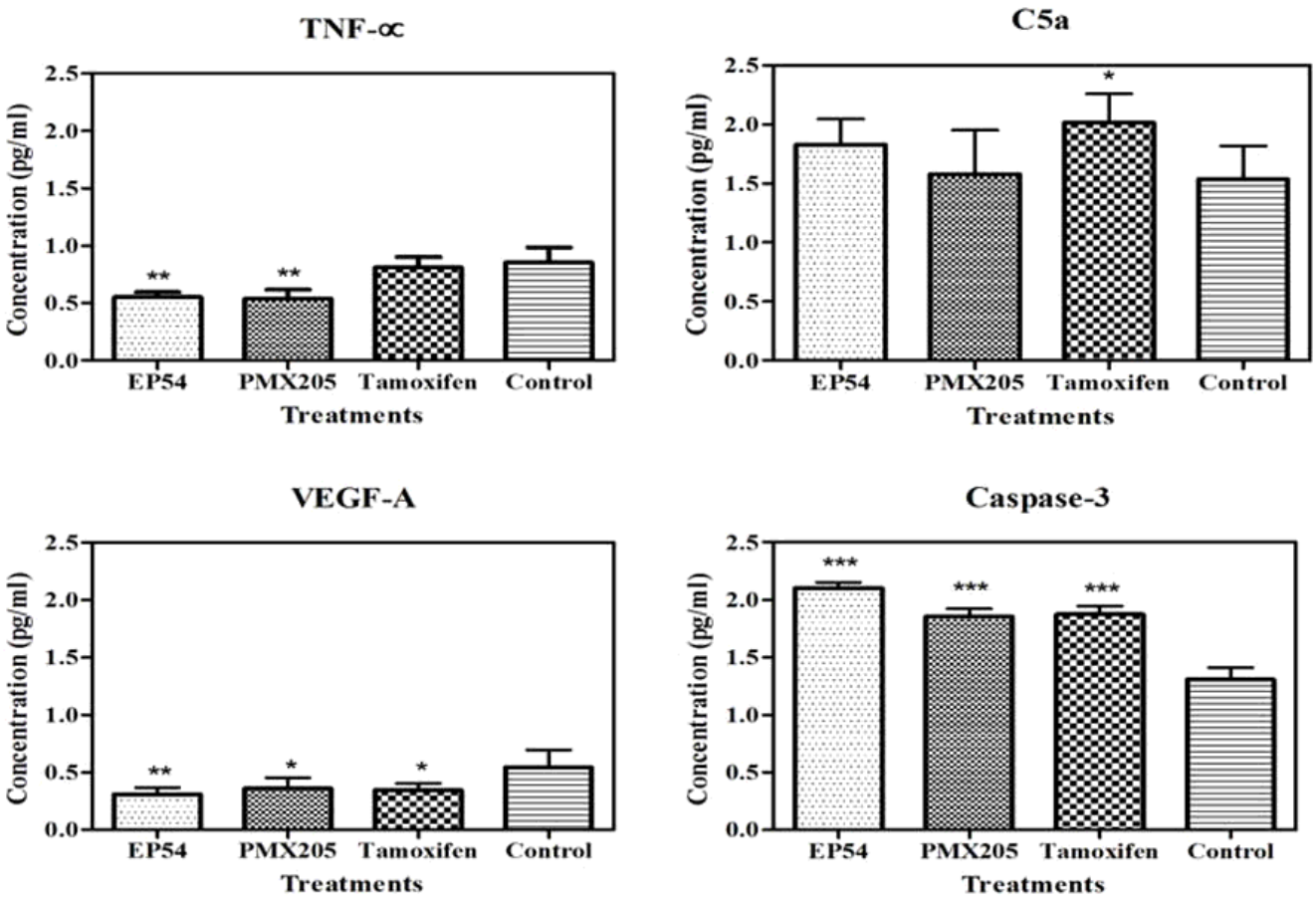

Figure 3

Production of mediators in liver tissue homegenates of TNF-alpha, C5a, VEGF-A and Caspase 3 in treatment group. The data represented as average (Mean ± SE).

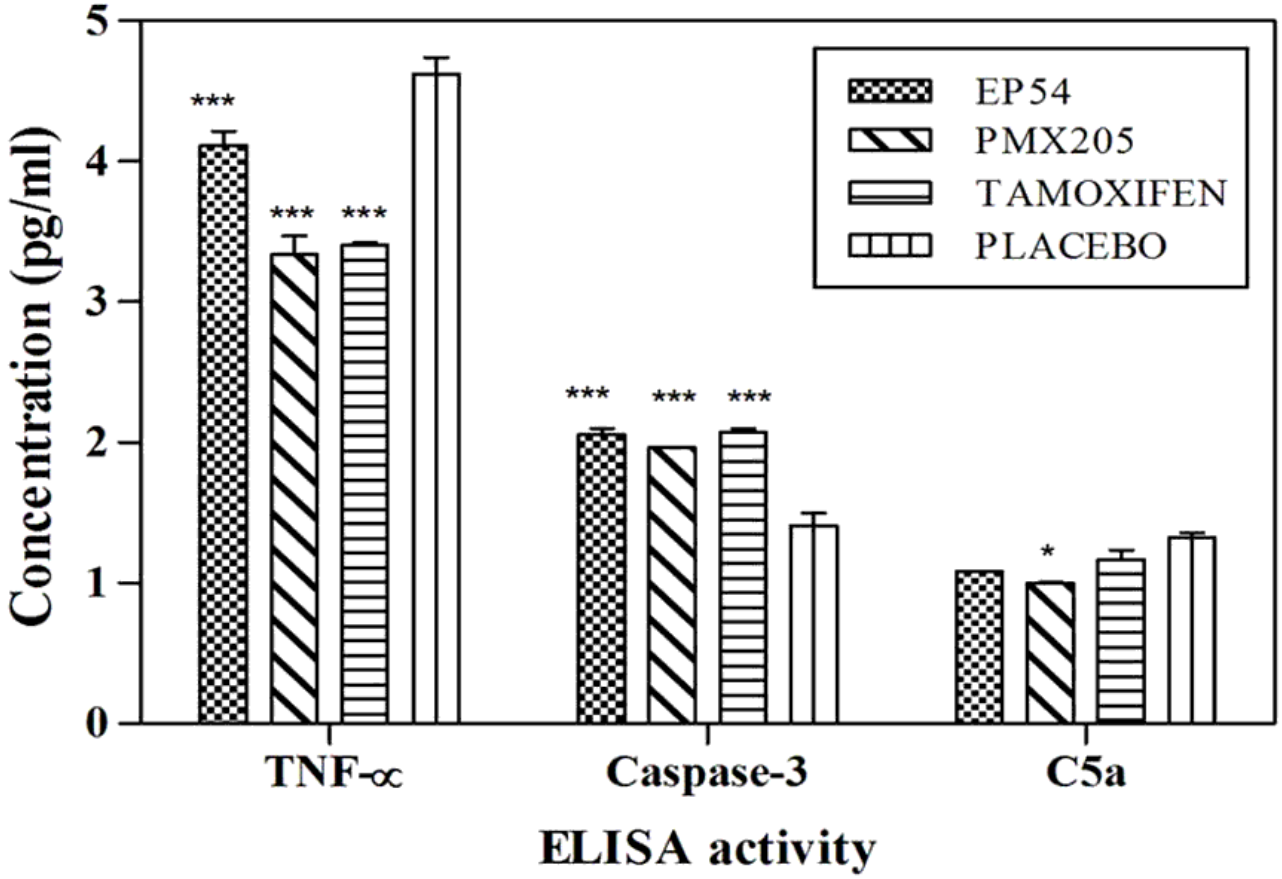

Figure 4

Concentration of TNF-alpha, Caspase-3 and C5a in EP54, PMX205, Tamoxifen and placebo in EMT6 cells. The data represented as average (Mean ± SE).

Supplementary Files

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