Video Article

Orthotopic Implantation and Peripheral Immune Cell Monitoring in the II-45 Syngeneic Rat Mesothelioma Model

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Abstract

The enormous upsurge of interest in immune-based treatments for cancer such as vaccines and immune checkpoint inhibitors, and increased understanding of the role of the tumor microenvironment in treatment response, collectively point to the need for immune-competent orthotopic models for pre-clinical testing of these new therapies. This paper demonstrates how to establish an orthotopic immune-competent rat model of pleural malignant mesothelioma. Monitoring disease progression in orthotopic models is confounded by the internal location of the tumors. To longitudinally monitor disease progression and its effect on circulating immune cells in this and other rat models of cancer, a single tube flow cytometry assay requiring only 25 µl whole blood is described. This provides accurate quantification of seven immune parameters: total lymphocytes, monocytes and neutrophils, as well as the T-cell subsets CD4 and CD8, B-cells and Natural Killer cells. Different subsets of these parameters are useful in different circumstances and models, with the neutrophil to lymphocyte ratio having the greatest utility for monitoring disease progression in the mesothelioma model. Analyzing circulating immune cell levels using this single tube method may also assist in monitoring the response to immune-based treatments and understanding the underlying mechanisms leading to success or failure of treatment.

Video Link

The video component of this article can be found at https://www.jove.com/video/53019/

Introduction

Malignant mesothelioma (MM) is an aggressive malignancy which arises from transformed cells in the membrane (mesothelium) that lines the lung and abdominal cavities, heart and internal reproductive organs, and is the most common primary tumor of the lung cavity or pleura[1,2]. Exposure to asbestos fibres accounts for 80% of all MM, and while bans on asbestos use were introduced decades ago in most western countries, its widespread use in the community has left a deadly legacy. The World Health Organization has estimated that 107,000 people worldwide die each year from asbestos related diseases, with mortality rates continuing to increase. A new non-occupational incidence wave is also emerging and there is little understanding of when, and at what level this will peak[3].

The majority of people with MM are diagnosed late when systemic chemotherapy represents one of the only viable options[4]. The most effective chemotherapy and current 'standard of care' (pemetrexed together with cisplatin[5]) was identified over 10 years ago. However failure of this treatment is inevitable and there are no proven second line options, leaving patients with a grim prognosis and median survival of only 12 months[2]. Therefore, there is an urgent unmet need for more effective treatments. Despite the examination of a number of novel therapies in clinical trials none has resulted in changes in practice. This is due in part to the low (5%) transference of pre-clinical results, generally performed in xenograft mouse models, to the clinical setting[6-8]. Such models do not faithfully recapitulate the complex aspects of the tumor microenvironment occurring in non-physiological locations, frequently in the absence of a functioning immune system[9].

Syngeneic orthotopic models create a significantly more realistic tumor environment than the commonly used subcutaneous xenograft models as the tumors occur in the correct physiological location with an intact immune system[10,11]. The larger size of the rat enhances its use as a rodent disease model, especially in drug studies where serial blood draws are required to assess treatment response and toxicity[12]. Furthermore, in models in which monitoring disease progression is difficult due to the location of the tumors (such as in the pleural cavity), the ability to monitor disease progression using factors found in the circulation is extremely attractive. The generation of a syngeneic orthotopic model of pleural mesothelioma using immune-competent rats is described. In addition, an easy and relatively non-invasive method for monitoring pleural disease progression by measuring circulating immune cells is also described.

Protocol

All procedures involving animals were carried out in accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The protocol for this study was approved by the Royal North Shore Hospital Animal Care and Ethics

Committee. Female Fischer 344 rats (F344, 150-200 g) were maintained at the Kearns Facility, Kolling Institute under standard conditions (12 hr light/dark cycles and free access to food and water).

Note: A flow chart for all experimental procedures is presented in **Figure 1.**

1. Preparation of Cells for Implantation

1. Culture the rat mesothelioma II-45 cell line (also known as IL-45; derived by peritoneal introduction of crocidolite asbestos) in RPMI 1640 (RPMI) media supplemented with 10% fetal bovine serum (FBS) and grow in standard conditions (37 °C humidified incubator with 5% CO_2). Maintain by passaging and sub-culturing at approximately 1:50 twice a week in a 75 cm^2 flask.
2. Prepare reagents for cell culture and warm aliquots at 37 °C. Required reagents include serum free RPMI media (SFM), RPMI with 10% FBS, phosphate buffered saline (PBS) and 0.5% trypsin-EDTA.
3. Culture cells for implantation to approximately 70-80% confluence. This ensures they are in the linear growth phase.
4. Harvest cells by discarding the media, washing once with 5 ml of sterile PBS and then adding 3 ml of 0.5% trypsin-EDTA.
5. Return flasks to the incubator for approximately 5 min until all cells become non-adherent.
6. Once cells are non-adherent, add 3 ml of RPMI with 10% FBS to inactivate the trypsin. Collect and centrifuge cells at 300 x g for 3 min.
7. Wash the cell pellet in 10 ml of SFM and centrifuge again at 300 x g for 3 min.
8. Wash cell pellet again with 10 ml of SFM and centrifuge as above.
9. Resuspend the cells in 10 ml of SFM and perform a cell count using a hemocytometer or similar instrumentation.
10. Dilute cells so that 100 µl contains the amount of cells to be implanted.
    Note: Tumor growth has been demonstrated at a dose as low as 100 cells in 100 µl but a standard dose is 500,000 cells in 100 µl.
11. Prepare sufficient cells in media for the number of rats to be implanted (*i.e.,* 100 µl/rat), plus at least 0.5 ml extra to compensate for losses from priming and the dead volume of the needle.
12. Prepare sufficient SFM (without cells) to be implanted into control rats (*i.e.,* 100 µl/rat), plus at least 0.5 ml extra.
    Note: The cells and SFM are now ready for implantation. They should be kept at 37 ^{o}C and implanted within 2 hr of harvesting to maintain viability.

2. *In vivo* Implantation of Cells

1. Place the F344 rat (> 13 weeks of age) into the induction chamber and anesthetize using 1.4% isoflurane inhalation (or the method in use in the facility). Once the rat appears to be asleep move it from the chamber to a nose cone (with 1.4% isoflurane flowing), place it on its back with chest facing up (ventral view). This allows the internal organs to settle away from the chest cavity. Check reflexes according to institutional protocols to ensure the rat is fully anesthetized.
2. Shave the right regio costalis (chest) area to remove the fur.
3. Clean the shaven area with 80% v/v ethanol.
4. Identify the injection site: on the right side, find the 2nd gland starting cranial. The injection site is 0.5 cm proximal to this, in between the 3rd and 4th rib from the caudal end of the rib cage. (**Figure 2A**).
5. Gently mix the II-45 cells to resuspend. Slowly draw the cell suspension (or SFM for control rats) into a 1 ml syringe without a needle attached. If a needle is attached for the drawing up of cells there is the potential for cells to grow along the needle injection line. Attach a 23G x 1¼ needle. Prime the needle and remove any air bubbles.
6. Once the syringe and needle are primed, place a 20 mm long and 5 mm diameter spacer over the needle shaft. This is used to prevent the needle from penetrating too deeply into the pleural cavity during the injection. Approximately 5 mm-12 mm of exposed needle is sufficient for penetration through the ribs without damaging any organs.
7. Slowly insert the needle between the ribs, draw back on the syringe to ensure a blood vessel has not been punctured (no blood should appear in the syringe) then inject 100 µl cells or SFM. (**Figure 2B**).
8. Remove the needle and gently roll the rat from side to side to spread cells in the chest cavity.
9. Place the rat into a cage and check for recovery. The rat should be awake within 1 min and starting to move around.
10. Repeat for each rat using a new needle. Reusing the same needle will result in cell growth along the injection line of the needle.
11. Monitor the well-being of the animals daily.
12. Euthanize animals at ethically defined endpoints as governed by the institutional animal ethics committee. The ethical endpoints for the rats in these experiments were weight loss of greater than 10% or labored breathing.

3. Tail Vein Blood Collection

1. If blood is to be collected immediately post-cell implantation, keep the rat anesthetized. If sampling blood at another time point, anesthetize the rat using 1.4% isoflurane inhalation. Check reflexes according to institutional protocols to ensure the rat is fully anaesthetized.
2. Place the rat onto its side and locate a lateral tail vein.
3. Sterilize the tail with 80% ethanol and label a 0.5 ml EDTA collection tube.
4. To collect blood, always start at the caudal end of the tail (approximately one third of the way along). This allows further attempts closer to the cranial end of the tail in case the first attempt is unsuccessful. Never resample caudally as this can cause a blood clot.
5. Position a 23G x 1¼ needle parallel to the lateral vein and slide it into the vein at a shallow angle so it penetrates approximately 10 mm (**Figure 3A**).
6. Note: If the vein has been successfully punctured blood will be visible in the attachment end of the needle (**Figure 3B**).
7. A drop of blood will form on the tail at the site of the needle puncture. Collect this blood using a pipette and transfer into the labelled 0.5 ml (or smaller) EDTA collection tube. For the immune cell assay 25 µl is sufficient. Apply gauze with pressure to puncture site until bleeding stops.
8. Flick the blood tube to mix the blood and EDTA to prevent clotting. Keep the time between blood collection and mixing with the EDTA as short as possible to prevent clotting.

9. When collecting blood from multiple rats store EDTA-blood samples in a rack at RT until analysis. Process blood within 2 hr of collection.

4. Sample Preparation for Immune Cell Profiling Using the Bead-based Method

Note: This single platform method relies on using commercially available absolute counting tubes that have a known number of beads for each sample. These tubes contain lyophilized pellets that dissolve during sample preparation, releasing the beads. The beads are fluorescently labelled and by gating on the bead population, absolute counts can be calculated.

1. Ensure the EDTA whole blood sample is well mixed by placing it on a slow rotary mixer for several min. Label one absolute counting tube for each sample. A pellet containing the beads should be visible underneath the metal bead holder at the bottom of the tube.
2. Transfer 25 µl of EDTA whole blood into a labelled absolute counting tube. The bead pellet will dissolve upon addition of the blood.
3. To each tube add 20 µl of anti-rat T/B/Natural Killer (NK) cell cocktail, 10 µl of anti-rat CD8a PE, 10 µl of anti-rat CD4 (domain 1) FITC and 10 µl of anti-rat CD45 PE/Cy7 (**Figure 4A**). Fluorophores are defined in **Table 1**.
4. Centrifuge the tube briefly (300 x g) to ensure the antibodies and cells are in the bottom of the tube and not stuck to the side of the tube. Vortex to mix and incubate for 15 min at RT.
5. To lyse red blood cells add 400 µl of 10 mM Tris, 0.15 M ammonium chloride buffer (pH 7.5) and vortex to mix. Lysis is complete when the sample appears translucent and not cloudy (**Figures 4B and C**). Failure to lyse the sample completely will lead to increased background and falsely elevated counts when analyzing by flow cytometry.

5. Flow Cytometric Processing of Samples

Note: Perform on a 4 color flow cytometer.

1. Open the software in acquisition mode and a new template with 8 plots as depicted in **Figure 5**.
2. Adjust instrument settings to those listed in Table 1 and set up gate R1 (FITC [FL-1] *versus* APC [FL-4]), **Figure 5Ai**) to count the fluorescent beads. The other gates are not as important at this acquisition stage but will be required for analysis. The absolute counting beads used in this protocol contain fluorescent dyes and can be detected in any channel although are weakest in the blue channel.
3. Using a prepared control blood sample, vortex and then load onto the cytometer and run at a low speed (12 µl/min) on setup mode so data acquisition gates can be adjusted.
4. Set the acquisition to collect 10,000 events in the R1 bead gate.
5. Set up a folder to record data and set file number and label sample file in acquisition menu.
6. Load the sample to be analyzed onto the cytometer and set the flow rate to medium (35 µl/min). Run each sample at the same flow rate. The flow rate may need to be varied to low (12 µl/min) or high (60 µl/min), but medium is generally appropriate. At this rate it takes approximately 90 to 120 sec to acquire 10,000 bead events for each sample.
7. Once the sample is loaded watch the scatter plots to make sure events are appearing in the R1 bead gate. Initially there can be some instability in the sample pressure causing drift in the scatter plots. Wait for this to stabilize.
8. Once stabilized, click on acquire and allow sample to run. Once the cytometer has finished acquiring 10,000 bead events in R1 the cytometer will stop acquiring and save all data.
9. Remove the sample and discard flow tube. The cytometer is now ready for the next sample. Run all samples and then proceed to analysis mode.

6. Immune Cell Analysis

Note: Gating strategies and Boolean algebra are used to define each cell population. Boolean algebra is a logic based analysis method that allows for multiple operations in a single definition. The analysis software of the flow cytometer (*e.g.*, BD CELLQuest) allows for the use of Boolean algebra. The equations are used to actively account for the significant negative reactivity that assists in defining the cell to more specifically identify each cell population. 'Regions' are used to define a 'gate'. Regions define a 2 dimensional space whereas gates can be composed of numerous regions connected by algebraic operators (+, *,-, defined in **Table 2**).

1. Switch the software to analysis mode. An analysis template should be generated to match **Figure 5** with the plots and gates shown.
2. Analyze each individual file (*i.e.,* each individual sample) separately. Set up gates R1 through to R9 and then set up the algorithms for each cell type as defined in **Table 2** (also shown in **Figure 5**) .
3. Use the cell statistics counter to calculate individual cell populations defined by gates and algorithms (**Table 2 and Figure 5**). The algorithms will adjust cell numbers automatically in the cell statistics counter.
4. Calculate cell subsets using the following equation:

$$\frac{Number\ of\ events\ for\ a\ defined\ cell\ type}{25\ \mu L} X \frac{number\ of\ bead\ events\ counted}{number\ of\ beads\ per\ tube\ (stated\ on\ product\ pouch)}$$

Note: Number of cell events counted (*e.g.*, CD4 T cell events) is enumerated using the above equation to give cell number per µl of blood. Examples are shown in **Figure 5**.

Representative Results

The method used in this paper for the generation of an orthotopic model of pleural mesothelioma using II-45 cells resulted in animals succumbing to mesothelioma in a reproducible and rapid timeframe, with no rats dying due to the implantation method. Titration of the number of cells implanted determined that 1x 10^3 cells was the minimum number required for a fully penetrant model (100% engraftment). The different

number of cells implanted in the rats changed the time course of the disease without appearing to affect the severity of the disease. Animals that received 1 x 10^3, 1 x 10^4 and 5 x 10^5 cells reached ethically defined endpoints by day 40, 30 and day 20 respectively **(Figure 6)**.

Blood was collected from rats approximately twice each week with no complications observed related to the collection. It is important to note that no more than 10% of the total blood volume (or approximately 2 ml) should be collected in a 2 week period. A flow cytometric assay which combined gating strategies and Boolean algebra was established to identify lymphocytes, monocytes and neutrophils and the lymphocyte subsets T, CD4 T, CD8 T, B and NK cells. The mean and range for each cell type was established using control rats (n=5) and was compared to the mean and range of II-45 implanted rats at endpoint **(Table 3)**. Endpoint values were first compared to determine whether differences existed in immune cell counts between healthy and diseased animals. In comparison to healthy control rats, total lymphocytes, CD4 T cells, B lymphocytes and NK cells decreased in II-45 implanted rats at endpoint whereas monocytes, neutrophils and neutrophil to lymphocyte ratio (NLR) increased.

To determine whether these immune cell parameters also changed with disease progression and thus could be used as surrogate markers for monitoring disease, longitudinal samples were also compared **(Figure 7)**. The most informative longitudinal parameter was NLR with increases detected up to 7 days before ethical endpoints **(Figure 7A)**. For rats with high dose cells, high NLRs were detected between day 7 and day 12 post implantation, whereas for rats with low dose cells, the NLR began to increase between day 18 and 22. Total lymphocyte counts decreased with disease progression in rats with a longer disease time course model (low cell dose: 1 x10^4, **Figure 7B**). This decrease was not evident in rats with a rapid time course (high cell dose: 5 x10^5). Monocytes remained at normal levels until the terminal collection when grossly increased counts were often observed **(Figure 7C)**. The other immune cell counts (neutrophils, NK, B cell and CD4 and CD8 T cells) were more variable and thus less informative.

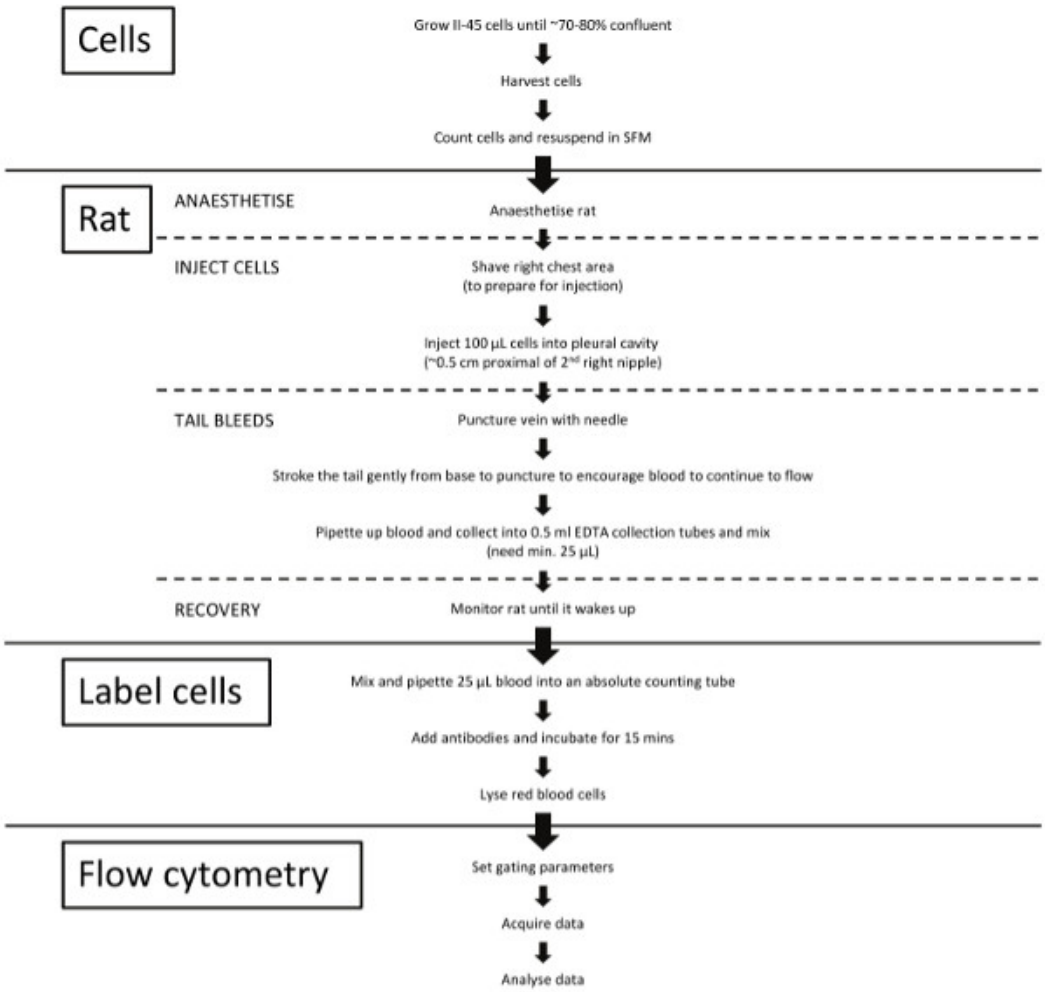

Figure 1. Flow chart of experimental procedure. Please click here to view a larger version of this figure.

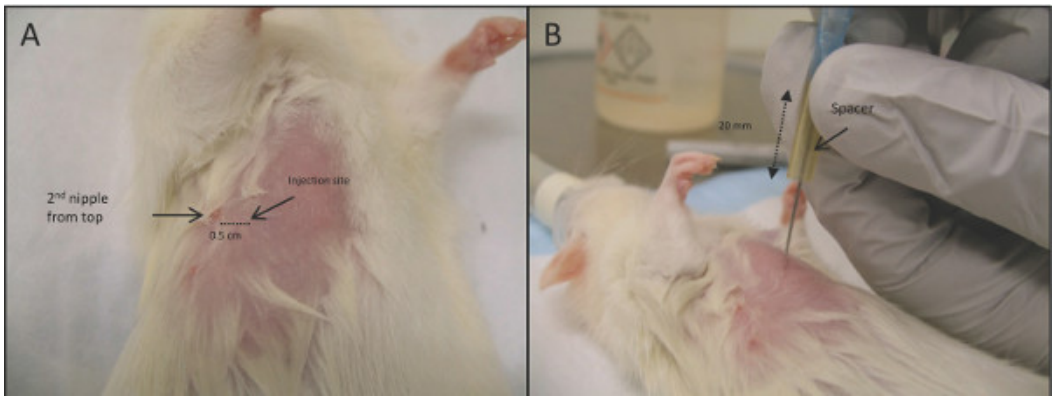

Figure 2. Location and site of implantation. The rat is first anesthetized, placed on its back and the right regio costalis (chest) area is shaved to remove fur. On the right side, the 2nd nipple from the top is identified and the injection site is located 0.5 cm proximal to this, in between the 3rd and 4th rib from the bottom of rib cage **(A)**. A spacer is then placed over the prepared needle and syringe to prevent the needle penetrating too deeply into the pleural cavity and 100 µl of cells or SFM is injected **(B).** Please click here to view a larger version of this figure.

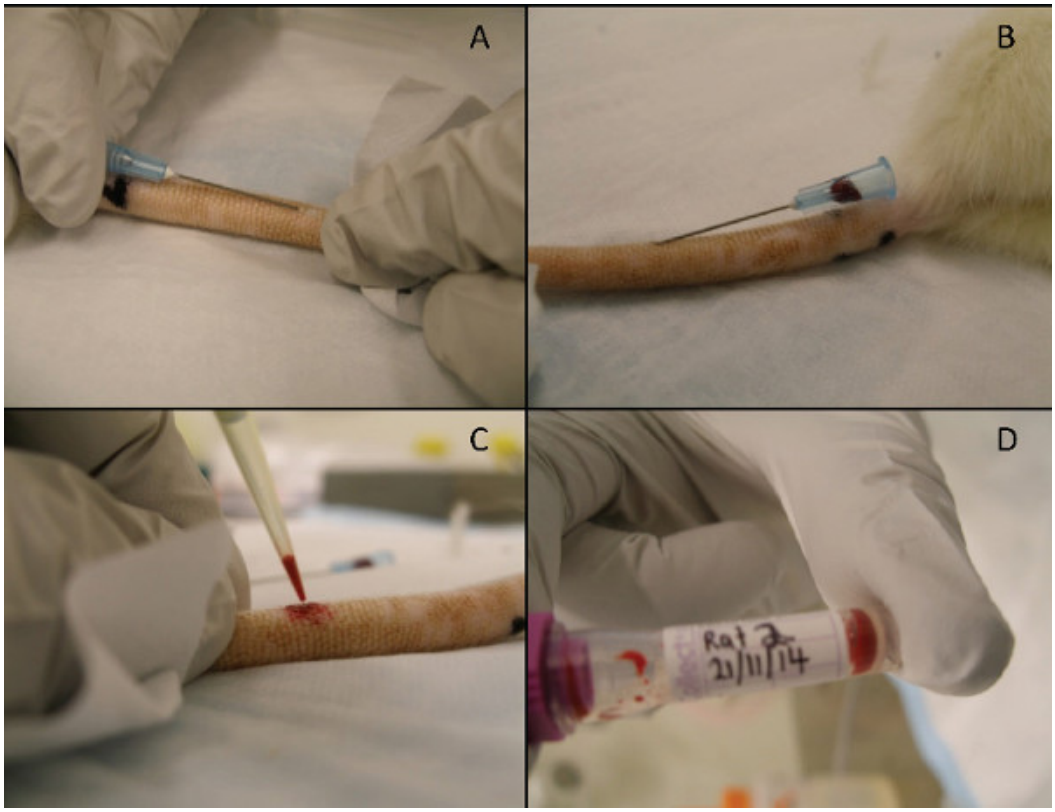

Figure 3. Sampling technique for collecting blood from tail. Position a 23G x 1¼ needle parallel to the lateral vein **(A)**. Slide the needle into the vein at a shallow angle, approximately 10 mm deep, and hold there for a few seconds until blood is visible **(B)** then remove needle. Collect blood using a pipette **(C)**. If necessary gently stroke tail vein to ensure blood continues to flow. Pipette blood into a 0.5 ml EDTA collection tube **(D)**, mixing as you go to prevent clotting. Please click here to view a larger version of this figure.

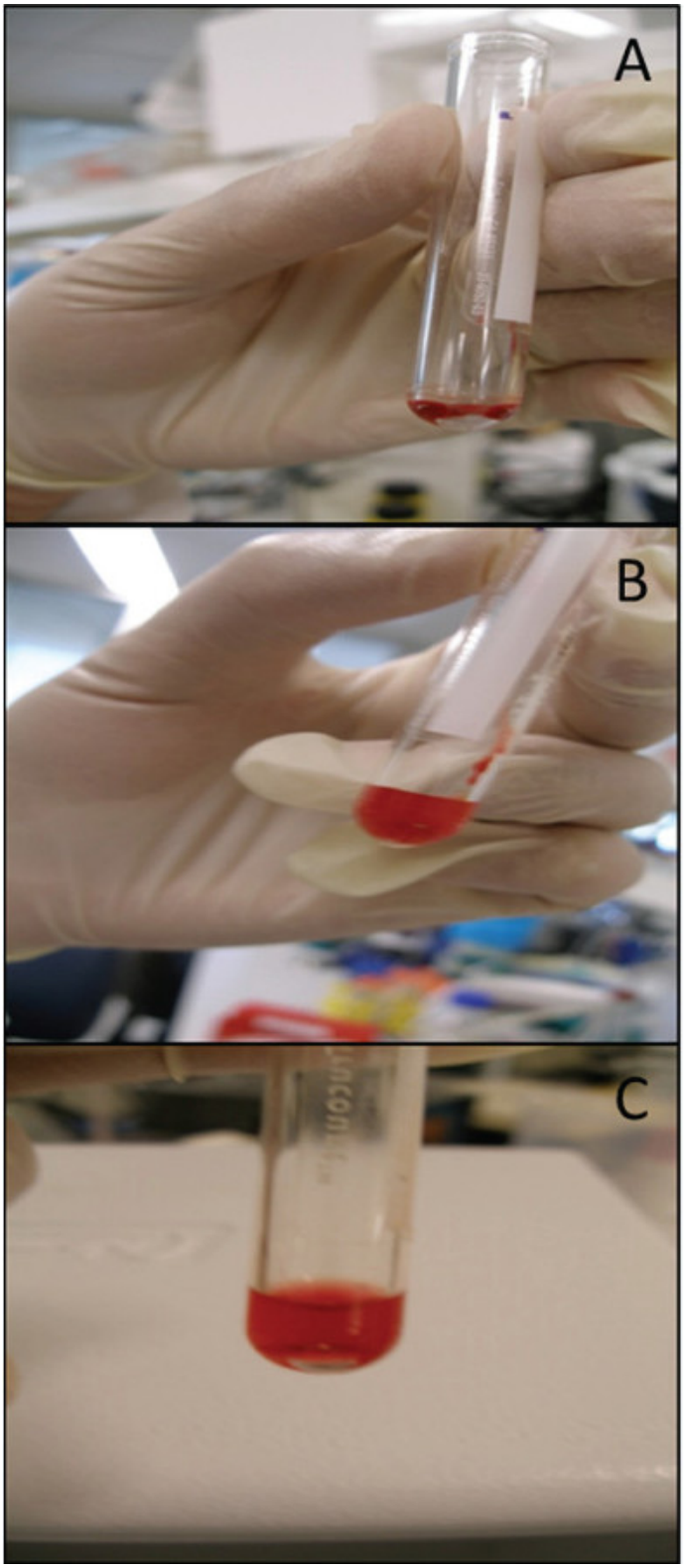

Figure 4. Labelling of blood sample in an absolute counting tube for flow cytometric analysis. Blood (25 µL) and antibodies are added to an absolute counting tube **(A)**. The tube is then vortexed and incubated for 15 min at room temperature. Before the addition of ammonium chloride buffer to lyse the red blood cells, the sample appears turbid or opaque **(B)**. Once lysed, the sample appears translucent and is ready for flow cytometry analysis **(C)**. Please click here to view a larger version of this figure.

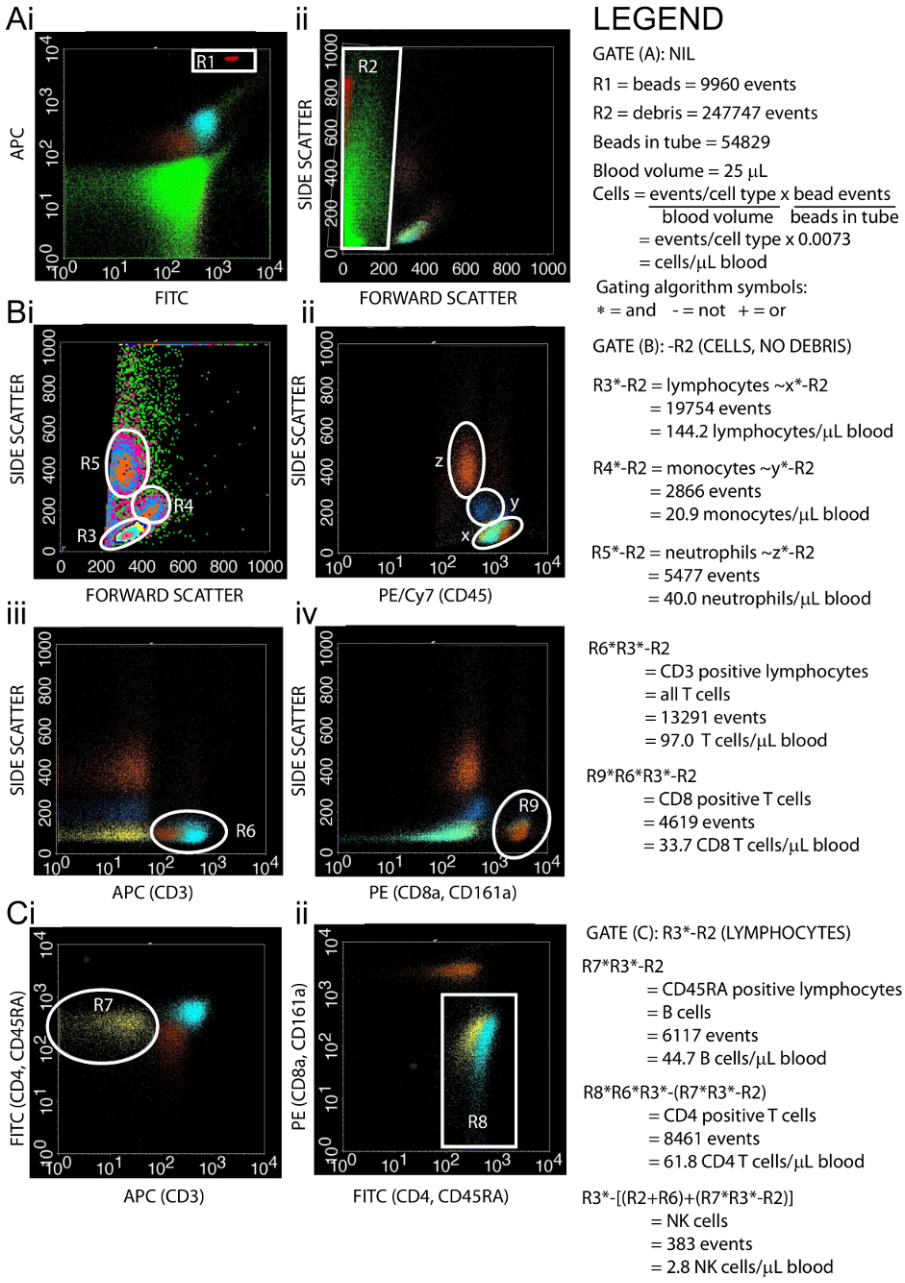

Figure 5. Gating strategy for flow cytometry analysis. A 6-dimensional dataset can identify lymphocytes, monocytes and neutrophils and the lymphocyte subsets T, CD4 T, CD8 T, B and NK cells. The defined gating strategies and algorithms incorporate both the negative and positive reactivity that define each cell type enabling specific identification. The number of beads in each absolute counting tube (found on the pouch for each tube) is recorded for each sample and is required for calculating the number of cells per µl blood for each subset as shown. The complete gating algorithm for each cell subset is listed to the right of the analysis plots.**(Ai)** Beads (R1) are initially counted by FITC (FL-1) *versus* APC (FL-4). Acquisition is set to approximately 10,000 events (9960 for this example). **(Aii)** Debris (R2) is identified by FSC *versus* SSC and removed from all subsequent gating. **(Bi)** Following removal of debris (R2) FSC *versus* SSC differentiates white blood cell population into lymphocytes (R3), monocytes (R4) and neutrophils (R5). **(Bii)** The cell populations identified in (Bi) are confirmed using the common leucocyte antigen CD45 gating on PE/Cy7 (FL-3) *versus* SSC. **(Biii)** The lymphocyte population (R3) is further differentiated into T cells (R6) using CD3 gating on APC (FL-4) *versus* SSC. **(Biv)** CD8 T cells are lymphocytes (R3) differentiated into T cell (R6) that also express CD8a (R9) and are gated on PE (FL-2) *versus* SSC. **(Ci)** B cells and NK cells are defined as lymphocytes (R3) which are CD3 negative. B cells (R7) are differentiated from NK cells as they express CD45RA and are gated by APC (FL-4) *versus* FITC (FL-1). **(Cii)** CD4 T cells are defined as lymphocytes (R3) that co-express CD3 (T cells, R6) and CD4 (R8) but do not express any of the other markers used. They are differentiated using FITC (FL-1) *versus* PE (FL-2) gating. NK cells are defined as any remaining lymphocyte that has not already been defined that also expresses CD161a. Please click here to view a larger version of this figure.

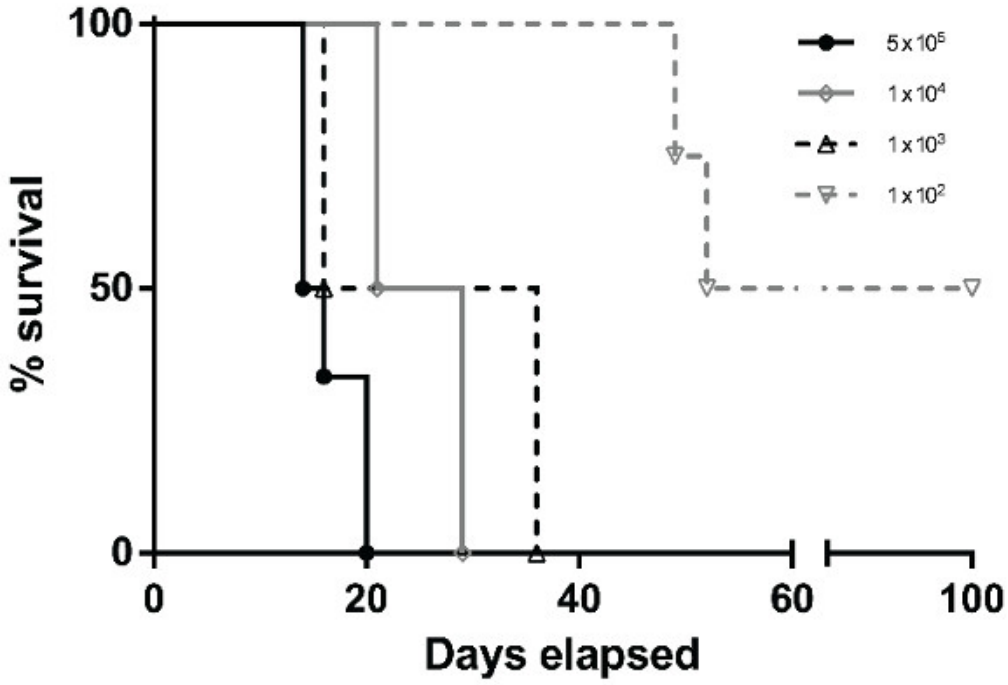

Figure 6. Survival curve for rats implanted with different doses of II-45 mesothelioma cells. Groups of rats were pleurally implanted with 5 x 10^5 (n=6), 1 x 10^4 (n=2), 1 x 10^3 (n=2) or 1 x 10^2 (n=4) II-45 mesothelioma cells in 100 µL volume and then monitored until ethical endpoint when they were euthanized. Survival was graphed using the log-rank (Mantel-Cox) Gehan-Breslow-Wilcoxon method.

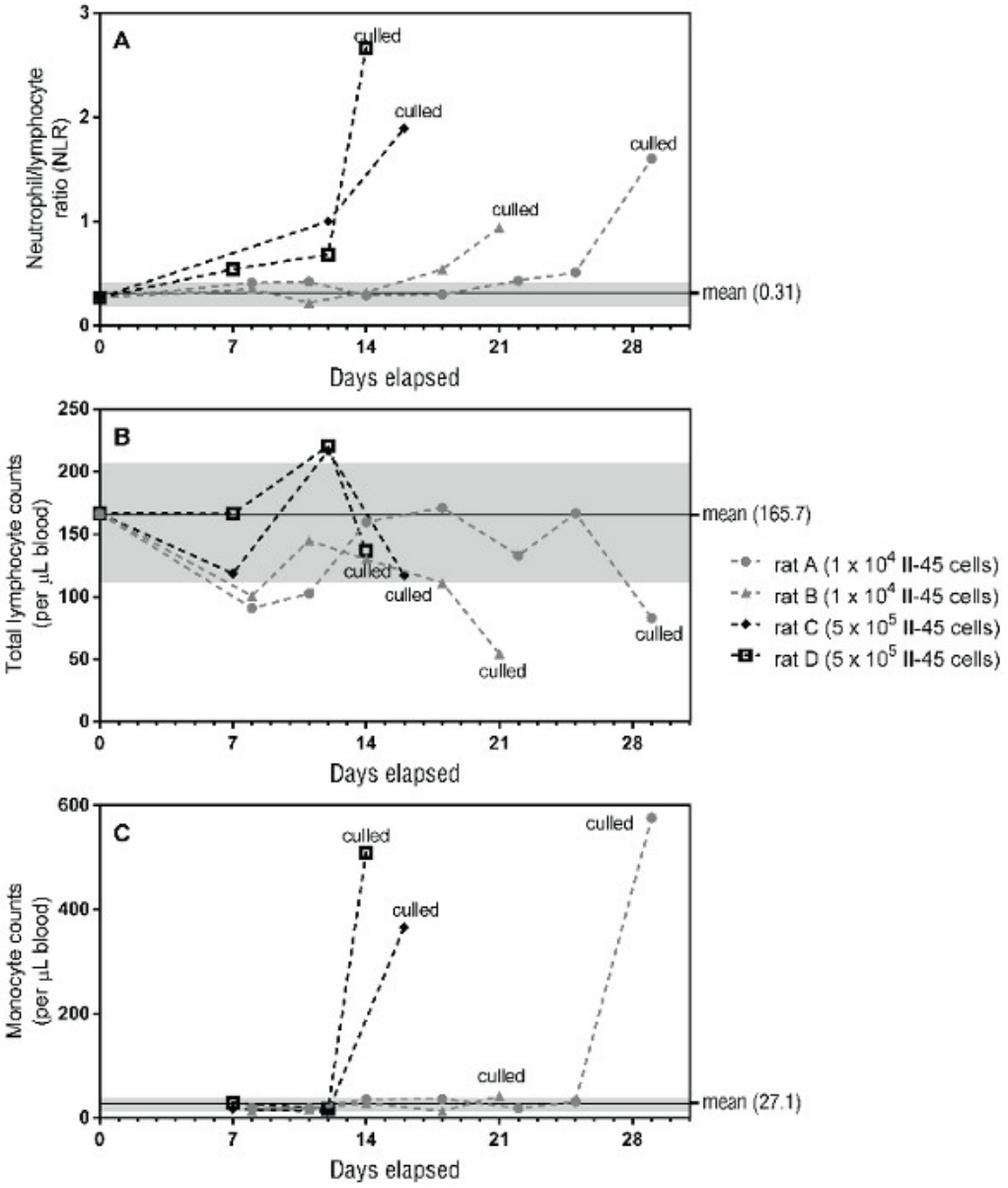

Figure 7. Longitudinal circulating immune cell counts from rats with mesothelioma relative to the mean and range for healthy control rats. The healthy control mean for each cell type is shown as a horizontal unbroken black line (value is shown on the right Y-axis of each graph) and the range is depicted by the grey shaded area. The Y-axis shows the counts per µl of blood for the stated cell type, or the value for NLR. The X-axis is the time course for disease progression, where day 0 is the day of implantation. Longitudinal results for 2 rats implanted with 1 x 10^4 cells (A, B) and 2 rats implanted with 5 x 10^5 cells (C, D) are shown. **(A)** NLR, **(B)** total lymphocyte counts, **(C)** monocyte counts. Please click here to view a larger version of this figure.

Detector	Fluorophore	Details	Voltage	Mode
FSC	---	---	E00	Linear
SSC	---	---	450	Linear
FL-1	FITC	533/30 nm	670	Log
FL-2	PE	585/40 nm	675	Log
FL-3	PE/Cy7	670nm (long pass)	600	Log
FL-4	APC	675/25 nm	735	Log

Table 1: Voltage detector settings for flow cytometric data acquisition using a 4 color flow cytometer. Amplitude gain is set at 1 for all detectors. FSC: forward scatter, SSC: side scatter, FITC: Fluorescein isothyocyanante, PE: Phycoerythrin, APC: Allophycocyanine.

Cell subset	Cell Markers	Gating Algorithm
Beads		R1
Debris		R2
Cells (pan-leukocytes)	CD45+	-R2
Lymphocytes	CD45+ FSC versus SSC	R3*-R2
Monocytes	CD45+ FSC versus SSC	R4*-R2
Neutrophils	CD45+ FSC versus SSC	R5*-R2
T cells (total)	CD45+/CD3+	R6*R3*-R2
CD8 positive T cells	CD45+/CD3+/CD8a+	R9*R6*R3*-R2
B cells	CD45+/CD3-/CD45RA+	R7*R3*-R2
CD4 positive T cells	CD45+/CD3+/CD4+	R8*R6*R3*-(R7*R3*-R2)
Natural Killer cells	CD45+/CD3-/CD161a+	R3*-[(R2+R6)+(R7*R3*-R2)]

Table 2: Cell markers and gating algorithms to characterize each immune cell subset. For the gating algorithm, the '+' symbol means 'or' and allows two separate populations to be added together even if their reactions do not overlap. The '-' symbol means 'not' and can subtract one population from within another defined population or the reverse of a previously described population. The '*' symbol means 'and' and defines a space that is present only when two reactions overlap.

Immune cell subsets	Control rats (n=5)		II-45 implanted rats at endpoint (n=4)	
	Mean	Range	Mean	Range
Total lymphocytes	165.7	111.5-207.0	97.9	54.4-137.0
CD4 T lymphocytes	72.9	45.4-111.4	38.4	25.6-50.0
CD8 T lymphocytes	36.6	26.0-48.3	32.1	16.1-46
B lymphocytes	56.6	36.0-83.9	24.7	11.2-36.0
NK cells	9.3	3.1-15.7	4.0	1.34-7.7
Monocytes	27.1	12.7-39.0	373.3	42.4-575.8
Neutrophils	51.4	24.3-87.0	159.2	51.1-366.0
NLR	0.3	0.18-0.42	1.8	0.94-2.66

Table 3: The mean and range of immune cells in healthy control rats (n=5) and II-45 implanted rats at endpoint (n=4). Results per µl of blood are shown.

Discussion

This paper details a method for the generation of a rat syngeneic orthotopic model of pleural mesothelioma and a simple method for monitoring disease progression through longitudinal blood sampling.

The II-45 model was developed by exposing Fischer 344 rats to asbestos fibers[13]. Although this exposure represents the true dynamics of host-asbestos-immune system interactions for mesothelioma pathogenesis, it has a long lag time (taking years to generate) and can be dangerous for the researchers due to the potential exposure to asbestos fibers. Orthotopic implantation of II-45 cells into the pleural cavity of animals provides a safe and rapid model of cancer progression which mimics the human disease in an immune-competent host[11]. However the exact methods and principles to adhere to during these experimental procedures are not well defined. The procedure described results in a highly reproducible model that is relatively independent of the experience of the operator. The critical steps are 1, harvesting II-45 cells in the exponential growth phase and accurate counting of viable cells; 2, correct positioning of the implantation site and ensuring the needle does not penetrate too deeply and puncture the lungs or damage the heart, and 3, maintaining and monitoring the well-being of the animals.

The II-45 cell line is fast growing and requires passaging every 3 to 4 days in culture, therefore when preparing for implantation, cells should be sub-cultured and growth monitored daily. Cells should be harvested and counted when in the exponential growth phase, *i.e.,* at approximately 70% confluence. Resuspension of cells in serum free media rather than PBS reduces the stress on the cells while counting and preparing for implantation. Accurate cell counts are crucial for reproducibility, especially when dosing with low cell numbers (*e.g.,* 100 to 1,000). The suspension should be homogeneous and consist of single cells (no clumps). An implantation volume of 100 µl enables accurate dosing without impairing lung expansion through excessive fluid in the pleural cavity. The key steps at implantation are correct positioning and limiting the needle penetration to a depth of no more than 12 mm. Correct positioning of the needle at the injection site is extremely important to ensure that the cells are implanted into the pleural cavity and not into the peritoneal cavity. Incorrectly implanting cells too low can result in mesothelioma tumors growing through the diaphragm, into the liver and peritoneal cavity. Restricting the penetration depth of the needle is also extremely important to prevent mortality as a result of internal organ damage. This restriction can be achieved through the use of a spacer over a long

needle (as shown) or the use of a shorter needle with a shaft length of 5 mm to 12 mm. In our hands, no adverse events have occurred during cell implantation. It is also essential to replace the needle after every implantation. Failure to do so can result in tumors growing out of the chest cavity along the injection line. Such growth outside of the pleural cavity is due to residual cells on the needle shaft and may result in extended survival.

In our hands this model has a 100% tumor engraftment rate when administering ≥ 1 x 10^3 cells. Disease progression in the II-45 mesothelioma model is quite rapid, being less than 40 days when 1 x 10^4 cells are implanted and less than 20 days for a dose of 5 x 10^5 cells. This may limit the usefulness of this model for pre-clinical testing of some therapies. The II-45 cells, while not commercially available can be sourced with permission from the originator[13]. However, the same implantation techniques and principles may also be applied to other rat mesothelioma cell lines available from cell line repositories when matched with the relevant syngeneic rat strains. The optimal cell dose may vary with cell line and would need to be determined for each line.

Monitoring disease progression and the well-being of the animals is difficult as the tumors are internal and cannot be monitored by size. Therefore we sought to develop a method of monitoring the animals that could predict deterioration of their condition such as an increase in NLR or other change in immune cell markers before physical symptoms became visible. The blood screen developed uses only 25 µl of peripheral blood so that regular sampling can be performed with minimal distress to the animal. The sampling technique described requires practice to gain the necessary expertise. However, once mastered it is fast and minimally invasive.

A critical step in the blood analysis is to prevent clotting of the blood sample by rapid transfer to an EDTA tube. Clotted samples should be discarded as the counts will be inaccurate. Another critical step is the red cell lysis prior to cell analysis. If the red cells are not fully lysed, the increased background will falsely elevate the counts. While the initial setup of dot plots and gating is time consuming, once the settings and template are saved, the assay requires little adjustment. The gating strategy employed uses a multi-parameter Boolean algebraic approach to deconvolute the data. By using known combinations of exclusive and shared antigens the algorithms for each line of the gate strategy define the discrete cell type in this six-dimensional data matrix[14]**.** This coupled with established accuracy of the ratiometric bead-based technology[15-17] has the advantage of enabling the enumeration of any defined cell type as both a proportion of cells and an absolute count per µl.

It is becoming increasingly recognized that the immune system plays an important role in the pathogenesis of cancer[18]. Both the failure of the immune system to recognize and destroy malignant cells and the suppression of immune cells by tumors can lead to uncontrolled tumor growth. Thus, the inclusion of an immune-competent tumor microenvironment is a critical consideration when modelling cancer and is a major advantage of syngeneic cancer models. These immune-competent models create a realistic and clinically relevant environment for tumor growth and immune interaction, which is deficient in xenogeneic models.

The immune parameters identified in this study that accompanied disease progression, *i.e.,* lymphocyte counts, monocyte counts and the NLR, have also been shown to correlate with poor prognosis in human mesothelioma[19-21] providing evidence for the relevance of the model and the blood test. In the models presented, the clinical utility of monitoring the immune parameters increased with the longer time course (lower dose) and may be further improved by more frequent sampling at later stages of the disease. While this paper focuses on a model of mesothelioma, the ability to monitor tumor progression through peripheral blood sampling can be utilized for all syngeneic cancer models. Further, the assay can also be used to assess the immunological response to different therapies. Previously we have used this assay to monitor the response to an anti-cancer vaccine therapy in a syngeneic glioma model (9L)[22]. In that study, CD4 and CD8 T cells were the most informative markers. We have also used this assay and the II-45 mesothelioma model to identify differences in the immune response to tumors resistant to different chemotherapeutics[11]. In that study, all parameters except CD8 T cells showed significant differences. The longitudinal monitoring during disease progression described here highlights the potential of different immune cell parameters to correlate with cancer progression and the well-being of animals when undertaking syngeneic orthotopic modelling of cancer in rats.

Disclosures

The authors have nothing to disclose.

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