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Assessment of IFN γ responsiveness in patient-derived xenografts

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Abstract

Patient-derived xenografts are a useful tool in cancer immunology, as they allow researchers to study human cancers in vivo when starting with a relatively small amount of human tumor tissue. These models make it possible to study tumor cell-intrinsic changes that occur in response to external stimuli including cytokines like interferon gamma (IFN γ) that are important for effective anti-tumor immune responses. IFN γ responsiveness can be measured by assessing surface expression of MHC class I on tumor cells, the molecule on which tumor antigens are presented to cytotoxic T cells in the tumor microenvironment. Low levels of MHC class I and lack of responsiveness have been associated with resistance to T-cell directed therapies like immune checkpoint inhibitors. In this chapter, we present a protocol for an assay to screen patient-derived xenografts for their responsiveness to IFN γ . The results of this assay can be used as a starting point for uncovering cancer cell-intrinsic mechanisms of resistance to immunotherapies in patient tumors.

1. Introduction

Preclinical models of human cancer are essential for gaining insight into this collection of diseases. Efforts to collect and propagate primary tumor samples from patients have led to the generation of patient-derived models of cancer, mostly engrafted as xenografts (patient-derived xenografts, PDXs) into immunodeficient mice. PDX models offer certain advantages over other mouse models of cancer, including the ability to expand and study a human tumor tissue in depth in vivo without the need for a large amount of the primary patient tissue. PDX models also recapitulate the variability that exists within human tumors, even among those of the same histological subtype. In contrast to human cancer cell lines cultured in vitro, by growing in the mouse, PDXs develop in the presence of other cell types (e.g., stromal cells and vasculature) which can influence tumor biology. As a result of these features, PDXs are valuable tools for identifying tumor-intrinsic factors relating to tumor

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progression and can be used as preclinical models for cancer therapies, especially those that target tumor cells (Uthamantil, Tinkey, & de Stanchina, 2017).

Cancer immunotherapies, such as immune checkpoint inhibitors (ICIs) that leverage a patient's own immune system to attack tumor cells, are transforming the cancer treatment landscape and their success has spurred research efforts in the field of cancer immunology. The immune system has already evolved many anti-tumor mechanisms, though transformed cells that develop into invasive tumors are able to circumvent these mechanisms in a process known as immune escape (Choi et al., 2018). The principle behind ICIs is to block inhibitory interactions on immune cells that dampen their ability to exert anti-tumor activity. Among these therapies, blockade of CTLA-4 and the PD-1/PD-L1 axis are the most broadly used, including as first- and second-line therapies in several cancers including melanoma and lung cancer (Webb et al., 2018). Despite the success of these therapies compared to chemotherapy, there is tremendous variability in response rates between different cancer types. Moreover, resistance to immunotherapy, whether it be primary or acquired (occurring after an initial response to therapy), is not well understood (Zaretsky et al., 2016). One roadblock to understanding resistance to ICIs is the challenge of developing preclinical models from human specimens, since human tumor tissue requires in vivo propagation in immunodeficient mice that cannot be used to test immunotherapeutics directly (Choi et al., 2018). However, PDXs can be useful models to study tumor-intrinsic mechanisms of resistance to therapy. For example, the ability of a tumor to respond to interferon gamma (IFN γ) stimulation is critical for response to immune checkpoint inhibitors (Sharma, Hu-Lieskovan, Wargo, & Ribas, 2017). Responsiveness to IFN γ can be examined by measuring upregulation of cell surface MHC-I by flow cytometry following stimulation with the cytokine (Gettinger et al., 2017). PDXs (and patient-derived tumor cells) that do not express surface MHC-I and do not upregulate MHC-I after stimulation with IFN γ are likely able to evade CD8⁺ T-cell recognition and thus be resistant to immunotherapy. Therefore, this approach allows the identification of PDXs unresponsive to IFN γ stimulation irrespective of the mechanism (genetic, epigenetic, or signaling) that underlies this defect.

In this chapter we present a protocol for the generation and maintenance of PDX lines that then can be used to study tumor-intrinsic mechanisms of resistance to immunotherapy. Additionally, we describe a protocol to measure the IFN γ responsiveness of PDXs. The use of PDX models for this purpose has the potential to foster a greater understanding of mechanisms of resistance to ICIs and ultimately determine strategies to overcome this resistance.

2. Establishment and maintenance of PDX lines

2.1 Primary tumor cell injection

There are numerous methods for establishing and maintaining PDX lines to study human cancers. The establishment of a PDX requires tumor cells directly from patients, most commonly pieces of human tumors obtained via biopsy or surgery or from fluid obtained from pleural effusions or ascites. The patient cells are then engrafted into a mouse, commonly subcutaneously in the flank of the mouse. These are referred to as subcutaneous PDX models. Orthotopic PDXs are generated when the tumor cells are implanted into the

same tissue as the tissue of origin of the patient tissue (Hidalgo et al., 2014). Here we describe studies in subcutaneous PDXs.

Methods for suspending human tumor pieces or cells for implantation vary greatly among labs that work with these models, and it is important to record the suspension and implantation method used for the establishment of each PDX. Fresh tumor pieces can be directly injected subcutaneously with a large-gauge needle or resuspended in Matrigel before injection. Alternatively, the pieces can be digested into a single-cell suspension using various methods, including the methods outlined later in this chapter. These single-cell suspensions can again be injected directly or resuspended in Matrigel. The use of any one method may impact the probability of successful engraftment; therefore, it is important to establish the optimal approach for the tumor type under investigation (Hidalgo et al., 2014).

A final important consideration when establishing a PDX line is the strain of mouse used. In order to prevent rejection of the human tumor tissue, an immunodeficient strain of mouse must be used. There are a number of immunodeficient strains that have been optimized throughout the years for the generation of PDXs (Shultz et al., 2014). The first strain used for engraftment of human tumor cells was the nude mouse which has a mutation in the *Foxn1* gene that causes the mice to be athymic and unable to develop T cells. However, these mice still have functional innate immune systems, including potent NK cells. Another strain that is still commonly used is the NOD-scid mouse, which has defects in the activity of innate cells such as NK cells, macrophages, and dendritic cells, as well as the absence of T and B adaptive immune cells. Further optimization of this strain by mutating the IL2 receptor-gamma, which plays a major role in cytokine signaling for both the innate and adaptive immune systems, yielded the NOD-scid-gamma (NSG) mouse. NSG mice are an extremely immunodeficient strain and have been valuable for increasing engraftment rates of PDXs.

2.2 Passaging and maintaining a PDX line

A successfully engrafted PDX will begin to grow as a subcutaneous tumor in the mouse. In order to maintain the line, the PDX must be passaged regularly. While institutional animal care policies may vary, a subcutaneous tumor of a volume of over 1cm³ is generally deemed to be the upper size limit for these tumors. Depending on the downstream analyses to be performed on the PDX and the amount of tumor tissue needed for each assay and passage, PDXs can be harvested at smaller tumor volumes. Indeed, PDXs can be passaged when the tumor volume is as small as 150–200mm³ by re-injecting tumor pieces or a single-cell suspension into a new mouse. Section 4.2 outlines a protocol for harvesting tumor tissue for passaging. Tumor pieces or single-cell suspensions can also be suspended in freezing media, allowed to slow-freeze then preserved in liquid nitrogen for injection at a later date. For each subsequent passage, it is important to record the passage number.

2.3 Critical data for fidelity of records associated with PDX lines

We have alluded to the vast amount of variation that exists in techniques dealing with the generation and study of PDX models of human cancer. To ensure that certain standard criteria are followed when generating and characterizing PDXs and that specific data are

collected about the models, the PDX models minimal information standard (PDX-MI) was developed (Meehan et al., 2017). The PDX-MI describes four different categories of information that should be collected and recorded when a PDX model is developed. These categories include: (1) clinical details (information such as patient demographics and tumor classification), (2) information on model creation (including mouse strain, engraftment rates, etc.), (3) model quality assurance data (e.g., assessment of the human origin of the PDX tumors), and (4) model study (e.g., assays performed, treatments administered, etc.). This information should be diligently kept for each PDX during analysis to ensure that the most appropriate PDXs are used for experiments and to avoid errors.

3. In vivo stimulation of PDX lines with interferon gamma

Interferon gamma (IFN γ) is the sole type II interferon and has diverse functions in the immune microenvironment. It is secreted and recognized by several different immune cells and has a wide array of immunostimulatory functions that include but are not limited to inducing T cell activation, antigen presentation and activating macrophages (Ni & Lu, 2018). While PDX models are not useful to study patient immune systems, these models can be used to study tumor-intrinsic factors that contribute to tumor immunogenicity and response to therapy, such as the responsiveness of a tumor to IFN γ stimulation. On non-immune cells IFN γ induces expression of antigen presentation machinery, leading to an increase in MHC class I on the cell surface, which is essential for recognition of cancer cells by cytotoxic T cells (Cheon, Borden, & Stark, 2014). Therefore, it is possible to assess the responsiveness of a PDX tumor to IFN γ (gamma-responsiveness) using fluorescence activated cell sorting (FACS) analysis to measure the levels of MHC class I on the surface of a cancer cell as a readout (Gettinger et al., 2017; Legrier et al., 2016).

4. Protocol

4.1 Equipment

1. 1-mL syringes and 18 and 27-G needles
2. Calipers
3. Dissecting tools (scissors and forceps)
4. Razor blades
5. 70- μ m filters
6. 15-mL conical tubes
7. 50-mL tubes
8. Laboratory centrifuge
9. 96-well, U-bottom plate
10. Titer tubes
11. Flow cytometer

4.2 Reagents and buffers

1. Phosphate-buffered saline (PBS)
2. Recombinant human interferon gamma (R&D, 285-IF)
3. Wash buffer (RPMI-1640+2% FBS)
4. Digest buffer (wash buffer+0.5mg/mL collagenase+1µg/mL DNase)
5. ACK lysis buffer
6. FACS buffer (PBS+2% FBS)
7. Fluorophore-conjugated antibodies for FACS staining:

Antibody/stain	Dilution	Vendor
Brilliant Violet 711 anti-mouse CD45	1:400	BioLegend #103147
APC/Cyanine7 anti-mouse CD45	1:400	BioLegend #103116
Alexa Fluor 488 anti-human EpCAM	1:400	BioLegend #324209
Brilliant Violet 605 anti-human MHC-I	1:100	BioLegend #311432
LIVE/DEAD fixable red dead cell stain	1:2000	Invitrogen #L23102

4.3 Intratumoral injection of interferon-γ (Fig. 1)

- 3.1. Begin with at least two subcutaneous tumors of the same PDX growing in separate NSG mice. Analysis can be performed once the tumor has grown large enough to have tissue to re-passage into a new mouse, to flash freeze, and to run FACS analysis, about 150–200mm³.
- 3.2. Measure and record the length and width of the tumor growing in the mouse using calipers. Calculate the tumor volume using the equation Tumor Volume=Length × Width² × 0.52, using two measurements the shorter of the as the width measurement (Euhus, Hudd, LaRegina, & Johnson, 1986; Tomayko & Reynolds, 1989).
- 3.3. Dilute stock recombinant human IFNγ in PBS to a concentration of 125,000 units per 100-µL injection.
- 3.4. Using a 27-G needle, inject 100µL of diluted IFNγ directly into the tumor of one of the two PDX tumors. Inject 100µL of PBS into the other PDX tumor as a negative control for IFNγ stimulation.
- 3.5. Administer a second dose of IFNγ/PBS after 24h.
- 3.6. 48h after the initial IFNγ injection, the PDX tumor is ready to be extracted for analysis.

4.4 Tumor digestion and single-cell suspension

- 4.1 Record tumor measurements in mm³.
- 4.2 Euthanize the mouse according to the appropriate institution-described protocol.

4.3 Pin the animal to a cork board, such that the tumor faces upward.

4.4 Spray the tumor and surrounding fur thoroughly with 70% ethanol.

4.5 Using forceps, lift the skin close to the tumor and make a small incision along one edge of the tumor. Insert scissors between the skin and the tumor, and carefully separate the tumor from the skin. Cut away separated skin, then cut along the bottom edge of the tumor to separate it from the mouse abdomen. Transfer tumor to PBS until ready to digest.

4.6 At this point, the tumor can be divided for desired analyses. A portion of the tumor should be finely chopped using a razor blade. If the PDX needs to be passaged this can be done using an 18-G needle to inject the minced tumor into a new NSG mouse. A portion of the tumor can be flash frozen in liquid nitrogen for further analysis. A piece of about 25mm³ or greater is necessary for FACS analysis.

4.7 Transfer the portion of the tumor devoted to FACS to a dry Petri dish. Using a razor blade, finely chop the tumor until no large pieces remain. Wash the razor blade with digest buffer and top the dish up to 3–5mL of digest buffer, depending on the size of the tumor.

4.8 Incubate the tumor for 45min at 37°C.

4.9 Transfer the contents of the Petri dish into a 70-µm filter in a 50-mL tube that has been pre-wet with wash buffer. Rinse the Petri dish with wash buffer to ensure that no tumor remains. Alternate pressing the remaining tumor sample through the filter using the plunger of a syringe and rinsing the filter with wash buffer. Do not exceed a total volume of 15mL.

4.10 Transfer the filtered sample to a 15-mL conical tube. Spin at 1500RPM for 5min at 4 °C. For large tumor samples (greater than 500mm³), centrifugation at 2500RPM for 8min can be performed instead.

4.11 Optional: If there are visible red blood cells in the cell pellet, lyse the sample with ACK buffer. Thoroughly resuspend the pellet in 1mL of ACK lysis buffer and incubate at room temperature for 2min. Top the volume up to 15mL with PBS, and spin at 1500RPM for 5min at 4 °C.

4.12 Resuspend the cell pellet in an appropriate volume of FACS buffer and pass the sample through a 70-µm filter.

4.5 Flow cytometry staining

5.1 Transfer approximately 1×10^6 cells (a minimum of 200,000 cells should be used to ensure that a sufficient number of cancer cells are stained since stromal cells are also present in the PDX digest) for each desired flow cytometry panel, as well an additional 1×10^6 cells for an unstained sample, into a 96-well U-bottom plate.

5.2 Wash the samples with FACS buffer: top up the volume in each well to 200µL with FACS buffer and spin the plate at 1650RPM for 2min at 4 °C.

5.3 Protecting samples from direct light exposure, prepare a stain mixture of all desired surface markers (see Section 4.1) for appropriate dilutions; each well of 1×10^6 cells will be stained with 100 μ L of stain mixture.

5.4 Protecting samples from direct light exposure, add 100 μ L of the prepared stain mixture to each sample and pipette up and down to mix. Stain for 30min on ice in the dark.

5.5 Wash the samples with FACS buffer: top up the volume in each well to 200 μ L with FACS buffer and spin the plate at 1650RPM for 2min at 4 °C. Repeat this step one time.

5.6 Resuspend each sample in 200 μ L FACS buffer and transfer the samples to flow cytometry tubes.

5.7 Analyze the samples on a flow cytometer.

5. Analysis

5.1 Markers (Fig. 2)

This IFN γ stimulation assay is a useful tool to analyze PDX samples. When investigating PDX lines established from immunotherapy-treated patients, the gamma-responsiveness of the tumor cells may be one indicator of whether the tumor is permissive to immune stimulation. Responsiveness to IFN γ is an indicator of a tumor-intrinsic factor that can affect sensitivity to immune checkpoint inhibitors. To perform this assay, it is essential that specific antibody stains are used to ensure that the correct population of cells within the PDX is captured for analysis.

The first consideration when performing FACS analysis of any sample is to ensure that the relative size of the cells, as seen by forward- and side scatter, is appropriate for the cell type being analyzed. In the case of PDX tumors, cell size tends to be less uniform and more variable. Therefore, a generous cell size gate is necessary to capture all of the tumor cells. This variation in tumor cell size makes it difficult to distinguish the tumor cell population based on size alone, so more markers are necessary to exclude unwanted cell types. In any flow cytometry analysis, a live/dead stain should be used to gate on the live cells from the tumor sample, as dead cells have the potential to auto-fluoresce in the analysis channels.

Despite the immunocompromised state of NSG mice, mouse CD45 positive cells (e.g., myeloid cells) may be present in PDX tumors. Therefore, staining for mouse CD45 will enable elimination of these cells from the population being analyzed. Similarly, since the tumors are taken directly from human tissue, it is possible for a small population of human immune cells to remain in the tumor, especially during early PDX passages. The likelihood of this occurring in subsequent passages is very low. Nonetheless, the samples should be stained with human CD45 in order to exclude these human immune cells. Another molecule that can be helpful for ensuring that only human tumor cells are being analyzed is to stain for mouse MHC class I. Any mouse stroma or other cells that have made it into the tumor before processing would be positive for mouse MHC class I, so exclusion of these cells from the population is useful. Furthermore, it is helpful to stain for human tumor markers in order

to positively identify the population of human tumor cells. However, it is important to be aware of the heterogeneity in expression of any marker used for a given cancer type so that no desired human tumor cells are excluded. For example, human EpCAM can be used to identify epithelial-derived tumor cells. However, some tumors can downregulate EpCAM expression so this marker is not helpful in these cases. Western blotting or immunohistochemistry for the marker of interest on the PDX can be performed prior to FACS analysis to establish whether the marker is expressed abundantly and uniformly in tumor cells.

The main readout for IFN γ responsiveness of a PDX is FACS analysis for cell surface human MHC class I. Under normal conditions, MHC class I surface expression is upregulated upon stimulation with IFN γ . When analyzing PDX samples, an IFN γ -responsive PDX will exhibit an increase in the fluorescence intensity of the fluorophore conjugated to the human MHC-I antibody in the IFN γ -stimulated PDX sample compared to the PBS-treated PDX. A PDX may be non-responsive to IFN γ for a variety of reasons. If the PDX is completely negative for MHC class I with and without IFN γ stimulation, this may indicate a defect in the IFN γ pathway. For example, loss-of-function mutations in JAK1 or JAK2 could prevent the interferon gamma receptor from signaling downstream in cells. Additionally, mutations in antigen presentation machinery genes, like β 2-microglobulin (B2M), could also prevent induction of MHC-I in a cell. Moreover, if the PDX expresses some level of MHC-I without stimulation and is non-responsive to IFN γ , this may indicate other defects in IFN γ signaling in the tumor that can then be further investigated.

5.2 Control cell lines

Proper control cell lines should be stained in tandem with the PDX samples as positive and negative controls for normal human MHC-I expression and IFN γ responsiveness (Fig. 2B). As a negative control for human MHC-I, the Daudi line can be used (ATCC® CCL-213™). This is a B cell lymphoma line that is negative for MHC-I. There are a variety of human cancer cell lines that are positive for MHC-I, such as H1975 (ATCC® CRL-5908™), a lung cancer cell line. It may prove useful to test human cancer cell lines that match the cancer type of the PDX samples being tested for their expression of human MHC-I, as well as their responsiveness to in vitro stimulation with IFN γ .

6. Summary

Patient-derived xenografts are a powerful tool for modeling human cancer. While the maintenance of PDX lines can be costly and time consuming, these models offer the advantage of being useful for a variety of investigative assays that generally cannot be conducted on limited amounts of primary patient tumor tissue. One such assay described in this chapter is an approach to analyze the responsiveness of patient tumors to IFN γ , a critical immunomodulatory molecule found in the tumor microenvironment. This assay provides a tool to functionally assess IFN γ signaling in a tumor and can be used to determine the frequency of defects in this pathway in a given tumor type. Moreover, wider application of this assay on an array of immunotherapy-resistant PDX samples has the

potential to reveal the role of IFN γ responsiveness in the response of tumors to immunotherapies and the mechanisms that underlie defects in the pathway.

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Fig. 1.

Schematic representation of the IFN γ stimulation protocol. Begin with at least two tumors of the same PDX growing in different NSG mice. Once tumors reach a sufficient volume, mice are injected intratumorally with either IFN γ or PBS every 24h. After 48h (two injections), mice are sacrificed and tumors are harvested. A single-cell suspension is prepared and stained for flow cytometric analysis. A portion of PDX tissue should be saved to re-passage into a new mouse or frozen down to maintain the line, either as tumor pieces or a single-cell suspension.

Fig. 2.

Representative flow cytometry gating of PDX samples. (A) First plot shows the forward scatter (FSC) on the X axis by the side scatter (SSC) on the Y axis (After gating out of doublets). Debris in the bottom-left corner of the plot is excluded, but cells are gated generously by FSC and SSC as tumor cells are not uniform in size. Generous gating here allows for all possible tumor cells to be captured. Within this initial gate, cells are plotted using the Live/Dead (LD) stain on the X axis while the SSC remains on the Y axis. Cells in the population that are positive in the Live/Dead channel are excluded from analysis, as these are dead cells. The live cell population is then plotted with both human and mouse CD45. Since the tumor cells are negative for both human and mouse CD45, the double-negative population is used from this plot. Within this population, additional tumor cell markers can be used, depending on the tumor type being analyzed. In this case, human EpCAM is used as a tumor marker, and the population of positive cells is analyzed for human MHC class I expression. (B) Example histogram of human MHC class I expression readout. The PDX sample is shown in blue. Human MHC-I positive (black) and negative (filled, gray) are shown on the same plot to compare expected MHC-I expression of known MHC-positive and -negative cell lines. In this example, H1975 cells are used as an MHC-I⁺ control, while Daudi cells are used as a MHC-I⁻ control. Examples of PDXs that upregulate MHC Class I as a result of stimulation with IFN γ can be found in Gettinger et al. (2017).