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Rapid Metastasis of Breast Cancer Cells from Primary Tumour to Liver

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Abstract: The aim of this study was to establish an animal model of mammary carcinoma metastasis to discern the *in vivo* effects of growth and spread of breast cancer. Six-week-old female BALB/c mice were inoculated with 4T1 murine breast cancer cells. Mice weight and primary tumour mass volume were regularly recorded to study the physical effects of a vigorously growing and spreading of cancer cell line. Gross and histological studies were carried out to determine the approximate day of metastatic onset. Production of IFN-gamma was assessed by ELISA to understand its role in tumour growth and metastasis. Lymphocyte markers such as $CD8^+$, CD25 and CD49b were analysed to elucidate its role in tumour growth and progression. Present study showed that the metastatic onset occurs approximately 11 days after the mice were inoculated with the 4T1 murine breast cancer cells. Gross studies showed hepatosplenomegaly. The breast cancer cells from primary tumour were found to spread rapidly to the liver on day 11. IFN-gamma production was higher in inoculated mice serum compared to control mice serum. Higher numbers of $CD8^+$, CD25 and CD49b cells were observed in the peripheral blood of inoculated mice, compared to control mice. In conclusion, the 4T1 murine breast cancer cells can migrate and metastasise rapidly to the liver, eliciting various immune responses.

Key words: 4T1, breast cancer metastasis, BALB/c mice, IFN-gamma, lymphocyte markers

INTRODUCTION

The most common cancer worldwide for males is lung cancer and for females is breast cancer (Parkin *et al*., 1999). A similar trend is also echoed in Malaysian health scenario where the Malaysian National Cancer Registry compiled by Chyw and Yahaya (2003) showed that in Peninsular Malaysia, 13.8% of male cancers were found to be of the lung, whilst breast cancer (31%) was the most common cancer amongst the females (Chyw and Yahaya, 2003). In the international scene, breast cancer is shown to be one of the major causes of cancer-related mortality in women (Hill and Iverson, 1997). Since many tumours are refractory to current therapies, majority of patients with breast cancer who receive therapy following the removal of primary tumour will general succumb to the disease. The lack of tumour-specific therapeutic agents to treat this condition is one of the major limiting factors that contribute to the high mortality rate in patients with breast cancer (Kershaw *et al*., 2004). In addition, many of the complications associated with breast cancer are due metastasis of the primary tumour mainly through the lymphatic spread to distant organs such as bone, liver, lung and brain (Weigelt *et al*., 2005). The metastatic process is made up of a sequence of events, namely invasion, intravasation, transport, arrest, extravasation and growth. The molecular changes that occur within both the tumour cells and the tumour microenvironment contribute to the detachment of tumour cells from the primary tumour site, which leads to the invasion into the tumour stroma, intravasation into nearby blood vessels or lymphatics, survival in the bloodstream, target organ extravasation and colonization and finally, metastatic outgrowth (Chambers *et al*., 2002; Hanahan and Weinberg, 2000). Several cytokines are known to promote the dissemination of breast cancer tumours to target organs and one of them is interferon-gamma (IFN-gamma). This cytokine has pleiotropic effects and can that regulate a variety of diverse activities. Recently, IFN-gamma has also been implicated to have a prominent role in immune responses to tumours (Elpek *et al*., 2007). Although, it has been discerned that IFN-gamma plays an important role in regulating tumour growth and spread, the mechanisms that it employs remain unclear. Tumour development and its progression is a complex process. The critical role of the immune system in immunosurveillance against tumours served as the basis to develop effective immunotherapy. However, many of these attempts have

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met with little or no success due to the existence of various immune evasion mechanisms in individuals with advanced tumours and the inability of current therapeutic approaches to override these immunoregulatory mechanisms (Elpek *et al*., 2007).

In recent studies, the understanding of mammary carcinoma progression and metastasis has greatly profited from advanced transplantation techniques and various types of mouse models. The generation of a mouse model of breast cancer is a critical step towards the understanding of many factors underlying mammary carcinogenesis. The knowledge on the ways breast tumours progress as been markedly improved by discerning the dynamics and key factors of mammary gland development (Fantozzi and Christofori, 2006). There are a number of studies on growth and progress ion breast cancer cell carried out in immunodeficient nude mice using human breast cancer cells (Fantozzi and Christofori, 2006). The immune system in the nude mice is very poorly developed due to lack of T-lymphocytes and hence is not a good model to study modulation of the immune system to the presence of tumour. Hence, in this study we used immunocompetent mice (BALB/c) and used a syngeneic mammary carcinoma cells (4T1), which were originally isolated as a subpopulation of 410.4 derived from a spontaneously arising mammary tumour in BALB/cfC3H mice (Dexter *et al*., 1978; Heppner *et al*., 1978) to induce tumour in these immunocompetent mice. The 4T1 cells are a poorly immunogenic cells and the primary tumour in a mice can metastasises via the hematogenous route to liver, lungs, bone and brain, making it a good model of human metastatic breast cancer (Heppner *et al*., 2000). The growth of tumour following inoculation of 4T1 cells in mice parallels highly to the invasive human metastatic mammary carcinoma (Pulaski *et al*., 2000) and the extent of this disease is comparable with human stage IV breast cancer (Pulaski *et al*., 2000). The 4T1 tumour therefore is an excellent metastatic breast cancer model mimicking clinical settings of human breast cancer. The aim of this study was to establish an animal model of mammary carcinoma metastasis to discern the *in vivo* effects of growth and spread of breast cancer in an immunocompetent mouse model.

MATERIALS AND METHODS

This study was carried out from December 2008 to March 2009.

Experimental model: Six to eight-week-old female BALB/c mice were obtained from the Animal Facility in Universiti Putra Malaysia, Serdang, Kuala Lumpur. The mice were chosen at that specific age as the recommended age for breast cancer establishment as reported previously (Pulaski *et al*., 2000) for optimum and efficient growth of tumours. During the entire duration of this study, the mice were house at the Animal Holding Facility at the International Medical University. They were housed in plastic ventilated cages (five in a cage) and were exposed to natural 12 hourly light and dark cycles. Animals were maintained on pelleted diet and water *ad libitum*. All of the animal procedures were subjected to review and approval by the Research and Ethics Committee of International Medical University, which also complies with the National Institutes of Health (NIH) guidelines for humane treatment of laboratory animals.

Cell line and reagents: The 4T1 cells (Pulaski *et al*., 2000) were purchased from ATCC. The cell line was cultured in 25 and 75 mL tissue culture flasks (Nunc, Denmark) in RPMI medium 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2% Hepes (Gibco, USA), 2% Sodium Pyruvate (Gibco, USA) and 1% Penicillin-Streptomycin antibiotics (Gibco, USA). The cells were cultured according to the protocol provided by ATCC and were found to be free of contamination throughout the experimental procedure.

Tumour challenge, documentation of weight changes and tumour volume: Mice were divided into 2 groups, inoculated mice that received the 4T1 cells and control mice that did not receive any treatment. The inoculated mice were challenged intramammarily in the right flank mammary pad with 100 μL of 4T1 cells at a concentration of 1×10^5 cells mL^{-1} using a 1 mL insulin Terumo® needle to prevent any damage to the internal organs of the mice. The weight of the mice in both groups was recorded every two days. The inoculated mice were palpated every day following the inoculation of the 4T1 tumour cells. The volume of the primary tumour was assessed morphometrically using an electronic vernier caliper (Eggert *et al*., 1999) and calculated using the formula previously shown below (Demaria *et al*., 2005):

$$V = (\pi/6)\times length\times width^2$$

Where:

V : Tumour volume (mm^3)
Length : Longer diameter (mm)
Width : Shorter diameter (mm)

Gross and histopathological studies: When the tumour was first palpable, three experimental mice and two control

mice were sacrificed every two days until day 23 i.e., when the last set of animals were sacrificed in both groups. Target organs for breast cancer metastasis (lungs, liver and spleen) were carefully removed using forceps to avoid shearing of the tissues. The morphology and conditions of the extracted organs were observed and noted down. The organs were stored in specimen containers containing 10% formalin solution for at least 48 hours and were retained as viable materials for histopathological studies. The tissues were processed overnight using an automatic tissue processor and embedded the next day using molten wax. They were sectioned into 4 μm slides using a rotary microtome knife (Leica RM2135) were dewaxed using hot air oven at 60°C for 45 min before subjecting through Haemotoxylin and Eosin (H and E) staining. The slides were then taken through a series of xylene and decreasing strengths of alcohol till exposure to distilled water. The slides were then stained with Haemotoxylin and then differentiated in acid-alcohol, immersed in sodium bicarbonate solution that acted as the bluing reagent before staining the slides with Eosin Y. Slides were mounted with cover slips using a drop of xylene and a drop of DPX Vecta Mount Mounting Medium. A section of relevant organs from control mice before the start of the experiment were used for comparison.

Analysis of sections: The metastastic tumour infiltrations were evaluated in the H and E stained slides. The morphology and the distribution pattern of the tumour infiltrations were identified in the primary tumour section as bigger than normal cells usually clumped as clusters, with deep blue or purple colouration. The tumour cell infiltrations were analysed using the Nikon Brightfield Compound Microscope at a magnification power of 100X using the NIS Elements BR 3.0 software.

Estimation of plasma levels of IFN-gamma: The IFN-gamma production level in mice plasma were analysed for both the inoculated group and control group using the cardiac puncture method. Blood was collected on day 0 and every 2 days after the tumour was palpable in heparin-coated tubes (BD Vacutainer coated with Lithium Heparin, BD USA). The tubes were then centrifuged using Sigma 2-16 KC's refrigerated centrifuge at 4°C, 5,000 rpm for 10 minutes. The plasma was transferred in to fresh tube and stored at -20°C until the enzyme-linked immunosorbent assay (ELISA) to estimate IFN-ganima production was carried out using a commercial ELISA kit as recommended by the manufacturer (eBioscience, USA). The results from the ELISA test were analysed using a microplate reader (Tecan, Switzerland) and the optical density used was 450 nm, with the reference optical density at 570 nm. For data analysis, the mean values of the samples read at 570 nm were subtracted from those of 450 nm. The determination of cytokine concentration estimation was achieved by plotting the mean absorbance against the standard concentration and this was compared to the standard curve.

Staining of cell surface markers: Briefly, peripheral blood of inoculated mice and control mice were collected in heparinised tubes when they were sacrificed using the cardiac puncture method. Following centrifugation to separate the component of blood, the cells were stained with various monoclonal antibodies using the protocol recommended by the manufacturer. Briefly, 100 μL of whole blood aliquots from each tube were then transferred to four different sterile 5 mL polystyrene round-bottom tubes (BD Falcon, USA). The tubes were single-stained with three antibodies, i.e., FITC-conjugated CD8 (BD Biosciences, USA), PE-conjugated CD25 (BD Biosciences, USA), PE-conjugated CD49b (BD Biosciences, USA). The last tube served as control, non-stained. The tubes were gently vortexed and allowed to stained in the dark for 20 min at room temperature. Then FACS Lysing Solution (BD Biosciences, USA) was added to the tubes and the tubes were incubated for 10 min at room temperature. After centrifugation for 5 min at 200x g, the supernatant was discarded and the tubes were washed twice with wash buffer (PBS). Cells were recovered after each wash by centrifugation (200x g for 5 min) and were resuspended in 0.5 mL of 1% paraformaldehyde solution mixed gently and thoroughly. The tubes were analysed using the flow cytometer as recommended by the manufacturer (FACSCALIBUR, BD Biosciences, USA).

Statistical analysis: The statistical tests were employed on the data using SPSS 15.0 software. The data were checked for normal distribution before proceeding to other tests. To determine the statistical significance of the weight changes, primary tumour volume and production of IFN-gamma and the percentages of lymphocyte markers the unpaired t-test was performed at a p-value set at less than 0.05 ($p<0.05$), which was considered significant. t test was used as we were comparing two separate independent and identically distributed parameters are obtained, one from each of the two populations (inoculated and control).

RESULTS

Weight changes between inoculated mice and control mice: The average weight changes for inoculated mice

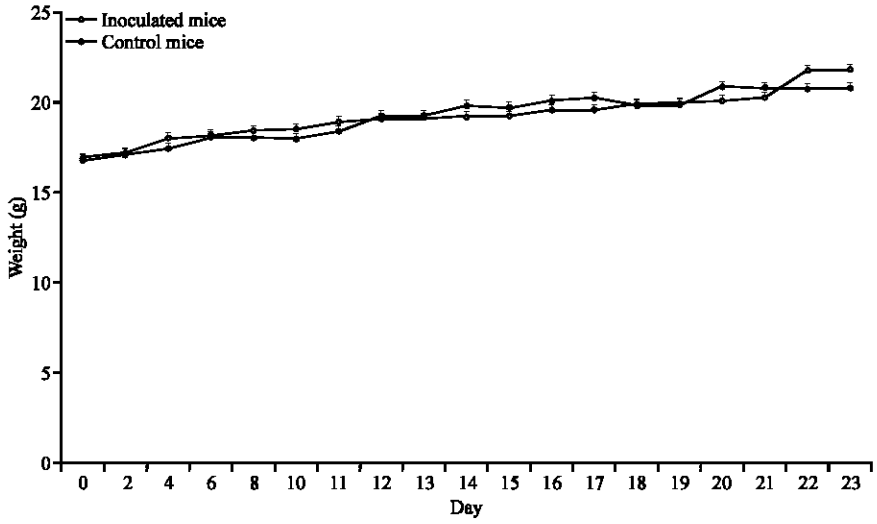

Fig. 1: Weight trend by the day for inoculated mice and control mice. Tumour was palpable from day 11 onwards. Each point represents three mice

and control mice were tabulated by the day, i.e., every two days starting from day 0 and every day subsequently after tumour was found palpable on day 11. As shown in Fig. 1, the weight trend among the two groups of mice did not vary too much from the starting point of experiment, mid-way through and at the end-point. There was a general trend of fluctuations for both groups but fluctuating weight difference did not bring about any obvious change. The weight changes were found to be statistically not significant ($p<0.05$) between the inoculated mice and control mice.

Mean tumour volume of inoculated mice: The average volume of the primary tumour in each animal was tabulated everyday starting from the day that the tumour mass was palpable (day 11). The mean volume of primary tumour showed an upward trend, gradually increasing until all the mice were sacrificed on day 23. As shown in Fig. 2, the mean tumour volume recorded on the day 11 was about 500 mm^3, whilst at the end of the experiment, it had increased at least four-fold to 2000 mm^3.

Gross analysis of harvested organs: Most of the organs from the 4T1-inoculated mice did not show any abnormal morphological features or any visible tumour nodules (Fig. 3). The organs looked similar to the organs of control mice. Lungs from both groups were of almost of the same size. However, the liver and spleen from the 4T1-inoculated mice were almost twice the size of the same organs from the control mice i.e. presence of hepatosplenomegaly in the tumour-laden animals.

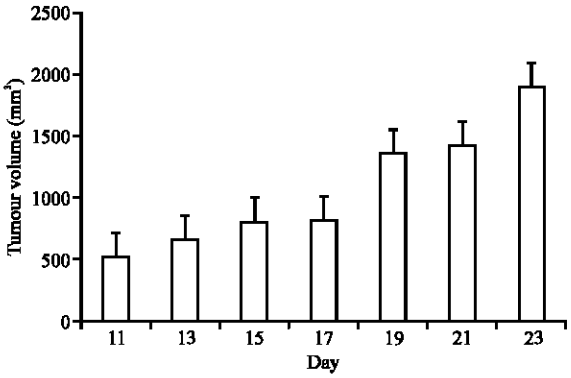

Fig. 2: Average volume of primary tumour excised from inoculated mice every two days starting from the day tumour mass was palpable (day 11). Each bar is a mean tumour volume from three mice

Gross analysis of excised primary tumour: The size of the primary tumours excised from the experimental mice was estimated using the digital vernier calliper. As shown in Fig. 4, tumour size gradually increased from day 11 to day 23 i.e., till the mice were sacrificed. The mean size of the tumours on day 23 was slightly than twice of the tumours excised on day 11.

Histopathological analysis of metastatic tumour infiltrates in target organs: The microscopical sections of the lungs, liver, spleen and primary tumour from the 4T1-inoculated and control mice were analysed using a compound microscope (Nikon Brightfield Compound Microscope) and analysed with the NIS BR 3.0 Software

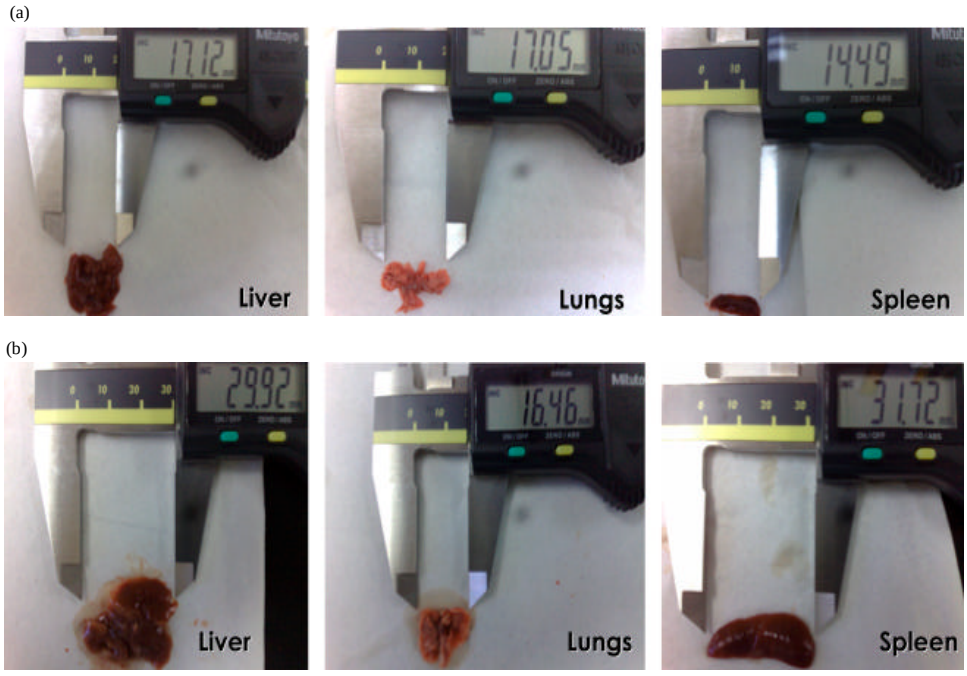

Fig. 3: Image showing the gross pictures of various organs (liver, lungs and spleen) taken from control and tumour-inoculated mice on day 17. (a) Control and (b) Inoculated

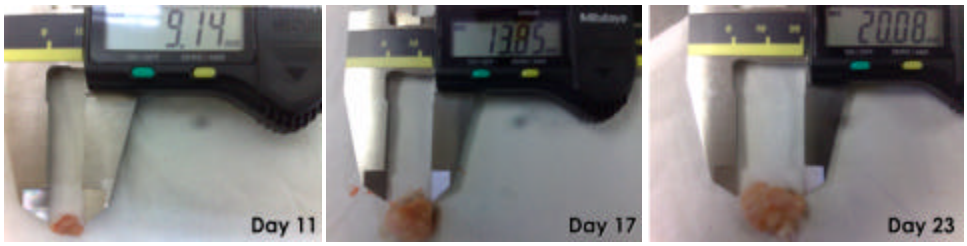

Fig. 4: Image showing the excised primary tumour of inoculated mice on day 11, 17 and 23, denoting the start, mid-way and end-point of the experimental duration

that was recommended by Nikon. The tumour cells are either deep blue or deep purple in color (Fig. 5). The cells were also noted to be on the larger side and usually appear as clumps or groups, forming different cluster sizes. The microscopic appearance of the tumour cells did not vary between the days, although there were two different distinct shapes. The tumour cells from the primary tumour excised on day 17 appear to be cuboidal and epithelial (Fig. 5a) while those from the tumour excised on day 21 appear to be spindle and fibroblastic (Fig. 5b). Microscopic sections of lungs and spleen of the 4T1-inoculated mice did not show presence of any metastatic tumour cells. However, the liver sections of all the 4T1-inoculated mice showed presence of metastastic tumour cells. Metastatic cells could already be detected in the liver harvested on day 11 i.e., the day tumour when was palpable. It was observed that the presentation of the clusters of metastatic cell infiltrations became more prominent on day 17, with the tumour cells appeared to be in clumps or groups (Fig. 6). The colour of the tumour cells were deep blue or purple, as opposed to the normal pink colour of normal hepatic cells. The size of the tumour cells was also bigger than the normal hepatic cells. They were easily identified due to their size and their distinctive physical features with the cuboidal or spindle shape. The tumour cells also were seen in the sinusoidal region, possibly restricting the blood flow and obstructing the fenestrated system of the vascular channels. In contrast,

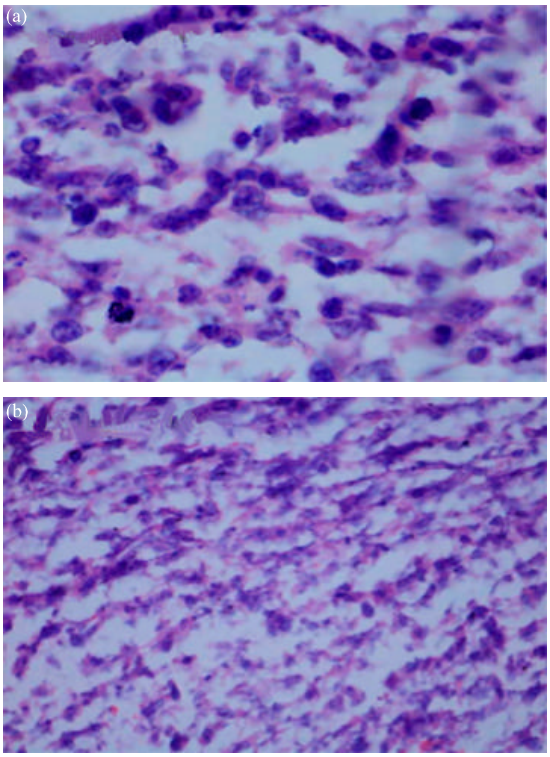

Fig. 5: Image showing a microscopical picture of H and E stained section of the primary tumour of a 4T1-inoculated mice that was sacrificed on (a) Day 17 and (b) Day 21. Tumour 100X

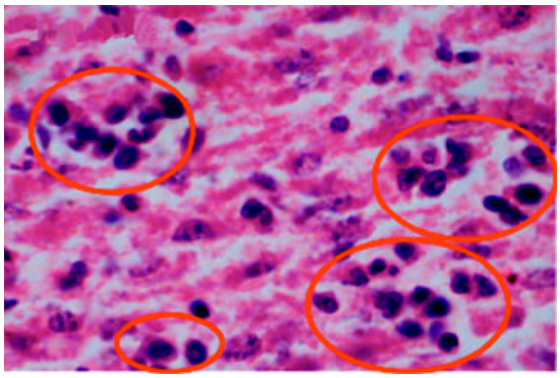

Fig. 6: Image showing a microscopic image of H and E stained liver section of a 4T1-inoculated mouse taken on day 17. Red circles denotes the cluster of metastatic tumour infiltrates appearing as deep blue and deep purple clumps or groups and are also larger in size compared to normal hepatic cells. Liver 100X

the lung sections from all the 4T1-inoculated mice did not show any presence of metastatic tumour infiltrates (data not shown).

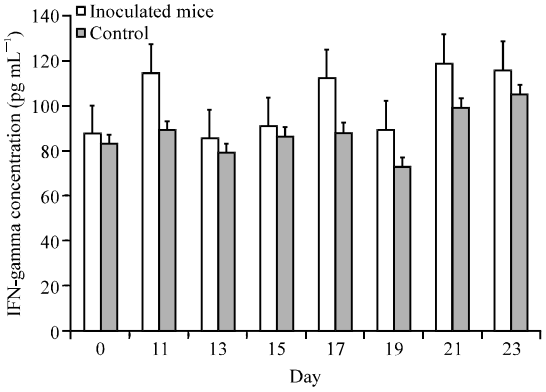

Fig. 7: Chart showing the IFN-gamma production in 4T1-inoculated mice and control mice on day 0 and every two days from day 11 (palpable tumour mass) onwards ($p>0.05$)

Production of IFN-gamma in serum of inoculated mice and control mice: The production of IFN-gamma was estimated as it is reported to promote the spread of metastatic tumour cells from primary site to distant organs such as liver, lungs, spleen, brain and kidneys (Aslakson and Miller, 1992; Lelekakis *et al*., 1999). As shown in Fig. 7, the amount of IFN-gamma in the plasma of 4T1-inoculated appears to fluctuate. However, the trend in plasma levels of IFN-gamma production between the inoculated and control mice was similar and statistically not significant ($p<0.05$).

Flow cytometry analysis of cell surface markers: Specific lymphocyte markers $CD8^+$, CD25 and CD49b were estimated in both control and inoculated mice, from the cardiac blood that was collected on day 0 and every 2 days from day 11 until the end of the experiment. The levels were estimated for day 0 to streamline the normal values for these markers, before the experimental mice were inoculated with 4T1 breast cancer cells. The results are shown in Fig. 8.

As shown in Fig. 8a, the gated percentage of $CD8^+$ for the control mice group ranged from 3.51% on day 0 and steadily escalated to 4.10% on the last day of experiment, whilst for the inoculated mice group the gated percentage ranged from 3.53% on day 0 and increased sharply to 14.59% on day 11 and was seen to rise steadily until the last day of experiment to 15.72%. The $CD8^+$ production between the inoculated and control mice was found to be statistically significant ($p<0.05$) (0.047). Figure 8b illustrates that the gated percentage of CD25 for the control group ranged from 3.25% on day 0 and the number was seen to rise gradually to 4.11% on the last day of experiment, whilst for the inoculated group the percentage was not too far away with the control group

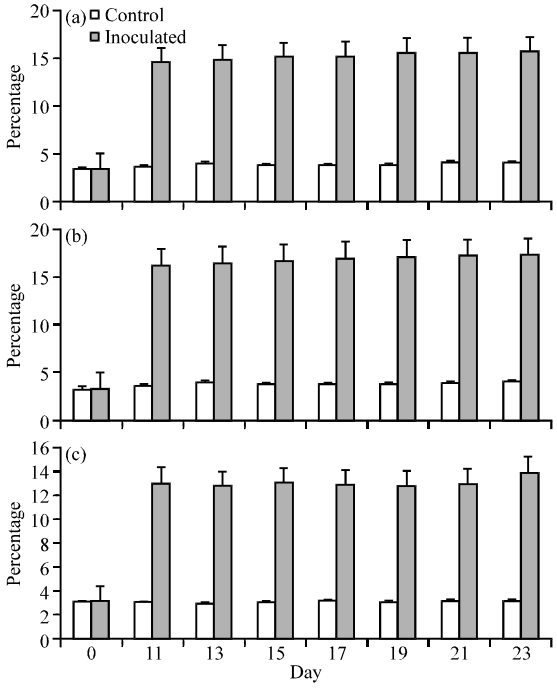

Fig. 8: Comparing the mean percentages of $CD8^+$, CD25 and CD49b cells between 4T1-inoculated and control mice from day 0 to 23 as determined by flow cytometry. (a) $CD8^+$ T-cells, (b) $CD25^+$ cells and (c) $CD49b^+$ cells

on day 0, raking up 3.49% and the number increased to 17.35% on the last day of experiment. The CD25 production between the inoculated and control mice was found to be statistically not significant ($p<0.05$) (0.051). Figure 8c elucidates that the gated percentage of CD49b for the control group ranged from 3.13% on day 0, rising up to 3.20% on the last day of experiment with slight fluctuations between, whilst for the inoculated group it starts off with 3.23% on day 0, increasing at least 4 times to 13.09% on the last day of experiment. The CD49b production between the inoculated and control mice was found to be statistically significant ($p<0.05$) (0.043).

DISCUSSION

Effect of breast cancer metastasis on weight of mice and tumour volume: The weight trend of the two populations of experimental mice, inoculated and control, were in similarly ascending fashion and there were no noticeable differences between the average weights of both groups. This is in agreement with studies conducted by others where it was reported there were no significant difference between the average body weights of control mice and tumour-induced mice (Birt, 1986; Welsch, 1987) (Freedman *et al*., 1990). This observation supports the contention that the diets fed and the amount of food intake by both groups of mice were found to be isocaloric. The increasing trend of the average weights observed in the 4T1-inoculated mice can be attributed to the fact that there was growing primary tumour burden by the day (Freedman *et al*., 1990). However, the weight gain itself was not in any way a source of any distress for the mice. Weight gains of both the inoculated mice and control mice also mirrors the findings in a comparative study between cancerous mice and control mice. It has been suggested that the slight increases in body weight for the cancerous mice could stem from a diet that provides more usable energy (Donato and Hegsted, 1985). In other words, it was found that the cancerous mice were more likely to indulge in increased calorie intake; hence more energy would be retained from the diet. This is possibly due to the internal changes taking place because of the growth and spread of tumour. However, it is important to note that this information may be discrepant in nature as there are several different methods that can be employed to measure body weight of experimental mice.

The mean tumour volume taken every 2 days from day 11 (i.e., from the day the tumour was palpable) showed a steadily increasing trend and the growing tumour burden was highlighted in the average body weight gains in inoculated mice. The 4T1 mammary carcinoma cells are reported to posses a unique primary growth kinetics and metastasis formation when they are injected in T-cell deficient nude mice versus immunocompetent BALB/c mice (Pulaski and Ostrand-Rosenberg, 1998). The increasing trend of the primary tumour in our study comes to suggest that T-lymphocytes are important effector cells in regulating primary tumour growth *in vivo* (Pulaski and Ostrand-Rosenberg, 1998).

Studies have shown that the ability of 4T1 tumour cells and metastatic mediators of the 4T1 cells to not only promote primary tumour growth, but also be selected within the primary site (Minn *et al*., 2007). This suggests that tumourigenesis and metastasis can be mechanistically linked and that primary tumour size *per se* does not independently influence 4T1-related metastasis. It has been postulated that while the primary tumour growth of these cells showed a steady incline in terms of volume, this factor alone may not necessarily contribute to the metastastic event solely by allowing for accumulation of metastasis genes, but rather may be required for the intravasation of pre-existing metastatically competent cells (Minn *et al*., 2007).

Effect of breast cancer metastasis on target organs, lungs, liver, spleen: gross and histopathological analysis: While previous studies have reported that the

breast cancer cells were able to migrate to certain target organs from the primary site, the time frame of the initial onset of metastasis have not been well-documented. The goal of our animal study was to pinpoint the approximate time frame for metastatic reference. The experimental mice were checked for palpable tumour from day 7 onwards, referring to previous studies that have noted that tumour mass is palpable around day 14 (Pulaski *et al*., 2000). For our batch of mice, tumour was found palpable on day 11. From the time the tumour was palpable, three mice were sacrificed every 2 days to investigate breast cancer metastasis. Lungs, liver and spleen were collected from the inoculated mice as these organs have been noted to be the target organs for breast cancer metastasis (Pulaski *et al*., 2000). Gross analysis revealed that whilst the lungs for both groups of mice remained the same average size, the liver and spleen extracted from inoculated mice were on average twice as large as the same organs extracted from control mice. This denoted a clear case of hepatosplenomegaly. Hepatosplenomegaly is a condition that is usually associated with increase workload, suggesting it is a response to hyperfunction. It is not surprising that hepatosplenomegaly is associated with any disease process that involves abnormal red blood cells being destroyed in the involved organs. Splenomegaly in a setting of metastatic carcinoma is usually caused by portal hypertension secondary to the obstruction of tumour deposits in the liver. Other common causes include congestion, also due to portal hypertension and infiltration by various carcinomas including breast cancer, leukemia and lymphoma (Boldt, 1998).

Histopathological analysis revealed that metastatic infiltrations started approximately on day 11 after the experimental mice were injected with viable 4T1 cells. Clear and marked metastatic tumour cells were seen in the sections of liver, but none were seen in spleen nor lungs. Spleen however showed signs of congestion, seen in the microscopic images as black spots (data not shown). These black spots were noted to grow in incidence and populated sporadically across the sections. The invasion of the congestion grew more apparent until day 23 when the sections were seen to be extremely congested. The congestion may be due to the infiltration of lymphocytes and macrophages, as a reaction to the breast cancer and its metastasis.

Animal models have been widely used in various pre-clinical tumour models, namely meningeal leukaemia (Perk *et al*., 1974) mammary carcinoma (Ostrand-Rosenberg *et al*., 2000; Russo and Russo, 1996), head and neck squamous cell carcinoma (Aubry *et al*., 2009), spinal tumours (Takahashi *et al*., 2004), prostate cancer (Drake, 2005) and many more. Mouse breast tissues undergo continuous changes throughout the lifespan of the active females, mediated mainly by interactions between the mammary epithelium and the sorrounding mesenchyme (Wiseman and Werb, 2002). The development and branching of mammary gland involve regulatory functions of several signaling pathways, including transforming growth factor-beta (TGF-beta) (Siegel *et al*., 2003) and Epidermal Growth Factor (EGF) (Pollak *et al*., 2004). These interactions between tumour cells and the sorrounding stroma, occurring via soluble growth factors, cytokines and chemokines as well as direct cell-cell adhesion are constantly modulating tumour development and are critical for tumour growth, migration, late-stage tumour progression and metastatic dissemination (Cavallaro and Christofori, 2004; Lopez *et al*., 2005).

The 4T1 cells are poorly immunogenic and share many characteristics with human breast tumours. It has been established as a model to study metastatic breast cancer (Pulaski and Ostrand-Rosenberg, 1998). In concurrence with our study that showed the 4T1 cells were able to grow into primary tumours and migrate rapidly to liver, various past studies have shown that inoculation of small quantities of 4T1 tumour cells in the abdominal mammary fat pad can cause growth of a primary tumour that will progressively grow into a nodule with the histology of a high-grade breast cancer and spontaneously metastasises to the lungs, liver, blood, lymph nodes, brain and bone marrow (Aslakson and Miller, 1992; Pulaski and Ostrand-Rosenberg, 1998; Ostrand-Rosenberg *et al*., 2000; Lelekakis *et al*., 1999). Analogous to human mammary carcinoma, metastatic cells proliferate at distant sites while the primary tumour is in place and continue to proliferate when the primary tumour is surgically removed (Pulaski *et al*., 2000). The animal model described in this study allows us to focus on the development of metastatic disease after the surgical removal of primary tumour. This scenario has not been previously explored in animal models and may be very relevant for human cancers such as breast cancer, in which metastatic disease after the excision of primary tumour is the principal cause of death.

Association of breast cancer metastasis and IFN-gamma production: The lack of experimental studies in metastatic tumour models means that even less is understood about the role of IFN-gamma in metastatic disease. In the present study, we have used a realistic metastatic mammary carcinoma model in which the primary tumour is excised to model the human disease situation and have examined some of the avenues of IFN-gamma activity. The

IFN-gamma has been implicated to be a critical mediator of metastatic tumour progression in BALB/c mice, with only marginal effects on primary tumour growth (Pulaski *et al*., 2000), as evidently seen in our study, whereby the production of IFN-gamma in inoculated mice was seen to increase with the size of the primary tumour volume. In general, IFN-gamma is produced predominantly by T-lymphocytes, natural killer T-cells (NKT) and natural killer (NK) cells, following activation with immune and inflammatory stimuli (Farrar and Schreiber, 1993; Boehm *et al*., 1997). The production of IFN-gamma in 4T1-inoculated mice was observed to be higher than the control mice, albeit with a fluctuating trend. Although we observed a slightly higher IFN-gamma production by the experimental mice, the difference between the two study groups was found to be not significant. A similar trend has been reported previously where the 4T1-inoculated mice were reported to produce slightly higher amount of IFN-gamma in the initial stages of tumour growth as compared to control mice (Ikeda *et al*., 2002). Ikeda *et al*. (2002) suggested that the initial increase in IFN-gamma levels may be due to the tumour being recognised first by NKT and NK cells that secrete this cytokine in the initial phase of tumour development and growth. Production of IFN-gamma at the tumour site subsequently induce production of more IFN-gamma by newly arrived cells of the innate immune system, such as NK cells and this is known to activate the cytotoxic functions of additional tumour infiltrating cells such as NK cells and activated macrophages. Whilst there are slight fluctuations in its production, primary tumours are exposed to a cytokine milieu, giving rise to anti-tumour responses by $CD4^+$ and $CD8^+$ T-cells. So the fluctuating trend of IFN-gamma in our study suggests that the continued production of IFN-gamma is a result of the immune system trying to reject the 4T1 cells by inducing and recruiting various leucocytes as well as in activating the MHC class I protein pathways to increase the tumour immunogenecity by promoting its recognition by tumour-specific T cells.

Effect and role of $CD8^+$, CD25 and CD49b in breast cancer metastasis: The net effects of the biological malignancy of tumour cells and the host factors serves as good determinants of the biological malignancy of cancer. Some of the representatives of the host factors include immune and vascular reactions and with this in mind, one of the presumptive mechanisms of breast cancer cell metastasis can be elucidated (Nakano *et al*., 2001). The functional characteristics of potentially tumour-reactive T-cells are being studied and analyzed as an important mechanism of immune evasion (Lee *et al*., 1999). Adaptive anti-tumour response is mediated by $CD8^+$ T-cells and to achieve initial recognition of cancers it exploits an indirect pathway, also known as cross-priming. The $CD8^+$ T-cells react with MHC-restricted tumour peptides derived from a number of different factors, including from mutated proteins and normal differentiations antigens that are produced by the cancer cells (Macchetti *et al*., 2006). In our study, we observed a marked increase in the percentage of $CD8^+$ lymphocytes in the 4T1-inoculated mice from day 0 and to the end of the experiment. The number of $CD8^+$ T-cells appears to steadily increase during the course of the experiment and was significant when compared to the control group.

The increase in the $CD8^+$ T-lymphocyte population suggests that activation of the host defence mechanism has occurred following infiltration of tumour cells. The marked increase in the $CD8^+$ T-lymphocyte population in the inoculated group from the day 11 (i.e., start of metastasis to liver) suggest possible initiation of an anti-tumour response. The increase of $CD8^+$ lymphocytes presence in our study is in agreement with the study conducted by Ekert and Vaux (1997), suggesting that the immune system recognises the infiltration of the tumour cells and might be trying to stimulate a wave of immunoediting and inducing cancer cell killing through some of the killing mechanism used by $CD8^+$ T-cells i.e., by direct exocytosis of granules that contain perforin and granzymes and by signalling by FasL. It has been suggested that the continuous migration and spread of the tumour cells may be due to the population of $CD8^+$ in the tumour microenvironment not having the ability to exert sufficient effector function or it could be an indication of the lack of lymphocyte maturation, given the step-wise functional maturation of cytotoxic T-cells (Gromo *et al*., 1987).

The T-lymphocytes that express high levels of CD25 is a subset of T-cells known as the T-regulatory cells, which also express CD4 and the transcription factor Fox P3. Collectively these cells have been shown to mediate suppressive function through cell-to-cell contact and soluble mediators such as anti-inflammatory cytokines IL-10 and TGF-beta (Gondek *et al*., 2005; Levings *et al*., 2002). A series of recent studies provided evidence that these cells, especially with a high number of CD25 are important players in immune evasion mechanisms used by tumours and the increase in the number of the cells as a function of tumour spread and growth has been demonstrated in various animal models as well as clinical settings (Ichihara *et al*., 2003; Ormandy *et al*., 2005; Wolf *et al*., 2003; Yu *et al*., 2005). In present study, the percentage of the CD25 population in the 4T1-inoculated mice showed a sharp increase from day 11 onwards and this upward trend continued until the end of experiment.

The percentages of the CD25 population were also higher when compared to the control group. Although we could establish that the difference in the CD25 population between the control and experimental group were statistically significant, we believe that there could be a positive correlation between the increased number of CD25 cells and tumour progression in experimental as well as clinical settings, providing a positive indirect evidence that this cell may play an important role in tumour immune evasion (Ichihara *et al*., 2003; Ormandy *et al*., 2005; Wolf *et al*., 2003). The increased population of CD25 as shown in our study has also been in agreement with suggestions by other researchers who showed that animals with tumours had increased percentages of systemic T-regulatory cells ($CD25^{high}$), playing a dominant role in early tumour progression (Elpek *et al*., 2007). An increase in the CD25 population was also associated with the increasing volume of tumour mass, where animals with large tumours had significantly higher percentages of $CD25^{high}$ cells compared with naive and small tumour-bearing animals. However, this does not in any way appear to change the kinetics of tumour growth.

Another set of cells in the immune system, CD49b which is found on various of cells including NKT cells, NK cells and fibroblasts, were first described as specialized lymphocytes of the innate immune system capable of eliciting cytotoxicity against MHC Class I-deficient tumour cells (Kiessling *et al*., 1975). Recent studies have focused on the role of NK cells in early defense against foreign cells and in tumour transformation. Both infections and tumour experimental models have shown that NK regulate adaptive immune responses. In our study, the gated percentage of CD49b was seen to steadily increase from day 11 onwards, also indicating a sharp rise from day 0. The percentages were also markedly increased when compared with the tabulated percentages of the control group. The results were statistically significant ($p = 0.043$). Marincola *et al.* (2000) reported that NK cells monitor for the loss of MHC Class I molecules from the surface of the tumour cells (Macchetti *et al*., 2006), which usually occurs during carcinogenesis (Diefenbach and Raulet, 2002; Dranoff, 2003). It is known that most breast cancers display diminished MHC Class I expression, which makes them vulnerable to NK cell-mediated killing. Present study showed a rather poor presence of NK cells when compared to T cells and this is agreement with the suggestion that MHC Class I might be associated with T-cell infiltration instead of NK cells (Marincola *et al*., 2000; Dranoff, 2003). The relatively high production of CD49b seen in the inoculated mice group is a probable indication of NK cells that were produced to generate an effective T-cell response, bringing about an anti-tumour effect (Kos and Engleman, 1996; Kurosawa *et al*., 1995). During ongoing immune responses, NK are rapidly recruited to produce IFN-gamma and IL-12 production mediated by dendritic cells, promoting a reciprocal cross-talk between these cells, leading to the initiation of efficient immune responses (Martín-Fontecha *et al*., 2004; Borg *et al*., 2004; Ferlazzo and Münz, 2004). However, the observation from our study showing the aggressive progression of tumour cells to target organs, as well as the growing tumour volume suggests that a single NK cells is not capable of lysing an excess of tumour cells *in vivo*, even though significant anti-tumour responses were seen (Hagenaars *et al*., 1998).

CONCLUSION

Present study has successfully demonstrated the growth and dissemination of 4T1 breast cancer cells from the primary site to the target organ, liver. The approximate time of metastatic onset in the BALB/c model was also discerned, which was on day 11. The *in vivo* study showed that a few immunological factors played into the spread of the tumour cells, including the increased production of IFN-gamma and a spike in T-cell marker like $CD8^+$, CD25 and natural killer cell marker like CD49b in the mice model.

Elucidation of the factors underlying breast cancer progression and metastasis has been tremendously fruitful from mouse models in which multiple stages of tumour progression are further confirmed. However, despite their obvious convenience in basic cancer research as well as in the testing of therapies, the use of mouse model carries several limitations. There are major discrepancies between human and mouse tumourigenesis, among which are the kinetics of carcinogenesis and the final size of the tumours, differences in cell intrinsic features and differences in physiology, metabolism, pathology and in the immune system (Fantozzi and Christofori, 2006). In-depth analysis of specific steps in the metastatic cascade would further the understanding of the basic biology of breast cancer metastasis, allowing a more critical evaluation of the roles of the many cellular and molecular factors implied in the environment and process of metastasis.

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