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Epic Battles in Myeloma: Deciphering the Role of PVR in the Fight Between Tumors and Cytotoxic Cells

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Epic Battles in Myeloma: Deciphering the Role of PVR in the Fight Between Tumors and Cytotoxic Cells

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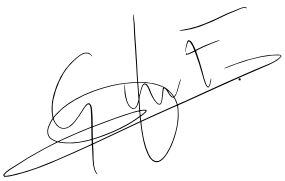

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Para los que están y siempre estuvieron,

para los que estuvieron y no pueden estar,

para los que estuvieron y ya no están.

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Abbreviations

A

AML	Acute Myeloid Leukemia
ASCT	Autologous Stem Cell Transplantation

B

B-ALL	B-cell Acute Lymphoblastic Leukemia
BCMA	B-cell Maturation Antigen (also known as TNFRSF17)
BCR	B-cell Receptor
BM	Bone Marrow
BSP	Bisulfite Sequencing PCR

C

CAR	Chimeric Antigen Receptor
CCND1	Cyclin D1
CCND3	Cyclin D3
CLL	Chronic Lymphoblastic Leukemia
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukemia
CMP	Common Myeloid Progenitor
CpG	CG site/Cytosine followed by a Guanine
CRAB	MM defining clinic symptoms (HyperCalcemia, Renal failure, Anemia and Bone disease)
CRS	Cytokine Release Syndrome
CSR	Class Switch Recombination
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4

D

DAMPs	Damage Associated Molecular Patterns
DAPK	Death-Associated Protein Kinase 1
DEC	Decitabine (5-aza-2'-desoxicitidine)
DNA	Deoxyribonucleic Acid
DNAM1	DNAX Accessory Molecule-1
DNMT	DNA Methyltransferase

E

E:T	Effector:Target ratio
EBF1	Early B-cell Factor 1
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELK.1	ETS-Like Gene 1

F

FBS	Fetal Bovine Serum
FGFR	Fibroblast Growth Factor Receptor

G

GATA-3	GATA Binding Protein 3
GC	Germinal Center
GMP	Granulocyte-Monocyte Progenitor
GZMB	Granzyme B

H

HC	Heavy Chain (Immunoglobulin)
HSC	Hematological Stem Cell

I

ICANS	Immune effector Cell-Associated Neurotoxicity Syndrome
IFNG	Interferon gamma
IGF-1	Insulin Like Growth Factor 1
IMDM	Iscove's Modified Dulbecco's Medium
IMiDS	Immunomodulatory Drugs
IRAE	Immune Related Adverse Effects

L

LAG3	Lymphocyte Activating 3
LC	Light Chain (Immunoglobulin)
LPMPP	Lymphoid Primed Multi-Potential Progenitor
LT-HSC	Long Term HSC

M

MDS	Myelodysplastic Syndrome
MEP	Megakaryocyte-Erythrocyte Progenitor
MGUS	Monoclonal Gammopathy of Undetermined Significance
MHC	Major Histocompatibility Complex
MIRNA	Micro RNA
MM	Multiple Myeloma
MMSET	MM Set domaining protein (also known NSD2)
MPN	Myeloproliferative Neoplasm
MPP	Multi-Potent Progenitor
MSC	Mesenchymal Stromal Cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZ	Marginal Zone

N

NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NOTCH1	Neurogenic Locus notch Homolog Protein 1
NPAT	Nuclear Protein, Coactivator Of Histone Transcription

O

OS	Overall Survival

P

PAX5	Paired Box 5
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
PI	Propidium Iodide
PVR	Poliovirus Receptor
PVRL2	Poliovirus Receptor-Related Protein 2

R

RNA	Ribonucleic Acid
RNA-SEQ	RNA sequencing
RT	Retro Transcription

S

S1P	Sphingosine-1-Phosphate
S1PR	Sphingosine-1-Phosphate Receptor
SCF	Stem Cell Factor
SHM	Somatic Hypermutation
SMM	Smoldering Multiple Myeloma
SOCS1	Suppressor of Cytokine Signaling 1
ST-HSC	Short Term HSC

T

T-ALL	T-cell Acute Lymphoblastic Leukemia
TCR	T-cell Receptor
TET	Ten-Eleven Translocation
TIGIT	T Cell Immunoreceptor with Ig and ITIM Domains
TIL	Tumor Infiltrating Lymphocyte
TIM3	T Cell Immunoglobulin Mucin 3
TSS	Transcription Strating Site

V

VISTA	V-Domain Ig Suppressor of T Cell Activation

W

WT	Wild Type

Introduction

Introduction

Hematopoiesis: how the blood is born

Before we start talking about this project, first we need to know the background and where the characters of this (epic) story are located. Since this thesis circles around blood biology and hematological malignancies, we will start by talking about how the blood cells are generated, the hematopoiesis. Then, we will introduce the different names that a blood cancer can receive, and finally, we will present the antagonist of the story and describe its characteristics.

Welcome to my thesis, I hope you enjoy the journey.

The origin of everything

Blood is one of the most important organs in our bodies. It distributes oxygen and nutrients through all our organism and gives origin to our active defense system. Blood is originated almost in its entirety from a multipotent cell commonly known as Hematopoietic Stem Cell (HSC). Although the system of how blood is formed has been heavily studied and the whole process is well described, we still use a model to describe it[1,2], you will soon understand why.

HSCs are cells with the capacity to self-renew and differentiate into any type of blood cell, they reside in the Bone Marrow (BM) inside the biggest bones of the body (i.e., femur, sternum, or hips). In a healthy BM, HSCs can find all the nutrients and the correct signals (cytokines and chemokines produced by Mesenchymal-Stroma Cells or MSCs) to remain there or to start the differentiation process[3]. Due to the topic of this thesis project, we will not go into how the balance is maintained, but it is important to mention that losing the balance may originate a disease, such as cancer.

HSCs can differentiate into several precursors which will give rise to more specific cell types. With each step down they take on the stair of differentiation, the cells lose their stemness and in most cases their capacity to self-renew[1]. As we said, the balance in the number of cells for each lineage is of high importance, as the work of the whole system depends on it[4]. The higher number of produced cells are, as you may imagine, erythrocytes, commonly referred to as red blood cells. They are 95% of the cells in the blood, have a short lifespan, and are in high demand due to their nutrient and oxygen transportation function, so the system cannot allow to sacrifice their production. And what about the rest? Well, mainly they are platelets, small cell fragments that take care of blood clotting upon vascular damage (around 5% of the cells in the blood). And finally, in the smallest percentage (less than 1%), leukocytes (commonly known as white blood cells). Leukocytes constitute a complex system that carries in its back the security of our body.

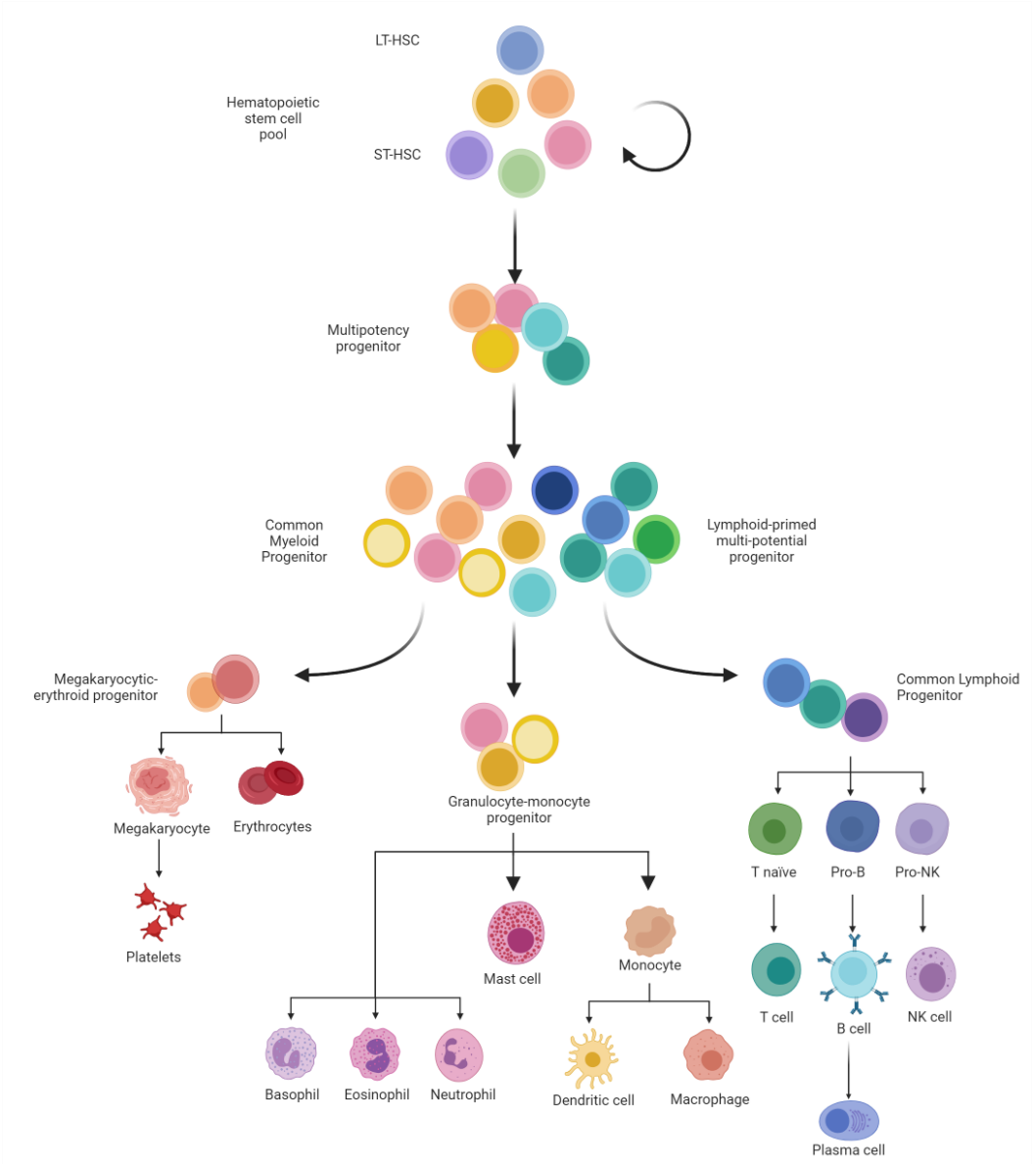


Figure 1: Hematopoiesis model combining the continuous and static models. Hematopoietic Stem Cells (HSC) are believed to be a pool of stem cells, much of which are dormant and considered Long-Term-HSC (LT-HSC). Some of these cells will start the differentiation machinery and be considered Short-Term-HSC (ST-HSC). After this stage, when the differentiation and division of the cells starts the pool of cells will go down until they become mature cells. Colors represent the theory that since the first stages of differentiations, HSCs are primed towards a certain lineage.

Following **Figure 1**, we will briefly describe one of the current models of hematopoiesis[1–3]. We are used to representing HSCs as a single population, but current technologies have allowed us to define HSCs as a heterogeneous group of cells, some deep into resting phase Long-Term HSCs (LT-HSCs), and others ready to exit this sleep state and start the division process (Short-Term HSCs or ST-HSC). What is described is that from a ST-HSC a multipotent progenitor will appear. This Multipotent Progenitor (MPP) will have much more restricted division capacity and lose the self-renewal capacity of HSCs. Further down, this cell will divide into a Common Myeloid Progenitor (CMP) or a Lymphoid-

Primed Multipotent Progenitor (LPMPP) which will give rise to the myeloid and lymphoid lineages, respectively. After this stage, the cell will enter a much more restricted division stage where it will be considered, on the myeloid side, a megakaryocyte-erythrocyte progenitor (MEP) or a granulocyte-monocyte progenitor (GMP). From these cells, the myeloid mature cells will be formed, including erythrocytes, megakaryocytes (which will break into platelets), granulocytes, monocytes, and dendritic cells. Myeloid cells constitute both the structural and the more innate part of the immune system. On the other side, common lymphoid progenitors (CLP) will give rise to the lymphocytes, T, B, and Natural Killer (NK) cells (and according to some researchers, dendritic cells too). The lymphocytes are part of the more specialized immune response, the so-called, adaptive response which we will talk about a little bit down the section. Keep an eye on our lymphocytes, as they are the villains and the heroes in this story.

As a side note, the current hierarchical model of hematopoiesis is not as rigid as shown here. For the sake of clarity, we will keep this representation for this project, but nowadays, most scientists have accepted hematopoiesis to be a dynamic entity[3]. Furthermore, it says when HSCs leave their inactive state cells have already a set destiny, a bias towards the final stage which controls the fate of that cell and the decisions it will take to reach it. Thanks to single-cell technologies, we now consider several hundreds of inter-stage cells and cells transitioning constantly, without forgetting other events that may mess up the up-to-down direction, such as trans-differentiation[5,6] and exponentially increase the complexity of deciphering how hematopoiesis actually works, which explain why we still use models to describe it.

B cell lineage

As we have established at the beginning of this section, it is important to set the basis before we engage in the main story. It is because of that we must make a stop here before continuing. Now we know where lymphocytes come from and that we have different types (B, T, and NK). To know where the hematological malignancies studied during this project originate, it is necessary to talk a little bit more about how B cells mature and differentiate. For sure most of the readers already know the big picture of B-cell maturation, but it is always good to revise concepts so you are ready to understand everything that will be discussed further down this story.

First of all, the stages of B-cell development are defined by the presence of sets of cell-surface markers (which include cytokine and chemokine receptors, and adhesion molecules), the expression of specific transcription regulators, and the rearrangement status of immunoglobulin genes. Here we will briefly mention each stage the cell goes through to finish the process and focus on the few characteristics defining each stage. I must remind the reader that as simple as these stages may be described here, biology is always much, much, more complex. B cells are the lineage responsible for creating

antibodies that will protect our bodies from infection. Since antibody formation is crucial for B-cell biology and has a deep relation with B-cell maturation, we will make a little stop here to revise the structure of a canonical antibody so all of us are able to fully understand all the stages of B-cell development. Antibodies are Y-shaped proteins that circulate through our system and participate in adaptive immunity (**Figure 2**). They are secreted by B cells and their configuration is quite characteristic. Antibodies, also known as immunoglobulins, are formed by two sets of identical chains, the heavy chain (HC) and the light chain (LC). The HC is (usually) composed of three constant domains and one variable domain, whereas the LC is formed by a single constant and a variable domain. Everything is linked together by disulfide bonds that make the structure quite stable. There are two types of LC (D and H) and five types of HC (IgA, IgD, IgE, IgG and IgM). By the combination of both, we have lots of variability and combinations that take care of specific functions.[7]

The variable region of the antibody (formed by the variable domain from the LC and the HC) is the part that takes the function of recognizing and attaching to specific antigens. The constant domain from the HC is the part recognizable by the cell in our system that will act depending on the interaction it makes. So far, the description is structural, but before continuing you may also want to know that antibodies can also be divided by their functional separation. The variable regions together with the constant region (the part that detects the antibody) are also known as the Fab region (Fragment antigen binding domain) while the constant region of the HC can also be known as the Fc region (Fragment crystallizable). The Fab is linked to the Fc region by a hinge which allows great mobility to the antigen-recognizing part and thus facilitates its function. Now that we have refreshed the antibody parts, we can go on with the B-cell maturation process.

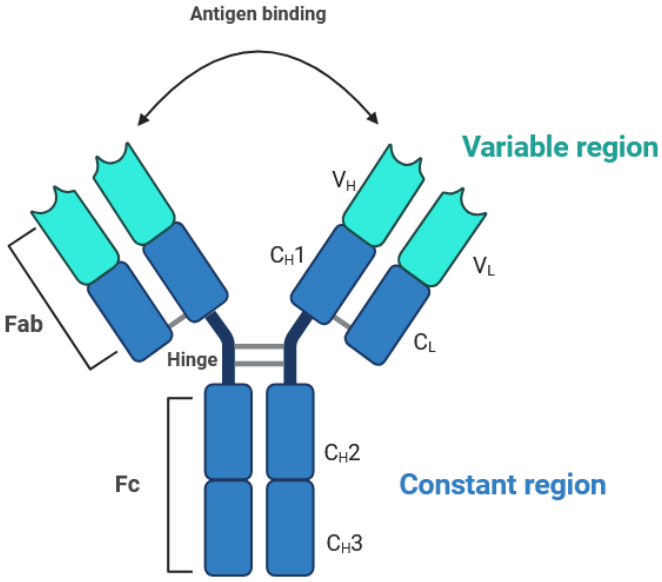


Figure 2. Antibody structure. The variable region of the antibody recognizes the antigen and binds it. Through the Fc end, immune cells can interact and act against the bound antigen.

Having said that, let's walk together through the B-cell maturation process. While you follow the explanation, in **Figure 3** you will find the representation of the stages described with the main characteristics indicated for each stage.

A given HSC residing on the BM through the expression of Stem Cell Factor (SCF), Vascular Cell Adhesion Molecule 1 (VCAM1) and CXCL4 among other receptors will interact with the BM-MSC, endothelial cells and others will maintain its self-renewal and full potential nature[8,9]. After a given signal, an ST-HSC will alter its expression and downregulate stemness factors, while Ikaros and PU.1 transcription factors will activate. This step will mark the first step towards LPMPP and CLP differentiation later. These lymphoid progenitors still hold the power to differentiate into both T and B lineages, but they lose their self-renewal capacity. CLP will further interact with MSCs in the BM, especially with the C-X-C motif chemokine 12 (CXCL12) expressing ones, and that will trigger the B-cell lineage markers to appear on their cell surface. Two main events define the transition from CLP towards the pro-/pre-B cell stage: (I) the upregulation of E2A transcription factor thanks to Ikaros and PU.1 and (II) the expression on the cell membrane of IL-7R. Pro-/pre-B cells will then be able to further interact with the BM MSCs and, through IL-7 signaling and E2A regulation, Early B cell Factor 1 (EBF1) will be expressed.[10,11] At this stage, B cell genes will start to be available for transcription, while other lineages genes, like Neurogenic locus notch homolog protein 1 (NOTCH-1) and GATA-binding protein 3(GATA-3) (T-cell genes) will be repressed without mutating the DNA (by ways that if you keep reading, you will discover soon).

EBF1 will then trigger Paired Box Protein 5 (PAX5) expression, considered one of the master transcription factors controlling B-cell maturation and differentiation. Here the cell is considered a pro-B cell. PAX5 expression will start, among other functions, to make available the expression of CD19 (a characteristic marker of B cells) and the start of the heavy chain recombination and expression on these still immature cells. Once the heavy chain rearrangement is completed, and if the resultant recombination is viable, the cell will transition into the pre-B cell stage. Pre-B cells will then proceed with further recombination and arrange possible light chain combinations and thus, pre-B-cell Receptor (BCR) markers will appear on their surface. The ability to produce a functional pre-BCR marker will determine if the cell can further proceed with the maturation process or if it is eliminated. If the cell overcomes this first B-cell checkpoint, IgMs will start being expressed on its surface. At this point, we consider that cell as an immature B cell, with a working BCR and IgM on its surface, immature B cells will be tested for self-recognition. Autoreactive cells will be eliminated, establishing the second B-cell checkpoint. Cells that are selected to go on the maturation process will exit the BM through the expression of Sphingosine-1-Phosphate Receptor (S1PR) which detects S1P in the blood. Once the cell exits the BM, it becomes a transition B cell and will travel to lymphoid tissue, usually the spleen, where it will finish the maturation process.

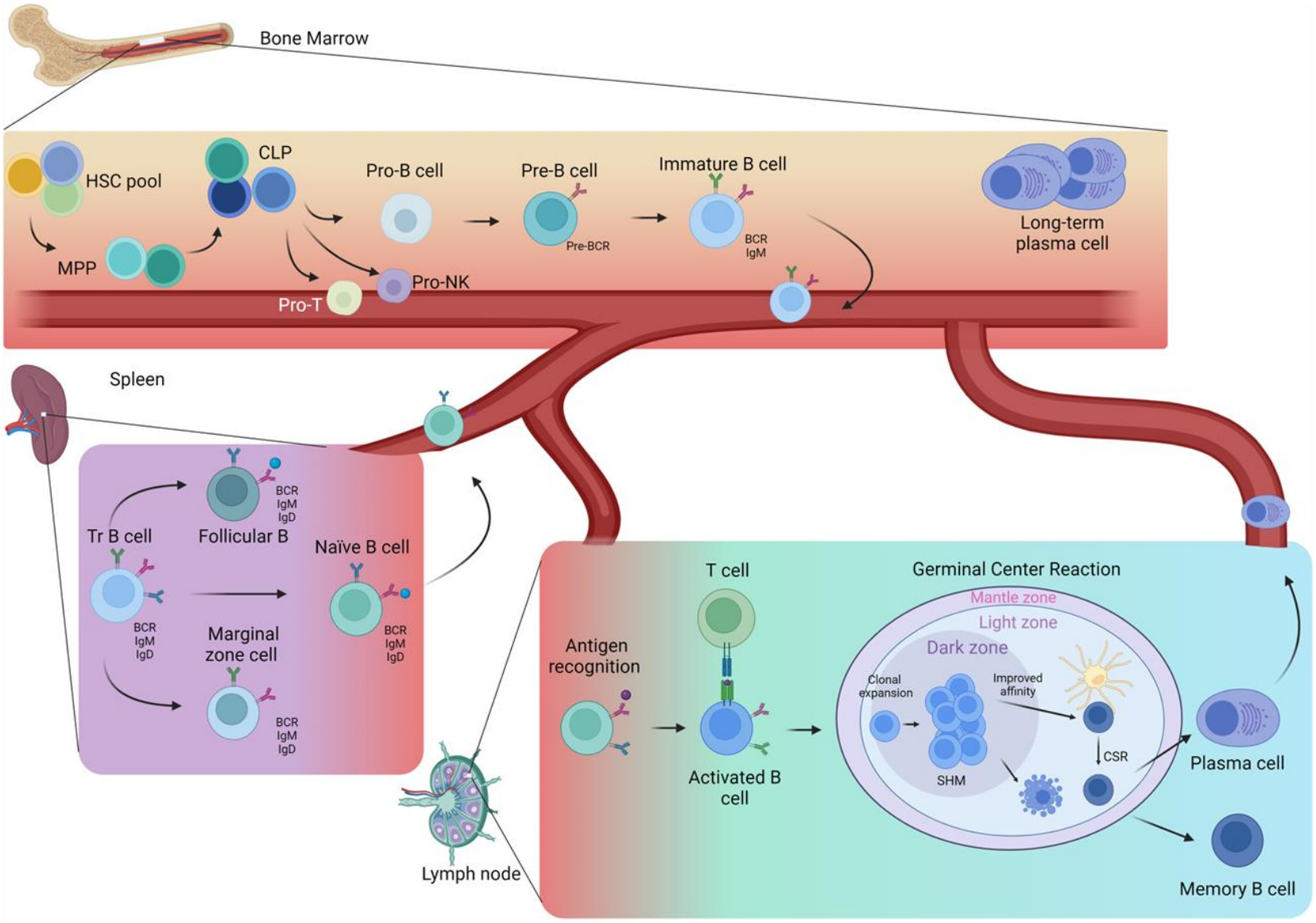


Figure 3. B-cell maturation and differentiation process. Graphic representation of the stages and the location where those stages take place. Once the Immature B cell leaves the BM it may vary the lymphoid organ where the differentiation occurs, regardless, the order of the event occurs in the same way. Remember this is an extreme simplification of the events and that the cells are in constant communication with the environment around them which determines the path to follow. Abbreviations: HSC- Hematopoietic Stem Cell, MPP- MultiPotent Progenitor, CLP-Common Lymphoid Progenitor.

Here a separation will be made, some populations will start expressing IgDs and overcome another self-recognition test while others will be established on the marginal zone of the follicles or remain on the follicles (known as marginal zone cells and follicular B cells), where their main activity will be help on the differentiation of other cells, help on building a response upon antigen presentation and produce unspecific antibodies to serve as first line of defense upon infection. From here, naïve B cells are ready to carry on their main immune function and to start their differentiation process.[10–12]

B cells will circulate through lymphatic tissues until an antigen is recognized through its BCR and the proper signals are given to its coreceptors (mainly CD21 and CD40). Antigen stimulation will trigger, among others, Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-kB), Nuclear Protein, Coactivator of Histone Transcription (NPAT), EG1 and ETS Like-1 protein Elk-1 (ELK.1) expression. The recognized antigen will be internalized and digested into small peptides which will then be presented on the cell surface through the Major Histocompatibility Complex (MHC) class II receptor together with costimulatory receptors like CD40, CD80, and CD86. The expression of the CCR7 receptor will then promote the move toward the T-cell zone in the follicle. Upon T cell encounter and interaction, IL-4 and IL-21 will be produced to trigger survival and differentiation programs on the presenting B cell.[11–13]

From here, two paths will be taken, some cells will immediately differentiate into primary plasma cells that will start producing IgM. They are short-lived and usually constitute one of the first lines of defense towards, now, a better-recognized enemy. Most of the population will retreat into the follicle and start the germinal center (GC) reaction. GC is formed by rapidly dividing B cells which will experience, thanks to the expression of Bcl-6, the two best-known events in B-cell biology: Somatic Hypermutation (SMH) and Class Switch Recombination (CSR). Through the SHM, cells will undergo affinity selection to become the perfect warrior against the impeding enemy trespassing the body's defenses. By CSR, the constant region of the heavy chain changes and becomes much more dangerous to the recognized antigen. These events are triggered thanks to the high level of recombination occurring on the antibody genes in their DNA. Only the best-specialized cells with the highest affinity will be able to exit the GC and differentiate towards the last stage of B-cell differentiation, the immunity long-term maintenance phase. Upon infection clearance, most of the cells produced here will be invited to die through apoptosis.[13,14]

A small percentage of cells will then establish the basis of immune memory. Generally, here we talk about two different types of cells: memory B cells and plasma cells. Memory B cells will enter circulation and reside in antigen drainage sites like follicles and mucosa where, upon antigen recognition will build a much faster response than naïve B cells. Another fraction of cells, through the expression of CXCR12, will be directed toward the BM where they will establish their long-term stay. Plasma cells are B cells specialized in

antibody production and are known to reside for undetermined but long periods in the BM. Inside the BM, plasma cells will be able to survive thanks to the signals sent by BM MSCs. They will produce antibodies that will be released into the bloodstream and like that, they will help keep the surveillance in the system..[11,13] Remember them, you never know from where your enemy may arise in biology. Both types (memory and plasma cells) are known to be able to persist up till the death of their host.

Leukemia, lymphoma, and myeloma

We have now learned a little bit more about the blood system and one of the most complex events taking place there but with still unexplained events in biology: B-cell maturation and differentiation. Having gone through all these events, don't you wonder, with all the recombination and changes in expression, and with the tight control that sends all default cells to die by apoptosis, is this system that perfect? Mostly, yes. But, of course, as with any other cell in our body, hematological cells are also susceptible to malignant transformation. In blood malignancies, the accumulation of errors leads to transformation. But, as you may as well know, cancer is not a single disease, and it may be one of the more complex diseases that we are facing. In the case of blood, any of the cells in the hematopoiesis tree can originate a different kind of disease (and in some particular cases, the same cell can originate several different malignancies). So, the scientific world has tried to classify hematological malignancies into big groups of similar characteristics, although the only common characteristic is the stage they originate from[15]. Depending on the cell of origin, we generally classify blood cancers as leukemia, lymphoma, or myeloma. In **Figure 4** you will see at which stage of B-cell differentiation each malignancy originates from.

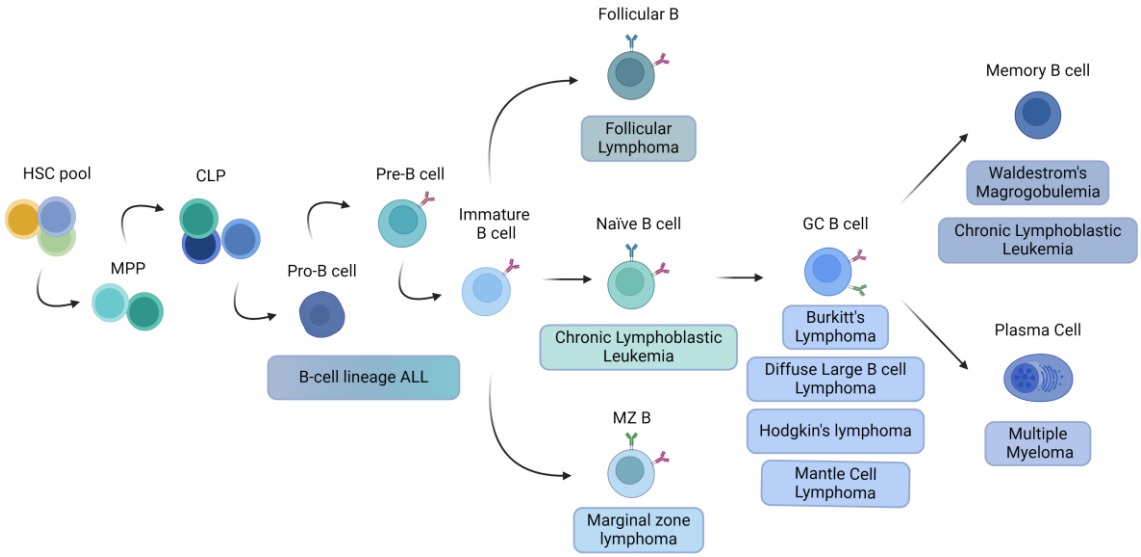


Figure 4. Malignancies related to B-cell lineage and their suspected stage of origin. Abbreviation: ALL – Acute Lymphoblastic Leukemia, CLP – Common Lymphoid Progenitor, MPP- Multipotent progenitor, MZ – Marginal zone, GC: Germinal center, HSC – Hematologic stem cell.

The classification is as follows:

Leukemia: Englobes a group of hematological malignancies characterized by the increment of leukocytes (white blood cells) in the bloodstream. They are classified depending on the lineage giving rise to the blasts (lymphoid or myeloid) and the aggressiveness of the disease, acute or chronic. The common types included here are: Acute Myeloid Leukemia (AML), Acute Lymphocytic Leukemia (can be of B or T origin, B-ALL or T-ALL), Chronic Myeloid Leukemia (CML), and Chronic Lymphocytic Leukemia (CLL). Depending on the characteristics of the disease, several subtypes appear for each of these.[16]

Lymphoma: Here we refer to all those malignancies where the cell of origin is a differentiated stage, usually of the lymphoid nature. Lymphomas are classified into Hodgkin (10%) and non-Hodgkin lymphomas. Most non-Hodgkin lymphomas originate from the B cell population (90%) given the several changes that the cell experiences through its maturations and differentiation process, each B-cell stage can give rise to several different malignancies. Usually, this type of malignancy is located in the lymph nodes and other secondary lymphatic organs.[17,18]

Myeloma: And you may say, "Oh, of course, since we have, myeloid and lymphoid lineages, myeloma will refer to the myeloid cells". And that will be a wrong assumption. Myeloid lineage malignancies usually are classified into Myelodysplastic Syndrome (MDS) or Myeloproliferative Neoplasm (MPN) and usually give rise to most of the AML cases. "Myeloma" is used for the disease originating from plasma B cells and usually is localized in the BM, where most plasma cells reside. In this group are included several different diseases all of which are caused by aberrant plasma cell presence and defective monoclonal immunoglobulin production, that eventually accumulate leading to clinical disease.[19]

As the title of this thesis may spoil, this project has been developed in the context of Multiple Myeloma (MM). Thus, we will dedicate a little bit more space to describe what is known about this disease and some of its main characteristics. Let's identify the big bad wolf in our story.

Multiple myeloma

MM is a disease caused by the abnormal clonal expansion of aberrant mature plasmatic B cells. The disease is defined by the presence of M-protein (defective monoclonal immunoglobulins) in urine or serum, the presence of BM clonal plasma cells, and related organ or tissue impairment. The problems related to this disease usually result from the abnormal protein production of M-protein which accumulates and gives rise to the characteristic phenotype of the disease. The clinical presentation of the disease is ruled

by hyperCalcemia, Renal failure, Anemia, and lytic Bone disease (CRAB). The median age of diagnosed patients is 65 years.[19]

MM accounts for 1% of all world cancers and in hematological malignancies accounts for around 10% of total cases.[20] The overall survival for the disease is around 6 years, but it varies greatly among subgroups. Regardless, it has been increasing thanks to the advances in immunotherapy which have greatly impacted the disease. Despite the advances, MM is considered an incurable disease where all patients eventually die due to complications and the failure of current treatments. Patients eligible for Autologous Stem Cell Transplantation (ASCT) have better prognostic scores as they survive more than 8 years. Sadly, as a big part of the diagnosed cases are in the elderly population (>65) and are not eligible for these more aggressive treatments, the OS decreases to 5 years.[19–22] These estimates are very general and as always with cancer, each individual case is different. In MM, the host characteristics, the tumor stage, and the cytogenetic abnormalities present in the malignant plasma cells are determinants of the success of the different treatment options. Let's discuss a little bit more about the pathogenesis of the disease, the cytogenetics involved, the effect of the tumor microenvironment (TME), the pre-malignant stages, and the current treatment options for the patients.

Pathogenesis

There is still work to do regarding the origin of the disease, but thanks to the advances in technology and single-cell strategies we are little by little deciphering the tumorigenesis of MM. What it is known until now is that the biological DNA recombination stages during B-cell maturation and differentiation may produce errors that may accumulate and contribute to the development of immunoglobulin heavy chain (IGH) gene chromosomal translocations, which are considered to be the starting point of the malignant transformation together with hyperdiploidy.[19,23]

What are these B-cell development events that trigger malignant transformation then? As we already mentioned in the previous section about B-cell differentiation, B cells need to overcome three DNA arrangement steps in order to become functional B cells, those three events are (I) VDJ rearrangement, which occurs on the pro-B cell stage and gives rise to heavy chain expression; (II) SHM and (III) CSR which occur much later on the GC reaction after encountering a reactive antigen. Because the disease originates from errors produced in these stages, it was discarded that this disease originates from a germline mutation.[19]

The genomic abnormalities generated during these events can be classified under big groups of changes that fall under the same error. Here we will mainly focus on the primary initiation events, and briefly mention other events that contribute to tumor development.

The first and more important primary event is IGH translocations. They occur during B-cell-specific DNA modification processes when breakpoints are introduced near IGH switch regions. Since these breakpoints occur near potent immunoglobulin enhancers, these aberrant translocations trigger disease initiation. Other IGH translocations may occur as secondary events and these are characterized to not necessarily occur on B-cell-specific DNA modification processes. MM is a very heterogeneous disease, and this can already be seen in the chromosomal loci involved in these translocations. Regardless, there are five regions that we find recurrently affected: 11q13 (CCND1), 4p16(FGFR), 16q23 (MAF), 6p21 (CCND3), and 20q11 (MAFB).[24–26]

Here are listed the main translocations observed in MM patients regarding the aforementioned chromosomal loci:

- t(11;14): This translocation is present in 15-20% of cases of MM patients and affects Cyclin D1 (CCND1), which as a result is juxtaposed to IGH enhancer. As a result, CCND1 is overexpressed (and this occurs in 100% of the patients presenting this translocation). CCND1 by coupling with a kinase ends up with the increased release of E2F. In turn, E2F increases cell proliferation by favoring G1 to S transition.[27]
- t(4;14): Is present in 15% of the cases and is not detectable by karyotyping. It affects the Fibroblast Growth Factor 3 gene (FGFR3) and the MM SER domain (MMSET) gene overexpressing them as a result. Neither of these genes is expressed in healthy plasma cells. MMSET overexpression is presented in most cases where the gene is affected while FGFR3 is only in 75% of the cases with this error. This indicates that the mutation of SET is an event needed for developing the disease whereas FGFR3 seems to contribute to disease progression. How these two genes contribute to pathogenesis remains elusive.[28,29]
- t(14;16): This translocation occurs in 5-10% of the patients. The breakpoint of the translocation occurs in MAF family members. The break occurs towards the centromeric region which makes it difficult to affect expression in expression by IGH enhancement, regardless, 50% of cases present MAF overexpression. MAF regulates the expression of several genes (i.e. CCND2, ITGB7, or CCR1) which contribute to malignant plasma cell survival, proliferation, and drug resistance.[30]
- t(6;14): Is presented in 3% of cases and is associated with high levels of CCND3, which acts similarly to CCND1 overexpression.[19,31]
- t(14;20); Occurs on the 1-2% of the MM cases and it involves MAFB, another transcription factor from the MAF family. In this case, the error is mostly structural and is considered a secondary translocation event.[19,31]

Another important cytogenetic event with a deep effect on MM pathogenesis is chromosomal material gain and loss. In MM, almost all cases are aneuploid (which means that patients have a different than normal number of chromosomes, which is 46). In MM,

the cases are divided into hyperdiploid (46/47 chromosomes) or non-hyperdiploid cases, which may mean that they are hypodiploid (44/45 chromosomes), pseudodiploid (44/45 to 46/47) or tetraploid (more than 74). The hyperdiploid cases are associated with trisomies of odd chromosomes (3,5,7,9,11,15 and 19) and a low incidence of structural chromosomal abnormalities. Non-hyperdiploid cases are associated with the high prevalence of IGH translocations on the five recurrent partners. The most common monosomy, deletion of 13, occurs in 40 to 50% of cases. Despite this, the most common abnormality in the group is gain on 1q. Deletion of the 17p chromosome arm involves the loss of TP53 and is associated with extramedullary MM. Occurs in 5-10% of cases. Other losses that may occur are the loss of 13, which is associated with t(4;14), t(14;16), del 17p, and 1q gain. Lately, 1p losses (1p22 and 1p32) have also been recognized as frequent errors in MM cases.[19]

Different from other well-known lymphomas, there is still a big heterogeneity in the mutations detected in the MM cases. Although it is a common occurrence to see mutations, only a few of them are considered recurrent and in their majority, they are defined as secondary events that help with malignant cell survival and proliferation. The mutations detected more frequently are those on the ERK pathway (RAS, RAF, or MAPK mutations). *MYC* is also described as dysregulated due to secondary translocations and in relapsed patients, *TP53* appears mutated (loss of function) in 75% of the patients.[31]

MM and the microenvironment

The B-cell microenvironment greatly contributes to MM pathogenesis. Due to plasma cell biology, long-term plasma cells are established in the BM and are retained there through adhesion molecules and BM stroma cell signals (trough Vascular Cell Adhesion Molecule 1 (VCAM-1) and Intercellular Adhesion Molecule 1 (ICAM-1) but also B1 integrin family genes). The homing is further increased by plasma cell adhesion molecules such as CD38, CD138, CD44, and CD106. In MM, this BM adhesion mediates the drug resistance phenotype. This adhesion also facilitates cytokine secretion such as Tumor Necrosis Factor-alpha (TNF-α), IL-6, and Insulin-Like Growth Factor 1 (IGF-1) which triggers signaling pathways (RAF/MEK/MAPK and JAK/STAT, among others) that promote cell proliferation and prevent apoptosis.[12,13] This means that a healthy BM microenvironment already favors plasma cell survival, even when they have transformed into something terrible.

There is much more to be said about MM TME and its contributions towards MM persistence but since this project did not focus on their interaction, we will have to keep moving on towards more disease characteristics.

Clinical manifestations

We briefly mentioned before that the clinical manifestation of MM can be defined by the acronym CRAB (HyperCalcemia, Renal failure, Anemia, and Bone disease). Now that we

know a little bit more about the pathogenesis of the disease, we can relate this to its clinical manifestation.

As malignant cells establish in the BM, the interactions described above produce osteolytic lesions which can result in bone pain hypercalcemia, and neurological compression. Furthermore, MM cells induce osteoclast activation and osteoblast function inhibition, increasing the severity of bone disease. Due to the clonal expansion and proliferation, normal hematopoiesis is impaired, and thus MM cases present anemia. Plasma cells are responsible for immunoglobulin production and thus, malignant cells keep up with abnormal immunoglobulin production, mainly M-protein, and light chains which are released into the bloodstream and end up leading to renal failure. Due to the lack of a proper immunoglobulin function, patients are exposed to life-threatening infections. Other clinical manifestations may appear depending on the host characteristics, but these are the symptoms considered MM-defining ones.

Non-symptomatic MM stages

MM develops through a multistep progression, during which plasma cells seek immortalization and expand clonally, leading toward end-organ damage. In **Figure 5** you will find the schematic representation of the disease progression. Most genetic lesions are already present at the first stages which will be discussed next.

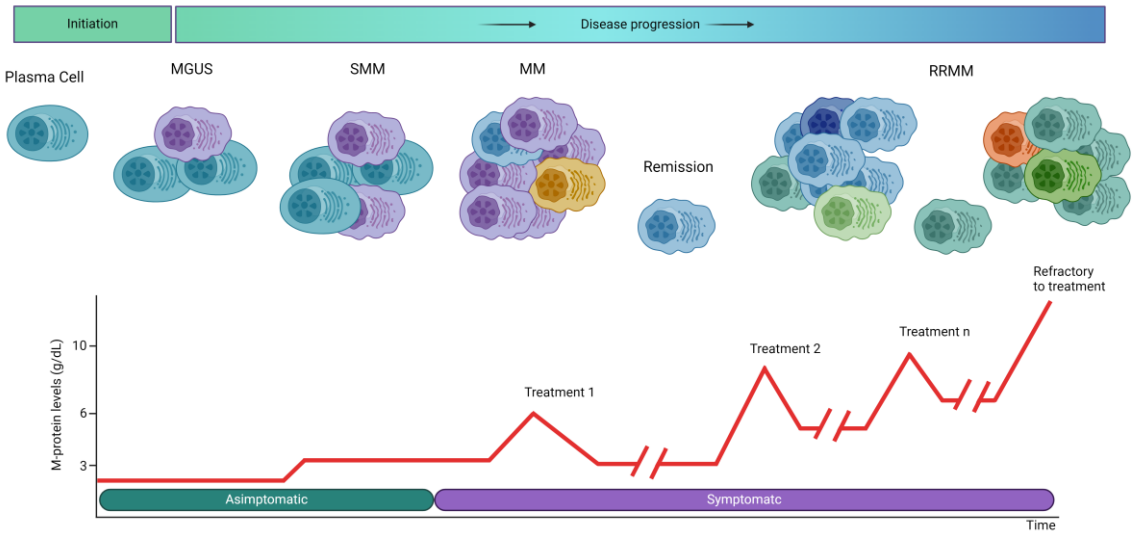


Figure 5. Pre-malignant and disease progression of MM. MGUS and SMM are considered asymptomatic and thus pre-malignant stages of MM with different risks of progression. The percentage of plasma cells in BM is below 10% in the asymptomatic stages and MM is considered when the abnormal proliferation of malignant cells takes place. After treatment, is believed that some malignant clones remain in the BM and there is a relapse after some time until the disease becomes refractory to treatment. With each new treatment line, the time to relapse decreases. The graph represents the increase of M-protein in serum in the patients in the different stages. Abbreviations: MGUS – Monoclonal Gammopathy of Undetermined Significance, SMM – Smoldering Multiple Myeloma, MM – Multiple Myeloma, RRMM – Relapsed/Refractory Multiple Myeloma.

MM is always preceded by a non-malignant condition called Monoclonal gammopathy of undetermined significance or MGUS. It is believed that around 5% of the population may develop this disease after the age of 50. MGUS is defined by the presence of less than 10% of clonal plasma cells in the BM and less than 3g/dL of M-protein circulating in blood bloodstream. MGUS patients have a risk of progressing towards MM. Clinicians usually define the risk of progression as a starting 1% chance, increasing each year. Aberrant plasma cells in MGUS malignancy already incorporate some characteristics and errors that will later appear in MM cases, but they do not present symptoms or organ or tissue impairment due to monoclonal gammopathy.[20]

Before symptomatic MM, some patients (around 10%) are considered to have an intermediate asymptomatic, but pre-malignant nonetheless, stage called smoldering MM or SMM. It is defined by the presence of more than 3g/dL of M-protein in serum and 10% or more plasma cells in the BM but with the absence of lytic bone lesions or any other clinical manifestation due to monoclonal gammopathy. In these cases, clinicians set the risk of transformations as 10% per year for the first 5 years, 3% for the next 5 and finally 1.5% thereafter. The rate of progression is deeply set by the cytogenetic defects of the disease as some types (i.e. t(4;14)) have increased risk of progression. The most recurrent cytogenetic errors occur in the same chromosomal region where immunoglobulin chain recombination sites are located.[19,20,22]

Currently, there is still a big controversy regarding whether the pre-malignant MM stages have to be addressed and/or treated to avoid progression[20]. International efforts are being made to reach a consensus on how to proceed with the pre-malignant cases. The Spanish Myeloma group has proposed a risk classification based on the percentage of aberrant plasma cells on the BM and immunoparesis (other immunoglobulins decrease which are not the main affected ones). By using this risk classification, high-risk patients may be considered early myeloma patients and thus receive early intervention, leading toward a more individualized and better disease management.[19]

Although this thesis project will not explore the different MM classifications, it is worth mentioning that malignant plasma cells may originate from different diseases depending on where the malignant plasma cells are detected (i.e. in circulation or establishing plasmacytomas outside the BM) and the type of protein or immunoglobulin being secreted.

Therapy strategy

One of the major issues that characterize this disease is the available treatment options. As we said before, malignant plasma cells giving rise to the disease are characterized by several genetic errors which create a great heterogeneity in the disease (even inside the same patient). Is because of that that immunotherapy has arisen and successfully brought some hope in MM cases, although a lot of work is yet to be done.

In the 1960s, two drugs appeared which were considered the standard of care for several decades. They are Melphalan and Prednisone, and although currently there are newer options, they are still included in the treatment options for some MM cases. Melphalan is a chemotherapeutic drug that produces elevated cytotoxicity to the cell and especially targets hematological cells (which is why this drug is also used in the induction phase before stem cell transplantation)[32]. Prednisone is another chemotherapeutic agent which is considered a corticosteroid and an immune-suppressor[33].

In the 2000s, novel drugs appeared which changed the MM treatment paradigm forever. Two immunomodulatory drugs (IMiDs) appeared which are now part of the main therapeutic options for MM patients, thalidomide and lenalidomide. Thalidomide[34,35] and lenalidomide act as cytotoxic agents for malignant cells and increase the ratio of effector cells in the immune system, which increases cytotoxicity attacks toward tumor cells. From this group of novel agents a proteasome inhibitor, bortezomib also was a success in MM treatment. Bortezomib[36] affects directly the malignant cell by inhibiting the proteasome 26S, which in turn affects several intracellular pathways leading to cell death. Another corticosteroid gained a position on MM treatment, dexamethasone[37]. Finally, another drug commonly used as first-line treatment is daratumumab, a monoclonal antibody targeting CD38 (one of the plasma cell surface markers)[38].

When evaluating the initial approach to newly diagnosed MM cases, physicians make a distinction between young (less than 65 years old) and old patients. This is because, in young patients, if they meet the criteria (such as not having comorbidities), they are eligible for ASCT which is considered one of the priorities and the best strategy to fight the disease. Old patients and those not eligible for ASCT are treated with different combinations of the aforementioned drugs. In Figure 6 you will find a very summarized scheme with the different pathways to follow for MM treatment. Since the treatment combinations for MM patients are complex and several characteristics both from the patients and the tumor need to be evaluated, we will not present here all the options, only mention the general guidelines followed in the different cases.[19,39]

MM is characterized by its relapses and refractory nature. After some time, a clone of the malignant cells becomes refractory to the treatment and gives rise again to cancer, making that new relapse unable to respond to the previous line of treatment (as the treatment has shaped the disease and the new clones are not affected by the drugs). After the initial relapse, the duration of the subsequent responses to rescue therapies is progressively shortened.[19,39]

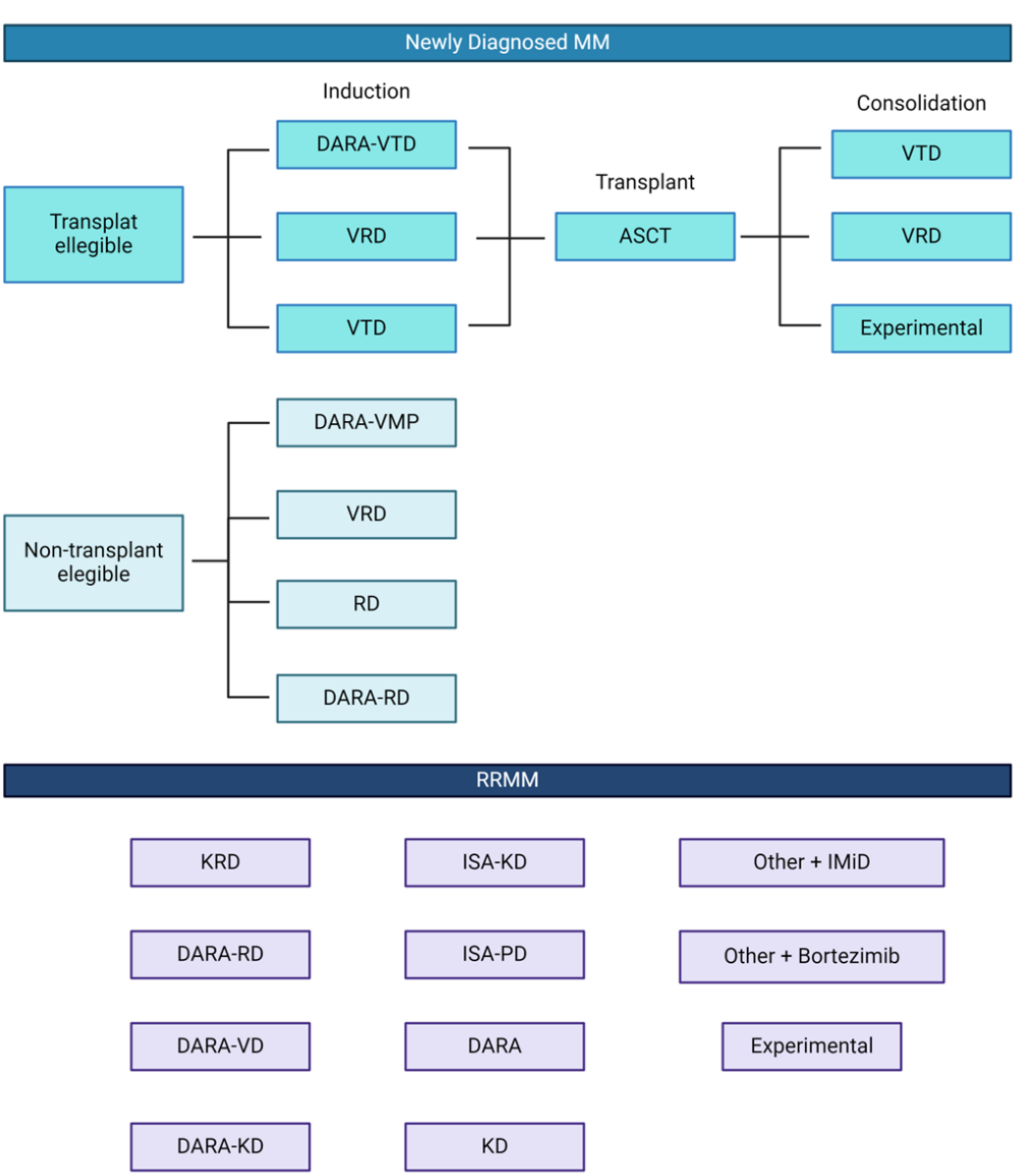


Figure 6. MM treatment options. This represents an oversimplification of the treatment paradigm in which the risk to progression and patient characteristics are considered when determining the best strategy. Abbreviations: DARA – Daratumumab, D – Dexamethasone, K – Carfilzomib, M – Melphalan, R – Lenalidomide, P – Prednisone, T – Thalidomide, V – Bortezomib, ISA – Isatuximab, IMiD – Immunomodulatory Drugs, ASCT - Autologous Stem Cell Transplant.

As MM is considered an incurable disease, all patients eventually relapse and the only actual way to consider a cure is through an allogenic SCT which is highly risky due to graft-versus-host-disease. After a relapse, again, depending on the characteristics of the patients (young or old) different approaches will be considered. Young individuals who relapsed before one year after ASCT are considered high-risk patients and thus are provided with the highest doses and considered for allogenic SCT. Those who relapse one to three years after ASCT are considered allogenic but usually treated with a different combination than the original one provided. Finally, those who relapse after three years from the ASCT are treated with the initial drug combination, incorporating, if necessary,

another drug to ensure the effectiveness of the treatment. Old individuals who relapse are encouraged to be included in clinical trials with novel drugs that will increase their chances of longer responses and help in the development and optimization of these novel drugs. In these promising new drugs, several immunotherapy options are arising in multiple myeloma treatment.[19,39]

We will further discuss immunotherapy in the following sections after talking a little bit more about immunity and its relations with cancer. Nevertheless, as stated before, this thesis project was developed in the setting of MM, a type of malignancy that as you have observed, needs to be tacked down by researchers in order to bring better opportunities to those suffering the malignancy.

Immune system and immune crosstalk

You know now where hematological malignancies arise from and which are the big differences between them. But what do other characters may we know before starting the actual story? Well, for that we will need to talk a little bit about the immune system, how our natural defenses play a part against cancer, and how thanks to research we are now able to enhance it to our advantage.

Immune system

Although we trust the reader to know enough about immunity to discuss this thesis, I wanted to set some definitions to lay out the context where this project was developed. We define the immune system as the group of cells and reactions that evolved to protect multicellular organisms from pathogens. In order to meet this need, as the intruders vary in size and infectivity, we have evolved a complicated and dynamic network of cells, molecules, and pathways to the point where, even today, new mechanisms are being described. Recent advances in cell imaging, genetics, epigenetics, bioinformatics, and cell and molecular biology have helped to understand better the individual players of this system. We usually divide immune response into two separate phases named innate and adaptive immunity. Pathogens exist in many forms and how the immune system responds to them is given by which pathogen is recognized and where it is found (i.e. immune-privileged areas are places where immune cells cannot access or how they will react if they find intestinal microbiota on the bloodstream).[40]

The initial response relies on the action of physical and chemical barriers to infection (i.e. skin and mucosa), as well as the recognition of conserved and common pathogen structures (named Pathogen-Associated Molecular Patterns or PAMPs). The innate response is rapid and is started within seconds of the start of the infection. This response is not specific, and they use both PAMP recognition and phagocytosis to control the infection. These strategies are inherited and always respond to the same set of patterns. This first line of defense prevents most infections from progressing. The cells taking care

of innate response are most of the myeloid lineage (macrophages, granulocytes, and dendritic cells among others). After 5 to 6 days of the initial exposure, the adaptive response will be formed.[40–42]

As the response progresses, the adaptive response, antigen-specific recognizing cells, will be generated through random DNA rearrangements. The adaptive response is slower but highly specialized in the encountered pathogen. It relies mainly on the ability of B and T lymphocytes to express this complex set of receptors able to match perfectly the encountered antigen. This adaptation will allow them to recognize and clear the pathogen and to remember it in case a new infection is produced. The full development of a correct adaptive response is dependent on the initial innate response, as, if dendritic cells do not bring the proper antigens to the lymph nodes, immature B cells will be unable to enter GC and thus enter their differentiation phase. [43,44]

Now, we have talked about hematological malignancies and know how the immune response is built. Before seeing the interaction of these two factors, we have to talk about one characteristic of immunity, crucial for our well-being and protection: Immune tolerance[45]. This is defined as the lack of immune response against the host tissues. Usually, tolerance is explained with a focus on the idea that immune cells can recognize antigens from our own cells versus patterns present in infectious pathogens. Nowadays, the model explaining how immune cells may differentiate is not based on the recognition of patterns but rather on the capacity of the recognized antigen to be a danger to the host. In order to differentiate them, they look for self-antigens accompanied by Damage-Associated Molecular Patterns or DAMPs. Before being released into the system, immature cells are tested for non-responsiveness to host antigens, for example, remember B-cell checkpoints during their maturation. Dysfunctional tolerance causes several health issues, for example, allergies and autoimmune diseases. The existence of this tolerance explains partially why the system ignores cancerous cells and sets an interesting background to develop new research.[46,47]

Immune system and cancer

Cancer is the term we use to refer to the group of diseases that arise from the malignant transformation and uncontrolled replication of any cell in our body. Since cells are originated in the host, tolerance mechanisms may interfere with their detection and elimination. Malignant cells are known to express unique or inappropriate proteins making them immune targets. This expression is due to this increased genetic instability. However, the instability of the rapidly dividing cells gives them an advantage in immune evasion and escape. But let’s talk a bit more about the relationship between the immune system and cancer.

Tumor cells present a unique challenge to the immune response (Figure 7). Environmental carcinogens, viral infection, chronic inflammation, and genetic predisposition may lead to malignant cell transformation. Upon malignant transformation, after the intrinsic tumor-suppressor mechanisms fail to control the malignant cell, they will be recognized by the immune system and usually be destroyed[46,48]. This phenomenon is known as immunosurveillance[45]. In homeostasis, T and NK cells are the main protagonists responsible for cancer immunosurveillance and their interaction with tumor cells is of utmost importance as they will determine which cell will survive the exchange. This exchange is termed immunoediting as the immune system has the ability to shape the tumor.

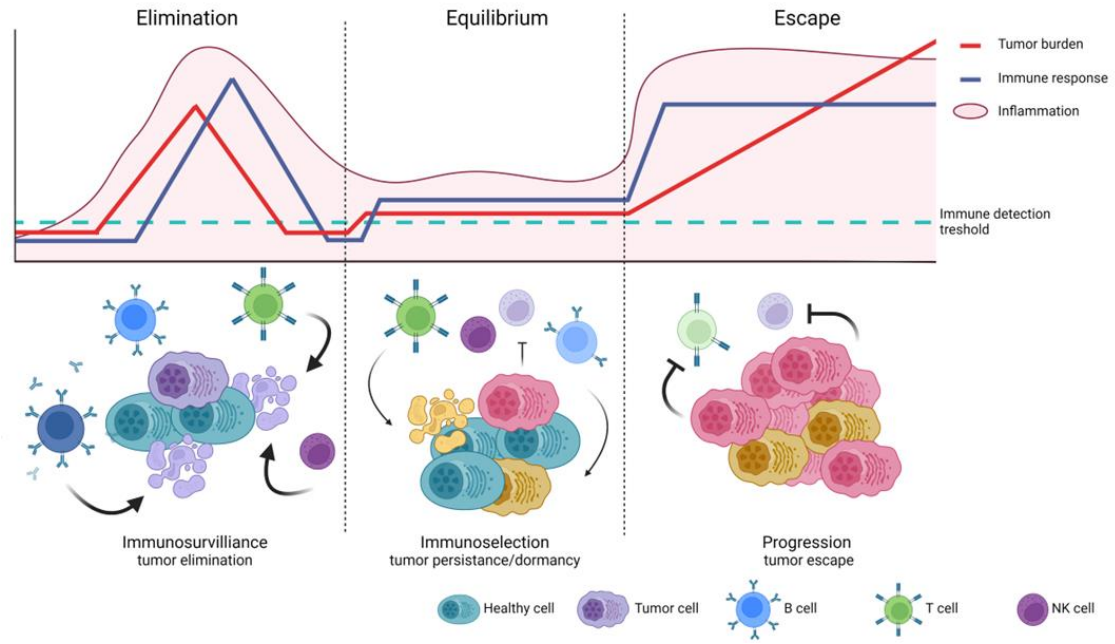


Figure 7. Immunoediting of tumoral cells. These are the three phases of cancer immunoediting of tumoral cells by the immune system. In the first phase, what occurs during homeostasis is that occasionally, tumoral cells appear due to a process of carcinogenesis. By intrinsic methods (repair and programmed death) or extrinsic (immune control) tumoral cells are eliminated. During the process of tumorigenesis, low immunogenetic clones or non-immunogenic clones may appear. Low immunogenic clones will resist death while non-immunogenic clones will escape the detection of the immune system or maintain it constantly activated. In the final phases, it is suspected that a tumor is developed when a clone successfully escapes detection, takes advantage of the environment, and replicates abnormally. In this phase, it is common to talk about an immune suppressive environment that contributes towards tumor survival.

What happens if the tumor cells can persist even after immune detection? Then we talk about the next phase in immunoediting which is equilibrium.[45] During the equilibrium phase, immune cells cannot eliminate all tumor clones and they are subjected to immune selection as the less immunogenic cells will be able to escape immune detection entering the final phase of immunoediting. Both intrinsic and extrinsic factors will act over the tumor cell to further succeed in immune escape, for example, cancer cells may downregulate MHC expression which will make T cells unable to recognize them or they

may upregulate inhibitors of apoptosis. Another well-known escape mechanism in tumor cells is the upregulation of inhibitory factors such as Programmed Death-Ligand 1 (PD-L1) or Fas.[49–51] And here, dear readers, is where we hit one of the main focuses of this thesis project: How do immune cells and tumor cells interact and what signals are exchanged to determine if the tumor cell must be eliminated or not?

If you want to know, keep reading, as the interesting part is just starting.

Immunity system-cell crosstalk: The Immune checkpoint of cytotoxic cell activation

Whereas the B-cell function is focused on antibody production, the T-cell function is mainly to monitor the status of the host cells and look for infection or malignant transformation. That is why, T cells, especially cytotoxic T cells or CD8+ due to the receptor expressed on their membrane, need a set of signals different from those given to B cells in order to become activated. For starters, B cells can recognize antigens in their soluble form whereas most T cells can only recognize antigens bound to an MHC class I molecule.

So, how is this cytotoxic cell activated, then? Is it as easy as recognizing an antigen bound to an MHC class I on the membrane of a cell and the elimination machinery will turn on? The immune system is much more refined than that. Classically we refer to the process of cytotoxic T lymphocyte (CTL) activation as a three-signal model[43]. The 1st signal is given by the T-cell receptor (TCR) upon binding to an MHC class I receptor with a bound antigen. Due to the risk that these cells (they are *cyto*toxic meaning they are cells with the capacity to kill other cells directly) a complementary activation signal is needed to proceed with the attack. So, once the TCR recognizes the antigen being presented, a 2nd signal will trigger. In order to become activated, this 2nd signal needs to be co-stimulatory and classically given by the CD28 receptor upon binding CD80 or CD86 on the presenting cell. Full T-cell activation requires also the 3rd signal which is provided by the local cytokines produced by other immune cells or the same presenting cell. Cytokines soluble proteins that mediate cell-to-cell communication. The range of signals is transmitted through englobe growth, differentiation, and inflammatory or anti-inflammatory cues. They are strong but very complex immune mediators. In **Figure 8** you will find a schematic representation of the three signals.

Although the 1st signal may be the trigger to build the activation and response to the antigen being presented, the 2nd signal is of utmost importance for this process. What happens if the antigen presented does not correspond to an infection, but rather is an innocuous peptide? And once the reaction is started, and consequently the infection cleared, how do we stop this cycle of activation and presentation? The answer relies on the role of this much-needed 2nd signal. We have mentioned that to become activated,

the CTL needs a "co-stimulatory" 2nd signal. This is because there are molecules producing a different type of 2nd signal, called co-inhibitory signals.

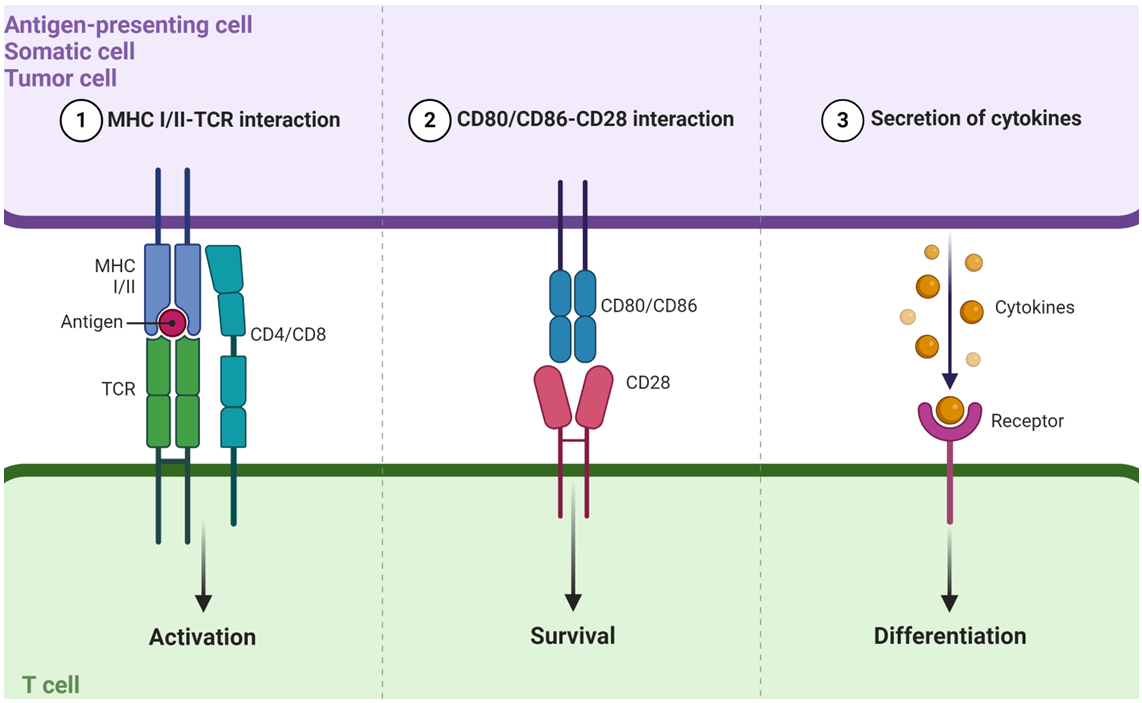


Figure 8. The three signals for T-cell activation. From left to right, the first signal consists of TCR stimulation by antigen presentation; the second signal is through the co-stimulation markers in the presenting cell, and the third signal is composed of the cytokines in the microenvironment which reinforces the stimulation. Abbreviations: TCR – T-Cell Receptor, MHC – Major Histocompatibility Complex.

This different set of markers, upon binding their receptors on the CTL, will induce signals to inhibit T-cell activation. This process is essential in maintaining periphery T-cell tolerance and reducing inflammation after the infection has been cleared. On the CTL surface the better known co-inhibitory signals are given by Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) and Programmed Death-1 (PD-1), which you may have heard off[52]. Given that this immune synapse can have an activation or inhibitory nature, this process is sometimes referred to immune checkpoint. And here you will see why we say: "In biology, nothing is as simple as it looks".

But, before focusing deeply on this set of interactions we must dedicate some sentences to another group of immune cells that strongly rely on stimulatory and inhibitory signals, NK cells. In a similar but completely different way, NK cells also can recognize MHC class I molecules but, in their case, they cannot recognize the antigen bound to them, and the signal produced upon this recognition is inhibitory. NK cytotoxicity is restricted to altered host cells rather than infected cells and the balance of signals received will be the ones fully determining if NK cells will kill or not. In a homeostatic setting where the recognized cell is healthy, MHC class I expression and other inhibitory signals will avoid NK activation. In front of an altered cell, due to stress, intracellular infection, or malignant

transformation, the cell will either stop MHC class I expression (which will trigger NK activation) or upregulate stress-induced molecules which will in the same way trigger NK cells to act. In this last case scenario, similar molecules to those regulating CTL are present on the NK surface.[42,43,53]

Now that we know that the immune checkpoint is important in different types of cytotoxic cells, let's dive into the myriads of signals that participate in this event.

Immune checkpoint: a tale on co-stimulation & co-inhibition balance

So, we refer to an immune checkpoint as the event that cytotoxic cells need to overcome to fully activate and perform their function. The checkpoint reaction is not dependent on a single interaction but instead, we are presented with a plethora of signals (in some cases bi-directional) which are part of the redundancy and safety needed for immune system control[52]. In **Figure 9** you will find some of the markers participating in the generation of co-stimulatory and co-inhibitory signals.

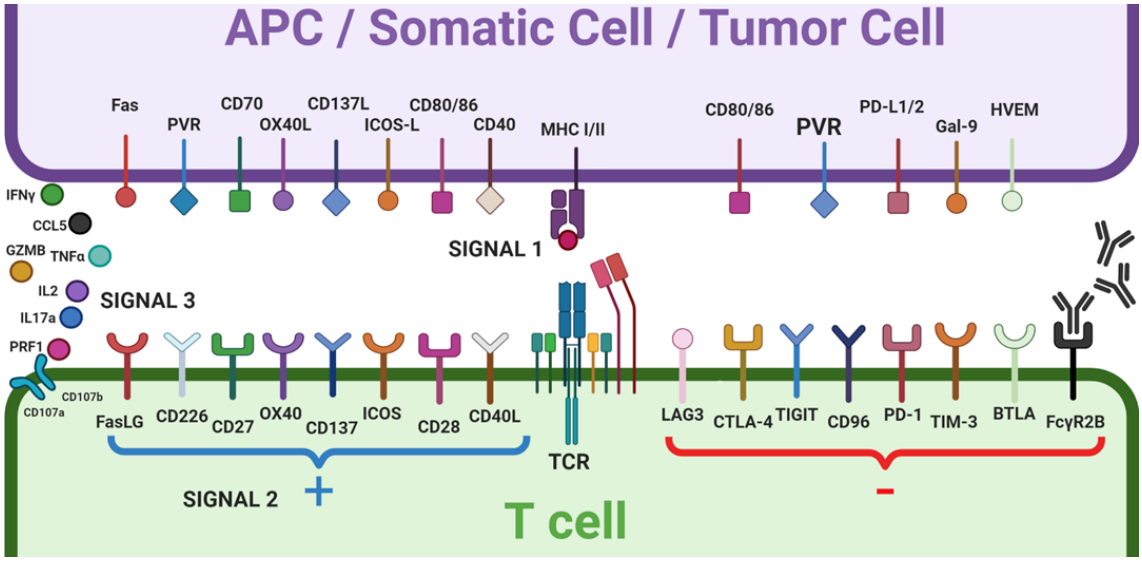


Figure 9. Immune checkpoint events. This is a small representation of the interactions occurring between a T cell and a presenting cell. The three signals for T-cell activations are also represented.

We refer to co-stimulatory receptors when talking about the group of markers responsible for co-stimulating and promoting cytotoxic cell activation. Different receptors have slightly different functions and are more prominent in different cell populations. Regardless, they promote cell proliferation and survival. The best-known co-stimulatory axes are CD28 with CD80/CD86 interaction, CD40 and CD40L, CD137 and CD137L, and CD70 with CD27 among several others, as shown in **Figure 9**. In this project, we focused mainly on co-inhibition, but it is important to know some of their names too.

Co-inhibitory receptors are the markers responsible for inhibiting TCR downstream signaling and promoting anergy (lack of response). In homeostatic conditions, they are as

relevant as co-stimulatory signals as they take care of maintaining immune system tolerance. Despite the importance of maintaining the balance and the redundancy of these signals, microenvironment changes have a strong influence on the type of signals favored. Co-inhibitory signals have been intensively targeted by immunotherapy developers, as their blockage strongly tilts the balance towards cytotoxic activation. In this group of markers, we have CTLA-4, which competes with CD80 and CD86 for the binding of CD28, blocking downstream TCR signaling, and PD-1 and its interaction with either PD-L1 or PD-L2, which may be the best described and known co-inhibitory marker. Regardless, several other co-inhibitory markers are gaining strong attention. Lymphocyte Activating 3 (LAG-3) which also interferes with TCR-MHC interaction or T Cell Immunoglobulin Mucin 3 (TIM3) that interacts with Galactosidase-9 or -Domain Ig Suppressor Of T Cell Activation (VISTA) and recently have been recognized as a PD-1-like receptor, among others.[54,55]

It is believed that co-signaling has a spatiotemporal regulation, at the start of the immune response, co-stimulatory signals are favored, and a pro-stimulatory environment is created. As the response progresses and the threat is cleared, the expression of co-inhibitory signals increases and leads the cytotoxic cells towards an anergic state[56]. This type of receptor is also common in exhausted cells, as they have been exposed for a long period of time to antigen presentation.

And, at this stage, you may wonder, how is this whole system important during malignant transformation? Upon detecting the errors in tumor cells, cytotoxic cells will eradicate them and keep on defending the host. But, of course, we wouldn't be in our current situation if this was enough for tumor clearance once the malignancy is established. We have talked previously about immunosurveillance and immunoediting and how at the final stage, malignant cells escape immune recognition. Well, a couple of strategies described of how tumor cells escape by taking benefit from immune checkpoint markers are: (I) Through the downregulation of co-stimulatory signals and (II) through the upregulation of co-inhibitory signals[55]. Although our knowledge of co-signaling molecules has increased considerably in the last decade, there is still a lot of discussion regarding the mechanisms occurring in this context and leading to immune suppression and regarding the signals cascade promoted by the interaction of the different markers[25].

For this thesis project, our main focus was on one receptor, considered inhibitory, which participates in immune checkpoint events in both T-cell and NK activation. I invite you to discover more about PVR in the following section. Let's characterize the main protagonist of this story.

PVR or the promiscuous receptor and its relatives

Among the co-signaling receptors known, there is a group of markers that have brought several headaches to experts in the area. The poliovirus family of receptors. This family is composed of a group of markers that interact with each other, changing partners and signals meanings easily.

Everything starts with PVR or Poliovirus Receptor, also known as CD155 or Nectin-5 receptor. Its name does not hide the main function of this receptor which is the entrance of Poliovirus cell infection. PVR has two other functions described in cells. The canonical isoform of the protein has an ITIM (Immunoreceptor Tyrosine-based Inhibitory Motif) which promotes signaling through SHP-2, a known enzyme that induces the signal transduction that promotes cell proliferation, migration, survival, and metastasis. Apart from the immune-related function, there has been some work on deciphering the relation of PVR with adhesion molecules as it can interact with integrins and Nectin-1. It has a soluble isoform which has been positively correlated with tumor growth, although its full potential is yet unknown the of this soluble form.[57]

There are four known ligands for PVR on cytotoxic cell membranes. The ligand that binds PVR with a higher affinity is TIGIT (T-cell immunoreceptor with immunoglobulin and ITIM domains) which produces an inhibitory signal to cytotoxic cell activation[58–60]. DNAM-1 (also known as CD226 or DNAX1 helper molecule 1) produces a stimulatory signal on the cytotoxic cell but does not render the same affinity for PVR as TIGIT does[57,61,62]. The third potential partner for PVR is CD96 which produces an inhibitory signal in both T and NK cells[57]. Thanks to the improvement of interaction technology in the last year another interactor was described for PVR, KIR2DL5A (killer cell immunoglobulin-like receptor 2DL5A), present on the surface of NK and T cells, and a known inhibitory marker[60]. At the same time, TIGIT and DNAM-1 can interact with another member of the family present on tumor cells, PVRL2. You will find the graphical representation of all these interactions in **Figure 10A**. On the cytotoxic cell surface, TIGIT has been deciphered to interact and interfere with DNAM-1 function (**Figure 10B**)[58,63].

The interactions among this family are not completely random and apart from the number of receptors expressed on the surface, there is an important factor which is attraction forces. Among all of them, PVR is most likely to bind TIGIT on the cytotoxic cell membrane and thus produce an inhibitory signal. Due to this evidence and our observations, here and on we will consider PVR as a co-inhibitory marker, mainly through its interaction with TIGIT.[58]

Remember PVR and TIGIT, they are in the title of this work for a reason.

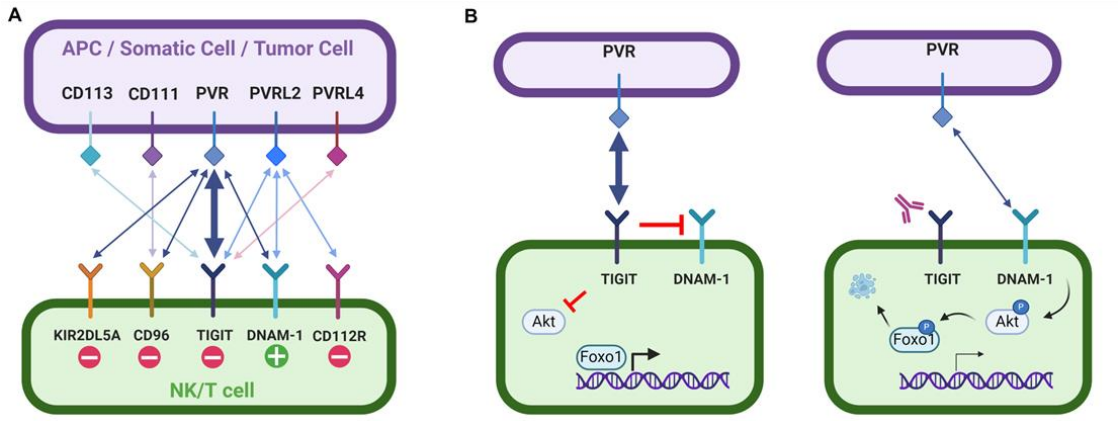


Figure 10. PVR's promiscuous nature. (A) Chart of interaction in the nectin family. PVR can interact with TIGIT, DNAM-1, CD96, or KIR2DL5A. The size of the arrow indicates affinity for that receptor. (B) PVR interaction with TIGIT is believed to interfere directly with the fitness of DNAM-1. TIGIT inhibits then Akt phosphorylation inhibiting its activity and allowing Foxo1 transcription factor activity inhibiting cytotoxic cell activation. If a neutralizing antibody is used, PVR will interact with DNAM-1 leading towards the destruction of Foxo1. This figure was adapted from the work of Chiang & Mellman, 2022[58].

Immunotherapy

During the last century, we have observed an increase in therapy options for cancer. In the beginning, chemotherapy and radiotherapy strategies led the therapy options for cancer, and still today, they are the first line of treatment for several malignancies. Despite their widespread use, these therapies are non-specific and associated with significant toxicity. In many cases, it is impossible to administer the doses required to fully eradicate tumor cells. Immunotherapy was born from the need of targeted therapies with lower toxicity relying on the biological antitumor response, the immune system.

We talk about immunotherapy when referring to any therapeutic strategy directed to increase, decrease, or direct immune response toward a better outcome. This line of strategies has been raised since 1998 leading towards a new concept of treatment, leaving the general chemotherapy and radiotherapy treatments and focusing on a much more personalized medicine strategy. Although it is still too early to forget about the strategies that have helped us for decades, a slow but strong change is bringing immunotherapy drugs to routine treatment options. In the case of MM, as stated before, the current options of treatment rely still on immunomodulators and proteasome inhibitors, but some antibodies are now being recommended as the first line of treatment in Spain (i.e Daratumumab).[64]

Here we will briefly introduce what kinds of treatment are considered to be immunotherapeutic, but ultimately, we will focus on the kind of therapies that were explored during the development of this thesis project.

Up-to-date reviews on immunotherapy classification are complex. Here we will classify them into six groups: (I) Vaccines, (II) Oncolytic virus (III) Cytokine treatments (IV) Adoptive cell transfer (V) Antibody treatments, and finally (VI) immune checkpoint blockers. You will find a representation of these different types in Figure 11.

In the first place, cancer vaccines. Vaccination is the first type of immunotherapy discovered and historically has been a very successful approach against infectious diseases. In the cases of tumors, things become more complex. As with other types of vaccines, the need for a defined cell target and antigen presentations pushes cancer vaccines toward a strong research need. Knowing that T cells carry the main role in tumor clearance, the required optimal antigen presentation, and the increasing knowledge on tumor antigens, current strategies are directed towards colocalization strategies of the desired antigen to be presented with dendritic cells, which will activate the CD8+ cytotoxic T cells, increasing tumor clearance rate. Different from other immunotherapy strategies, oncolytic vaccines can be directed against intracellular tumor antigens, but the complexity of delivering the antigen to the right APC complicates further their development. Due to the success of other therapies (i.e. CAR constructs and cell engagers), and the lack of favorable results on clinical trials, has pushed vaccine development towards a much slower pace. Nevertheless, there is still a lot of potential to be explored.[65,66]

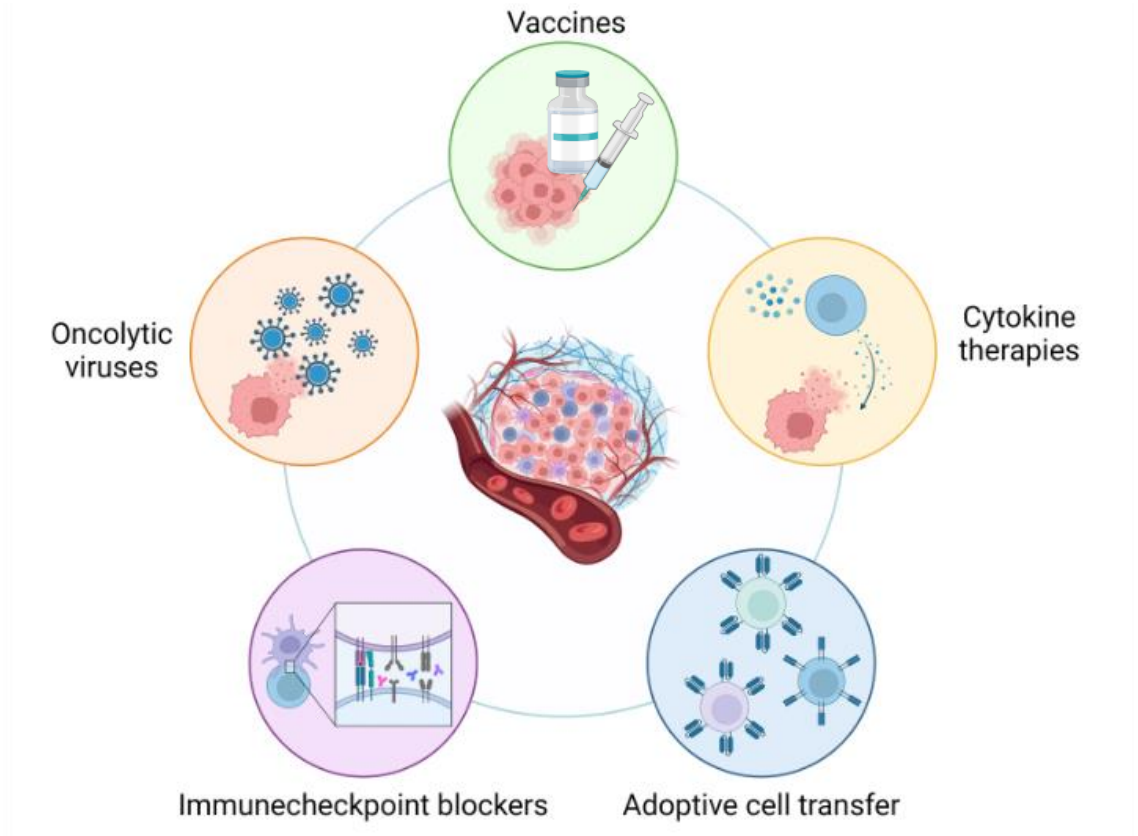


Figure 11. Immunotherapy general classification.

Oncolytic viruses started to gain attention as a therapeutic option for solid tumor treatment. For now, is still a growing field with only one treatment approved but the potential for oncolytic viruses. The recent clinical trials have highlighted the benefits of this type of therapy. Oncolytic viruses can be directed for selective replication in tumor cells and induce immunogenic cell death while promoting antitumor immunity and a low toxicity profile that does not overlap with other immunotherapies. Since toxicity profiles are limited, the best approach to maximize effectivity relies on combination therapies with other current oncolytic strategies. There is still a great field to explore through research as using this strategy, which is another form of infection, in a setting where immunity is already compromised, may be detrimental to the success of therapy. So, research is needed to develop a virus with enough immunogenic profile to recruit immune cells to the site of the tumor but low enough to be able to persist in the time needed to develop an antitumor response, not merely an antiviral. Nevertheless, this strategy is focused mainly on solid tumors, disregarding, once again, hematological malignancies.[67]

Cytokines as we said are small soluble proteins that act as cell-to-cell communicators. Their function strongly directs immune response and can have a direct anti-proliferative effect on tumor cells. Their strong effect is compensated by their limited half-life in circulation. Cytokine-based therapies are complex to develop and require a high degree of knowledge regarding cytokine biology and pharmacokinetics. It seems that the best approach to take maximum advantage of cytokines relies on the combination with other types of immunotherapies that can benefit from their ability to expand and reactivate effector NK and T lymphocytes and increase persistence and infiltration rates on the TME. That stated this type of therapy requires a high level of research as imbalanced administrations may lead to promoting systemic pro-inflammatory effects and undesirable side effects. Nowadays, the main strategies to incorporate this approach are through combination with checkpoint inhibitors and through the incorporation of CAR constructs as a way to increase permanency.[68,69]

As this project involved the other types of immunotherapies, we will dedicate an entire section to properly focus on them.

Antibodies, antibody-drug conjugates, cell engagers, and immune checkpoint blockers

For the sake of clarity, we grouped antibody therapies under the same title. We will briefly introduce the different types and then focus on cell engagers and their relevance for this project.

Monoclonal antibodies were one of the first targeted therapies to succeed in the fight against tumors as they are highly specific to their target antigen. Through a wide range

of pathways antibody binding leads to tumor cell death. The advances in biology and immunity have led to the development of better antibody products such as immune checkpoint inhibiting antibodies and the so-called cell engagers.[70]

Monospecific antibodies are full-length immunoglobulins that recognize and bind tumor surface epitopes. The most common isotype used in these drugs is IgG1 antibodies due to their strong capacity to promote antibody-dependent cytotoxicity and antibody-dependent phagocytosis by interacting with NK cells and macrophages.[71]

Antibody-drug conjugates appeared after the technology for antibody modification improved. Several molecules can be attached to a directed antibody but here we will briefly mention drug conjugated ones. They rely on the internalization of the antibody after binding its target, where, after being inside the target cell, will release its cargo. Although some degree of toxicity is experimented with (as we are relaying once again in chemotherapy), the fact that the drug can be directed to the specific target increases its rate of success.[69,71]

From monospecific antibodies, a group of drugs differentiates itself due to their high success and particular specificity: immune checkpoint inhibitors (ICI). There are currently 11 ICIs approved by the European Medicament Association. This type of antibody targets (as their name indicates) immune checkpoint markers and neutralizes co-inhibitory signals (2nd signal) detrimental to cytotoxic clearance. Around 30% of patients respond to this kind of treatment across cancers but the number of successes is much higher in immune-inhibiting TME and when co-inhibitory markers (like PD-1) are highly expressed. We may not forget how, despite the success of this type of therapy, there are still high toxicities associated with their administration. They are commonly referred to as Immune-related adverse events or irAEs. Thankfully, they are infrequent and can be managed safely.[54,58,66,72–74]

Finally, and most recently, a new type of antibody appeared from the bench. Bispecific antibodies. We will refer to them as cell engagers as they have overcome their own name and can now target several more epitopes. The key for this construct relies on their capacity to bind two targets simultaneously by being constructed from the light chain fraction from a canonical antibody. These two targets don't necessarily need to be on the same cell, here is why they are called cell engagers, as the main strategy has been to attract cytotoxic T cells towards tumoral cells. This approach improves cytotoxic response and has been shown to be highly effective. As in other cases, we cannot forget the toxicity associated with this strategy as the fact that we are yet again stimulating immune cells, inevitably patients experiment toxicity associated with immune activation. Luckily, these toxicities can be managed and balanced.[72,75–77]

For this project, we were able to work with immune checkpoint inhibitors, T-cell engagers, and monoclonal antibodies. Furthermore, we were able to know more about this type of therapy on MM.

Adoptive cell therapy

Adoptive cell therapy is one of the most personalized methods developed recently to fight against cancer. The principle defining this group of treatments is the modification or enhancement of effector cells (T cells, NK cells, and most recently, macrophages to prime them towards direct anticancer activity[78,79]. For that different strategies have appeared. The first strategies that arose, contrary to what nowadays has become the rule, came from solid tumor observations. Autologous Tumor Infiltrating Lymphocytes (TIL) and CTL therapies rely on extracting T cells from the tumor site and expanding the best-fitting ones to then proceed to the adoptive transfer back into the patient. Although there is still research to be done regarding the improvement of these types of therapies, they were completely outshone by the success of gene modification adoptive therapies. Commonly we talk about two groups of modification, TCR therapy and CAR therapy.

As the name indicates, TCR therapies focus on the genetic modification of the TCR on T cells, again, obtained from the patient. This genetic modification is usually accomplished through viral vector transduction and ex-vivo expansion. The TCR modification usually leads to an expression of TCR antigen-specific recognition and better signal transduction which leads to successful cancer eradication. Major technological advances have facilitated the identification and optimization of TCR specificity and affinity. Although TCR therapy has produced successful responses, limitations have rendered the success of this strategy in the clinic. For starters, TCR therapies are only useful for individuals who share the same HLA. Secondly, the modified T cells still rely on antigen recognition through MHC presentation and are susceptible to failure due to the tumor strategy of MHC downregulation. Finally, if the TCR target is not expressed only on tumor cells, on-target/off-tumor toxicities are most likely to occur.[79,80]

Chimeric Antigen Receptor (CAR) modified cells have revolutionized cancer immunotherapy and have been one of the most successful strategies in cancer in this century. In contrast with TCR therapies, CAR constructs can be incorporated into a myriad of effector cells. Until now, researchers have successfully modified T cells, NK cells, and macrophages[78]. CAR constructs are built by linking the variable region of the antibody heavy and light chains to intracellular signaling chains which will promote effector cell efficiency and direct its killing capacity towards specific tumor targets. They do not require MHC recognition in order to attack. Several hematological malignancies have benefitted from the apparition of CAR constructs as therapeutic options, but there is still a long way to go. CAR-T cells are, for now, the most successful strategy, although several limitations are appearing which are changing the winds of CAR therapy. Target specificity

has brought a high on-target/off-tumor toxicity. Furthermore, in CAR-T treatments, it is usual to observe CRS (Cytokine Release Syndrome) and Immune-effector Cell Associated Neurotoxicity Syndrome (ICANS) due to the high degree of activation and inflammatory signals that CAR-T cells reach.[81–84]

Recently, more CAR-NK research is changing the paradigm towards a better option. Despite their persistence problems, CAR-NK cells have several benefits compared to CAR-T cells, making them an attractive research target. CAR-NK cells can mount a rapid response to non-self-cells and have different cytokine profiles which have proved to be much safer for patients as less CRS and ICANS are observed on CAR-NK clinical trials. The fact that NK cells can be obtained from several sources (and not only from the patient) ensures an "off-the-shelf" product, ready to use without delay once this therapy is required in the clinic. Finally, NK cells, as indicated by their name, have other mechanisms to eliminate tumor cells, additionally to the CAR pathway. Persistence and development are right now the two major disadvantages of this type of therapy but a big effort is being made to overcome these setbacks and bring CAR-NK products to the clinic.[78,82,85,86]

For this project, we had the luck to work alongside with CAR therapy leaders in CAR-T and CAR-NK development[82,85–88]. You will discover more in the Results section but just in short, it was very interesting to know more about this technology and be able to closely work with such amazing groups.

Immunotherapy in MM

After this extensive explanation of the different types of treatment categorized as immunotherapies, you may still wonder how all this applies to this current thesis project. During the explanations, I have let you know that in the experiments composing this thesis, we have been able to work with different constructs in the MM setting. But what immunotherapies are being used in the clinics? Are they successful in MM? Let's set the basis regarding the disease.

As you may remember, we exposed the treatment options currently available for MM patients, and if you noticed, we have explained the main drugs that so far have improved MM patients' options (i.e. IMiDs and proteasome inhibitors). We also mentioned Daratumumab (a CD38 monoclonal antibody) which is used as the first line of treatment in newly diagnosed MM cases. There is currently a list of targeted immunotherapies for MM. They are: (I) monoclonal antibodies, (II) antibody-drug conjugates, (III) bispecific antibodies, (IV) checkpoint inhibitors, and (V) CAR constructs, both on T and NK cells.

Monoclonal antibodies targeting CD38

CD38 is expressed in a myriad of cells but compared to normal cells, malignant plasma cells have much more elevated surface expression. Thus, it has been some time since it

emerged as a candidate for MM treatment[89]. Daratumumab has been used for MM since 2015. It triggers cell death mainly by several Fc-dependent pathways such as complement-dependent cytotoxicity or antigen-dependent cell-mediated cytotoxicity.[38]

Another anti-CD38 that can be used on MM treatment is Isatuximab which triggers the apoptosis cascade in malignant plasma cells directly, but regardless targets the same element as Dara. There are currently other anti-CD38 being tested in monotherapy and combination exploring different cytotoxic pathways, some of them (i.e SAR442085) seem promising substitutes for Dara, but only time will show which antibodies will be more useful for the patients.[90]

Despite the success of CD38 monoclonal antibodies, there is still an area of improvement and because of that other markers are being studied, such as SLAMF7 (Signaling Lymphocyte Activation Molecule 7) which already has a FDA accepted drug called Elotuzumab and is used in some countries[91,92]. Other targets are CD47 (the "don't eat me receptor" in macrophages), CD138 or CD74, which appears to be overexpressed on CD138+ plasma cells in higher numbers than B-cell maturation antigen (BCMA), or CD38[38].

Antibody-drug conjugates

Although not as successful as anti-CD38 antibodies, antibody-drug conjugates have also been explored in the MM setting. The focus is on Belantamab mafodotin which targets BCMA and delivers MMAF, a cytotoxic drug, towards its target. It was approved by the FDA in 2020, and it is recommended for RRMM patients after the fourth line of treatment. Other antibody-drug conjugates target CD38 or CD74 with varying grades of success[93].

Bispecific antibodies

In MM, there are currently four strategies focused on bispecific antibodies, such as bringing together T cells and MM cells. All of them target CD3 with a MM receptor. The most successful combination so far is the Anti-CD3/BCMA bispecific antibodies with BiTE® and DuoBody® currently in clinical trials. Other combinations being explored in MM setting are CD3/GPRC5D, CD3/FcRH5 and of course, CD3/CD38 bispecific antibodies.[71]

Immune checkpoint blockers

Immune checkpoint inhibitors in MM do not seem as successful as in other malignancies (such as melanoma). Despite this, anti-PD1 neutralizing antibody (which you already know is one of the main inhibitory axes acting on immune effector cells) has been deeply studied in the MM setting. The two drugs most studied are Nivolumab and Pembrolizumab, and despite all efforts, the clinical trials have shown so far little improvement, especially in monotherapy[90]

CAR constructs

Finally, what may be the most successful immunotherapy strategy in MM apart from anti-CD38 antibodies? Anti-BCMA CAR-T cells completely changed the paradigm of MM. CAR-T therapy improves the survival and prognosis of patients with MM and its function has changed the options for RRMM cases[75]. Despite this, CAR-T treatment needs more optimization, as addressing the severe side effects is crucial for the patients being treated. As a side note, several groups have started focusing on anti-BCMA CAR-NK constructs, as they bring some advantages compared to CAR-T constructs. CAR-NK cells can be synthesized from allogenic sources, do not secrete inflammatory factors such as IL-1, and can rely on their natural ability to kill as an additional path to cause cytotoxicity. Another fact that needs optimization is that until now this kind of treatment has been set for late lines of treatment as a last-option attack.[78,90] CAR constructs rely on the fitness of the effector cells already on the host, is because of that advancing these strategies towards early lines of treatment may benefit all the parties involved. Despite this, the time-consuming process, the side effects of the treatment, and other aspects such as the short persistence of the constructs in the host make this area a very promising prospect that still needs research.

Using the current guidelines for MM treatment, the most novel immunotherapeutic options are relegated towards late lines of treatment (after the patients have become refractory to currently available treatments) and always in the setting of clinical trials, as it is complicated to show the effectiveness of this drugs upfront. Despite this, in Spain, several MM physicians are making a point towards treating with the most effective technique standard risk patients and including directly high-risk patients in the most novel clinical trials in order to fight when there are options still available for them.[19]

New strategies for CAR technologies

As successful as immunotherapy is, CAR constructs are maybe the best studied, and where more efforts are destined to optimize the treatment and eliminate thesis-associated toxicities, which sadly, are several (**Figure 12**).

Several approaches have been taken towards improving the fitness and manufacturing of CAR-T cells. There is also research towards decreasing the time to develop the CAR cells and improving the transduction efficiency. All in the same direction, decrease the time to have a final product and increase by this way the chances of the patient receiving the product. Although there is an important factor and is the ability of the cells to expand rapidly, persist in the host, and control the disease. For example, there is intensive research on transitioning CAR-T products towards an allogenic origin, similar to CAR-NKs, to benefit from the “off-the-shelf” characteristic that will allow for rapid response once this type of therapy is selected.[94]

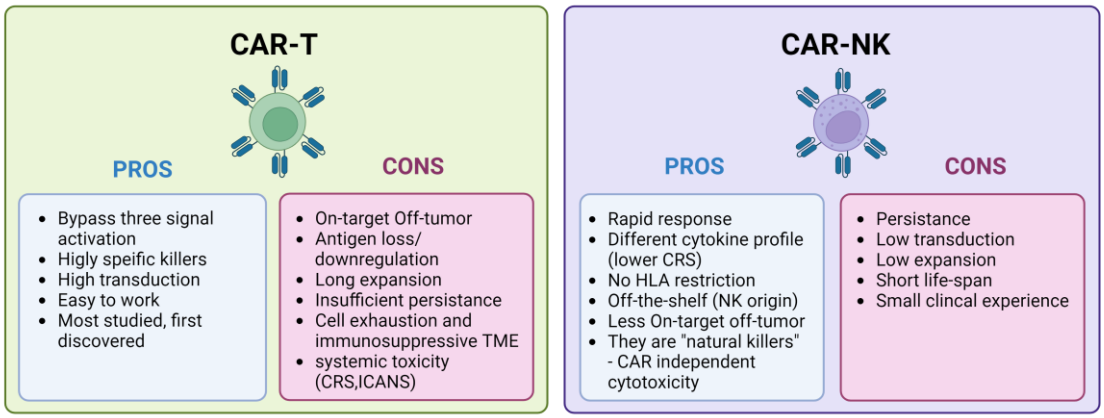


Figure 12. Pros and cons of CAR-T and CAR-NK cells.

In the same way that researchers are focusing on improving the therapies that have been already described and are being used in the clinic, there are also new strategies designed to decrease the toxicity associated with CAR constructs but more importantly, expanding its use towards solid tumors and improving the persistence of the CAR cells in the host. Among the new strategies being investigated, CAR-macrophages have appeared as an alternative to treat solid tumors, as macrophages infiltrate the tumor in greater numbers than lymphocytes and can survive in the immunosuppressive TME (with a pro-inflammatory profile M2 but with a phagocytic capacity nonetheless)[78]. There are already CAR-macrophages in clinical trials, for now for solid tumors overexpressing HER2, but for sure new therapies will appear soon[95].

One problem associated with CAR products, apart from the toxicity they can produce at a systemic level, is the ability of something worse to develop in the host, as already there have been cases of CAR-T cell lymphoma appearing in some cases[96]. A safety switch is being tested for high-risk cases where a lymphoma may appear due to the activity of the CAR. There is already some publication where an inducible caspase 9 has been coupled with Rimiducid which allows it to activate the caspase and destroy the CAR in case of high toxicity or secondary events that may develop because of the treatment[97].

Another crucial aspect of CAR therapies' success is the persistence of the cells in the host. To tackle this issue, armored CAR-cells are being studied. The most successful case so far is the presence of IL-15 on CAR-T cells which increases survival and enhances anti-tumor activity in the cells.[94,98]

In line with other strategies to increase persistence, a new concept appeared some years ago. The modification of the most persistent cell in the hematological system as a way to control CAR persistence in the host, the modification of HSCs.

As a part of my program, I was able to complete a short stay at the Synthetic Biology and Cellular Engineering Group at Dana-Farber Cancer Institute in Boston under Eric L. Smith's

direction. There I was able to participate in top-notch studies and learn about CAR construct development. My work there focused on helping with the development of CAR constructs led by specific NK promoters to overcome CAR-NK weak attributes and create better strategies to improve CAR persistence in the host and guarantee its function.

As was mentioned before, CAR-T and CAR-NK have a list of pros and cons that make them very successful but at the same time highly complicated to deal with. One of the issues that both strategies share is the short persistence that the modified cells have in the host. CAR-modified cells are highly specific constructs with one goal and one objective to fulfill, kill cells. To do that, these cells employ all their resources in the mechanisms prepared to fulfill their objective and because of that, little energy is left to maintain cell fitness over time. Furthermore, these cells are usually found in environments where everything acts against them, low nutrient concentrations are available for intake[50], most of the interactions they establish encourage them to give up[82], and they are not meant to last[78].

Upon induction, CAR cells make a difference, they can eradicate most of the malignant cells in a short period of time and although the secondary effects from the treatment are not ideal, their management has reached a point where it is worth taking on this treatment option as the benefits highly surpass the disadvantages. Despite this, and especially in the context of diseases similar to MM, some clones can resist the CAR attack. By for example, downregulation of the CAR target. With time, as we mentioned a few lines above, CAR cells tend to disappear, even though CAR cells have been found to last for years in some cases, this does not mean that they can be as effective as they were just after induction. Because of that, and other intra-tumoral factors, what we experience is a relapse, where the CAR cells that once were able to fight against the tumor now see themselves overcome by the tumor growth.

To tackle this issue, some groups have decided that maybe the solution resides in modifying those cells that are most persistent in the host, and those are HSCs. But, by modifying HSCs, we will express the CAR constructs in all the cells originating from that cell, and that is not of interest. Furthermore, we will lose the randomness and the diversity that protect us and allow each cell to perform its function correctly. What should be the strategy then? Well, indeed, to modify the HSC, able to differentiate into any cell. But, in the same fashion as certain lineage genes are only expressed when that cell has reached the right stage, the strategy is to make the CAR construct unavailable until and only, if the modified HSC reaches the right mature cell, which in the case we were studying, was a mature NK cell.

During my stay, I was able to learn the different strategies that scientists follow to generate a CAR-NK cell. My work was focused on optimizing the CAR insertion by lentivirus transduction. The strategy followed was to generate an NK promoter (exclusive

of NK, only expressed in that lineage) so the CAR construct expression would be restricted. Although the theory seems something easy to perform, the reality is that promoters are tricky and like to be expressed in different lineages, which produces the main issue of this strategy which is leakiness into other lineages. Furthermore, we are working with immune cells that change state upon encountering their target and become activated, changing their expression programs and bringing even more variables into an already set of conditions. My work focused on testing several activation markers to differentiate between inactivated and activated cells, finding the right activation molecule, and testing the leakiness of the construct. As this part was developed separately from the project, the results have been gathered under **Annex I.**

Epigenetics or how to mess with everything without breaking the strand

Epigenetics describes all the heritable changes in the DNA that do not change its code and are reversible[99]. However, they do not have to be able to be transmitted to the inheritance and they can be quite permanent.

What are we talking about then? We refer to everything that can modify gene expression without incorporating errors in the genome. These biochemical/conformational changes are an important part of cell adaptation to environmental changes. They are also very important in biological processes, such as cell differentiation and cell lineage commitment (as a neuron should not express muscle cell genes). Indeed, all these states are reversible, although to avoid misconceptions, set cells toughly silence genes (the word used by the epigenetic field when referring to something not expressing due to epigenetic action) that are not of interest.[100,101] Epigenetics is an attractive environment due to the "easiness" of changing from one form to another and the capability of rescuing the expression of (wrongly) silenced genes in a malignant environment.

Epigenetic mechanisms

Is common to treat epigenetics as a group holding the same identity, but we are in front of several different changes that ultimately affect gene expression (**Figure 13**). Here we will briefly describe some of the best representatives for the group so that together we can set the background from which this thesis project started.

The first and better-known epigenetic mechanism is DNA methylation. This occurs when a methyl-transferase enzyme incorporates into a cytosine a methyl group on its fifth ring[99]. You may wonder, are all cytosines in the DNA chain eligible for this modification? And of course, they are not. Only cytosines forming a dimer with guanine (CpG) are candidates to receive this mark, and even in that situation, not all of them are methylated. It will depend on the exact location of that CpG. CpGs like to group and it is common to

find them in groups all along the DNA chain. Neighbor CpGs usually behave similarly and receive or do not the mark, together. We refer to them as CpG islands (because we love to refer to the big unknown DNA chain as a big ocean). CpG islands are located on non-coding DNA regions and usually are concentrated either on gene bodies or on promoter regions (usually, the neighboring part to the Transcription Starting Site (TSS) of a gene, which controls transcription machinery attachment). If the promoter region of a given gene is methylated, that given gene, most likely, cannot be expressed and thus, it is silenced. Unmethylated CpGs allow transcription machinery to attach to the DNA chain and read the given gene information.[101,102]

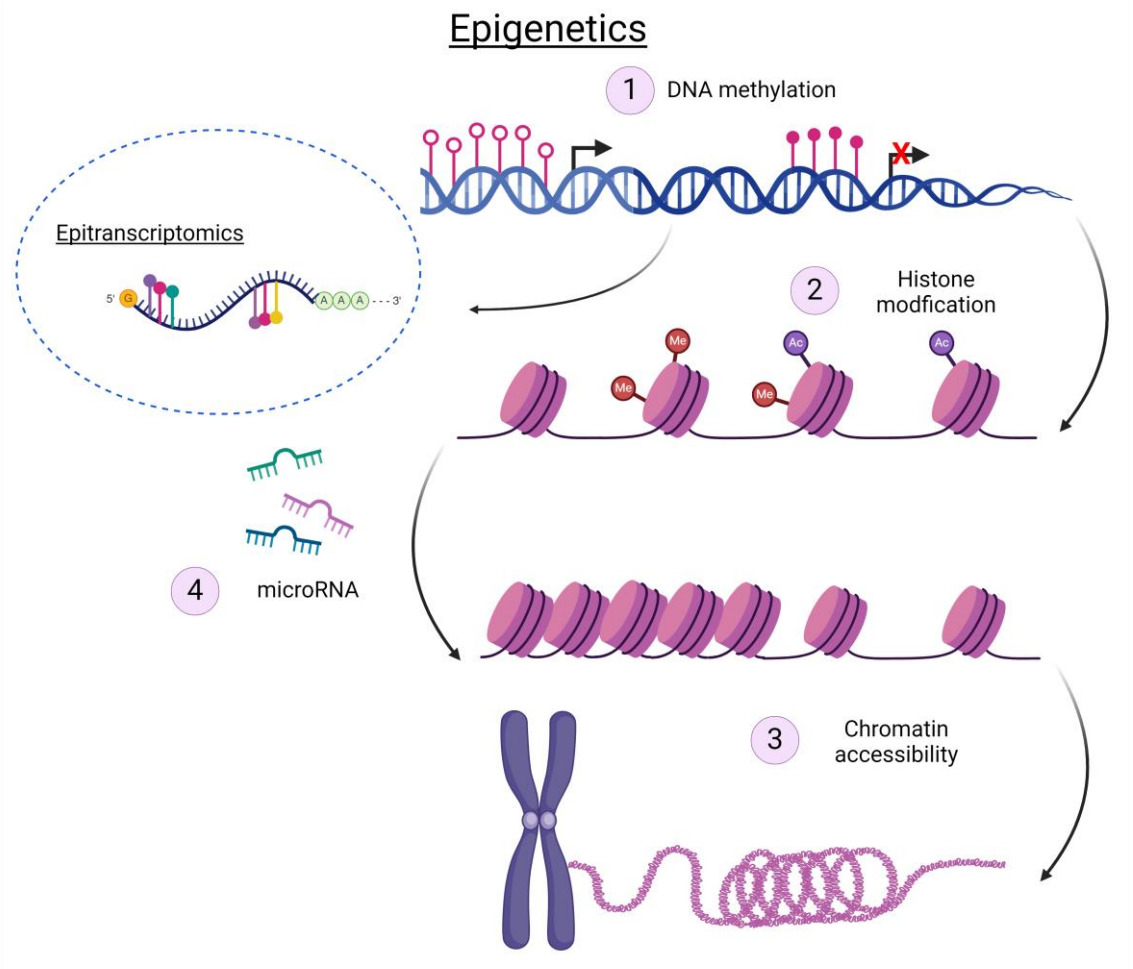


Figure 13. Main epigenetic mechanisms involved in the regulation of gene expression. There is a part of epigenetic regulation that consists of the biochemical modification of different RNA (coding and non-coding) which is considered separate from the main theme and has been given its own name, RNA modifications are considered epitranscriptomics.

DNA methylation can be actively (TET enzyme family) or passively (through replication) be lost. The balance between methyltransferases (DNMTs family) and demethylation machinery maintains cellular function and avoids malignant transformation[102].

As with everything in biology, this system is not exclusive and is rather redundant, as we have other systems to control gene expression above DNA modification. The second mechanism is histone modification. Histones are the proteins located all along the DNA chain that serve as anchors and help with DNA organization. We have known since some years ago that the interaction with the DNA is not completely random and that the number and type of biochemical modifications in these proteins have a strong effect on DNA access, thus interfering with or facilitating gene transcription[99,101]. Since this thesis project did not explore histone modifications, we will only mention that there are several different histone proteins and several different histone modifiers. Among the possible changes, we know now that histone may be acetylated, methylated, ubiquitinated, and phosphorylated among others[103]. I hope with this at least to have picked your curiosity.

Chromatin organization plays an important role in DNA accessibility and is thus considered another epigenetic mechanism. Through euchromatin and heterochromatin conformations, DNA becomes or not accessible. Through its folds and interactions, distant DNA regions can find each other and be regulated. Through its loops and TADs (Topologically associating domains), transcription machinery can access genes ready and primed to be expressed in a matter of seconds.[104]

Finally, this listing will finish with the last type of epigenetic regulation which is non-coding RNA. There are several types of non-coding RNA, but scientists only consider microRNA as part of the epigenetics family. MicroRNA are small fragment of around 20 base pairs that bind messenger RNA and can have different effects depending on the type and the site of the RNA they bind, usually interfering with its expression. Every year new microRNAs are described, and it has been broadly demonstrated their important role in health and disease.[99,105]

Epigenetics in immunity

At this point of the introduction, this idea may have already been said enough, but just in case, immunity is a tightly regulated process. Thanks to technological advances and the job of several researchers, we know now that epigenetics plays a central role in immune cell function and differentiation process[106]. Here I will just mention some cases of how this process occurs, nevertheless, this is another important fact demonstrating how epigenetics participates in every aspect of our bodies. Concerning immunity, epigenetics has been best studied in lymphocytes: T cells, B cells, NK cells, and macrophages.

The maturation process of T cells, from naïve to active to memory differentiation (passing through exhaustion phenotypes) is led by epigenetic changes. From DNA methylation control to go from naïve to effector T cell, we also see the effect on demethylation and the uprise of expression of exhaustion in those same cells. Histone modifiers also play a crucial role in the balance between long-term memory T cells, or exhaustion phenotypes

where the state of methylation and acetylation of a given set of histones (H3K27 or H3K79 marks) have a deep influence on them.[106,107]

For B cells, we know that HDAC (histone deacetylases) rule over B development and GC formation, crucial steps for the correct maturation of B cells. As explained in the corresponding section, the B-cell maturation and differentiation process requires rapid changes in transcription factor expression and proteins. To be able to apply the changes when needed, epigenetic mechanisms (especially DNA methylation and microRNA regulation) have been found to have a crucial role during the different stages of B maturation.[100,101,105]

As mentioned in previous sections, NK cells are part of the innate immune response and as such, their function is governed by more simple mechanisms. NK activation is led by the balance of positive and negative signals given by the interacting cell, well, DNA and histone methylation regulate NKG2D ligands which are responsible for inhibiting NK cell activation. Another example is how EZH2-mediated H3K27 methylation downregulates the expression of such markers.[100,101,105]

Finally, in macrophages, myeloid cells which shine by their plastic capacity and the ability to take different roles depending on the influence of the microenvironment, methylation status of key genes, and acetylation of histones 3 and 4 are important elements in the fate of monocytes which will derive towards M1 (inflammatory profiles) or M2 phenotype (Immunosuppressive) depending on its methylation status.[106]

Epigenetics and cancer

Since this thesis project is developed in the field of malignant transformation, we could not have forgotten to talk a bit about what role plays epigenetics on cancer.

It has been widely described and demonstrated that virtually all types of cancer follow the same rule regarding DNA methylation status. Malignant cells are known to hypermethylate the promoter regions of key tumor suppressor genes and hypomethylate oncogenes to increase their survival[100,101,105]. Furthermore, together with the increasing number of mutations registered as biomarkers for malignant transformation, generalized hypomethylation on the cell increases significantly DNA instability, increasing the chances of new beneficial mutations appearing. In hematological malignancies, it is common to find in a big percentage of patients mutations in DNA methyltransferases (as is the case of AML, where DNMT3 appears mutated in 40% of the patient population) and also on DNA demethylases (TET2 is mutated in MDS patients 30%).[101]

B-cell malignancies, tumors originating from any of the stages of B-cell differentiation, are known to have dysregulated expression on histone modifiers in a large percentage of

patients. For example, HDAC7 appears downregulated in pro-B-ALL cases and thanks to basic research now we know that its lack of expression contributes to arresting B cells on the pro-B stage and cells cannot proliferate to pre-B[108,109]. Another case will be the sirtuin family, another family of histone deacetylase, have an important role in maintaining the correct function of healthy cells and its mutations give rise to lung and hepatocellular cancer[110].

Finally, as mentioned before, microRNAs have strong implications for cell cycle, proliferation, and differentiation. It is why dysregulations in microRNA biology have been proven to lead towards tumorigenesis (among many other types of disease, such as autoimmunities or other immune disorders). To name an example, miR-126 is known to be dysregulated in several malignancies, in the case of AML its overexpression inhibits apoptosis pathways in leukemia cells.[101,108]

Aberrant methylation in MM

MM is a disease originating from plasmatic cell malignant transformation and although there are no two identical cases of cancer, here we will mention some of the characteristics observed in newly diagnosed cases and what happens further down disease development, once the patient relapses.

As said before, B cell development is a marked path where both genetic and epigenetic mechanisms rule over B cell progression and maturation. In the case of plasma cells, the B cell transcriptional program changes towards the plasma cell-specific program, where antigen presentation is relegated to antibody production and long-term endurance. In newly diagnosed MM, the changes are already evident. During MGUS and SMM phases of the disease, genome instability starts to increase the number of translocations associated with the disease, as global methylation levels go down. It has been deeply characterized how two important transcription factors on the plasma cell differentiation are silenced through promoter hypermethylation, Suppressor of Cytokine Signaling 1 (SOCS1), and Death Associated Protein Kinase (DAPK). Due to MGUS and SMM being considered premalignant conditions (despite the evidence showing the start of accumulation of errors), there is little work done on histone modifications for these conditions. [111]

Going down the malignancy progression, in relapsed cases, hypomethylation is strictly increased over time, further increasing the genome instability of the malignant cells and increasing the chances of acquiring better mutations and best-fit translocations. Here, the malignancy behaves as many other cancers, hypermethylation tumor suppressor genes and hypomethylating oncogenic regions which will increase the chances of survival. At this stage is also common to have epigenetic modifiers (DNMTs, TET enzymes, and histone modifiers) with several loss of function mutations. Studies regarding histone

modifications demonstrate a marked increase of H3K27 acetylation and H3K4 trimethylation leads towards activation of specific tumor-associated genes, and repression marks like trimethylation of the H3K27 or trimethylation of the H3K20 increases double-strand breaks on the DNA.[106,111,112]

And finally, how is everything in these sections related to the start of this project? Well, several sources validate and demonstrate the global hypomethylation upon malignant transformation and the specific hypermethylation of promoter regions of tumor suppressor genes. And what happens with immune-related genes? Malignant cells take advantage of every aspect available to escape immune detection, including the dysregulation of expression in presenting machinery a.k.a. immune checkpoint proteins.

Lately, more and more evidence has been observed in different solid tumors regarding the hypomethylation of promoter regions of genes related to immune checkpoint events[112]. And what happens in hematological malignancies? Do they follow this incongruency also? And is MM like all the others?

After these questions, this thesis project was born and with the background set, we can finally move towards the origin of the history, or rather, with our objectives.

Objectives

Objectives

Hypothesis

Epigenetics influence cancer and recently epigenetic plasticity has been included as a new hallmark of cancer described in the latest published work of Hanahan[113]. Growing scientific evidence has pointed out the importance of epigenetic regulation in immunology106 and the important role of immune checkpoint regulation in tumor recognition and clearance. As observed in clinical settings, therapeutic strategies for solid and hematological tumors often build upon each other's successes. Solid tumors have shown greater therapeutic success in the context of immune checkpoint targeting. This is mainly due to solid tumors' well-defined TME, facilitating more effective inhibition of pathways regulating immune cell activity. Based on this, we hypothesized that an epigenetically regulated gene plays a key role in immune checkpoint regulation, which could be critical in hematological malignancies. Despite the less distinct TME in these cancers, the mechanisms of immune cell recognition remain significant. With this foundation, the following objectives were established for this thesis project.

Objectives

We have decided to divide this thesis project into three different objectives.

The overarching objective of this thesis was to study the regulation of PVR and its relation to cytotoxic activation in the context of MM. The specific objectives of this thesis are:

- Objective I: To characterize PVR's epigenetic regulation and expression in multiple myeloma.

- Objective II: To evaluate the role of PVR in cytotoxic cell function and its impact on immunotherapy.

- Objective III: To study the effect of PVR expression alteration and its relationship with other immune checkpoint markers.

Materials and Methods

Materials and methods

In this section, you will learn the protocols we followed to develop this thesis project. Probably this will also be the least-read section, as many of the people involved in science already have mastered most of these techniques. Regardless, for the sake of a good discussion, I recommend you go through them. Let's go together through what may be the most interesting part of a project: How the magic in the laboratory occurs.

Cell lines

RPMI-8226 and HEK-293T were acquired from the American Type Culture Collection (ATCC) and AMO-1, KMS-12-BM, JJN-3, EJM, and ATN-1 from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). PVR models were generated for MM cell lines. Overexpression models were generated for AMO-1 and KMS-12-BM trough lentivirus transduction of lentiviral plasmid (pLVX-IRES-ZsGreen from TakaraBio) containing ampicillin resistance cassette and ZsGreen as tracker. For PVR depletion, short-hairpin RNA interference was used through lentivirus production and pLVX-shRNA2 infection (Takara Bio). Cell lines were cultured in RPMI 1640 media (Gibco) supplemented with 10% v/v Fetal Bovine Serum (FBS) (Gibco) and 1% v/v Penicillin/Streptomicyn antibiotic mixture (Biowest). For JJN-3 and EJM, Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) was used, supplemented with 20% v/v FBS and 1% v/v of antibiotic mixture. All cell lines were maintained in incubators at 37°C in an atmosphere of 5% carbon dioxide and 90% humidity. Cells were tested regularly for the absence of mycoplasma.

For the DNA demethylation experiments, AMO-1 and KMS-12-BM were treated with 5-aza-2'-deoxycytidine (Sigma) for 120h at a final concentration of [1 µM], with medium renewal after 48 hours.

Cell line characterization was performed by consulting data from the commercial provider, from the Keat Labs web page (https://www.keatslab.org/myeloma-cell-lines/hmcl-characteristics), and from Guide to LL Cell Lines[114].

DNA methylation analysis

DNA methylation microarrays

CpG islands were identified *in silico* using the USCS genome browser (www.genome.ucsc.edu/) and DNA methylation microarray Infinitum HumanMethylation450 Beadchip kit or Infinium MethylationEPIC 850k BeadChip Kit (Illumina). Normalized β-values were used for the analysis which range from 0 to 1. Cell lines were considered hypermethylated when the average β-value of the selected CpGs in its promoter region (1500 bp upstream of the TSS of the gene) was higher than 0.66.

In human primary tumors, this number is reduced to 0.33 due to the potential contamination of the sample by normal adjacent tissues.

DNA extraction

For *in vitro* methylation validation, bisulfite PCR sequencing was performed on the wild-type cell lines.

Genomic DNA was extracted from cell pellets. First cells were incubated in DNA lysis buffer (10 mM Tris pH 8.0, 100 mM NaCl, 5 mM EDTA, 1% SDS, and 10 mg/mL proteinase K) overnight at 37ºC. Cell lysates were added 5M NaCl and centrifuged for 15 minutes at maximum speed (~4000rpm). Supernatants were collected and purified. For DNA precipitation isopropanol incubation was performed followed by 70% ethanol washes. Air-dried pellets were then resuspended in nuclease-free water and stored at -20°C. DNA concentration was measured using Nanodrop.

Bisulfite-sequencing PCR (BSP)

Genomic DNA was converted using EZ DNA Methylation-Gold Kit (Zymo Research). By bisulfite incubation, unmethylated cytosines are converted into uracil and later amplified into thymine, which allows the study of the methylation status of a known CpG site. The converted DNA was amplified using primers targeting promoter regions of the genes of interest (primers are listed in **Table 1**). BSP products were purified using a 1.5% agarose gel and NucleoSpin Gen and PCR Clean-Up (Macherey-Nagel) and then cloned into pGEM®-T vector (Promega). Plasmids were transformed into competent bacteria and incubated overnight. At least eight clones were picked and sequenced. Sequencing PCR was performed using BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems) and sequenced in a 3730 DNA Analyzer (Applied Biosystems). The reads were aligned using BioEdit software and the methylation frequency was represented using the software BSMap package for R.

Pyrosequencing

Bisulfite-converted DNA was used as a template to amplify by PCR the promoter region of PVR from primary samples (**Table 1**). The amplification was then processed and analyzed using PyroMark Q48 Autoprep System (Qiagen).

Expression analysis

In silico correlation

For correlation analysis, the COSMIC cell line database (https://cancer.sanger.ac.uk/cell_lines) was used and paired with the methylation information from our database.

Table 1. List of primers employed for methylation analysis.

Bisulfite PCR	
Primer	Sequence (5'-3')
PVR_BSP_Forward	GTTTTTTTATTTGGAATGTGG
PVR_BSP_Reverse	CTTCAAACTCCAAACAAATAACT
Pyrosequencing	
Primer	Sequence (5'-3')
PVR_pyroseq_primer1	GTTTTATTAAGAGTTGGAATTTTAG
PVR_ pyroseq_primer2	CCTACCCCTACCCAAATCTCTCCC
PVR_pyroPCR_Forward	AGTTTAGGTTGAGTGGAAGGATAGT
PVR_pyroPCR_Reverse	AACCACCCAAACTAACCC

RNA extraction, retro transcription, and quantitative PCR

Total RNA was extracted from cell pellets using Maxwell simplyRNA Tissue Kit (Promega) and Maxwell instrument (Promega). Extracted RNA concentration was measured using NanoDrop One (Thermo Fisher Scientific). 2µg of RNA were retrotranscribed to cDNA using RevetAid RT (Thermo Fisher Scientific) and adding Oligo(dT) primers to enrich coding RNA retro transcription following manufacturer's instructions. Quantitative (qPCR) was performed to measure the mRNA expression of the genes in the corresponding samples (primers listed in **Table 2**). SYBR Green PCR Master Mix (SIGMA) and 384-well plates (Applied Biosciences) were used for the analysis. 1:100 dilution of cDNA was used. Analysis was performed using QuantiStudio 5 PCR machine (Thermo Fisher Scientific) and data was analyzed by 2^-(ddCt) method using GAPDH as the control gene. Technical triplicates were analyzed of triplicate biological samples. The graphs represent mean values from three independent experiments.

Table 2: Primers used for qPCR.

Quantitative PCR	
Primer	Sequence (5'-3')
PVR_qPCR_Forward	CTACACCTGCCTGTTCGTCA
PVR_qPCR_Reverse	GGTCTGAGTGCCAGGTGATT
GAPDH_qPCR_Forward	GAAGGTGAAGGTCGGAGTC
GAPDH_qPCR-Reverse	TGGACTCCACGACGTACTCA

Protein expression by flow cytometry

PVR protein expression was studied by flow cytometry. FACS buffer (0.5% BSA, 0.05% NaN_3) was used for the washes and the incubation with the chosen antibody. The antibodies used were PE anti-human PVR Antibody (BioLegend). After removing the media, cells were washed once and centrifuged for 5 min at 1200rpm. Then, cells were incubated with a solution of 1:200 of the antibody for 15 min in the dark at room temperature. After that, another wash was performed before resuspending the cells in FACS buffer. Flow cytometry data were acquired using FACSCanto™ II (BD Biosciences)

and analyzed with FlowJo v10.7.2. The representative flow cytometry strategy is shown in **Figure 14.**

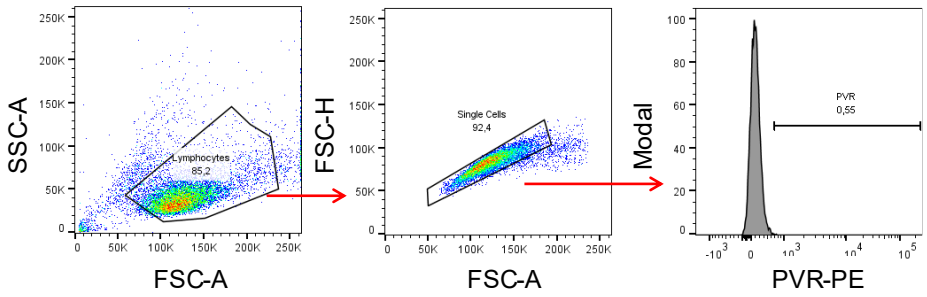


Figure 14. Gating strategy for flow cytometry experiments assessing PVR levels. Single cells were plotted from all events as indicated. Then, tumoral cells were selected as live single events. Finally, tumoral cells were stained by PVR-PE (for wild-type cells) or PVR-APC (for cell models).

Primary samples

Buffy coats from healthy donors from the Banc de Sang i Teixits (Blood and Tissue Bank, BST). Gene expression and clinical data from 776 newly diagnosed MM patients were obtained from publicly available CoMMpass project[115]. Restricted clinical data from a subset of patients (n=615) was evaluated by Dr. Aguirre and Dr. Prósper. Frozen DNA and RNA were kindly provided by Dr. Martínez-López (Madrid's cohort) and Dr. Gutierrez (Salamanca's cohort). Written informed consent was received from participants before enrollment as approved by the ethics committee of each institution.

Healthy bone marrow from four donors was obtained from the IJC-Campus ICO-GTP Biological Sample Collection and were analyzed to obtain the methylation status in normal controls of CD138+ plasmatic B cells.

Methylation from MM primary cases

The already normalized Infinium HumanMethylation450 BeadChip DNA methylation matrix from 104 MM patients (Navarra's cohort) was obtained from Duran-Ferrer et al. [5] at http://resources.idibaps.org/paper/the-proliferative-history-shapes-the-DNA-methylome-of-B-cell-tumors-and-predicts-clinical-outcome, also available in the European Genome-phenome Archive (EGA) under the accession number EGAS00001000841). CpGs from the PVR CpG island of interest were selected and mean methylation values for each patient were used to determine the methylation status of the CpG island.

Survival curves and multivariate analysis

From the publicly available data CoMMpass project (NCT01454297), we obtained clinic information and expression values using R software v. 4.0.3 run in RStudio software and the TCGAbiolinks package. From there we established two groups of patients by dividing

by the median value and establishing a high-expression quartile and low-expression group respectively. We performed a log-rank test, and a Kaplan-Meyer graph followed by a Hazard Ratio and Coefficiency Index analysis to test the impact on overall survival.

Generation of cellular models

Overexpression models

The PVR wild-type cDNA sequence was amplified from RPMI-8226, an expressing unmethylated cell line. The plasmids were altered to incorporate into the full PVR cDNA sequence the EcoRI restriction site and the Kozak sequence to the 5' region of the transcript and a NotI restriction site and a FLAG-Tag to the 3' terminal end. The primers used for this amplification can be found in **Table 3**. For the amplification PCR, Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used. After PCR purification using NucleoSpin Gen and PCR Clean-Up (Macherey-Nagel), the sequence was cloned into the pLVX-IRES-ZsGreen1 vector (Takara Bio) using restriction enzymes and T4 ligase. Vectors were transformed into competent bacteria. DNA was extracted and sequenced for validation before PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific) plasmid preparation. In parallel, empty vector (EV) preparation was being made as vehicle control for the experiments.

For the stable expression of the gene, a third-generation lentivirus system was used. For lentivirus production, co-transfection of HEK-293T cells with the recombinant plasmid, psPAX2 (Addgene) and pMD2.G (Addgene) using JetPrime® Transfection Reagent (Polyplus) was performed. After 72 hours, viral-containing media was collected, filtered, and delivered to AMO-1 and KMS-12-BM cell lines. Spinoculation, centrifugation of the cells at 1000g for 90 minutes, was performed to ensure virus infection. After 5 passages, green cells were purified by cell sorting or clone selection by limiting dilution. The models were validated by qPCR and Western Blot.

For the Western Blots, total protein extracts from cell lines were obtained with RIPA buffer (SDS 0.1%, deoxycholate 0.5%, NP40 1%, Tris-HCl pH 8.0 50 mM, NaCl 150 mM) with cOmplete™ EDTA-free protease inhibitor cocktail and phosSTOP™ (Roche) and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Western Blot was used for the protein analysis detecting β-actin as loading control. Primary and secondary antibodies used for the analysis are listed in **Table 3.**

PVR depletion models

For the genes' knockdowns, four different short-hairpin RNA (shRNA) sequence pairs were designed and cloned into pLVX-shRNA2-ZsGreen plasmid (Takara Bio). For the control construct, a Scramble sequence was used. This sequence is a shRNA that targets MSS2 yeast mRNA which is absent in mammal's genome. Sense and antisense oligonucleotides were created and annealed for 20 min in a descending temperature

ramp from 95°C to 5°C. The oligonucleotides contain the EcoRI target sequence in their 5' end and the NotI target sequence in their 3' end which allows the integration of the shRNA sequence into the plasmid by restriction enzyme digestion. The shRNA sequences are listed in Table 3.

Table 3. Primers and antibodies used for PVR overexpression and depletion.

Overexpression		
Primer	Sequence (5'-3')	
cPVR_a_Fd	AAAAAAACTAGTGCCGCCACCCTGAGCTCCGGGAGCTGGACTCGC AGCGACCGC	
cPVR_a_Rv	AAAAAAGCGGCCGCTCACTTATCGTCGTCATCCTTGTAATCGCCG GAGCCATTACGGCAGCTCTGGTGATGCTCACTCGAGGGACACAG ATGACAGTGCC	
Western Blot		
Antibody	Company	Reference
Anti-PVR	Cell Signaling	D8A5G
anti-rabbit HRP-conjugated secondary antibody	Sigma	A0545
anti-β-Actin HRP-conjugated	Sigma	A3854
Depletion		
Primer	Sequence (5'-3')	
PVR_shRNA_Sense	gatccGCGCAGAACAAATTCGTCCATTCAAGAGATGGACGAATTTG TTCTGCGTTTTTTACGCGTg	
PVR_shRNA_Antisense	aattcACGCGTAAAAAACGCAGAACAAATTCGTCCATCTCTTGAATG GACGAATTTGTTCTGCGCg	

After this, the protocol followed was the same as how we obtained the overexpression constructs.

After lentivirus generation, the cells infected were RPMI-8226, JJN-3, and EJM. Once again, infection was performed by spinoculation and five passes were made before selecting green positive cells. The models were validated by qPCR and flow cytometry using APC anti-human PVR Antibody (BioLegend).

Functional analysis

Cell proliferation assay

Cell proliferation was determined by MTT assay. The optimal concentration of cells was seeded in 96-well plates. On 6 consecutive days, cells were added 10 µL of MTT at 5mg/mL (Sigma-Aldrich) and incubated for 3h at 37°C. After the incubation, 100µL of lysis buffer were added and the cells were incubated for 24h at 37°C. Absorbance at 560nm was measured to assess cell viability using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific).

Cell cycle by propidium iodide (PI)

Cell cycle was analyzed by PI incorporation, following the protocol published by Zbigniew Darzynkiewicz and Gloria Juan in 1997 in *Current Protocols in cytometry*[116]. First, we fixed the cells in ethanol. Briefly, the desired number of cells around 5 million cells, were pelleted and suspended in 5mL of PBS and centrifuged for 6 min at 200xg. Then, the cells were resuspended in half a milliliter of PBS and incorporated into 4.5 mL of 70% ethanol (cold) previously prepared. The cells were left overnight at -20°C. Fixed cells were then washed twice with 5 mL of PBS at 200xg for 5 min. A fresh PI solution was prepared for each experiment (10mL of solution containing 0.1% (v/v) Triton X-100 (Sigma) in PBS, 2 mg DNase-free RNase A, and 200 µl of 1 mg/ml PI). We added 1mL of PI solution to the cells and incubated them in the dark for 30 minutes at room temperature. After the incubations, the cells were acquired with the cytometer and analyzed using FlowJo v10.7.2 software.

Apoptosis

Cell apoptosis was measured using Annexin V (Thermo Fisher Scientific). Cells were washed for 5 min at 200 x g with FACS buffer. Fresh Annexin V staining solution was prepared by mixing Binding buffer 1x (120uL/sample) and 2uL of fluorochrome-conjugated Annexin V for each milliliter of the buffer.

Cells were incubated for 10 minutes before acquisition and then were analyzed using BDFacs Canto II and the software FACsDiva. The results were exported and analyzed using FlowJo v10.7.2 software.

Co-culture experiments

Cell isolation

We obtained buffy coats from healthy donors from the Banc de Sang i Teixits of Hospital Universitari Germans Trias i Pujol. Immediately after receiving the samples, PBMCs were isolated by ficoll density gradient separation using LymphoPrep™ (StemCell Biotechnologies). Buffy coats were diluted with PBS 1:2 and were transferred to a 50mL tube containing around 20 mL of LymphoPrep™ maintaining the different phases. The tubes were centrifuged for 20 min at 2000g and the PBMC layer was aspirated with Pasteur pipettes and washed twice with PBS.

Cells were counted and PBMCs were resuspended in freeze medium at a concentration of 20-50M cells/mL depending on the number of total cells obtained from the separation.

Co-culture experiment

Frozen PBMCs from healthy donors were washed once with PBS and counted. To increase the number of T cells for the coculture experiments, an activation period was performed

before the experiment. PBMCs were seeded in the presence of the control cell line (the empty vector or scramble cell line) on a scale of 10 to 1 (Effector:Target, E:T). Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher Scientific) and 40U/mL of Recombinant Human IL-2 (Peprotech) were added to further potentiate the T-cell activation of the PBMCs. We incubated the coculture for 120h and medium was added, if necessary. You will find a graphical representation of the workflow in **Figure 15.**

To obtain the purified T cells, we used EasySep™ Human T Cell Isolation Kit (StemCell Technologies) to obtain isolated T cells by negative selection.

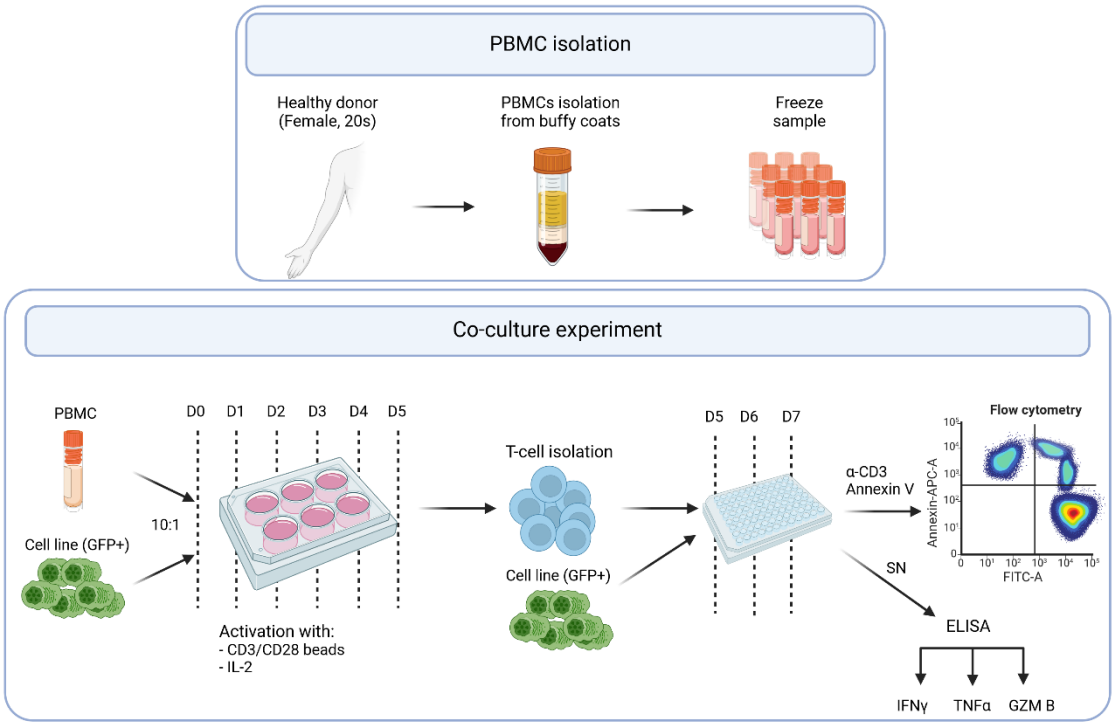


Figure 15. Co-culture workflow representation.

Flow cytometry

PVR protein expression was studied by flow cytometry. FACS buffer (0.5% BSA, 0.05% NaN_3) was used for the washes and the incubation with the chosen antibody. The antibodies used were APC anti-human PVR Antibody (BioLegend). For the analysis, cells were labeled with Pacific Blue™ anti-human CD3 Antibody (BioLegend) and APC Annexin V Apoptosis Detection Kit (BioLegend). Furthermore, we added to all the samples CountBright™ Absolute Counting Beads (Thermo Fisher Scientific) before the acquisition to study the distribution of the population and quantify the tumoral surviving cells. Flow cytometry data were acquired using FACSCanto™ II (BD Biosciences) and analyzed with FlowJo v10.7.2. The representative flow cytometry strategy is shown in **Figure 16.**

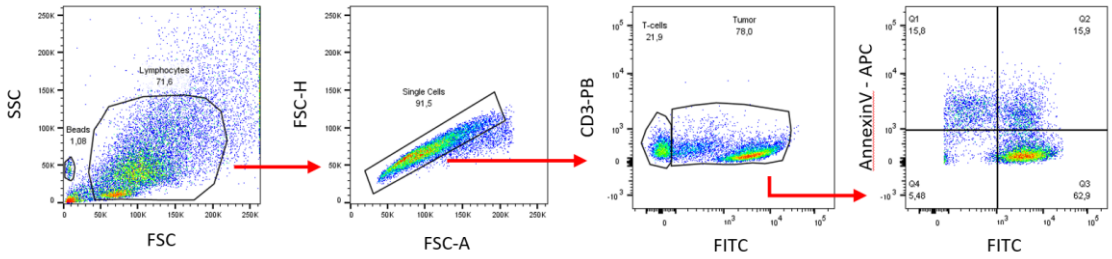


Figure 16. Gating strategy for flow cytometry experiments assessing tumoral cell population survival after coculture experiment. Single cells were plotted from all events as indicated. Then, tumoral cells were selected as GFP$^+$ population vs PacificBlue-A which depicted the T cell population. Finally, tumoral cells stained by Annexin V-APC were plotted against GFP levels to assess survival. Q1 and Q3 quadrants were used for the quantification experiments.

Interferon Gamma (IFNγ), Tumor Necrosis Factor Alpha (TNFα) and Granzyme B (GZMB) ELISA

To validate cytotoxicity as the cause for tumor apoptosis, cytokine ELISAs were performed. At the final point of co-culture experiments, plates were centrifuged for 5min at 300g and 150µL of supernatant was collected and stored for ELISA detection. Human IFN-gamma and Granzyme B DuoSet ELISA kits (R&D systems) were used to detect their respective cytokines in the coculture supernatant following manufacturer instructions. If not stated otherwise, graphics represent biological replicates of four different donors.

Fluorescent microscopy image acquisition and cell quantification

Cellular coculture of JJN-3 cell line and T cells was acquired through optical fluorescence microscopy using an EVOS^{TM} M5000 Imaging System (Invitrogen). Briefly, as JJN-3 already had incorporated GFP reporter gene, T cells were stained using CellTrace™ Violet kit (Thermo Fisher Scientific) following manufacturer instructions. Cells were seeded following the same procedure as the other coculture experiments. Images were acquired at time 0h and 48h. Plates were placed in the microscope, kept at room temperature, and images were captured using a 10x objective and GFP channel fluorescence (470/22 nm excitation filter and 510/42 nm emission filter) for JJN-3 cells and DAPI channel fluorescence (357/44nm excitation filter and 447/60nm emission filter) as it is suitable for CellTrace™ Violet for T cells.

After the acquisition, images were adjusted using Adobe Lightroom Classic software version 12.5, and cell counting was performed using ImageJ software version 1.54h.

T-cell co-culture conditions and TIGIT inhibition

Using purified T cells, we used different effector/target ratios depending on the cell line being evaluated. The cells were incubated at 37ºC and 5% CO_2 for 48 hours. We included 10µg/ml of BMS-986207 anti-TIGIT Neutralizing Antibody (Bristol Myer Squibb). Human IgG Isotype Control (Thermo Fisher Scientific) was used as negative isotype control.

T cell Engager and CAR cells

Co-culture in the presence of T-cell Engager Bispecific antibody

Anti-BCMA/CD3 Bispecific T cell Engager was acquired from BPS biosciences. Following the described system, 10pg/mL of the drug was added after the T-cell isolation and left for 48 hours incubation at 37°C 5% CO_2.

CAR-T co-culture

Anti-BCMA CAR-T cells were kindly provided by Dr. Fernández de Larrea or Dr. Martínez-López. CAR-T cells provided by Dr. Fernández de Larrea were transduced with the ARI0002h CAR [3] and the one from Dr. Martínez-López with a BCMA-CAR containing the scFv sequence of the anti-BCMA antibody J22.9, CD8a receptor hinge and transmembrane domain, followed by 41BB co-stimulatory domain and CD3ζ chain. From frozen CAR-T samples, cells were thawed and left for 48 hours in RPMI 1640 media supplemented with 10% human AB serum, 1% Penicillin/Streptavidin mixture, and 100U/mL of human IL-2. The co-culture was performed following the same conditions as stated previously at a ratio of 1:4 (E:T) for 24 hours. Results were obtained by flow cytometry and ELISA following the protocols described above.

CAR-NK co-culture

Anti-BCMA CAR-NK were kindly provided by Dr. Martínez-López. In the first two weeks of the experiment, they generate and expand CAR-NK cells from healthy donor peripheral blood samples by transduction with the same BCMA-CAR lentivector [4]. The cells were analyzed by flow cytometry to evaluate the levels of CAR expression, and they were sent to our laboratory for the experiment on an overnight 4°C journey. Upon reception, cells were incubated in RPMI 1640 media with 10% human AB serum and 1% Penicillin/Streptavidin solution. After 24 to 48 hours of acclimation, the cells were directly seeded for the coculture. The coculture was performed following the same conditions as stated previously at a ratio of 1:8 (E:T) for 24 hours. Results were obtained by flow cytometry and ELISA following the protocols described above.

Immune checkpoint expression panel

qPCR panel

In order to determine the expression of different immune checkpoint markers, we analyzed both pre-activated T cells and activated ones in the same way that we studied the expression of 30 genes in our MM cell line models. Primers are listed in Table 4-5. Briefly, CD138+ cells were sorted from peripheral blood of a healthy donor. CD138+ cells were sorted using Brilliant Violet -510™ anti-human CD138+. Cells were pelleted and the same protocol as in expression analysis was followed for RNA extraction and RT-qPCR expression analysis.

Correlation matrix

For the correlation analysis, expression information from the qPCR panel or RNA-seq data from the CoMMpass project was used, from this project STAR-counts were used. For methylation data, β-values from EPIC array were used. In the array, 180 cell lines were analyzed. For each correlation, we calculated Spearman's correlation for all pairs using R software and R studio to calculate and visualize the data. FDR was used to calculate adjusted P values. To identify clusters of expression or methylation we next applied in the script a hierarchical clustering.

Table 4. Primer list for T-cell immune checkpoint markers expression.

T cell panel			
Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
TIM3_qPCR_Forward	GGAATACAGAGCGGAGGTCG	GZMB_qPCR_Forward	ACTGCAGCTGGAGAGAAAGG
TIM3_qPCR_Reverse	AGGGACACATCTCCTTTGCG	GZMB_qPCR_Reverse	TTCGCACTTTCGATCTTCCT
LAG3_qPCR_Forward	ACCCCATCCCAGAGGAGTTT	IFNg_qPCR_Forward	TGACCAGAGCATCCAAAAGA
LAG3_qPCR_Reverse	GTCGCCACTGTCTTCTCCAA	IFNg_qPCR_Reverse	CTCTTCGACCTCGAAACAGC
CTLA4_qPCR_Forward	CCGTGCCCAGATTCTGACTT	TNFa_qPCR_Forward	CTATCTGGGAGGGGTCTTCC
CTLA4_qPCR_Reverse	ACATTCTGGCTCTGTTGGGG	TNFa_qPCR_Reverse	GGTTGAGGGTGTCTGAAGGA
CD28_qPCR_Forward	ACAATGCGGTCAACCTTAGC	CCL5_qPCR_Forward	GAGGCTTCCCCTCACTATCC
CD28_qPCR_Reverse	ACAGTTGAACCCCGTTTTTG	CCL5_qPCR_Reverse	CTCAAGTGATCCACCCACCT
ICOS_qPCR_Forward	GGACCATTCTCATGCCAACT	IL2_qPCR_Forward	GAATCCCAAACTCACCAGGA
ICOS_qPCR_Reverse	AAAGGCTGCACATCCTATGG	IL2_qPCR_Reverse	ATGGTTGCTGTCTCATCAGC
CD137_qPCR_Forward	CACTCTGTTGCTGGTCCTCA	TIGIT_qPCR_Forward	CGTGAACGATACAGGGGAGT
CD137_qPCR_Reverse	CACAGGTCCTTTGTCCACCT	TIGIT_qPCR_Reverse	ACTGCTGTGCAGATGACCAC
PD1_qPCR_Forward	GTGCCTGTGTTCTCTGTGGA	CD96_qPCR_Forward	AACCAGCCCAATCAGACAAC
PD1_qPCR_Reverse	CCAAGAGCAGTGTCCATCCT	CD96_qPCR_Reverse	GGTTGGGTGTCAAGGGTAGA
OX40_qPCR_Forward	CCTCAGAAGTGGGAGTGAGC	DNAM1_qPCR_Forward	CTTTCGGAATGCCTCTGAAG
OX40_qPCR_Reverse	AGATTGCGTCCGAGCTATTG	DNAM1_qPCR_Reverse	CTGGATCTTTTCCCACCTCA
CD27_qPCR_Forward	CAGCCCACCCACTTACCTTA	MKI67_qPCR_Forward	GGGCGAAGTTCACAGTCAAT
CD27_qPCR_Reverse	TCCTTCGTTGATGGAGGAAC	MKI67_qPCR_Reverse	CTCCTTCACTGGGGTCTTGA
CD40L_qPCR_Forward	AAGCCAGTTTGAAGGCTTTG	FasLG_qPCR_Forward	ATGGTTCTGGTTGCCTTGGT
CD40L_qPCR_Reverse	TCAGCTGTTTCCCATTTTCC	FasLG_qPCR_Reverse	GCATCTGGCTGGTAGACTCTC
CD47_qPCR_Forward	TATTGCGGCGTGTATACCAA	IL10_qPCR_Forward	GTTGCCTGGTCCTCCTGACT
CD47_qPCR_Reverse	TCTCCAAATCGGAGTCCATC	IL10_qPCR_Reverse	CACTCTGCTGAAGGCATCTCG
CD107a_qPCR_Forward	CTGCCTTTAAAGCTGCCAAC	CD107b_qPCR_Forward	GTGCAACAAAGAGCAGACTGT
CD107a_qPCR_Reverse	TGTTCTCGTCCAGCAGACAC	CD107b_qPCR_Reverse	CGCTATGGGCACAAGGAAGT
PRF1_qPCR_Forward	AGTCTGGTTCCCATGAGGTG	FcgR2B_qPCR_Forward	GTGGACAGCTGTGCTATTCCT
PRF1_qPCR_Reverse	TGTGAGAACCCCTTCAGTCC	FcgR2B_qPCR_Reverse	GAATGGAGTCGCTCTCAGGG
BTLA_qPCR_Forward	CCCTGACCTTTGTTTCAGGA		
BTLA_qPCR_Reverse	ATGGTCCCTGTTGGAGTCAG		

RNA-sequencing analysis

Total RNA was extracted from cell pellets following the methods described previously. Total RNA from 3 biological replicates from all the cell lines, wild type and models, were sequenced. Whole transcriptome sequencing was performed in order to examine the

gene expression profiles using GRCh37 as reference. Libraries were prepared with TruSeq Stranded Total RNA Library Prep Gold Kit (Illumina) and sequenced using the TruSeq Stranded Total RNA Reference Guide in Illumina Platform. Paired-end reads were prepared from around 150 base pairs each. An average of 60 million pairs of 150bp paired end read per sample were generated.

Differential expression was analyzed using DESeq2 package (v1.16.1)[117] with default options, in R (version: 4.3.2). Genes were considered to be differentially expressed with an absolute log2 fold change bigger than 1 and an FDR adjusted *P* value < to 0.01.

Table 5. Primer list for MM cell line model immune checkpoint markers expression.

Tumor panel			
Primer	**Sequence (5'-3')**	**Primer**	**Sequence (5'-3')**
PVRL2_qPCR_Forward	CGCTACCCTCCTGAAGTGTC	**CD70_qPCR_Forward**	GACACACTCTGCACCAACCT
PVRL2_qPCR_Reverse	CTGTGCAGACGAAGGTGGTA	**CD70_qPCR_Reverse**	TAATCAGCAGCAGTGGTCAGG
CD276_qPCR_Forward	CGTGTGCTGGAGAAAGATCA	**OX40L_qPCR_Forward**	AGGCCAAGATTCGAGAGGAAC
CD276_qPCR_Reverse	GGAGCTGTAGGGAGGGGTAG	**OX40L_qPCR_Reverse**	TGGGAAGTGAGGATGAAACC
PDL1_qPCR_Forward	CGAAGTCATCTGGACAAGCA	**IDO1_qPCR_Forward**	GCGCTGTTGGAAATAGCTTC
PDL1_qPCR_Reverse	ATTTGGAGGATGTGCCAGAG	**IDO1_qPCR_Reverse**	ATGTCCTCCACCAGCAGTC
PDL2_qPCR_Forward	CAGCAATGTGACCCTGGAAT	**ICOSL_qPCR_Forward**	GTTGGCTGTGATCCTGGAAT
PDL2_qPCR_Reverse	GGACTTGAGGTATGTGGAACG	**ICOSL_qPCR_Reverse**	GAAGAGCCTGGTGCTGAATC
CD80_qPCR_Forward	AGGGAACATCACCATCCAAG	**GAL9_qPCR_Forward**	GTGTGAAGCTCACTGCCTCA
CD80_qPCR_Reverse	TGCCAGTAGATGCGAGTTTG	**GAL9_qPCR_Reverse**	GGATGATGAGAGGACCCAGA
CD86_qPCR_Forward	GTATTTTGGCAGGACCAGGA	**CD40_qPCR_Forward**	GCAGGCACAAACAAGACTGA
CD86_qPCR_Reverse	ATTCCTGTGGGCTTTTTGTG	**CD40_qPCR_Reverse**	TCGGGAAAATTGATCTCCTG
HVEM_qPCR_Forward	CCACTGGGTATGGTGGTTTC	**BCMA_qPCR_Forward**	GGGCAGTGCTCCCAAAATGA
HVEM_qPCR_Forward	TCACCTTCTGCCTCCTGTCT	**BCMA_qPCR_Reverse**	CACTGAATTGGTCACACTTGCAT
CD137L_qPCR_Forward	GCCCAAAATGTTCTGCTGAT	**Fas_qPCR_Forward**	GGACCCTCCTACCTCTGGTT
CD137L_qPCR_Reverse	GCCGCAGCTCTAGTTGAAAG	**Fas_qPCR_Reverse**	ACCTGGAGGACAGGGCTTAT

Statistical analysis

Data are presented as mean ± standard deviation if not stated otherwise and P>0.05 was considered as significant. Survival was analyzed by log-rank test and multivariate Cox regression using the "survival" R package and Kaplan-Meyer curves and the forest plot were then represented. Correlations were analyzed using Spearman rho analysis. For coculture experiment analysis, we performed paired Wilcoxon test between the scramble-PVR shRNA models to investigate their significance. Three to five biological replicates were used in at least four different donors in all the cocultures performed. If not stated otherwise, the scramble population was set as reference for the graphics. Statistical analyses were performed with GraphPad Prism software and R (version: 4.3.2) on RStudio (version: 2023.06.0).

Results

Results

Objective I: To characterize PVR's epigenetic regulation and expression in multiple myeloma

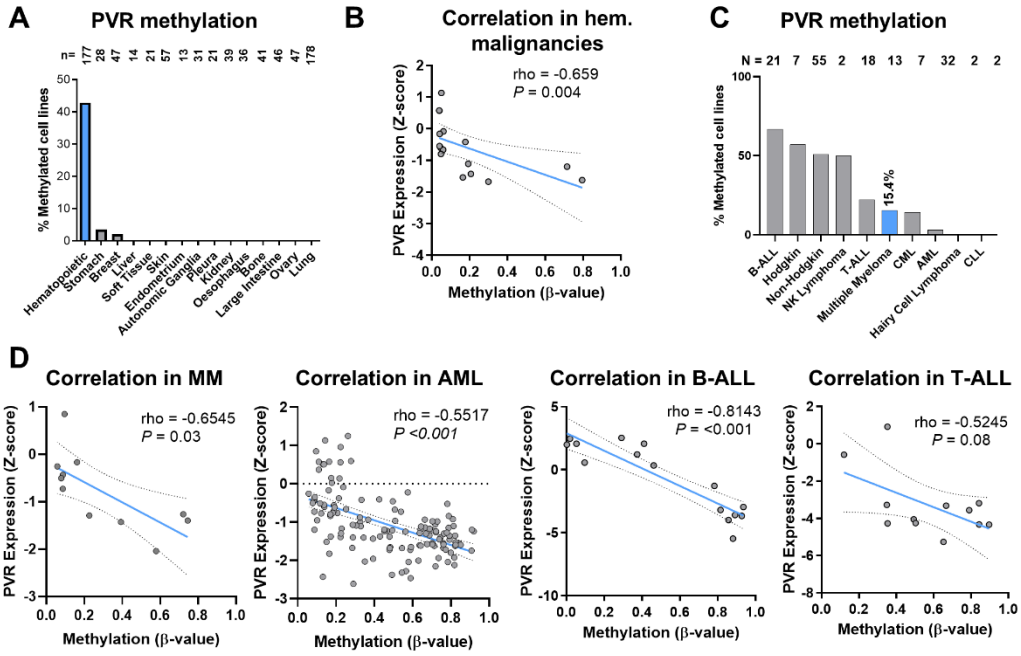
PVR is methylated in cell lines from hematological malignancies

After studying the methylation profiles for several genes involved in the cytotoxic cell immune checkpoint in hematological cancer cell lines, we observed that PVR was hypermethylated almost exclusively in hematological malignancies (**Figure 17A**). Using the methylation data and correlating with expression levels obtained in COSMIC database, we could observe the correlation between expression and promoter methylation levels in hematological cell lines (**Figure 17B**). Further characterization of the data allowed us to observe how the ranges of methylation levels varied substantially among hematological diseases (**Figure 17C**).

We explored the relation of methylation and expression on a selection of gene candidates (*PVR*, *PVRL2,* and *CD276*) all part of immune checkpoint presentation. This relation was studied in several hematological malignancies (**Figure 17D**). We have already disclosed the dire nature of MM and the need to study immune events in the context of the disease, furthermore, in MM, our first *in silico* analysis pointed towards a strong correlation between PVR promoter methylation and PVR expression and thus we selected this topic to explore further and develop this thesis project. The levels of methylation in MM cell lines are represented in **Figure 17E.**

PVR is functionally regulated by promoter methylation in multiple myeloma cell lines

A panel representing MM cell lines with different promoter methylation levels was selected for the *in vitro* experiments (**Figure 18A**). First, we validated the *in silico* data by Bisulfite Genomic Sequencing of the region 200 bp upstream of the transcription starting site of *PVR* gene. We validated how JJN-3, EJM, and RPMI-8226 presented low levels of methylation in the CpGs located on the promoter island, whereas AMO-1 and KMS-12-BM appeared hypermethylated in the same region (**Figure 18B**), in accordance with the *in silico* data obtained. In order to determine if the promoter methylation of PVR had any active role in transcription regulation, we performed expression analysis at the mRNA level by qPCR, and at the protein level by flow cytometry. As the data from the *in silico* analysis suggested, we could validate that the promoter methylation of *PVR* was functional and thus was enough to regulate gene expression. Methylated cell lines lacked PVR expression while unmethylated cell lines presented different levels of PVR expression (**Figure 18C**). Methylated cell lines were treated for 120 hours with the DNMT3 inhibitor, 5-aza-2′-deoxycytidine (DEC), and PVR expression was partially restored (**Figure 18D**). In summary, these data validated the DNA promoter methylation role in PVR expression in malignant plasmatic cell lines.



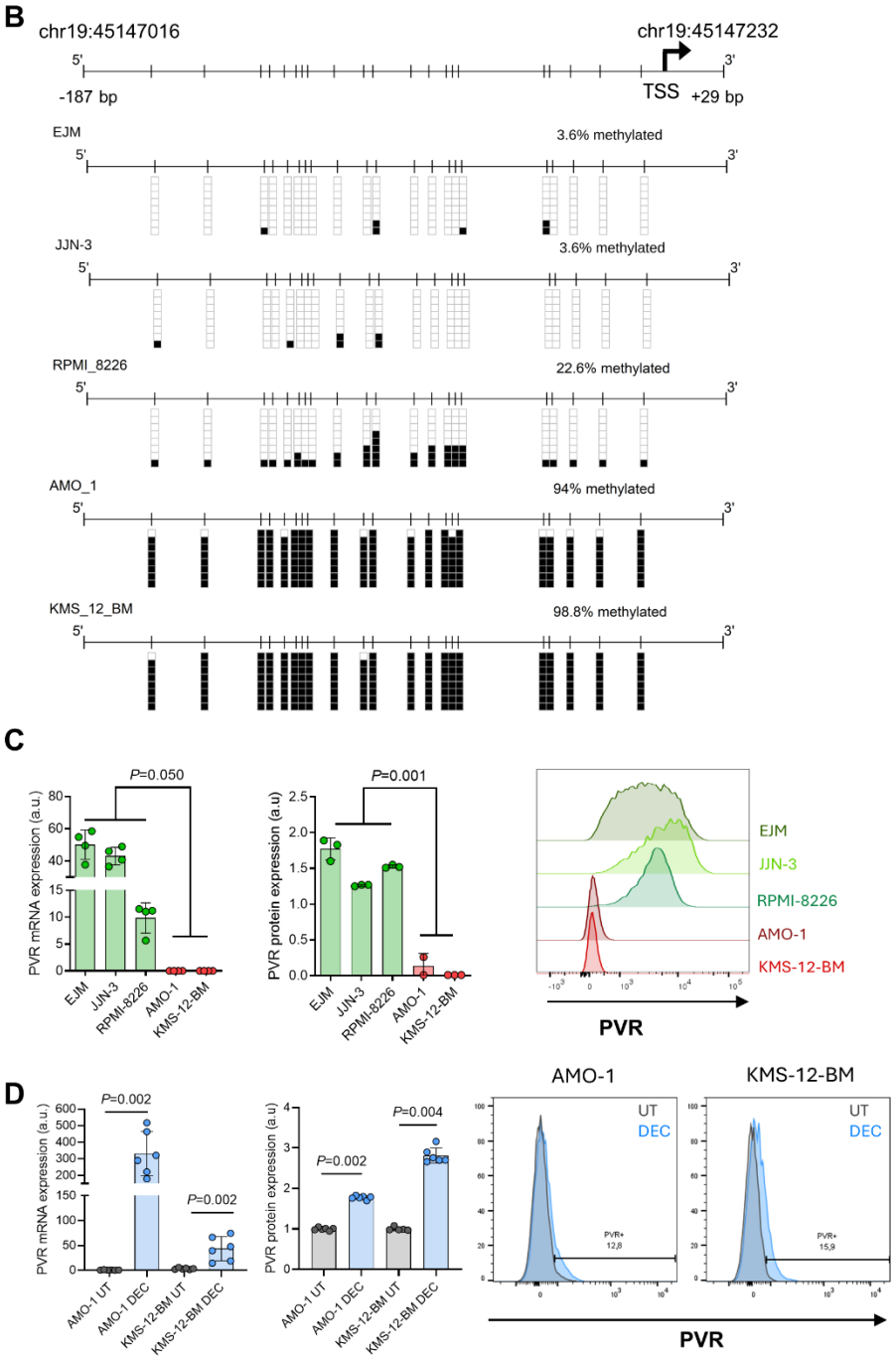
E

	CpG ID	cg02415834	cg21521892	cg08150575	cg01396723	cg22580353	cg04566018	cg14538146	cg07917289	cg05012825	cg13906416
	Chromosome	chr19	chr19	chr19	chr19	chr19	chr19	chr19	chr19	chr19	chr19
	Position (hg19)	45146246	45146289	45146784	45146828	45146900	45146967	45146976	45147056	45147078	45147440
	Gene region	TSS1500	TSS1500	TSS1500	TSS1500	TSS200	TSS200	TSS200	TSS200	TSS200	1stExon
CD138+ healthy control	HC_1	0.94	0.94	0.94	0.17	0.44	0.10	0.01	0.02	0.02	0.07
	HC_2	0.91	0.93	0.94	0.18	0.44	0.11	0.02	0.02	0.03	0.09
	HC_3	0.92	0.93	0.94	0.15	0.39	0.11	0.02	0.03	0.03	0.10
	HC_4	0.92	0.92	0.93	0.19	0.38	0.11	0.04	0.02	0.07	0.11
MM cell lines	AMO1	0.86	0.95	0.95	0.52	0.60	0.72	0.92	0.74	0.98	0.70
	EJM	0.53	0.64	0.95	0.09	0.22	0.15	0.02	0.02	0.02	0.13
	JJN-3	0.84	0.83	0.95	0.12	0.66	0.13	0.02	0.03	0.02	0.11
	KMS-12-BM	0.44	0.79	0.96	0.21	0.72	0.69	0.95	0.77	0.98	0.84
	L-363	0.67	0.89	0.91	0.12	0.52	0.24	0.26	0.05	0.16	0.12
	ME-1	0.96	0.96	0.96	0.52	0.91	0.23	0.07	0.19	0.43	0.23
	MM.1	0.93	0.95	0.96	0.19	0.74	0.12	0.03	0.03	0.08	0.17
	MM1.R	0.58	0.98	0.96	0.05	0.14	0.14	0.01	0.01	0.02	0.07
	MM1.S	0.87	0.96	0.93	0.13	0.16	0.13	0.02	0.02	0.02	0.10
	NCI-H929	0.48	0.95	0.87	0.42	0.51	0.60	0.67	0.47	0.80	0.64
	OPM-2	0.91	0.96	0.96	0.09	0.26	0.14	0.03	0.02	0.03	0.09
	RPMI-8226	0.74	0.94	0.95	0.14	0.13	0.16	0.03	0.03	0.03	0.14
	SK-MM-2	0.60	0.10	0.95	0.08	0.08	0.11	0.02	0.03	0.02	0.10

Figure 17. *In silico* data from *PVR* promoter CpG island shows the correlation between promoter methylation and gene expression in hematological cell lines. **(A)** Frequency of *PVR* hypermethylation in cancer cell lines derived from the Sanger panel according to tumor type. A promoter region is considered hypermethylated when the mean value of its promoter CpGs has a β-value above 0.66. **(B)** *PVR* methylation correlates with a loss of expression of the PVR transcript in hematological cancer cell lines from the Sanger panel. Spearman's correlation. **(C)** Frequency of *PVR* hypermethylation in hematological malignancies' cancer cell lines derived from the Sanger panel according to malignancy type. **(D)** *PVR* methylation correlates with a loss of expression of the *PVR* transcript in cancer cell lines from Multiple myeloma (MM), Acute Myeloid Leukemia (AML), B-Acute Lymphocytic Leukemia (B-ALL), and T-ALL. Spearman's correlation was used for the analysis. (E) DNA methylation profile of the *PVR* promoter CpG island analyzed by the Infinium EPIC DNA methylation array. Single CpG absolute methylation B-values are shown (0 to 1). Red, methylated; Green, unmethylated. Thirteen cell lines from multiple myeloma are shown in the figure together with four samples from normal plasmatic B-cells.

A

CpG ID	cg04566018	cg14538146	cg07917289	cg05012825	cg13906416
Chromosome	chr19	chr19	chr19	chr19	chr19
Position (hg19)	45146967	45146976	45147056	45147078	45147440
Gene region	TSS200	TSS200	TSS200	TSS200	1stExon
AMO1	0,72	0,92	0,74	0,98	0,70
EJM	0,15	0,02	0,02	0,02	0,13
JJN-3	0,13	0,02	0,03	0,02	0,11
KMS-12-BM	0,69	0,95	0,77	0,98	0,84
RPMI-8226	0,16	0,03	0,03	0,03	0,14



(Figure legend on the next page)

Figure 18. PVR methylation and expression characterization in a panel of MM cell lines. (A) DNA methylation profile of the *PVR* promoter CpG island analyzed by the Infinium EPIC DNA methylation array. Single CpG absolute methylation β-values are shown (0 to 1). Red, methylated; Green, unmethylated. Five cell lines from multiple myeloma were selected for the following experiments. **(B)** Bisulfite genomic sequencing of *PVR* promoter CpG island in multiple myeloma cell lines. CpG dinucleotides are represented as short vertical lines; the TSS is indicated with a black arrow. Single clones are shown for each sample. Black squares represent methylated CpG dinucleotides, whereas white squares denote unmethylated positions. **(C)** PVR expression levels in multiple myeloma cell lines determined by qRT-PCR and flow cytometry protein quantification. The bar plots represent the mean and SD of at least three biological replicates, each dot represents a replicate. The results were compared using One-way ANOVA. Representative flow cytometry histograms of PVR are shown for each cell line. **(D)** Recovery of *PVR* transcript and PVR protein expression after 1μM DEC treatment for 120h. The graphics represent the mean and SD of at least three biological replicates. The results were compared using unpaired two-tailed Student's t-test. Representative flow cytometry histograms show the recovery at the protein level in AMO-1 and KMS-12-BM cell lines. The number indicates the percentages of positive cells for PVR.

PVR presents a similar pattern of methylation in MM primary cases and has clinical relevance in MM

After establishing the epigenetic regulation by promoter methylation of *PVR* on MM cell lines, we directed our search toward healthy plasma cell status and PVR methylation on MM primary cases.

Although the importance of epigenetic regulation was recognized long ago, is still far from being incorporated into routine analysis and diagnosis work in the hematology area of medicine. Despite our efforts, our access to complete databases, with clinical, expression, and methylation information has been unavailable. It is because of that that we have used several sources to study PVR methylation and expression on primary MM samples. Thanks to our collaborators and the international effort of the Multiple Myeloma Research Foundation, we were able to interrogate four different sources of clinical data. For the sake of clarity, we will refer to them as Madrid's cohort (n=7), Salamanca's cohort (n=12), Navarra's cohort (n=104), and International cohort (n=776) (Table 6, Figure 19 - 20).

Starting by directing our focus toward methylation data, only Madrid, Salamanca, and Navarra had information and none of them were able to disclose clinical data from the patients. In the Madrid cohort, 14,3% were considered methylated (cut-off value of ≥0.2) with a good correlation with gene expression (rho=0,94, *P*=0.02) (Table 6, Figure 19A), whereas the Salamanca cohort presented a higher percentage of methylated samples 18.2% but not strong relation to its expression levels (rho=0.127, *P*=0.45) (Figure 19B). Despite the mixed results from these two cohorts, bigger cohorts like Navarra's showed that the percentage of methylated samples seems stable across patients' databases, as 14.6% of the samples were methylated (Figure 19C-D).

Table 6. Clinic-biological characteristics and PVR methylation status of primary MM samples.

Sample ID	Age	Sex	R-ISS	Isotype	Sample timepoint	Treatment	Methylation Status
MM_IJC_ID_21	62	F	II	IgGK	Dx	Isatuximab VRD+ ASCT	Unmethylated
MM_IJC_ID_22	76	F	III	CL kappa	Dx	Dara-VD	Unmethylated
MM_IJC_ID_23	60	F	II	CL lambda	Dx	Dara-VD+ ASCT	Unmethylated
MM_IJC_ID_25	84	M	Unk	CL kappa	Dx	Dara-RD	Unmethylated
MM_IJC_ID_26	56	F	I	IgG kappa	Dx	VRD + ASCT	Methylated
MM_IJC_ID_27	86	F	III	IgG kappa	Dx	Iberdomide + Dexamethasone	Unmethylated
MM_IJC_ID_29	64	M	II	IgA kappa	Dx	VRd + ASCT	Methylated

Unk: Unknown.
Dx: Diagnosis
V: Velcade
R: lenalidomida
D: dexamethasone
Dara: daratumumab
ASCT: Autologous stem cell transplantation

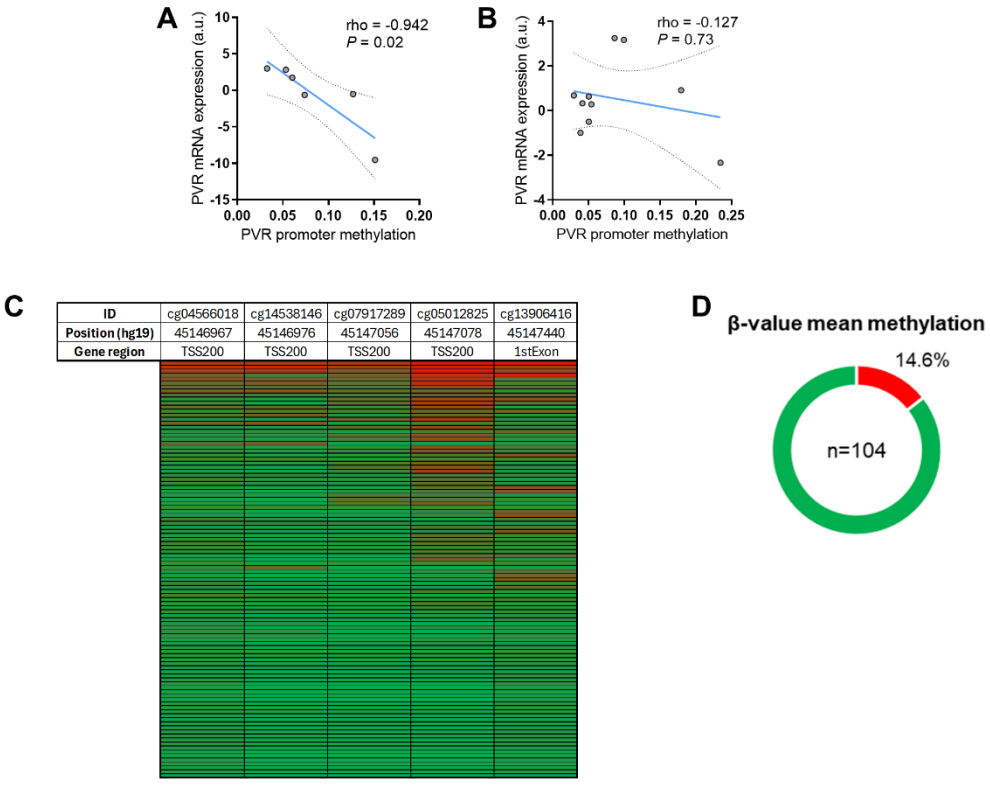


Figure 19. DNA methylation in primary MM cases. (A) Spearman's correlation of Madrid cohort cases. Expression levels were obtained by RT-qPCR while pyrosequencing was performed to obtain the levels of methylation of the different cases. **(B)** Spearman's correlation of the Salamanca cohort cases. The same procedure was followed as in (A). **(C)** DNA methylation heatmap of the *PVR* promoter CpG island showing the methylation levels from 104 patients from MM samples from *Duran-Ferrer et al*.[118] Single CpG absolute methylation β-values obtained with Infinium HumanMethylation450 BeadChip were used (0 to 1). Red, methylated; Green, unmethylated. **(D)** Percentage of methylated samples in the MM cohort. Methylation cut-off ≥0.20.

The biggest cohort, available thanks to the MM Research Foundation, lacks methylation data and although there are plans to incorporate this information in the future, it was not available at the time of writing this report. However, expression and clinical data can be found in the database. Analyzing these data, we found that expression levels (from RNA-seq) vary greatly in the cohort, which agrees with what we have observed previously. We divided the cases into quartiles (**Figure 20A**) and analyzed several group combinations. The PVR highest expression cohort (Q4) significantly presented worse OS when compared to any of the other groups (Log-rank test=0.006) (**Figure 20B**). A multivariate analysis (with the available data) showed that PVR is an independent risk factor [HR=1.591 (1.140-2.220), *P*=0.006] (**Figure 20C**).

These data imply the importance of PVR regulation in the context of MM and suggest epigenetic regulation by promoter methylation. Altogether, this data validates the epigenetic regulation of PVR and its relation to overall survival in MM patients.

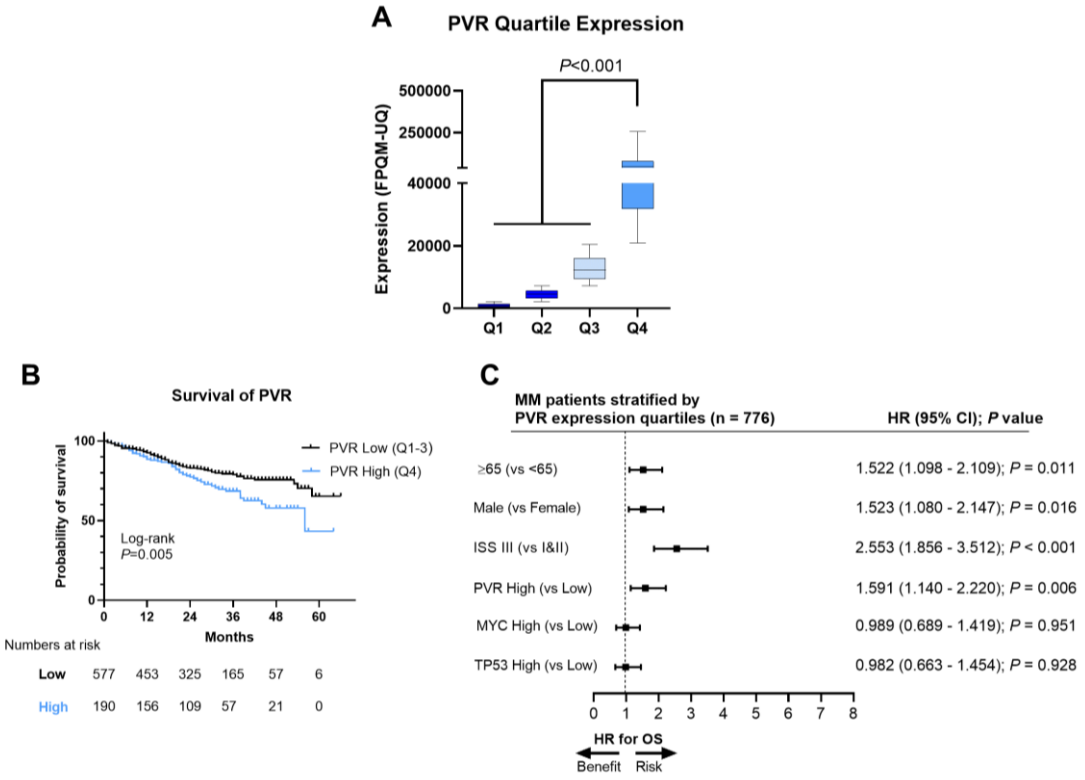


Figure 20. International cohort by CoMMpass project newly diagnosed data expression analysis. (A) PVR expression levels were analyzed by normalizing RNA-seq STAR counts available from the project. **(B)** Napierian logarithm of expression levels of PVR. **(C)** Kaplan-Meyer survival curves for n=776 patients of newly diagnosed MM divided by expression levels of PVR. **(D)** Forest plot of overall survival (OS) in subgroups stratified by clinical factors and PVR expression quartile.

PVR has higher expression in high-risk cytogenetic groups

Thanks to our collaborators we were able to access some of the closed cytogenetic data from newly diagnosed cases of MM. They provided us with the data appearing in **Figure 21**. In **Figure 21A** we can observe the distribution of cases depending on their PVR

expression levels classified by the different cytogenetic abnormalities registered in each of them. In Figure 21B we collected some of the comparisons carried out. PVR expression was higher in the group of cases that had cytogenetic errors associated with high-risk disease as are t(4:14) or del 13q. On the other hand, intermediate of standard risk profiles presented in some cases lower PVR expression compared when compared with the fraction of patients that did not present the cytogenetic error.

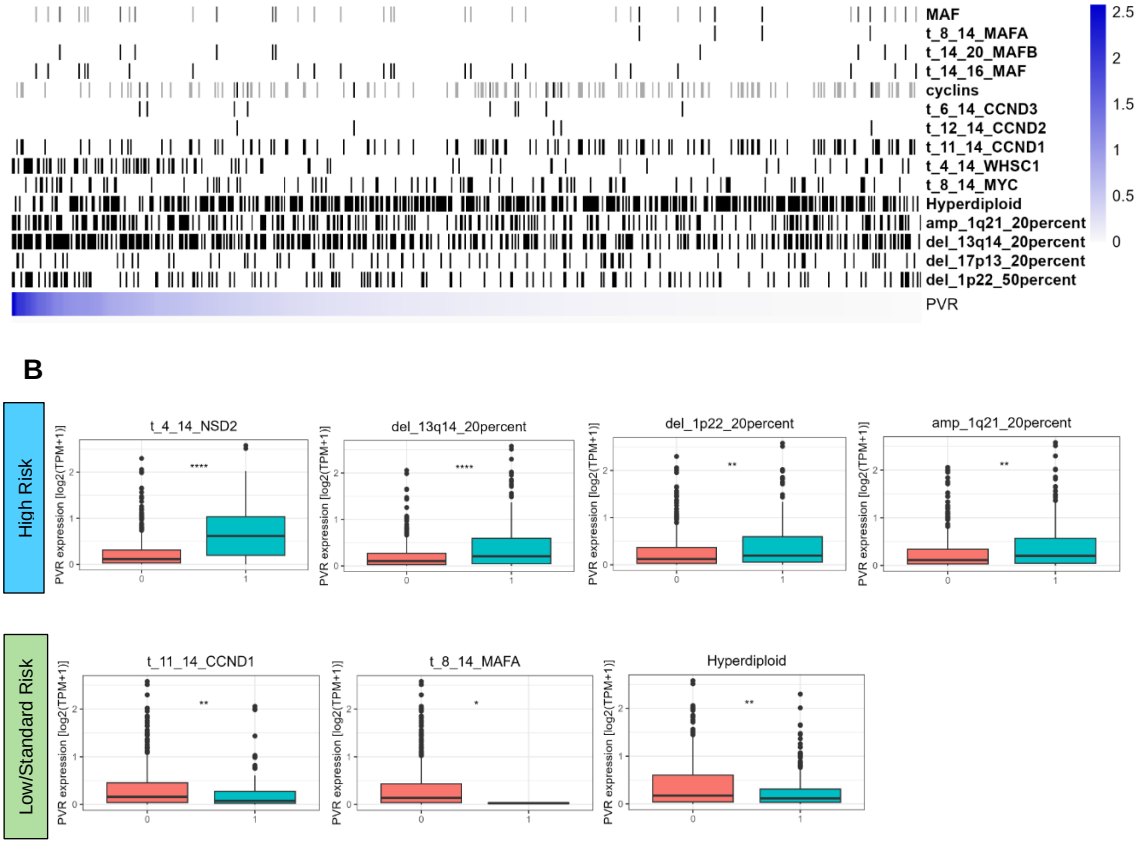


Figure 21. PVR expression in MM subtypes. (A) Oncoprint representing the cases for each MM subtype about PVR expression. **(B)** Dot plots representing PVR expression levels in CoMMpass Newly diagnosed cases subtypes (n=615). In red population without the error, in blue the population with the error. The results were compared using unpaired two-tailed Student's t-test. * *P*<0.05; ** *P*<0.01; *** *P*<0.001; **** *P*<0.0001

MM cell lines represent the heterogeneity of the disease

The primary samples that were used for the project came from newly diagnosed patients and thus the results do not show the effects that the therapy had on them. Despite this, another clinical factor may have affected the methylation status of the samples, and that was the cytogenetic aberrations that gave rise to the disease. Since we did not have access to all the clinical data from the patients, we wanted to explore the data that was available to us, and that was the genetic information from the cell lines representing the disease in the laboratory.

With information obtained from different sources, we elaborated a graphical representation of the cytogenetic errors detected on 13 cell lines derived from MM to see patterns of methylation on them (Figure 22).

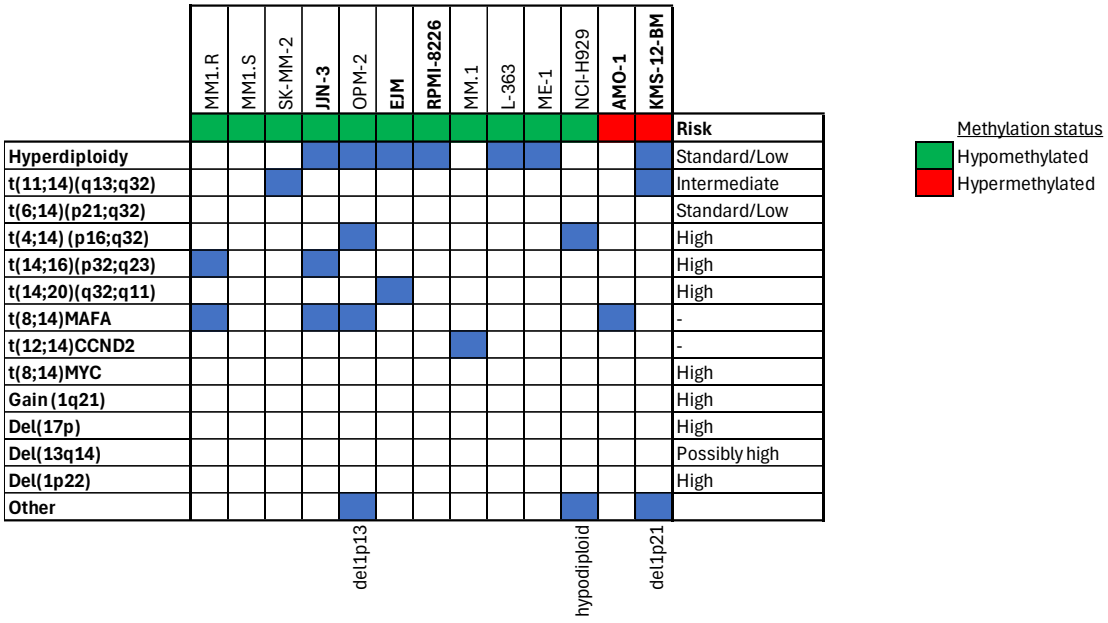


Figure 22. Oncoprint representation of the MM cell lines incorporated in our panel and relation with MM cytogenetic subtypes. In bold are the cell lines used for the project.

PVR does not affect cell biology by itself

In order to progress with the project, we developed cellular models where we altered PVR expression on both methylated and unmethylated cell lines (Figure 23-24). We analyzed several different biological processes (i.e. proliferation, apoptosis levels, and cell cycle dynamics) but we found no differences whatsoever between the models and their respective controls (cells where we transduced empty vector or scrambled constructs).

All in all, we demonstrate that PVR is regulated by the methylation status of its promoter region and that its expression has an effect on the OS of patients independently of other factors. The percentage of methylation observed in our methylation in silico data was similar to the percentage of methylated cases observed in data from patient cohorts, validating the use of cell lines to further explore the relation of PVR with MM.

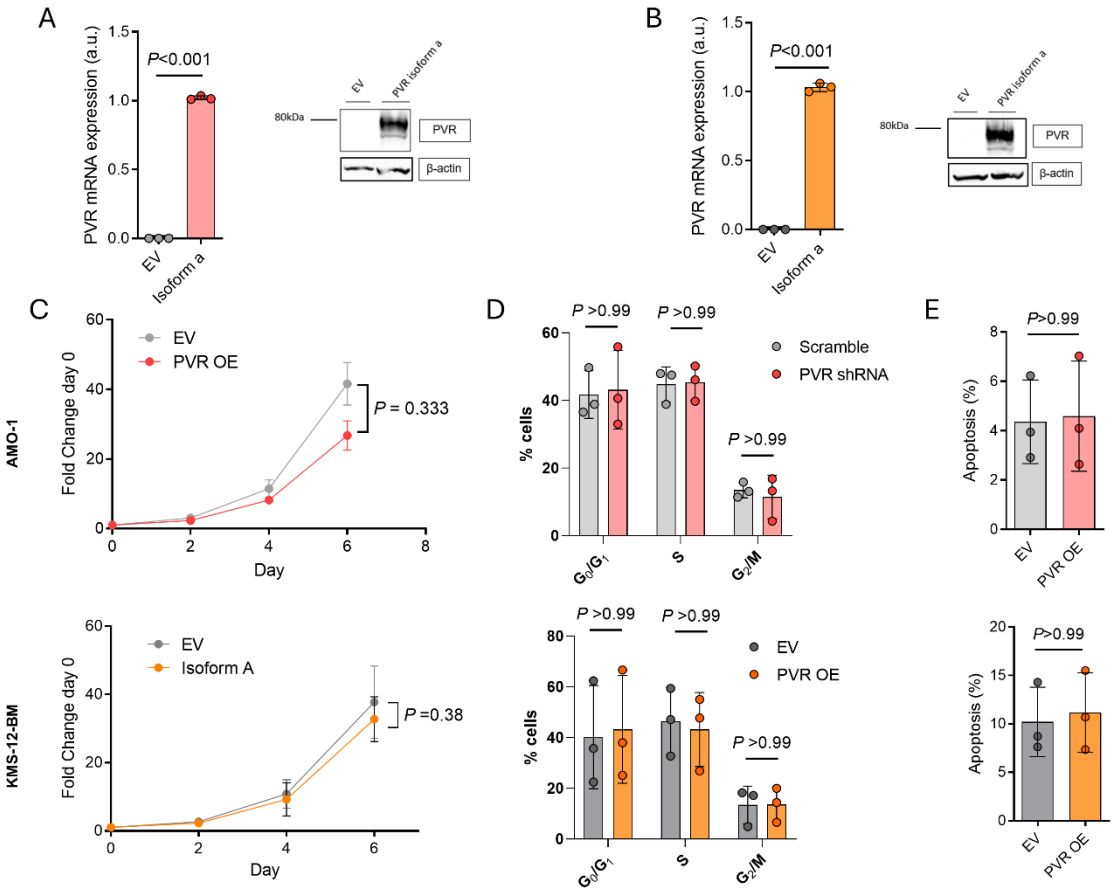


Figure 23. Overexpression of PVR in the multiple myeloma cell lines AMO-1 and KMS-12-BM and functional characterization of models. (A-B) Efficient overexpression of PVR at transcript level determined by qRT-PCR (*left*) and Western blot (*right*) in AMO-1 (A) and KMS-12-BM (B) from multiple myeloma. The graph represents the mean and SD of at least three biological replicates. The results were compared using unpaired two-tailed Student's t-test. ** p<0.01. **(C)** MTT assay (*left*) shows that PVR shRNA-mediated depletion does not affect the growth of two of the models (left) and it does not affect the apoptosis percentage of cells in culture (determined by flow cytometry). These graphs are representative of 3 independent experiments. PVR overexpression does not affect the apoptosis percentage of cells in culture (determined by flow cytometry) or cell cycle percentages *(right*). These graphs are representative of 3 independent experiments. For the growth assay, each data point represents mean ± SD. Statistical differences were determined using a 2-tailed Student's t-test, for the growth assay, at the day 6 time point. **(D)** Cell cycle percentages of cells determined by PI incorporation. **(E)** Apoptotic cell percentage measured by Annexin V incorporation using flow cytometry.

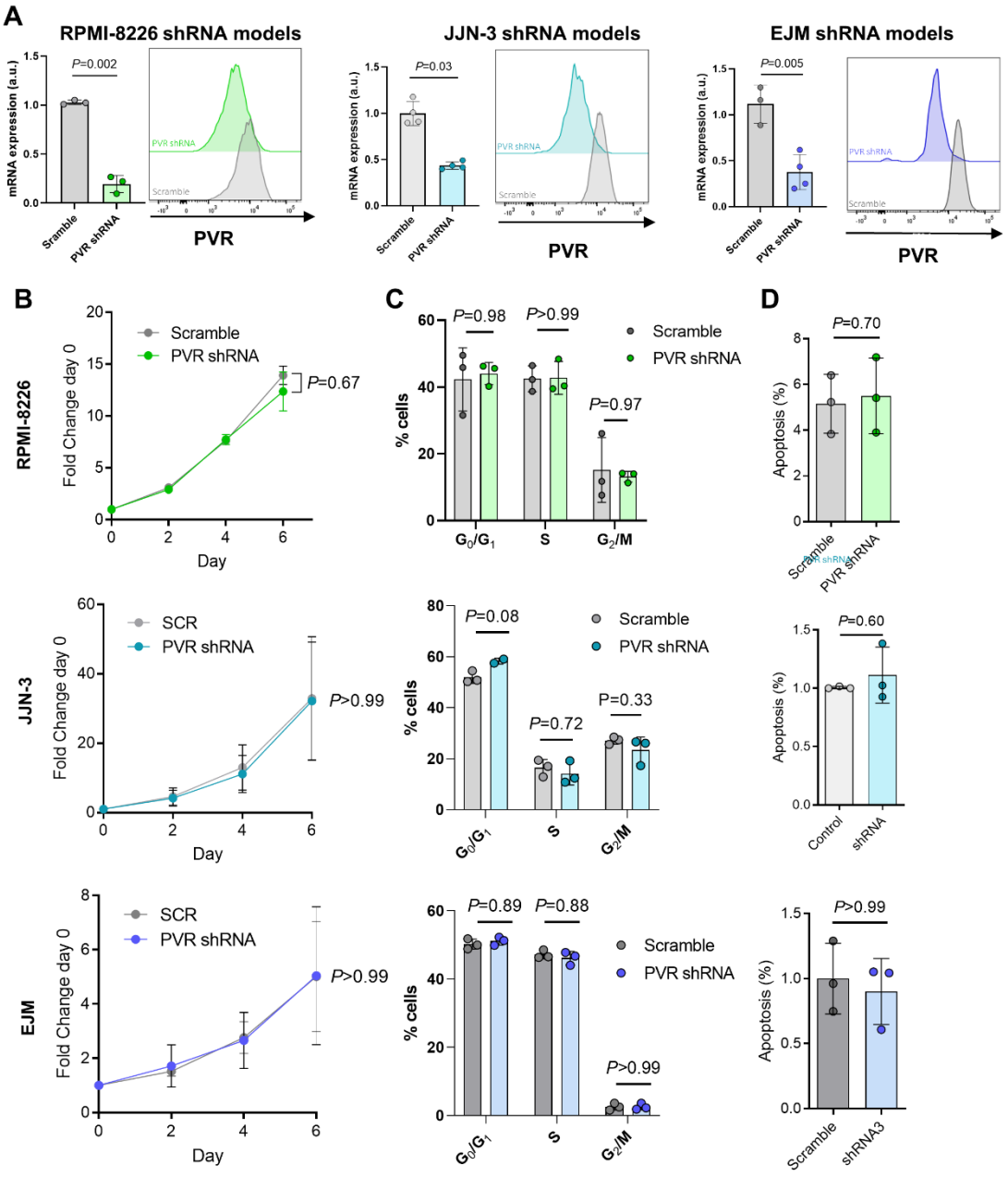


Figure 24. Depletion of PVR in the multiple myeloma cell line RPMI-8226, functional characterization of models and co-culture experiments. (A) Efficient shRNA-mediated depletion of PVR at transcript level determined by qRT-PCR (*left*) and protein levels by flow cytometry (*right*) in the unmethylated cell lines RPMI-8226, JJN-3, and EJM. The graph represents the mean and SD of at least two biological replicates. The results were compared using unpaired two-tailed Student's t-test. **(B)** MTT assay shows that PVR shRNA-mediated depletion does not affect the growth of two of the cells. These graphs are representative of 3 independent experiments. For the growth assay, each data point represents mean ± SD. Statistical differences were determined using a 2-tailed Student's t-test, for the growth assay, at the day 6 time point. **(C)** Cell cycle analysis by PI incorporation measured by flow cytometry. The percentages were obtained using Watson algorithm in Flow Jo v10.7. Three biological replicates were used for each sample. Results were compared using two-way ANOVA multiple comparisons. **(D)** The percentage of surviving cells determined by Annexin V was measured by flow cytometry. The graphs represent the mean and SD of three biological replicates The results were compared using unpaired two-tailed Student's t-test.

Objective II: To evaluate the role of PVR in cytotoxic cell function and its impact on immunotherapy

PVR affects T-cell cytotoxicity towards MM cell lines

Since we were interested in the immune function of PVR for this thesis project, we developed a co-culture system in order to test this role. Briefly, after PBMC isolation from healthy donor buffy coats, we activated the cells in the presence of CD3/CD8 Activation Dynabeads and 40U/mL of recombinant human IL-2. By activating T cells without isolating them first, we relied on the other cells present in the PBMC population (monocytes, B cells, and NKs) to contribute towards a better environment for the T cells to activate. After five days, we isolated the whole T cell population and set the co-culture experiment with the models described in the previous section.

Initially, both overexpression (methylated cell lines) and depletion (unmethylated) models were tested. We always performed the experiments with at least four different donors, always with the objective of taking into account individual biology variability. The co-culture experiment lasted 48 hours since we determined it was the amount of time necessary to be able to appreciate differences in T cytotoxic activity. After that time, supernatants were collected, and cells were stained for flow cytometry acquisition. Since our models presented GFP expression, by measuring the loss of GFP and Annexin V incorporation, we were able to measure the remaining surviving cells in the culture. Supernatants were analyzed to confirm the correlation between survival and cytotoxic cytokines, Interferon-gamma (IFNγ), Tumor Necrosis Factor-alpha (TNFα), and Granzyme B (GZMB), to validate cytotoxicity.

First, we evaluated how the wild type (WT) cells reacted to the presence of the cytotoxic cells. When compared to normal culture, we could observe how unmethylated cells (PVR expressing) showed different degrees of sensitivity to T-cell action, whereas methylated cells remained unaffected by them. Further validation through ELISA analysis of co-culture supernatants revealed a positive correlation between the survival percentage of tumoral cells versus cytokine level. A more exhaustive analysis dividing by cell line indicated that this effect was mostly produced by the influence of KMS-12-BM, whereas in all the other cell lines the relation was always a negative correlation. Although AMO-1 is also a methylated cell line and presented some level of sensitivity towards cytotoxicity we treated it as a resistant cell line (Figure 25).

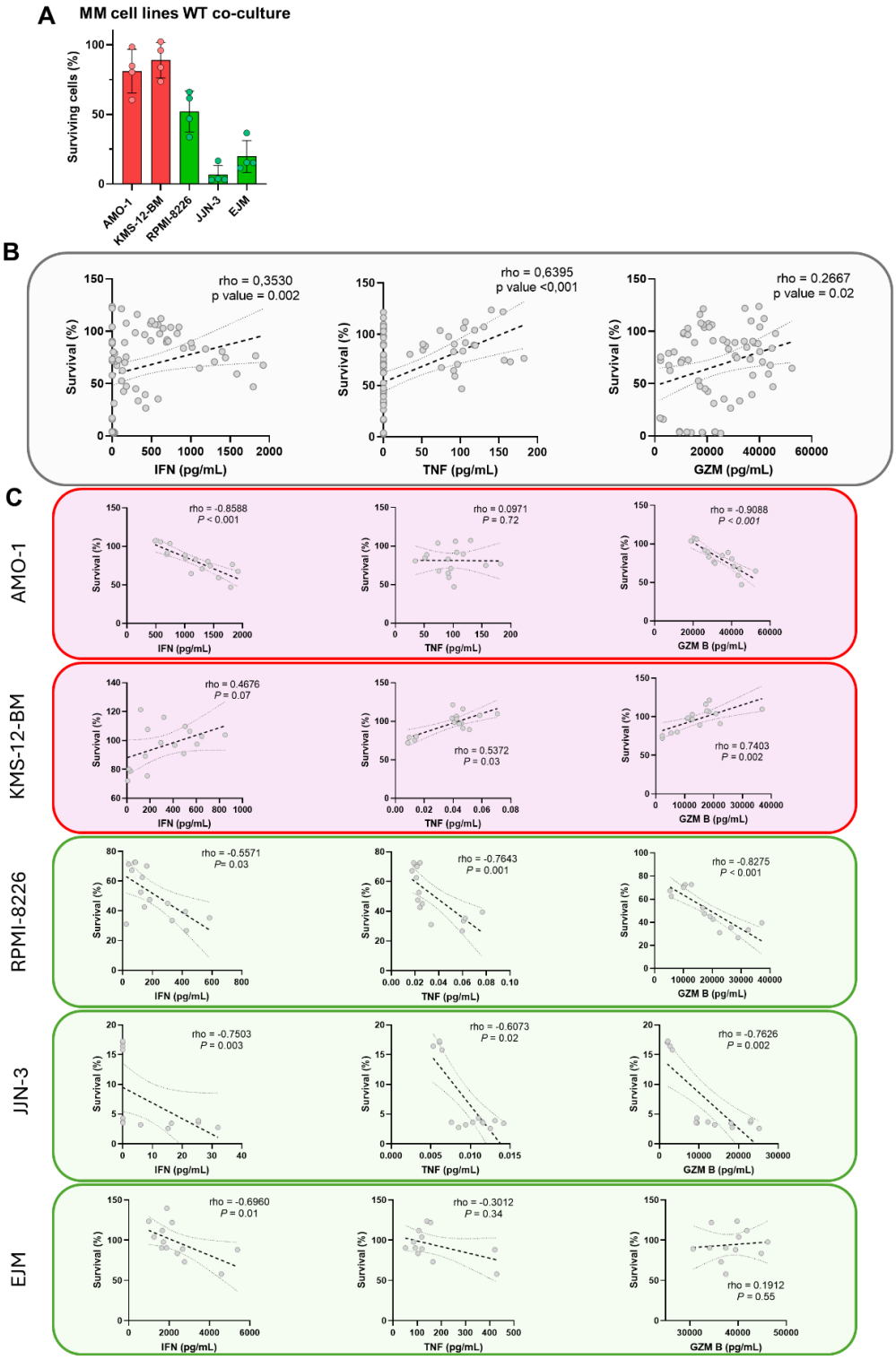


Figure 25. MM cell lines can be resistant to T-cell cytotoxicity independently of PVR methylation status. (A) Percentage of MM surviving cells after 48h co-culture assay. **(B)** IFNγ, TNFα, and granzyme B levels were measured in the supernatant (at 48 hours) of the co-culture systems determined by ELISA assay. Spearman correlation rho and p values are represented in each graphic. **(C)** Subdivision of the different cytokines by cell lines. Each dot represents a biological replicate of the coculture experiment. At least four replicates were carried out in four different healthy donors.

Then we proceeded to compare models with their respective control, either empty vector cells (EV) or scramble infected cells (SCR). Methylated cell line models did not respond to cytotoxic attack independently of the genetic modification whereas unmethylated cell line models were susceptible to the attack and this susceptibility increased upon PVR depletion (**Figure 26**).

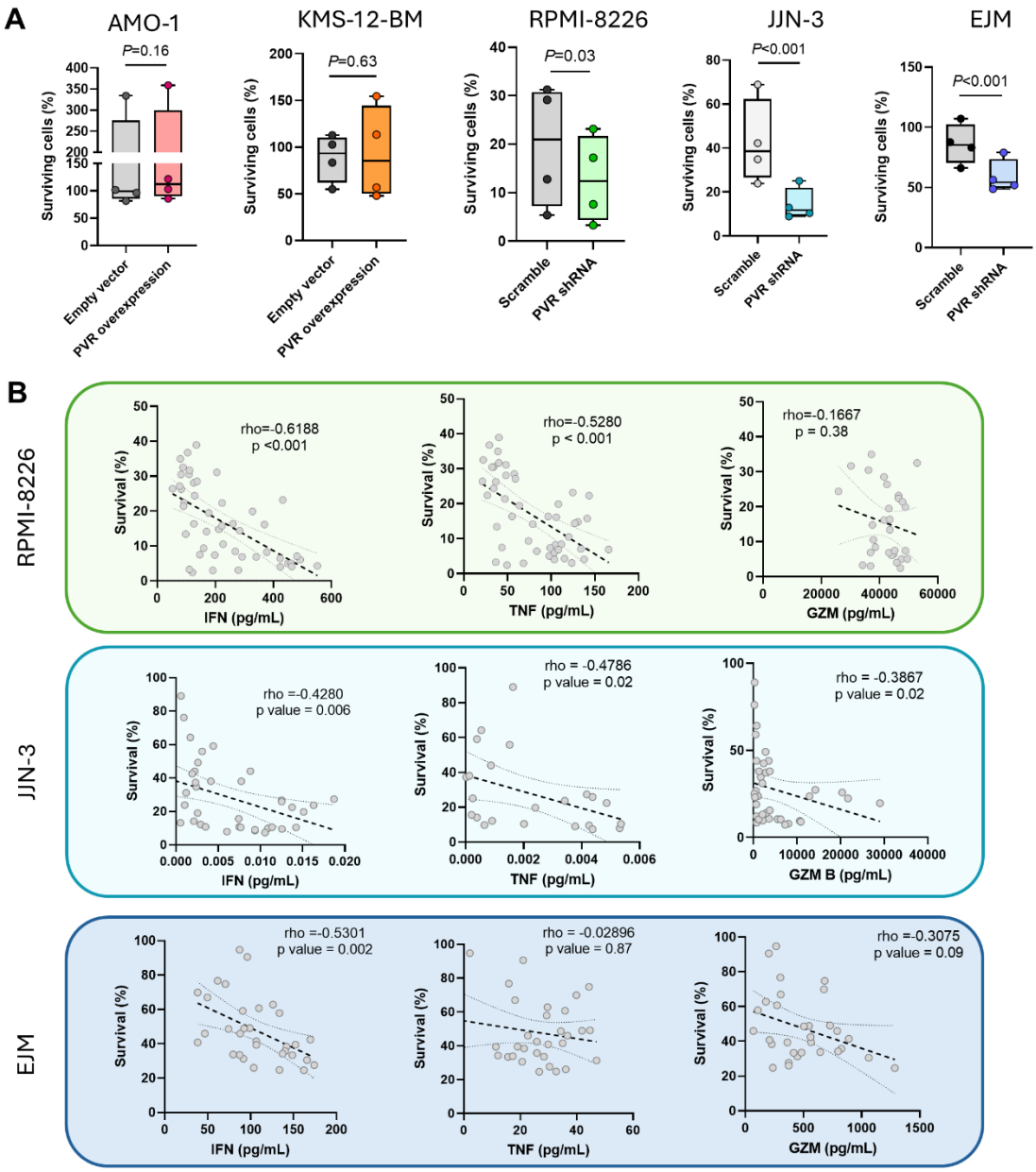


Figure 26. Modified PVR multiple myeloma cell lines co-culture experiments, and cytokine detection. (A) Percentage of MM surviving cells determined by Annexin V incorporation in co-culture assay for 48 hours between healthy donor T cells and PVR-depleted cells. The ratios to control cells were represented as the surviving portion of cells. **(B)** Representative pro-inflammatory cytokines (IFNγ, TNFα, and GZMB) levels were measured in the supernatant (at 48 hours) of the co-culture systems with the three cell lines determined by ELISA assay. Spearman correlation was used to measure statistical significance. Each dot represents a biological replicate. At least three biological replicates and T cells from four different healthy donors were used in the experiments. The dotted lines represent a 95% confidence interval.

Although we were able to observe the increment of cytotoxic cytokines with the decrease in tumor survival, we wanted to capture this phenomenon in a much more visual way. We were able to elaborate several time-lapse videos where we could record, first, the apoptosis of tumoral cells (green) by T-cells (either red or blue) (Movie 1). Furthermore, we were able to observe how PVR-expressing controls were able to resist cytotoxicity by aggregating and avoiding T cell attack (Movie 2). When we performed the experiment in parallel for methylated (expressing) cell lines and their PVR-depleted counterparts, we were able to observe the resistance that expressing controls had to cytotoxicity (**Figure 27**, Movie 3).

These results demonstrate the effect of PVR depletion on MM cell lines and confirm that methylated cell lines can resist T-cell cytotoxicity.

TIGIT-neutralizing antibodies successfully inhibit tumor escape *in vitro*

We have scientific evidence of the attraction of PVR and TIGIT and how PVR immune function seems to be through this interaction. Since the inhibitory action of PVR cannot be studied without its interaction with TIGIT we decided to validate this interaction in our co-culture system. We added to our co-culture systems neutralizing antibodies targeting TIGIT (BMS-986207) or human IgG control (**Figure 28**).

On RPMI-8226 co-culture, neutralizing TIGIT antibody restored cytotoxicity in T-cells faced by PVR-expressing cells (P=0.01) while depleted models remained unaffected. Cytokines reinforced these results. For JJN-3 a similar trend was observed, scrambled (expressing) models became sensible to cytotoxicity after the addition of anti-TIGIT. Although the effect was not enough to reduce survival to 0%, a significant effect was observed. PVR shRNA models remained unaffected and again, cytokines levels were higher in lower surviving populations. For EJM, we were able to observe a small shift and although the same trend was observed, this cell line presented much more resistance towards cytotoxicity than RPMI-8226 or JJN-3.

With these data, we were able to confirm the action of PVR-TIGIT as one of the mechanisms involved in cytotoxicity resistance.

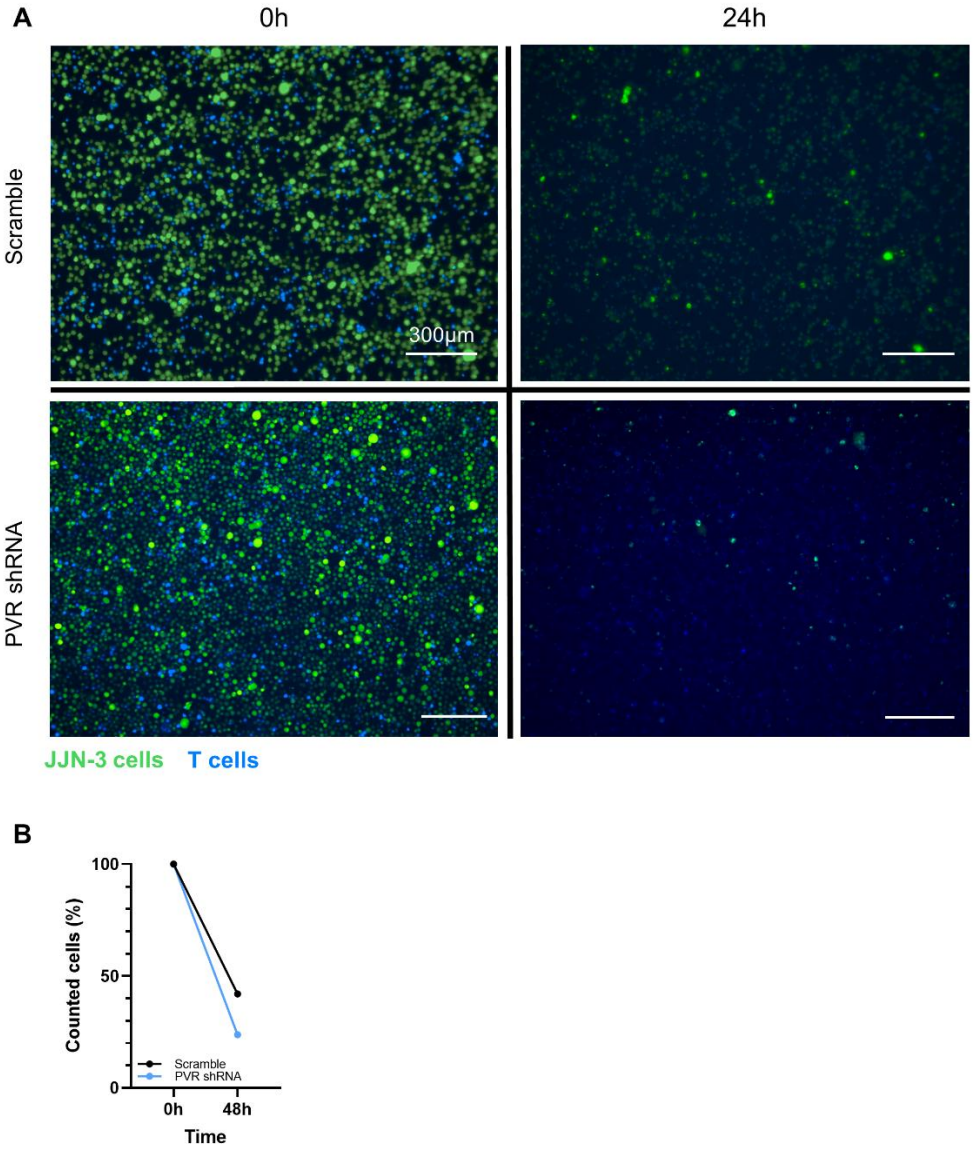


Figure 27. Fluorescent microscopy images representing the time 0h and 48h of coculture in JJN-3 models. (A) First and last picture for the time-lapse experiment. The two experiments were taken one immediately after the other. (B) Graph representing the percentage of counted cells in each picture.

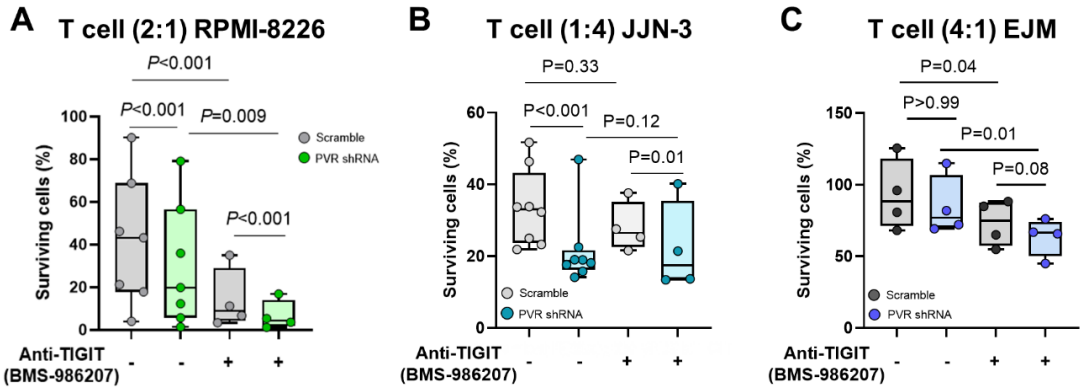


Figure 28. Depletion of PVR in the multiple myeloma cell lines co-culture experiments. (A-C) Percentage of MM surviving cells determined by Annexin V incorporation in co-culture assay for 48 hours between healthy donor T cells and PVR-depleted cells (A) RPMI-8226, (B) JJN-3 and (C) EJM. The ratios to control cells were represented as the surviving portion of cells.

Incorporating T-cell engagers into the co-culture system potentiates effect in cytotoxicity

Bispecific antibodies seem to be one of the promising novel immunotherapies that will help in the fight against MM. We decided to repeat once more, our co-culture experiment with healthy T cells but furthermore, we added a commercially available drug simulating and anti-CD3/BCMA bispecific T-cell Engager, T-engager from here and on. The first reason to use this drug was to test how an antibody similar to the drug currently being studied in the context of MM will affect the co-culture experiment[77]. The second reason was to validate that the cell death we were observing in our experiments was due to the presence of T cells. For that, we added 10pg/mL of the T-engager to our co-cultures with our depleted models.

We tested the effectivity of the Bispecific T-cell Engager first on a co-culture between healthy T cells and a T-ALL cell line not expressing one of the receptors of the drug (BCMA). The presence of the drug did not affect cytotoxicity towards the malignant cells (**Figure 29**). Upon performing the experiment with the PVR-depleted models, we again observed that they were more efficiently targeted compared to normal co-culture conditions and PVR depletion was detrimental to their survival. This trend was observed in RPMI-8226, JJN-3, and EJM.

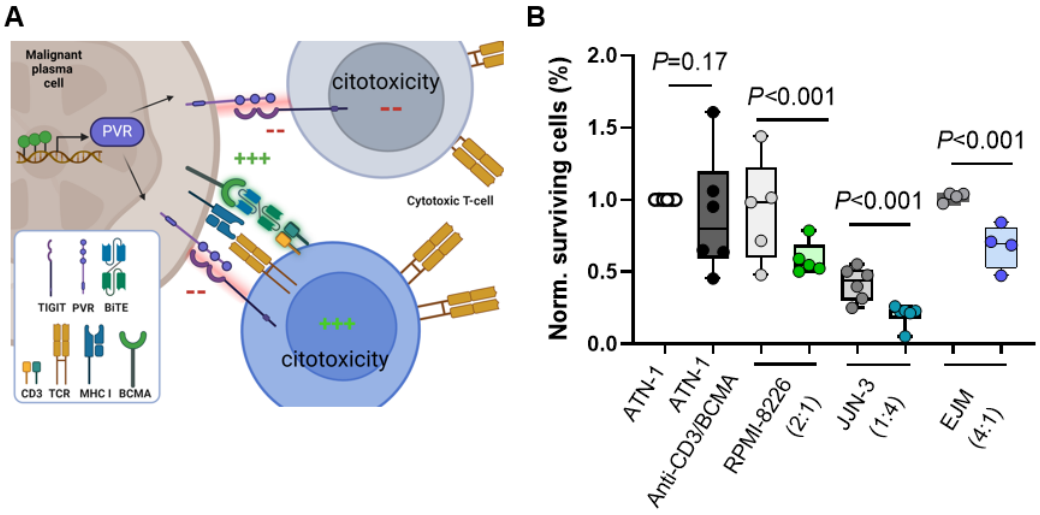


Figure 29. Bispecific T-cell Engager addition to our co-culture system resulted again in an advantage in cells expressing PVR. (A) Graphical representation of the influence of PVR in the interaction between malignant B plasmatic cells and different immunotherapy approaches. **(B)** Percentage of surviving cells determined by Annexin V incorporation in the co-culture assay for 48 hours between healthy donor T cells and PVR-depleted cells in the presence of the Bispecific T cell Engager at 10ng/ml. ATN-1 is a T-ALL cell line not expressing one of the targets for the Bispecific T cell Engager.

PVR/TIGIT axis interferes with the action of CAR-T and CAR-NK cells in MM

MM patients usually experience several lines of treatment and, when possible, eventually are introduced in clinical trials of novel immunotherapy approaches since a large percentage of patients become refractory to all available treatments[37]. CAR-modified

cells have become an important part of the path towards control management in RRMM with clinical trials showing overall response rates of 73-98%[84], with FDA-approved CAR-T products[119], and other promising constructs[85]. Because of this, we decided to use our co-culture system and confront anti-BCMA CAR cells with our models.

We evaluated how PVR-expressing and PVR-depleted cells behaved in the presence of CAR-T cells. We observed how EJM and RPMI-8226 were more sensible to CAR-T action, whereas JJN-3 although presenting the lowest percentage of survival, no differences were observed between PVR expressing and depleted models (**Figure 30A-B**). To test if the PVR/TIGIT axis could increase CAR-T treatment success, we added 10µg/mL of BMS-986207 to the co-culture. As expected, neutralizing the interaction of TIGIT and PVR, successfully sensitized malignant cells to T-cell cytotoxicity.

Although CAR-T therapies are currently the most studied novel immunotherapies, CAR-NK has become attractive from the biology point of view as they lack several of the complications associated with CAR-T treatments (i.e. ICANS or CRS). We confronted anti-BCMA CAR-NK cells against our malignant models and observed that the treatment didn't seem optimal in our *in vitro* conditions (**Figure 30C-D**). The addition of BMS-986207 stabilized the cell culture, and the same trends as described before were observed in all our replicates (**Figure 30C-D**). On both occasions, we could observe how donor variability had a huge impact on therapy success. Although independent, PVR/TIGIT interaction always played a key role in the results.

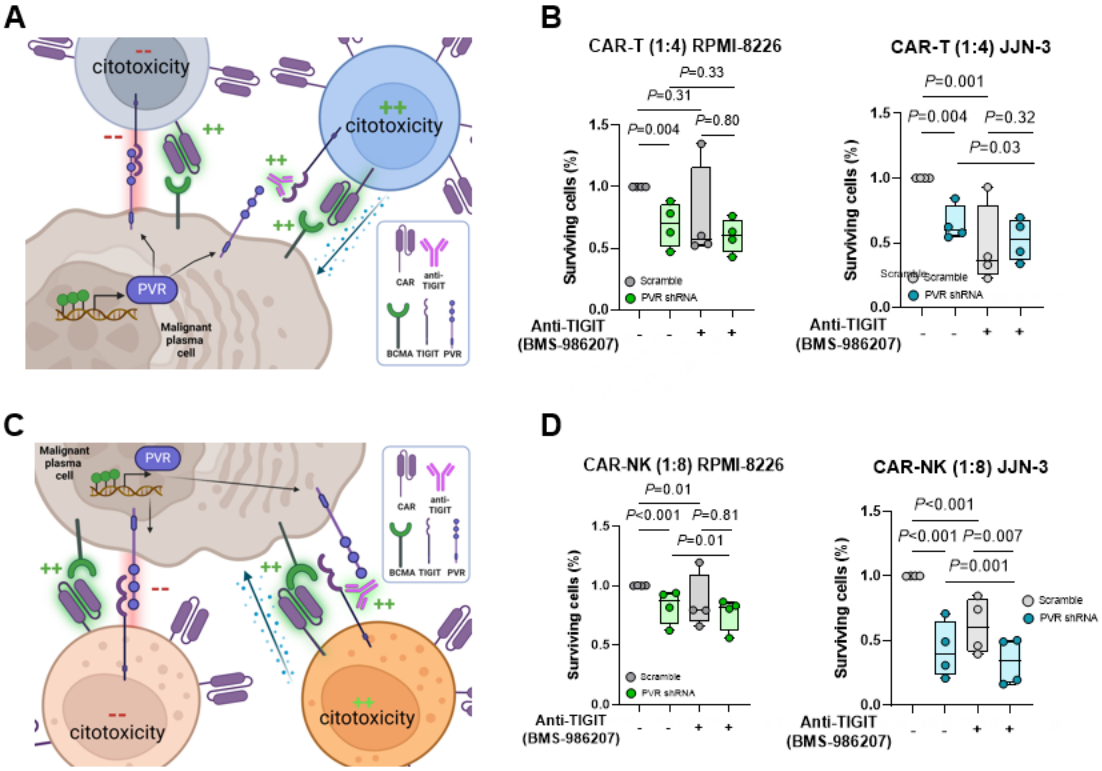


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Figure 30. Co-culture experiment using CAR-modified cytotoxic cells. (A) Representation of the effect of anti-BCMA CAR-T cells and the addition of anti-TIGIT neutralizing antibody to the culture. **(B)** Percentage of JJN-3 and RPMI-8226 surviving cells determined by Annexin V incorporation in co-culture assay for 24 hours between PVR-depleted and anti-BCMA CAR-NK at 1:4 ratio. Statistics: Each dot represents the mean of each of the donors with at least three biological replicates from four different healthy donors. The results were compared using non-parametric paired Wilcoxon test. **(C)** Representation of the effect of anti-BCMA CAR-T cells and the addition of anti-TIGIT neutralizing antibody to the culture. **(D)** Percentage of JJN-3 and RPMI-8226 surviving cells determined by Annexin V incorporation in co-culture assay for 24 hours between PVR-depleted and anti-BCMA CAR-NK at 1:8 ratio. Statistics: Each dot represents the mean of each of the donors with at least three biological replicates from four different healthy donors. The results were compared using non-parametric paired Wilcoxon test.

Objective III: To study the effect of PVR expression alteration and its relationship with other immune checkpoint markers

PVR/TIGIT axis and PD-1/PD-L1 axis are expressed in different cohorts at expression and methylation level

Based on the information found in the original research paper from Lee et al.[120] and supported by the findings in Worboys et al.[121] we decided to incorporate in our study a qPCR panel to determine the expression of different genes involved in the immune checkpoint. Here we sought to shed more light on the interactions occurring between malignant and immune cells during our co-culture system. We extracted the RNA of T cells from 12 different healthy donors and evaluated their levels of expression upon activation. We also extracted RNA from the malignant cells used from the co-cultures. We elaborated a graph representing the expression level of 26 proteins involved in T-cell activation and 17 co-stimulatory or co-inhibitory ligands present in our tumor cells. Replicating the correlation analysis performed in the mentioned manuscripts, we develop unsupervised correlation analysis of the expression levels found in both groups (immune cells and malignant cells).

On one hand, we were able to observe how, upon activation, genes related to this process increased their expression levels in T cells (i.e. Granzyme B, KI67, or IFNγ) (**Figure 31A**). Correlation analysis revealed that as described previously, PD-1 and TIGIT were expressed in different cohorts **(Figure 31B).**

On the other hand, we wanted to compare the expression of the different markers to the levels of healthy plasmatic B cells. Here, we could observe how some co-stimulatory markers like CD137L, CD70, or ICOSL were being expressed at higher levels than in normal B plasmatic cells, although the same happened with co-inhibitory proteins like CD276, PD-L1, PD-L2 or PVRL2 (**Figure 32A**). Correlation analysis revealed that at least in our studied models, PD-L1, PD-L2, and PVR were found in two clear distinct expression cohorts (**Figure32B**).

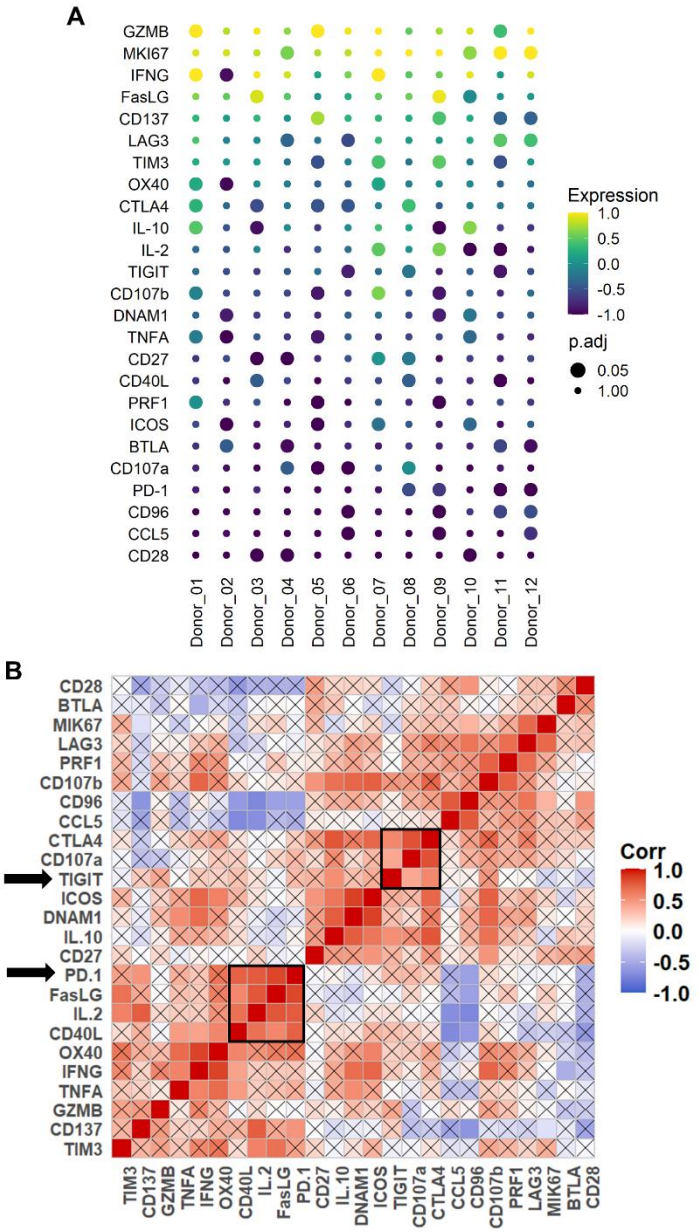

Figure 31. Expression levels of immune-checkpoint markers present in the T cell surface. (A) Dot plot depicting changes in a panel of IC markers present in T cells. Each dot represents fold change of the expression taken by reference preactivated T cells. Wilcoxon test was used to look for statistical significance and FDR method was used to adjust *P* values. For the dot plot, FDR *P-adjusted* values were used for representation. Colors and size indicate the number of times, direction, and significance. **(B)** Correlation matrix of the markers studied in the expression panel. Unsupervised clustering was performed, and Spearman's correlation was used. Significant correlation values are empty squares.

As part of the EPIC v2 test, our group collected methylation data from 180 hematological cell lines data. Using that information together with data publicly available from COSMIC cell line database, we elaborated a series of analyses first looking at the correlation value of expression and promoter methylation at all cell lines (n=156), at B-cell malignancy level (n=73) and MM cell line level (n=13) (Figure 33A, Annex II). Then, for the same three groups we looked at the correlation between gene expression and methylation (Figure 33B-D). Due to the small n in some of the analyses, significance was hard to obtain.

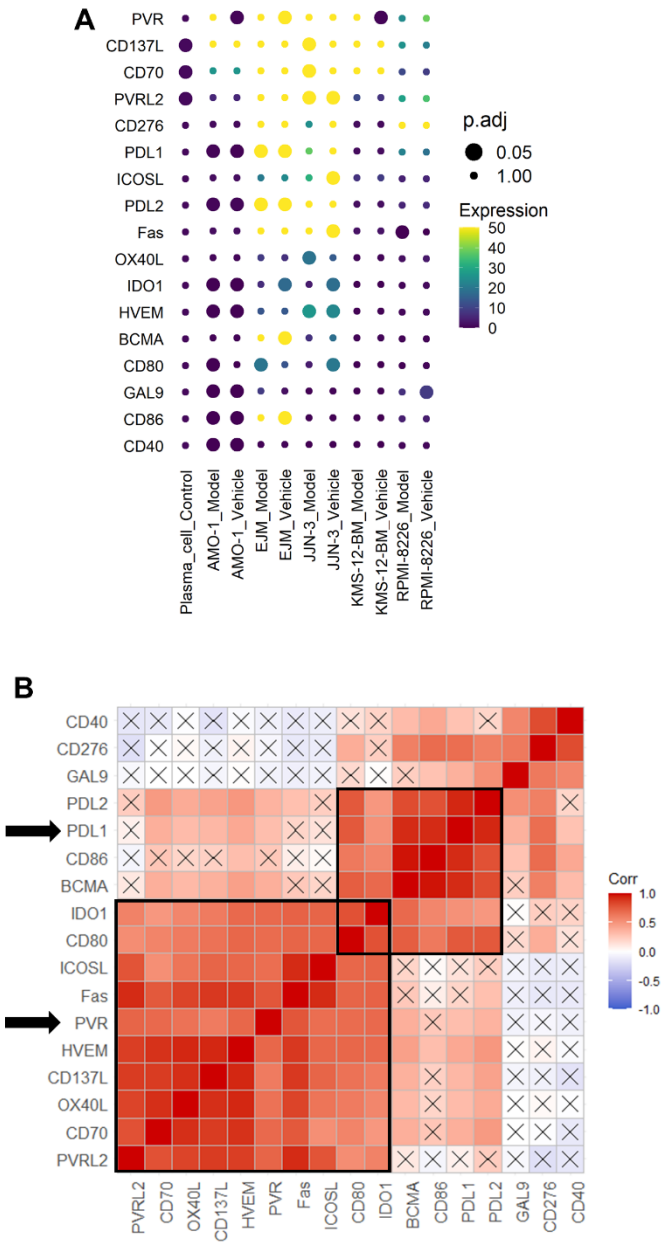

Figure 32. Expression levels of immune-checkpoint markers present in MM cellular models. (A) Dot plot depicting changes in a panel of IC markers present in plasmatic B cell surface. Each dot represents fold change of the expression taken by reference CD138+ plasmatic cells expression. Wilcoxon test was used to look for statistical significance and FDR method was used to adjust P values. For the dot plot, FDR *P*-adjusted values were used for representation. Colors and size indicate the number of times and significance. (B) Correlation matrix of the markers studied in the expression panel. Unsupervised clustering was performed, and Spearman's correlation was used. Significant correlation values are empty squares.

All in all, these expression analyses revealed how the PD-1/PD-L1/PD-L2 axis and PVR/TIGIT axis are expressed in separate cohorts and are two parallel pathways of co-inhibition.

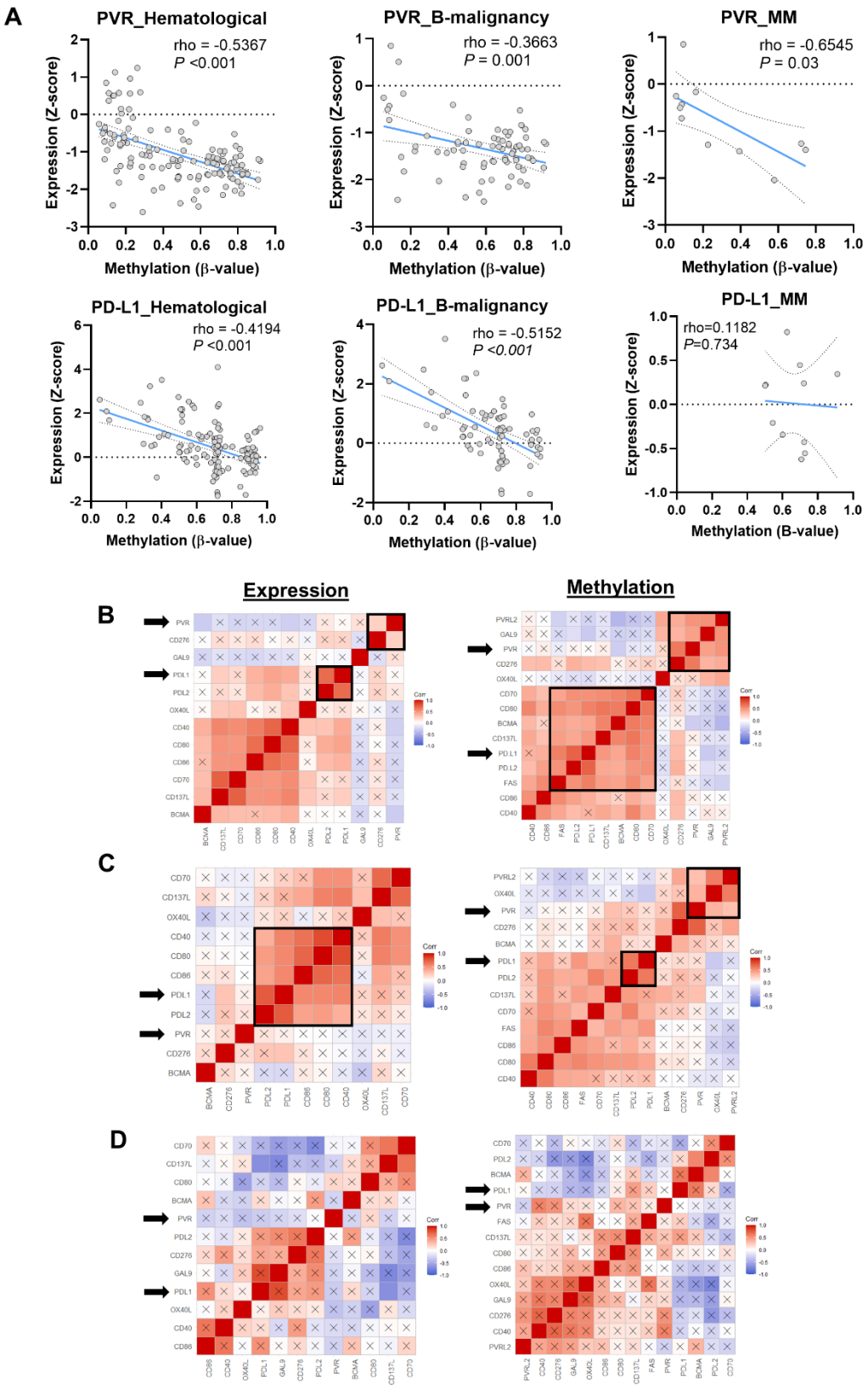

Figure 33. Expression and methylation correlation and clustering of immune checkpoint markers in hematological malignancies, B-malignancies, and MM. (A) Correlation matrix of PVR and PD-L1 in hematological malignancies cell lines, B-cell malignancies cell lines, and MM cell lines. **(B-D)** Correlation matrix for significant IC markers for methylation (*left*) and expression (*right*) in hematological malignancies cell lines (B), B-malignancies (C), and MM (D).

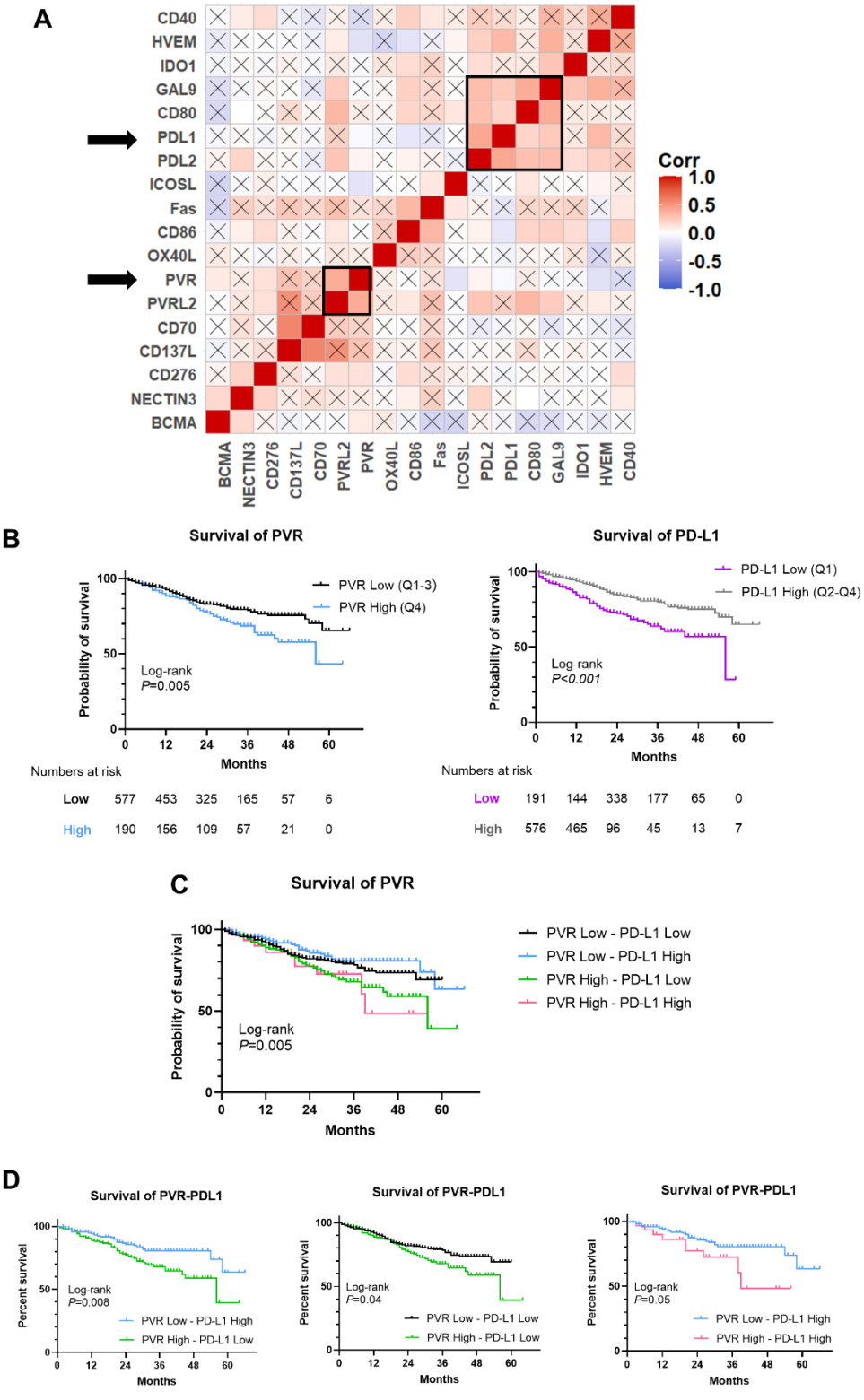

Figure 34. PVR and PD-L1 expression in newly diagnosed MM cases and their relation to survival. (A) Correlation matrix of the markers studied in the expression panel in 776 samples from the CoMMpass project. Unsupervised clustering was performed, and Spearman's correlation was used. Significant correlation values are empty squares. **(B)** Survival curves for PVR and PD-L1 divided by quartiles. **(C)** Survival group of patients grouped by their levels of PVR and PD-L1 expression. **(D)** Survival curves with significant log-rank *P* value that resulted from groups from (C).

PVR in primary cases and PD1 distribution

The separated expression cohorts described in the previous section were also observed in the International cohort from the CoMMpass project (**Figure 34A**). Using the clinical data available for this cohort, we investigated if primary MM follows this pattern (PVR and PD-L1 as separated clusters). Patients were subdivided into four groups based on low and high PVR and PD-L1 expression and found that high PVR expression was detrimental independently of PD-L1 expression for OS (**Figure 34B-D**).

These data support (I) the previous knowledge that IC genes are regulated by promoter methylation and (II) as in solid tumors, hematological malignant cells also present PVR and PD-L1 in different clusters of both gene expression and methylation levels.

Blocking antibodies for TIGIT and PD1 successfully inhibits tumor escape in vitro in an independent manner

To study the role of PVR and TIGIT interaction in T-cell activation and its independence from PD1/PD-L1/PD-L2 interaction, we added to our co-culture systems neutralizing antibodies targeting TIGIT, PD-1, or human IgG control, we also performed the experiment combining both neutralizing antibodies.

On RPMI- 8226 co-culture, neutralizing TIGIT antibody restored cytotoxicity in T-cells faced by PVR-expressing cells (P=0.01) while depleted models remained unaffected. PD-1 antibody affected both expressing and depleted models (P=0.002 and P>0.001, respectively) and a synergic effect was observed when combining both antibodies (expressing with a P<0.001 and depleted cells with P=0.001). Cytokines reinforced these results (**Figure 35**).

RNA-sequencing reveals new insights into PVR in cell biology

Because we wanted to study if *PVR* changes were influencing any other genes or pathways previously unknown to us, we performed RNA-sequencing analysis in our cell line models. The results show significant differences among the models while replicates maintain their similarities.

For the analysis, we performed DEseq2 analysis and considered only those genes with a differential expression greater than 1 with an FDR-adjusted P value lower than 0.01. We were able to perform this analysis in the wild type of cell lines used for this project on the overexpression models, and on two of our depletion models, RPMI-8226 and JJN-3 (**Figure 20, Annex III**). We focused on further analyzing the results of the depletion models as they were the cells we had used for most of the work in this thesis.

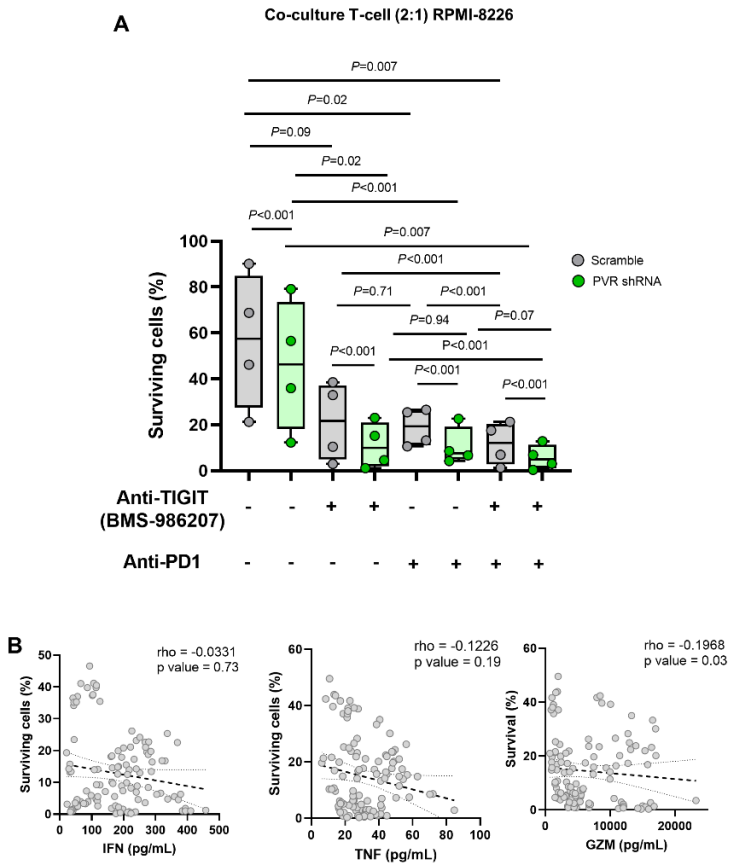

Figure 35. Co-culture experiment with anti-TIGIT and anti-PD1 shows a synergic effect in RPMI-8226. (A) Percentage of RPMI-8226 surviving cells determined by Annexin V incorporation in the co-culture assay for 48 hours between PVR-depleted and healthy T cells at a 2:1 ratio in the presence of control IgG, neutralizing anti-TIGIT or neutralizing anti-PD-1 antibodies. Statistics: Each dot represents the mean of each of the donors with at least three biological replicates from four different healthy donors. The results were compared using non-parametric paired Wilcoxon test. **(B)** Representative pro-inflammatory cytokines (IFNγ, TNFα, and GZMB) levels were measured in the supernatant (at 48 hours) of the co-culture systems with the three cell lines determined by ELISA assay. Spearman correlation was used to measure statistical significance. Each dot represents a biological replicate. At least three biological replicates and T cells from four different healthy donors were used in the experiments. The dotted lines represent a 95% confidence interval.

From the genes that meet the conditions stated above, we selected two lists, one with the biggest changes in downregulation when comparing controls to PVR depletion models and another list with the biggest changes in upregulation (Figure 36A). For RPMI-8226 we found 72 genes upregulated and 94 downregulated when comparing controls to PVR-depleted models whereas in JJN-3 we found 189 genes upregulated and 230 downregulated on the same comparison. From there we performed Gene Ontology (GO) analysis in biological process and KEGG pathway analysis (Figure 36B-C). We found that for RPMI, the biggest changes seem to affect cell adhesion and motility, and, curiously, functions where PVR has been described to be implicated before. We also found effects on signaling molecules (especially intracellular signaling and transduction) and neurogenesis. On the KEGG analysis, we found the most significant changes to be affecting gap junctions and again, neuronal implication and several indications that

cardiovascular effects too. On the other hand, we found that in JJN-3 cells, the biological processes most affected were, again, cell adhesion and motility ("morphogenesis" and "projection morphogenesis"), and surprisingly, neurogenesis once again. For KEGG pathways, JJN-3 changes seemed to replicate RPMI-8226 effects once again as intracellular signaling appeared to be affected by PVR downregulation and neurological involvement appeared once again.

Finally, from the lists of downregulated and upregulated genes, we decided to perform a Venn diagram and look for possible genes that appeared affected in both sets of models. We found a common number of genes (n= 6) were downregulated both in RPMI-8226 and JJN-3 (Figure 36D) and only 2 genes were upregulated in both of them (Figure 36E) despite the similar results obtained from the GO and KEGG analysis.

While the commonly downregulated genes seemed to fit right in the categories found on the GO analysis (i.e *GHR* is a type I cytokine receptor involved in cellular growth or *RRAD* and *CDHR1* which are involved in Calcium metabolism), the upregulated genes did not bring any significant information as they are receptors of epithelial and epidermal activity. You will find the full list of genes and their function described in Table 7.

CoMMpass project made available raw counts for the expression of several thousands of genes, so we performed a similar analysis as the one we performed on our samples. We divided the patients into 4 quartiles depending on PVR expression. From the beginning, it was clear that Q4 contained the most different cases, so we focused on comparing high-expression patients (Q4) versus low-expression patients (Q1 to Q3).

We performed Gene Ontology analysis on the list of genes significantly upregulated (>1 Fold change) or significantly downregulated (<-1 Fold change). Although the results were very heterogeneous, some similarities were found among the three studied groups. On the upregulated genes, we found some related to adhesion and plasma membrane components, and inorganic ion transport. For downregulated genes, they were related to transmembrane kinase activity and signal transduction. These results warrant further investigation on the effect of PVR expression changes and their relation to other cellular functions (further from immunologic activity) (Figure 37).

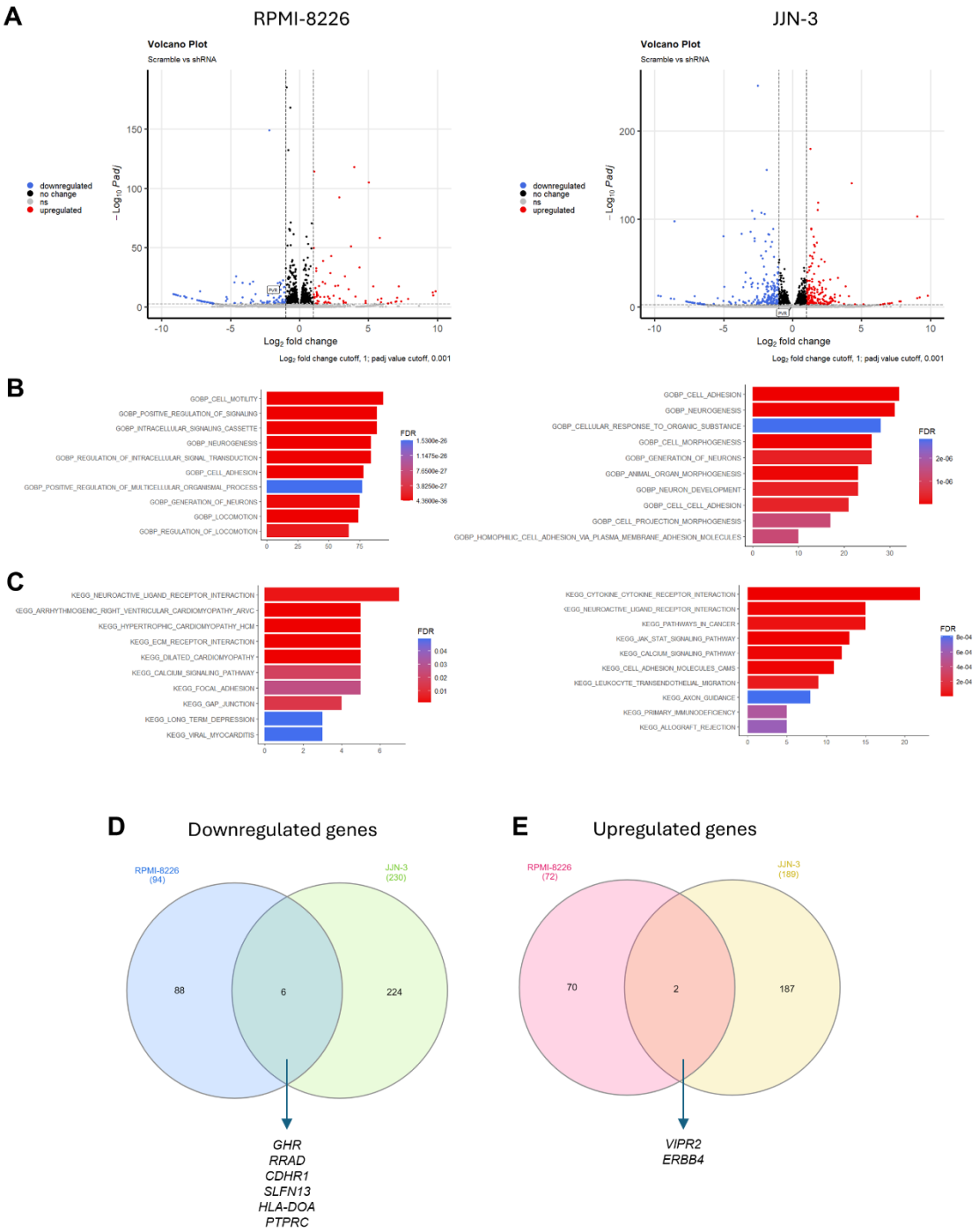

Figure 36. RNA-seq results from RPMI-8226 and JJN-3 comparison. (A) Volcano plot of RPMI-8226 (*left*) and JJN-3 (*right*). **(B)** GO biological process analysis for the genes changed (both downregulated and upregulated) with more of a differential expression value of 2 with a bigger FDR *P*-adjusted value of 0.01 for RPMI-8226 (*left*) and JJN-3 (*right*). **(C)** KEGG pathway analysis of the genes found in the list mentioned in (B). **(D)** Venn's diagram of the genes that were found downregulated in both RPMI-8226 analysis and JJN-3. **(E)** Venn's diagram of the genes found to be upregulated in the RPMI-8226 and JJN-3 analyses.

Table 7. RNA-seq downregulated and upregulated genes common in RPMI-8226 and JJN-3.

Gene	Function
GHR	This gene belongs to the type I cytokine receptor family. It is a transmembrane receptor for growth hormone. The binding of growth hormone to the receptor leads to receptor dimerization and the activation of an intra- and intercellular signal transduction pathway leading to growth.
RRAD	Ras Related Glycolysis Inhibitor and Calcium Channel Regulator. This protein enables GTP binding activity and calcium channel regulator activity.
CDHR1	Potential calcium-dependent cell-adhesion protein. May be required for the structural integrity of the outer segment (OS) of photoreceptor cells (By similarity).
SLFN13	Enables endoribonuclease activity. Involved in rRNA catabolic process and tRNA catabolic process. Located in cytoplasm.
HLA-DOA	HLA-DOA belongs to the HLA class II alpha chain paralogues. HLA-DOA forms a heterodimer with HLA-DOB. The heterodimer, HLA-DO, is found in lysosomes in B cells and regulates HLA-DM-mediated peptide loading on MHC class II molecules. In comparison with classical HLA class II molecules, this gene exhibits very little sequence variation, especially at the protein level.
PTPRC	The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitosis, and oncogenic transformation.
VIPR2	This gene encodes a receptor for vasoactive intestinal peptide, a small neuropeptide. The vasoactive intestinal peptide is involved in smooth muscle relaxation, exocrine and endocrine secretion, and water and ion flux in lung and intestinal epithelia.
ERBB4	This gene is a member of the Tyr protein kinase family and the epidermal growth factor receptor subfamily.

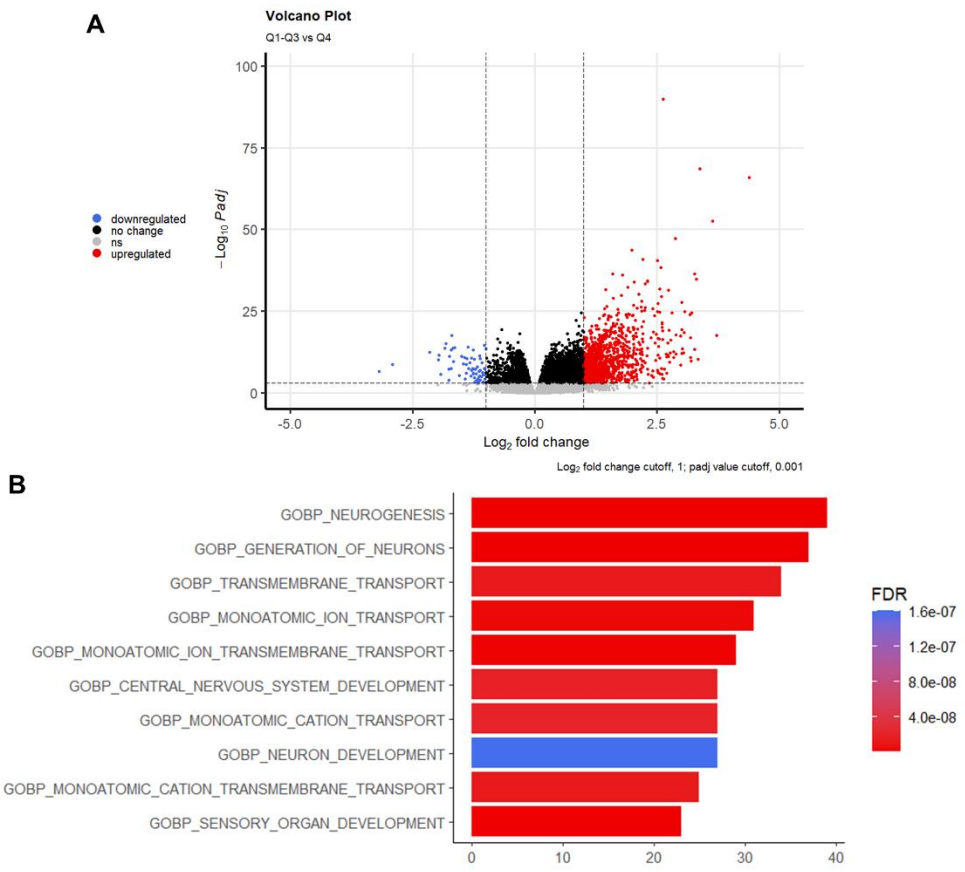

Figure 37. Changes in the expression of newly diagnosed patients separated by the low and high PVR expression. (A) Volcano plot showing the differentially expressed genes between the groups. (B) GO biological process analysis with the genes most changed.

Discussion

Discussion

At this point in the thesis, we have laid down all the chapters composing this project. We have set the background, presented the protagonists and the antagonists, and shown all the data collected through this journey, and now we reach the climax, the part of the story where everything makes sense and fits into its place.

As established before, for the sake of clarity, we will structure this section following the order set, by doing that we avoid leaving important data behind and connecting everything found in this project to our objectives.

Having said that, let's begin the end of this story.

Objective I: To characterize PVR's epigenetic regulation and expression in multiple myeloma

The project started in 2019. At that time, immune checkpoint inhibitors were beginning to gain attention in cancer research. New discoveries were leading toward novel and promising results, especially promising in specific areas like melanoma and anti-PD1 treatment. From then on, two things happened: (I) We experienced a revolution both in immunotherapy options and immune checkpoint research, and (II) this PhD student learned how to do science.

This project started with a broad study of how promoter DNA methylation affected the expression of immune checkpoint markers on the surface of several tumoral cells coming from hematological cell lines. Our intention here was to screen *in silico* data to detect potential candidates in the arising field of immune checkpoint control that would benefit the fight against hematological malignancies.

As **Figure 17** shows, we successfully found a promising candidate to explore in hematological malignancies, PVR. PVR was only methylated in cell lines originating from blood cancers (**Figure 17A**), which gave us the indication that it may be behaving like many other genes[105] and acting as a tumor suppressor in hematological malignancies. Then, we subdivided the data and looked at the distribution of these methylated cell lines across all the hematological malignancies we had available in our *in silico* data (**Figure 17C**).

As we explored the data available to us, we discovered that the correlation between promoter methylation and gene expression was observed in several different diseases. Finally, we decided to further explore the role of PVR in MM as the disease remains incurable and immunotherapy seems a promising strategy against the malignancy.

Epigenetics affect gene expression without changing its information (i.e. mutation) and they can be at times a little bit tricky as not all modifications have a direct effect on a

specific gene expression, sometimes the regulation comes from a combination of several mechanisms and epigenetic modification alone cannot fully control gene expression. It was because of this fact that we needed to validate the *in silico* data on a representative panel of MM cell lines. In order to do that we explored the methylation status of the promoter region of *PVR* and also studied different expression levels, mRNA and protein, to see if the correlation was true. Across Figure 18 you will be able to check that we were able to confirm that the *in silico* promoter methylation data was correct, and furthermore that the MM cell lines that appeared hypermethylated lacked PVR expression, both at mRNA and protein levels. These data confirmed that promoter methylation in PVR was in fact, functional in MM.

So far into the project, we were working with *in silico* and *in vitro* data, and then we wondered, what is happening in primary MM cases? Does this correlation still exist in patients' samples?

Well, this was maybe one of the hardest points to investigate in this project. Thanks to our collaborators and the international effort of the Multiple Myeloma Research Foundation we had access to primary data and primary samples from newly diagnosed patients (as treated patients may have changed their methylation/expression status and thus could not give us data on the relation in the disease). Depending on the origin, we divided the data into cohorts. Due to data protection policies and data availability in the different sources, we were unable to gather from the same cohort methylation, expression, and clinical data. Regardless, we had some data to explore. For example, in Navarra's cohort, we had available methylation data from newly diagnosed patients, but we did not have access to expression or clinical data from them. From this group, we observed a methylated percentage of cases (>0.2) of 14,3%. This percentage was similar to the one observed on our panel of cell lines (15,7%) and other cohorts where methylation status was available (Madrid: 14,7%, Salamanca: 18.1%). This led us to believe that at the methylation level, our panel was a good representation of what was observed in real-world data. We were also able to study both expression and methylation status in thirty samples from Salamanca and Madrid. In the Madrid cohort (n=6), methylation and expression data were correlated whereas in Salamanca's cohort (n=10) only a tendency could be observed (Figure 19A-B), but we were unable to obtain all the data necessary to reach significance. Due to the small population sample available regarding expression and methylation relation, it is difficult to draw strong conclusions from this data but taking into account the *in vitro* results and the relation already observed between cell lines and primary samples, we decided to consider this relation (methylation/expression) to be true also in primary samples.

Finally, we asked what may be the most interesting open questions regarding PVR and primary samples: Is PVR expression related to patient disease? Do we see any clinical relevance in PVR expression in MM? What does the data say? Regarding expression and clinical data, we only had available the International cohort, and publicly available data from the CoMMpass project. As shown in Figure 20, after dividing the patients into

quartiles, we were able to observe how the highest expression group (Q4) had significantly worse overall survival than the rest of the patients. In a multivariate analysis, we could see how high PVR expression was an independent risk factor (when MYC or TP53 were not). Thanks to our collaborators in Navarra, we were able to see how PVR expression was related to the different MM cytogenetic subtypes. We observed that some of the patients with the cytogenetic profile of highest risk had also a higher PVR expression. Despite the initial disconnection between these two factors, cytogenetic instability may be the cause of the increase in PVR expression which in turn is related to higher-risk disease. Taking all into consideration, PVR expression (or methylation status if available) may be helpful in risk stratification.

Since we saw a relation between cytogenetic subtypes and PVR expression, we wanted to further characterize the cell lines in which we were going to develop the project. The only conclusion reached after analyzing the data was that in fact, *in vitro* cell lines represented the heterogeneity of the disease as we found not two identical cell lines.

We have briefly mentioned that from the beginning we wanted to observe if PVR was behaving as a tumor suppressor gene which may explain the percentage of methylated cases that we detected in the first part of the discussion. Seeing the results and the methylation pattern, it seemed that demethylation was associated with a higher risk than hypermethylation. Upon malignant transformation, although it has been demonstrated once and once again that tumor cell hypomethylated promoter regions of tumor suppressor genes and hypermethylated oncogenes[105,111], in this case, immune checkpoint markers behave as beneficial for the disease and it has been observed that it is common to see hypomethylation of immune checkpoint markers upon malignant transformation[112,122], which was the same we were observing in our data.

Finally, to close this first discussion section, since for the project we needed to see how the changes in expression affected the different cell lines, we developed overexpression and depletion models for the selected cell lines. Since PVR has been described as having an endogenous function in the cells, we wanted to check if our altered models were affected at the functional level. We checked for apoptosis changes, proliferation, and cell cycle alterations but we could not find any significant changes between the controls and the models (regardless of if the alteration was towards overexpression or depletion).

Objective II: To evaluate the role of PVR in cytotoxic cell function and its impact on immunotherapy

So, in this segment, we studied the expression profile of PVR and left it on after developing cell line models where this expression was altered. Apart from the function that gives its name to our protagonist, the main function of the protein is in the immunity area. Although PVR is presented as a promiscuous receptor, it seems to have a strong predilection for its inhibitory receptor, TIGIT, as the forces of evil are always stronger than those to make good.

In homeostasis, PVR is part of an important structure that prevents the immune system from overreacting (which we know it tends to) and stops the attack when it is not necessary (as in when we present our perfectly healthy proteins to the system as a check routine). In this context then, the strong attraction of PVR for TIGIT makes total sense. But then malignant transformation occurs and what was once ideal now turns up to be undesirable and needs to be stopped. And so, our project proceeded toward how PVR played a role in the interaction between tumors and the immune system.

At this point of research, immune checkpoint markers have been quite recognized and although lots of work is yet to be done to take full advantage of their potential both as biomarkers and as treatment targets, we are going in the right direction. So, with this work, we did not pretend to explore new undescribed paths of inhibition, but rather to demonstrate what was happening in MM, what role immune checkpoint markers were playing (focused on PVR) in the disease, and if this effect went further than what we may have hypothesized at the beginning of the project.

And who suffers the effect of their negative interaction? Mainly affects T cells and NK cells which are the cytotoxic cells in part responsible for tumor clearance. Since this project grew while the laboratory went into immunity, most of our experiments were performed in the presence of T cells and not NK cells. After extensive research in T cells, where we became acquainted with primary cell culture and immune primary culture. We also learned that these types of cells were indeed the easiest to culture *in vitro*.

First, we studied how unaffected PVR-expressing cells behaved in the presence of allogenic-activated T cells. In our experiments, we did not select specifically for CD8+ cells, as we wanted to have a good representation as possible of the T cell population in the different donors. We activated the T cells using commercially available materials which allowed us to overcome the HLA restrictions, and although this system is still a small representation of what may actually be happening in MM patients, we were interested to see the interaction between healthy T cells and malignant cells and if the action of PVR made any difference at all.

We co-cultured our panel of cell lines with healthy T cells activated and isolated from at least four different donors. Nevertheless, the variability was huge among the cell lines, but even more so among the donors. The cells behaved singularly. Our hypothesis that PVR acts as an inhibitory marker was completely revoked as the expressing cell lines (unmethylated) were sensible to T cell attack and non-expressing (methylated) resisted the attack and proliferated. To confirm that in fact, the cells were acting through the expected pathway we analyzed the cytokines release into the supernatant of our cultures. We were able to observe how in all the cases, more cytokine detection was related to less survival, in all cases, except KMS-12-BM (Figure 25). If you are still up to discussion after a couple more points in the lists, you will see that we did not drop those problematic

cell lines just yet as we wanted to explore what was happening here. We will leave this temporary in order to keep the order of how the events succeed.

We then repeated the co-culture experiments with our model cell lines (Figure 26), where we altered the WT PVR expression by either overexpressing PVR (in methylated, non-expressing cell lines) or depleting PVR (in unmethylated, expressing cell lines). And from here we confirmed the results from the WT experiments. PVR overexpression models, although seemed to be slightly affected by the co-culture with the T cells, were completely unresponsive to cytotoxicity (Figure 26A), the alteration of PVR expression did nothing to change the sensibility of the cells towards T cell cytotoxicity, neither by increasing nor decreasing it. This indicated that these cell lines may have other mechanisms not described as resistance to cytotoxicity. In fact, several works have been published pointing towards several strategies found on tumor cells where they have evolved to be protected by the mechanisms used by cytotoxic cells. For example, the classic way of cytotoxicity involves the formation of pores by the perforins secreted by the cytotoxic cells and the internalization of granzyme B which cleaves several targets intracellularly, both leading towards apoptosis. Well, mechanisms have been described where cells can resist perforin action (i.e. ESCRT machinery use and vesicle formation)[123] or activate DNA repair programs to avoid granzyme damage[124]. Since we did not explore how our cell lines models resisted cytotoxicity here, we can only hypothesize possible ways for them to do so but seeing that other researchers have found several mechanisms to resist specifically cytotoxic activity by T and NK cells in hematological malignancy cells[125–127] led us to believe that this was probably the explanation for what we encountered in our experiments.

Despite what we observed in the PVR overexpression models, we still had functional PVR-depleted models where we successfully reduced PVR expression, we saw no effect on endogenous cell function, and that was sensible to cytotoxicity. And what happened when we compared controls and PVR-depleted models? As you can see in Figure 26A, in our three different cell lines, upon PVR depletion we were able to observe how cells became more susceptible to T cell attack, indicating that PVR was acting as an inhibitor towards cytotoxic activity in T cells. Again, as in the WT experiments, we corroborated that it was cytotoxicity that we were seeing and again we saw the same correlation between cytokine presence and decrease in survival.

One important aspect to bring up in this discussion is that as you may observe, different ratios were used in the different models. This was because, in the same way that we found that some cell lines seemed to resist T-cell cytotoxic attack, we also found that some cells were extremely sensible to them. By adjusting the ratio of effector cells and tumoral cells we were able to always move between the range of survival (20-80%) that our collaborators recommended us to be able to believe the results and be able to compare them among experiments and between donors.

Thanks to the characteristics of our models (which incorporated the production of GFP) we were able to track, using an EVOS M5000 fluorescent microscope, how the tumor cells were behaving in the presence of the T cells. In order to be able to differentiate them, we stained also the immune cells in blue and produced a time-lapse for 24 hours. As seen in Figure 27A, the number of cells (green) appreciated in the image is less in the PVR shRNA culture (depleted model) than in the scramble (control). Using ImageJ, we were able to count the "live" (green) cells after 24h, and as you can see the decrease was bigger in the depleted cells than in the scramble ones (Figure 27B, Movie 3). Since we had to learn to work with the machine, we also produced as another validation experiment some time-lapse movies where we could appreciate two facts that bring interesting points to the discussion. In Movie 1 you will see how cells die by apoptosis, most likely after they interact with a T cell. In Movie 2 you can see how tumoral cells tend to aggregate once the T cells are added to the co-culture. So, with these two facts we can say that: (I) Our MM cells die by cytotoxicity and (II) as a mechanism of resistance, cells tend to aggregate, which was observed in most of the experiments performed by this PhD student and where cells behave as they do in the BM, aggregating to be protected from immune cell attack[20].

In the co-culture experiments where we successfully saw differences, we wanted to confirm the action of PVR to be involved in the decrease of the cell survival we were observing. Since the rise of ICI was on the rise, we were able to work on an agreement with Bristol Myers Squibb Company and they provided us with a limited amount of one of their drugs in development BMS-986207 which has reported positive results in the last years[128,129]. This drug acted as a neutralizing antibody for TIGIT and all of its interactors. Following what was published, we added 10ug/mL of antibody to our cocultures. In Figure 28, you can see that in two of the three tested cell lines, the addition of anti-TIGIT successfully reduced the survival of our control models (and in the third one we observed a tendency). In some of the cultures, we also saw a decrease in our depleted models. To explain that you have to remember that depleted models are not knockouts, so the protein PVR is still expressed in lower numbers, and that the neutralizing antibody blocks all of TIGIT interactions, which if you remember from the background story, has also several ligands (although none as strong as PVR).

The results pointed towards a confirmation that in fact, PVR was acting as an inhibitor of T-cell activity in the context of MM and that by blocking its interaction with its main ligand (TIGIT) we would be able to somewhat modulate its effect. But was this everything that the interaction of PVR and TIGIT had to offer? At the end of the day, ICI are rarely used in monotherapy in the clinics since their success has been proven once and again to be limited, probably because we are fighting with one agent three hundred ninety different negative interactions acting at the same time (Figure 9).

So, seeing that the answer is the combination, we worked towards exploring how immunotherapy agents, which in some cases were being studied in clinical trials behave with our models. We started by incorporating a bispecific T-cell engager into our co-

culture system. By doing that we were warrantee that it was T-cell activity that was killing the cells and we wanted to explore if PVR depletion could also affect a forced interaction between the immune cell and the MM cell. As you can see in Figure 29, we observed how PVR-depleted cells were always more sensitive to cytotoxicity, even when the T-cell interaction was being forced.

Regardless, this was not the only novel immunotherapy that we were able to incorporate in our experiments. Thanks to our collaborators, we were able to receive from important hospital research groups from Spain two constructs that have been changing MM treatment options in the country.

On one hand, Dr. Fernández de Larrea's group sent us some CAR-T cells modified with the construct that recently have been approved for RRMM as the first construct produced exclusively by a public institution[130,131].

Before returning to the experiments performed for the project, I wanted to mention that during the starting phases of the collaboration, some samples were sent to be tested with these CAR-T products. Among the samples sent, we analyzed how the CAR-T cells behaved in the presence of AMO-1, KMS-12-BM, and RPMI-8226 models (our two PVR overexpression models and a PVR depletion one). This data is not included in this document as the replicates were small, the experiments were not performed by us, and this was part of preliminary experiments to explore if we could detect any cytotoxicity towards our models. Surprisingly, while RPMI-8226 (PVR depletion model) was sensible towards cytotoxic attack by CAR-T construct, the overexpression models, which if you may remember resisted T cell attack, were not affected by CAR-T cells either. What we saw in this preliminary data would be explained by the same reason that these cell lines resisted T-cell cytotoxicity, if they were able to resist apoptosis by cytotoxicity, they would be able to resist it independently from the cell delivering the effect.

After this small observation, let’s go back on track and continue with how PVR/TIGIT interaction affected CAR constructs. Since the modified cells were limited, we could only perform these experiments in two of our models. As you can see in Figure 30B, the depletion of PVR allowed cells to be more sensible to CAR-T cytotoxic attack. When we added anti-TIGIT, in our pursuit of how combination therapies could work against MM cells, we were only able to get significant results in one of our models, but a strong tendency was observed in three of the four donors studied on the other cell line model. This indicates again, the importance of taking biological variability when designing the experiments and how this may represent how this kind of treatment can also fail in some of the cases observed in the clinic.

Thanks to Dr. Martinez in Madrid, we were able to perform our experiments in the presence of CAR-NK products, also targeting BCMA, and that are currently being explored in the hospital setting in real MM cases[82,85]. In our co-cultures, again with the same two models, we could obtain in both cases significant results when depleting PVR and when

adding our anti-TIGIT neutralizing antibody (Figure 30D). These results indicate that while PVR depletion (PVR low expression) seems to be the best strategy towards avoiding cytotoxicity resistance, neutralizing the interaction between PVR and TIGIT could (in most cases) recapitulate these results and imitate in cases with high PVR expression the benefit that PVR low expression cases have in this setting.

Altogether these experiments led us to believe that again as it has already been described, using a combination strategy with a novel immunotherapy option and adding anti-TIGIT neutralizing antibody to the equation could bring promising results to explore in the MM setting. Since this project did not explore the microenvironment in which the disease occurs, is important not to forget about the fact that our results are just a small representation of several other interactions that may be affecting the fitness of both the CAR construct cells and the survival of the tumor. Regardless, I think it is important to take into account these results when designing new strategies to fight against MM.

Finally, this is the picture (Figure 38) depicting our theory summarized.

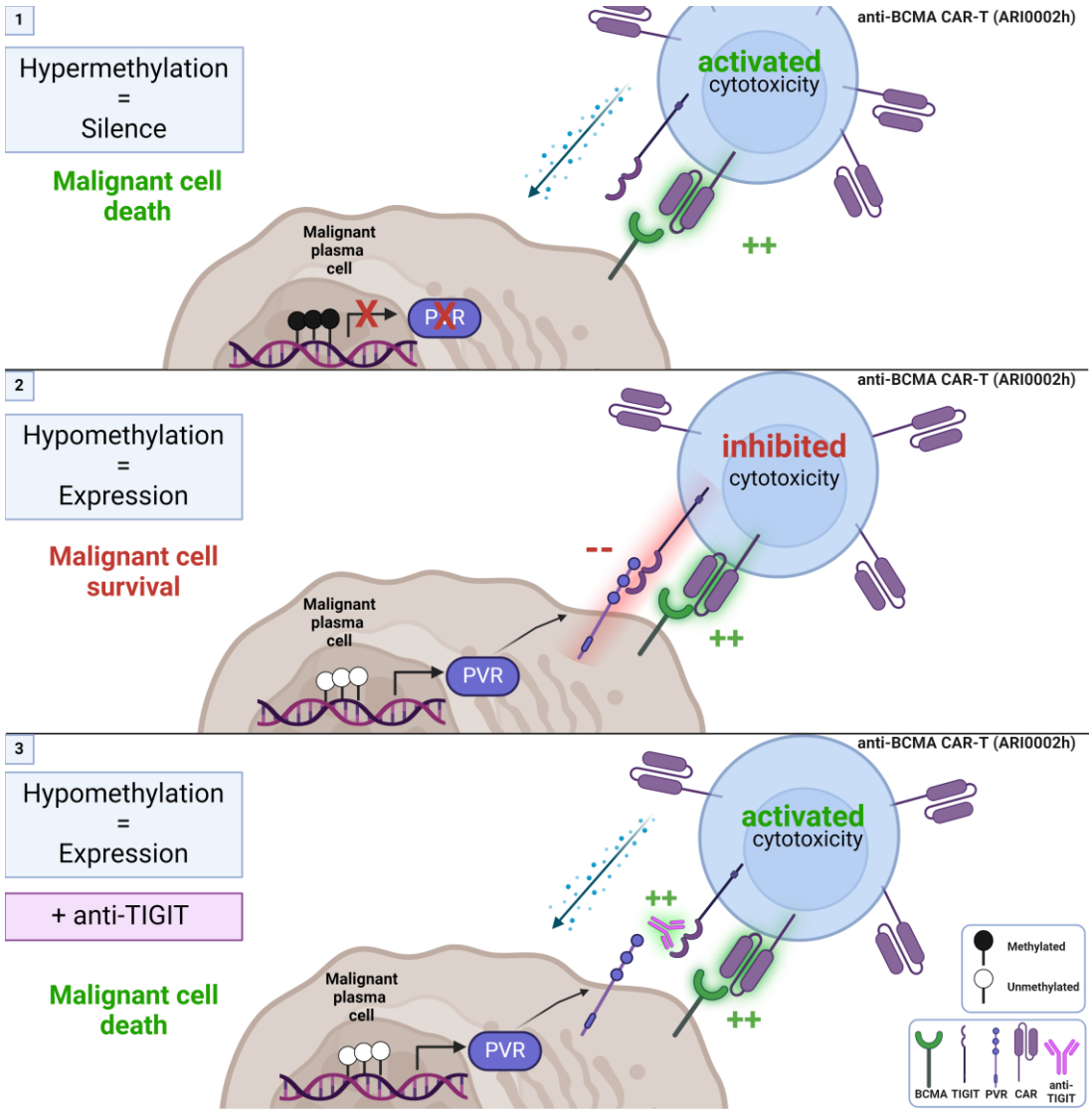

Figure 38. Graphical resume of the findings in this thesis.

In this context and reaching the final lines of this part of the discussion regarding the second objective of this project, I was able to work for a period of three months in an international lab in the USA, under the supervision of Dr. Smith at Dana Farber Cancer Institute, where I was able to work more deeply on the development of CAR-NK products and learned cloning techniques. Annex I are just a sneak peek of the work I performed in other to help advance their project. There are several other small issues to be addressed as CAR constructs are big chunks of DNA that need to be incorporated into small-size viruses so reducing the promoter towards its minimal functional expression is one way to go, other strategies may be added to the NK promoter specificity, as for example the use of suicide genes to block the expression of the construct in other lineages or microRNA target sides which may destroy the mRNA if that is expressed on an undesired cell, or altogether, change the strategy and make a direct modification of the HSC by using CRISPR technology, which, as you may wonder, comes with a different set of issues that needs to be resolved before continuing with the project.

Objective III: To study the effect of PVR expression alteration and its relationship with other immune checkpoint markers

We have demonstrated that PVR expression can modulate how MM cells interact with cytotoxic cells. But as we have already mentioned something that I thought was important to bring up for discussion was how PVR expression was affected by other immune checkpoint markers. At this point in the text, we are experts on immune checkpoint markers, and we know that there is not a unique interaction determining if the cytotoxic cell will attack or not.

In 2019, when we experienced the rise of knowledge in immune checkpoint markers, some groups dedicated their efforts to studying how the different immune checkpoint proteins were expressed or in other words, if they were forming cohorts of expression, something that is known to happen in a family of proteins with similar functions. In fact, Lee et al.[120] described that in several different solid tumors, PD-L1 was forming an expression cohort with other markers and they demonstrated that it was most likely that they were expressed together.

First, we studied the expression profile of our cells (both from T cells from healthy donors and from the models we were using for our experiments). In T cells (Figure 31) we could see how upon activation some proliferation genes increased their transcription, (like *MKI67*) and others related to cytotoxic activity (like *GZMB*, *IFNG,* or *FasL)*, those were the biggest changes observed (although due to the size of our sample, those changes were not always significant). Other changes where transcription was increased were in genes related to immune checkpoint proteins like *CD137, LAG3,* or *TIM3*. When we created a correlation matrix with Spearman's R, we could clearly see how *PD-1* was forming an expression cohort different from *TIGIT*, indicating that their expression, in our samples, was independent of each other.

On the other hand, in our models (Figure 32), we saw a great variety of transcriptional changes when comparing them to CD138+ plasmatic cells. Here we could see changes mainly in inhibitory immune checkpoint markers (like *PD-L1, PD-L2,* or *CD276*) with the same issue that we encountered before about sample size and significance. When we used these data to create Spearman's correlation, we observed how, as described in Lee et al.[120], *PVR* and *PD-L1/PD-L2* were present in different cohorts of expression also in the models.

Altogether, these experiments told us that the inhibitory axis for PVR/TIGIT and PD-L1/PD-L2/PD-1 were independent of each other, at least in our samples, but following a trend that has been observed before in other solid tumors. This fact brings up the point that we have been discussing before which is that by blocking one inhibitory axis, most likely we will have others acting in parallel in an independent manner which may nullify our efforts towards avoiding cytotoxic cell inhibition.

Following our first objective and going back briefly, since we saw that the expression of IC markers was correlated, we wanted to explore if the methylation status of their promoter regions would also maintain this relation. In fact, we were able to access several promoter methylation statuses. In some of them, we were able to confirm the relation between promoter DNA methylation status and expression. When we analyzed the DNA methylation of the IC markers, we could see how at Hematological malignancy, B-cell malignancy, and MM level (Figure 33), *PVR, PD-L1,* and *PD-L2* were always situated at different cohorts of methylation and expression, confirming the conclusion reached in the previous paragraph.

Since so far, we have been working with healthy T cells and our models, we wanted to explore if this relation was still true in primary MM cases, at least at the expression level. Once again, thanks to CoMMpass and the RNA-seq data available from the patients we were able to normalize their counts and create an expression matrix (Figure 34A). Once again, *PVR* was not included in the expression cohort that *PD-L1* and *PD-L2*, meaning that what we so far observed *in vitro* was being translated into the clinic and supporting our idea of combination therapies as the best approach to the disease. Since the CoMMpass project had survival data associated, we took advantage and performed some survival analysis by dividing the patients into groups depending on their PVR and PD-L1 expression. To our surprise, while PVR was significantly independent from PDL1 (high or low), PD-L1 was not enough to separate the patients into different groups (Figure 34B-C).

We had evidence pointing towards the independent nature of PVR and PD-L1, and in the case of our samples, also of PD-1 and TIGIT. It was because of this fact that we wanted to test *in vitro* what we had observed *in silico*. To one of our co-cultures, we added, apart from the 10µM of anti-TIGIT neutralizing antibody, a commercially available anti-PD1 neutralizing antibody (by this way we were blocking both PD-L1 and PD-L2 interactions with PD-1). As shown in the graph in Figure 35, the addition of both antibodies to the co-

culture created a synergy effect, both in the scramble and the PVR-depleted models. With this experiment, we confirmed that the axis was being expressed independently and that a combination strategy would be the best approach in this setting.

Finally, as a more exploratory strategy, we performed RNA-seq in some of our models. Due to the limited time left to develop my thesis project, we were only able to explore the data obtained from the PVR depletion models although we also obtained results for the wild type of cell line comparison and the overexpression models. We chose the PVR depletion models since the results obtained with their experiments were compositing the larger part of this project. First, we performed a differential expression analysis from the raw counts. We decided to be permissive and considered all the changes above a log2 fold change of 1 (positive or negative) with a significance lower than 0.01. RNA-seq data is tricky to analyze since usually a large number of results is obtained and scientists must select which way to look at that data. Using the list of genes that were in the fractions shown in Figure 36A, we performed two separate analyses the GO biologic process analysis and the KEGG pathway analysis. These types of analyses take into consideration the given list of genes and look for associations among them to a set database from the program used for the analysis. In our case, we were comparing our list of genes to the database of biological processes that fall into the Gene Ontology Human 2023 collection and under the KEGG Legacy compendium. We analyzed several different sets of genes and decided that the best approach was to look at the list of genes that had a differential expression bigger than a log2 fold change of 1. The results (listed in Figure 36B-C) were surprising. PVR has been described to be actively participating in the ability of cells in motility and adhesion capacity[57,61]. It seems that altering the expression of PVR also influences other genes related to that as cell adhesion and cell motility were collections that were greatly affected. Although this finding may lack novelty, it is important for us as it confirms the role that PVR has, further away from immunity than what we initially believed. Another important group of functions that appeared affected after PVR depletion were intracellular pathways. This may indicate that PVR, being a receptor itself, has also an effect on other transporters. One of the results that surprised us, was the appearance of neurological-related development being affected by PVR depletion. Curiously, it seems that neurons and malignant transformation have a lot in common and this brings up something that previously may have been obscured by other discoveries which is the involvement of CNS in tumorigenesis.

Autonomous nervous system (ANS) innervates the bone. It is believed that both the sympathetic (SNS) and the parasympathetic systems (PNS) play a role in controlling bone function and orchestrating bone remodeling (formation and destruction)[132]. But concerning the topic, we have been discussing until now, nerves also play a role in BM regulation, and it has been described to interact (although not directly) with the cells in the microenvironment. Several sources confirm the role of peripheral sympathetic nerves in tumor progression and although the work has focused on leukemia and myeloid syndromes[132–136], the fact that the model has been presented to participate in BM (where MM is developed) and to affect the cells residing on it (promoting a tumor surviving

environment and increasing the expression of co-inhibitory markers such as PD-L1[135]) indicates that although not tested yet, MM may benefit from neurogenesis in the BM and make sense to our findings on the RNA-seq analysis.

Finally, to this topic, we also performed differential analysis and gene ontology analysis on a comparison of the CoMMpass newly diagnosed cases (Figure 37). Once again, to our surprise, the genes that registered bigger changes were related to neurogenesis and ion transport which may or may not be related to neurons anyway and their changes in potential. These results were reminiscent of the pathways that were also altered when comparing our PVR depletion models, validating the results found in our samples.

And with these words, this journey comes to an end. I hope you enjoyed this little story which now is part of the world thanks to our publication Martinez-Verbo *et al*.[137] I hope you learned something new and enjoyed the journey as much as I did.

Conclusions

CONCLUSIONS

Taken together, our findings prove that in the context of MM, PVR has a role in the interaction between immune cells and tumors. PVR interaction with its receptors leads to an immunosuppressive effect that can be decreased by the application of neutralizing antibodies and new immune therapies benefit from this blockage. Globally, the results of this thesis project contribute to increasing the understanding of the role immune checkpoint events play in cytotoxicity inhibition in the context of multiple myeloma but that sets the basis to decipher if this knowledge can be extended to other hematological malignancies.

The specific conclusions reached for each of our objectives are listed:

Objective I: To characterize PVR's epigenetic regulation and expression in multiple myeloma.

I. Promote methylation of PVR controls its expression in hematological cancers, and this is demonstrated in multiple myeloma.

II. The percentage of hypermethylated cases is similar in multiple myeloma primary cases and multiple myeloma cell lines.

III. High PVR expression is connected with worse overall survival in newly diagnosed multiple myeloma cases, independent of age, sex, and risk stratification.

IV. High PVR expression is significantly higher in high-risk cytogenetic groups, t(4:20), thjaks.

V. Multiple myeloma cell lines represent the heterogeneity of the disease.

VI. In our models, no differences were found in apoptosis, cell cycle, and proliferation of tumoral cells upon differences in PVR expression.

Objective II: To evaluate the role of PVR in cytotoxic cell function and its impact on immunotherapy.

I. PVR expression inhibits T-cell activation.

II. PVR expression effect on immune cell activation can be, in part, controlled with anti-TIGIT neutralizing antibody.

III. In the context of novel immunotherapies, PVR expression is enough to change the effect of bispecific T-cell engagers.

IV. CAR-T and CAR-NK products, directed towards multiple myeloma also experience a detriment in their function upon PVR expression.

V. This effect can be ameliorated by the presence of an anti-TIGIT neutralizing antibody, although is not enough by itself as PVR depletion.

VI. A combination strategy against multiple myeloma seems the best approach that will increase the efficacy of novel immunotherapies.

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Objective III: To study the effect of PVR expression alteration and its relationship with other immune checkpoint markers.

I. PVR and PD-L1 are expressed in different cohorts of expression in multiple myeloma samples.

II. PVR and PD-L1 inhibitory axis act in parallel inhibiting T-cell activation.

III. PVR expression seems to be related to other elements in the multiple myeloma tumor microenvironment inside the bone marrow as neurogenesis pathways are affected by its downregulation.

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Annex

Annex

Annex I: CAR construct design and optimization

Defining activation states in different lineages

As part of the HSC-CAR project, we worked on determining which markers would indicate an activated state in different immune cells. We were able to determine that the best activation markers for NK, T cell, and THP-1 (as representation for myeloid cell lineage) were CD25, 4-1BB, and CD40 respectively (Figure A1).

Testing specific NK promoters into NK and T cells

To work with HSCs in the laboratory requires spending a high number of resources for an experiment that may go wrong. It was because of this that before developing an experiment where HSCs would have to be used, we needed to develop constructs where we were sure that (I) did not present any leakiness and (II) we knew the right concentrations and methods to measure the results and be able to detect the differences. Before going on and not bringing up your hopes, let me say that this is a clear example of negative results are also results.

Using the constructs shown in Figure A2A, we transduced them into lentiviral particles. We proceeded then to expand the culture for 72 hours before measuring the presence of the marker, in our case GFP protein, in the cells. To our surprise, it seemed like the CAR construct was not expressed, while this experiment was a replicate of a previous one (Figure A2B). The synthetic protein used for measuring the activity of the CAR was denaturalized, a new one could not be generated in time and thus, my experiment ran short. Despite this, we were encouraged to continue by seeing the results of the previous experiment.

These data, together with the information recollected from the activation experiment, were used to set the basis for future experiments where the results from this work will be combined.

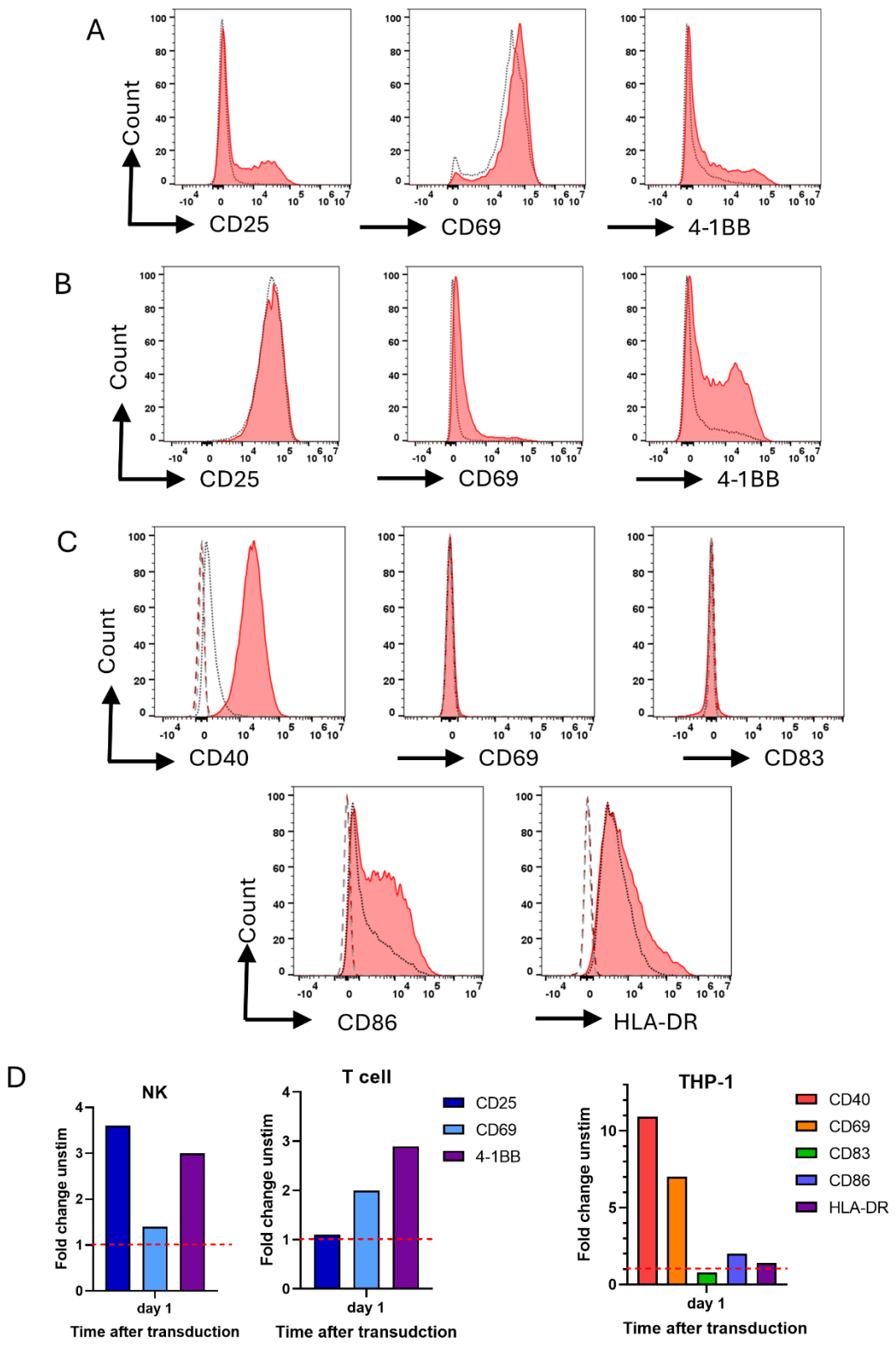


Figure A1. Expression levels of activation markers in NK, T cells, and THP-1 cell line. (A-B) Expression levels by flow cytometry of activations markers for lymphocytes in primary NK cells (A) and primary T cells (B). (C) Expression levels by flow cytometry of activation markers for myeloid lineage cells in THP-1 cell line. (D) Quantification of (A-C) as fold change of unstimulated cell levels.

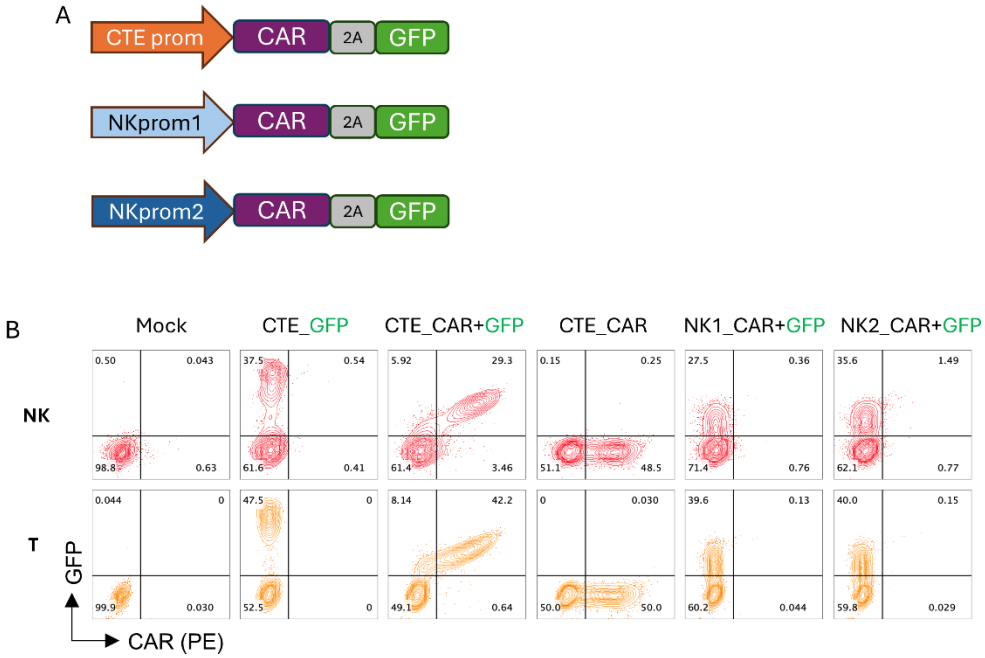
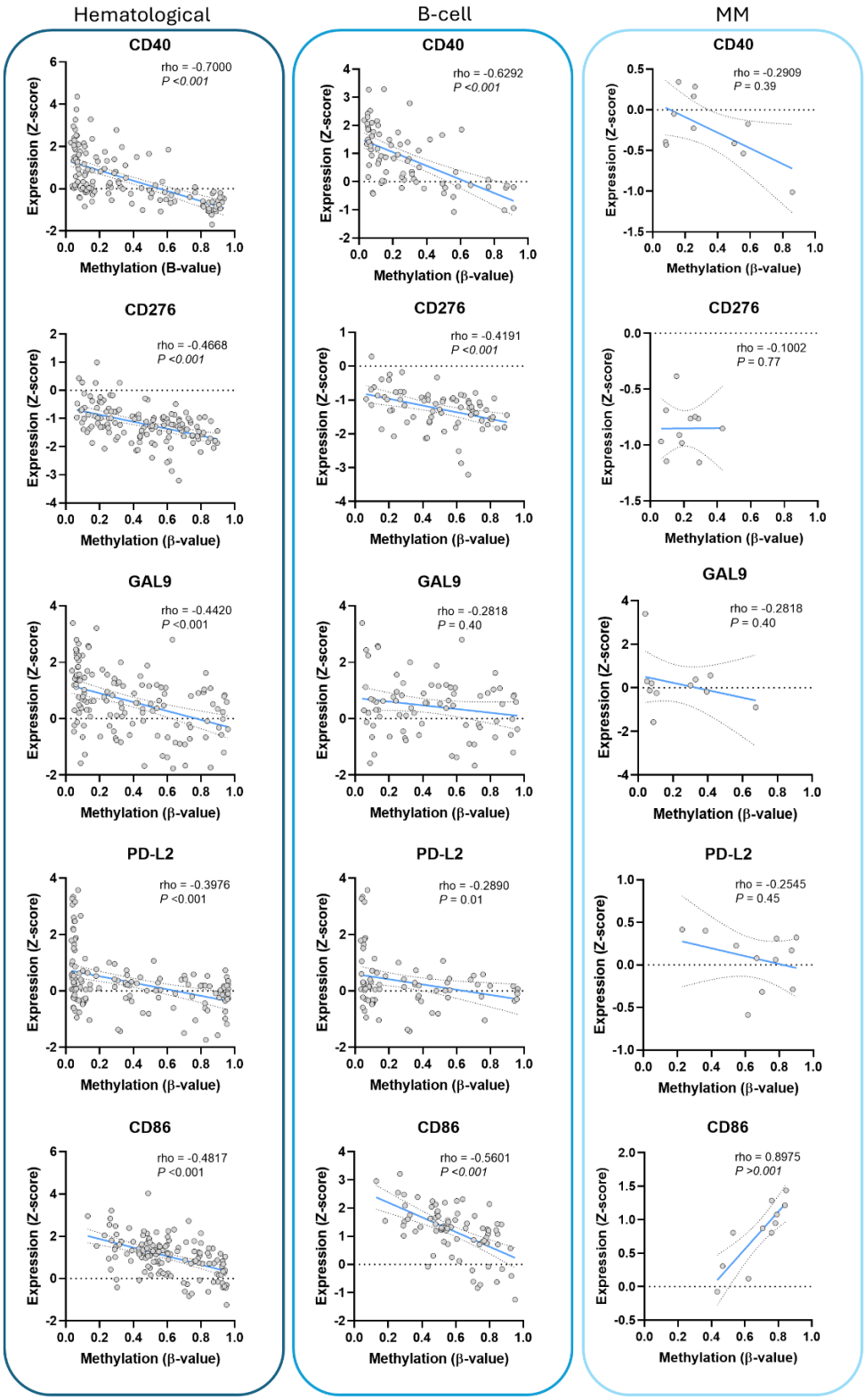


Figure A2. CAR-constructs transduction into primary NK and T cells. (A) Constructs cloned into lentivirus particles to test NK promoter lineage specificity). (B) Flow cytometry graphs showing the CAR (PE) and reporter levels to see transduced positive cells.

Annex II: Methylation-expression correlation analysis of immune checkpoint markers in hematological malignancies, B-cell malignancies, and MM

In Figure A3 we show the correlation graphics from expression and methylation values from cell lines from hematological malignancies, B-cell malignancies, and Multiple myeloma.



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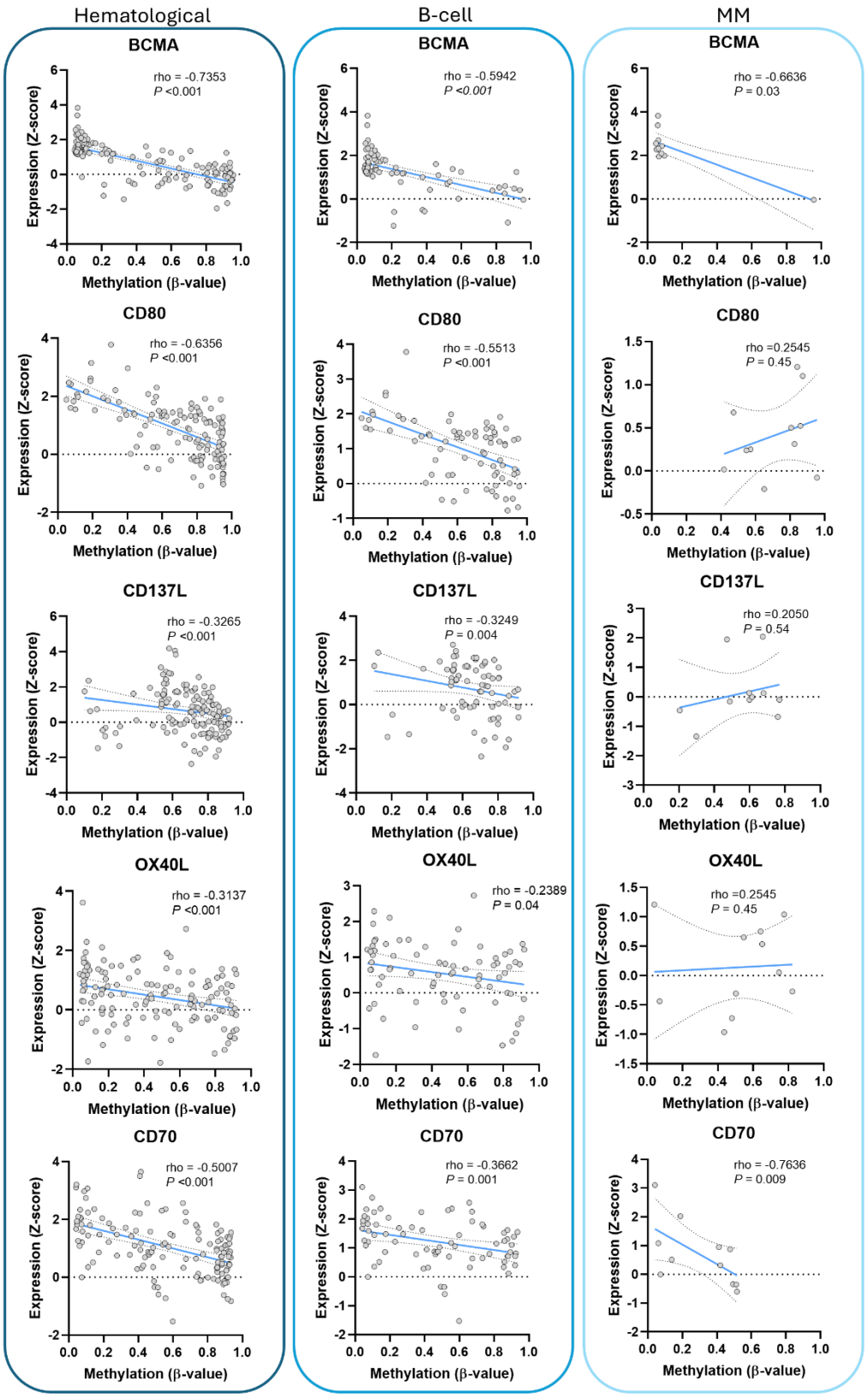
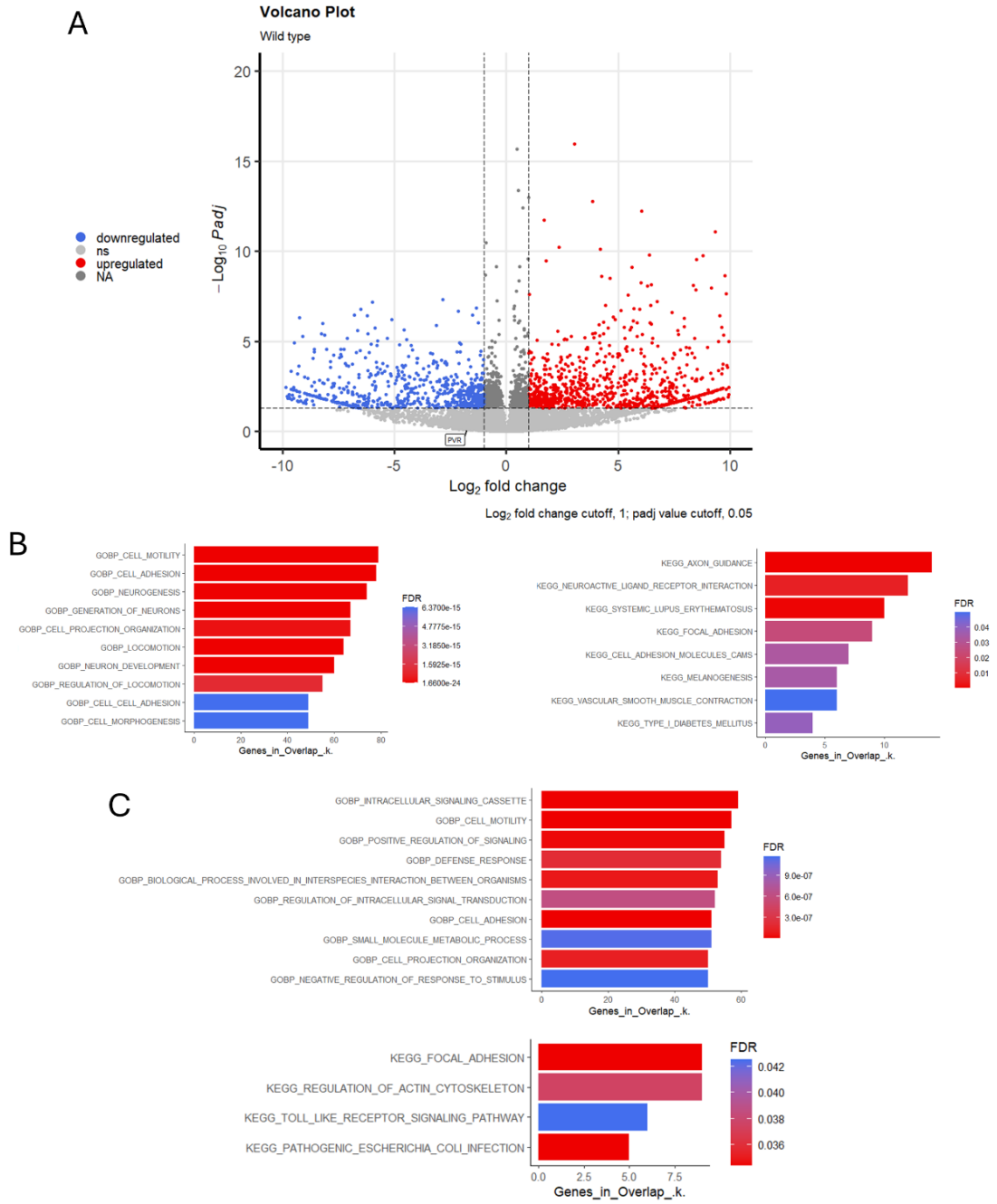


Figure A3. Correlation graphics for promoter methylation and expression level of immune checkpoint markers in hematological cell lines, B-cell malignancy cell lines, and MM cell lines.

Annex III: RNA-seq analysis results

The full list of Differentially Expressed (DE) genes can be found in the following link or following this link.

In this section, you will find the results from the gene ontologies generated from the genes with a log2 DE higher or lower than 1 with a *P*-adjusted value of <0.001 for the comparisons of WT cell lines, PVR overexpression models and a more detailed representation of PVR-depleted model and CoMMpass results.



Annex A4. Representation of RNA-seq results comparing WT MM cell lines. The analysis was performed by comparing methylated cell lines (AMO-1 and KMS-12-BM) against unmethylated cell lines (RPMI-8226, JJN-3, and EJM). (A) Volcano plot. (B) GO biological analysis and KEGG pathway for the downregulated genes of the analysis. (C) KEGG pathway analysis for overexpressed genes of the analysis.

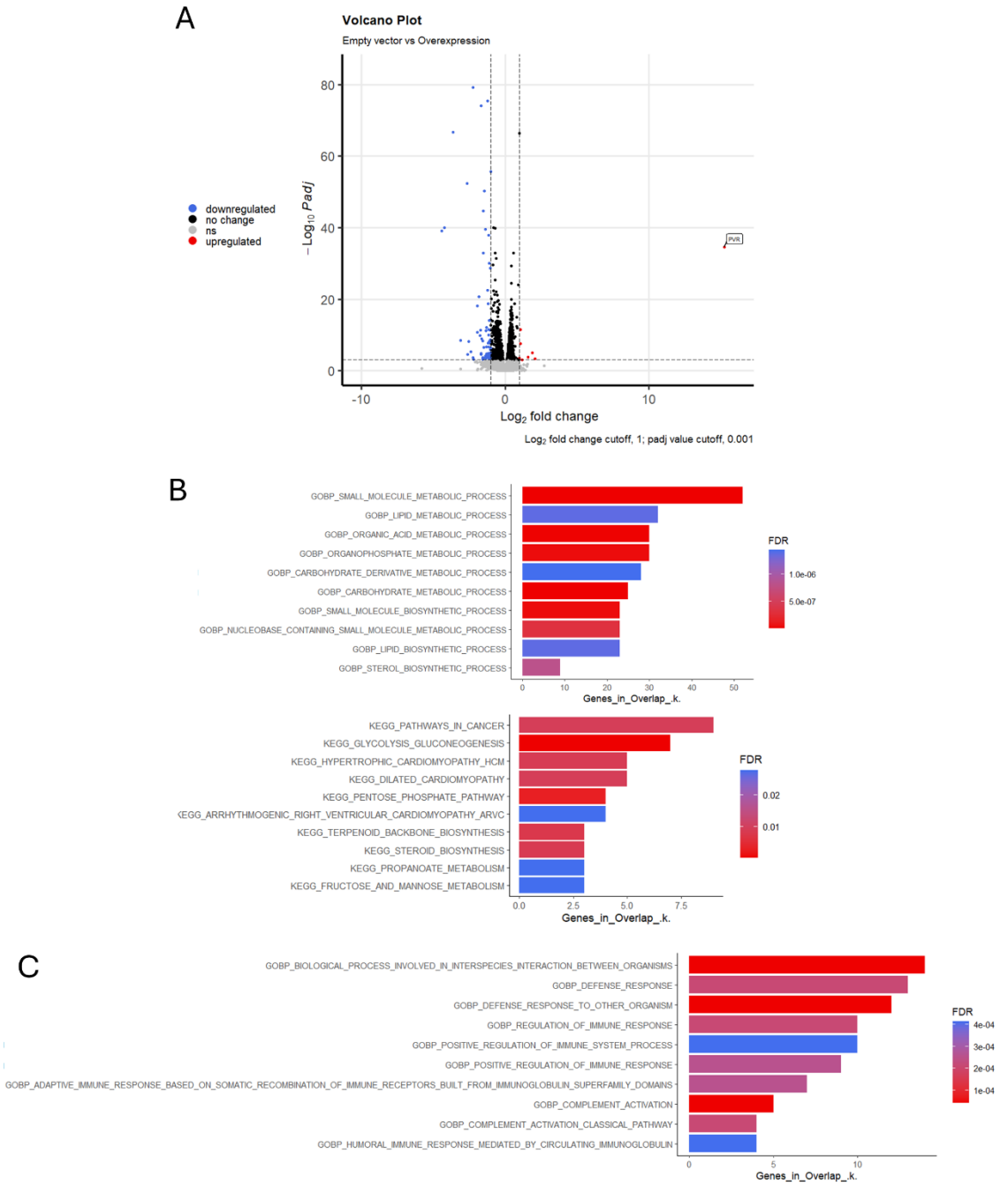


Figure A5. Representation of RNA-seq results comparing PVR overexpression AMO-1 models. The analysis was performed by comparing the PVR overexpression model in a methylated cell line (AMO-1) versus its respective empty vector control. (A) Volcano plot. (B) GO biological analysis and KEGG pathway for the downregulated genes of the analysis. (C) GO biological analysis for overexpressed genes of the analysis.

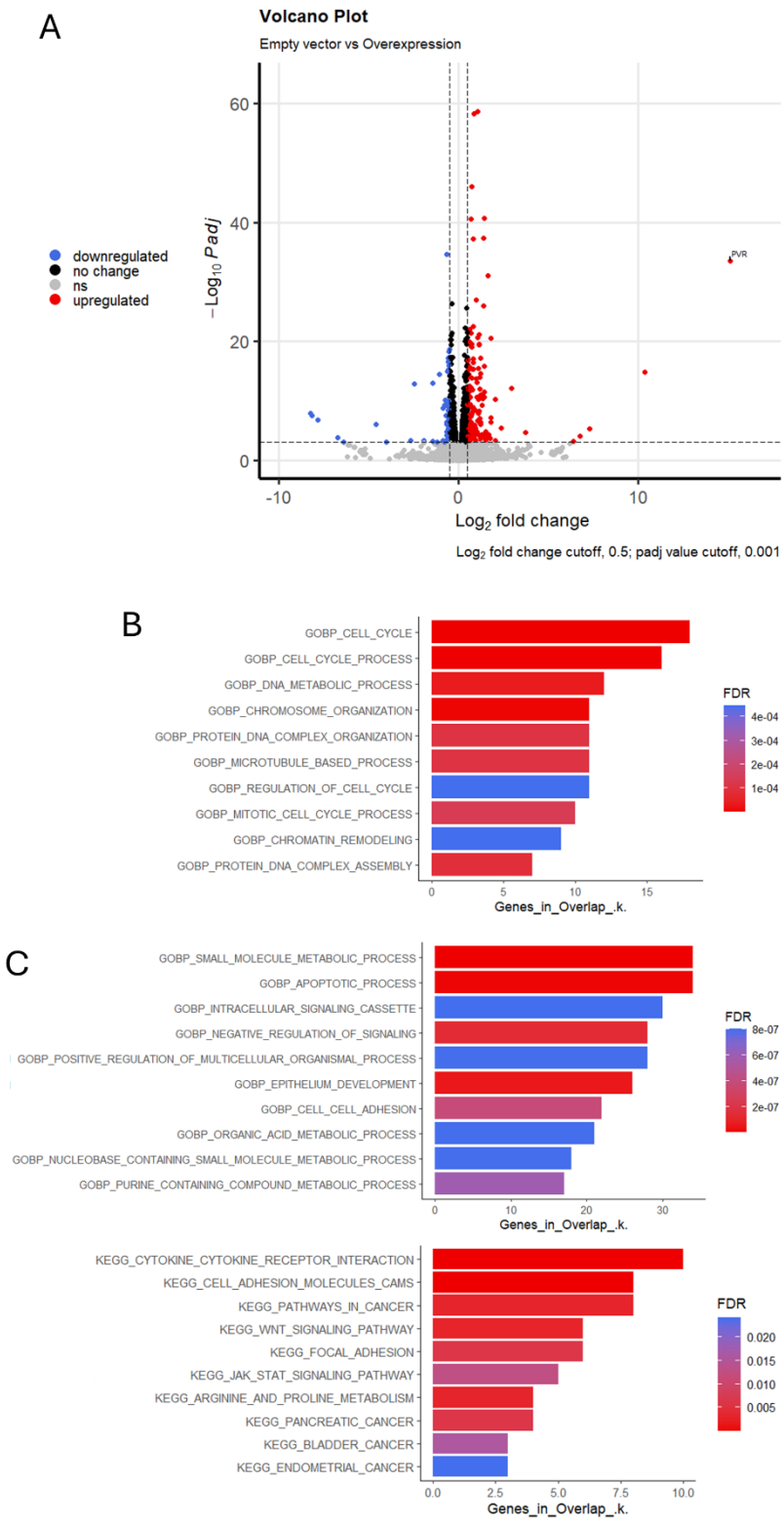


Figure A6. Representation of RNA-seq results comparing PVR overexpression KMS-12-BM models. The analysis was performed by comparing the PVR overexpression model in a methylated cell line (AMO-1) versus its respective empty vector control. (A) Volcano plot. (B) GO biological analysis for the downregulated genes of the analysis. (C) GO biological analysis and KEGG pathway analysis for overexpressed genes of the analysis.

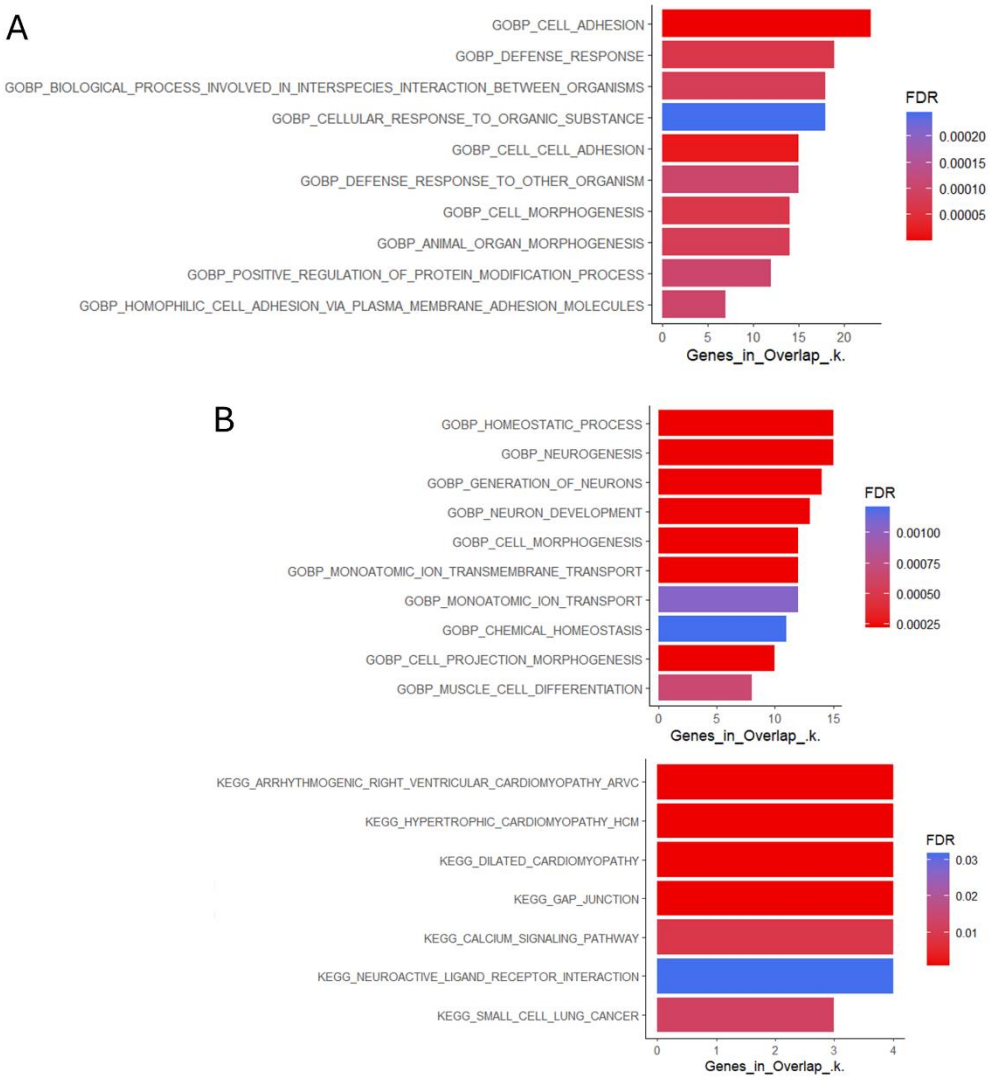


Figure A7. Representation of RNA-seq results comparing PVR-depleted RPMI-8226 models. The analysis was performed by comparing the PVR depletion model in an unmethylated cell line (RPMI-8226) versus its respective scramble control. (A) GO biological analysis for the downregulated genes of the analysis. (B) GO biological analysis and KEGG pathway analysis for overexpressed genes of the analysis.

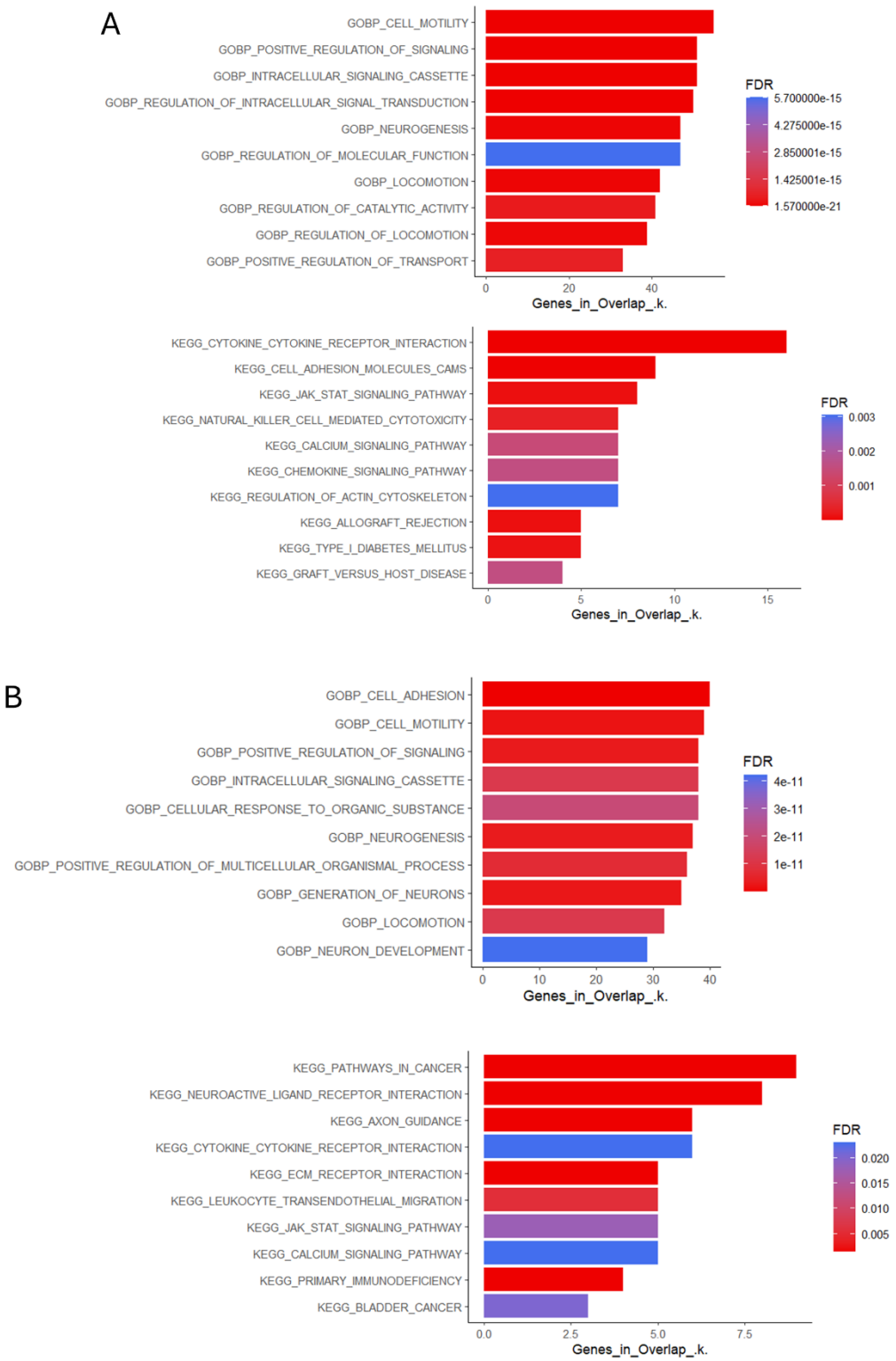


Figure A8. Representation of RNA-seq results comparing PVR-depleted JJN-3 models. The analysis was performed by comparing the PVR depletion model in an unmethylated cell line (JJN-3) versus its respective scramble control. (A) GO biological analysis and KEGG pathway analysis for the downregulated genes of the analysis. (B) GO biological analysis and KEGG pathway analysis for overexpressed genes of the analysis.

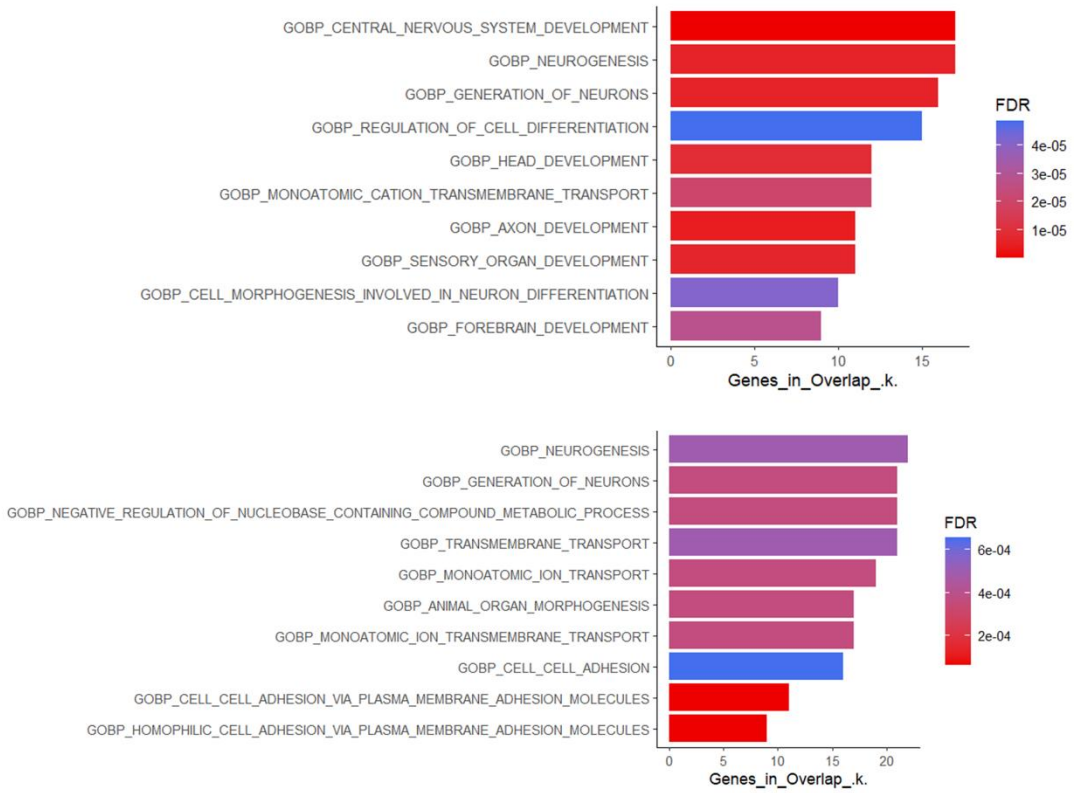


Figure A9. Representation of RNA-seq results comparing PVR-high expression (Q4) against PVR-low (Q1-Q3) expression patients from the CoMMpass project. GO biological analysis and KEGG pathway analysis for the downregulated genes of the analysis. (*top)* GO biological analysis and KEGG pathway analysis for overexpressed genes of the analysis. (*bottom*).

Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.

Antonio Machado

Proverbios y Cantares, *Campos de Castilla*, 1912