UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

Final project degree

Cancer immunotherapy and application of PPRHs for PD-L1 silencing in lymphomas

Main subject:	Biochemistry and Molecular Biology
Secondary subjects:	Immunology
	Pharmacology and therapeutics

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1 Summary/ Abstract

Cancer is a collection of related diseases characterized by an abnormal cell growth that tend to proliferate in an uncontrolled way in any part of the human body. There are many types of cancers but we will focus our study in Non-Hodgkin Lymphomas. The current cancer treatments include chemotherapy, radiotherapy and sometimes surgery. However, the greatest knowledge of cancer related genes has led to an improvement of targeted tumour therapies such as cancer immunotherapy or gene therapy. Cancer immunotherapy is based on understanding how to use the body's adaptive immune system to destroy cancer. Cancer has the ability to evade the immune system by up regulating PD-L1. For this reason, PD-L1 is considered a potential target in cancer research. In the experimental part of the project, the aim was to validate the application *in vitro* of Polypurine Reverse Hoogsten Hairpins (PPRHs) for PD-L1 silencing in a cell line derived from a follicular lymphoma. PPPRHs are non-modified DNA molecules able to decrease gene expression, such as PDL-1 in our case.

El càncer engloba un gran nombre de malalties que es caracteritzen pel desenvolupament de cèl·lules anormals, que es divideixen i creixen sense control a qualsevol part del cos. Existeixen molts tipus de càncers però en aquest treball ens centrarem en els Limfomes no Hodgkin. Avui en dia, el tractament d'aquesta malaltia inclou quimioteràpia, radioteràpia i de vegades cirurgia. No obstant, l'ampli coneixement actual dels gens implicats en el càncer, ha permès desenvolupar noves teràpies dirigides com la immunoteràpia del càncer o teràpies gèniques per millorar el tractament d'aquesta. La immunoteràpia es basa en entendre com utilitzar el sistema immune del pacient per lluitar contra el càncer i destruir-lo. Les cèl·lules cancerígenes són capaces de sobre expressar una proteïna anomenada PD-L1 per tal d'evadir el sistema immunitari. És per això que PD-L1 es considera una potencial diana en la recerca de teràpies anticanceroses. L'altre meitat del treball es tracta d'una part experimental en la qual es pretén validar l'aplicació dels Polypurine Reverse Hoogsten Hairpins (PPRHs) pel silenciament de *PD-L1 in vitro* en una línia cel·lular provinent d'un

limfoma fol·licular. Els PPRHs són molècules de DNA no modificat capaces de disminuir l'expressió d'un gen, en aquest cas PD-L1.

2 Initial discussion

Cancer is a group of diseases characterized by an abnormal cell growth that tend to proliferate in an uncontrolled way in any part of the human body. Cancer spreads to nearby tissues and organs through a process of invasion. It can also invade blood and lymphatic vessels, and travel to other organs or tissues where it can be implemented. This process is called metastasis. This project will be focused in Non-Hodgkin lymphoma. Lymphoma is a cancer that begins in cells of the lymphatic system and it can involve other lymphoid organs such as the lymph, lymph vessels, lymph nodes, spleen, tonsils, thymus and bone marrow.

Current cancer therapies count on surgery, systemic treatments such as chemotherapy or radiotherapy, and biological therapies with monoclonal antibodies or gene therapies directed to a specific gene. The present knowledge of tumour's molecular biology is making it possible to develop new-targeted treatments such as immunotherapy or gene therapy. The first one marks an entirely different way of treating cancer, by targeting the immune system and not the tumour itself. Its aim is to enhance and stimulate the patient's immune system to attack and destroy the tumour. Immunotherapy is becoming a powerful tool to fight cancer either alone or in combination with other therapies. In the same way, gene therapy is becoming really important for the treatment of a wide range of illnesses. Its aim is to create a treatment adapted to the genetic profile of the patient. In oncology, it basically consists on targeting a protein which is responsible for tumour cells proliferation and survival.

In the present study a protein called programmed-death-ligand 1(PD-L1) which is broadly expressed in multiple cancers cells and tumour infiltrating cells was selected as the target. PD-L1 protects tumours from cytotoxic T cells by inhibiting the antitumor immune response. Therefore it enables cancers to evade the immune system.

2.1 Subjects integration

Three subjects are included in this project: Molecular Biology, Immunology and Pharmacology. Molecular biology was useful to understand the molecular causes of cancer, the blockade of signalling pathways, basically the PD-L1/PD-1 pathway, and the role of PPRHs in gene silencing. Secondly, Immunology was basic to figure out the cancer immunity cycle and the way immunotherapy was able to enhance the immune system in order to attack and destroy cancer cells. Finally, Pharmacology is also involved since we were evaluating the current therapies for cancer treatment such as chemotherapy, radiotherapy and directed therapies with monoclonal antibodies.

2.2 Contextualisation

The self-therapeutic use of the immune system resources has always been a challenge for oncology researchers. Nevertheless, until now, anticancer treatments weren't powerful enough to generate an important stimulus of the immune system. Fortunately, nowadays a new drug generation is available and capable of changing the treatment perspective. Currently anticancer immunotherapy is one of the main goals for worldwide researchers especially since when the prestigious magazine "Science" in 2013 has chosen cancer immunotherapy as Breakthrough of the Year for 2013 (1).

The origin of cancer's immunotherapy started at late 1800s when William Coley, a famous American surgeon, observed that a bacterial infection generated spontaneous tumour regression. Coley took advantage of this natural phenomenon, developing a killed bacterial vaccine, "toxin Coley", for cancer patients. Unfortunately, this therapy was not well accepted by medical institutions given that the toxin induced extremely high fevers. Clearly, Coley's approach has a place in the past, present and future. It offered an opportunity for the development of a broadly applicable therapy for cancer (2).

A century later, anticancer antibodies such as rituximab succeeded and created great interest in anti-body based therapies for lymphomas, leukaemia and solid tumours.

Currently, monoclonal antibodies are standard therapeutics for several cancers such as the anti-CD20 antibody, Rituximab, for B cell non-Hodgkin lymphoma (NHL). However, antibodies are not curative alone and they must be combined with cytotoxic chemotherapy for clinical benefit. This treatment combination really improves the cancer treatment in comparison to chemotherapy regimens alone (3).

There has been an important progress in cancer research during 2014. This progress is directed towards a better understanding of cancer molecular biology (4). The greatest knowledge of cancer related genes and cancer molecular pathways led to an improvement of the targeted tumour and immune system therapies. This project will be focused in PD-L1, a target for immunotherapy with monoclonal antibodies and a target for gene silencing. PD-L1 is an overexpressed protein in a lot of cancers that enables cancer to evade the host immunity system.

2.3 Objectives

The main goal of this project is to make a bibliographical research about the current situation of immunotherapy, especially the use of monoclonal antibodies and gene therapies, for cancer treatment. Nowadays, PD-L1 is a potential target under investigation for cancer immunotherapy treatments.

The second goal is to evaluate the possibility of using PD-L1 for targeted therapies in lymphomas. PD-L1 is an inhibitory ligand overexpressed in lots of cancers that enables cancer to evade the immune system. Silencing this protein with Polypurine Reverse Hoogsteen Hairpin (PPRHs) could be a way of decreasing PD-L1 expression in cancer cells to reduce this suppressing signalling. If PD-L1 silencing is effective, cancer cells could be well detected and destroyed by the immune system.

The last aim of this project is to discuss whether the PPRH technology could be an appropriate treatment option for lymphomas in combination to chemotherapy regimens.

3 Introduction

3.1 Cancer and lymphomas

Cancer is a leading cause of disease worldwide. An estimated 14.1 million new cancer cases occurred in 2012 and it is also the leading cause of death worldwide, with 8.2 million deaths in 2012 (5). Nowadays almost half of cancers occur in countries with a medium or low level of Human Development Index. The development in these countries will result in a reduction of infection related cancers, outweighed by an increase of cancers associated with reproductive, dietary and hormonal risk factors. This will represent an increase of 68% compared to 2012.(5)

Cancer is a collection of related diseases that can start almost anywhere in the human body. They are an abnormal growth of cells that tend to proliferate in an uncontrolled way. Cancerous tumours can be malignant or benign. The malignant ones tend to invade other tissues and to metastasize, because cancer cells have different mechanisms that enable them to grow up and become invasive. After years of research, scientists have defined the different biological principles operating in a tumour cell. Cancer cells are able to evade cell death, immune destruction and growth suppressors to survive. They are also able to reprogram energy metabolism, to induce angiogenesis, to sustain proliferative signalling and above all to create genome instability and mutations. Thanks to these features we can distinguish a tumour cell from its non-malignant counterpart. Therefore, cancer research is focused on exploring innovative ways to target the biological mechanism of cancer.

There are many different types of cancer and they are usually named after the organs or tissues where they originate. We will focus all our study on the most common blood cancer: **Lymphoma**.

Lymphoma is a cancer that begins in cells of the lymph system. It occurs when cells of the immune system called lymphocytes grow and multiply uncontrollably. These cancerous lymphocytes can travel to many parts of the body, including the lymph nodes, spleen, bone marrow, blood, or other organs and form a tumour. The two

main forms of lymphoma are Hodgkin lymphoma and non-Hodgkin lymphoma (NHL). Hodgkin lymphoma occurs when there are abnormal B-lymphocytes called Reed-Sternberg cells; this type can usually be cured. However, NHL is a heterogeneous group of lymphoproliferative malignancies with different behaviour and responses to treatment. These diseases have been classified as B-cell and T-cell neoplasms based on the World Health Organisation (Annex 1). B-cell lymphomas represent 90% of all lymphomas and the most common types are diffuse large B-cell lymphoma (DLBCL), which is a usually aggressive and follicular lymphoma, which is usually indolent. (6) Our study will mainly be focused on Follicular Lymphoma since the cell lines used in this work are derived from this type of lymphoma. In Spain, NHL is the seventh leading tumour in both men and women and there are 3100 new cases for men and 2400 for women every year (7).

Non Hodgkin Lymphoma can cause many different signs and symptoms depending on the type, the speed of growing, the body location and the age of the patient. In more than two from three patients, non-Hodgkin lymphoma is presented as an enlarged palpable lymph node. 40% of NHL patients present general symptoms such as fever, weight loss and night sweats. These symptoms are called “B symptoms” and they are more common in rapidly growing lymphomas. B symptoms are really useful for the diagnosis and for determining the stage and prognosis of the lymphoma. NHL can also cause anaemia, cough, chest pain and fatigue. However, in some cases NHL might not cause any symptoms until it grows quite large (7).

The treatment and the chance of cure depend on the stage and the type of lymphoma. The treatment usually includes surgery, chemotherapy, radiotherapy, and sometimes stem cell transplant. The standard chemotherapy treatment for lymphomas is called CHOP and it consists of cyclophosphamide (an alkylating agent), hydroxydaunorubicin/doxorubicin (an intercalating agent), oncovin/vincristine (to prevent cell duplication) and prednisolone (corticosteroids). This regimen can also be combined with the monoclonal antibody rituximab (R-CHOP) directed against the protein CD20 found in pre-lymphocytes B and mature lymphocytes B. R-CHOP is generally given in 21-day intervals during 3 or 6 cycles in standard doses.

Therefore, the treatment for indolent Non-Hodgkin Lymphoma in early stage disease (Stage I and II) is based on radiation therapy and/or CHOP chemotherapy. For stage III and IV cases the treatment includes radiotherapy, chemotherapy with R-CHOP and/or radio immunotherapy. For aggressive Non-Hodgkin Lymphoma the treatment for early stages consists on R-CHOP chemotherapy for 3 or 6 cycles and radiotherapy. And in III and IV stages the treatment may include a monoclonal antibody therapy in combination with chemotherapy (R-CHOP) during 6 cycles (8).

New types of treatments are being tested in clinical trials, such as vaccine therapies, gene therapy or new therapies with monoclonal antibodies like cancer immunotherapy with anti-PD-L1. For some patients, taking part in a clinical trial may be the best treatment choice. Clinical trials are part of the research process and they are really useful to improve the way cancer will be treated in the future.

3.2 Cancer immunotherapy

Immunotherapy is a cancer treatment different from other available therapies because it is not directed to destroy cancer cells. Its aim is to enhance and stimulate the patient's immune system to attack and destroy the tumour. So, it is based on understanding how to use the body's adaptive immune defence against cancer's ability to evolve and evade destruction. This treatment will initiate or reinitiate a self-sustaining cycle of cancer immunity (Figure 1), enabling it to amplify and propagate, but not so much as to generate unrestrained autoimmune inflammatory responses (9). This type of therapy is very specific for tumour cells and less harmful for healthy tissues. Once the immune system is enhanced, it has the potential to be self-propagating. However, it may require quite a bit of time due to the fact that the immune system needs to get ready to fight against the tumour.

a) Cancer immunity cycle

It is important to understand the cancer immunity cycle before envisaging cancer immunotherapy (Figure 1). The cancer immunity cycle is the natural process by which

our immune system protects the body against cancer. Oncogenesis leads to the expression of antigens that can be captured by dendritic cells. Once captured, dendritic cells present antigens to T-cells in order to prime and activate cytotoxic T-cells. Activated T-cells infiltrate the tumour microenvironment to recognise and kill cancer cells. Dying cancer cells release additional cancer antigens, propagating the cycle. Immune surveillance is an essential cellular process that prevents tumour formation in the human body. However, there are some cancer cells not effectively detected and not destroyed by T-cells. These cells continue to divide and grow (10).

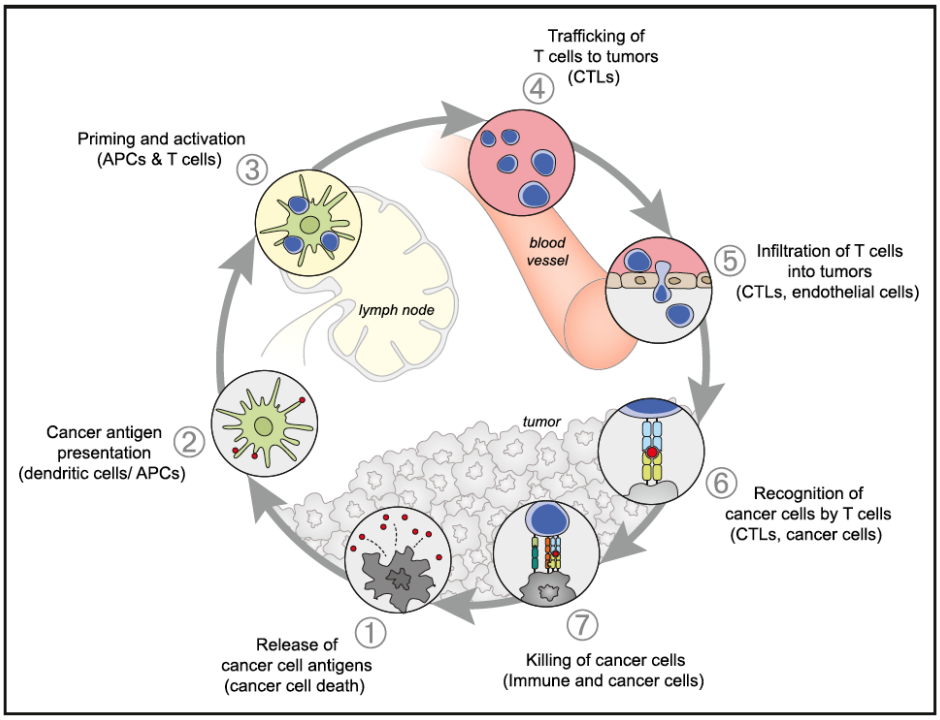


Figure 1: The Cancer-Immunity Cycle. (9)

This cycle can be divided into seven major steps. Each step of the cycle requires the coordination of stimulatory and inhibitory factors. Stimulatory factors promote immunity whereas inhibitors reduce immune activity. By disrupting the processes of this cycle, tumours can avoid detection of the immune system and limit the extent of immune destruction. One way of doing so is through the up regulation of inhibitory molecules such as **programmed death ligand** (**PD-L1**) in tumour cells and tumour-infiltrating immune cells, like macrophages and dendritic cells (11).

b) Different types of immunological treatments for cancer

There are lots of immunological treatment types and they can be classified into two major groups, non-specific immunotherapies and specific immunotherapies. The specific ones, basically vaccines and adoptive cell therapy, are directed towards a specific antigen or cell. Meanwhile, non-specific immunotherapy looks for a stimulation of the whole immune system. In this group we can include cytokines and immune control proteins. Moreover, monoclonal antibodies are designed to recognize tumour cells and create a passive immune response (12).

- Monoclonal antibodies: are specific antibodies that target a tumour receptor expressed on cancer cells in order to modulate the tumour proliferation. They are periodically administered intravenously and sometimes attached to a toxin to enhance their effect. Some of these drugs are: Rituximab, Avastin, Herceptin, Erbitux…
- Vaccines: there are two types of vaccines preventives and therapeutics. The first type is used to prevent the development of the tumour such as papillomavirus and hepatitis B vaccine. Therapeutics vaccines are made from a patient's own tumour cells with the goal of strengthening the body's immune system against cancer. The only one accepted in USA is the sipuleucel-T vaccine for metastatic prostate cancer.
- Adoptive cell therapy: lymphocytes cells are extracted from the patient, cultivated, reproduced and re-infused to the patient. Nowadays this type of treatment is only under experimental observation. However, a similar approach called CAR (chimeric antigen receptors) is becoming really successful in refractory leukaemia treatment.
- Cytokines: two types of cytokines are used to treat patients with cancer interleukins and interferon. They are broadly used to generate a non-specific stimulation of the immune system. Nowadays recent advances are enabling the development of more effective immunotherapies with cytokines or in combination of these agents.

- Immune control proteins: they have the ability to block certain proteins, called immune checkpoints proteins that limit the strength and duration of the immune responses. The blockade of these proteins increases the ability of the patient's immune system to detect and destroy cancer cells. Some of these are Ipilinumab and anti PD-L1/PD1 drugs basically used in melanoma and lung cancer (12)(13).

c) PD-L1 pathway

Under normal conditions, the PD-L1 pathway (Figure 2) can play an important role in maintaining immune homeostasis and protecting normal cells from autoimmunity (14).

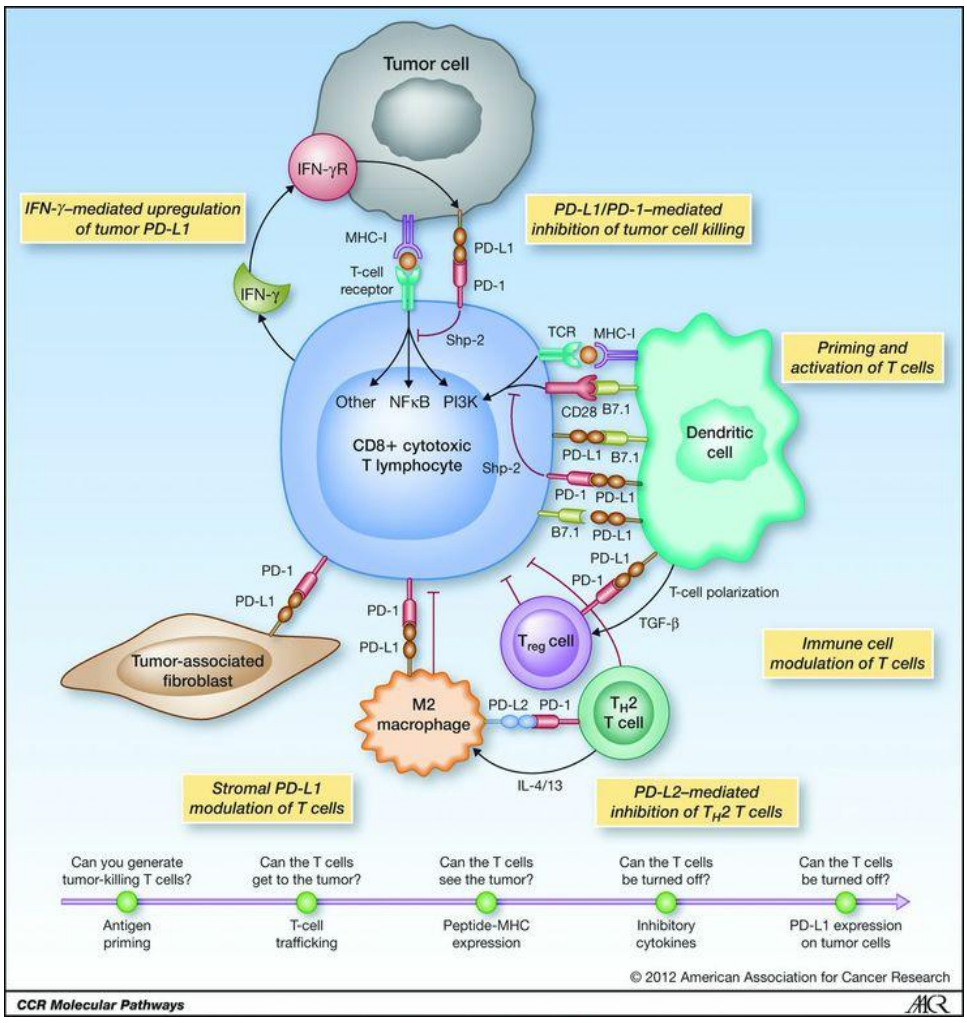


Figure 2. Tumour immunology and the PD-L1/PD-1 pathway (15)

However, cancer uses the PD-L1 pathway to inhibit cytotoxic T-cell activity in the tumour microenvironment and to inhibit cancer immunity cycle propagation in the lymph nodes. PD-L1 is found in the surface of cancer cells and in tumour infiltrating immune cells such as T and B cells, dendritic cells and macrophages. PD-L1 binds to PD-1 and B7.1 receptors leading to the deactivation of cytotoxic T-cells. PD-1 is an inhibitory receptor expressed on the surface of T cells that works as an immune checkpoint. This binding releases an inhibitory signal that reduces the production of cytokines and the proliferation of T cells. Cancer uses PD-L1 up-regulation to evade the host immune response during progression of the disease. (15)

Preventing PD-L1 from binding to its receptors on T cells (B7.1 and PD-1) may allow T-cells to remain active (15)(16). In these conditions, T-cells may be able to respond and kill cancer cells. Thus, the aim of cancer immunotherapy is to block the PD-L1/PD-1 and PD-L1/B7.1 pathways to let the body better detect and fight cancer.

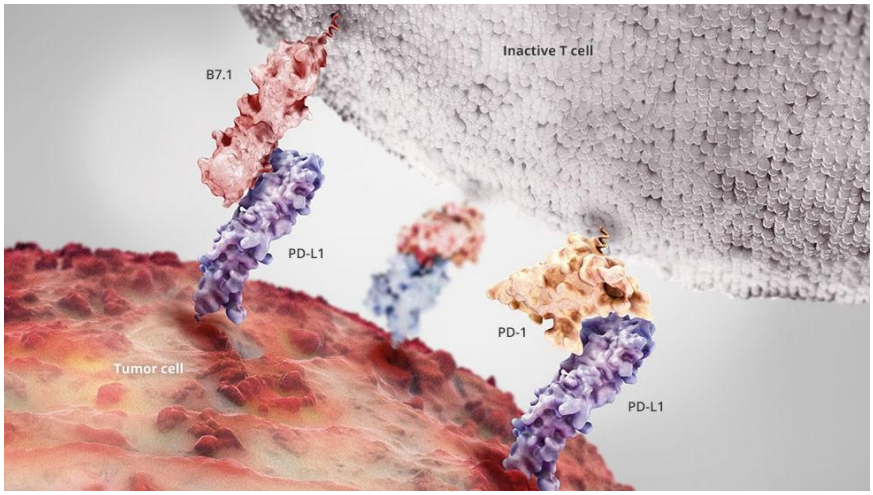


Image 1. PD-L1 binds to B7.1 and PD-1 on cytotoxic T cells and disables their anticancer immune response (17)

d) PD-L1 expression

Researchers are investigating whether high amounts of PD-L1 may indicate a better response to immunotherapies that interfere with PD-L1. Measuring the amount of PD-L1 on both tumour cells and tumour-infiltrating cells may help to predict the effect of certain medicines. This could contribute to determine which people are

more likely to respond to cancer immunotherapy alone or in combination with other cancer therapies (15). Overexpression of PD-L1 has been observed in multiple cancer types, making PD-L1 a potential target and a potential biomarker in cancer research.

Cancer Type	% PD-L1 expression
Melanoma	40-100
Non- small cell lung cancer	35-95
Nasopharyngeal cancer	68-100
Glioblastoma/mixed glioma	100
Colon adenocarcinoma	53
Hepatocellular carcinoma	45-93
Urothelial cancer	28-100
Multiple myeloma	93
Ovarian cancer	33-80
Gastric carcinoma	42
Esophageal cancer	42
Pancreatic cancer	39
Renal cell carcinoma	15-24
Breast cancer	31-34
Lymphomas	17-94
Leukemias	11-42

Table 1: Expression of PD-L1 by tumour type according to Chen DS, a current review of PD-L1 expression (15)

Tumour expression of PD-L1 has been reported to be associated with positive responses to PD-L1/PD-1 pathway inhibition in clinical studies (15). As shown in Table 1, PD-L1 is highly expressed in many human cancer types. However, in lymphomas, PD-L1 expression varies from 17% to 94% because peripheral T-cell lymphomas, diffuse large cell lymphomas and small lymphocytic lymphoma are included in this category. In consequence targeting PD-L1/PD-1 pathway in lymphoma patients could lead to

different responses. PD-L1 expression should be measured before deciding whether immunotherapy is the appropriate treatment.

e) Anti PD-L1 drugs

Nowadays, there are research cancer immunotherapy studies with monoclonal antibodies designed to interfere with PD-L1 and PD-1. Antibodies against PD-1 inhibit this receptor from binding to PD-L1 and PD-L2 so they could be appropriate for tumours with a high expression of both ligands. But the direct inhibition of PD-L1 can lead to serious autoimmune adverse events (18). Otherwise, anti-PD-L1 molecules are designed to prevent PD-L1 from binding to PD-1 and B7.1 on the surface of T cells allowing cytotoxic T cells to kill cancer cells expressing PD-L1. In this direction, Atezolizumab (MPDL3280A) from Genentech, is an anti-PD-L1 for solid tumours under clinical trial evaluation. In Table 2 some anti PD-L1 and PD-1 currently being developed in oncology are shown.

Therapeutic	Blockade	Company	Antibody Type	Development
MPDL3280A	Anti PD-L1	Genentech	IgG1	Phase II
MEDI-4736	Anti PD-L1	AstraZeneca	IgG1	Phase I
Nivolumab	Anti PD-1	Bristol-Myers	IgG4	Phase III
Lambrolizumab	Anti PD-1	Merck & Co	IgG4	Phase III
Pidilizumab	Anti PD-1	Cure Tech	IgG4	Phase II
AMP 224	Anti PD-1	GSK	IgG4	Phase I

Table 2: Inhibitors of PD-L1 or PD-1 currently being developed in Oncology (9)

Other new therapies are focused on targeting overexpressed cancer proteins such as BCL-2 or p53 that are required for cancer cell survival. These therapies are highly active but they are incapable of changing with cancer's ability to mutate and evolve. Nevertheless, anti PD-L1/PD-1 agents are able to enhance the host immune response to generate durable and adaptable antitumor immunity. Once immune response is enhanced, it has the potential to be self-propagating. PD-L1/PD-1 targeted therapy may not be enough to treat the majority of cancers. Combination approaches may

probably be the best treatment choice to induce cancer durable regression. These combined therapies could include radiation therapy or chemotherapy, antiangiogenic therapy and tumour-targeted therapies. The combination of PD-L1/PD-1 with other targeted therapies will also improve cancer treatment responses. (15)

Immunotherapy with anti PD-L1/PD-1 molecules would help patients to better fight this highly aggressive evolving disease.

3.3 Gene therapy

Gene therapy is a treatment that involves the transfer of genetic material into the patient's cells in order to cure a disease or to improve the clinical status of the patient (19). Gene therapy may be a promising treatment option for a wide range of diseases including cancer, cystic fibrosis, heart disease, diabetes and haemophilia (20).

The development of this type of therapies requires a long process that starts with the determination of the molecular defect and the mutated gene or genes that are causing the disease. Once the therapeutic gene is identified, a functional copy that corrects the problem will replace it. It works by introducing DNA with a healthy copy of the defective gene into the patient cell.

In cancer, it is difficult to find the appropriate genomic change since it is a polygenic disease caused by a huge variety of gene alterations. These alterations can be functional or structural including mutations, insertions, deletions, amplifications, fusions and translocations.

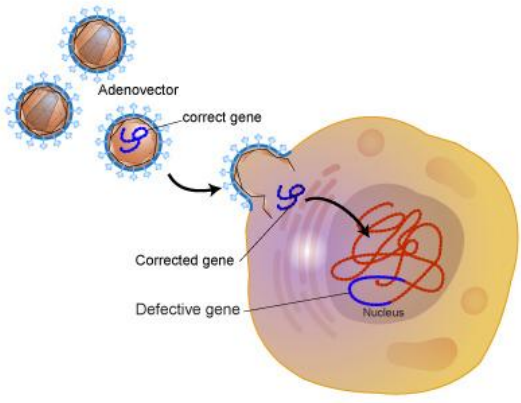


Figure 3: introduction of DNA with the corrected gene (21)

Cancer gene therapy nowadays is based in the following strategies:

- To replace the mutated genes. Some cells have genes that gain or lose their function. Replacing the altered or mutated genes could be helpful to treat some diseases. For example, many cancers have mutated versions of the p53 protein, a proto-oncogene that interferes with the apoptosis process. The introduction of a normal p53 gene could make cancer cells more sensitive to standard chemotherapy than cancer cells with the altered protein.
- To silence or "knock out" a mutated gene. Activated, overexpressed or mutated genes that cause the disease can be silenced to avoid tumour growth and tumour proliferation. Otherwise, we can stimulate the expression of genes that help prevent the disease.
- To make the immune system more sensitive to disease cells. Gene therapy could be used to train the immune system to recognise disease cells by promoting cytokines or by stimulating tumour immunity cycle.
- Other strategies such as blocking oncogenes (genes that promote tumour formation) or stimulating tumour supressing genes.

There are two different types of gene therapy depending on which types of cells are treated, either germline or somatic. In germline gene therapy DNA is transferred to reproductive cells (cells responsible of producing eggs or sperm). Therefore, the effects of the therapy will be transferred to the following generation. Germinal gene therapy it is currently not legal.

Somatic gene therapy targets all the cells of the body except the germinal ones. This means that the effects of this gene therapy will not be inherited. It can be done *in vivo* or *ex vivo*. The *ex vivo* strategy consists on removing the cells from the body to transfect them while *in vivo* gene therapy consists on doing the genetic modification inside the patient's body.

A key factor for gene therapy is the development of a delivery system capable of transferring the gene to the patient's cells. The vector that delivers the DNA is usually a virus (retrovirus, adenovirus or virus associated to adenovirus) or a liposome. Naked DNA transfer is also viable for gene delivery. The type of delivery vehicle depends on

the specificity of the transfection, the size of the gene and the target cells. Once the altered genes are carried inside the cells of the patient they will be integrated into the genome of the patient and they will ideally produce the therapeutic protein (20).

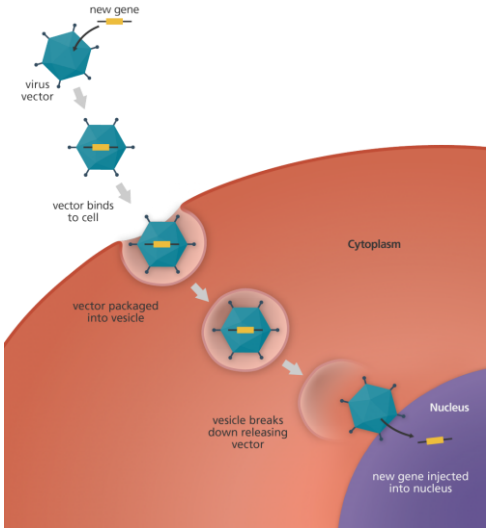


Figure 4: gene transfection with viral vector (22)

Gene therapy allows for an incredible diversity of cancer treatment options. It can be used to complement and increase the effectiveness of current chemotherapeutic regimens as well as to develop personalized cancer therapies. With the current information of the molecular causes of cancer and the knowledge of the implied genes, gene therapy can be a huge opportunity for creating more sophisticated and personalized treatments for the patients.

3.4 Silencing with PPRHs

Gene silencing consists on decreasing the expression of a gene in order to treat a disease. Nowadays, molecules like antisense oligonucleotides (aODNs), small-interference RNA (siRNA) or Triplex Forming Oligonucleotides are being used to silence gene expression in biomedical research. However, they present some disadvantages such as instability and activation of the immune response (23). The research group that I've been working with has developed a new type of molecule capable of decreasing the gene expression. These molecules are Polypurine Reverse Hoogsteen Hairpins (PPRHs). PPRHs are nonmodified DNA molecules formed by two antiparallel

polypurine domains linked by a pentathymidine loop that allows the formation of intramolecular reverse Hoogsten bonds (23). These hairpins bind to polypirimidine targets in the DNA through Watson-Crick bonds and maintaining their original structure. PPRHs molecules can target both coding and template stretches. Coding-PPRHs directed against the DNA coding strand can also interact with the mRNA because they have the same sequence and orientation. In this direction, coding-PPRHs can interfere with the splicing process leading to a decrease in mRNA levels (24). On the other hand, Template PPRHs are directed to the template strand of the DNA and have the ability to decrease mRNA levels by inhibiting gene transcription (25). Therefore, PPRHs are polypurine sequences that bind to polypirimidine sequences in the DNA to knock down gene expression. This property allows us to selectively modulate the activity of a specific gene, in this case PD-L1.

For this project we have designed a PPRH against PD-L1 (programmed cell death ligand) in order to reduce its expression and make cancer more vulnerable to the patient's immunity system. So, instead of using anti-PD-L1 monoclonal antibodies for cancer immunotherapy, we have used PPRHs against PD-L1 in the same direction.

4 Objectives/ hypothesis of the experimental design

I've developed this project under the supervision of Doctors Veronica Noé and Carles Ciudad, from the research group of Cancer Therapy in the Department of Biochemistry and Molecular Biology of the School of Pharmacy in the University of Barcelona.

In this research project, our main goal was to evaluate the possibility of using PD-L1 as a target to treat Lymphomas or almost reduce their progression. PD-L1 is a trans membrane protein overexpressed in a lot of cancers, responsible of protecting tumours from cytotoxic T cells. We targeted PD-L1 with Polypurine Reverse-Hoogsten Hairpins (PPRHs) to silence the gene and induce lymphoma remission. Targeting PD-L1 is probably not enough to kill all cancer cells, and for this reason we also wanted to evaluate the effect of combining gene therapy (PPRHs) with chemotherapy regimens.

A secondary goal was to demonstrate if the tumour expression of PD-L1 is associated to a better response to PPRHs treatment.

5 Experimental design

We designed this experiment with two main objectives:

- To incubate the lymphoma cell line (WSU) used as a model with PPRHs against PD-L1 in order to validate their effects, to determine their cytotoxicity and to evaluate the effect of combined therapies (PPRHs + chemotherapy).
- To determine the levels of PD-L1 expression in WSU cells

PPRHs were incubated either naked or complexed with a cationic liposome, such as DOTAP or Viromer®Green, alone or in combination with Bendamustine. In all cases we analysed whether PPRHs had the ability to knock down the expression of PD-L1, by a decrease of the mRNA levels of PD-L1, using the MTT assay. A lower amount of mRNA meant the silencing process had been effective. We also tried to determine the cytotoxicity of PPRHs and its vehicles.

In addition we determined PD-L1 levels in WSU cells using RT-Real Time PCR and comparing it to other cell lines with reported PD-L1 overexpression. In this direction, we have used PC3, a human prostate cancer cell line, and MiaPaca-2, a human pancreatic cancer cell line. PD-L1 expression could vary from 17% to 94% in Lymphomas.

6 Materials and methods

6.1 Design of PPRHs

The PPRHs used in this study were formed by two antiparallel polypurine domains, bound by intramolecular reverse Hoogsten bonds, and linked by a pentathymidine loop (24). Triplex Forming Oligonucleotide Target Secuence Search Software was used to find polypirimidine sequences in the *PD-L1* gene in order to design the corresponding PPRH against PD-L1 (26). A sequence of 28 bases with only three

pyrimidine interruptions was found in intron 1 of the gene located in chromosome 9 (Annex II). This sequence was used for the design of the Wild Type Coding PD-L1 hairpin.

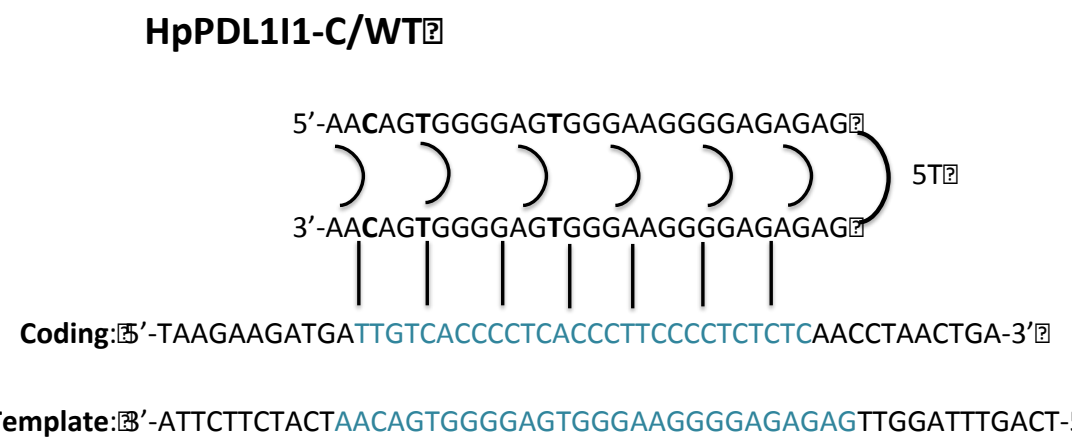


Figure 5: Coding-PPRHs binding to its target sequence in the PD-L1 gene. Curved lines represent Hoogsteen bonds and straight lines Watson-Crick bonds. The blue sequence corresponds to the polypirimidine ssDNA target in the gene.

In Figure 4 we can see a schematic representation of the Wild type Coding-PPRH and its binding to its polypirimidine DNA target in intron 1 of the *PD-L1* gene. As shown, the Coding-PPRH hairpin forms a triplex structure with the DNA that knocks down the expression of the *PD-L1* gene.

PPRHs were synthetized as non-modified oligodeoxynucleotides by Sigma-Aldrich. After that, they were dissolved in sterile RNase-free TE buffer and stored at -20ºC.

6.2 Cell line (WSU)

All the experiments were performed with a human B cell line called WSU-FSCCL (WSU), a generous gift from researchers at IDIBAPS, Hospital Clinic of Barcelona. It was established from a patient with low-grade follicular (small cleaved cell) lymphoma in leukemic phase. These cells presented two chromosomal translocations t(14;18) and t(8;11). The first translocation is common in follicular lymphoma while the second one is not usual in this type of lymphomas and it may probably confer the aggressive

behaviour seen in the terminal phase of the disease. The t(8,11) translocation is a juxtaposition of the region of the *BCL-2* gene leading to an overexpression of BCL-2 another PPRH target possibility studied by my team mate. Nevertheless, this cell line does not present any anomaly towards PD-L1. WSU cells reacts with monoclonal antibodies to B-cell antigens.

6.3 Cell culture

WSU-FSCCL cells were grown in RPMI medium containing 10% growth factors (FCS), two antibiotics and a pH regulator to avoid the acidification of the medium. This medium has only one-month validity. While most cancer cells get attached to the plate, lymphoma cells live in suspension. This means that, it is necessary to centrifuge them before changing the culture. Lymphoma cells grow and multiply really easily however they are quite fragile and difficult to transfect.

RPMI + 10% FCS for 500mL

RPMI……5,215g

Streptomycin……50mg

Penicillin G……31,25 mg

$NaHCO_3$ ……1g

FCS……50mL

Sodium pyruvate……5mL solution 100mM

HEPES……500µL solution 1M

H_2O……444,5mL

6.4 PPRH transfection and liposomes

PPRH transfection needs a delivery system capable of transferring the hairpin to the cells. As we said, the delivery system can be viral or non-viral including electroporation, cationic liposomes or microinjections. Most of the delivery methods present problems with the DNA delivery and sometimes they present cytotoxicity

problems. Two cationic liposomes DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) and Viromer Green were chosen in order to reduce these problems. The transfection process is different depending on the vehicle.

When DOTAP was used as a vehicle, PPRHs were first mixed with DOTAP for 20 minutes. Then, cells were transfected and the plate was incubated for 3 to 7 days. However, in case of using Viromer®GREEN the transfection process was more complex. First, PPRHs were diluted with Buffer GREEN obtaining an 11µM dilution (Tube 1). In Tube 2, Buffer GREEN was added to Viromer®GREEN and vortexed for 3 to 5 seconds. After that, 1µL of Tube 1 was mixed swiftly with 9µL of Tube 2 and incubated for 15 minutes at room temperature. 15 minutes later, the transfection complexes were added to the cells and incubated as usual.

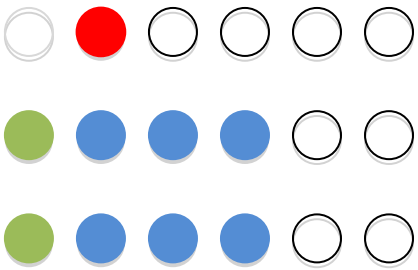
6.5 MTT assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to evaluate the effect of PPRHs in our cell line (WSU). MTT measures the cell proliferation rate or the reduction in cell viability by the determination of the total mitochondrial activity (27).

MTT assays were performed in a 12, 24 or 96 well dishes. The assay always included blank wells with only medium, negative control wells with cells and medium, positive control wells with untreated cells and test cells treated with PPRHs. Treated cells were usually present in duplicate or triplicate. Once the assay was designed, we selected the optimal number of cells, usually between 10.000 and 40.000 cells per well. The optimal cell number was plated in 12 or 24 well dishes with 500µL of RPMI medium or in 96 well dishes with 100µL of medium.

After the incubation period, for both transfection vehicles, the MTT reagent was added to the culture medium and allowed to react 2h at 37ºC. Once a purple precipitate was visible, we started with the addition of the lysis solution (24V Isopropanol + 1V HCl 1M) and we pipet up & down. Cell viability was measured at 570nm in a

Spectrophotometer. Absorbance values that were lower than the untreated control cells (negative control) indicate a reduction in cell viability. While higher absorbance values indicate an increase in cell proliferation. Therefore, we wanted to see a decrease in the absorbance values to correlate it with an increase in cell death due to PPRH transfection.



- Blank: Medium
- Negative control: cells and medium
- Positive control: cells, medium and DOTAP
- Treated cells: cells, medium, DOTAP and PPRHs in different concentrations

Figure 6: MTT assay example with DOTAP

6.6 mRNA analyses, RT-PCR and Real Time PCR

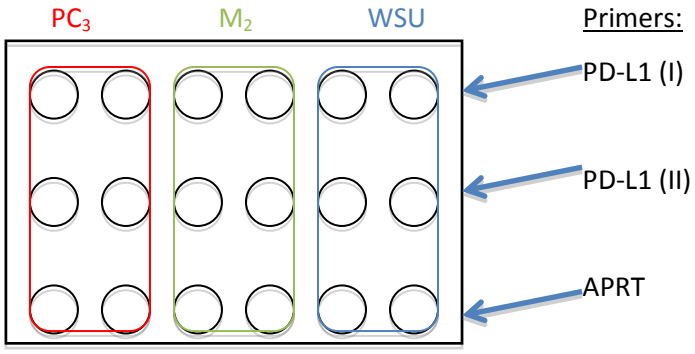
Real-time PCR is a simple method to determine the amount of a target sequence or gene present in a sample (28). Before performing a Real Time PCR reaction, it is needed to extract the RNA from the sample with Trizol and convert the RNA into cDNA by reverse transcription (RT). This method was used to quantify PD-L1 levels in the untreated and the treated WSU cells in order to predict the effects of PPRHs transfection and to evaluate the effect on gene silencing.

For mRNA analyses, total RNA from 50.000 WSU cells was extracted using Trizol according to the lab PNT. The amount of RNA was determined by measuring its absorbance in a Nanodrop spectrophotometer. RNA quality was correct when the Ratio 260/280 (RNA/proteins) was close to 2 in the Nanodrop spectrophotometer.

Once RNA was extracted, cDNA was synthetized with the reverse transcriptase mixture. This mixture contained 0,25µL of the random hexamers 125µg/µL dilution (primers), 1µL of the dNTPs 500µM dilution, 2µL of Buffer 10x, 20 units of RNAse inhibitor and 200 units of Moloney-Murine Leukemia Virus Reverse Transcriptase (M-

MuLV). 4,75µL of the reaction mixture were added to 15,25µL of the RNA solution and incubated at 42º for 1h.

3µL of the cDNA mixture were used for the real time PCR amplification. The reaction was performed using the ABI-Prism 7000 Sequence Detection System (Applied Biosystems). The final volume for the reaction was 20µL, 3µL of cDNA and 17µL of the PCR mixture. The PCR mixture contained 1xSYBR Universal PCR Mastermix, 0,25µM of reverse and forward primers and 6µL of H_2O MQ. The mixture and the cDNA were placed in the PCR plate in order to start the Real Time-PCR. PCR cycling conditions were 10 minutes denaturation at 50ºC, 10-minute denaturation at 95ºC, followed by 40 cycles of 15s at 95ºC and 1 min at 60ºC. The mRNA amount of PD-L1 was determined by the $\Delta\Delta C_T$ method, where C_T is the cycle in which the amplified product reaches the fluorescence threshold (25).



The primers sequences for PD-L1 were:

Forward (I): 5'-ACAATTAGACCTGGCTGCAC-3'

Reverse (I): 5'-GTATGGGGCATTGACTTTCAC-3'

Forward (II): 5'-CATGACCTACTGGCATTTGCTG-3'

Reverse (II): 5'ACATATAGGTCCTTGGGAACCG-3'

APRT mRNA was used as an endogenous control.

Therefore, two pairs of PD-L1 primers were used to determine PD-L1 levels in WSU treated and untreated cells, in MiaPaca-2 and in PC-3 cell lines using the *APRT* gene as a control. It is known that MiaPaca-2 and PC-3 cell lines overexpress PD-L1.

7 Results and discussion

7.1 Incubation with PPRHs against PD-L1

a) Effects of PPRHs against PD-L1 using DOTAP as a vehicle

WSU cells were incubated with HrpPD-L1-C WT for 6 days. After that period, cell viability was analysed with the MTT assay. A decrease in the absorbance of the transfected cells in relation to the control was observed (Figure 7). This could probably mean that WSU cells were being transfected and killed by the combination PPRH+DOTAP. Thus, when the higher hairpin concentration was used, the most reduction in cell viability was obtained.

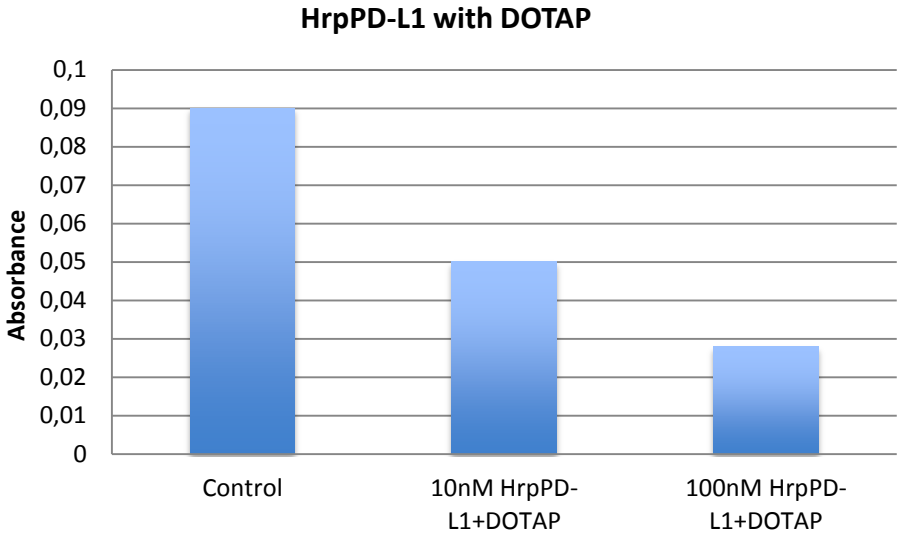


Figure 7: Cell viability of WSU cells incubated with PPRHs against PD-L1 (10nM and 100nM) using DOTAP as a vehicle.

We repeated the experiment with 20.000 and 30.000 cells in 96 well dishes for 72h. The main aim of it was to see if the cells were being killed by HrpPD-L1. A control hairpin (HrpLac) was also included in the experiment. Theoretically, the control hairpin should not affect cell viability because its target gene is not present in cancer cells.

Unfortunately, a decrease in cell viability could be observed with both PPRHs as shown in the following graphs (Figure 8 and 9). Consequently, we started to think that either the vehicle (DOTAP) or the hairpin were cytotoxic by themselves.

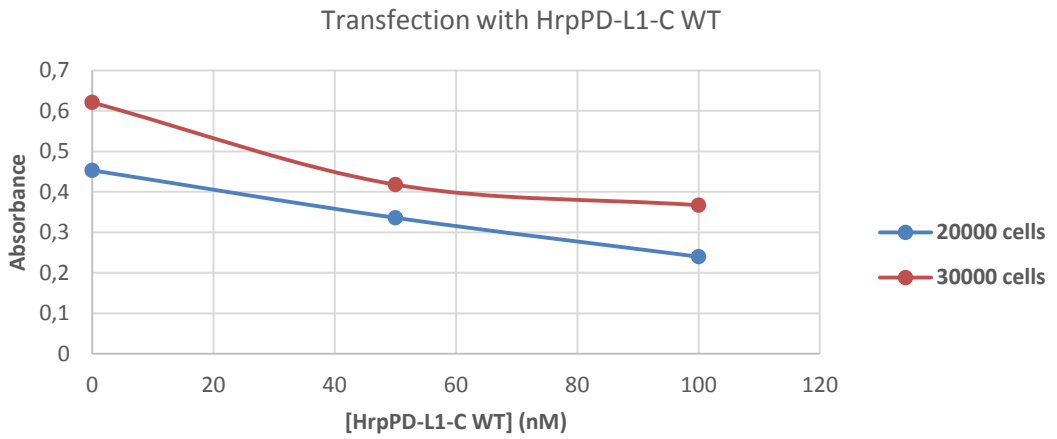


Figure 8: Cell viability of WSU cells incubated with PPRHs against PD-L1 (0nM, 50nM and 100nM) using DOTAP as a vehicle.

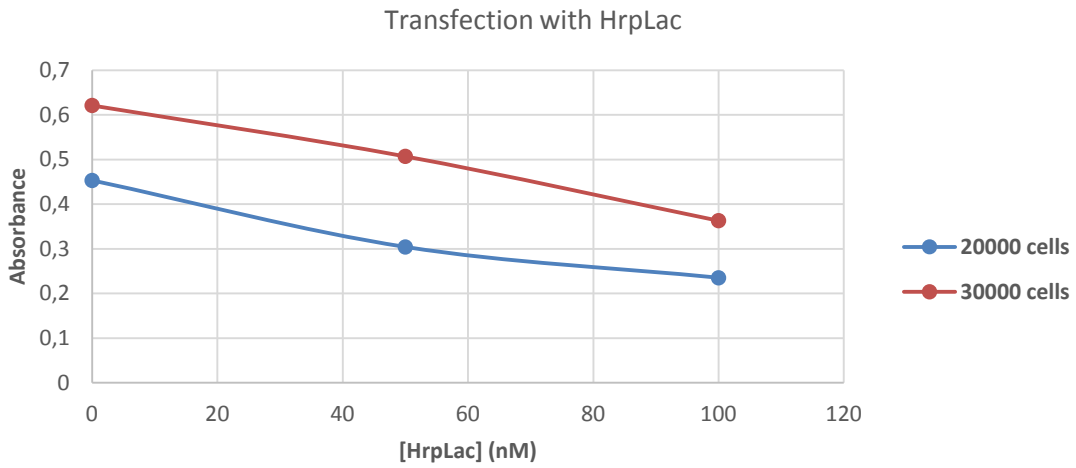


Figure 9: Cell viability of WSU cells incubated with PPRHs against Lac (0nM, 10nM and 100nM) using DOTAP as a vehicle.

b) PPRH and DOTAP cytotoxicity

40.000 cells were incubated during 48 hours with increased concentrations of DOTAP, 1/10 dilution of DOTAP and Hairpin PD-L1-C WT with DOTAP. After the MTT assay a similar tendency in cell death was observed in both treated and untreated cells (Figure 10, 11 and 12). Therefore, we concluded that DOTAP was toxic for WSU cells. The effects observed in previous experiments were probably due to the toxicity of the vehicle and not to gene silencing. Consequently, another vehicle called Viromer Green was chosen to transfect the hairpin.

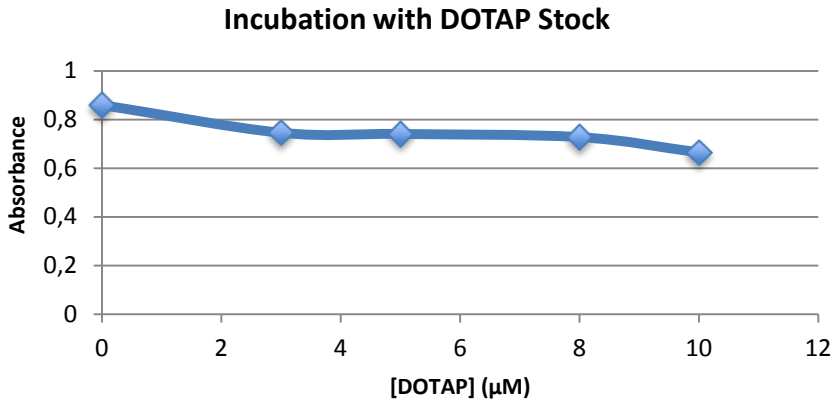


Figure 10: Cell viability of WSU cells treated with increased concentrations of DOTAP (0µM, 3µM, 5µM, 8µM and 10µM)

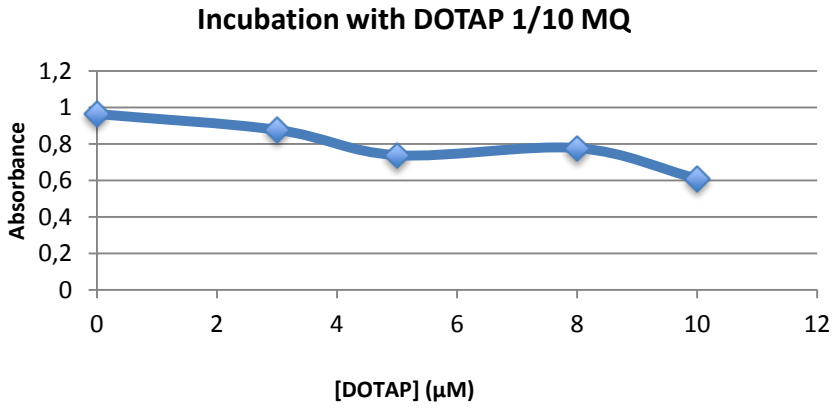


Figure 11: Cell viability of WSU cells treated with increased concentrations of 1/10 dilution of DOTAP (0µM, 3µM, 5µM 8µM and 10µM)

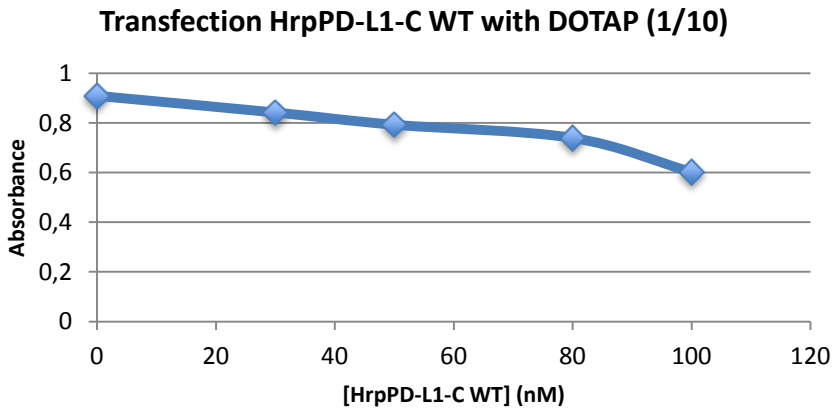


Figure 12: Cell viability of WSU cells incubated with increased concentrations of Hrp PD-L1 (0µM, 30µM, 50µM and 10µM) and DOTAP as vehicle.

c) Effects of PPRHs against PD-L1 with Viromer Green as vehicle

60.000 cells were incubated in 96 well dishes for 48 hours with three different hairpins: HrpPD-L1-C WT, HrpBCL-2-C WT and HrpLAC. A negative control with only cells and culture, and a positive control with cells, culture and the vehicle (Viromer Green) were also included. After analysing the results, a reduction in cell viability was observed for treated cells as well as for positive control (Figure 13). This meant that cells were not being well transfected and Viromer Green was probably killing them. Apart from that, there were no significant differences between the transfection with the studied hairpins (HrpPD-L1 and HrpBCL-3) and the control hairpin (HrpLAC). This confirmed us the fact that the hairpin was not knocking down gene expression (PD-L1 and BCL-2) in order to kill the cancer cells.

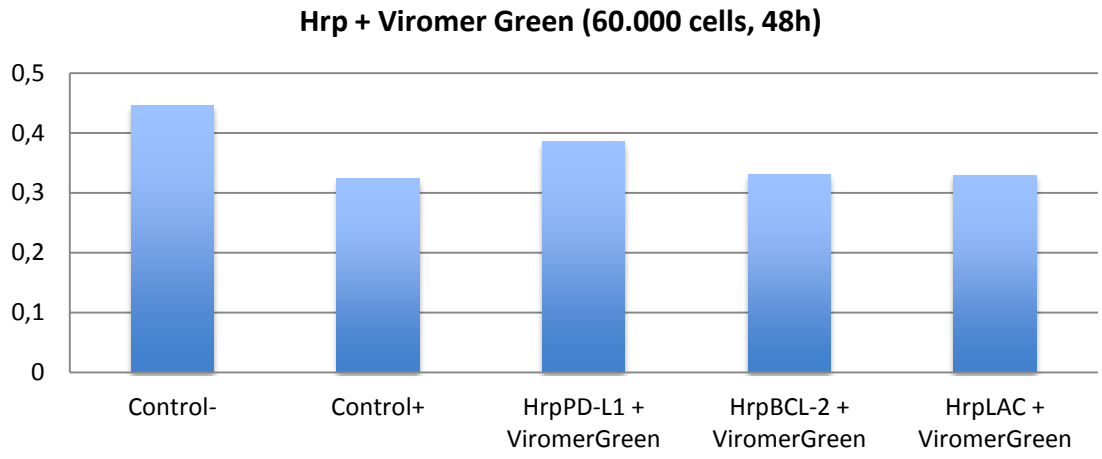


Figure 13: Cell viability of WSU cells incubated with HrpPD-L1, HrpBCL-2 and HrpLAC using Viromer Green as vehicle.

d) Effects of naked PPRHs against PD-L1

20.000 and 30.000 cells were incubated during 72 hours with increased concentrations (1µM, 3µM and 5µM) of HrpPD-L1-C WT and HrpLAC without vehicle. Much more hairpin concentration was needed because any delivery system was used. In these conditions we observed that the treated cells presented the same cell viability than the

controls. This was due to the inability of the naked hairpins to get inside the cells so they could not silence the genes.

e) Determination of PPRHs internalization by flow cytometry

500.000 cells were incubated during 24h in a final volume of 1mL with increasing amounts of a Fluorescent Hairpin (50nM, 100nM or 150nM) and a vehicle: DOTAP (5µM or 10µM) or ViromerGreen (1µL, 2µL or 3µL). After 24h incubation the efficacy of hairpin delivery was evaluated by cytometry at the Parc Científic of Barcelona (Annex III).

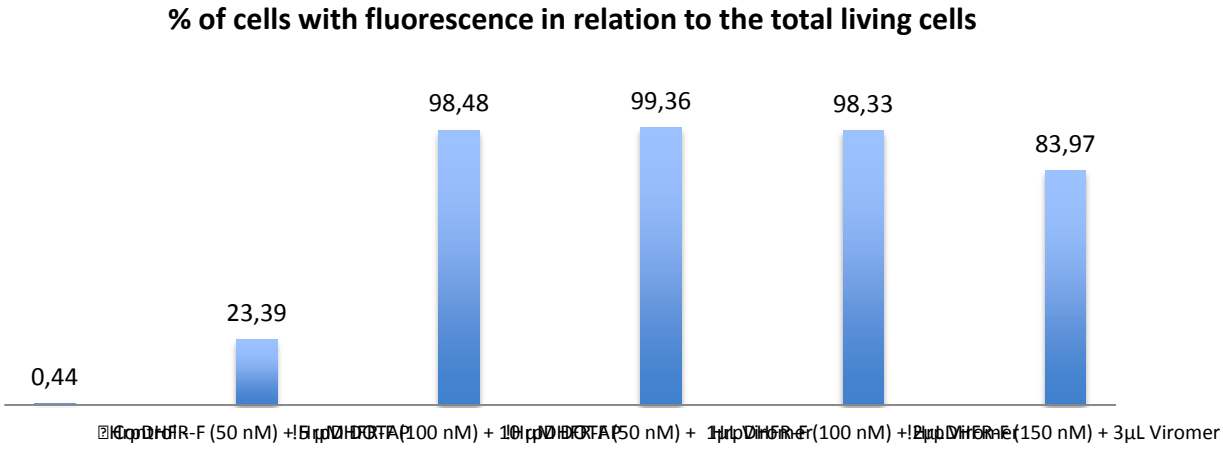


Figure 14: % of WSU cells with fluorescence in relation to the total living WSU cells incubated with a Fluorescent Hairpin (DHFR) using DOTAP or Viromer Green as vehicle.

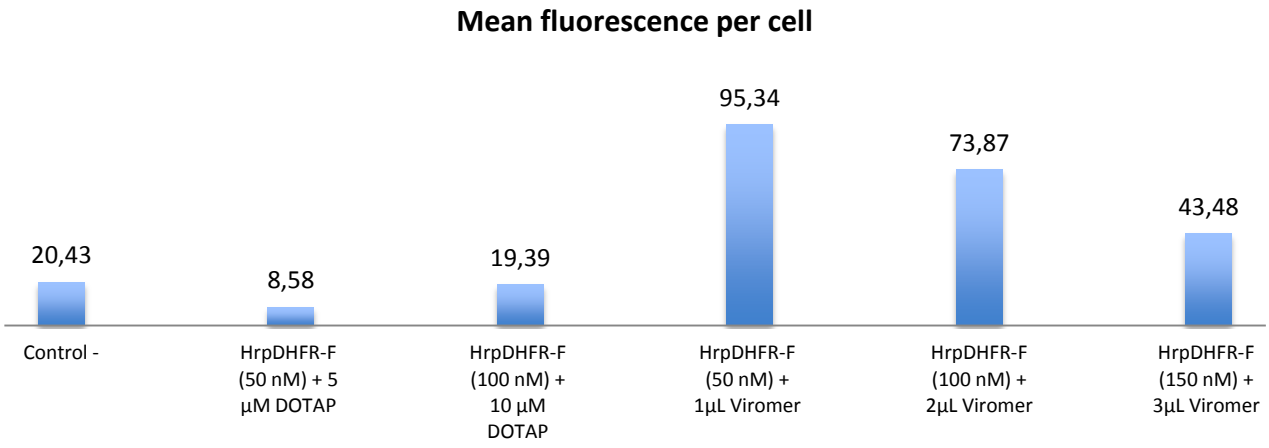


Figure 15: Mean fluorescence per WSU cell incubated with a Fluorescent Hairpin (DHFR) using DOTAP or Viromer Green as vehicle.

The samples treated with 100 nM PPRH with 10µM DOTAP and 50nM PPRH with 1µL Viromer were the ones with higher values of fluorescence in relation to total living cells (Figure 14). However, the transfection with 1µL of Viromer green showed the best results in relation to the mean fluorescence per cell (Figure 15). This meant that Viromer green transfected WSU cells better than DOTAP.

Despite the fact that Viromer Green was a better agent for transfection, it was extremely toxic for WSU cells. For this reason DOTAP was chosen for the following experiments even though it was not the best vehicle.

f) Effects of Bendamustine

40.000 and 30.000 WSU cells were incubated with Bendamustine during 48h and 72h respectively. Bendamustine is an alkylating agent currently used as a single agent or in combination with other anti-cancer agents for indolent non-Hodgkin lymphoma's treatment (29). It was observed that Bendamustine killed lymphoma cells in both assays and its effect was dose dependent.

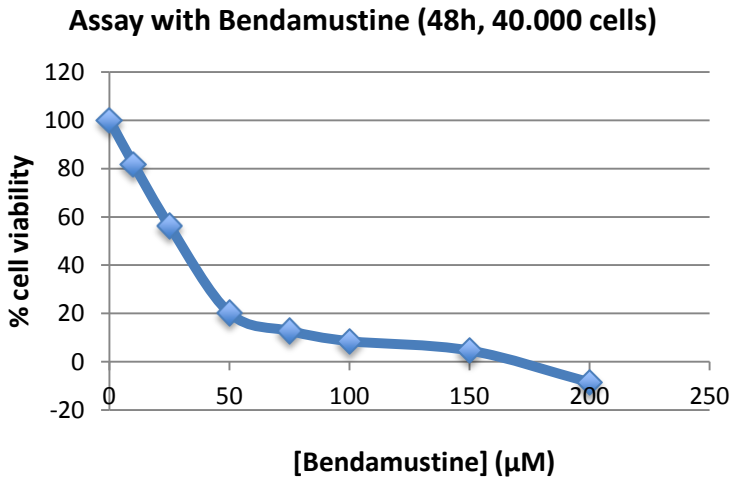


Figure 16: Cell viability of WSU cells treated with increased concentrations of Bendamustine (0, 10, 25, 50, 75, 100, 150, 200 µM)

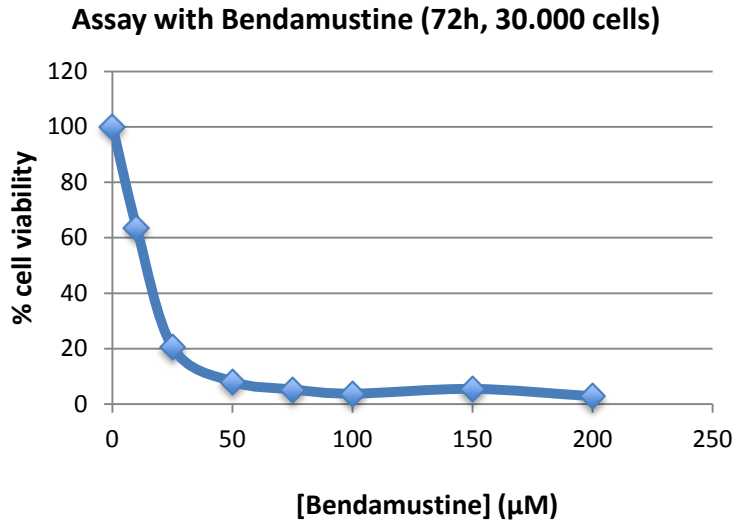
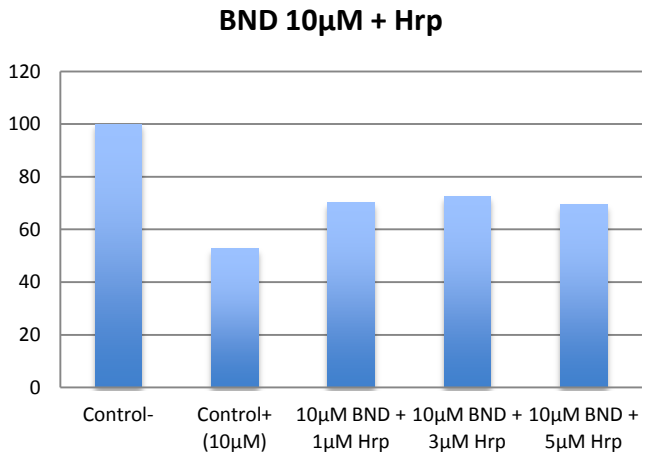
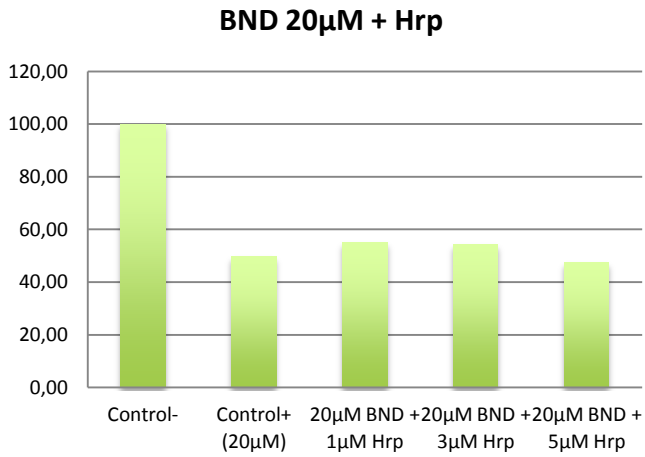


Figure 17: Cell viability of WSU cells treated with increased concentrations of Bendamustine (0, 10, 25, 50, 75, 100, 150, 200 µM)

g) Effects of the combination of Bendamustine with naked PPRHs against PD-L1

20.000 cells were incubated for 72h and 30.000 cells for 96 hours with Bendamustine and naked PPRHs. The experiment was carried out with three different Bendamustine concentrations (10µM, 20µM and 30µM). For each concentration we performed a negative control, a positive control and three incubations with 1µM, 2µM and 3µM of Hairpin.





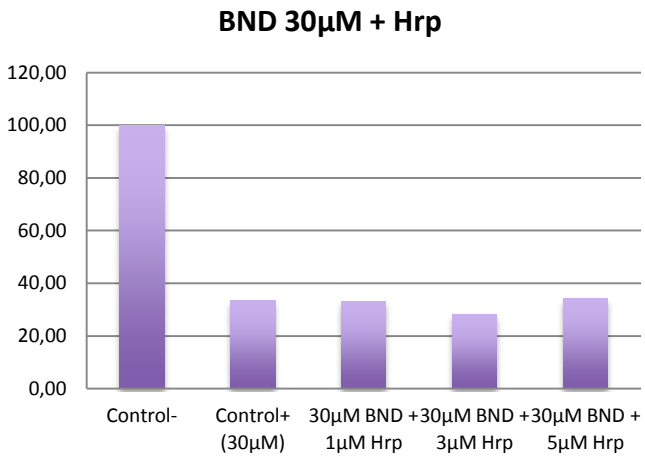


Figure 18, 19, 20: Cell viability of WSU cells treated with Bendamustine (10µM, 20µM and 30µM) and naked HrpPD-L1.

As shown in the results above (Figure 18, 19, 20), there was no significant difference in cell viability between cells treated with Bendamustine and cells treated with Bendamustine and the Hairpin. PPRHs transfection without vehicle was not effective.

7.2 PD-L1 levels

Cell lines	Ct specific	Ct endogenous	_Ct	__Ct	2^(-__Ct)	%
PC3	30,56	24,35	6,21	0	1,00	100,00
PC3	30,59	24,01	6,58	0,37	0,77	77,38
M2	32,62	23,82	8,8	2,59	0,17	16,61
M2	32,84	24,21	8,63	2,42	0,19	18,69
WSU	37,31	24,52	12,79	6,58	0,01	1,05
WSU	36,45	24,33	12,12	5,91	0,02	1,66

Table 3: Real Time PCR values of the amount of PD-L1 gene in PC3, M2 and WSU cell lines.

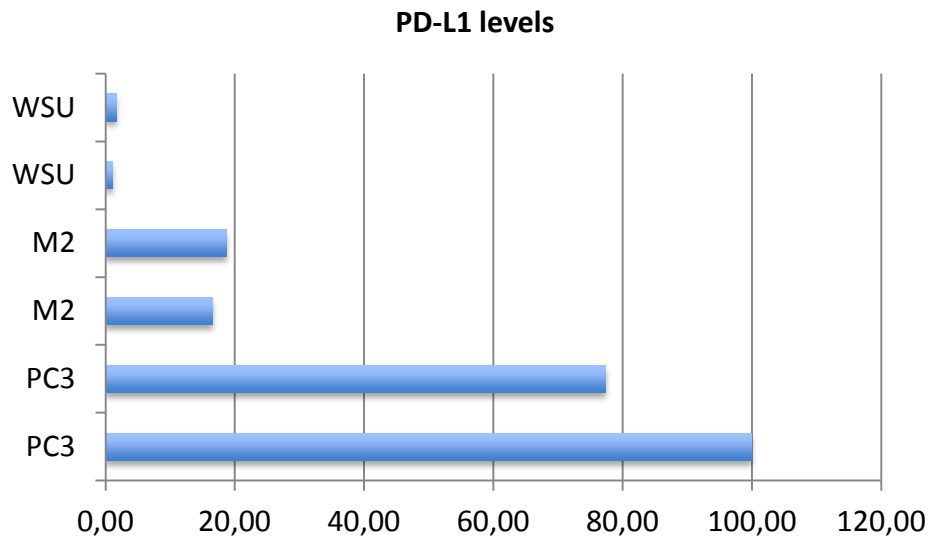


Figure 21: PD-L1 levels in PC3, M2 and WSU cell lines.

PC3 cell line was taken as reference, that's why its PD-L1 values represent the 100%. The results of the RT-PCR analyses reported that PC3 cell line overexpress PD-L1 more than MiaPaca-2 or WSU (Figure 21). PD-L1 levels in WSU cells were almost negligible.

7.3 Discussion

As we have seen, non-Hodgkin lymphomas are a type of cancer with high prevalence and with a high mortality rate in Spain. That's why every day, the research of new therapeutic alternatives to treat NHL, keeps going. Until now, chemotherapy (CHOP) combined with Rituximab (R-CHOP) has considerably increased the survival rate of these patients. However, there are lots of therapeutic possibilities under research that could improve even more the regression of lymphomas. Thanks to the current knowledge of tumour's molecular biology, lymphomas subtypes are accurately classified today. Thus a better treatment adaptation based on each lymphoma type, its aggressiveness and stage can be designed. Furthermore, this progress has lead to the development of new-targeted therapies that could complement or strengthen chemotherapy regimens. Targeted therapies such as monoclonal antibodies or gene therapy produce less systemic effects and reduce the tumour progression.

Nowadays Biotechnology and Bioinformatics are promoting the development of personalized treatments for cancer patients. Using an adapted medication regimen for each tumour we can optimize the lymphoma treatments. Thus, a good tumour diagnosis and classification is the key to decide which one of the targeted or classical treatments best fits each tumour.

Cancer immunotherapy can be a very good therapeutic alternative to be combined with chemotherapy and targeted therapies. Differently from other anti cancer therapies, immunotherapy is based on providing to our body the necessary tools to fight against the tumour. With this, our immune system can get adapted to the tumour changes and destroy all the tumour-spread cells as if they were a foreign antigen. The potential is very promising. Could one imagine a better treatment option than stimulating the patient's immune system to fight itself against cancer?

Immunotherapy opens therefore, a new great opportunity for cancer treatment. It is currently showing good results for solid tumours, whether it could also be a solution for lymphomas and leukaemia still remains unknown. Theoretically, it should be a good option for lymphomas as they are always widely scattered and they affect to the immune system itself.

Our results show that PPRHs application for WSU cells was quite difficult to carry out. WSU cells are very sensitive and really difficult to transfect. As these cells were really difficult to transfect, we couldn't quantify PPRHs transfection against PD-L1 effect for WSU. Therefore, we don't know if PPRHs against PD-L1 would have been effective for these cells. The first problem was that none of the tested vehicles were useful for these cells, as they resulted to be too cytotoxic. It would be interesting to consider the use of other delivery methods such as nanoparticles, biopolymers as alginate or protamine or electroporation. As far as I'm concerned, with the appropriate vehicle these cells could have been well transfected and PD-L1 could have been silenced. It is already proved that PPRHs work well in other cell lines such as breast cancer or pancreas cancer. That's why I strongly believe that this therapy for lymphomas could

have worked and it would have reduced cell viability in combination with Bendamustine. Unfortunately this cell line had this extra complication.

Apart from that, PD-L1 levels in WSU cells were almost negligible. This meant that targeting PD-L1 in this cell line was difficult to perform. PD-L1 is probably not the best target option in this type of cells. However, the fact that there is little PD-L1 does not have to be negative because it would be easier to reduce their levels to zero.

We have studied all the effects for WSU cells but we could not generalize to all the lymphoma types because as we have seen PD-L1 expression can vary a lot from one type to another. I consider that PD-L1 levels should be quantified before deciding the therapeutic strategy.

Under my point of view decreasing PD-L1 expression with PPRHs of a lymphoma would avoid the immune system evasion by the tumours. If we avoid the overexpression of PD-L1 from the beginning in lymphomas, our immune system would be able to recognise and kill them. This would obviously be a way of enhancing our immune system as with the monoclonal antibodies.

8 Conclusions

- Cancer immunotherapy opens a great opportunity for cancer treatment by stimulating the immune system to fight itself against tumour cells.

- PD-L1 is a potential target in cancer research. Preventing PD-L1 from binding to its receptors may prevent T-cell suppression.

- Gene therapy allows for an incredible diversity of cancer treatment options according to the patient and the type of tumour.

- The combination of targeted therapies such as immunotherapy or gene therapy with chemotherapy would improve lymphoma's patients survival rate.

- Transfection of Polypurine Revers Hoogsteen Hairpins against PD-L1 with the liposomes DOTAP or Viromer Green was too toxic for WSU cells. That's why another delivery method such as nanoparticles or biopolymers needs to be tested.

- Gene silencing with PPRHs seems to be an *in vitro* great approach to reproduce cancer immunotherapy with monoclonal antibodies. Avoiding PD-L1 overexpression from the start would reduce the cancer's immune system evasion.

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10 Annex

Annex I

THE REVISED EUROPEAN AMERICAN LYMPHOMA CLASSIFICATION (REAL)

a) Non-Hodgkin Lymphoma

- Precursor B-cell neoplasm:
  - Precursor B-lymphoblastic leukemia/lymphoma

- Mature (peripheral) B-cell neoplasms
  - B-cell chronic lymphocytic leukemia / small lymphocytic lymphoma
  - B-cell prolymphocytic leukemia
  - Lymphoplasmacytic lymphoma
  - Splenic marginal zone B-cell lymphoma (+/- villous lymphocytes)
  - Hairy cell leuekmia
  - Plasma cell myeloma/plasmacytoma
  - Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type
  - Nodal marginal zone lymphoma (+/- monocytoid B-cells)
  - Follicle center lymphoma, follicular,
  - Mantle cell lymphoma
  - Diffuse large cell B-cell lymphoma
    - Mediastinal large B-cell lymphoma
    - Primary effusion lymphoma
  - Burkitt's lymphoma/Burkitt's cell leukemia

- Precursor T cell neoplasm:
  - Precursor T-lymphoblastic lymphoma/leukemia

- Mature (peripheral) T cell and NK-cell neoplasms
  - T cell prolymphocytic leukemia
  - T-cell granular lymphocytic leukemia
  - Aggressive NK-Cell leukemia
  - Adult T cell lymphoma/leukemia (HTLV1+)
  - Extranodal NK/T-cell lymphoma, nasal type
  - Enteropathy-type T-cell lymphoma
  - Hepatosplenic gamma-delta T-cell lymphoma
  - Subcutaneous panniculitis-like T-cell lymphoma
  - Mycosis fungoides/Sézary's syndrome
  - Anaplastic large cell lymphoma, T/null cell, primary cutaneous type

- Peripheral T cell lymphoma, not otherwise characterized
- Angioimmunoblastic T cell lymphoma
- Anaplastic large cell lymphoma, T/null cell, primary systemic type

b) Hodgkin lymphoma

- Nodular lymphocyte predominance Hodgkin's lymphoma
- Classical Hodgkin's lymphoma
    - Nodular sclerosis Hodgkin's lymphoma
    - Lymphocyte-rich classical Hodgkin's lymphoma
    - Mixed cellularity Hodgkin's lymphoma
    - Lymphocyte depletion Hodgkin's lymphoma

Annex II

Triplex-Forming Oligonucleotide Target Sequence Search
The University of Texas MD Anderson Cancer Center

About | Search | Results

Gene information

Species	Gene Name	Entrez Gene ID	Alternate Symbols	Description	Chromosome	Transcription Direction
Hs	CD274	29126	B7-H, B7H1, PD-L1, PDCD1L1, PDCD1LG1, PDL1	CD274 molecule	9	forward

Transcript information[1]

Transcript (link is to NCBI GenBank)	Click the Search link below to use region data from a different transcript	Transcript Length	Location on Chromosome
NM_001267706.1	region data[1] from this transcript were used in the search	20065	5450503-5470567
NM_014143.3	search	20065	5450503-5470567
NR_052005.1	search	20065	5450503-5470567

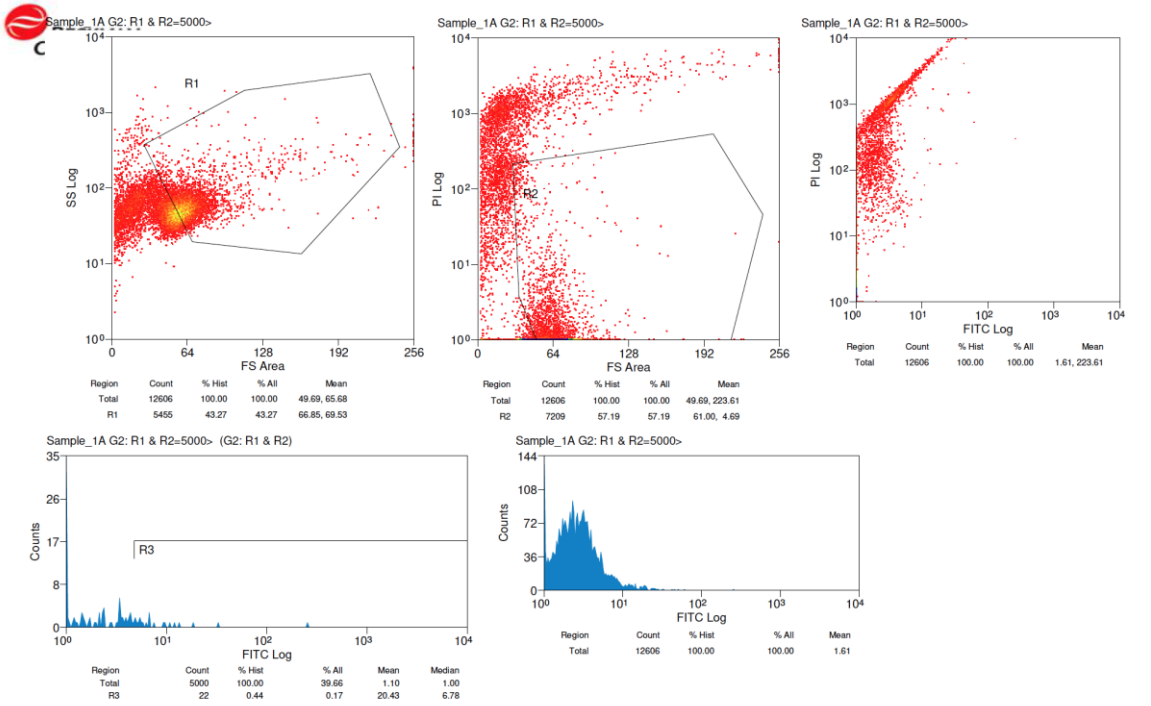
TFO Target Sequence Search Parameters and Results

Minimum Length	Maximum Length	Minimum %G	Allowable Number of Pyrimidines	Putative Promoter Region Length	Downstream Region Length	Number of TFO Target Sequences Found	Mask for repeat sequences prior to searching
15	no limit	50	3	2000	0	41	Yes

TFO target sequences

TFO Target Sequence[2]	Length	% G	Strand	Start[3] in Chromosome	Start[4] in Gene	Region[4]	BLAST[5]	Repeat Sequence[6]	Overlapping Gene[7]	PubMed ID[8,9]
GGGATGGAGAGAGGAGAAGGGAAAGGGAACGCGA	34	52.9	forward	5450693	191	intron_1	BLAST			
AACAGTGGGGAGTGGGAAGGGGAGAGAG	28	57.1	reverse	5455505	5003	intron_1	BLAST			
AAGGGGTGAGGGGCAGGAGAATGGG	25	60.0	forward	5465779	15277	intron_4	BLAST			
GGGGGTGAGGAAGGGGTGAGAA	22	63.6	forward	5465639	15137	intron_4	BLAST			
AGGAGCAGGGAAGGGGAAGGAG	22	59.1	forward	5466996	16494	intron_5	BLAST			
AGTGGTGAAGGGAGGAGGGACA	22	54.5	reverse	5457783	7281	intron_2	BLAST			
GACAGATAGGAGGAGGTGGGAG	22	54.5	forward	5464686	14184	intron_3	BLAST			
GGGAAGTAGAATATGGAAGGGG	22	50.0	forward	5466217	15715	intron_4	BLAST			
GGGGGAGGGGGCAGTGAGCAG	21	66.7	reverse	5464006	13504	intron_3	BLAST			
GTGAGAGGGGCAGAAGAGGAG	21	57.1	forward	5455554	5052	intron_1	BLAST			
AGGGAGTGTGTAGGGAAGGGA	21	57.1	forward	5459023	8521	intron_2	BLAST			
GGGTAGGAGGATGAGATAGAG	21	52.4	reverse	5452349	1847	intron_1	BLAST			
AGAAGTAGGTAGGGTGGGAGA	21	52.4	forward	5463876	13374	intron_3	BLAST			
GGATGAATGGAGGTGAGGAA	20	50.0	reverse	5463282	12780	intron_3	BLAST			
GGGCAGAGGTGGGCGGGA	18	66.7	forward	5450469	-33	putative-promoter	BLAST			
AAGGGTGAGAGGTGGGAG	18	61.1	forward	5464510	14008	intron_3	BLAST			
ATGCAGGAGGAAAGGGGG	18	55.6	reverse	5464578	14076	intron_3	BLAST			
AGAGGGAGAACAGGTAGG	18	50.0	reverse	5454344	3842	intron_1	BLAST			
GAAAGTGAGGCGAGTGAG	18	50.0	forward	5463928	13426	intron_3	BLAST			
GTGAACAGAAAGAGGGGG	18	50.0	forward	5464107	13605	intron_3	BLAST			
GAGTGGAGGCAAAGGGCA	18	50.0	reverse	5468243	17741	exon_6	BLAST			
GGGATGGAAAGGAAGAG	17	52.9	forward	5458929	8427	intron_2	BLAST			
GGAGGATGAAGTGGAGA	17	52.9	reverse	5469384	18882	exon_6	BLAST			
GGGGAAGAAGTGATGG	16	56.2	reverse	5452459	1957	intron_1	BLAST			
AAGAGGAATGGAGGAG	16	50.0	forward	5451096	594	intron_1	BLAST			
AGTAGGTAGGCAGAGG	16	50.0	forward	5451623	1121	intron_1	BLAST			
GTGAGGGACGAAAAGG	16	50.0	reverse	5452645	2143	intron_1	BLAST			
GTGCATGGAGAGGAAG	16	50.0	forward	5457228	6726	intron_2	BLAST			
GTGATGGGAAGTGAAG	16	50.0	reverse	5459523	9021	intron_2	BLAST			
GGAAAGAGGGAAGCAG	16	50.0	reverse	5463568	13066	intron_3	BLAST			
GGGTGAAGATGAAGCG	16	50.0	forward	5465762	15260	intron_4	BLAST			
ATGGGGGAAAGTAGTG	16	50.0	forward	5466278	15776	intron_4	BLAST			

Annex III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FS Area
Region Count % Hist % All Mean
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R2
PI Log
FS Area
Region Count % Hist % All Mean
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R2 7209 57.19 57.19 61.00, 4.69
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PI Log
FITC Log
Region Count % Hist % All Mean
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R3
Counts
FITC Log
Region Count % Hist % All Mean Median
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R3 22 0.44 0.17 20.43 6.78
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FITC Log
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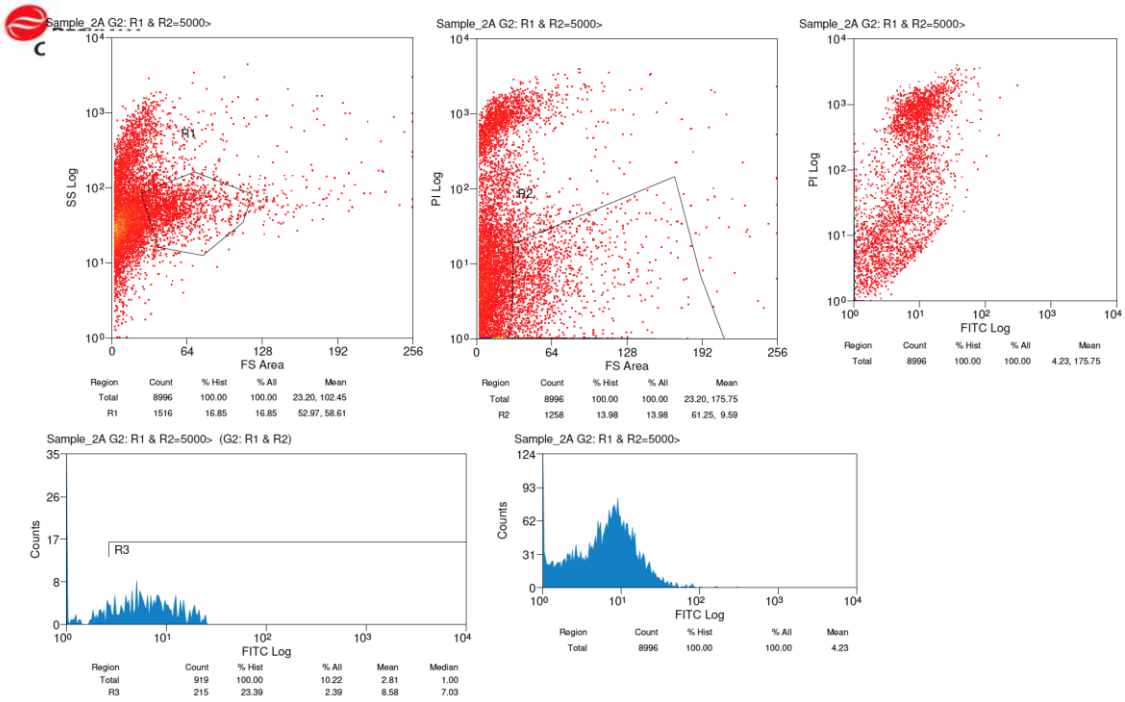
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FS Area
Region Count % Hist % All Mean
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Sample_2A G2: R1 & R2=5000>
R2
PI Log
FS Area
Region Count % Hist % All Mean
Total 8996 100.00 100.00 23.20, 175.75
R2 1258 13.98 13.98 61.25, 9.59
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PI Log
FITC Log
Region Count % Hist % All Mean
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Sample_2A G2: R1 & R2=5000> (G2: R1 & R2)
R3
Counts
FITC Log
Region Count % Hist % All Mean Median
Total 919 100.00 10.22 2.81 1.00
R3 215 23.39 2.39 8.58 7.03
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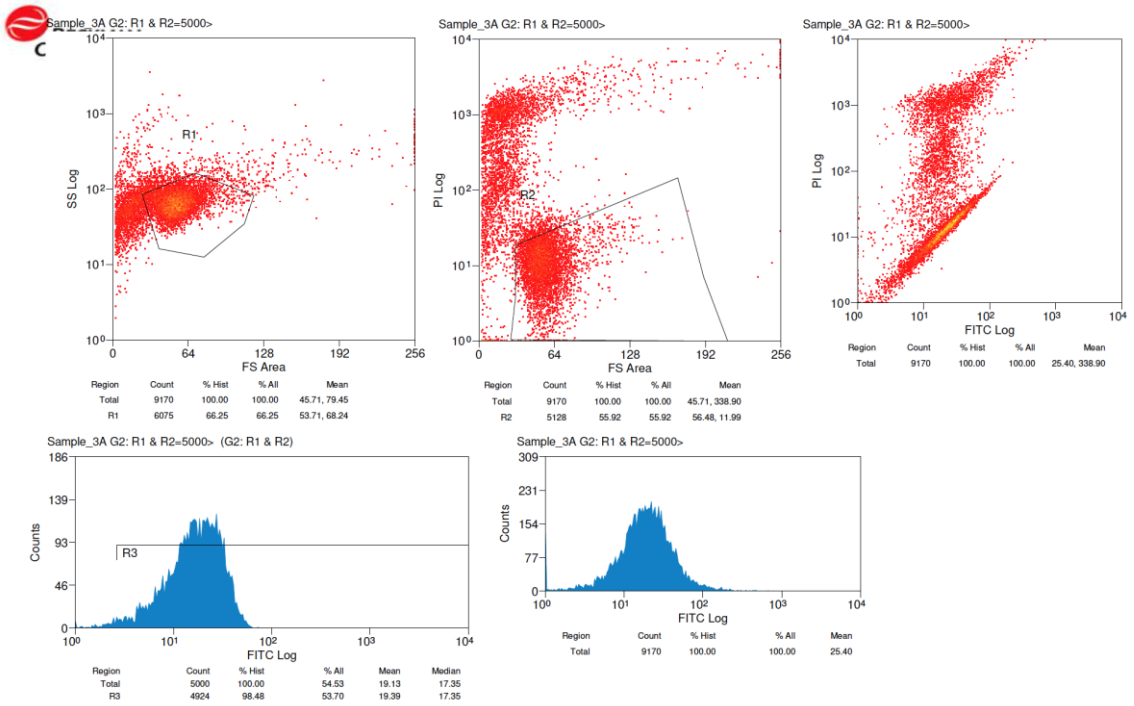
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FS Area
R1
Region Count % Hist % All Mean
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R1 6075 66.25 66.25 53.71, 68.24
Sample_3A G2: R1 & R2=5000>
PI Log
FS Area
R2
Region Count % Hist % All Mean
Total 9170 100.00 100.00 45.71, 338.90
R2 5128 55.92 55.92 56.48, 11.99
Sample_3A G2: R1 & R2=5000>
PI Log
FITC Log
Region Count % Hist % All Mean
Total 9170 100.00 100.00 25.40, 338.90
Sample_3A G2: R1 & R2=5000> (G2: R1 & R2)
Counts
FITC Log
R3
Region Count % Hist % All Mean Median
Total 5000 100.00 54.53 19.13 17.35
R3 4924 98.48 53.70 19.39 17.35
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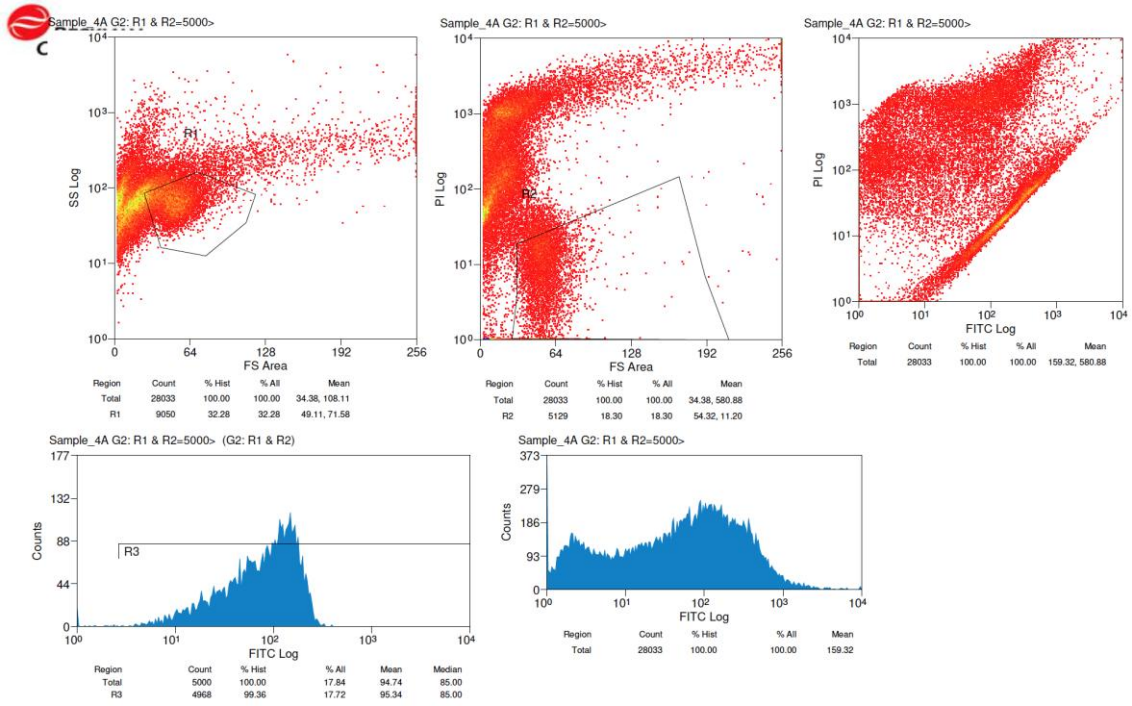
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FS Area
R1
Region Count % Hist % All Mean
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R1 9050 32.28 32.28 49.11, 71.58
Sample_4A G2: R1 & R2=5000>
PI Log
FS Area
R2
Region Count % Hist % All Mean
Total 28033 100.00 100.00 34.38, 580.88
R2 5129 18.30 18.30 54.32, 11.20
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PI Log
FITC Log
Region Count % Hist % All Mean
Total 28033 100.00 100.00 159.32, 580.88
Sample_4A G2: R1 & R2=5000> (G2: R1 & R2)
Counts
FITC Log
R3
Region Count % Hist % All Mean Median
Total 5000 100.00 17.84 94.74 85.00
R3 4968 99.36 17.72 95.34 85.00
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Counts
FITC Log
Region Count % Hist % All Mean
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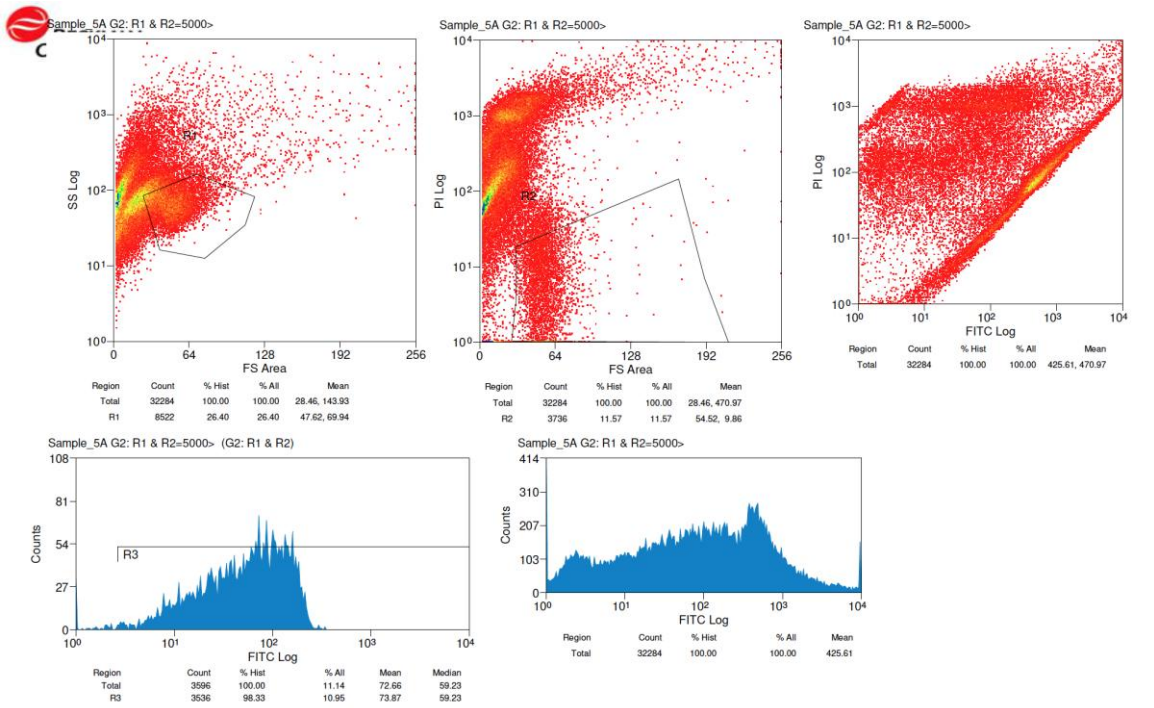
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FS Area
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Region Count % Hist % All Mean
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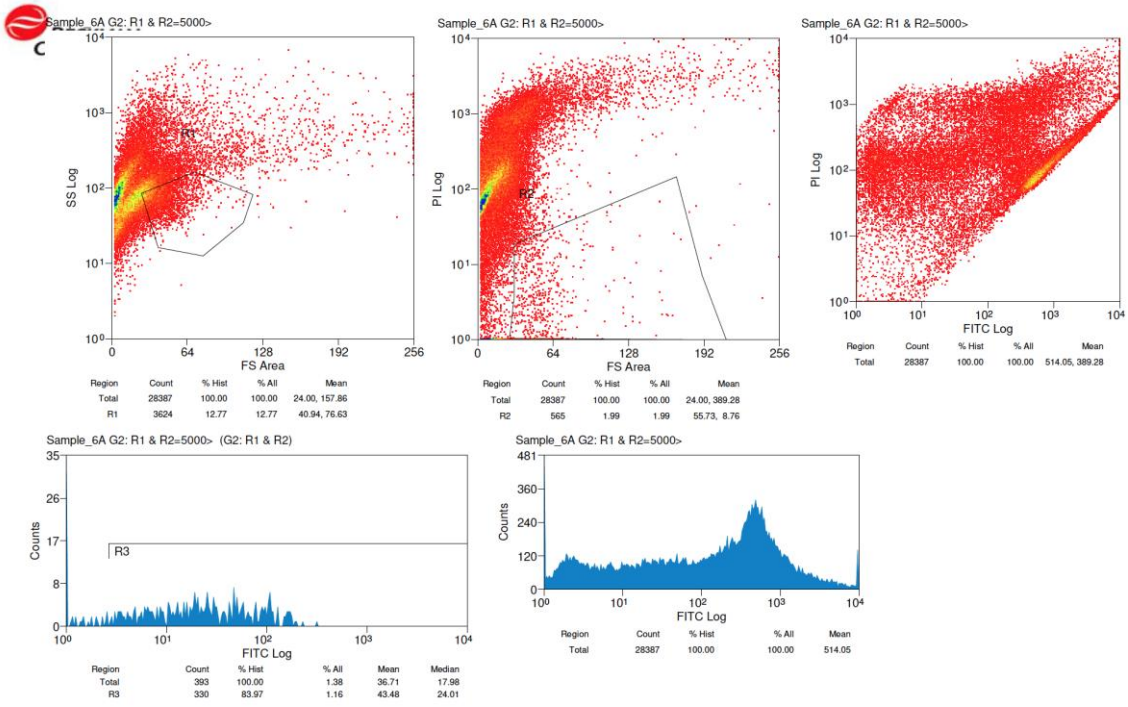
R1 8522 26.40 26.40 47.62, 69.94
Sample_5A G2: R1 & R2=5000>
PI Log
FS Area
R2
Region Count % Hist % All Mean
Total 32284 100.00 100.00 28.46, 470.97
R2 3736 11.57 11.57 54.52, 9.86
Sample_5A G2: R1 & R2=5000>
PI Log
FITC Log
Region Count % Hist % All Mean
Total 32284 100.00 100.00 425.61, 470.97
Sample_5A G2: R1 & R2=5000> (G2: R1 & R2)
Counts
R3
FITC Log
Region Count % Hist % All Mean Median
Total 3596 100.00 11.14 72.66 59.23
R3 3536 98.33 10.95 73.87 59.23
Sample_5A G2: R1 & R2=5000>
Counts
FITC Log
Region Count % Hist % All Mean
Total 32284 100.00 100.00 425.61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FS Area
R1
Region Count % Hist % All Mean
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R1 3624 12.77 12.77 40.94, 76.63
Sample_6A G2: R1 & R2=5000>
PI Log
FS Area
R2
Region Count % Hist % All Mean
Total 28387 100.00 100.00 24.00, 389.28
R2 565 1.99 1.99 55.73, 8.76
Sample_6A G2: R1 & R2=5000>
PI Log
FITC Log
Region Count % Hist % All Mean
Total 28387 100.00 100.00 514.05, 389.28
Sample_6A G2: R1 & R2=5000> (G2: R1 & R2)
Counts
R3
FITC Log
Region Count % Hist % All Mean Median
Total 393 100.00 1.38 36.71 17.98
R3 330 83.97 1.16 43.48 24.01
Sample_6A G2: R1 & R2=5000>
Counts
FITC Log
Region Count % Hist % All Mean
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