Egypt. Acad. J. biolog. Sci., 2 (2): 19- 26 (2010) **B. Zoology**
Email: egyptianacademic@yahoo.com **ISSN: 2090 – 0759**
Received: 21/4/2009 **www.eajbs.eg.net**

Immune responses in the microenvironment of a metastatic 4T1 mouse model

Shalini S. Kumar[1]; Radhakrishnan A. K.[1]; Cheong S. K.[2]
1- Department of Pathology , Faculty of Medicine and Health, International Medical University, 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia
2- Department of Medicine, Faculty of Medicine and Health, International Medical University, 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia

ABSTRACT

The 4T1 murine mammary carcinoma cells have the ability to spread to target organs in BALB/c mice breast cancer model. The spread of 4T1 cells mimics human stage IV breast cancer and elicits immune responses. The aim of this study is to establish an animal model of mammary carcinoma metastasis to discern the *in vivo* effects of growth and spread of breast cancer. Six-weeks-old female BALB/c mice were inoculated with 4T1 murine breast cancer cells. 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. The metastatic onset occurs approximately 11 days after inoculation and accompanied with hepatosplenomegaly. The breast cancer cells from primary tumour were found to spread rapidly to the liver on day 11. IFN-γ production was higher in inoculated mice serum compared to control. Higher numbers of $CD8^+$, CD25 and CD49b cells were observed in the peripheral blood of inoculated mice, compared to control. In conclusion, the 4T1 murine breast cancer cells can migrate and metastasise rapidly to the liver, eliciting various immune responses.

Keywords: 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), with breast cancer shown to be one of the major causes of cancer-related mortality in women (Hill and Iverson, 1997). The metastatic process is made up of a sequence of events, namely invasion, intravasation, transport, arrest, extravasation and growth. Several cytokines are known to promote the dissemination of breast cancer tumours to target organs and one of them is interferon-gamma (IFN- γ). Recently, IFN- γ has also been implicated to have a prominent role in immune responses to tumours (Elpek *et al.*, 2007).

The generation of a mouse model of breast cancer is a critical step towards the understanding of many factors underlying mammary carcinogenesis. In this study immunocompetent mice (BALB/c) and a syngeneic mammary carcinoma cells (4T1).

The 4T1 were used poorly immunogenic and the primary tumour in 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 aim of this study is 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

Cell line and reagents

The 4T1 cells (Pulaski *et al.*, 2000) were purchased from ATCC (P.O. Box 1549, Manassas, Virginia 20108, USA). The cell line was cultured 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 antibiotic mixture (Gibco, USA). The cells were cultured according to the protocol provided by ATCC (ATCC Tissue Culture Protocol).

Experimental model

Six to eight-weeks-old female BALB/c mice were obtained from the Animal Facility in Universiti Putra Malaysia. (Serdang, Kuala Lumpur, Malaysia). 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. Mice were housed at the Animal Holding Facility at the International Medical University and all animal procedures were subjected to review and approval by the Research and Ethics Committee of International Medical University.

Gross and histopathological studies

Lungs, liver and spleen were carefully removed when tumour was first palpable (day 11 after inoculation). The morphology and conditions of the extracted organs were observed. The organs were stored in 10% formalin solution for at least 48 hours and were retained as materials for histopathological studies, subjecting through Haemotoxylin and Eosin (H&E) staining. The metastastic tumour infiltrations were evaluated using the Nikon Brightfield Compound Microscope at a magnification power of 100X using the NIS Elements BR 3.0 software. Sections from control mice (did not receive 4T1 cells) were used for comparison.

Estimation of on serum levels of IFN-γ

The IFN- γ production level in mice plasma was estimated using a commercial ELISA kit (eBioscience, USA). The results were analysed using a microplate reader (Tecan, Switzerland) at 450 nm. The determination of cytokine concentration estimation was achieved by plotting the mean absorbance values against the standard concentrations and this was compared to the standard curve.

Staining of cell surface markers

Briefly, 100 µl of whole blood aliquots were single-stained with three antibodies: BD Biosciences, USA, FITC-conjugated CD8, PE-conjugated CD25, PE-conjugated CD49b, with the last tube serving as control (non-stained). After 20 minutes of staining, the tubes were washed once with PBS using centrifugation and resuspended with 200 µl of PBS after the supernatant was discarded. They were analysed using the flow cytometer (FACSCALIBUR, BD Biosciences, USA).

Statistical analysis

The statistical tests were employed on the data using SPSS 15.0 software and data were checked for normal distribution before proceeding to other tests. The unpaired *t*- test was performed at a *p-value* set at less than 0.05.

RESULTS

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. 1). The liver and spleen from the 4T1-inoculated mice were almost twice the size of the same organs from the control mice denoting hepatosplenomegaly. This morphological abnormality indicates tumour infiltration in the liver and the hyperfunction of the spleen following the spread of the tumour.

Histolopathological analysis of metastatic tumour infiltrates in target organs. The microscopical sections of the liver showed the tumour cells were either deep blue or deep purple in colour (Fig. 2). The cells were also noted to be on the larger side and usually appear as clumps or groups.

The tumour cells from the primary tumour excised on day 17 appear to be cuboidal and epithelial (Fig. 3a) while those from the tumour excised on day 21 appear to be spindle and fibroblastic (Fig. 3b).

The tumour cells also were seen in the sinusoidal region, possibly restricting the blood flow and obstructing the fenestrated system of the vascular channels. Microscopic sections of lungs and spleen of the 4T1-inoculated mice did not show presence of any metastatic tumour cells.

Production of IFN- γ in serum of inoculated mice and control mice

As shown in Fig.4, the amount of IFN- γ in the plasma of 4T1-inoculated appears to fluctuate. However, the trend in plasma levels of IFN- γ production between the inoculated and control mice was similar and statistically not significant (p=0.368).

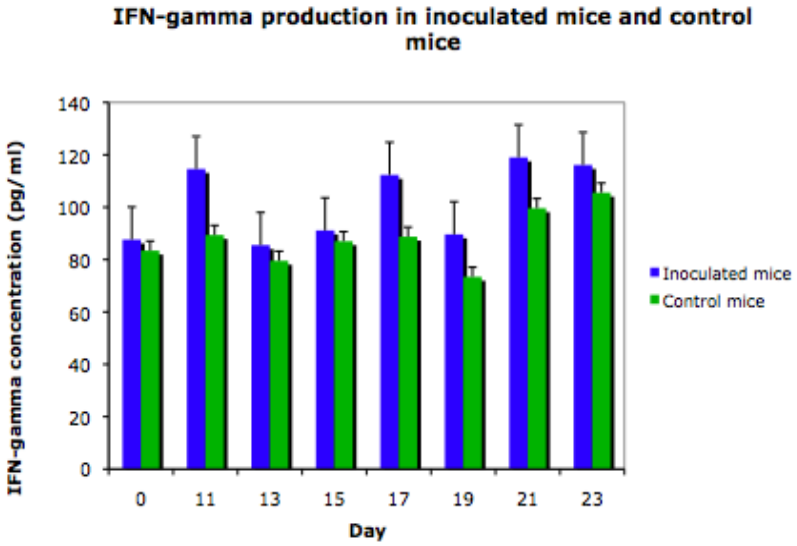
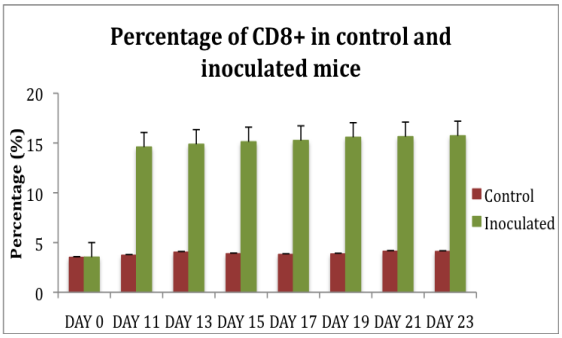


Fig. 4: Chart showing the IFN-gamma production in 4T1-inoculated mice and control mice on day 0 and every two days from day 11 (palpable tumour mass) onwards (p>0.05).

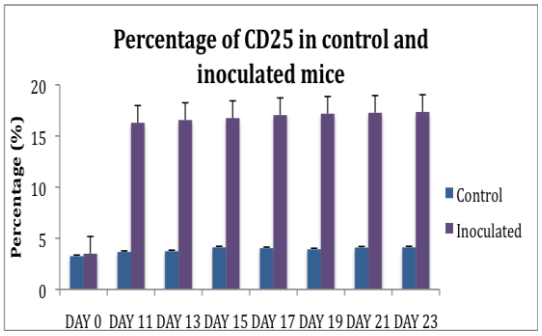
Flow cytometry analysis of cell surface markers

As shown in fig. 5a, the gated percentage of $CD8^+$ for the inoculated mice was higher than control mice from day 11 onwards and its production was found to be statistically significant ($p<0.05$) (0.047). Similarly in Figure 5b the gated percentage of CD25 for the inoculated group was almost 3 times higher than of the control mice, with its production being statistically not significant (p=0.051). Figure 5c elucidates that the gated percentage of CD49b for inoculated mice was 3 times higher than the control mice, the production being statistically significant (p=0.043).

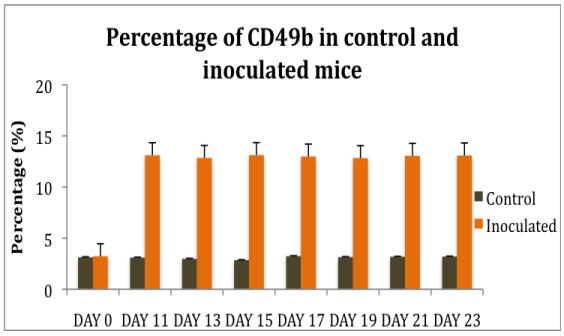
a) $CD8^+$ T-cells



b) $CD25^+$ cells



c) $CD49b^+$ cells



Figs. 5: 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.

DISCUSSION

Effect of breast cancer metastasis on target organs, lungs, liver, spleen: *gross and histopathological analysis*

There was a clear case of hepatosplenomegaly and this can be associated with increase workload, suggesting it is a response to hyperfunction. One of the goals of our animal study was to pinpoint the approximate time frame for metastatic reference. Histopathological analysis revealed metastatic infiltrations in the liver, starting approximately on day 11 after the experimental mice were injected with viable 4T1 cells.

Association of breast cancer metastasis and IFN- γ production

The IFN-gamma has been implicated to be a critical mediator of metastatic tumour progression in BALB/c mice (Pulaski *et al.*, 2000). Production of IFN- γ at the tumour site subsequently induce production of more IFN- γ 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. 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 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 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.

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 (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).

The study showed a rather poor presence of CD49b when compared to T cells, and this is in agreement with the suggestion that MHC Class I might be associated with T-cell infiltration instead of NK cells (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 and also promoting a reciprocal cross-talk between immune cells, initiating efficient immune responses (Ferlazzo and Münz, 2004).

CONCLUDING REMARKS

The interrelationships of the immune cells and tumour can be considered important, especially in view of the role played by the immune system in the pathogenesis and progression of the breast cancer. Further understanding of the metastasis process allows a more critical evaluation of the roles of the many cellular and molecular factors implied in the microenvironment of a 4T1 mouse model.

REFERENCES

ATCC Tissue Culture Protocol, sourced from: http://www.atcc.org/common/products/CellImmortOverview.cfm

Dranoff, G. (2003). Coordinated tumour immunity. J. Clin. Invest., 111:1116-1118.

Ekert, P.G. and Vaux, D.L. (1997). Apoptosis and the immune system. Br. Med. Bull., 53: 591-603.

Elpek, K.G.; Lacelle, C.; Singh, N.P.; Yolcu, E.S. and Shirwan, H. (2007). CD4+CD25+ T regulatory cells dominate multiple immune evasion mechanisms in early but not late phases of tumour development in a B cell lymphoma model. J. Immunol., 178: 6840-6848.

Ferlazzo, G. and Münz, C. (2004). NK cell compartments and their activation by dendritic cells. J. Immunol., 172: 1333-1339.

Heppner, G. H.; Miller, F. R. and Shekhar, P. M. (2000). Nontransgenic models of breast cancer. Breast Cancer Res., 2: 331–334.

Hill, D. and Iverson, D. (1997). World Conference for Cancer Organisations: March 3-7, 1996, Melbourne Australia. Cancer, 79: 619-625.

Ormandy, L. A.; Hillemann, T.; Wedemeyer, H.; Manns, M.P.; Greten, T.F. and Korangy, F. (2005). Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res., 65: 2457-2464.

Parkin, M.D.; Pisani, P. and Ferlay, J. (1999). Global Cancer Statistics. CA Cancer J Clin, 49: 33-64.

Pulaski, B.A.; Terman, D.S.; Khan, S.; Muller, E. and Ostrand-Rosenberg, S. (2000). Cooperativity of Staphylococcal aureus Enterotoxin B. Superantigen, Major Histocompatibility Complex Class II, and CD80 for immunotherapy of advanced spontaneous metastases in a clinically relevant postoperative mouse breast cancer model. Cancer Res., 60: 2710-2715.

Wolf, A. M.; Wolf, D.; Steurer, M.; Gastl, G.; Gunsilius, E. and Grubeck- Loebenstein, B. (2003). Increase of regulatory T cells in the peripheral blood of cancer patients. Clin. Cancer Res., 9: 606-612.

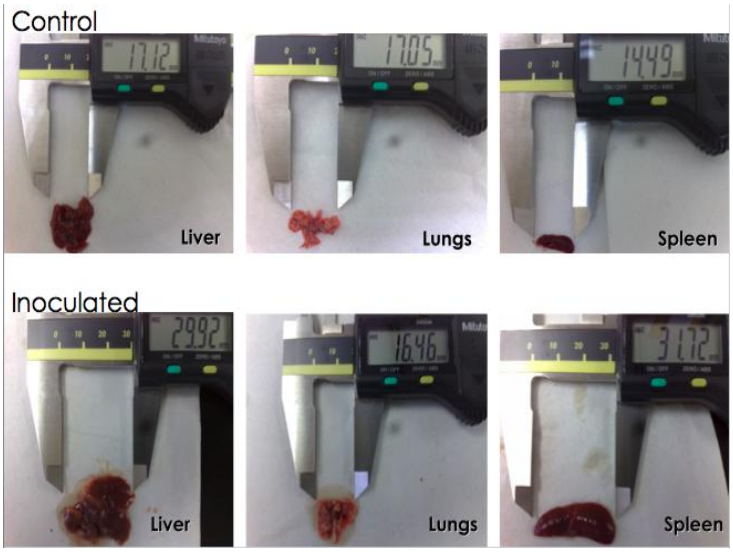


Fig. 1: Image showing the gross pictures of various organs (liver, lungs and spleen) taken from control and tumour-inoculated mice on day 17.

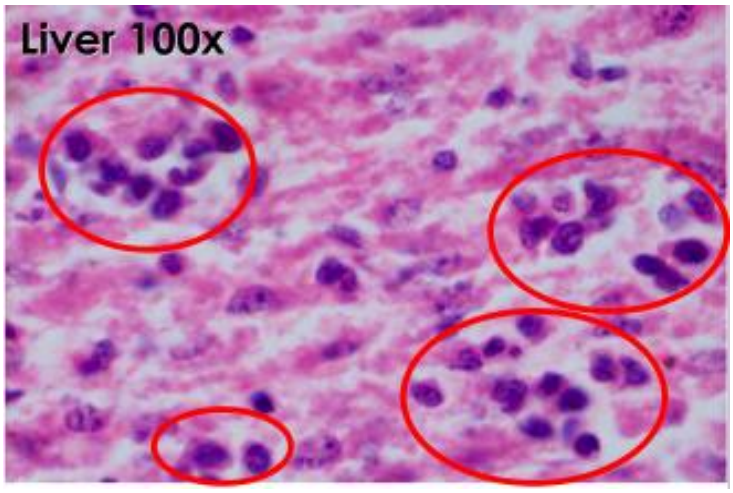


Fig. 2: Image showing a microscopic image of H&E stained liver section of a 4T1-inoculated mouse taken on day 17. Red circles denotes the cluster of metastatic tumour infiltrates appearing as deep blue and deep purple clumps or groups and are also larger in size compared to normal hepatic cells.

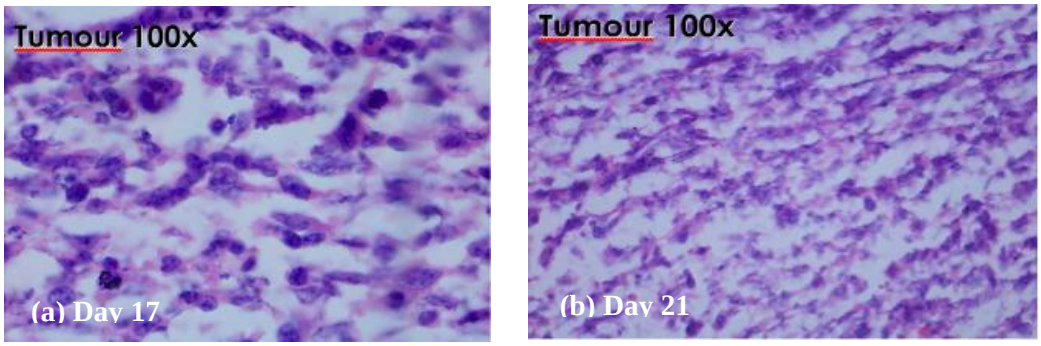


Fig. 3: Image showing a microscopical picture of H&E stained section of the primary tumour of a 4T1-inoculated mice that was sacrificed on a) Day 17 and b) Day 21.

ARABIC SUMMARY

Immune responses in the microenvironment of a metastatic 4T1 mouse model

Shalini S. Kumar[1]; A.K. Radhakrishnan[1], S.K. Cheong[2]

1- Department of Pathology , Faculty of Medicine and Health, International Medical University, 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia

2- Department of Medicine, Faculty of Medicine and Health, International Medical University, 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia

إنّ خلايا سرطان الثدي 4T1 لها القدرة على الانتشار الى اعضاء معينه في الفئران ،من سلالة BALB/c، المصابة بسرطان الثدي. ان انتشار خلايا ال 4T1 مشابها" للمرحلة الرابعة لسرطان الثدي لدا الانسان ويحفز الجهاز المناعي لديه. ان الهدف من هذه الدراسة هو لإنشاء نموذج حيواني من سرطان الثدي للتبين من اثر نموه وانتشاره في الجسم الحي. تم تلقيح فئران من نوع BALB/c ذات ستة اسابيع من العمر بخلايا سرطان الثدي 4T1. اجريت دراسات نسيجية لتحديد اليوم التقريبي لبدء الانتشار . وجرى تقييم انتاج الانترفيرون gamma بواسطة ELISA لفهم تأثيرها على نمو وانتشار الورم . تم تحليل علامات الخلايا اللمفاوية مثل: +CD8، CD25، CD49 وذلك لاستيضاح دورها في نمو وتطور الورم . عملية الانتشار بدأت بعد 11 يوم تقريبا من تأريخ الحقن وكان مصحوبا بتضخم الكبد . لقد وجد إنّ الخلايا السرطانية تنتشر بسرعة الى الكبد في اليوم الحادي عشر . ووجد ايضا زيادة انتاج الانترفيرون y من قبل الفئران المحقونة مقارنتا" بالفئران غير المحقونة بخلايا سرطان الثدي، كما شوهد ارتفا ع اعداد $CD8^+$، CD49b، CD25 في الدم لدى الفئران المحقونة بالمقارنة مع غير المحقونة . في المحصلة وجد انه لايمكن ل خلايا سرطان الثدي 4T1 -في الفئران- بالانتشار بسرعة الى الكبد، وذلك بسبب تحفيز الاستجابات المناعية المختلفة.