Video Article

Tumor Engraftment in a Xenograft Mouse Model of Human Mantle Cell Lymphoma

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Abstract

B lymphocytes are key players in immune cell circulation and they mainly home to and reside in lymphoid organs. While normal B cells only proliferate when stimulated by T lymphocytes, oncogenic B cells survive and expand autonomously in undefined organ niches. Mantle cell lymphoma (MCL) is one such B cell disorder, where the median survival rate of patients is 4 - 5 years. This calls for the need of effective mechanisms by which the homing and engraftment of these cells are blocked in order to increase the survival and longevity of patients. Therefore, the effort to develop a xenograft mouse model to study the efficacy of MCL therapeutics by blocking the homing mechanism *in vivo* is of utmost importance. Development of animal recipients for human cell xenotransplantation to test early stage drugs have long been pursued, as relevant preclinical mouse models are crucial to screen new therapeutic agents. This animal model is developed to avoid human graft rejection and to establish a model for human diseases, and it may be an extremely useful tool to study disease progression of different lymphoma types and to perform preclinical testing of candidate drugs for hematologic malignancies, like MCL. We established a xenograft mouse model that will serve as an excellent resource to study and develop novel therapeutic approaches for MCL.

Video Link

The video component of this article can be found at https://www.jove.com/video/56023/

Introduction

Lymphocytes by nature play a major role in immune surveillance, and lymphocyte trafficking is a critical step in mounting antigen specific immunity[1,2]. This process includes migration of naïve T lymphocytes from the thymus to the blood stream, and from there to secondary lymphoid organs, including lymph nodes, Peyer's patches, or spleen, where they meet cognate antigens. The B lymphocytes differentiate in the bone marrow and migrate as naïve cells into follicles of secondary lymphoid organs[3]. Some of these B cells bind antigen with their receptor and are activated by specific T cells. Proliferation and differentiation of these B cells pushes the non-activated, naive B cells into the mantle zone of the follicle. Activated cells can then differentiate into memory B cells, which patrol the body, or mature into immunoglobulin secreting plasma cells that migrate to the bone marrow[4].

MCL occurs when naïve B lymphocytes in the mantle zone transform into a tumor. These lymphoma cells reside in the microenvironment of the lymphoid organs and proliferate independently of specific T lymphocyte control. However, at a certain stage of density they escape from this niche and recirculate in the bloodstream in search for niches in other organs. Considering the complexity of adhesion molecules and the promiscuity of chemokines and their receptors, the mechanism of this cellular trafficking *in vivo* is poorly understood and therefore hampers therapy. Novel methods are needed to effectively block this migration process to prevent the lymphoma B cells from reaching new microenvironments.

MCL is one of the most difficult to treat B cell malignancies. The development of a neoplastic phenotype of MCL is the result of a multistep cascade, characterized by the acquisition of unique biologic properties. At the time of diagnosis, most patients (70%) already present with a disseminated disease, with a majority of cases exhibiting extranodal involvement in spleen, bone marrow, and/or the gastrointestinal tract[5,6]. In treated patients, relapse by resistant tumors within a few years is common, even though conventional chemotherapy induces high remission rates at short term[7,8]. Here we present a new disease model that can help understand MCL dissemination and its underlying biology: we established a human MCL xenograft mouse model that originated from primary tumor cells of patients. We hope that this model will help develop therapeutic strategies against MCL dissemination, and possibly provide new clinical perspectives for optimal diagnosis and treatment of relapsed patients.

Protocol

The human blood samples were used according to procedures approved by the local ethics and human experimentation committees of the Geneva University Hospital.

Animal procedures were performed in accordance with the Institutional Ethical Committee of Animal Care in Geneva, Switzerland and the Cantonal Veterinary Office (authorization number: GE/26/15).

1. Preparation of Primary Peripheral Blood Mononuclear Cells (PBMCs) by Density Gradient Separation

NOTE: 3 - 5 mL of peripheral blood was obtained from patients presenting with MCL in a leukemic phase. The diagnosis was established according to standard diagnostic criteria for flow cytometry (CD5+, CD23−, CD200+, monoclonal B cell population), and subsequently confirmed by presence of the chromosomal translocation t(11;14) and overexpression of cyclin D1. In all cases, monoclonal B cells constituted to >90% of the total B cell population. The remaining cells were mainly monocytes and some T cells.

1. Dilute (1:1) 5 mL of MCL blood sample with 5 mL of Roswell Park Memorial Institute medium 1640 (RPMI) (**Figure 1A**).
2. Invert the density gradient media (**Table of Materials**) several times before use to ensure thorough mixing.
3. Add 5 mL of density gradient media into a 15 mL centrifuge tube using a pipette aspirator.
4. Gently layer the diluted blood sample over the density gradient media in the 15 mL centrifuge tube using an aspirator (the blood to density gradient media ratio should be 2:1, *e.g.*, 10 mL of blood to 5 mL of density gradient media). Care should be taken to not mix the two layers (**Figure 1B, C**).
5. Centrifuge the sample at 400 x g for 40 min at room temperature with the centrifuge brakes turned off.
6. Using a sterile Pasteur pipette, gently aspirate the middle layer (whitish, thin ring marked with an arrow (**Figure 1D, E**)) containing the mononuclear cells and transfer them into a clean tube.
7. Washing of mononuclear cells
    1. Estimate the volume of the transferred cell suspension and add to it 3 volumes of sterile 1x phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA).
    2. Mix the suspension by pipetting up and down several times.
    3. Centrifuge the suspension at 400 - 500 x g for 10 - 15 min at room temperature.
    4. Discard the supernatant to obtain the cell pellet and repeat the wash step again.
    5. Remove the supernatant and resuspend the cells in the desired volume, *e.g.*, 1 or 2 mL of PBS.
    6. Count the number of cells by an automated cell counter or manually using a hemocytometer (Neubauer chamber).

2. Enrichment of B Cells by B Cell Negative Selection

1. Dilute the cells in PBS to a final concentration of 50 x 10^6 cells/mL.
2. Transfer the cells to a 5 mL round bottom polystyrene tube. Add 100 µL of antibody cocktail from the B cell enrichment kit to 2 mL of cell suspension, and mix gently by pipetting up and down (**Figure 2-1**).
3. Incubate the cells at room temperature for 10 min.
4. Briefly vortex the magnetic particles provided in the B cell enrichment kit for 30 s (**Figure 2-2**). Add 150 µL of the magnetic particles to 2 mL of the sample .
5. Incubate the sample for another 5 min at room temperature.
6. Bring the sample volume to 2.5 mL with 1x PBS containing 2% fetal calf serum (FCS) and 1 mM ethylenediaminetetraacetic acid (EDTA). Place the tube in the magnet and incubate the tube for another 3 - 5 min (**Figure 2-3**).
7. Pick up the magnet with the tube in place, and in one continuous motion, decant the tube so that the enriched cell suspension is collected in a fresh tube (**Figure 2-4**). Count the cells by an automated cell counter or manually using a hemocytometer to obtain the total yield of B cells. NOTE: Typically, the yield is ~10^5 B cells/mL for normal B cells and 5 - 50 x 10^6 cells/mL for MCL.
8. Confirming the purity of the B cell enrichment
    1. Take ~ 50,000 cells and incubate the cells with anti-CD19/CD20 antibodies (1:20 dilution) and anti-CD45 antibody coupled to different fluorochromes (1:10 dilution) at room temperature for 15 - 30 min.
    2. Wash with 1 mL of 1x PBS + 1% BSA, centrifuge the cells at 400 - 500 x g for 5 min, and resuspend the cells in ~200 µL of PBS.
    3. Acquire the cell samples by flow cytometry and analyze for the purity of B cells. Purity is mostly >90% by this method (**Figure 3**).

3. Development of the Xenograft Mouse Model

1. Suspend ~40 - 60 x 10^6 cells in 150 - 200 µL of 1x PBS (volume required to inject per mouse).
2. Inject 150 µL of the cell suspension intravenously (i.v.) into each tail vein of 6 - 7 week old immunodeficient NOD scid gamma (NSG) mice irrespective of the gender (**Figure 4A**).
3. Allow the mice to develop human lymphoma for ~10 weeks.
   NOTE: Primary human lymphoma cells take much longer to develop tumor compared to human lymphoma cell lines (~3 weeks).
4. When the animals start to show symptoms of fatal illness like weight loss, ruffled hair, decreased activity, hind limb paralysis, *etc.*, sacrifice the mice by CO_2 asphyxiation followed by cardiac heart puncture to obtain blood.

5. Collect different organs like bone marrow, spleen, and liver by dissecting the mice and collect peripheral blood by drawing blood from the heart at the time of sacrifice for tumor engraftment analysis (**Figure 4B - F**).

4. Chimerism Analysis of Tumor Cell Engrafted in Different Organs

1. After collecting the different organs, process them by mechanical disruption to generate single cell suspensions following red blood cell (RBC) lysis using 200 µL of 1x ammonium chloride potassium buffer for blood and bone marrow, 500 µL for spleen, and 2 mL for liver.
2. Count and stain the cells using human B cell specific antibodies, *i.e.*, anti-CD19, anti-CD20, and anti-CD45.
   NOTE: NSG mice lack B cells and hence for analysis, cells are stained with human specific B cell markers.
3. Analyze the cells derived from each organ by flow cytometry by gating on B cells (CD19+, CD20+, CD45+).
4. Quantify the engrafted tumor cells in different organs derived from MCL injected mice based on the gated cells as indicated in step 4.3 (**Figure 4G**) .
   NOTE: See **Table of Materials** for details.

Representative Results

The manuscript describes an optimized protocol for the successful development of a xenograft mouse model for engraftment of MCL cells. Preparation of a pure cell population (in this case MCL cells), is very critical to develop successful MCL xenografts. **Figure 1** represents the preparative steps for mononuclear cell isolation from MCL patient's blood by density gradient separation. The mononuclear cells are further processed to obtain pure B cells using a negative B cell enrichment kit to obtain a pure cell population for xenograft injection into mice. Care should be taken to obtain maximum purity in order to have successful MCL engraftment. The purity obtained using this method is usually >90%. The remaining cells were mainly monocytes and some T cells (data not shown).

Figure 2 represents the different steps of the B cell purification protocol as described in the methods section. The enriched cells are further analyzed for their purity by flow cytometer using different markers (CD45, CD19, CD20, CD23, CD200, CD5, kappa, and lambda). Sequential gating as shown in **Figure 3** allows characterizing of MCL cells: CD45+, CD19+, CD20+, CD5+, CD23−, and CD200− are selected. Compensation for multicolor staining was carried out by using single stained controls for each of the fluorochromes used, according to standard cytometry set-up.

Figure 3E, G represents the typical dot plot of B cells before and after enrichment. In this case, >95% of the enriched cells using this kit are B cells. The cells are suspended in PBS at a concentration of 40 - 60 $\times 10^6$ cells in 150 - 200 µL PBS as mentioned in the protocol. They were immediately injected i.v. to NSG mice. After 10 weeks, >90% of the injected animals developed lymphoma shows signs of terminal illness (weight loss, ruffled hair, decreased activity, *etc.*). After sacrifice, spleen and liver are removed and processed to obtain single cell suspensions by mechanical disruption (**Figure 4D, F**); blood is drawn carefully by cardiac heart puncture and the bone marrow is processed by cutting both ends of the femur and flushing it using a 2 mL syringe filled with RPMI medium or PBS (**Figure 4C**). The cells are further processed by flow cytometry using human B cell specific markers like CD19/CD20 and CD45. We used human B cell specific markers here because the recipient mouse strain (NSG) lacks B Cells. **Figure 4B-F** represents the collection of organs after sacrifice and the steps of further processing. The degree of engraftment of MCL cells derived from two patients is represented in **Figure 4G**. The engraftment pattern in different organs and between patients can be compared following this technique.

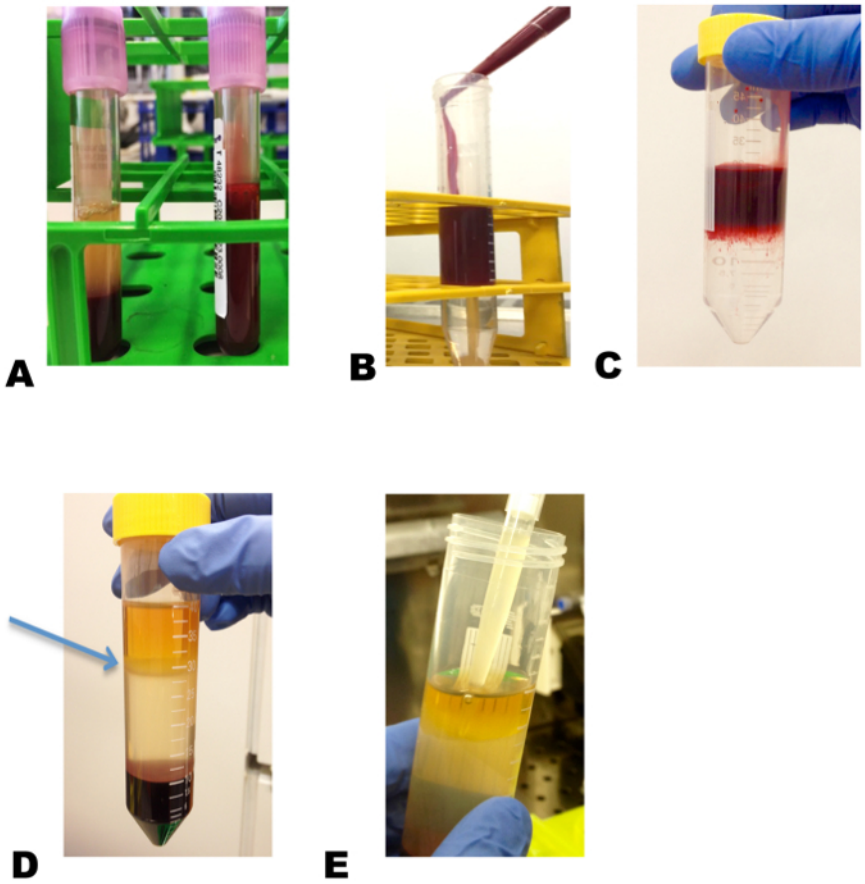


Figure 1: Isolation of PBMCs from whole blood. Blood drawn from a MCL patient is diluted 1:1 in RPMI medium (**A**), and placed carefully on top of the density gradient media layer (**B**) without mixing the blood into this density gradient media (**C**). Centrifugation at 400 x g for 45 min separates the mononuclear cells, which appear as whitish ring (arrow indicates the mononuclear cell layer) (**D**). This layer is pipetted gently without mixing with other layers into a clean tube for further processing (**E**). Please click here to view a larger version of this figure.

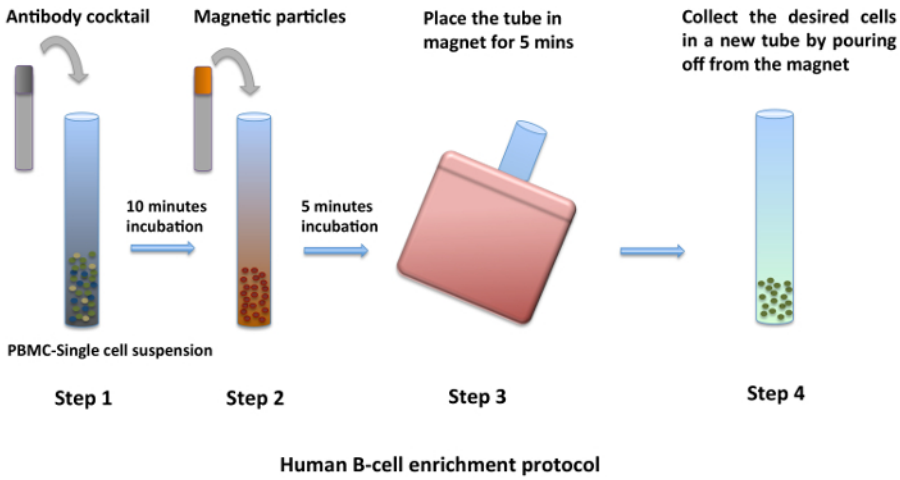


Figure 2: Enrichment of B cells from PBMCs. PBMCs isolated by density gradient centrifugation are washed twice with PBS. Using the negative B cell enrichment kit and by following the manufacturer's protocol, a pure population of B cells is obtained (**1-4**). Please click here to view a larger version of this figure.

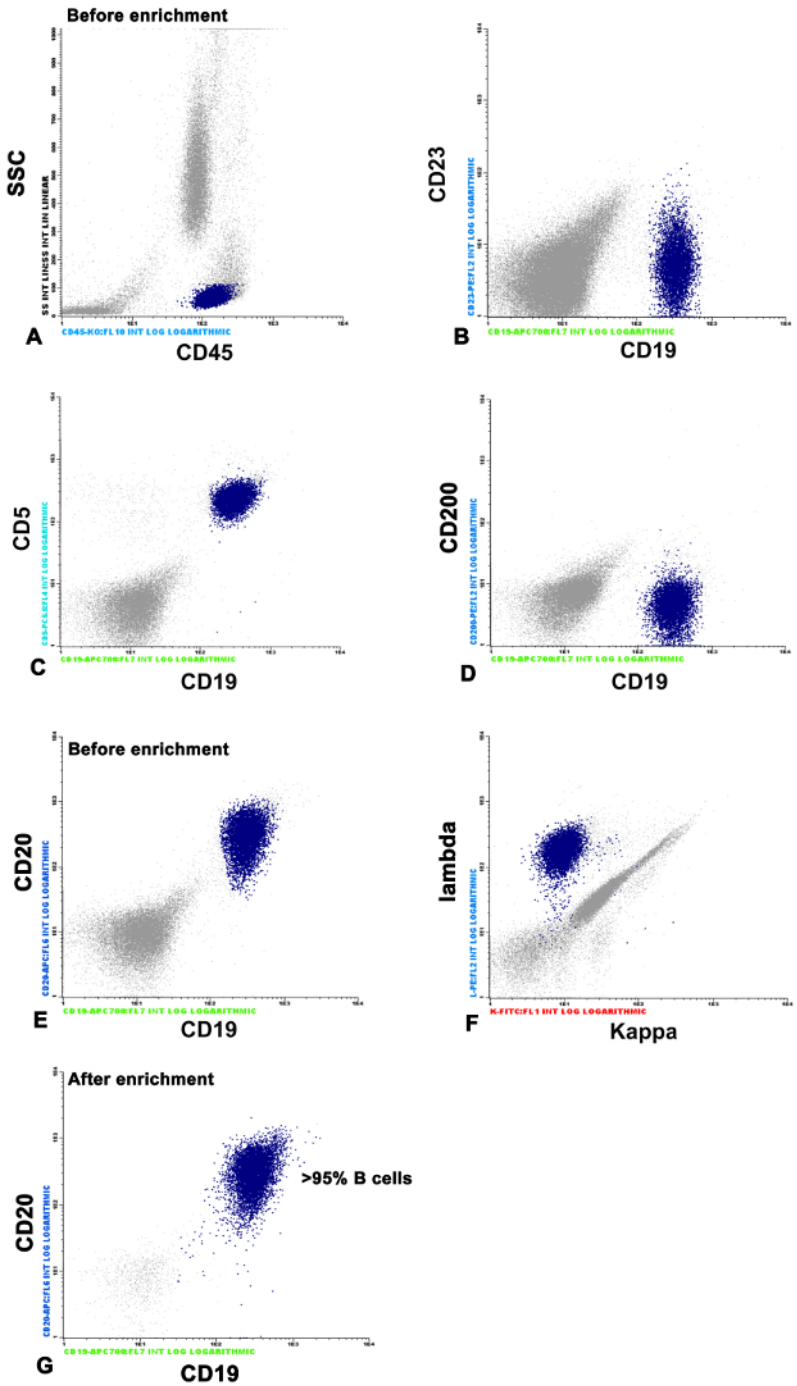
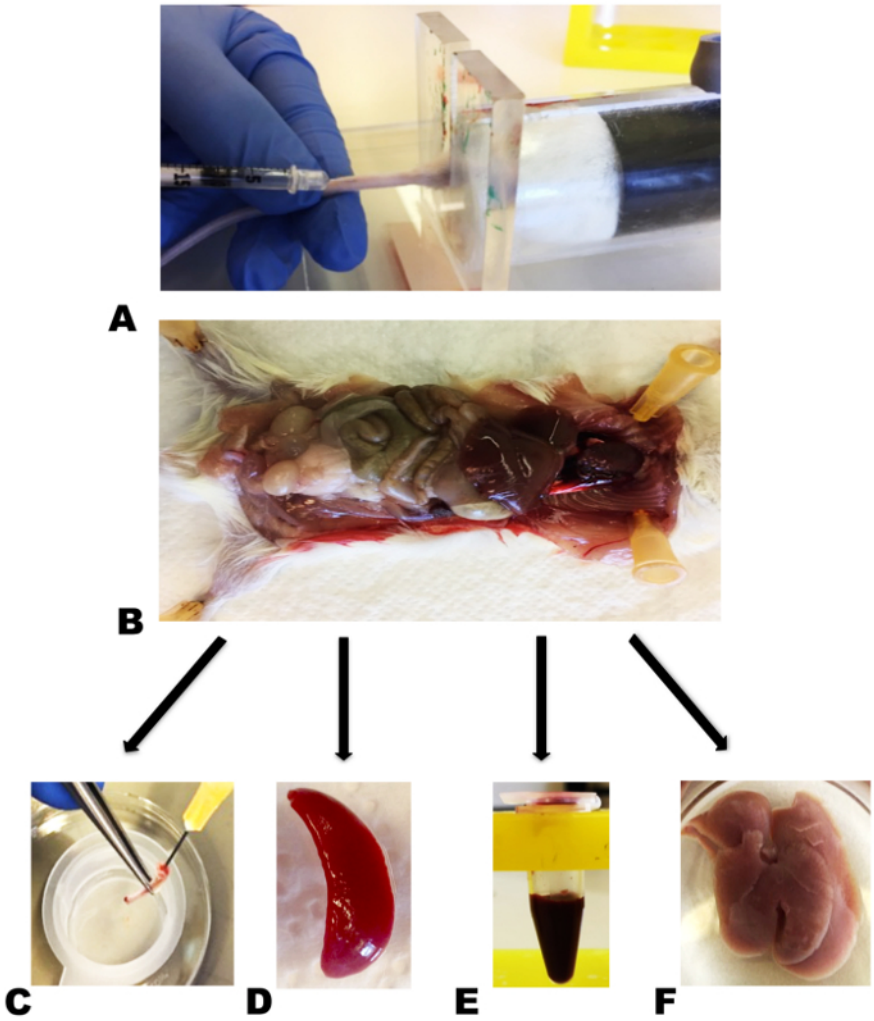


Figure 3: FACS analysis of MCL cells and purity of B cells before and after enrichment. The mononuclear MCL cells are characterized by FACS analysis using different markers like CD45, CD19, CD20, CD23, CD200, CD5, kappa, and lambda. Cells that are positive for CD45, CD19, CD20, CD5, and negative for CD23 and CD200 are selected (**A-F**). **G** represents the cell population after enrichment using the negative selection kit compared to **E**, which is before enrichment. Please click here to view a larger version of this figure.



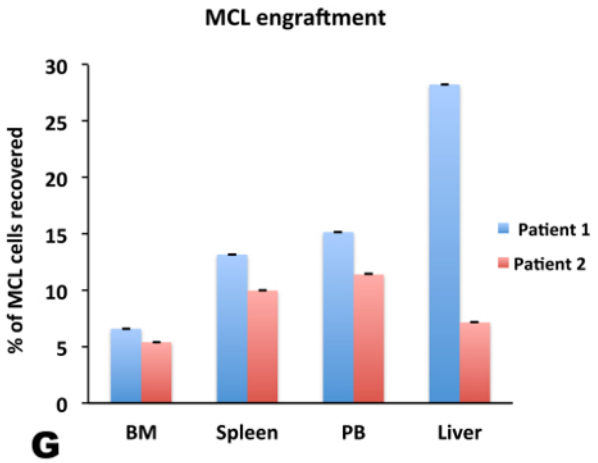


Figure 4: NSG mice as a xenograft model for engraftment of MCL cells. B cells derived from MCL patients were injected i.v. via the lateral tail vein of NSG mice (**A**). After allowing several days for the tumor to engraft, the mice were sacrificed, and dissected (**B**) to collect different organs like bone marrow (**C**), spleen (**D**), peripheral blood (**E**) (drawn by cardiac heart puncture), and liver (**F**). These organs were processed further and analyzed by flow cytometry for the presence of B cells. **G** represents the engraftment pattern of MCL cells in different organs derived from MCL injected NSG mice. Results from two patients are shown. Data are shown as mean ± SEM, n = 3. Please click here to view a larger version of this figure.

Discussion

Clinical trials are possible for drugs that are in an advanced stage of development but cannot be used for drug discovery. Efforts to develop animal recipients for human cell xenotransplantation in order to test early stage drugs have long been pursued. Here we present an animal model that avoids human graft rejection and can establish a model for human diseases, such as MCL. This is at present a state of the art xenograft model to study the mechanisms of human tumor engraftment and tumor growth. Here we use NSG mice, one of the most immune deficient mouse models to date in order to achieve greater success of lymphoma engraftment. NSG mice lack mature T cells, B cells, and NK cells. They also have impaired cytokine signaling and have defects in innate immune response.

Lymphocyte preparation from the primary MCL sample by density gradient is an important step to remove other cell types like red blood cells and platelets. Traditionally, a density gradient centrifugation allows successful isolation of lymphocytes. Further processing of these lymphocytes to enrich the B cell population is attained by the use of a B cell enrichment kit following the protocol as described within this manuscript. Care should be taken to attain a high purity of B lymphocyte populations to reach optimal engraftment of the lymphoma cells. The MCL cells are injected intravenously (at least 40 - 60 x 10^6 cells/mouse) through the lateral tail vein, and mice were allowed to develop lymphoma for up to 10 weeks. The mice were closely examined every day for symptoms of illness including, weight loss, ruffled hair, decreased activity, hind limb paralysis, *etc.* Analysis of different organs like spleen, liver, bone marrow, and blood by flow cytometry revealed MCL engraftment. Recently, it has been shown that primary MCL cells engraft in bone marrow and spleen of irradiated NSG mice at 20 weeks of injection[9]. Independent of this finding, we have successfully developed our own xenograft model of primary MCL with more rapid tumor formation.

This xenograft mouse model may become an extremely useful tool for the study of disease progression of different lymphoma types. Xenograft models also provide powerful tools to perform preclinical testing of candidate drugs for hematologic malignancies like MCL, however, with certain limitations for example, the dominant clone present at relapse in a patient is not necessarily the clone emerging on xenotransplantation[10]. This could be due to the different selective pressure that the xenograft undergoes in different host systems. The hematopoietic composition in this strain does not fully recapitulate human hematopoiesis, thus, limiting the long-term maintenance of human cells.The advantages in using human tumor xenografts overrides the limitations as results are obtained in a few weeks from a human tumor biopsy regarding response to therapy. Drug responses do not often correlate with clinical activities in patients[11] when human cell lines instead of primary tumor cells are used as xenografts, but they help form the groundwork for possible therapeutic responses. The use of primary tumors as an orthotropic xenograft has a stronger predictive response value, particularly when a clinically relevant drug dosage is used[11,12,13]. Homing of lymphoma cells to their niches is an important pre-requisite in order to survive and to establish tumor engraftment. This process is majorly regulated via the lymphatic system, and the entry of circulating lymphocytes through the high endothelial venules maintains the lymphocyte homeostasis in lymph nodes.

Circulation of B lymphocytes through lymph nodes requires crossing endothelial barriers and chemoattractant-triggered cell migration by coordinated interaction with different adhesion molecules. Therefore, targeting the molecules that control the homing of lymphocytes to their survival niches could constitute a new treatment strategy for B cell lymphomas as they behave similar to normal lymphocytes. Our aim was to target the molecule JAM-C, a junctional adhesion molecule, which is present on MCL. This xenograft model will be used to study the therapeutic effects of JAM-C antibody on homing and engraftment of primary lymphoma cells to their survival niches. We have recently shown an effect of anti-JAM-C antibody in mice that received a MCL cell line, Jeko-1[14]. This antibody had a blocking effect on homing of Jeko-1 cells and also when administered in regular intervals, it efficiently prevented the engraftment of Jeko-1 cells. In addition to its effect on homing, the antibody treatment eradicated the lymphoma engrafted in most of the organs tested in this mice[14].

In the light of the scientific and clinical question being addressed, these xenografted tumors represent at present, an interesting model to study the therapeutic outcome of MCL.

Disclosures

The authors declare that they have no competing financial interests.

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