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Induced Pluripotent Stem Cells (iPSC) in Chronic Myeloid Leukemia (CML) : Modelling Blast Crisis and Designing a CAR-Cell Therapy Model

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Induced Pluripotent Stem Cells (iPSC) in Chronic Myeloid Leukemia (CML): Modelling Blast Crisis and Designing a CAR-Cell Therapy Model

*Les cellules souches induites à la pluripotence (CSIP) dans la Leucémie Myéloïde
Chronique (LMC) : Modélisation de la crise blastique et design d'un modèle de
thérapie à base de cellules CAR*

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le co-encadrement de **Annelise BENNACEUR-GRISCELLI**, PU-PH

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SYNTHESE

La leucémie myéloïde chronique (LMC), hémopathie clonale maligne de la cellule souche hématopoïétique est un modèle d'immunothérapies et de thérapies ciblées. L'histoire naturelle de cette maladie, constamment mortelle jusqu'au début des années 1970, a été transformée d'abord par la pratique des greffes de moelle allogénique avec un effet immunitaire démontré des cellules du greffon sur la leucémie. Ce traitement était cependant applicable à une minorité de patients en raison de l'âge et de l'absence de donneurs. La véritable révolution a été l'introduction à la thérapie, des Inhibiteurs de tyrosines kinases (ITK) dans les années 2000. Ces thérapies ont changé le pronostic et la prise en charge des patients: Ainsi, les patients ayant une réponse moléculaire aux ITK, ont une espérance de vie normale, identique à celle de la population générale. Toutefois, les résistances aux ITK et la progression de la LMC surviennent avec apparition d'une phase de leucémie aiguë, appelée « crise blastique » notamment chez les patients non-répondeurs, nécessitant des approches de greffe allogénique.

La compréhension des mécanismes impliqués dans la progression de la LMC est donc essentielle et nécessite l'utilisation de nouveaux modèles reproductibles *in vitro*. L'utilisation des cellules souches induites à la pluripotence (CSIP) pour la modélisation de la LMC a permis de reproduire certains aspects de la phase chronique de la maladie à partir de CSIP patient-spécifiques Ph1+ (Telliarn, 2016). L'évolution de la maladie vers une leucémie aiguë est liée à l'instabilité génétique des cellules, avec apparition au cours du temps, de mutations supplémentaires dans le clone Ph1+. Actuellement, il n'existe aucun modèle permettant de créer cette évolution *in vitro*.

Mon travail de Thèse a consisté d'une part en l'établissement d'un modèle d'instabilité génétique de cellules leucémiques dérivées des CSIP Ph1+ et d'autre part de démontrer la faisabilité d'identifier des cibles thérapeutiques. J'avais par ailleurs comme objectif, de démontrer la possibilité d'utiliser une approche d'immunothérapie par utilisation des cellules « Natural Killers » (NK) pour cibler les cibles identifiées par notre approche de mutagénèse.

Nous avons dans un premier temps démontré que la mutagénèse des CSIP par l'agent alkylant (*N*-nitroso-*N*-éthylurée) provoquait une instabilité génétique globale, identique à celle observée chez les patients (Article 1). La comparaison transcriptomique entre les cellules hématopoïétiques générées à partir de CSIP mutées et la base de données GSE4107 (Radich et al. 2016) montrent que 125 des 249 aberrations identifiées sont déjà décrites dans les phases accélérées et blastiques de la LMC. Dans le premier article nous avons ainsi démontré que notre protocole de mutagénèse permettait de générer *in vitro* un modèle d'instabilité génétique récapitulant les événements génétiques décrites dans les cellules leucémiques primaires de patients présentant une crise blastique.

Nous avons par la suite étudié de plus près l'effet de la mutagénèse au niveau transcriptionnel. Nous avons ainsi utilisé trois lignées de CSIP Ph1+ générées dans notre laboratoire à partir de trois patients atteints de LMC. La mutagénèse a permis une signature transcriptomique globale de progression. Chez un patient, nous avons pu montrer, la génération de cellules de leucémie aiguë de phénotype monoblastique *in vitro*, avec une signature transcriptomique identique à celle des patients atteints de leucémie aiguë. Parmi les marqueurs observés, nous avons identifié le *CD25* comme un marqueur de la progression de la LMC et l'avons validé dans une cohorte de patients atteints de LMC en phase chronique et blastique.

Dans la troisième partie de ma thèse nous avons exploré la possibilité de génération de cellules de type NK ciblant spécifiquement le CD25 par une approche de CAR-NK.

Nous avons ainsi généré des vecteurs permettant de transférer le CD25 CAR aux cellules NK. Dans les travaux décrits dans l'article 3, j'ai validé la possibilité de générer cette approche dans une lignée ayant une activité NK (NK92). Ces cellules devant cibler les cellules leucémiques exprimant CD25, j'ai généré une lignée « cible » leucémique surexprimant le récepteur CD25 via transfert du gène CD25 dans la lignée K562.

Nous avons ainsi pu identifier l'expression du CAR à la surface des cellules NK92. Les cellules CD25 CAR-NK92 ont montré des niveaux plus élevés de Granzyme B et de Perforine ainsi qu'une cytotoxicité plus accrue envers des K562 exprimant l'antigène CD25. Les résultats préliminaires chez la souris ont aussi montré une efficacité plus élevée des CD25 CAR-NK92 comparée aux NK92 sauvages. La perspective de cet article en préparation est de renouveler les expériences *in vivo* avec des cohortes plus grandes et de différencier les CSIP CD25 CAR caractérisées en NK.

Les résultats que nous avons générés ouvrent de nouvelles perspectives dans la modélisation de crises blastiques par utilisation de CSIP et proposent pour la première fois une thérapie potentielle à base de CAR-NK pour la crise blastique chez patients atteints de LMC.

ABBREVIATIONS:

AAV – Adeno-associated Viruses
ACA - additional cytogenetic abnormalities
Ad- Adenoviruses
ADCC – Antibody-Dependent Cellular Cytotoxicity
ALL – Acute Lymphocytic Leukemia
AML – Acute Myeloid Leukemia
Ang1 – Angiopoietin 1
AOG – Aorta-Gonad-Mesonephros
AP-CML – Advanced Phase Chronic Myeloid Leukemia
APC – Antigen presenting cell
ASCT – Autologous Stem Cell Transplant
ATP – Adenosine Triphosphate
B2M – Beta-2 microglobulin
BaEV-LV – Baboon Envelope Pseudo-Type Lentivector
BC-CML – Blast Crisis – CML
Bcl – B-cell Lymphoma gene
BCR – Breakpoint Cluster Region Protein
BFU-E – Burst-Forming Unit
BLI – Bioluminescence Imaging
BM – Bone Marrow
BMI 1 – B lymphoma Mo-MLLV insertion region 1 homolog
BMP4 – Bone Morphogenetic Protein 4
BMT – Bone Marrow Transplantation
BRCA1 – Breast cancer 1
CAR – Chimeric Antigen Receptor
Cas9 - CRISPR-associated protein 9
CD – Cluster of Differentiation
CFC – Colony Forming cell
CFU – Colony-Forming Unit
CFU-GEMM – Colony Forming Unit-Granulocyte Erythrocyte Monocyte and Megakaryocytes
CFU-GM – Colony Forming Unit-Granulocytes Macrophage
CLP – Common Lymphoid Progenitor
CML – Chronic Myeloid Leukemia
CMP – Common Myeloid Progenitor
CMR – Complete Molecular Response
CP-CML – Chronic Phase-Chronic Myeloid Leukemia
CRISPR - Clustered regularly interspaced short palindromic repeats
CTL – Cytotoxic T Lymphocytes
DC – Dendritic Cells
DLI - Donor Lymphocyte Infusion
DMR – Deep Molecular Response

DNA - Desoxyribonucleic acid
DNAM – DNAX accessory molecule
DSB – Double Strand Breakages
EB – Embryoid body
EHT – Endothelial-to-hematopoietic transition
EMT – Epithelial-Mesenchymal Transition
EPH – Ephrin protein
EPO – Erythropoietin
ESC – Embryonic Stem Cell
ETH – Endothelial-to-hematopoietic transition
FABD - F-actin binding domain
FBS – Fetal Bovine Serum
FDA – Food and Drug Administration
FGFR – Fibroblast Growth Factor Receptor
Flt3L – FMS-Like Tyrosine Kinase 3 Ligand
FoX – Forkhead box
FV – Foamy Viruses
G-CSF – Granulocyte-Colony Stimulating Factor
GM-CSF – Granulocyte and Macrophage Progenitor
GPCR - G-Protein-Coupled Receptors
GR - Growth Factors
GSK – Glycogen Synthase Kinase
GvHD – Graft Versus Host Disease
GvL – Graft Versus Leukemia
HDAC – Histone deacetylase
HE – Hemogenic Endothelium
hESC – Human Embryonic Stem Cell
HOXB4 – Homeobox Protein 4
hPSC – Human pluripotent stem cells
HSC – Hematopoietic Stem Cell
ICAM – Intercellular Adhesion Molecule
ICM – Internal Cell Mass
IFN – Interferon
Ig – Immunoglobulin
IL – Interleukin
IM – Imatinib
INCA – Institut National du Cancer
iPSC – Induced Pluripotent Stem Cell
IVF – *In vitro* Fertilization
JAK – Janus Kinase
kDa – Kilo Dalton
KIR – Killer Immunoglobulin-like Receptor
KO – Knock Out
LAA – Leukemia Associated Antigens
LDL – Low-Density Lipids
LDLR – Low-Density Lipids Receptor
LIF – Leukemia Inhibitory Factor

LILRB1 - Leukocyte immunoglobulin-like receptor subfamily B member 1
LMMP – Lymphoid Primed MMP
LRP – Lipoprotein
LSC – Leukemic Stem Cell
LT-HSCs – Long-Term HSC
LTC-IC – Long-Term Culture Initiating Cells
LV – Lentivirus
M-CSF – Macrophage Colony Stimulating Factor
MDS - Myelodysplastic syndrome
MDSC – Myeloid Derived Suppressive Cell
MEF - Mouse Embryonic Fibroblasts
mESC - Mouse Embryonic Stem Cell
MHC – Major Histocompatibility Complex
MICA – MHC class I polypeptide-related sequence A
MMP – Multipotent Progenitors
MP – Macrophage Progenitor
MPN – Myeloproliferative Neoplasm
MPP – Multipotent Progenitors
MSC – Mesenchymal Stem Cells
NAD – Nicotinamide Adenine Dinucleotide
NCI – National Cancer Institute
NCR - Natural Cytotoxicity Receptors
NF κ B – Nuclear Factor – kappa B
NFAT - Nuclear Factor of the activated T-cell
NK cells – Natural Killer cells
NKG2 – Natural Killer Group 2
NOD/SCID – Non-obese diabetic/Severe combined immunodeficiency
NSG – NOD Scid Gamma Mouse
OCT4 – Octamer-binding transcription factor 4
OSKM – OCT4, SOX2, KLF4, cMYC
PB - Peripheral Blood
PBMC – Peripheral Blood Mononuclear Cells
PcG – Polycomb Repressive Complexes
PCP – Planar Cell polarity
PDGF – Platelet-Derived Growth Factor Receptor
Ph – Philadelphia
PI3K – Phosphoinositide 3-kinase
PIGF – Placental Growth Factor
PMA – Phorbol Myristate Acetate
PNN – Polymorphonuclear Neutrophils
PR3 - Proteinase 3

PRAME – Preferentially Expressed Antigen Melanoma
PTC – Patched 1
PTEN – Phosphatase and TENsin Homolog
RAC1 - Ras-related C3 Botulinum Toxin Substrate 1
RET – Rearrangement During Transfection Protein
ROS – Reactive Oxygen Species
RT-PCR – Reverse Transcription Polymerase Chain Reaction
SAHA - Suberoylanilide hydroxamic acid
SB – Sleeping Beauty
SCF – Stem Cell Factor
scFV – Single-Chain Variable Fragment
SCT – Stem Cell Transplantation
SeV – Sendai virus
SHH – Sonic hedgehog
SLAM – Signaling Lymphocytic Activation Molecules
SMO pathway – Smoothed Pathway
ST-HSC – Short Term HSC
STAT – Signal Transducers and Activators of Transcription
TCR – T Cell Receptor
TD – Transmembrane Domain
TF -Transcription Factors
TFR – Treatment-Free Remission
TGF- β - Transforming Growth Factor β
TIGIT – T cell immunoreceptor with Ig and ITIM domains
TKI – Tyrosine Kinase Inhibitor
TME – Tumor Microenvironment
TNFR – Tumor Necrosis Factor Receptor
TPO – Thrombopoietin
Treg – T Regulatory Cells
Tyk2 – Tyrosine Kinase 2
UCB – Umbilical Cord Blood
VCAM – Vascular Cell Adhesion Molecule
VEGF – Vascular Endothelial Growth Factor
VH – Variable Fragment Heavy Chain
VL – Variable Fragment Light Chain
VSV-G – Vesicular Stomatitis Virus G Protein
WHO – World Health Organization
WT1 – Wilms Tumor 1
ZFN – Zinc Finger

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INTRODUCTION

1. Chronic Myeloid Leukemia

1.1. General overview

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm (MPN) representing between 15 and 20 % of leukemic diseases in adults. In high-income countries, CML is a relatively rare disease with an incidence of 1/100 000 and a median age at diagnosis of 55 years. The incidence is higher in males as compared to females (ratio: 1.3-1.5/1) (Jabbour et Kantarjian 2014).

CML is characterized by the presence of the *BCR::ABL1* fusion gene generated by the reciprocal translocation between chromosomes 9 and 22, t(9;22) (q34.1;q11.2). The BCR::ABL fusion protein generated from this translocation is highly oncogenic and characterized by an enhanced tyrosine kinase (TK) activity.

The natural history of CML is characterized by the evolution from a chronic phase (CP), (most cases diagnosed at this phase) that can last between 3 and 5 years towards a more aggressive, accelerated (AP-CML) and blastic transformation phases (BC-CML). Contrary to the chronic phase, during the accelerated phase, the differentiation level of the cells is altered and this can lead to a differentiation arrest giving rise to acute leukemia designed as a “blast crisis”. Blast crisis (BC) is characterized by high-rate cell proliferation, a differentiation arrest, and an accumulation of blast cells in the bone marrow and the peripheral blood. BC is life-threatening and remains of dismal prognosis even in the era of tyrosine kinase inhibitors (TKI).

In general, CML is diagnosed fortuitously during a checkup. However, in some cases, symptoms like fatigue or abdominal pain related to splenomegaly can occur requiring medical attention. The etiology of the disease remains still unknown, but some factors have already been described such as the exposition to ionizing radiations e.g., the use of historic radiotherapeutics for the treatment of ankylosing spondylitis (Wick et al. 2008) or the number of CML cases observed among the survivors of atomic bombs in Hiroshima and Nagasaki in 1945 (Ichimaru et al. 1991; Lange, Moloney, et Yamawaki 1954).

Since the introduction of TKI to therapy in early 2000, the natural history of CML has changed. Before this revolutionary therapy, the median survival of patients was invariably low, between 3-5 years from the time of diagnosis. Nowadays, patients in CP responding to TKI therapies have a survival expectancy similar to that of persons of the same age and sex. However, treatment-refractory cases occur when the disease has progressed to AP-CML or BC-CML, this represents between 25 and 30% of CML patients which raises the need for new therapeutic approaches against CML.

Cell therapy has been historically the mainstay of CML therapy before TKI, as the only curative therapy of CML before the TKI era was allogeneic bone marrow transplantation (E. D. Thomas et Blume 1999). It has also become very clear that upon the relapse of patients after bone marrow transplantation (BMT), the use of donor-derived lymphocytes could be used to obtain remission. This donor lymphocyte infusion (DLI) still used today, has paved the way for many more sophisticated cell therapies approaches today such as chimeric antigen receptor-T (CAR-T)

therapies. Indeed, cell therapy based on cell engineering of T cells CARs is an emerging therapy for cancer treatment. Although not applied currently to CML, the identification of targets in advanced phases of the disease could find an application in this pathology. CAR-T cells targeting CD19 were recently approved by the US Food and Drug Administration (FDA) for the treatment of relapsed or refractory acute lymphoblastic leukemia (ALL) in pediatric and young adults (Kymriah), and for the treatment of relapsed or refractory diffuse large B cell lymphoma in adults (Kymriah and Yescarta). CAR-T cells might be responsible for some adverse effects in patients such as CRS (cytokine-releasing syndrome) or graft-versus-host disease (GvHD). To overcome these effects, scientists are currently interested in Natural Killer (NK)-CAR-based therapies. Moreover, CAR-NKs are related also to 'off-the-shelf' manufacturing, thanks to their major histocompatibility complex (MHC)-independent activity.

1.2. History of CML

CML has been described for the first time independently in 1845 by John Bennett in Scotland and Rudolph Virchow in Germany (Schwalbe, 1901.)

A case described by Alfred Donne in France was published only a year before (1844) and was probably also a CML. Alfred Done described this case in a book in which he noticed that the blood was thick with a major proliferation of white blood cells (Ballière, 1844).

The infectious origin of the disease was also suspected in all three cases, explaining the term "leucocythemia" by Bennett (1852). In 1960, Peter Nowell and David Hungerford identified for the first time in two male CML patients, a short chromosome 22, due to the translocation t(9:22) (q34;q11) and they named this chromosome as Philadelphia 1 (Ph1) chromosome. Ph1 was suspected to be related to damages due to the treatment that those patients received. However, Baikie et coll. (Baikie et al. 1960) describe during the same year a small chromosome in bone marrow cells in CML patients that have not received any treatment. Moreover, this was specific to bone marrow cells and was absent in skin fibroblasts. In the '70s of the past century, Janet Rowley identified this translocation as a reciprocal event between the long arms of the chr. 9 and the chr. 22. The next major discovery was the identification of BCR::ABL (Gale et Canaani 1984) allowing cloning of the fusion gene (Witte et al. 1978) and generating later experimental data leading to CML models and the development of targeted therapies (see details below).

From a pathophysiologic point of view, the clonal nature of CML such as any other myeloproliferative neoplasm (MPN) was described by Fialkow using the X chromosome inactivation technology in female CML patients, showing that the isoenzymes of G6PD were always from one type (A or B) in leukemic cells whereas both A and B isoenzymes were expressed in normal tissues. This allowed him to describe for the first time, the clonal nature of CML (Fialkow, Gartler, et Yoshida 1967).

CML is one of the highly studied leukemia and over the last 20 years, many advances have been achieved from cloning of BCR::ABL (Witte et al. 1978) to CML models (Chomel et Turhan 2011). **Figure 1** summarizes the main steps of CML discovery.

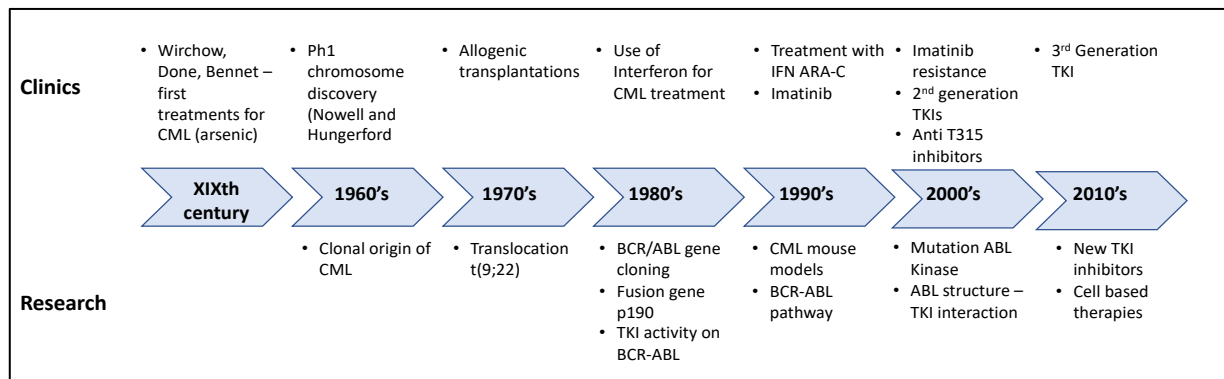


Figure 1: Historical evolution of CML-related discoveries and therapeutics from its discovery in the XIXth century until nowadays.

1.3. Epidemiology

As mentioned previously, CML is a rare disease. At a global level, it represents 1-2 cases/100 000. In France, around 12 000 people are living with CML, mostly among people over 50 years old, and in France, like in other countries, there is a male predominance. According to the National Cancer Institute (NCI) the prevalence of this cancer in the USA in 2018 has been estimated at around 61 700 people. CML is typically found in patients over 50-60 and it is quite rare in young people, representing only 3% of newly diagnosed cases of infantile leukemias (according to the Canadian Society of Leukemia and Lymphoma). The annual incidence in France is around 700 cases, and according to the National French Cancer Institute (INCA), the number of CML cases in 2018 was 872 (480 males and 392 females) (Gautier Defossez et al. 2019). According to the NCI there have been 9110 diagnosed cases of CML in 2021 in the United States, representing 0.5% of all new cancer cases. For the same year, 1220 patients died from CML which represents 0,2 % of all cancer deaths.

1.4. CML Pathophysiology and its clinical evolution

CML is a hematopoietic stem cell malignancy originating from a single transformed stem cell clone (Fialkow, Jacobson, et Papayannopoulou 1977). As opposed to acute leukemias, the acquisition of the Ph1 chromosome gives rise to the proliferation of cells of myeloid lineage with an accumulation of myeloid precursor cells in the BM and spleen. This major proliferative potential is accompanied by an antiapoptotic effect related to *BCR::ABL* expression, leading to the clinical presentation with splenomegaly, leukocytosis, and thrombocytosis but rarely also with anemia. In rare cases, especially in young female patients, there is a clinical presentation with exclusive thrombocytosis with hyperleukocytosis. The diagnosis is made in all cases by the bone marrow aspiration with cytogenetic analysis demonstrating the presence of t(9;22) translocation. The cytogenetic analysis is of crucial importance as it can reveal additional cytogenetic abnormalities (ACA) which can be present at diagnosis and are of prognostic importance. Molecular analyses are performed in BM but also in the peripheral blood according to standardized techniques revealing the presence of *BCR::ABL* transcript which is quantified according to an international standard (ELN Leukemia recommendations). The first-line therapies currently involve the use of TKIs, the efficacy of which is followed by regular molecular analyses every 3 months (Hochhaus et al. 2020).

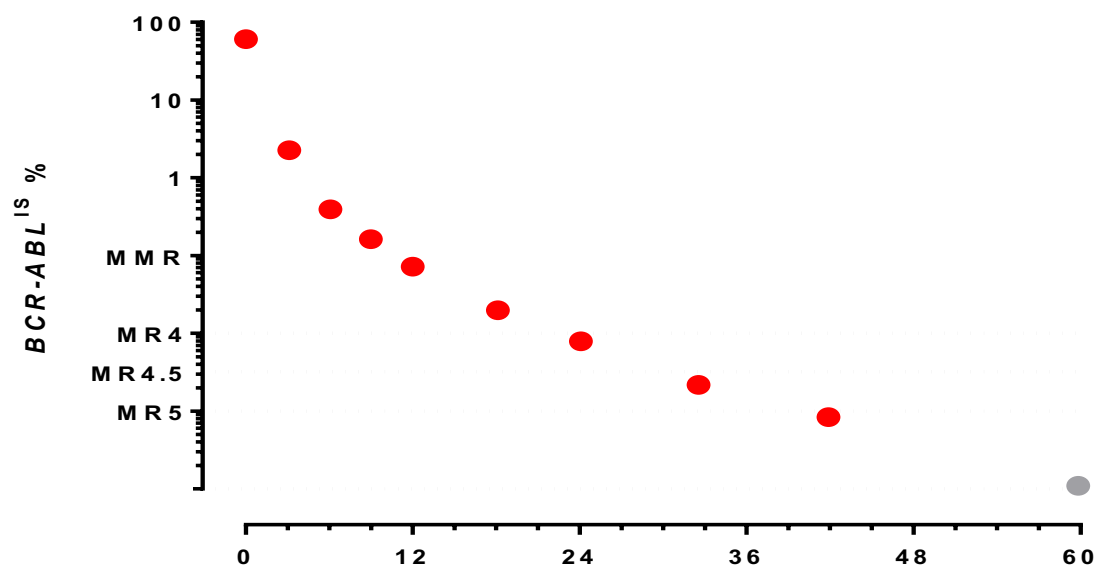


Figure 2: Evolution of the percentage of BCR::ABL during TKI treatment (Cross et al. 2012)

As mentioned previously, the natural history of CML involves a progression from chronic phase to accelerated and blast phases. Clinical and cytological features allow for determining the phase of CML.

- a. **The chronic phase (CP)** – is the phase where most patients with CML are diagnosed. At this step, there is hyperleukocytosis and very often splenomegaly. There is a major hypercellularity in the bone marrow with blast cells not exceeding 10%.
- b. **The accelerated phase (AP)** is suspected during the therapy of a patient, with the absence of response to therapy on molecular analyses and progressive thrombocytopenia and splenomegaly. Bone marrow analyses reveal an increase in the number of blast cells by 10-30% of blast cells in BM.
- c. **Blast crisis (BC)** – is a life-threatening step of CML. Despite the major success of TKI therapies to control the disease, BC indeed still occurs even after several years of TKI therapies, especially in patients with TKI-resistant disease. Patients present with the appearance of increased numbers of blast cells in peripheral blood (PB) and bone marrow (BM) aspirates show more than 30% of blast cells. The diagnosis is made by cytological analysis of circulating and bone marrow blasts, and the phenotype of the cells is evaluated using flow cytometry allowing to determine the lineage of cell proliferation. Therapy at this stage depends on the previous TKI that the patients received, the phenotype of blast cells (myeloid or lymphoid), and the possibility of performing a stem cell transplantation if a donor is available.

In the majority of the cases, BC is of myeloid lineage with AML phenotype (70% of cases) but a lymphoid BC can occur in 30% of cases with typical features of an ALL). The differential diagnosis between de novo ALL and CML in lymphoid BC remains on the analysis of molecular workup which shows a transcript of minor BCR encoding a

fusion protein of 190 kDa, shorter than BCR::ABL P210 (see below). **Figure 3** shows the evolution of CML.

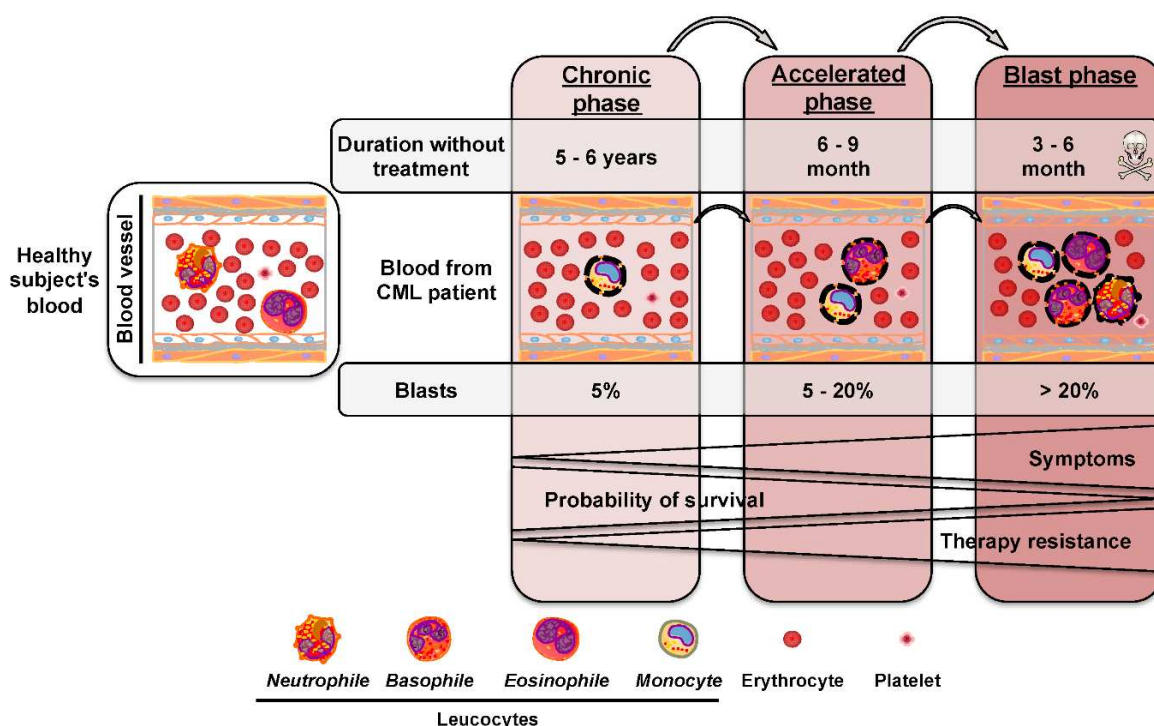


Figure 3: Progression of CML – Schematic representation of CML evolution from chronic phase to accelerated and blast phase and its duration without treatment. (Losson et al. 2020)

1.5. t(9;22) Translocation and BCR::ABL fusion protein

1.5.1. 22q- Chromosome

As described in the previous chapter, CML is characterized by the appearance of a pathognomonic chromosomal abnormality which is the Philadelphia (Ph) Chromosome in a very primitive hematopoietic stem cell. Ph chromosome results from a translocation between the long arm of chr. 9 (region q34) and the long arm of chr. 22 (region q11) (**Figure 4**). This event generates in the chr. 22 (which is shortened following the fusion between genes BCR (Breakpoint Cluster Region)) and ABL (Abelson murine leukemia 1). BCR is a region of 4 to 5 kb which includes all breaking points between exon 12 and 16 in CML (b1 to b5) whereas, in the ABL gene, the breaking points could take place in a large region of 200 kb (Groffen et al. 1984) (**Figure 5**). The hybrid gene is located at chr. 22 q- and after splicing it gives rise to an RNA transcript of 8 kb which translates into a fusion protein of 210 kDa. According to the loci of breaking BCR, different variants can be generated (**Figure 5**)

Major BCR::ABL1 is the most common form. It consists of a 210 kDa (p210) protein and is resulting from the recombination of BCR exon 13 or 14 with exon 2 from ABL1. The transcripts are named e13a2 and e14a2 respectively or **M-BCR** for **major BCR::ABL1**. This form is found in 95% of CML Ph+, in 5% of AML, in 15-30% of ALL, and in 5% of ALL in children. The expression of p210 is the molecular marker of reference for CML (Deininger, Goldman, et Melo 2000).

Minor BCR::ABL1 is resulting from the break between intron 1 in BCR and exon 2 of ABL1. It results in a protein of 190 kDa (p190) and is named **minor BCR::ABL1 (mBCR::ABL1)** or **e1a2**. It represents 25% of B-ALL in adults and more rarely in AML. It can also be present in CML (only 0,4%) following alternative splicing. It can be expressed simultaneously by lymphoid blast crisis cells following a CP-

CML. p190 BCR::ABL has a very important tyrosine kinase activity compared to p210 which is related to a high phosphorylation level associated with a weak auto-inhibition (J. Radich et al. 1997).

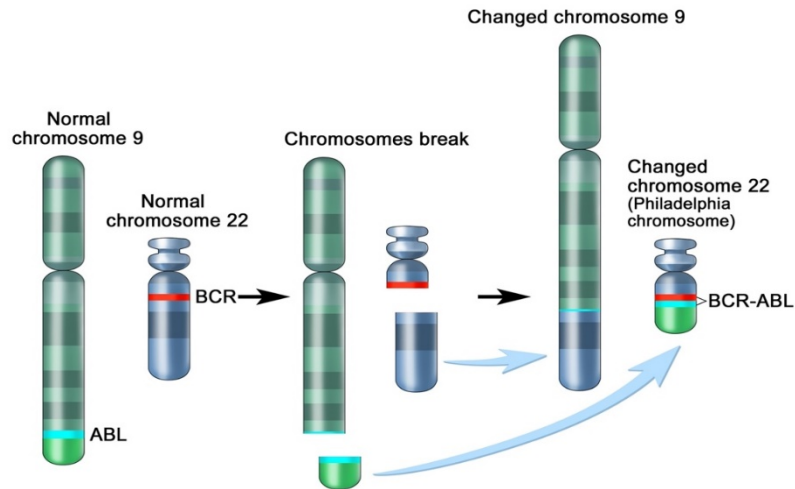


Figure 4 : BCR::ABL translocation between chr. 9 and 22 (Terese Winslow, 2007)

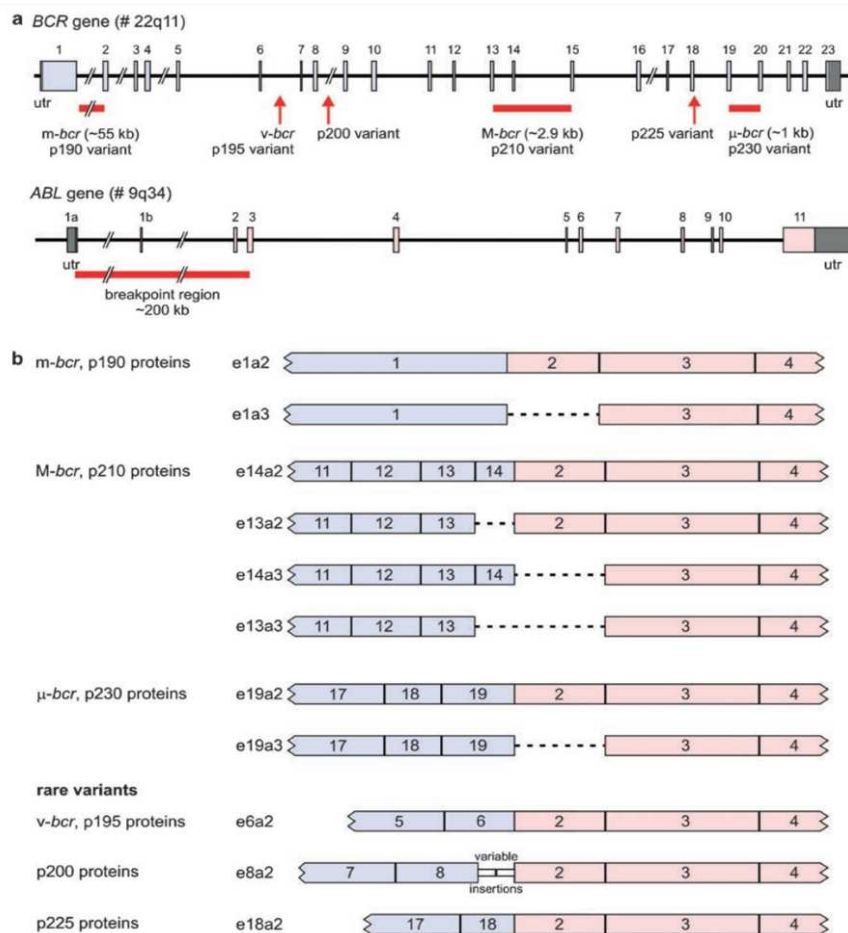


Figure 5: Structure of BCR and ABL genes with the breakpoint regions and corresponding fusion gene transcripts (Weerkamp et al. 2009).

μ-BCR (microtranscript BCR::ABL) is resulting from the break between exome 19 of BCR and exon 2 of ABL1. Chronic neutrophilic leukemia and/or thrombocytosis may be associated with this breakpoint region (Pane et al. 1996). The evolution of the disease and the response to treatment are identical to CML p210 (Pasternak et al. 1998). It is found in less than 0.1% of CML cases. This combination is responsible for the formation of e19a2 ARN which codes for p230 BCR::ABL.

1.5.2. Chromosome 9q+ and ABL-BCR protein

In 70% of CML cases, a reciprocal translocation ABL::BCR in chr. 9q+ is also transcribed. (Melo et al. 1993). This translocation is responsible for the generation of 4 transcripts: ABL (1a or 1b)-b3 and ABL (1a or 1b)-b4. b3 and b4 associated with exon 1b of ABL are longer and are represented in 2/3 of CML Ph+ cases. The proteins generated contain the region GAP of BCR. The deletion of some genes in this region might have a deleterious effect on the complex Swi/Snf (Grand et al. 1999). The role of this deletion remains still not fully understood. Different studies show that it might have an important role in BC-CML (Huntly et al. 2002).

1.5.3. ABL gene

The role of the normal ABL gene remains still not well characterized compared to the fusion gene *BCR::ABL*. ABL is the human equivalent of murine transforming gene *v-Abelson (A MLV)* (Abelson et Rabstein 1970) (Shields et al. 1979). It plays a major role in cell and developmental biology together with p53 as p53 and ABL double K/O mice are not viable (Whang et al. 2000). In physiological conditions, ABL plays also a role in the cell cycle during which it shuttles between the nucleus and cytoplasm (Wang., 1993). Even before its participation in the *BCR::ABL* fusion gene, the role of *v-ABL* (a viral form of *c-ABL*) in cell transformation was established. Indeed *v-ABL* can induce a cell transformation of B-lymphoid cells and fibroblasts *in vitro* (Shields et al. 1979) (Witte et al. 1978). The proto-oncogene *ABL (c-Abi)* codes for a ubiquitarian protein of 145 kDa present in the nucleus. Following alternative splicing, the isoform has an NH2-terminal glycine domain which can be myristoylated and thus send the protein to the membrane (Van Etten, Jackson, et Baltimore 1989).

ABL contains different domains highly conserved and able to bind to the DNA or the actin FABD (F-actin binding domain) (J. Y. J. Wang 2014), an import/export nuclear signal, and 3 Src-domains (Figure 6).

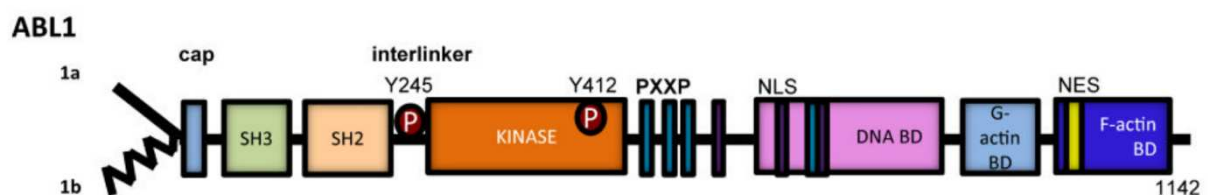


Figure 6: ABL structure (Greuber et al. 2013).

1.5.4. BCR (Breakpoint Cluster Region) gene

BCR gene codes for an ubiquitarian cytoplasmic protein mostly expressed in the brain and the hematopoietic tissue. There are three isoforms of BCR, 130 kDa, 190 kDa, and 160 kDa (Ali G Turhan 2001). BCR plays two main functions including immunomodulation and an indirect function as a negative regulator of the cell cycle. It plays also a role as a shuttle between the cytoplasm and the

apical membrane and intercellular by interacting with vesicular membranes via Mint3 (E. K. Malmberg et al. 2004).

As described above, the precise reasons for the occurrence of *BCR::ABL* recombination in the exact genomic locations in all patients remain still unknown. Nevertheless, endogenous and exogenous factors might be responsible for chromosomal rearrangement and abnormal genomic replication. 90% of CML patients are Ph+ (Stefan Faderl, Hagop M. Kantarjian, et Moshe Talpaz 1999). Data are suggesting the presence of fragile sites facilitating chromosomal breakpoints in different leukemias (Gümüş et al. 2002) but no precise reproducible genomic sites have been identified in patients with CML.

1.5.5. *BCR::ABL* activity and its transforming potential

The fusion gene *BCR::ABL* is responsible for the generation of a protein (210 kDa) with an exacerbated tyrosine kinase activity. Its activity is unregulated and provokes an uncontrolled proliferation of the cells where it appears (myeloid progenitors and mature hematopoietic cells). Firstly, this unregulated proliferation does not affect the differentiation level of the cells but with the progression of the disease, there is an inversely proportional ratio between differentiation and proliferation.

All three isoforms described above (p190, p210, p230) show a common structure through different domains: SH3, 2, 1, and NLS and actin-binding domains (**Figure 7**).

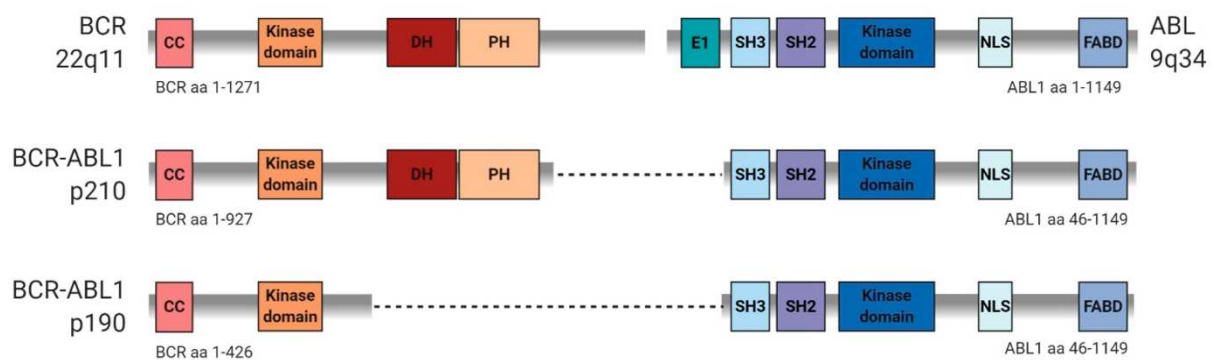


Figure 7: Structural differences between *BCR::ABL1* isoforms. (CC: coiled-coil; DH: Dbl-homology; E1, ABL1 exon1; FABD: F-actin binding domain; NLS: nuclear localization signal; PH: Pleckstrin-homology; SH2/3: SRC homology 2/3) (Komorowski et al. 2020).

BCR::ABL is necessary and sufficient for establishing and maintaining the CML phenotype and it will change profoundly the cell function (Daley, Van Etten, et Baltimore 1990; Kelliher et al. 1990) (**Figure 8**). The constitutive activation of *BCR::ABL* occurs at the same time as the activation of BCR and there is a dimerization or tetramerization of the protein leading to its autophosphorylation. The different phosphorylation sites and the cytoplasmic position of *BCR::ABL* enable the recruitment of different proteins involving and deregulating thus different pathways such as the cell cycle, the cell migration, the cell proliferation, the apoptosis, the response to different signals, altered genomic repairing mechanism and other.

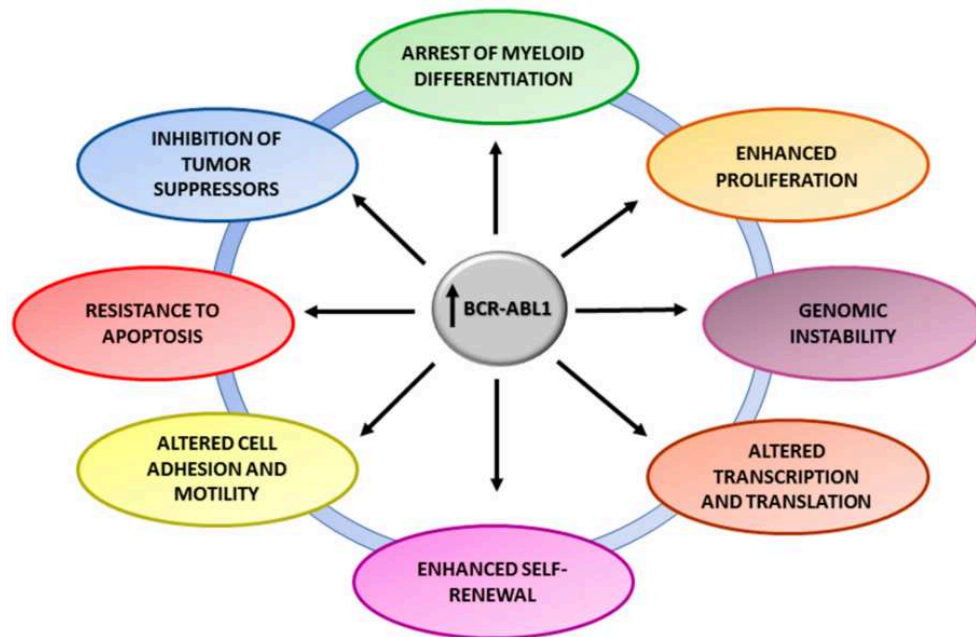


Figure 8: Overview of BCR::ABL1-dependent mechanisms (Bavaro et al. 2019)

1.6. Overview of normal and leukemic hematopoiesis

1.6.1. Normal hematopoiesis

Mature blood cells cannot proliferate and have a very limited lifespan (120 days for erythrocytes, 7-10 days for platelets, and 24 hours for granulocytes). Hematopoiesis is the process of fabrication and replacing blood cells in a continuous and controlled way during the lifetime of the individual (daily production 10^{11} to 10^{12} mature cells). Hematopoiesis is a complex and hierarchized process that can be presented as a pyramid with at the top, the HSC (**Figure 9**). Akashi et al described in 2000 the “bifurcation” model (**Figure 9**) where the HSC can differentiate progressively into multipotent progenitors (MPP) by conserving their differentiation abilities but not the self-renewal capacity (Akashi et al. 2000). The hierarchized organization of hematopoiesis implies different levels:

HSCs – are multipotent cells with self-renewal ability and the capacity to differentiate into all hematopoietic lineages (under the effect of transcription factors (FT) or growth factors (GF))

Progenitors- are undifferentiated cells with a high mitotic potential giving rise to, specialized differentiated cells with gradually reduced proliferation capacity.

Precursors – can be identified under a microscope thanks to their morphology. They do not have any self-renewal ability. During their maturation, they divide and acquire markers specific to hematopoietic lineages into what they are differentiating to and finish by becoming mature cells.

Mature cells- are the cells that migrate into the blood.

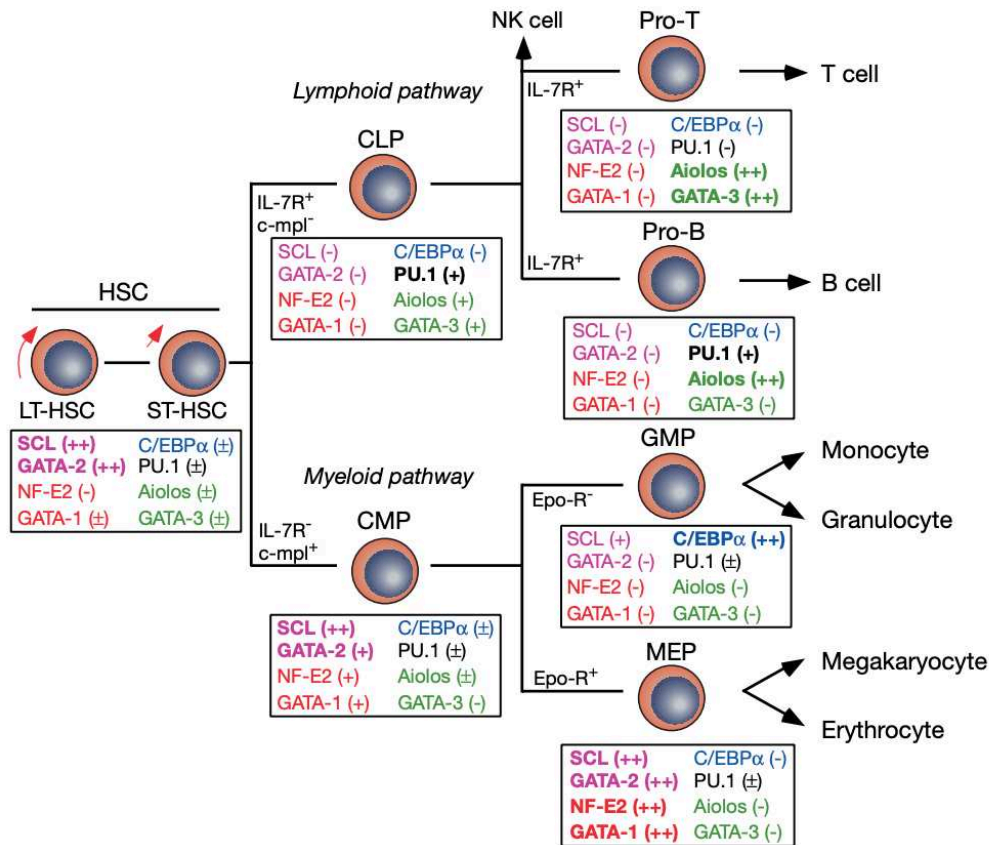


Figure 9: Proposed model of major hematopoietic differentiation pathways from HSCs.

Long-term HSCs (LT-HSCs) give rise to short-term HSCs (ST-HSCs). ST-HSCs give rise to Common Lymphoid Progenitors (CLPs), which can form all cells of the lymphoid lineages, and Common Myeloid Progenitors (CMPs), which can differentiate into either GMPs or MEPs which form the cells of the granulocyte/macrophage or megakaryocyte/erythroid lineages, respectively. Progenitor; MP: Macrophagic Progenitor; GP: Granulocytic Progenitor; T cells Progenitor NKP: Natural Killer Progenitor. IL: Interleukin; SCF: Stem Cell Factor; GM-CSF: Granulocyte and Macrophage Colony Stimulating Factor; MCSF: Macrophage Colony Stimulating Factor; EPO: Erythropoietin; TPO: Thrombopoietin ((Akashi et al. 2000).

These last years, different models of HSC differentiation have been proposed thanks to many studies done in mice and challenging the initial model proposed by Akashi. These new models suggest variations in the threshold of cell engagement and differentiation.

In 2001, Kawamoto, propose a biased model where the hematopoietic progenitors keep their plasticity and have a myeloid potential until the latest steps of differentiation (Kawamoto et al. 2010). Therefore, in this model none of the progenitors are lymphoid, they all have a myeloid potential.

In 2005, Adolfsson et al suggests that there is a population of MMPs named LMMP (Lymphoid-primed MMP) that can give all mature blood cells except erythrocytes suggesting thus an early bifurcation of the MMP (Adolfsson et al. 2005).

More recently, studies show the existence of different populations of stem cells, with self-renewal ability but able to differentiate directly into a cell lineage. In this model, granulocytes and macrophages can result exclusively from myeloid progenitors named rCMP (for restricted Common multipotent progenitors) but also from myeloid progenitors with lymphoid potential (B or T) (Ema, Morita, et Suda 2014).

New models are based on the hypothesis that erythrocytes and megakaryocytes have a particular progenitor, and are more immature that can give only myeloid cells. All the other blood cells such as granulocytes, monocytes, and lymphocytes (T, B, and NK) originate from less specialized progenitors that differentiate progressively. These progenitors lose their multipotent potential but keep their ability to give myeloid cells (granulocytes or monocytes) (Woolthuis et Park 2016).

During their maturation and differentiation, HSCs progressively lose their specific surface markers and acquire markers of maturity corresponding to the lineage where cells are differentiating. Characterizing the markers in these cells, tests in cell culture (semi-solid), and the engraftment of irradiated receivers, enable to determine which cells can reconstitute all mature cells of the hematopoietic system. Mouse HSC have been characterized in 1988 and 1991 (Spangrude, Heimfeld, et Weissman 1988; Ogawa et al. 1991) by the following markers:

- 1) "Negative lineage" (Lin-), HSC are negative for TER199 (erythroid marker), Mac1 (macrophage), Gr1 (myeloid), CD45R/B220 (B lineage), CD3 (T lineage), CD4 (T lymphocytes, macrophage, monocytes) and CD8 (cytotoxic T lymphocytes)
- 2) Sca1 positive (Sca1+), which is a proper marker of HSC and immature progenitors (MMPs).
- 3) KIT positive, which is the receptor of the Stem Cell Factor (SCF).

Later on, HSC has been further characterized notably by the use of CD34 markers and markers of the SLAM family (Signaling Lymphocytic Activation Molecules). Including CD150, CD48, and CD224 (Oguro, Ding, et Morrison 2013). In a nutshell, HSC is characterized as following Lin⁻Sca1⁺c-KIT⁺CD34⁻CD150⁺CD48⁻ and the MMPs become progressively CD34⁺, CD48⁺ and CD150⁻. These markers enable to distinguish the HSC from progenitors (Pietras et al. 2015).

In humans, the characterization of hematopoiesis is not as well progressed as it is in mice. This is mostly because cell surface markers are rarer and it is difficult to define precisely the mechanisms of human hematopoiesis. Nevertheless, one of the main markers for the detection of immature cells is the CD34. Some other markers such as lineage-specific markers (CD2, CD3, CD11b, CD11c, CD14, CD16, CD19, CD24, CD56, CD66b, CD235), CD45RA, CD38, CD90 (Thy1) or CD49f are used for the characterization of HSC and to distinguish it from progenitors and precursors. Other markers such as CD117/KIT, CD135 (cytokine receptor), or markers of specific lineages (myeloid: CD41, CD71, CD105, or lymphoid: CD7, CD10) can be used to increase the precision of characterization for more differentiated progenitors (Notta et al. 2011; 2016; Doulatov et al. 2010; Mori et al. 2015; Hao et al. 2001).

1.6.1.1. Regulation of hematopoiesis

In adults, hematopoiesis occurs only in the bone marrow found in the proximal epiphyses of the long bones, skull vertebrae, pelvis, ribs, and sternum. Hematopoiesis is a highly regulated process. Regulation occurs at the stromal level through cell-cell interaction but also different soluble factors, hormones, cytokines (erythropoietin), interleukin 3, granulocyte-monocyte colony-stimulating factor (GM-CSF), Monocyte-Macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF) (Smith 1990). In general, the regulation of hematopoiesis is based on two different regulatory mechanisms:

- 1) The extrinsic regulation (microenvironment)
- 2) Regulation by transcription factors

1.6.1.1.1. The extrinsic regulation of hematopoiesis

In 1975, Brian Lord described the architectural organization of the bone marrow demonstrating the regionalization of stem and progenitor cells *in vivo* (Lord, Testa, et Hendry 1975). Three years later, in 1978, Raymond Schofield proposed for the first time the concept of stem cell niche and the role of a specialized microenvironment for stem cell function *in vivo* (Schofield 1978). According to Schofield, stem cells are located in physical sites where different signals regulate their stemness and impose stem cell features on daughter cells through a mechanism of self-renewal. Therefore, the niche confers to the HSC a self-renewal capacity and there are features of the niche that prevent the accumulation of genetic damages that might come from the self-renewal.

Stromal cells of the BM are responsible for activating pathways such as Hedgehog, Wnt, and Notch via secretion of different cytokines (TPO, Ang1, TGF- β) and chemokines. They also express adhesion molecules but also factors such as Stem Cell Factor (SCF), Interleukine-7 (IL-7) and other (Cordeiro Gomes et al. 2016) (Figure 10).

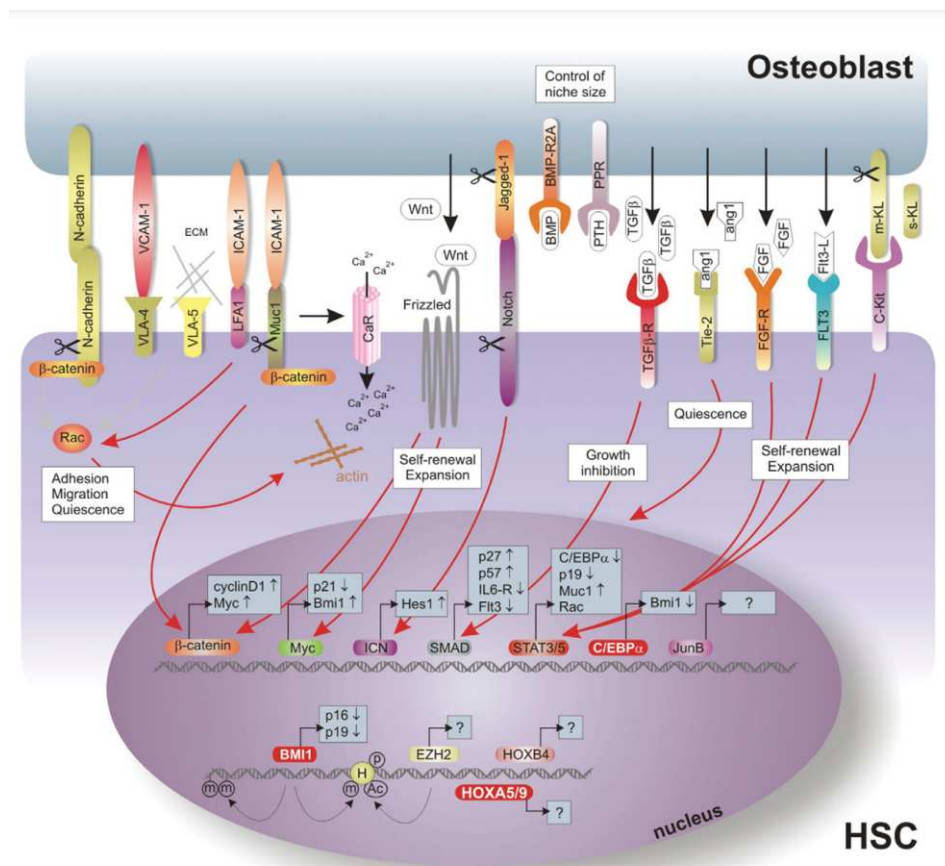


Figure 10: Signal transduction pathways in the HSC niche (Rizo et al. 2006)

Experiments on mice have enabled the description of two types of niches including medullary and osteoblastic niches. The medullary niche is a complex tissue containing stromal cells, endothelial cells, osteoblasts, and adipocytes but also mature hematopoietic cells such as monocytes, and macrophages. They are also innervated by non-myelinating Schwann cells (Kiel et Morrison 2008; Frenette et al. 2013) whose importance in the regulation of hematopoiesis has recently been recognized (Maryanovich et al. 2018).

Through its chemokines such as CXCL12 or CXCR4, the hematopoietic niche can attract HSCs and keep them attached to the niche components such as selectins, different adhering molecules such as ICAM1, VCAM1, CD44, and integrins. Different pathways involved in HSC quiescence or self-renewal have already been described, among them Wnt is one of the most important. The number of HSCs and their size is controlled by proteins such as BMP, TGF- β , and parathormone (PTH) (Chomel et al. 2014). At the same time, different receptors and ligands have been described to be involved in the self-renewal ability of the HSC, such as FGF-R, c-KIT, and NOTCH (Stier et al. 2002; Coutu et Galipeau 2011; Shin et al. 2014).

An important role is shown to be played also by the mesenchymal stem cells (MSC) that can differentiate into osteoblasts, chondrocytes, adipocytes, and stromal cells (Frenette et al. 2013). These cells furnish many factors indispensable for HSCs and progenitors' survival, proliferation and differentiation. Among them, the Stem Cell Factor (SCF) and the chemokine CXCL12 play an essential role for the maintenance of the HSC in the bone marrow (Calvi et al. 2003; Sugiyama et al. 2006; Greenbaum et al. 2013).

In case of an immune response, cytotoxic T cells can produce IFN- γ enabling thus the MSCs to produce IL-6-inducing myeloid differentiation of HSCs by repressing RUNX1 and CEBP α . (Riether, Schürch, et Ochsenein 2015).

Macrophages have also been shown to play an important role in erythroid terminal differentiation (Klei et al. 2017) but also in the maintenance of the HSCs (Kaur et al. 2018).

In addition to this intra-hematopoietic regulation, the nervous system plays also a role in the regulation of hematopoiesis. Synaptic nerves are shown to connect with MSCs and secrete noradrenalin and norepinephrine based on a circadian rhythm enabling thus the control of CXCL12 production and participating in the maintenance of the HSC (Méndez-Ferrer et al. 2008; Méndez-Ferrer, Battista, et Frenette 2010).

Hormones such as estrogens are also involved in the regulation of hematopoiesis. HSCs express the ER α (Estrogen Receptor α), which is shown to have a positive impact on the self-renewing of the HSCs and erythropoiesis, especially during pregnancy (Nakada et al. 2014).

Vitamins or their metabolites such as retinoic acid (vitamine A metabolite) play also a role in the regulation of HSCs. Recently, it has been shown that retinoic acid is involved in the maintenance of the HSCs pool by inducing quiescence (Cabezas-Wallscheid et al. 2017). Vitamin C is also shown to have a similar effect (Agathocleous et al. 2017; Cimmino et al. 2017).

Figure 11 shows the heterogeneity of populations and different factors involved in the regulation of HSC in the bone marrow.

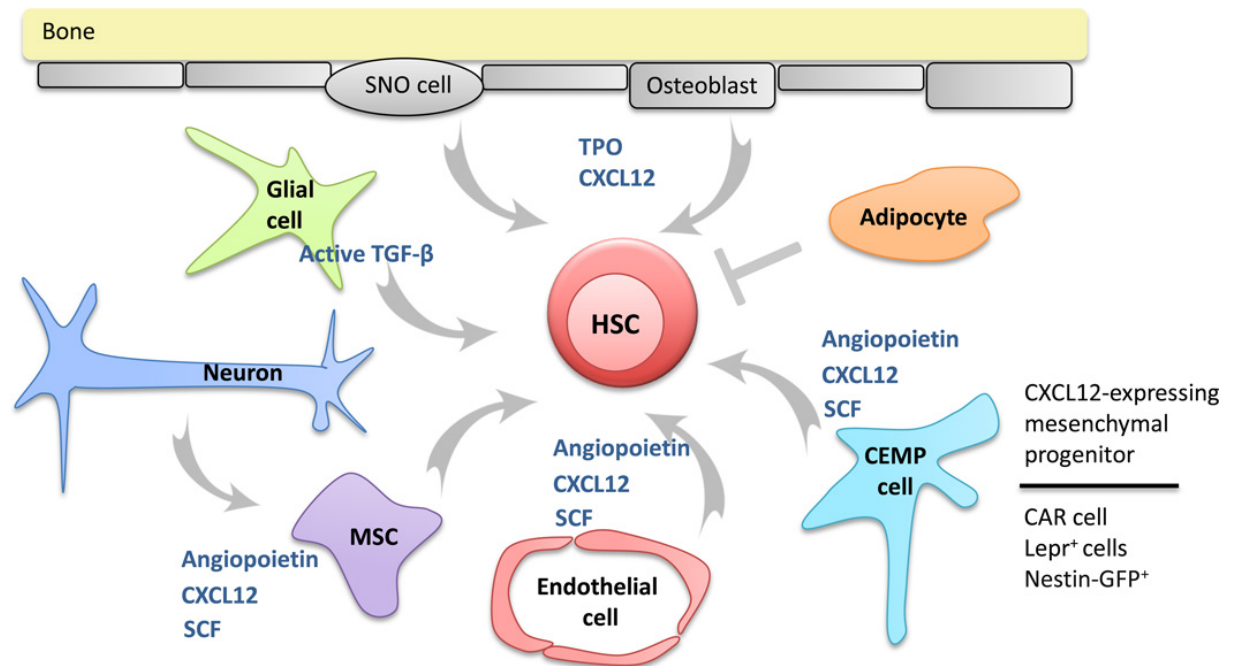


Figure 11: Distinct stromal cell populations in the bone marrow contribute to the regulation of hematopoiesis (Anthony et Link 2014).

1.6.1.1.2. Regulation by transcription factors

The maintenance of the HSCs pool is highly regulated by an equilibrium between proliferation, quiescence and differentiation. This is regulated by a network of transcription factors including essential genes for hematopoietic differentiation. Among these factors, we can enumerate the Stem Cell Leukemia/T-ALL 1 (SCL/TAL-1), LIM domain only 2 (LMO2), and Friend Leukemia Integration 1 (FLI1/ERG). The knockout of these genes in mice is lethal due to a total absence of HSCs and endothelial issues showing the close relationship between these two tissues (Shivdasani, Mayer, et Orkin 1995; Landry et al. 2009).

Among transcription factors involved in the regulation of hematopoiesis, RUNX1 (*Runt-Related Transcription Factor 1*) and GATA2 are crucial. Their deletion in mice is related to embryonic death, due to a lack of HSCs and consequently anemia but their vasculogenic system is normal (Ling et al. 2004; Q. Wang et al. 1996). It has been shown that GATA2 is not only involved in the endothelial-to-hematopoietic transition (ETH) but also the maintenance of the HSCs during life (de Pater et al. 2013).

Other transcription factors have been shown to play an important role in the regulation of hematopoiesis such as LYL1, LMO2, ERG, and FL1 but also their protein interactions (between GATA2, RUNX1, SCL, and ERG) (Wilson et al. 2010) showing the complexity of the regulation of hematopoiesis (Figure 12).

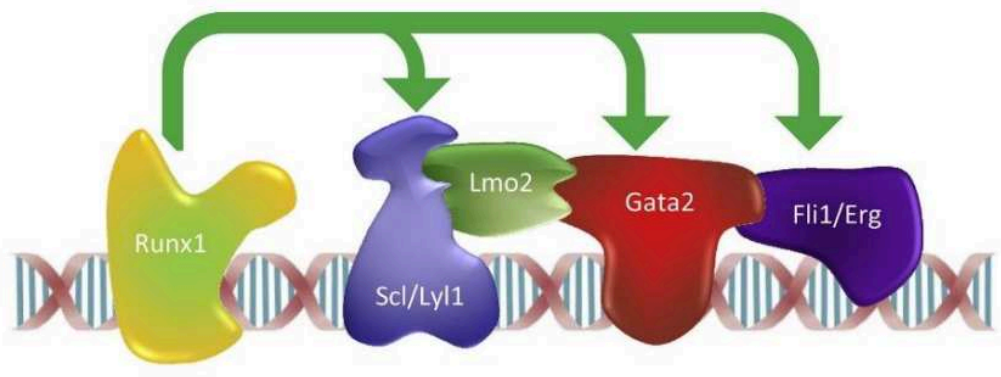


Figure 12: Schematic representation of transcription factor interaction bound to DNA. Runx1 interacts with other proteic complexes during hematopoietic regulation (Wilson et al. 2010).

1.6.2. Leukemic hematopoiesis: CML model

1.6.2.1. Leukemic stem cells (LSC) in CML

1.6.2.1.1. LSC General Overview

CML arises from a single transformed, primitive HSC which is highly quiescent and resistant to TKI (S. M. Graham et al. 2002; Holyoake et al. 1999). This evidence has been obtained by the cell-sorted highly quiescent cells exposed to large concentrations of Imatinib (10 μ M) as compared to cells in the cycle showing that the quiescent population survived whereas cells in division underwent apoptotic cell death (S. M. Graham et al. 2002). The importance of LSC has become very apparent with the attempts of TKI arrest in patients in deep molecular response (DMR) showing a relapse of the disease in the majority of patients, suggesting the persistence of LSC resistant to TKI therapies (Konig et al. 2008). Clear evidence for the persistence of the LSC in the BM of patients in DMR has rapidly been obtained (S. Chu et al. 2011; Chomel et al. 2011) The LSC preservation in the bone marrow is felt to be responsible for the relapse when the patient will stop the treatment which is now possible in patients in deep molecular response with the control of *BCR::ABL* transcripts in the peripheral blood, as a molecular relapse occurs in 50-60% of patients. Indeed, since the initial TKI cessation trials in France (Etienne et al. 2017), several other trials have shown that 50-60% of the patients will experience a molecular relapse requiring to resume the TKI therapy (Ross et al. 2013; Shah et al. 2020; Hochhaus et al. 2016; Saussele et al. 2018). Remarkably, molecular relapses occur almost exclusively during the first 6 months suggesting the rapid entry into the cell cycle of a very quiescent stem cell.

All trials have also analyzed different host and disease-related factors influencing relapse. It has been shown that the most important factors to impact the relapse are the duration of TKI therapy (>5 years is the best), the duration of deep molecular response (>3 years is the best), and the disease status at diagnosis evaluated by the Sokal score (low score is the best). It has also been shown that the immune status could be of major importance such as the presence of increased numbers of NK cells in patients without relapse as compared to patients with relapse (Ohyashiki et al. 2012; A. Hughes et al. 2017). The negative regulatory factors such as the presence of increased numbers of regulatory T-cells (Tregs) and Myeloid-derived suppressor cells (MDSC) could favor the relapse occurring after TKI cessation (T. P. Hughes et Ross 2016). **Table 1** shows the summary of immunological studies performed in TKI cessation trials.

Table 1: Immunological studies on TKI stopping trials

	STIM	DADI trial	EURO-SKI	ISAV	STOP	Case series
TKI	Imatinib	2 nd line dasatinib	Imatinib (small cohort of 2 nd gen TKI treated)	Imatinib	Imatinib/ Imatinib+IFN	Imatinib
Patient number in immunology	51	59	132 (50)/ 122	36	12	42 (10 patients stopped)
Analysis	Lymphocyte subsets and function	Lymphocyte subsets	Lymphocyte subsets and function (Nordic) DCs (German)	KIR (killer immunoglobulin receptor) genotype	Lymphocyte subsets and function	Lymphocyte subsets
Relapse criteria	MR4.5	MR4.0	MR3.0	MR4.5	MMR	CMR
Positive predictors	⇒ High NK count • NK function impaired	⇒ High NK count • High NK LGLs • Low Tregs	⇒ High NK count • Low CD86 expression on DCs	KIR profile (KIR A haplotype)	⇒ High NK count	⇒ High NK count • Effector memory CD8+ T-cells

To detect this relapse, it is necessary to perform a molecular analysis every month for 1 year. The same TKI therapy started at relapse is almost always effective allowing one to obtain a second DMR (Atallah et Schiffer 2020). The mechanisms of the persistence of the LSC at the origin of the relapse are the subject of extensive research in several centers worldwide (Chomel et Turhan 2011).

The first approach was to discover novel LSC markers in CML and to develop different ways to eradicate TKI-resistant LSC. This phenotypical difference has been widely studied over the last decade (Chomel et Turhan 2011), nevertheless few phenotypical differences have been identified. Indeed, recently, it has been shown that LSCs express IL1RAP (K. Zhao et al. 2014), CD26 (Herrmann et al. 2014), CD93 (Kinstrie et al. 2015) as well as CD25 (Sadovnik et al. 2016). CD25 (J. Zhou et Chng 2014) and IL1-RAP (Ågerstam et al. 2015) are also expressed in the AML stem cell while CD26 and CD93 are more specific to LSC.

Newly diagnosed patients with CML show a higher percentage of CD26 both in PB and BM (in the progenitor fraction CD34+/CD38+ or stem cell fraction CD34+/CD38-) (Houshmand et al. 2021). These cells could theoretically be targeted by the clinically available monoclonal antibody anti-CD26 (Begelomab).

The second approach for LSC eradication came from the discovery that CML LSCs do not rely on the TK activity for their survival. Indeed, it has been shown that following TKI-induced inhibition of TK activity, LSC could survive in the presence of growth factors, suggesting that they do not need TK activity for their survival (Corbin et al. 2011). It has also been shown that the most primitive LSC detected by LTC-IC assays express reduced levels of *BCR::ABL* transcript, suggesting that they will not be eradicated by TKI therapies (Chomel et al. 2012). This “oncogene-independence” phenomenon stimulated a major research interest to uncover novel ways of eradicating CML LSC in different ways such as by targeting the LSC heterogeneity, LSC quiescence, signaling pathways that confer independent survival to LSC form *BCR::ABL* (RAF/ERK/MEF) (Corbin et al. 2011; Hamilton et al. 2012), epigenetic events, etc. **Table 2** shows the most significant advances in that field with the identification of some druggable targets.

Table 2: Current studies on targeting LSC through an oncogene-independent way. (Ruggiu et al. 2018; Scott et al. 2016)

Target	Drug	Authors/Clinical trial
JAK2-TYK2-STAT3 pathway	Ruxolitinib+Nilotinib	NCT01702064
Epigenome	TKI+Decitabine or Azacytidine	Ruggiu et al., 2018
H3K27me3 inhibition)/epigenome	EZH inhibitors	Scott et al., 2016
PPAR- agonists	Pioglitazone	EudraCT 2009-011675-79

1.6.2.1.2. Expression of *CD25* in CML LSC

The specific identification of LSC and the ability to distinguish it from the HSC remains a challenge in the perspective of LSC eradication therapies. However, currently, only a few clinically relevant targets expressed specifically have been identified on CML LSCs (van Rhenen et al. 2007; Hosen et al. 2007; Herrmann et al. 2014).

As discussed above, recent studies show that CML LSCs specifically express some aberrant markers. Among these aberrant markers, recently, gene array studies have shown that CML LSCs express *CD25* in excess compared to normal HSCs (Gerber et al. 2013; Kobayashi et al. 2014; Sadovnik et al. 2016).

The IL-2 receptor complex consists of an alpha chain (IL-2RA or *CD25*), a functional beta chain (*CD122*), and a gamma chain (*CD132*). CML LSCs do not express the two other subunits of IL-2, making thus impossible for the stimulation of LSC by IL-2 cytokine (Sadovnik et al. 2016). It has been shown that *CD34+CD38-* LSCs express *CD25* in more than 90% of patients with untreated CML (Sadovnik et al. 2016).

The expression of *CD25* on CML LSC is regulated by BCR::ABL1-dependent STAT5 activation (Sadovnik et al. 2016). The same authors have shown that *CD25* expression is restricted to LSC, although *BCR::ABL* and *STAT5* are expressed in most clonal cells. *CD25* expression is also found in other myeloid neoplasms such as AML (Herrmann et al. 2020; Saito et al. 2010), but also Ph+ ALL and some patients with primary myelofibrosis.

The real functions of *CD25* on LSC remain still unclear. In a murine model of CML, *CD25+LSK+* cells exhibit higher leukemia-initiating capacity (Kobayashi et al. 2014). Administration of an anti-*CD25* antibody improved the survival of mice transplanted with *CD25+KSL* cells (Kobayashi et al. 2014). The same authors have then analyzed the expression of *CD25* in human CML and demonstrated that *CD25* was expressed predominantly in *CD34+CD38-* a fraction of CML bone marrow but was found to be absent in *CD34+CD38+* fraction and absent in normal *CD34+CD38-/+* cells. Interestingly, *CD25* expression was found to be increased in leukemic cells of patients progressing to blast crisis (Kobayashi et al. 2014). According to Sadovnik et al, *CD25* could be a growth regulator of CML LSC as drugs inducing its expression combined with TKI could be an effective way of treatment strategy (Sadovnik et al. 2016; 2017). The expression of *CD25* is a STAT5-dependent event and TKIs such as Ponatinib and Nilotinib down-regulate STAT5 in LSCs and consequently the *CD25* expression (Sadovnik et al. 2016). Interestingly, *CD25* knock-down in CML cell lines (KU812) and *CD34+* cells from two CML patients led to increased proliferation (Sadovnik et al. 2016). Similarly, overexpression of *CD25* in *CD25*-negative

K562 cells led to reduced growth of transduced cells. They then asked whether upregulating *CD25* combined with TKI could have a synergistic effect. PI3K inhibitors and mTOR blocker BEZ235 as well as the mTOR inhibitor rapamycin are shown to increase the level of *CD25* expression. In this context, they have been shown to inhibit the growth of LSCs when combined with TKI therapy (Sadovnik et al. 2016). These results suggest that in the context of CML, especially in advanced phases, targeting *CD25* could be a novel therapeutic approach combined with TKI therapy.

1.6.2.1.3. Detection and quantification of CML LSCs

Up to now, it has been very difficult to characterize CML LSCs during the remission period, due to their low frequency in the bone marrow, and to the inability to distinguish them from their normal counterparts. One of the biggest challenges while studying LSCs is to determine the number of cell surface antigens that define the phenotype of the LSCs, which are stably expressed (including during the different steps of the cell cycle), and which are different from normal HSCs. **Table 3** recapitulates surface markers that are currently used for LSC identification and as compared to normal HSCs.

Table 3: Differential expression pattern of surface markers on normal HSCs and CML LSCs (Houshmand et al. 2019)

Marker	CD34+/CD38- CML	CD34+/CD38- Normal	CD34+/CD38+ CML	CD34+/CD38+ Normal	References
IL2R α (CD25)	++	–	+/-	+/-	Sadovnik et al., 2017
DPP4 (CD26)	++	-	+/-	–	Herrmann et al., 2014
Siglec-3 (CD33)	++	+	+	++	Herrmann et al., 2012
SCARB3 (CD36)	++	+/-	++	++	Landberg et al., 2018
IL1RAP	+	-	+	+	Landberg et al., 2016

Single-cell genomics data have shown an important heterogeneity among the CML LSC population, heterogeneity which is exacerbated under TKI therapies rendering thus the identification of these cells even harder (Warfvinge et al. 2017). Indeed, in the study of Warfvinge et al, although TKIs are not able to eradicate CML LSCs, they can induce phenotypic changes with a reduction of the expression of LSCs markers *CD25*, *CD26*, and *IL1RAP* after TKI treatment (Warfvinge et al. 2017). As mentioned above, the identification and quantification of LSCs remain still a difficult task, nevertheless, aberrant markers can be used for the identification of CML LSCs by flow cytometry assays (Sadovnik et al. 2016; Soverini et al. 2021) and for more precise quantification, molecular techniques are required. RT-qPCR tests are not sensitive enough to be used for LSCs identification and quantification because LSCs do not transcribe detectable levels of *BCR::ABL1*. Therefore, more sensitive techniques such as nanofluidic digital PCR (RT-dPCR) are currently used (Jennings et al. 2014; Goh et al. 2011).

Another classical technique for LSC evaluation is the long-term culture-initiating cells assays (LTC-IC) where cells are cultured in stroma favoring the adherence of LSC while non-leukemic progenitors are eliminated with the medium changes during the 5 weeks of culture (Chomel et al. 2011). LTC-IC-derived progenitors are then assayed in semi-solid culture for colony forming cell (CFC) assays allowing to quantify the numbers of LTC-IC initiating progenitors present in the initial population. It is possible

that the quantification of LSC to be a major predictor of leukemia recurrence in patients stopping TKI therapies but this evaluation has never been performed prospectively so far.

1.6.2.1.4. LSC quiescence

In normal conditions, preservation of the HSC pool from exhaustion throughout the lifetime of an individual is ensured by their highly quiescent status. Similar to their normal counterpart, LSCs are also characterized by quiescence which enables them to be protected from the action of TKI therapies. Quiescence is associated with the upregulation of pro-survival mechanisms such as the expression of *Bcl-2*, reduced metabolism, and differential reliance on glycolytic versus mitochondrial ATP production (Nakada et al. 2014).

Quiescence is an important property of HSCs in case of leukemic treatment because it allows them to resist therapies and favor recovery after treatment. Therefore, selective targeting of quiescent LSCs but not normal HSCs remains a major question that needs to be elucidated. The regulation of LSCs quiescence is still not fully understood, nevertheless, it is known that it is regulated by intrinsic (cell-autonomous) and extrinsic (niche-driven) mechanisms (O'Reilly, Zeinabad, et Szegezdi 2021).

1.6.2.1.5. LSC and microenvironment

CML LSCs like normal HSCs, are present in the bone marrow and localized in the stem cell niche. We know from the study of normal hematopoiesis that marrow niches are complex, heterotypic, and dynamic structures (Crane, Jeffery, et Morrison 2017). As described above, medullary niche factors include a variety of cellular and secreted factors, extracellular matrixes, physical factors and metabolites. Interactions between stem cells and the niche are reciprocal and bi-directional (Lane, Williams, et Watt 2014).

The bone marrow niche is a complex structure including osteoblasts, adipocytes, endothelial cells as well as beta-adrenergic components the importance of which has recently been discovered (Fielding et Méndez-Ferrer 2020).

In this complex microenvironment, the most primitive HSC reside essentially in contact with the osteoblastic niche but recent data suggest that the vascular niche also harbours HSC with a major role in their trafficking and homeostasis (Comazzetto, Shen, et Morrison 2021; Mangialardi, Cordaro, et Madeddu 2016).

As described in the previous paragraph, bone marrow MSCs represent a major component of HSC niches (Kfoury et Scadden 2015) by expressing CXCL12 (Agarwal et al. 2019) but also for the LSCs. Targeted deletion of CXCL12 from MSC disrupts MSC-LSC clusters and enhances LSC cycling (Greenbaum et al. 2013). This explains that MSC expressing CXCL12 is a niche that keeps stem cells in a quiescent form and the removal of CXCL12 from MSC, makes CML LSC more sensitive to TKI. Recently, it has been shown that CXCL12 deletion from MSC expands LSC by undergoing increased self-renewing divisions (Agarwal et al. 2019)

Changes in adherent molecules and a loss of chemotaxis due to CXCL12 is responsible for the decrease of cell homing in the hematopoietic niche in CML. These adhesion defaults are responsible for the “escaping” of the leukemic cell from the niche (Foudi et al. 2006), followed by an increase in the proliferation of stem and progenitor cells.

In CML, there are several lines of data suggesting the existence of a cross-talk between the components of the niche and leukemic cells. It has been established that BM stromal cells protect the niche from the toxicity of TKI (Parmar et al. 2011). Similarly, the marrow niche can maintain LSC in a quiescent state (O'Reilly, Zeinabad, et Szegezdi 2021). Conversely, there are data showing that leukemic cells could transform the normal BM niche into an abnormal one via this cross-talk during the development of CML. In this context, it has been shown that leukemic cells induce BM to secrete placental growth factor (PIGF) which can then promote leukemogenesis (Schmidt et al. 2011). **Figure 13** shows potential interactions between LSC and niche.

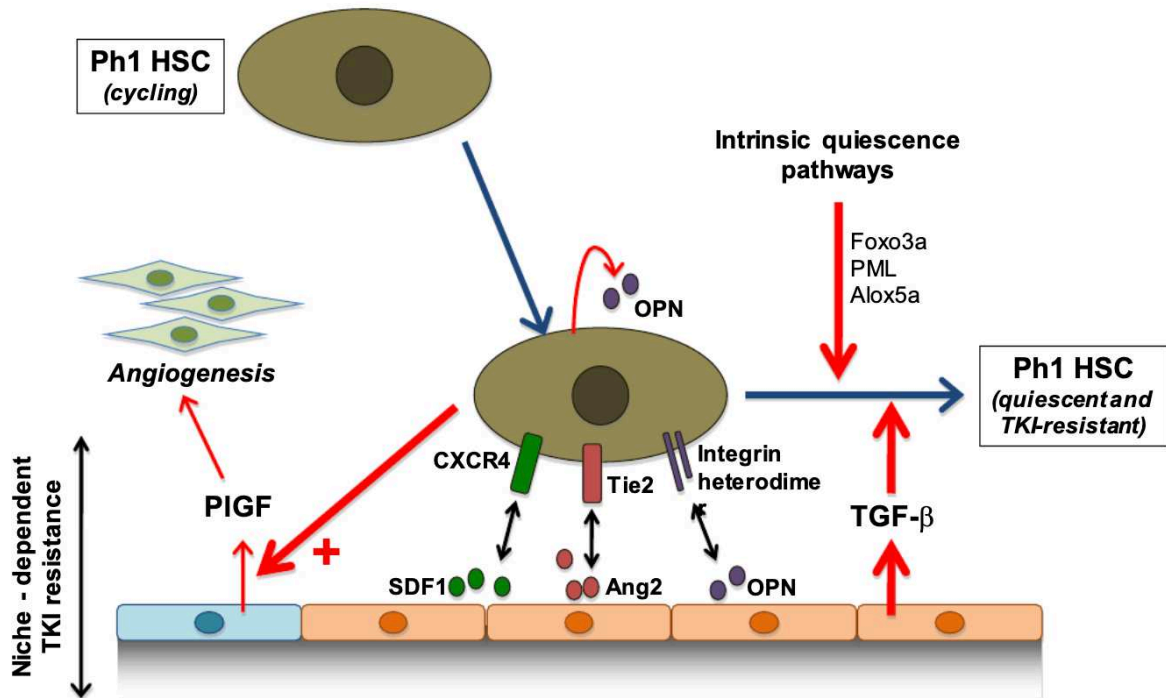


Figure 13: CML stem cells in the context of bone marrow microenvironmental niche. HSC, hematopoietic stem cell; PIGF, placental growth factor; CXCR4, C-X-C chemokine receptor type 4; SDF-1 α , stromal cell-derived factor-1alpha; Tie2, tyrosine kinase with immunoglobulin and EGF homology domains; ang2, angiopoietin 2; OPN, osteopontin; TGF- β , Transforming growth factor-beta (Chomel et Turhan 2011).

Interestingly, transcriptome analysis of BM MSC in CML patients in DMR show the persistence of an aberrant molecular signature found in the chronic phase of the disease suggesting a pathological “memory” (Aggoune et al. 2017).

It has been shown that in CML BM, there is an increased expression of a variety of chemokines and cytokines. These factors that derive from leukemic cells may persist after TKI treatment and during remission (B. Zhang et al. 2012; Aggoune et al. 2017). This effect is mediated in part by soluble factors because it can be reproduced by using BM supernatants. Cytokines such as IL-1a, IL-1b, and TNF- α can lead to enhanced growth of leukemic cells versus normal cells (B. Zhang et al. 2012).

1.6.2.2. Signaling pathways involved in BCR::ABL-induced leukemogenesis

1.6.2.2.1. PI3K/Akt/mTOR pathway

Phosphatidylyl 3-kinases (PI3Ks) are cytosolic proteins that regulate many cell functions such as differentiation, proliferation, cell survival, and malignant transformation (Martini et al. 2013). PI3K family includes three different groups (I, II, and III) according to their primary structure, their specificity to their substrate, and their regulation. One of the most well-known targets of class I PI3K is Akt. Akt is a serine/threonine kinase, with an important role in cell survival, cell growth, proliferation, differentiation, as well as in glucose metabolism. Akt protein has different substrates and one of them is mTOR which gets activated via tuberous sclerosis complex (TSC1/2). mTOR is a serine/threonine kinase involved in the regulation of metabolism, cell growth, and cell survival. Loewith and colleagues described in 2002 two different complexes of mTOR (mTORC1 and mTORC2) (Loewith et al. 2002). mTORC1 acts mainly on eukaryotic translation initiation factor (eIF) 4E binding protein (4E-BP1) and S6K1 (Wullschleger, Loewith, et Hall 2006). The activation of this protein by mTORC1 is essential for the translation of different proteins such as c-Myc and cyclin D1 which are involved in the control of the cell cycle. mTOR is also involved in the regulation of protein synthesis, cell size, and autophagy. PI3K signals are often antagonized by the tumor suppressor protein PTEN (phosphatase and tensin homolog). Its deletion provokes an increase in STAT5 and Akt (**Figure 14**).

In CML, BCR::ABL activates this pathway due to its high tyrosine kinase phosphorylating ability which results in PI3K phosphorylation and activation. BCR::ABL also down-regulates PTEN *in vivo* inducing thus a cell cycle arrest in the leukemic cells. Nilotinib is a specific inhibitor of this pathway and enables the sensitization of LSC and progenitor cells to TKI effect (Airiau et al. 2013).

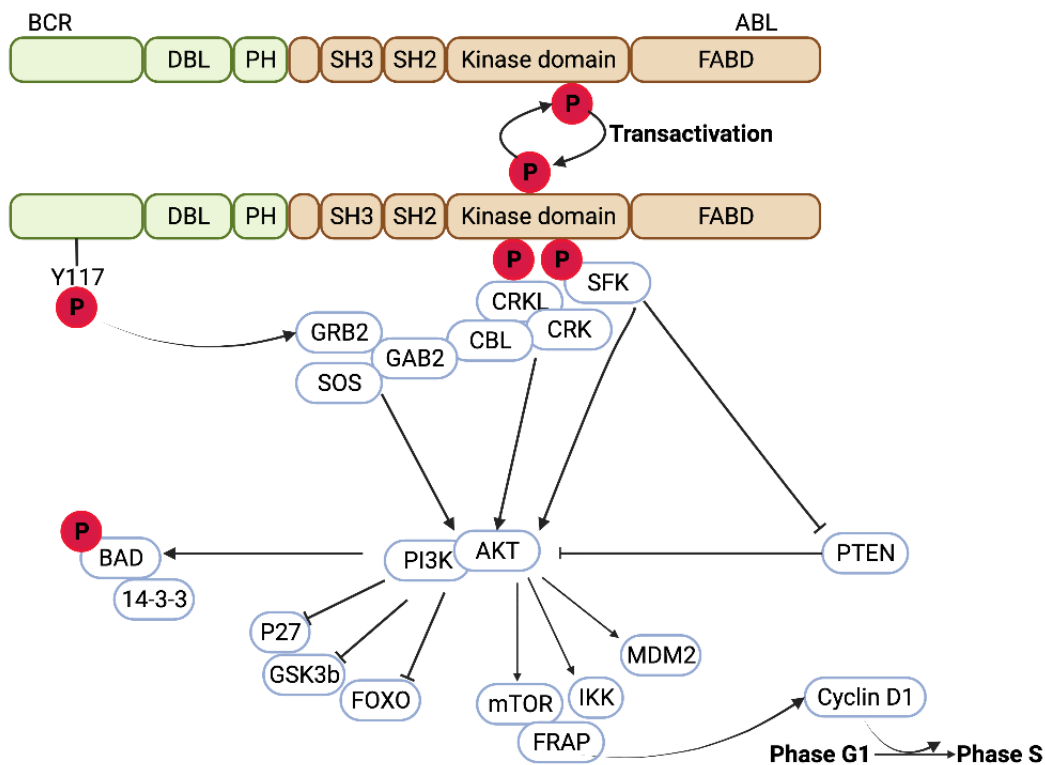


Figure 14: Pi3K/Akt pathways and their deregulation in CML.

1.6.2.2.2. JAK/STAT pathway

Janus kinases/signal transducers and activators of transcription (JAK-STAT) represent major players in the regulation of cell differentiation, proliferation, survival, apoptosis and migration (Schindler et Plumlee 2008). The JAK family includes 4 members (JAK1, JAK2, JAK3, and Tyrosine kinase 2 (Tyk2)) whereas STAT protein family contains 7 members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6). This pathway is involved in the transmission of extracellular signals through the cell membrane. In absence of stimuli, JAK proteins are inactive and STAT proteins are located in the cytosol. In presence of extracellular stimuli, JAK proteins change their conformation and phosphorylate STAT proteins by activating them. The activation of STAT proteins results in their dimerization and activation and their migration to nuclei where they activate different target genes.

Abnormal STAT5 activation has been described in different hematopoietic malignancies such as acute myeloid leukemia, B-cell lymphoma, myelodysplastic syndrome, and CML (Valentino et Pierre 2006). Activation of STAT5 in the context of BCR::ABL-induced leukemogenesis has been the subject of intense investigation during the last two decades (Carlesso, Frank, et Griffin 1996; Danial et Rothman 2000; Casetti et al. 2013). It has also been shown that STAT3 is activated by BCR::ABL via a pathway involving JAK and MEK, not only in murine embryonic stem cells (Coppo et al. 2003) but also in primary CML CD34+ cells (Coppo et al. 2006).

In normal conditions, JAK2 is associated with the EPO receptor which is the first step of the activation of this pathway. An oncogenic mutation of *JAK2* has been identified in 2005 by James et al in the French team of Vainchenker W (James et al. 2005). This mutation is present in 95% of patients with Polycythaemia vera (Ganly et al. 2007; A. V. Jones et al. 2005; Verstovsek et al. 2006). It is occurring in the JH2 pseudokinase domain of the gene is responsible for the substitution of valine by phenylalanine in position 617 (V617F) with the constitutive JAK2 activation. This is a unique mutation and clonal (present in all myeloid cells of the patient (PNN, erythroblasts, thrombocytes), but not in their T lymphocytes)). This mutation is responsible for constitutive activation of JAK2 protein capable to induce a transcriptional activity of STAT5 in absence of Epo (James et al. 2005).

In CML, STAT5 can be activated by p190 and p210 isoforms (Carlesso, Frank, et Griffin 1996; Casetti et al. 2013) and it increases the resistance of leukemic cells to apoptosis (activation of anti-apoptotic signals such as Bcl-xL) and a cell cycle arrest through cyclin D1 (de Groot et al. 2000).

The JAK/STAT pathway has also been described recently to be involved in the resistance of TKI (A. M. Eiring et al. 2014). STAT3 has been shown to stimulate the self-renewal of embryonic stem cells (ESC) expressing BCR::ABL (Coppo et al. 2003).

Thus, both STAT5 and STAT3 have been evaluated as targets in CML cells. The STAT5 phosphorylation inhibitor Pimozide allows specific inhibition of CML clonogenic growth (Nelson et al. 2011) with a synergistic activity with TKI. A combined STAT3 and BCR::ABL inhibition has also been shown to lead to an inhibitory effect in TKI-resistant CML progenitors (Anna M. Eiring et al. 2015).

1.6.2.2.3. Sonic Hedgehog (SHH) pathway

SHH pathway is involved in the self-renewal of several types of stem cells including neural stem cells. It has been shown that it plays also a role in human hematopoiesis by inducing the proliferation of primitive hematopoietic cells in adults (Bhardwaj et al. 2001) and primitive hematopoiesis (Pethe, Noel, et Kale 2021). It plays an important role in embryonic development, tissue regeneration, and maintenance of LSC. Hedgehog is a ligand whose receptor is Patched 1 (PTC). When PTC binds

Hedgehog, inhibitory protein Smoothed (SMO) is released activating thus different transcriptional factors such as Gli1 and Gli2 that are responsible for the activation of BCL2, Ptch1, Cyclin D1, and angiogenesis (through VEGF). This pathway is very important for the expansion of LSC.

In CML, the SMO pathway has been identified as a factor allowing the maintenance and expansion of LSC (Dierks et al. 2008). Specific inhibitors have been identified to target this pathway such as cyclopamine (SMO antagonist) which has shown interesting results in AML (Kobune et al. 2009). SHH pathways plays also a role in the progression of CML toward blast crisis (Su et al. 2012) and its therapeutic potential has been suggested by Zhao and colleagues (C. Zhao et al. 2009). Similarly, the expression of PTCH has been evaluated as a predictor of imatinib response (Alonso-Dominguez et al. 2017). A clinical-grade SMO inhibitor (Sonidegib) has been shown to inhibit CML stem cell growth but the toxicity of this product hampered its clinical development (Irvine et al. 2016).

1.6.2.2.4. Proteins of the polycomb family (PcG)

Proteins of the polycomb family are part of the Polycomb Repressive Complexes 1 and 2 (PRC1/PRC2) and their function consists of the repression of gene expression via chromatin modification (Simon et Kingston 2009). These proteins are highly conserved during evolution, highlighting their important role. The epigenetic signature of LSC has been reported to be different from its normal counterparts (Jung et al. 2015) and that is one of the reasons for the important role that it plays in the alteration of the epigenetic program in cancer progression. In the PRC1 complex, BMI1 is involved in the self-renewal of both HSC and LSC (Lessard et Sauvageau 2003). Rizo and colleagues showed in 2010 that BMI1 proteins interact with BCR::ABL when introduced in CD34+ CML cells and stimulate proliferation and self-renewal (Rizo et al. 2010). Other proteins from the PcG family such as EZH2 (PRC2) are involved directly in CML LSC maintenance (H. Xie et al. 2016) and are potential targets for TKI sensitization.

1.6.2.2.5. Histone deacetylase (HDAC)

Another family of epigenetic regulators highly involved in HSC and LSC regulation and maintenance is the histone deacetylase (HDAC) family. In mammals, there have been described more than 18 histone deacetylases. They are classified into different types from type I to type IV. Among different types, there are sub-types e.g., in type I there are 4 subtypes (HDAC1, HDAC2, HDAC3, and HDAC8). In the context of CML, several targets have been identified and some clinical-grade HDAC inhibitors have progressed toward clinical trials. For instance, the first HDAC inhibitor used in combination with a TKI to target LSCs is the Vorinostat (SAHA – Suberoylanilide hydroxamic acid) which is a class I and II dual-HDAC inhibitor. It enhances the Imatinib-induced apoptosis of CD34+ (Nimmanapalli et al. 2003). Other HDAC inhibitors can be used in combination with Imatinib or Nilotinib to eliminate Imatinib-refractory primary CML or CML cells harboring the T315I mutation (Nimmanapalli et al. 2003; Bali et al. 2005).

Amongst the SIRT group that belongs to the HDAC family, SIRT1, is a NAD-dependent deacetylase that is overexpressed in human CML-LSC (L. Li et al. 2012) and involved in the regulation of HSC and LSC (Z. Wang et al. 2013; Yuan et al. 2012). Inhibiting SIRT1 by specific drugs such as Tenovin-6 or TV390H has been shown to enhance p53 activity via increased acetylation suggesting that combining those inhibitors with TKIs could be of interest for LSCs targeting (L. Li et al. 2012; Abraham et al. 2019).

1.6.2.2.6. Wnt pathway

Wnt ligands are secreted glycoproteins that bind to Frizzled (Fzd) receptors which are G-protein-coupled receptors (GPCRs) (Schulte 2015). The ligand binding activates canonical and noncanonical

pathways (Clevers et Nusse 2012). The canonical pathways consist of the cytoplasmic stabilization of β -catenin which is the key effector of Wnt. Wnt ligands are also able to bind to lipoprotein receptors (LRP5/6) in addition to Fzd receptors and activate pathways such as the Wnt/Planar Cell polarity (PCP) pathway or Wnt/ Ca^{2+} pathway (Figure 7). In the absence of Wnt ligands, β -catenin is incorporated into the adenomatous polyposis coli (APC)-Axin-CK1 α destruction complex, resulting in its phosphorylation by glycogen synthase kinase 3 β (GSK-3 β) leading to its degradation (Figure 15)

In the presence of Wnt-Fzd receptor interaction association with lipoprotein-receptor-related protein 5/6 (LRP5/6) coreceptor, the destruction complex is disassembled, and β -catenin is translocated to the nucleus activating different pathways. Noncanonical Wnt pathways interact with Fzd and other coreceptors to recruit a disheveled segment polarity protein (Dvl). Noncanonical Wnt activation pathways include the PCP pathway and Wnt- Ca^{2+} . Their role in regulating leukemic cells remains largely unexplored. In Wnt/PCP pathway, there is an activation of GTPases RhoA and Ras-related C3 botulinum toxin substrate 1 (RAC1) via Dvl which is responsible for triggering the expression of downstream genes. The calcium pathway is also an important way to activate different proteins such as the Nuclear Factor of the activated T-cell (NFAT).

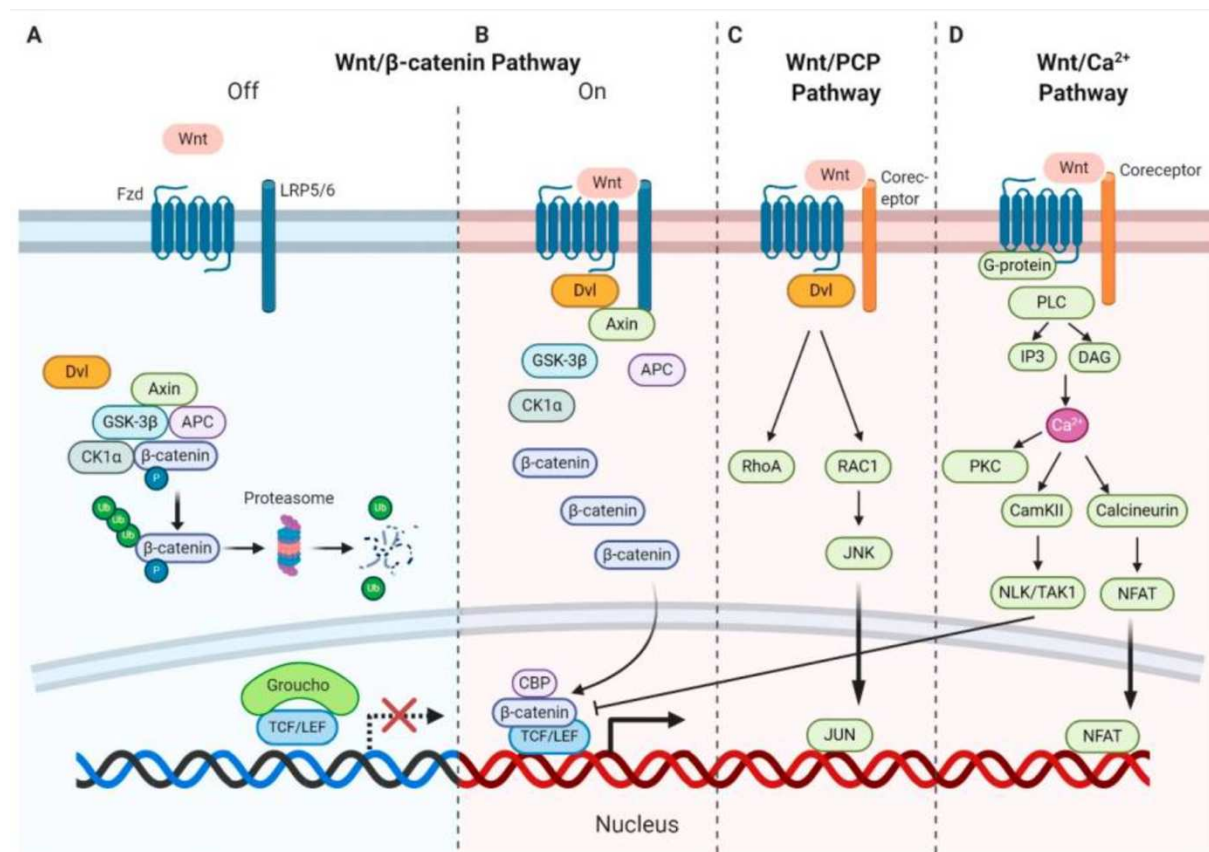


Figure 15: Wnt signaling pathways. (A) In absence of Wnt, the formation of the destruction complex and β -catenin degradation. (B) Wnt/Fzd activation through LRP5/6 coreceptor recruitment (C) In the Wnt/PCP pathway, Dvl activates the small GTPases RhoA and RAC1 resulting in activating c-Jun N-terminal kinase (JNK). (D) In the Wnt- Ca^{2+} pathway, the activation of Phospholipase C (PLC) results in a cytosolic calcium flux which promotes the nuclear factor of the activated T-cell (NFAT) transcription (Ruan et al. 2020).

Wang et al reported in 2010 that the activation of the Wnt/ β -catenin pathway led to the increase of self-renewal activity of LSC (Y. Wang et al. 2010). In CML, a direct relation has been shown between

the BCR::ABL and Wnt/ β -catenin pathway and the inhibition of the BCR::ABL-PI3K-AKT pathway leading to β -catenin degradation (J. Hu et al. 2016). The interaction of NF κ B-p65 with FoXM1/ β -catenin is highly important for the LSC (Jin et al. 2017).

1.7. The Immune Landscape of CML

In the field of onco-hematology, CML is also a model of immunotherapy. Indeed, before the introduction of TKI therapies to the clinical armamentarium, bone marrow transplantation (BMT) was the only curative therapy for CML (Martin et al. 1988; Appelbaum 1995). BMT for leukemia therapy was developed in Seattle in Fred Hutchison Cancer Center thanks to major experimental innovations from the group of Prof. E. Donnall Thomas (winner of the Nobel Prize in 1995). Indeed, the Seattle group was the first to demonstrate the disappearance of the Ph1 chromosome after syngeneic bone marrow transplantation in CML (Fefer et al. 1977). BMT became the first curative therapy widely applied to young patients in whom an HLA-compatible donor was available until the development of TKI therapies (Goldman et al. 1982; Speck et al. 1984; Thompson et Thomas 1986; Clift et Storb 1996; Gratwohl et al. 2004).

Currently, allogeneic stem cell transplantation remains a second-line therapy performed essentially in patients failing TKI therapies or in patients in advanced CML (Craddock 2018).

The first successful BMT have shown that the T cells transplanted along with the bone marrow were responsible for the graft-versus-host (GvH) effect but also for graft versus leukemia (GvL) effect that contributes to the cure (D. W. Barnes et al. 1956; Mathe et al. 1965). The role of T cells to control leukemia became also clear with the T-cell-depleted transplantation experiments (Ho et Soiffer 2001). In some of these early clinical trials, T-cells were depleted from the grafts to reduce the GvH effect (Ho et Soiffer 2001). In all these trials, it has been observed an increased number of leukemic relapses (Martin et al. 1988; van der Straaten et al. 2001). Interestingly, it has been shown that the GvL effect could also be performed by the autologous NK cells present in the graft (Cervantes et al. 1996). It has therefore been rapidly obvious that the immune factor was a very important step in understanding LSC eradication and disease progression.

It has been established that BCR::ABL can be immunogenic by the expression of small junction peptides at the surface of leukemic cells. This knowledge led to clinical trials using BCR::ABL junction peptides that have been loaded into autologous dendritic cells in patients with CML (Bocchia et al. 2010). Some of these trials have shown activity in terms of BCR::ABL expression in the peripheral blood but none of them has been conclusive. BCR::ABL could also induce the expression of immunogenic proteins such as WT1 (Svensson et al. 2007) and PR3 (Šmahel 2011). This adaptive immunity (T cells and T-mediated anti-leukemic responses) is, therefore, BCR::ABL dependent. Indeed, T cell-specific form PR3 depends on the expression of BCR::ABL in CML but also in Antigen-presenting cells (APC). It has been acknowledged that CD4+ and CD8+ T cells reactive with CML are detectable in the PB of CML patients (Brauer et al. 2007). Inflammatory variables are important for predicting DMR. The loss of CD62L on the surface of immune cells at diagnosis and the increase of soluble CD62L which is a homing receptor for T cells are shown to predict molecular response to TKIs (Sopper et al. 2017).

Thus, CML is a model for immunotherapy by the immunogenicity of BCR::ABL itself, but also the generation of neoantigens in BCR::ABL-expressing cells such as WT1, PR3n PRAME, and BMI-1. Different clinical trials have been performed aiming to validate a “vaccination effect” in CML (Jeffrey J. Molldrem 2006).

In the TKI era, several questions remain regarding immunity in CML at diagnosis, progression as well as during the attempts of TKI discontinuation. The identification of effectors of immune response in CML, the role of TKI (immunomodulation effect), and others pave the way for new therapeutic perspectives in CML. Among these perspectives, NK cells play a pivotal role.

1.7.1. Immune effectors on CML

1.7.1.1. NK Cells and the missing self

NK cells are the founding member of innate lymphoid cells. They comprise between 5-20% of blood lymphocytes and traffic into most tissues in the body including the bone marrow. NK cells were originally identified as cells able to kill tumor cells non-specifically. Since then, they are shown to exhibit potent anti-tumor immunity through multiple mechanisms. NK cells are functionally similar to CD8⁺ cytotoxic T cells and kill target cells through similar cytotoxic mechanisms, but lack a somatically rearranged and antigen-specific T-cell receptor (TCR). They differ from T-cells in the way how they see their targets. Indeed, unlike T-cells, NK cells stochastically express different germline DNA encoded inhibitory receptors, activating receptors, and cytokine receptors (**Figure 16**).

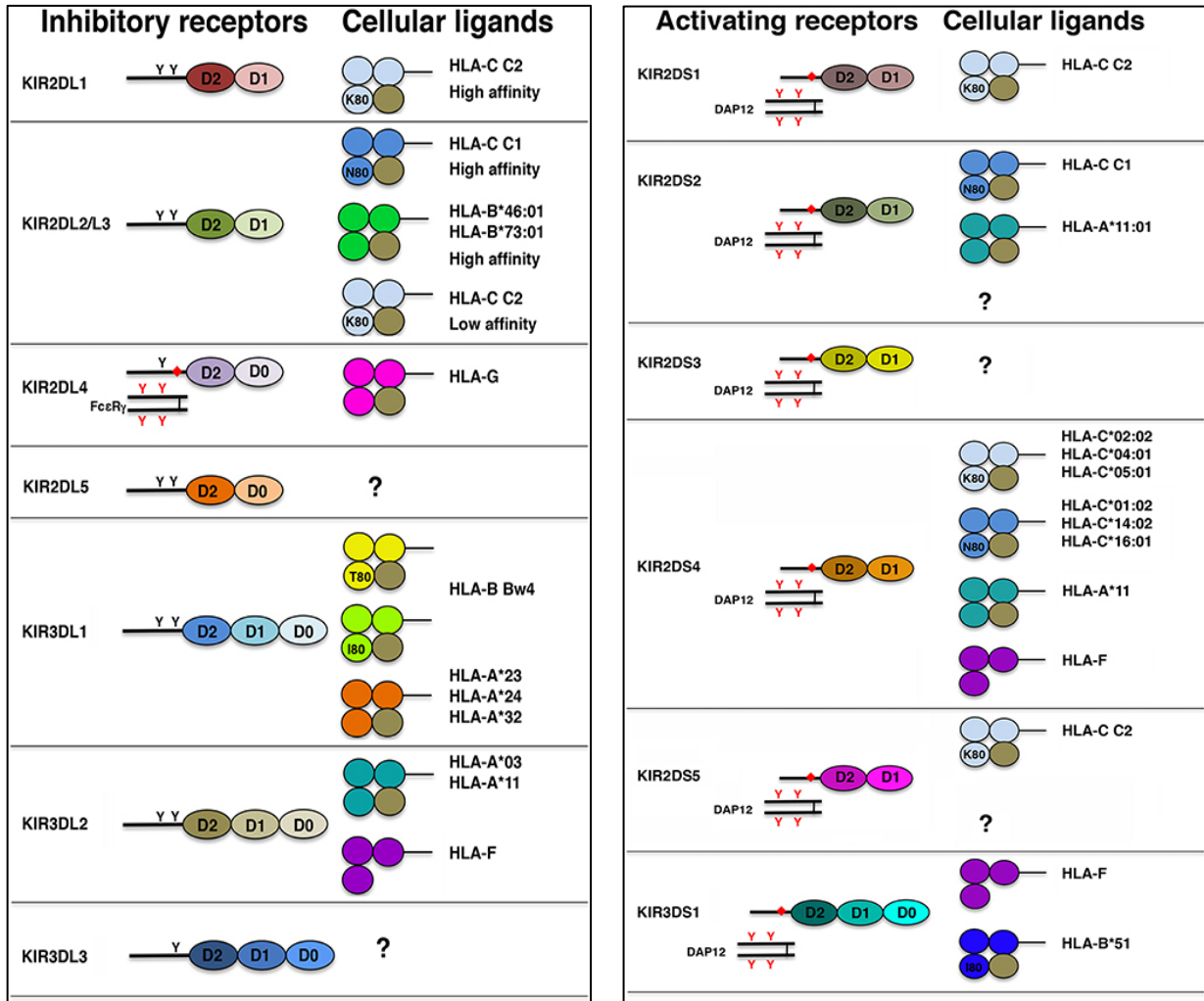


Figure 16: Schematic representation of the structure of inhibitory and activating receptors and their HLA class I ligand. Each Ig domain is depicted as an oval and, according to its structural features, labeled as D0, D1, and D2. (Pende D. 2019)

It is the balance of these receptors that will control whether NK cells will respond to targets or not. NK cell education is mediated through the engagement of their MHC class I-specific inhibitory receptors. To maintain homeostasis, NK cells, using their receptors, identify the MHC on the surface of tumor cells and subsequently induce apoptosis. The loss of MHC molecule expression on tumor cells promotes NK cytotoxicity (Vivier et al. 2012). They also lyse target cells via antibody-dependent cellular cytotoxicity, a critical mode of action of several therapeutic antibodies used in cancer.

The key receptors controlling self-recognition by human NK cells are HLA class I-binding receptors, including the Killer Immunoglobulin-like Receptor (KIR) family as well as the Natural Killer Group 2A (NKG2A) and Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1, also referred to as LIR-1) (Vivier et al. 2008). The inhibitory KIRs and the NKG2A receptor have also been shown to be involved in NK cell education, a functional maturation process that allows self-inhibited NK cells to become potent killers upon interaction with cells losing self-HLA class I expression (Anfossi et al. 2006). In contrast to the inhibitory receptors, an array of activation, co-activation, and adhesion receptors such as the natural cytotoxicity receptors (NCRs) NKp30 and NKp46 and the NKG2D, 2B4, and DNAM-1 receptor trigger NK cell activation following binding to ligands up-regulated on cells undergoing stress and/or infection (Vivier et al. 2008). The NKG2D receptor in NK cells recognizes the transmembrane proteins related to MHC complex I chain-related genes A and B (MICA and MICB), and by the interaction between NKG2D and MICA, the NK cells have been shown to exert cytotoxic activity against CML (Danier et al. 2011) (**Figure 16**).

The role of NK cells in cancer has been explored since the discovery of their important role in immune surveillance (K.-J. Malmberg et al. 2017). Individuals with poor NK cell function have been shown to exhibit a higher risk to develop cancer (K. Imai et al. 2000). Similarly, an NK-activated profile is directly linked to a better outcome in patients undergoing chemotherapy (Mastaglio et al. 2018). In the same perspective, allogeneic stem cell transplantation (SCT) and adoptive NK cell infusion can be of interest in cancer therapy (Miller et al. 2005).

NK cells have two main functions:

- 1) Cytotoxicity via perforin, granzyme, or the death receptor pathways, to recognize and kill tumor targets, similarly to CD8+ cytotoxic cells.
- 2) Intercellular communication via secretion of cytokines such as IFN- γ , chemokines, recruitment of immune cells, and promotion of an anti-tumor microenvironment.

Activation of NK cells by some cytokines is responsible for inducing memory-like NK cells (Romee et al. 2012). Indeed, cytokines such as IL-12, IL-15, and IL-18 give a highly activated profile to NK cells (Gang et al. 2020). They can then differentiate into different types of NK cells and among them, into memory NK cells. In the case of a restimulation of NK cells through other stimuli such as cancer cells, NK exhibit enhanced responses in terms of cytotoxicity, IFN- γ production, and proliferation. Epigenetic factors such as methylation play also an important role in that phenomenon (Ni et al. 2016)

In 1983, just after the discovery of NK cells, Pattengale et al. showed for the first time that freshly explanted CML blasts can be lysed by IFN-activated NK cells from healthy donors (Pattengale et al. 1983). A few years later in 1989, Ronald Herberman showed the importance of cell-to-cell interaction and soluble factors in NK response (Herberman 1989) More recently, immunotherapy strategies have been developed to explore the potential of *ex-vivo* expanded NK cells for anti-cancer therapy (Torelli et al. 2002; Romee et al. 2016).

1.7.1.1.1. Harnessing and Enhancing NK cell activity for cancer therapy

It has been shown that NK receptors such as DNAM-1, NKG2D, and NCRs play a central role in the response of NK cells to AML and CML blasts (Schlegel et al. 2015).

In general, there are different strategies to enhance the effects of NK cells against cancer (**Figure 17**). The first is the reduction of NK cell suppression through blocking inhibitor receptors, blocking inhibitory cytokines, and targeting suppressive cells. Blockades of inhibitory KIR, CD94/NKG2A, and LIR-1 have been shown to increase NK cell cytotoxicity of blast cells (Godal et al. 2010).

The second possibility is to improve the recognition of tumor targeting through monoclonal antibodies, BiKE and TRIKE Specific engagers (Felices et al. 2016), or CAR engineering. The CAR strategy as a leukemia therapy will be developed in Chapter 3.

The third possibility is to enhance the effector response through cytokine response: IL-12, IL-15, and IL-18 induced memory NK cells.

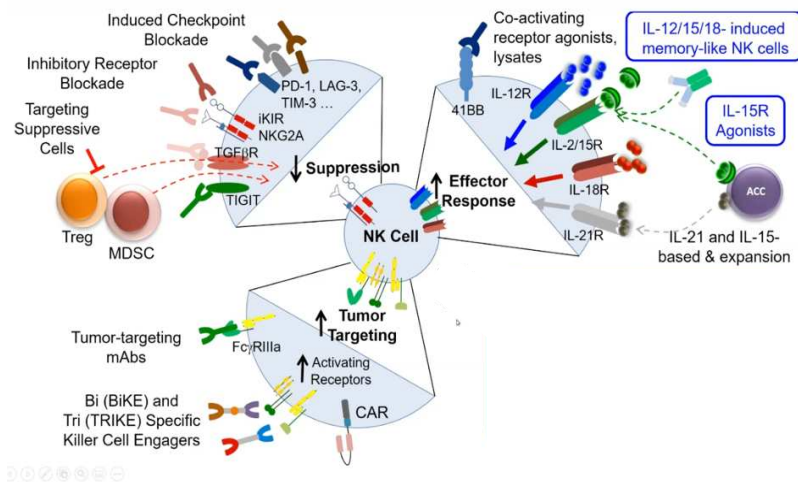


Figure 17: Strategies for improving NK cell activity (Berrien-Elliott M et al., 2015)

1.7.1.1.2. NK cell function in CML

As discussed previously, myeloid malignancies, and in particular CML, originate from a normal HSC that acquires a single genetic aberration leading to the oncogenic transformation and to the expansion of a myeloid cell mass able to differentiate and carry the same time this oncogenic alteration (Shlush et al. 2014). There has been a controversy about the involvement of T and NK cells in these stem cell malignancies. In particular, NK cells isolated from chronic CML patients are BCR::ABL negative but NK from more advanced CML stages can be BCR::ABL positive (Nakajima et al. 2002). Introducing artificially BCR::ABL to NK92 or umbilical cord blood (UCB) CD34+ cells has shown that in the first case it provokes a loss of NK cytotoxicity and in the second case a lack of differentiation (Nakajima et al. 2002; J. H. Gong, Maki, et Klingemann 1994; Chiorean et al. 2003). In 1996, Silla and colleagues showed that NK cells isolated from CML patients grown in culture have a reduced cytotoxic and proliferative capacity (Silla et al. 1996). The reason why BCR::ABL+ NK cells are found only in advanced phases of CML remains still unclear. It might be related to an expansion of the malignant stem cell pool during disease progression that gradually outcompetes normal HSC (Mattias Carlsten et Järås 2019).

NK cells are not a homogenous population and their effector function depends on their maturation stage. Immature NK cells are responsible for producing cytokines and mature NK cells are highly cytotoxic. The inflammatory cytokines levels such as that of IL-1 β , IL-6, or TNF- α have been measured and found to be elevated in CML, potentially promoting the growth of leukemic cells (Camacho, Kuznetsova, et Welner 2021). The leukemic cytokine milieu affects the terminal maturation of NK cells and cytotoxicity with a possible shift toward a cytokine-producing phenotype (immature NK cells). The CML microenvironment is also responsible for changing the NK phenotype by downregulating the activating receptors in CML-exposed NK cells (reduction of NKp46) (Rea et al. 2017). In the same perspective, CML-exposed NK cells express inhibitory molecules such as Lag-3 and TIGIT and have a higher apoptosis rate. Degranulation of NK cells is also affected in CML patients with a decreased level of degranulation (Rea et al. 2017; C. I.-U. Chen et al. 2012).

Multiple factors including the cancer microenvironment, soluble factors, or genetic aberrations can be responsible for the loss of function of NK cells in CML. Several studies show the relationship between altered NK and poor clinical outcomes (Fauriat et al. 2007; M. Carlsten et al. 2010). Most of them have highlighted the down-regulation of NK activation receptors such as DNAM-1, NKG2D, and the NCRs (Fauriat et al. 2007; M. Carlsten et al. 2010; Costello et al. 2002; Sanchez-Correa et al. 2011). Moreover, the progression of the disease is directly correlated with the loss of these receptors (Fauriat et al. 2007; M. Carlsten et al. 2010).

One of the mechanisms already described is the loss of activation receptors by internalization following contact between cancer cells and NK cells (Fauriat et al. 2007; M. Carlsten et al. 2010; Coudert et al. 2008). Soluble factors such as soluble NKG2D ligands (MICA, MICB, ULBP1) produced by the microenvironment or the cancer cell can also be an inhibition signal for NK cells (Pende et al. 2005). Cytokines such as TGF- β can induce down-regulation of NKG2D (Lee et al. 2004).

In the same perspective, the down-regulation of ligands for DNAM-1 on the cancer cell surface makes them resistant to NK cells (Kearney et al. 2016). Nowbakht et al showed that blast cells characterized by an undifferentiated profile, express low amounts of NCRs and NKG2D ligands but following IFN- γ treatment and induction of blast differentiation there is an increase in the level of expression of these ligands (Nowbakht et al. 2005).

Moreover, the expression of CD200 interacts with CD200 expressed in NK cells and induces an escape of cell lysis (Coles et al. 2011).

Other factors related to the microenvironment such as reactive oxygen species (ROS) can induce a reduction of NK cell activity but also apoptosis of NK cells (Mellqvist et al. 2000; Aurelius et al. 2017).

1.7.1.1.3. Impact of TKIs on NK cell receptors

As described above (see chapter "CML therapies"), TKIs have revolutionized the therapy of CML by their direct cytotoxic activity based on the BCR::ABL-dependence of these cells. Nevertheless, these drugs could have also a significant effect on the immune system, especially on NK cells. TKIs such as Imatinib are shown to increase the expression of activating receptors NKp30, NKp46, NKp80, and NKG2D (Binotto et al. 2014), whereas Dasatinib has been shown to increase the expression of inhibitory receptors KIR2DL1 on NK cells. Dasatinib is also shown to decrease the level of expression of inhibitory receptors such as NKG2A and therefore induce a rapid treatment response in CML patients (Chang et al. 2018). Even though it exists different polymorphisms of KIRs, no difference has

been observed in KIR genotypes (KIR2DL2, KIRDL5, and KIE3DS1) in non-relapsing, early and late relapsing groups in patients from the TFR clinical trial EURO-SKI (Illander et al. 2017).

The loss of expression of NKG2DL in LSC is one of the reasons that these cells survive selectively chemotherapy and escape from immune surveillance (Paczulla et al. 2019).

NK cell immunity remains an important component to take into consideration while treating CML. The phenotyping of CML NK cells to evaluate the level of activating and inhibitory receptors is an interesting tool to understand the progression of the disease. Enhancing NK cell numbers and/or activities as described above, provides a promising therapy to achieve treatment free-remission (TFR). Lenalidomide and other NK cell-modulating agents show real potential in achieving TFR through the activation and proliferation of T and NK cells (J. R. Jones et al. 2016).

1.7.1.2. Role of T cells in CML immune response - CD8+ cytotoxic T-cells

CD8+ CTL plays an important role in the appearance and control of CML. It has been shown that CD8+ CTLs are functionally exhausted in CML at diagnosis (Reuben et al. 2000). Moreover, there is a direct correlation between CD8+ CTL and the ability of patients to reach a DMR. Patients in DMR exhibit a restored CD8+ CTL pool with decreased inhibitory molecule PD1 (A. Hughes et al. 2017).

1.7.1.3. Role of T cells in CML immune response Regulatory T-cells (T-regs)

The involvement of these immune modulatory cells in CML is now supported by recent experimental data. Indeed, the T-reg population is increased in CML patients at diagnosis and is responsible for an immune-modulatory effect on immune effectors (T and NK cell anergy). Same as CD8+ CTL, their number is low in patients achieving deep molecular response status following TKI treatment (A. Hughes et al. 2017). An inverse correlation with NK cell status has also been shown in this context (Najima et al. 2018). The reduction of Tregs is also correlated with the achievement of TFR in CML (Okada et al. 2018).

CML cells, like other malignancies, express aberrant antigens (Leukemia-associated antigens - LAA) that are targeted by T cells. These LAAs include among others, BCR::ABL1, WT1, and PR1. All of them can induce a T-cell response enhancing the antileukemic immune response. Hughes and colleagues have described in 2017 that these immune effector responses are restored in patients in DMR. Molldrem and colleagues showed in 2000 the involvement of PR1 in CML immune response. In a cohort of 38 CML patients treated with allogeneic BMT, IFN-alpha2b, or chemotherapy, they found a strong correlation between the presence of PR1-specific T cells and clinical responses after IFN- α and allogeneic BMT (J. J. Molldrem et al. 2000).

WT1 is also an interesting antigen highly expressed in myeloid malignancies. Different vaccination strategies have been designed by using WT1 as a cancer antigen (Oji et al. 2010). Several other vaccination strategies using BCR::ABL and BCR::ABL-induced antigens have been reported with reduction of BCR::ABL transcripts but little clinical effect on CML progression (Bocchia, Forconi, et Lauria 2006).

1.7.1.4. Dendritic cells (DC)

Blood and marrow DC originate from a common myeloid precursor and can therefore be originated also from the Ph1+ clone. Thus, in CML patients, there is a co-existence of cytogenetically healthy DC

and leukemia-derived DC expressing *BCR::ABL*. These *BCR::ABL* DC can be isolated in the PB of CML patients, suggesting that CML-derived DCs may contribute to anti-leukemic activity (Qin et al. 2013). They can capture and process antigens and show defective cell migration compared to DCs from healthy donors (Dong et al. 2003). Regarding the *BCR::ABL1* expression in CML DCs, different studies have shown contradictory results, in one hand DCs from CML patients have been shown to induce an anti-CML immune response (Eibl et al. 1997) and on the other hand, CML DCs are shown to have a low maturation status and a reduced antigen-presenting ability but also impaired homing to secondary lymphoid organs (Mumprecht et al. 2009). CML-DCs have been also used as a vaccine therapy to induce an anti-leukemic immune response. Due to the advent of TKIs, few CML patients have received CML-DC, nevertheless, data indicate that CML-DC vaccination triggered T cell responses in most CML patients, and, in some of them, cytogenetic or clinical responses (Fujii et al. 1999; Ossenkoppele et al. 2003; Litzow et al. 2006; Westermann et al. 2007).

Even though TKIs have revolutionized the treatment of CML, the financial burden and side effects associated with lifelong adherence to TKIs might make the DC vaccination approach an interesting therapy in the future.

1.7.1.5. Interaction between NK cells and DC

Bi-directional cross-talk between NK cells and DC results in the development of an efficient innate response, through NK-mediate DC editing and maturation (Ferlazzo et Morandi 2014). Following interaction between DC and NK cells, there is a reciprocal activation: mature DCs release cytokines able to promote NK cell activation, $IFN-\gamma$ is released by activated NK cells and promotes Th1 polarization, and together with TNK, DC maturation and migration to draining lymph nodes. *In vitro* studies have shown that the culture of activated NK cells with immature DCs at low NK/DC ratios promotes DC maturation, whereas a higher NK/DC ratio can result in NK cell-mediated killing DCs (Piccioli et al. 2002).

1.8. CML Therapies

The first data on CML treatment exist since the 19th century. At this time, the main therapy for CML was based on arsenic, Fowler's solution. At the beginning of the 20th century, CML therapy was based mainly on splenic radiotherapy and by the middle of the 20th century, alkylating agents such as busulfan were used (Galton 1953). Busulfan had for effect the reduction of leukocytes but in any case, couldn't stop the progression of the disease. Furthermore, many adverse effects were described for busulfan such as pulmonary fibrosis and severe myelosuppression (Hehlmann et al. 1993).

Later on, hydroxyurea was introduced to therapeutic use and it is still used as initial therapy in patients presenting with very high blood counts while waiting for the results of *BCR:ABL* determination. It is a ribonucleotide reductase inhibitor shown to have better results in the overall survival of patients than busulfan (Hehlmann et al. 1993) but without the ability to stop the disease progression.

$IFN-\alpha$ represented a major therapeutic tool in the last decades of the past century allowing for the first time an improvement of survival, especially in association with cytosine arabinoside (Guilhot et al. 1997). This association remained the best therapy until the introduction of Imatinib to the clinical use.

1.8.1. Current therapies for CML

At the beginning of the XXst century, a real revolution in CML treatment was achieved by the use of specific tyrosine kinase inhibitors (TKI).

Nowadays, TKIs are grouped in three different groups:

1. 1st generation – Imatinib
2. 2nd generation – Nilotinib, Dasatinib, Bosutinib
3. 3rd generation – Ponatinib, Asciminib

Imatinib (Gleevec®) – is a competitive inhibitor of Adenosine Tri-Phosphate (ATP). It occupies the Tyrosine Kinase (TK) active site of BCR::ABL leading to a decrease in its activity. The active sites of TK have a binding site for ATP. Imatinib binds close to the ATP binding site of BCR::ABL inducing thus a locked conformation and therefore inhibiting the enzyme activity of the protein. Imatinib remains today the reference treatment for CML, nevertheless, it can show different adverse effects such as vomiting, nausea, and diarrhea (O'Brien et al. 2003).

Since imatinib is a lifelong treatment, different mutations can occur in 20-30 % of patients, especially in the kinase domain of BCR::ABL (Jabbour et al. 2011), other patients might develop relapse through alternative resistance (Shah et Sawyers 2003) or should switch to other TKI because of intolerance (Druker et al. 2006).

For all those reasons, other TKIs have been developed, such as 2nd generation TKIs which are more efficient than Imatinib (between 30 and 200 times).

Nilotinib – in the market since 2007 is used for CP-CML and AP-CML. Structurally it is very close to Imatinib and can inhibit BCR::ABL 50 times more efficiently than Imatinib (Manley et al. 2004). Nilotinib is efficient against F359V, Y253H, and E255V/K mutations (T. Hughes et al. 2009). Nevertheless, it is not the case against T315I mutation.

Dasatinib – is a 2nd generation TKI used to treat unresponsive or intolerant patients to Imatinib. Dasatinib is efficient against the majority of mutations of the kinase domain but not for T315I (M. C. Müller et al. 2009). This is due to its ability to inhibit both active and inactive conformation of the kinase domain of BCR::ABL (Lombardo et al. 2004; Tokarski et al. 2006). Dasatinib is also shown to inhibit other tyrosine kinases in the cell such as Src proteins or c-KIT (Lombardo et al. 2004). Src proteins are involved in the progression of the disease (S. Li et Li 2007; S. Li 2008) therefore their inhibition by Dasatinib makes this drug an interesting tool for the treatment of these patients.

Bosutinib – is a 2nd generation TKI able to inhibit BCR::ABL and platelet-derived growth factor receptor (PDGF), c-Kit, and Src proteins (Remsing Rix et al. 2009; Amsberg et Koschmieder 2013).

Ponatinib – is used in 2nd line treatment in case of unresponsiveness to 1st generation TKI or first-line treatment in case of T315I mutation (Cortes et Kantarjian 2012). Same as Dasatinib, it can bind both active and inactive conformation of BCR::ABL (Modugno 2014). It is one of the most efficient TKIs against T315I mutation and its activity goes through the substitution of a threonine with an isoleucine in the P loop of the kinase domain of ABL. It can also inhibit other kinases such as VEGF, FGFR1, cKIT, Src, PDGF, RET, EPH, and FLT. Despite its interesting efficiency in CML, Dasatinib is responsible for different adverse effects on patients such as cardiac thrombosis, and for that reason, Ponatinib is used as a last resort for CML patients.

Asciminib – is the newest TKI in the market. Developed by Novartis as ABL001, it is an allosteric inhibitor of BCR::ABL that binds to the myristoyl binding site of BCR::ABL, which confers a high specificity against BCR::ABL (Wylie et al. 2017). Contrary to other TKIs described here, Asciminib does not bind to the ATP binding site, making it an interesting tool against T315I mutation.

1.8.2. Drug resistance in CML

TKI resistance can be defined from a clinical and biological point of view. Here will be described only the biological mechanisms.

BCR::ABL is a highly oncogenic protein that is responsible for generating an important disequilibrium in the cells that will be higher and higher with the evolution of the disease. The resistance mechanisms in CML can be classified into two groups: dependent or independent of BCR::ABL.

1.8.2.1. Resistance mechanisms dependent of BCR::ABL

1.8.2.1.1. ABL kinase domain mutations

The most common resistance mechanisms to TKIs are related to point mutations of the sequence coding for BCR::ABL (Gorre et al. 2001; Soverini et al. 2005). These mutations can occur in any stage of the disease through modification of an amino acid of the kinase domain of BCR::ABL inducing thus a loss of efficacy of TKIs (Quintás-Cardama, Kantarjian, et Cortes 2009). Not all of these point mutations of ABL are responsible for resistance but some of them such as the T315I mutation can lead to TKI resistance to all licensed TKIs except Ponatinib and Asciminib (**Figure 18**).

T315I mutation occurs at the binding site of imatinib same as the F317L mutation. The binding site to Imatinib is located in the P loop which is also the binding site for ATP as described previously. Other mutations such as G250E, Q252E/H, Y253F/H, or E225K/V can also occur at this site. In the activation loop mutations such as M351T, and F359C/V can happen and in the catalytic site, the mutations H396P/R have been described (Chomel et Turhan 2011).

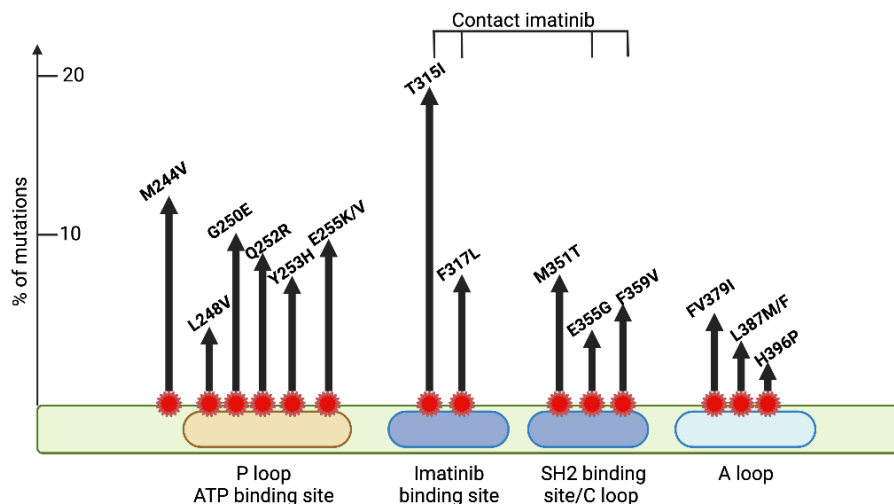


Figure 18: Representation of mutations occurring in the kinase domain of BCR::ABL. Red stars represent proteic sites generated by mutations. The height of the arrows shows the percentage of patients carrying that mutation. (Adapted from Chomel and Turhan, 2011)

The T315I mutation is also named as “gatekeeper”. The substitution of the threonine by an isoleucine at position 315 provokes the elimination of an oxygen residue needed for the formation of a hydrogen bond between imatinib and the kinase domain. This will also prevent the binding of other TKIs to BCR::ABL because of the steric hindrance, but not for the Ponatinib which binds to the BCR::ABL through an ethynyl bound between methylphenyl and purines groups (O’Hare et al. 2009).

Currently, it is very difficult to determine whether these mutations are the consequence of the genomic instability related to the disease progression or whether they are responsible for the progression (Khorashad et al. 2006). Mutations in the kinase domain do not provoke relapsing systematically, this is proof that supplementary factors are involved in the real resistant phenotype (Willis et al. 2005).

1.8.2.1.2. BCR::ABL amplification

Roughly 10% of resistances to Imatinib are related to the acquisition of a second Ph1 chromosome. The increasing levels of BCR::ABL due to the amplification of the gene or to the duplication of the Ph1 chromosome are less involved in TKI resistances compared to kinase domain mutations. The increase in BCR::ABL expression is found in patients in accelerated phases and it is suspected to favor mutations of the kinase domain (D. J. Barnes et al. 2005).

1.8.2.1.3. Resistance mechanisms independent of BCR::ABL

The resistance to TKIs might also be independent of BCR::ABL but related to other reasons. Among these reasons, the most known are:

1.8.2.1.3.1. The TKI bioavailability

The bioavailability of TKIs is one of the main factors for the efficient anti-cancerous effect of these drugs. TKIs are metabolized by P450 3A4 (CYP3A4) in the liver. Therefore, the levels of this cytochrome are directly correlated with the bioavailability of TKIs.

Once TKIs have crossed different physiological barriers, they join the systemic circulatory system where they bind to different transporters. In the case of imatinib, it binds to the albumin and the α 1-glycoprotein (C. B. Gambacorti-Passerini et al. 2003). That allows the drug to circulate through the organism but the overexpression of these transporters provokes sequestration of Imatinib in the plasma (C. Gambacorti-Passerini et al. 2000).

1.8.2.1.3.2. The Efflux transporter (ABCB1/P-gp (P-glycoprotein))

The overexpression of efflux pumps ABCB1/P-gp and ABCBG2/BCRP (breast cancer-related protein) is one of the major factors for resistance development in CML (Chomel et Turhan 2011; Nestal de Moraes et al. 2012). Those transporters control the absorption of different molecules such as xenobiotics in an active manner (ATP-dependent). In CML they are involved in the efflux of imatinib (J. Thomas et al. 2004). The ability of cells to adapt to treatment goes through the overexpression of these proteins and it enables the cancer cell to resist treatment. It has been shown that ABCB1/P-gp pumps are overexpressed in BC-CML (J. P. Radich et al. 2006).

1.8.2.1.3.3. The influx transporter (OCT1/SLC22A1)

OCT1/SLC22A1 is a soluble transporter that enables the influx of molecules and xenobiotics in a passive manner (independent of ATP). It is involved in the transport of neurotransmitters, nutrients, and metabolites. It has been shown that OCT1 enables the entry of imatinib into the cells, and the decrease of its expression is a bad prognostic marker (Chomel et Turhan 2011; Nestal de Moraes et al. 2012). **Figure 19** shows the principal resistance mechanisms to TKI (Apperley 2007).

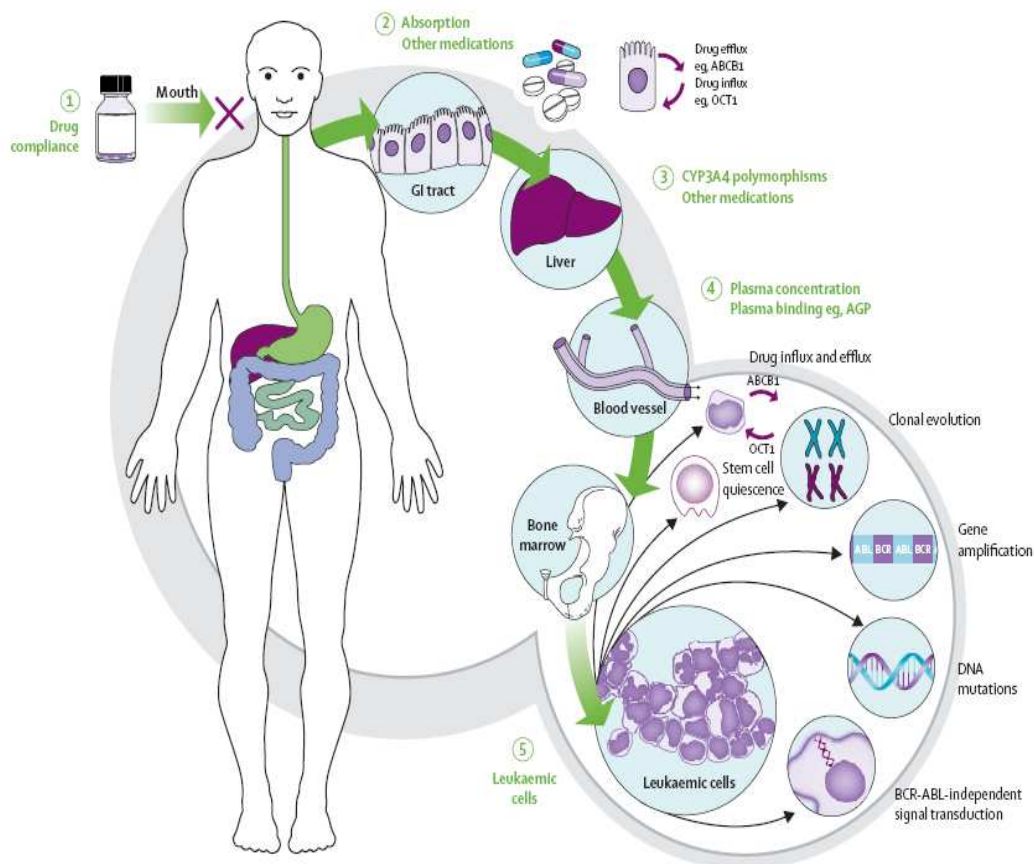


Figure 19: Principal mechanisms to TKIs resistance (Apperley, 2007)

1.8.3. Immunomodulatory effects of TKIs

TKIs have been shown to have an impact on the immune system and induce immunomodulatory effects with anti-tumor and anti-viral activity. Those effects can be on T, NK, and B cells (Stegmann et al. 2012). Imatinib is shown to cause lymphopenia and decrease immunoglobulin levels (Cwynarski et al. 2004), and nilotinib inhibits CD8+ T cells (J. Chen et al. 2008). As shown previously, they are also effects shown on NK cells e.g., Dasatinib suppresses the cytotoxic activity of NK cells (Damele et al. 2018).

Suppressor cells are also affected by TKIs. The Treg level is higher in patients with CML at diagnosis compared to healthy controls and when the patient reaches the DMR, the number of Tregs is lower (Fujioka et al. 2021).

NK cells have been linked to the likelihood to stay in remission upon TKI cessation. Patients that have early relapses after TKI discontinuation have a lower percentage of NK cells as compared to patients non-relapsing. The NK subpopulation CD56+ Dim/CD16- secrete cytokines such as IFN- γ and TNF- α . If these cells are in abundance, the likelihood of staying in remission is higher (Vivier et al. 2008;

Lewis L. Lanier 2008; Ilander et al. 2017). Altogether, this shows that NK cells are eradicators predictive of successful TKI discontinuation (See also Table 1).

1.9. Pathophysiology of Blast crisis (BC)

As described above, BC is a life-threatening step of CML and still occurs in the TKI era, especially in patients with TKI-resistant therapies with an incidence of 2-3%. According to the World Health Organization (WHO), CML has reached BC when the number of blasts in the PB is 30% or more. BC is characterized by a blockage of differentiation of leukemic cells and an accumulation of these blasts in the PB or the BM. BC is often but not always preceded by an AP-CML.

BC-CML is fundamentally different from CP-CML in terms of proliferation, differentiation, apoptosis, and adhesion (Calabretta et Perrotti 2004; Shet, Jahagirdar, et Verfaillie 2002). Patients in BC-CML require more aggressive therapy, especially if there is a T315I mutation. The first goal in this phase is to obtain the return to CP-CML using TKI therapy (such as Ponatinib) and then search for an HLA-compatible donor for an allogeneic stem cell transplant (ELN recommendations 2020 (Hochhaus et al. 2020)).

BC-CML is characterized by specific biological events such as genetic events, DNA repair abnormalities, epigenetic modifications, etc.

1.9.1. Genetic events involved in BC-CML

The most frequent additional chromosomal abnormalities in BC-CML are classified into two groups (Mitelman et al. 1976).

- Major-route abnormalities include an additional 8 chromosome, a second Ph chromosome, an isochromosome of the long arm of chromosome 17 i[17q], and an additional 19 chromosome. These abnormalities are found in 70-80% of patients.
- Minor-route abnormalities include -7; -17, -Y, +17 et +21 and are observed in 15% of patients.

The t(3;21) additional translocation generates the fusion protein AML/EVI1 and is involved in the blockage of cell differentiation and the increase of cell proliferation in BC-CML (Kurokawa et al. 1998; Phan et al. 2008).

At the molecular level, the most common gene mutation in BC-CML is the deletion of p53 located in the short arm of chromosome 17, while its mutation is uncommon in CP-CML. Stuppia and colleagues showed in 1997 that the loss of the p53 gene is directly correlated with a reduction of apoptosis and blast transformation of CML (Stuppia et al. 1997). p53 is mutated in 25-30% of BC-CML (Calabretta et Perrotti 2004).

MYC is another important gene shown to be upregulated in BC-CML (Chereda et Melo 2015). The overexpression of MYC is related to mitochondrial genome instability including abnormal DNA replication and aberrant DNA synthesis (Albajar et al. 2011).

Mutations in other genes such as RUNX1, CBL, CBLB, TET2, ASXL1, and IDH1/2 are also shown to be involved in BC-CML (Makishima et al. 2011).

1.9.2. DNA repair abnormalities

The progression of the disease from CP-CML to BC-CML is due to an accumulation of anomalies responsible for the appearance and selection of malignant clones. BCR::ABL expression prevents many

DNA repairing systems and it is considered the major reason for genetic instability (Calabretta et Perrotti 2004; Shet, Jahagirdar, et Verfaillie 2002).

As early as 2000, it has been established that BCR::ABL was able to induce major oxidative stress in the cells in which it is expressed (Sattler et al. 2000; Koptyra et al. 2008). This increased oxidative stress may induce double-strand breakages (DSB) in DNA and a delay in repair (Tomasz Skorski 2002) and it gives rise to a “mutator phenotype” demonstrated for the first time in 1999 in cells expressing BCR::ABL p210 and p190 (Canitrot et al. 1999). Interestingly, BCR::ABL-expressing cells are also doomed by a second molecular event: the abnormalities of DNA repair mechanisms.

Data from our laboratory have shown in 2003, the DNA repair gene modifications induced by BCR::ABL. Indeed, it has been demonstrated that BCR::ABL was directly responsible for the reduced DNA-PKcs expression due to the repair of protein degradation under the influence of BCR::ABL TK activity (Deutsch et al. 2003). Similarly, BCR::ABL has been shown to reduce transcriptionally the expression of BRCA1 in leukemic cells (Deutsch et al. 2003), exposing them to the occurrence of unrepaired mutagenic events. Therefore BCR::ABL-expressing cells cannot use BRCA1 for DNA repair (Deutsch et al. 2003). Thus, in this perspective, BCR::ABL not only induces the mutator phenotype via oxidative stress but also interferes with repairing pathways such as non-homologous end-joining (NHEJ) and Homologous recombination repair (HRR) and induces unfaithful repair of DSB (Tomasz Skorski 2002; Slupianek et al. 2006).

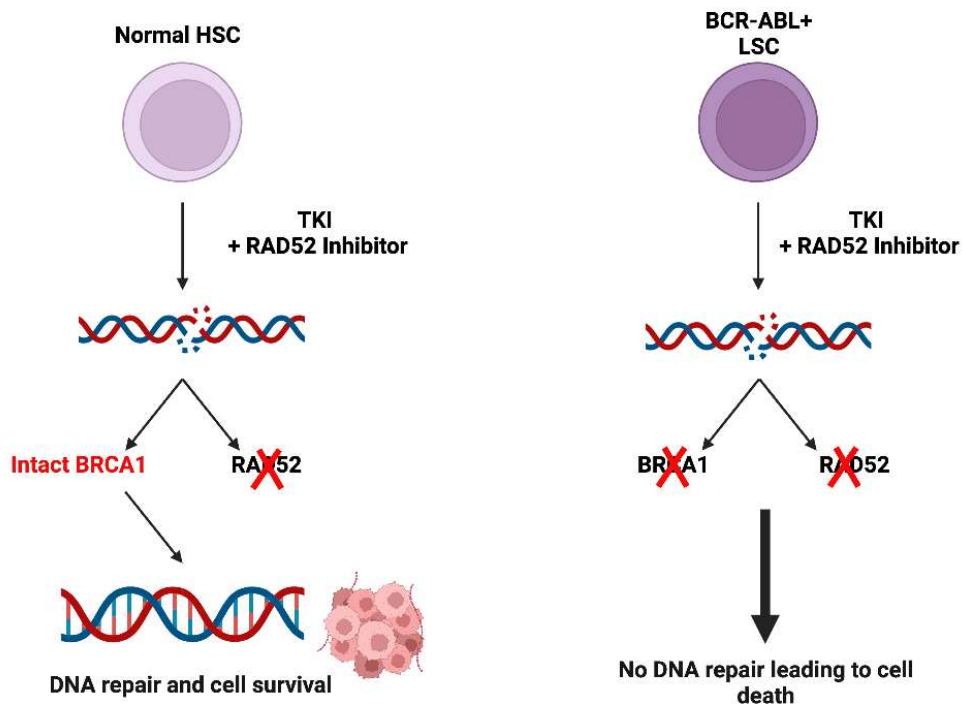


Figure 20: Strategy for the use of RAD52 inhibition as a therapy to eradicate BCR::ABL+ LSC

The DNA repair abnormalities are correlated with the progression of the disease towards aggressive phases but the modelling of these events has been so far difficult due to the requirement of collection and patient samples at different phases of the disease and therefore has been characterized sporadically in patient samples and cell lines described previously. Interestingly, DNA

repair abnormalities could represent the “Achilles Heel” of the leukemic cells: Indeed, the group of Skorski has taken advantage of the BRCA1 deficiency (Deutsch et al. 2003) to design a therapeutic strategy to target CML cells. As In BRCA1 deficient cells, the activation of RAD52 can partially complement this defect, they have designed a strategy to inhibit RAD52 via aptamers, leading to the inability of BCR::ABL-expressing cells to repair DNA damage induced by TKIs (Slupianek et al. 2011) (Figure 20).

1.9.3. Epigenetic modification

Epigenetic abnormalities, including DNA methylations and histone modifications, have been shown to play an important role in CML progression (Yin et al. 2019). Methylation of genes such as tumor suppressor genes (EPB41L3 and PRDX2) have been identified in BC-CML but also genes involved in cell proliferation such as (BCL11B, NDRG2, and PID1) (Heller et al. 2016). The hyperphosphorylation of JAK2 and STAT pathways due to a low level (high degree of methylation) of long non-coding RNA (MEG3) provokes cell proliferation and apoptosis escape (Z.-Y. Li et al. 2018).

At histone level modifications, the expression of retinoblastoma-binding protein 2 (RBP2) is shown to be inhibited in BC-CML (Yin et al. 2019). RBP2 is a gene mediating the dephosphorylation of BCR::ABL by directly downregulating PTEN expression, while PTEN targets the protein phosphatase activity of BCR::ABL. Under-expression of RBP2 promotes blast crisis by activating an RBP2/PTEN/BCR::ABL cascade (Yin et al. 2019).

1.9.4. Increased resistance to apoptosis

The resistance to apoptosis is one of the most important mechanisms that leukemic cells exhibit to resist treatments and lead to tumor progression (Calabretta et Perrotti 2004). Among these antiapoptotic pathways involved in BC-CML, the most well-described are RAS, PI-3K/Akt, and STAT5. Down-regulation of the RAS pathway is shown to suppress cell proliferation and sensitize cells to apoptotic stimuli (Sawyers, McLaughlin, et Witte 1995; T. Skorski et al. 1994). Similar effects have been observed for PI3K/Akt inhibition that leads to suppression of *in vitro* colony formation and *in vivo* leukemogenesis of BCR::ABL (T. Skorski et al. 1995). STAT5 is known to be activated by BCR::ABL as described in the previous chapter. A STAT5 target gene is the antiapoptotic *BCL-X_L* (Horita et al. 2000), through which it increases the antiapoptotic resistance of BCR::ABL expressing cells.

1.9.5. Acute transformation of CML

BC-CML is an acute leukemia, occurring in most cases (60-80%) as an acute myeloblastic leukemia (AML) and less often as an acute lymphoblastic leukemia (ALL) (20-30% of cases) (Ivanov et al. 2020).

Among the types of AML, almost all phenotypes have been reported, including myeloblastic megakaryoblastic (Khemka, Gupta, et Jena 2019) and monoblastic (Nemoto et al. 1986) blast crisis. Acute T-cell transformation of CML occurs but very rarely (Nakase et al. 2017). Also in some rare cases, BC-CML can be bi-phenotypic expressing both myeloid and lymphoid blasts (6-10%). The bi-phenotypic blast crisis is characterized by a poorer prognosis than the monophenotypic blast crisis, especially in the case of extramedullary involvement. Biphasic transformation is fortunately very rare, less than 10 of cases have been described in the literature. Nevertheless, most cases are in pediatric patients and mortality rates are near 100% despite aggressive chemotherapy.

CD25 is expressed in de novo AML and its expression is correlated with poor prognosis (Kuhn et Dou 2005). As described previously, CD25 is aberrantly highly expressed on LSCs, and interestingly it has been shown that CD25-AML blasts have an LSC-like gene expression profile (Gönen et al. 2012).

Nakase and colleagues describe that CD25 is also expressed in BC-CML and BC-CML transformed to AML (Nakase et al. 2017). They show also for the first time that the level of CD25 expression is significantly higher in MDS/AML (myelodysplastic transformed AML) and BC-CML compared to de novo AML making this marker an interesting target for both LSC and BC-CML.

1.10. CML models

1.10.1. CML cell lines

The generation of CML-derived cell lines has been a real challenge for modelling CML and studying potential treatments. The main issue in generating such cell lines is the risk of losing the Ph1 chromosome after a serial sub-culture for longer periods (Miles et al. 1968; Belpomme, Minowada, et Moore 1972). In 1975, the first CML cell line was successfully generated and named K562. This cell line was derived from the pleural effusion of a 53-year-old female CML patient in blast crisis (C. B. Lozzio et Lozzio 1975). K562 cell line is characterized by a lack of MHC making them highly vulnerable to NK cytotoxicity (Drexler et Matsuo 2000).

In 1983, another interesting CML cell line (KCL-22) has been generated from a 32 years-old female patient in lymphoid blast crisis (e13a2 fusion transcript type) (Kubonishi et al. 1983). In the KCL-22 cell line, the karyotype shows a double Ph1 chromosome and additional chromosome abnormalities and also mutated p53 gene.

In 1985, Ogura and colleagues publish a new megakaryoblastic Ph+ cell line, the MEG-01 (Ogura et al. 1985). It was generated from the bone marrow of a 55-year-old male patient in a megakaryoblastic crisis of CML.

In 1995, KBM-7, a CML line from a patient in blast crisis has been published (Andersson et al. 1995). This cell line is characterized by a near-haploidy, meaning that it contains only one copy for most of its chromosomes. Also, in 1995, Wada H and colleagues publish the AR230 cell line (Wada et al. 1995). New subclones of AR230 have been generated such as AR230-r resistant to Imatinib mesylate.

Later on, new CML cell lines have been generated especially by introducing artificially *BCR::ABL* on cells by viral transduction. That was the case of the UT-7 cell line, an erythro-megakaryocytic cell line isolated from AML blast cells and dependent on GM-CSF (Issaad et al. 2000; Dugray et al. 2001).

1.10.2. CML mouse models

One of the earliest attempts to model CML *in vivo* dates back to the '70s when the K562 cell line generated from the myeloid blast crisis of a patient was injected into nude mice (B. B. Lozzio, Lozzi, et Machado 1976). Since then, a wide variety of *in vivo* models have been developed to better understand the molecular pathology of CML and develop new treatments. Among these models, we can find conventional models, conditional transgenic models, knock-in models, and murine bone marrow retroviral transduction models.

Mouse models have been a very interesting tool for the understanding self-renewal ability of LSC. By engrafting SCID (severe combined immunodeficient) mice, Cobaleda and colleagues showed similar behavior of LSC and HSC (Cobaleda et al. 2000).

The first CML *in vivo* models were generated at the end of the '80s by introducing the synthetic *BCR::ABL* gene into the germ line (Hariharan, Adams, et Cory 1988). In 1990, the team of David Baltimore in Boston showed for the first time that the engraftment of *BCR::ABL* retroviral transduced murine HSC leads to the development of myeloproliferative syndromes in mice (Daley, Van Etten, et

Baltimore 1990). To generate this model, murine bone marrow has been transduced with retrovirus encoding p210bcr/abl and transplanted into irradiated syngeneic recipients. The transplant recipients developed a myeloproliferative syndrome closely resembling CML (Daley, Van Etten, et Baltimore 1990). This model is one of the most used for understanding CML, identifying new genes involved in the diseases, and characterizing LSCs. Nevertheless, there are some disadvantages related to this model. The induced CML is highly aggressive leading to the death of the animals a few weeks after transplantation since the expression of BCR::ABL is not controlled. This paved the way for the development of new models especially inducible models expressing BCR::ABL under the tetracycline response element (TRE) (Furth et al. 1994; Huettner et al. 2000). The generation of this model has enabled us to understand the importance of the type of promoter for BCR::ABL. They have shown that promoters like MMTV induce pre-B lymphoid leukemia (Huettner et al. 2000) but the CD34 promoter induces a megakaryocytic myeloproliferative syndrome (Huettner et al. 2003).

In other studies, xenotransplant models have been developed by injecting cells from CML patients at different stages of the disease in SCID mice (Clutterbuck et al. 1997) or into NOD/SCID (Eisterer et al. 2005). These models have enabled the elucidation of different pathways and mechanisms of CML.

1.10.3. CML modelling using embryonic stem cells

As shown before, CML is characterized by the LSCs (resistant to TKIs) which are responsible for relapses under therapy or after stopping TKIs. These rare stem cells are highly quiescent (S. M. Graham et al. 2002) and therefore difficult to isolate and characterize. The use of murine CML models require overexpression of BCR::ABL in primitive bone marrow cells but they are very aggressive and do not recapitulate the disease. To uncover the characteristics of the most primitive HSC involved, several groups developed murine ESC-based models (Perlingeiro, Kyba, et Daley 2001; Melkus et al. 2013). One major interest of these models is the fact that BCR::ABL expression in ESC-derived hematopoietic cells endows them with long-term reconstitution potential (Perlingeiro, Kyba, et Daley 2001; Melkus et al. 2013) This potential seems to be correlated in mice, with the activation of STAT5 which is also able to induce a long-term repopulation potential by itself (Kyba et al. 2003; Era et Witte 2000).

Several studies in rodents have shown that BCR::ABL is involved at the pluripotent level and it activates STAT3 (Coppo et al. 2003), which is responsible for the maintenance of the “stem” phenotype of mouse embryonic stem cells (mESC). Transduction of mESC with retroviruses carrying BCR::ABL with the T315I mutation has been shown to enable these cells to undergo long-term hematopoiesis (Melkus et al. 2013).

There are currently no data about the overexpression of BCR::ABL in hESC, the availability of iPSC which could be derived from CML patients made this approach more “physiological” and has now been pursued in several laboratories.

1.10.4. Induced pluripotent stem cell (iPSC) CML models

Induced pluripotent stem cells (iPSC) have revolutionized disease modelling (these cells will be described further in the following chapter). Briefly, iPSCs were derived in 2007 (Takahashi et al. 2007) and opened a new era in the field of disease modelling including hematopoietic diseases. Whereas monogenic inherited blood diseases were among the first to be modelled with iPSC (Hanna et al. 2007), malignant hematologic disorders are more challenging. In inherited genetic diseases, the mutation responsible for the disease is present in all somatic cells, that is not the case for *de novo* cancerous genetic lesions which occur often in HSC and are responsible for blood cancers. The first challenge in

reprogramming cells from a CML patient is the heterogeneity of the initial sample. Usually, PBMCs are used as initial cells and the fact that not all of these cells are mutated leads often to the reprogramming of Ph-negative cells, especially because nowadays there is no specific marker able to distinguish between mutated and healthy cells in the blood.

The other issue in generating myeloid malignancy iPSCs is their refractoriness to reprogramming (this will be detailed further in the following chapter).

In 2010, Carette and colleagues showed that it is possible to reprogram Ph⁺ cells by using an OSKM retrovirus (Carette et al. 2010). One year later, K. Hu published an iPSC cell line derived from PBMCs of a CML patient (K. Hu et al. 2011). The interest of this iPSC was its ability to differentiate into hematopoietic cells with better efficiency compared to iPSC derived from fibroblasts. Hematopoietic cells generated from Ph⁺ iPSCs were able to give hematopoietic progenitors (CD34⁺ and CD43⁺) and among them a population of erythro-megakaryocytic multipotent progenitors that can produce hematopoietic colonies in semi-liquid cultures such as CFU-GM, BFU-E, and CFU-GEMM (K. Hu et al. 2011).

As expected, CML-iPSC are insensitive to imatinib because of the activation of many survival pathways in iPSC (Kumano et al. 2012). This has prompted investigators to suggest the use of iPSC models to study drug resistance but this can be studied only when the cells are differentiated to the hematopoietic lineage (K. Hu et al. 2011; Bedel et al. 2013). Another interesting question is whether CML-iPSC-derived hematopoietic cells could lead to the generation of cells with long-term repopulating ability, i.e., *bona fide* HSC. In light of this question, we and others have reprogrammed leukemic cells from several patients with CML (Sloma et al. 2017; G. Telliam et al. 2016). Among these iPSC cells line, one with particular interest is a double mutated iPSC carrying BCR::ABL and V617F JAK2 (Sloma et al. 2017) which exhibits an increased LTC-IC potential and thus an increased stem potential. This cell line has been used also for drug screening and Pimozide, a STAT5 inhibitor shows synergistic inhibitory activity with Imatinib (Sloma et al. 2017). Suknuntha K and colleagues showed also the possibility of generating “LSC-like” cells from CML-iPSC (Suknuntha et al. 2015).

Despite the ability to generate “LSC-like” cells from CML-iPSC, so far, no data is showing that CML-iPSC could generate repopulating LSC in immunodeficient mice. One of the reasons for that might be the fact that CML-iPSC misses a signaling link that is present in murine pluripotent cells transduced with BCR::ABL. In murine experiment systems, BCR::ABL expression is under the control of strong promoters which is not the case in human CML-iPSC.

The other reason might also be the lack of a “niche” allowing the emergence of a primitive LSC potential. Suzuki N and colleagues showed in 2013, that teratomas generated by injection of iPSC in NSG can be an interesting microenvironment for spontaneous *in vivo* generation of hematopoietic cells that can also exhibit a 2nd transplantation ability (N. Suzuki et al. 2013).

2. iPSC and hematopoietic differentiation

2.1. Stem cells - general overview

Stem cells are characterized by their ability of self-renewal and differentiation, generating functional and differentiated cells according to their tissues of origin. They exist both in the embryonic and adult stages. In the embryonic stage, they participate in the generation of all tissues in which they remain specialized and still able to self-renew when the individual reaches the adult stage. Besides the embryonic stem cells (ESC) which are obtained from the culture in the internal cell mass of an embryo, (ICM) in defined conditions, the presence of stem cells specialized for maintaining the perpetuation of adult tissues during the lifetime, has been recognized in several organs, the first and most widely characterized is the hematopoietic system.

From the hierarchical point of view, several types of stem cells can therefore be individualized:

- 1) **Totipotent stem cells** – have the highest differentiation potential and can differentiate into tissues from both embryo and extra-embryonic structures. It consists of the first steps of the zygote just after sperm fertilization of the egg. On day 4 after fertilization, the blastocysts will lose their totipotency and become pluripotent
- 2) **Pluripotent stem cells** – can differentiate into all germ layers but not extraembryonic structures (e.g., placenta). The prototype of PSC are the embryonic stem cell lines (ESC) obtained initially in mice in 1981 (Evans et Kaufman 1981) and later in humans (Thomson et al. 1998). Murine ESCs have been highly used to uncover pathways involved in the differentiation of ESC towards different tissues and they have been of major interest for disease modelling (Sukoyan et al. 2002). Human ESCs have also been used for disease modelling and more recently for therapeutic purposes (cardiomyocyte transplantation derived from ESC – (Burt et al. 2012; Menasché et Vanneaux 2016). Although ESCs have been a very interesting tool in research and therapy, their use is highly regulated by health authorities (in France, Law for Biomedicine n°2021-1017). Shinya Yamanaka and Kazutoshi Takahashi showed in 2006 in mice and 2007 in humans, the possibility to generate pluripotent cells from adult somatic cells. This discovery has revolutionized and democratized the use of pluripotent stem cells (Nobel Prize 2012) and avoids most ethical issues.
- 3) **Multipotent stem cells** – have a narrower spectrum of differentiation compared to pluripotent stem cells. They conserve their capacity to self-renew by dividing and can develop into multiple specialized cell types present in a specific tissue or organ. They represent the highest number of stem cells in adults. One typical example is the hematopoietic stem cell (HSC) which can differentiate into different types of blood cells.
- 4) **Oligopotent stem cells** – have an even more narrow spectrum of differentiation compared to multipotent stem cells (e.g., myeloid stem cells can divide into leukocytes but not erythrocytes).

2.1.1. Pluripotent stem cells:

2.1.1.1. Embryonic stem cells

In mice, ES cell research dates back to the early 1970's when teratocarcinoma (stem cell of germline tumor) was first generated (Stevens 1966) and showed that they could produce teratomas after transplantation (Stevens 1970). ES cell lines were initially developed in mice in 1981 (Evans et Kaufman 1981). These freshly generated mESCs were kept in a feeder based on mouse embryonic

fibroblast cells (MEFs) because it was reasoned that fibroblasts provide some critical factors either to promote self-renewal or to suppress differentiation. The pluripotency of these cells was demonstrated *in vivo* by the induction of ES cells into blastocysts in mice. The resulting mouse chimeras showed that ES cells could contribute to all three cell lineages including the germ line (Bradley et al. 1984). Once generated, these cell lines showed a stable karyotype even after several passaging and a short generation time (between 12 and 15 hours) characterized by a short G1 cell cycle phase (Smith 2001; Rohwedel et al. 1996). Among factors needed for pluripotency preservation, two different teams independently showed the involvement of the leukemia inhibitory factor (LIF) (Williams et al. 1988; Smith et al. 1988, 19). LIF is a soluble glycoprotein of the IL-6 family of cytokines and its activity is based on the bounding of the gp130 signaling complex and the regulation of the STAT pathway (Burdon et al. 1999).

In 1995 Thomson et al published the first ES cell line isolated from non-human primates, and in 1998 from humans (Thomson et al. 1995; 1998). That was the first time where scientists were able to derive human embryonic stem cells from an embryo and maintain the pluripotent state. For the generation of hESC, researchers used 36 embryos from patients who underwent *in vitro* fertilization (IVF) and donated their embryos. From these embryos, 14 inner cell masses were isolated and five hESC cell lines were derived. Generated cell lines showed important similarities with murine lines, such as Oct-3/4 expression, telomerase activity, and the formation of teratomas (Richards et al. 2002; Thomson et al. 1998).

Nevertheless, it is important to underline that some important differences exist between mouse and human ES cells. Compared to mESC, hESC show a longer average population doubling time (between 30 and 35h vs. 12-15 h in mESC) (Amit et al. 2000), and contrary to mESC, in hESC, LIF is insufficient to inhibit their differentiation (Dahéron et al. 2004; Reubinoff et al. 2000; Thomson et al. 1998).

As for mESC derivation, the first methods described for the derivation of hESCs involved the use of mouse feeder cell layers (MEFs) and a medium containing fetal bovine serum (FBS), two factors that may contain animal pathogens and immunogens rendering these cells inappropriate for potential clinical use. For that reason, in the following years, the FBS was replaced with Knock-Out serum which contains several vitamins, amino acids, antioxidants, trace elements, and proteins including lipid-rich albumin which plays an important role in hPSC (Garcia-Gonzalo et Izpisúa Belmonte 2008), aiming to tend through a feeder-free culture. The first feeder-free hESC was published in 2005 (Klimanskaya et al. 2005), where scientists used high concentrations of basic fibroblast growth factor (bFGF) for the hESC growth. In 2007, Ludwig and colleagues showed the possibility to use defined culture media, named TeSR1 for hESC culturing (T. Ludwig et A Thomson 2007). In the following years, the use of different coatings products was reported, based on a complex mixture of proteins at the beginning and more defined and xeno-free later on (laminin, fibronectin, vitronectin, etc) (Klimanskaya et al. 2005; T. E. Ludwig et al. 2006; C. S. Hughes, Postovit, et Lajoie 2010; Braam et al. 2008; Rodin et al. 2010).

The refinement of cell culture conditions over the years has enabled the use of hESC for clinical purposes. Ongoing clinical trials that use hESC are described in the chapter “iPSC/hESC-based therapies”.

2.1.1.2. Induced pluripotent stem cells (iPSC)

Until very recently, the dogma in the field of biology was the fact that the cells which have reached a given differentiated stage, could not return to a more primitive stage (Raff M., 2003) and even less

probably to an embryonic stage. This concept has been reversed by the revolutionary experiments performed by Shinya Yamanaka, who took advantage of the transcriptome analyses of the ESC in which several pluripotency genes had been identified. Using a combination of 24 pluripotency genes, Takahashi and Yamanaka showed in 2006 that, overexpressing 4 factors of pluripotency Oct4, Sox2, Klf4, and cMYC (known as OKSM) were sufficient to “reprogram” a differentiated adult cell into a pluripotent stem cell identical to ESC in mouse (Takahashi et al. 2006) and human cells (Takahashi et al. 2007). Later on, other teams have shown the reprogramming of other murine and human somatic cells such as fibroblasts (Lowry et al. 2008), blood cells (Haase et al. 2009), neurons (H. W. Choi et al. 2011), and keratinocytes (Aasen et al. 2008).

Reprogramming consists mainly on three steps: initiation, maturation, and stabilization. It implies phenotypic, metabolic, genomic, as well as major epigenetic changes in the somatic cell (Hawkins, Joy, et McKay 2014).

The initiation step is responsible for the methylation of somatic genes and the expression of ectopic transgenes introduced via different methods (detailed below). It follows by an increase in cell proliferation, telomerase activity, epithelial-mesenchymal transition (EMT), and an epigenetic change (Hawkins, Joy, et McKay 2014).

The maturation step in humans is characterized by Lin28 (Tanabe et al. 2013) and by the expression of endogenous genes such as Fbxo15, Sall4, Esrrb, Oct4, and Nanog.

Stabilization is the last step of cell reprogramming and is characterized by very few numbers of cells able to reach this step (only 1%). It consists of the suppression of transgenes through DNA inhibitors and histone methyltransferases.

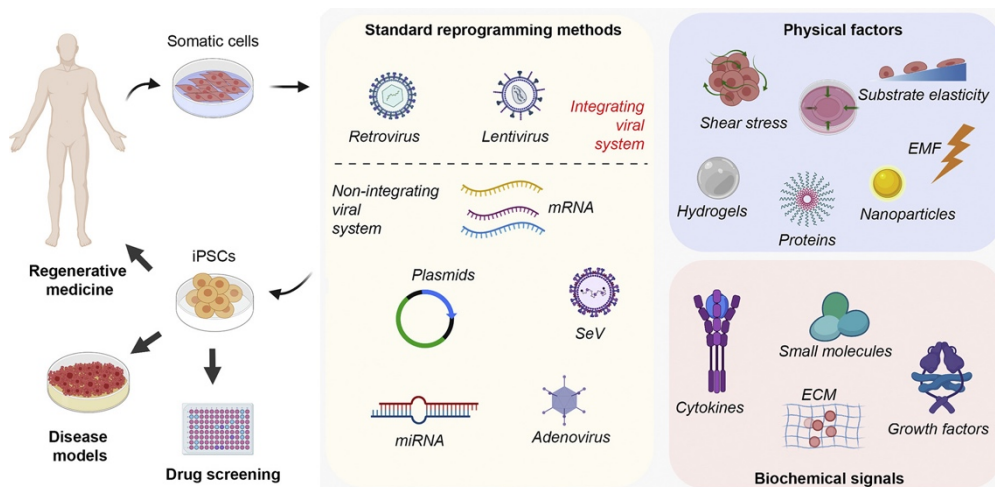


Figure 21: Schematic representation of different techniques of cell reprogramming (Romanazzo et al., 2020)

Different techniques are used for cell reprogramming and they are more or less efficient according to the species or cell types. Generally, it is possible to classify reprogramming techniques into two main groups: integrative and nonintegrative (**Figure 21**). Viruses have been the main vehicle for carrying transgenes, among them integrative lentiviruses. The integration of transgenes in the genome and the number of copies introduced are determinants for the tumorigenic potential of iPSC. Therefore, other techniques have been developed based on non-integrative transgene expressions such as adenoviruses (Stadtfield et al. 2008) or Sendai Virus (SeV) (Fusaki et al. 2009). Currently, SeV is the most commonly used technique for cell reprogramming, it consists of a murine virus coupled with a human

Parainfluenza virus. SeV is an enveloped, single-stranded, negative-sense RNA virus whose replication occurs in the cytoplasm (**Figure 22**). Since it is not integrated, its expression decreases when cells proliferate. SeV is considered as the gold standard method for cell reprogramming. In all cases, externally overexpressed pluripotency genes do not persist in successfully reprogrammed cells and this is the endogenous pluripotency gene expression that maintains the pluripotency phenotype in the target cell

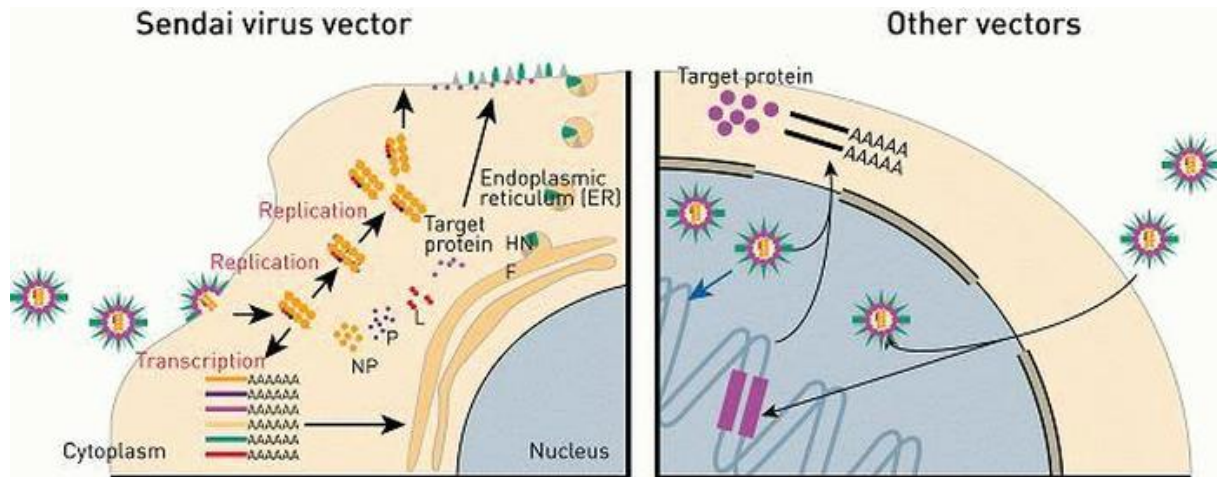


Figure 22: Schematic representation of cell reprogramming with Sendai vector (non-integrative) versus integrative techniques (adapted from ThermoFisher Scientific)

Other techniques for cell reprogramming (**Figure 21**) consist of the use of the Epstein-Barr virus (J. Yu et al. 2009), synthetic RNA (Warren et al. 2010; Yakubov et al. 2010), episomal vectors (J. Yu et al. 2011), protein delivery (H. Zhou et al. 2009), RNA delivery and chemical molecules (Hou et al. 2013; Ye et al. 2016).

Although chemical reprogramming is very challenging since human somatic cells are refractory to chemical stimulation owing to their stable epigenome (Barrero, Boué, et Izpisua Belmonte 2010; Nicetto et Zaret 2019), Guan and colleagues showed recently that chemical reprogramming can be reached by creating of an intermediate plastic state (Guan et al. 2022). Moreover, they have identified the JNK pathway as a major barrier to chemical reprogramming and reported that its inhibition was indispensable for inducing cell plasticity and reprogramming. The epigenetic status of the cells is also of major importance as several types of molecular abnormalities have been identified as “barriers” to reprogramming such as TET2 gene mutations (Secardin et al. 2020) as well as TP53 gene expression (Brosh et al. 2013). The inhibition of TP53 has been shown to facilitate reprogramming (Brosh et al. 2013).

Pluripotent cells exhibit a major proliferative potential due to a G1 phase shorter than in somatic cells and grow in colonies (Becker et al. 2006). They can be cultured either in feeder conditions (inactivated Mouse Embryonic Fibroblasts – iMEF) or in feeder-free conditions (Laminin, Vitronectin, Matrigel, etc). They express at their surface specific markers related directly to their pluripotent state such as TRA-1-60, TRA-1-80, SSEA-3, SSEA-4, and these markers together with their intracellular counterparts (Nanog, Sox2) are used to define the pluripotent state of a cell. They can be identified either by flow cytometry or by RT-PCR.

As mentioned above, the main characteristic of a pluripotent stem cell is its ability to differentiate into all three germ layers (endoderm, mesoderm, and ectoderm). This is validated through the

xenograft of pluripotent cells in immunodeficient mice and the development of teratomas (Dressel 2011). Tissues generated from the teratoma are in general normal tissues nevertheless, in some cases, teratocarcinoma development has been identified. For that reason, teratoma assay can also identify pluripotent stem cell lines with a lower intrinsic tumorigenic risk compared to others and remains the gold standard technique for pluripotency validation. Although teratoma has been widely used for pluripotency characterization, some issues such as standardization or animal welfare remain to be solved. Thereby, new techniques based on defined media such as STEMdiff™ Trilineage Differentiation Kit from STEMCELL Technologies have been recently developed.

One of the major requirements before the validation of a newly reprogrammed cell is the karyotype. The tumorigenic potential of iPSC mentioned above is directly related to their genomic instability (Catalina et al. 2007). Moreover, Ben-David and colleagues showed in 2014 that aneuploidy can induce profound changes in gene expression, proliferation, and tumorigenicity in human pluripotent stem cells, and in disease models. Altered cells do not always reproduce the same characteristics as the original cells that give rise to them (Ben-David 2015). The genomic integrity of iPSC is mainly evaluated by karyotyping and many karyotypic abnormalities have been repeatedly reported in human iPSCs, such as trisomy 12 (Sm et al. 2011; Ben-David 2015; Lamm et Kerem 2016) and trisomy 17 (Ben-David et Benvenisty 2011; Sm et al. 2011).

2.1.1.3. Use of iPSC for disease modelling and therapy

2.1.1.3.1. iPSC-Based Disease Modelling

The emergence of cell reprogramming allowed the use of disease-specific iPSC in directed differentiation protocols, avoiding many limitations of disease modelling with ESCs. Until then, ESC-based disease modelling consisted mainly in introducing mutations via gene editing or overexpressing a given gene (such as an oncogene) in ESCs. Cells differentiated from patient-derived iPSC reflect the disease's intrinsic specificities and are robust and reproducible. For instance, motor neurons differentiated from iPSC derived from patients with spinal muscular atrophy, have shown a decreased neuronal connectivity (Ebert et al. 2009) or reduced colony formation observed in hematopoietic cells generated from iPSC derived from patients with Fanconi anemia (Tulpule et al. 2010).

The introduction of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) technology has facilitated the generation of isogenic iPSC lines (correction or insertion of mutations) that differ only at the genome-edited loci from the original cell (Seeger, Porteus, et Wu 2017; Hadoux et al. 2018). These isogenic controls allow studying the disease phenotype without influences from the genetic background.

“Personalized medicine” consists of an approach where each patient would receive a tailored treatment and it is becoming more and more attractive in nowadays clinical approaches. Its main aim is to avoid possible adverse side effects and to minimize the frequency of non-responders. Side effects are a real health issue for public authorities because millions of people are hospitalized annually due to adverse side effects of their medications leading to thousands of deaths per year, this highlights the need for precision medicine (van den Berg et al. 2019). For this purpose, models from patient-derived iPSC are a very interesting tool thanks to their accessibility, scalability, and mutations related to the disease.

Despite the advances in understanding diseases, 2D iPSC models remain very limiting because they do not take into account the tissue complexity where cancer occurs, the cancer niche is capital for

reproducing the real conditions of cancer. For that reason, more complex models based on 3D (organoids) are being developed currently to faithfully recapitulate human tissue and organ dysfunction (Developed in the paragraph “Current protocols for hematopoietic differentiation”).

2.1.1.3.2. iPSC/hESCs-based therapies

From a therapeutic point of view, pluripotent stem cells have been studied for many years to develop new therapeutic tools for incurable diseases. The use of hESC for this purpose implies two concerns: ethical issues regarding the use of human embryos and immune rejection after transplantation (Yamanaka 2020). The derivation of iPSC paved the way for new tools circumventing these issues and iPSC-based clinical trials have already started in several countries (Figure 23)

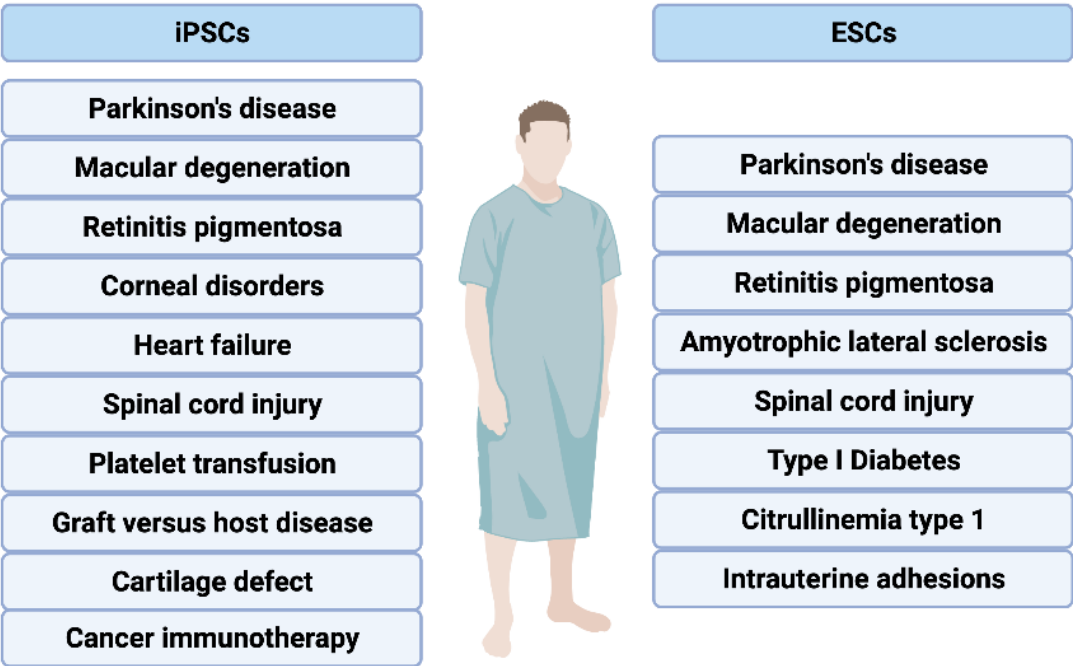


Figure 23: Clinical Trials for cell therapies using PSCs (Adapted from Yamanaka, 2020)

Although iPSC represents a huge potential for therapy, several challenges remain to be addressed before making these cells fully applicable for therapy. The three major issues among these challenges are their tumorigenicity, immunogenicity, and heterogeneity (Yamanaka 2020).

Tumorigenicity: As described above, iPSCs in undifferentiated stages generate benign tumors in immunodeficient mice detected as teratomas in which differentiated cells belonging to three embryonic lineages can be identified. In some situations, especially if an oncogenic mutation occurred in the iPSC, a carcinoma can be detected by pathological analysis. Another reason for iPSC tumorigenicity is related to reprogramming factors. Reprogramming factors have been associated with tumorigenicity and c-Myc is one of the most frequently mutated genes in human cancers. Other factors might also be involved such as a dominant negative mutant of p53 used to increase the reprogramming efficiency (Hong et al. 2009).

The third reason for iPSC tumorigenicity are the genetic abnormalities. As mentioned before, iPSC should be checked regularly for genetic abnormalities, and not only just after reprogramming. Cell culture *in vitro* inevitably causes genetic alterations, such as chromosomal abnormality, copy number variation, and single nucleotide mutations.

The other aspect of the tumorigenicity is related to that of the iPSC-derived differentiated cells. This can be due to different mechanisms among which the most common ones are incorrect patterning and incomplete reprogramming. Indeed, residual PSCs can result in teratoma formation or existing lineage-specific stem cells within the transplant may also form tumors. This highlights the need for a highly purified cell population before the validation of these iPSC-derived cell products for clinical applications and highlights the major interest in quality control procedures developed (Sullivan et al. 2018).

Immunogenicity: Immune rejection of iPSC is also another problem to be taken into consideration when developing an iPSC-based therapy. Rejection is due to a T cell response provoked by iPSC in the host organism as it has been observed in mice when B6-derived mice iPSC failed to form teratoma in syngeneic host mice where a T cell infiltration was observed (T. Zhao et al. 2011). The use of immunosuppressors is not a lasting solution because it would imply life-long immunosuppression and as consequence severe side effects. Another solution for this problem has been autologous transplantation, nevertheless, for acute diseases such as heart failure or spinal cord injury, the extended time needed for the production of clinical-grade autologous cell products would not be compatible with the urgent therapy that needs to be provided to these patients. Another approach to circumvent iPSC immunogenicity is the generation of HLA haplotype banks of iPSCs which is widely used in hematopoietic stem cell transplantation. However, preparing iPSC lines with thousands of unique HLA haplotypes is technically complicated, therefore Taylor and colleagues proposed in 2012 (Cj et al. 2012) that 10 hiPSCs lines homozygous for HLA types selected from 10 000 donors provide a complete HLA-A, HLA-B, and HLA-DR match for 37.7% of recipients and a beneficial match for 64.7% in the UK population. Besides these results, other teams have shown that although HLA matching is an interesting approach, immunosuppressors will still be required (Kawamura et al. 2016).

Another approach for circumventing iPSC immunogenicity is the HLA cloaking approach which is also known as the “missing self-recognition” and consists of deleting (by CRISPR technology) all class I MHC (HLA-A, HLA-B, and HLA-C) by deleting their common component, the beta 2-microglobulin. However, this approach can make cells vulnerable to NK attack as these lymphocytes are specialized to eliminate cells with missing self. Indeed, Gornalusse et al showed in 2017, that introducing HLA-E (non-classical MHC class I molecule with limited polymorphisms) in B2M deleted iPSC can circumvent the NK activation since HLA-E can activate NKG2A and thus inhibit NK cell activation (Gornalusse et al. 2017).

Heterogeneity represents a typical characteristic of pluripotent stem cell lines. Although all these cell lines share pluripotency and theoretically unlimited proliferation as common features, they differ in their growth rate, gene expression, and their capacity to differentiate into different cell lineages. It is currently difficult to identify beforehand, which cell line will be more prone to exhibit efficiency to generate blood, pancreas, or neural cells. The heterogeneity of PSC to give rise to hematopoiesis is related to the expression of the mir-206 which regulates negatively the hematopoietic potential (Flamant et al. 2019).

One of the major reasons for this heterogeneity is directly related to the genetic background of PSC (J. Choi et al. 2015). To overcome this problem, one solution might be the “priming” of PSC. Human iPSCs mimic post-implantation epiblast both in gene expression and epigenetic status, and this is known as “primed status”. Mouse iPSC, mimic the inner mass of blastocyst or pre-implantation epiblast and this is known as “naïve status”. According to the status of the PSCs, their differentiation potential

is not the same, primed PSCs are characterized by a more limited differentiation potential compared to naïve PSCs (Brons et al. 2007; Pj et al. 2007).

Currently, the aim is to convert primed PSC to naïve status and many teams have proposed different approaches to reach this goal. One of the most promising strategies has been developed by Theunissen et al who achieved the conversion of primed human PSC to a naïve state using inhibitors of ROCK, BRAF, and SRC in the presence of hLIF (Theunissen et al. 2014).

Even though primed state induction of PSC might be an interesting approach for solving the PSC heterogeneity, some concerns such as the increased chromosomal abnormalities and epigenetic regulations should be addressed before generalizing this approach for clinical applications.

2.2. Developmental hematopoiesis

Our current understanding of fetal hematopoiesis is based mainly on data from small animal models because of ethical concerns related to the use of human fetal tissue. It implies sequential events occurring in multiple and partially overlapping but spatiotemporally separated waves (Demirci, Leonard, et Tisdale 2020). Currently, three hematopoietic waves in embryonic development have been described, the primitive wave, the pro-definitive wave, and the definitive wave.

2.2.1. The primitive hematopoietic wave

The primitive wave is derived from the mesoderm and occurs in the yolk sac from the blood islands. In humans, it occurs in the second week of gestation and it is characterized by a lack of lymphoid potential but it is rich in transitory primitive blood cells such as erythroblasts, megakaryoblasts, and macrophages. This is related to its physiological role consisting of the first irrigation and immune defense of the embryo (Palis et al. 2010). Therefore, it consists of a fast differentiation, i.e., macrophages arise from their progenitors without monocyte intermediate (Tavian et al. 1999). Myeloid progenitor cells generated in the yolk sac are also the origin of microglial cells in the brain, Langerhans cells, and Kupfer cells in the liver (Blériot et al. 2020). This is related to the fact that hematopoietic primitive cells are derived from the same progenitor, the hemangioblast (Kyunghae Choi 2002). Its existence in adults is still controversial but some teams have described its involvement in the generation of hematopoietic and endothelial cells but also smooth muscles in adult mice (Huber et al. 2004).

The concept of hemangioblast emerged at the beginning of the past century from the observations of Sabin, His, and Murray in chick embryonic explants, and the generation of hematopoietic cells from endothelial cells. This emerged the theory of a common progenitor between these two lineages. This hypothesis has been validated in *ex-vivo* explants of the yolk sac (Moore et Metcalf 1970), from FLK1+ cells from chick embryos (Eichmann et al. 1993), and *in vitro* from murine ESC models (K. Choi 1998; Nishikawa et al. 1998). All these data have shown the ability of a mesodermal cell to produce either endothelial or hematopoietic cells.

In humans, this has been validated in 2007, *in vitro* by the teams of Kennedy and Lu (Kennedy et al. 2007; Lu et al. 2007).

The potential of hemangioblast has been shown also *in vivo* in mouse embryonic models (Huber et al. 2004), in zebrafish (Vogeli et al. 2006), in drosophila (Mandal, Banerjee, et Hartenstein 2004), and in humans (Oberlin et al. 2002).

2.2.2. The pro-definitive hematopoietic wave

The pro-definitive wave occurs in the yolk sac vessels from a subset of endothelial cells named the hemogenic endothelium (HE). In the HE, a change in the cellular identity provokes the conversion of endothelial cells into hematopoietic cells and this process is known as the “Endothelial-to-hematopoietic transition (EHT)”. This transition is different from the one that occurs in definitive hematopoiesis (described above) because hematopoietic cells generated during the pro-definitive wave can reconstitute hematopoiesis only transiently after transplantation contrary to hematopoietic cells from the definitive wave. The pro-definitive hematopoietic wave has been described in mouse (Hoeffel et al. 2015; Frame, McGrath, et al. 2013) at E8.25 and produce *bona fide* erythromyeloid progenitors with a dual erythroid and myeloid fate. These cells colonize thereafter the liver and infiltrate other organs. Ivanovs and colleagues have suggested that the pro-definitive hematopoietic wave should also exist in humans (Ivanovs et al. 2017).

2.2.3. The definitive hematopoietic wave

Definitive hematopoiesis originates from hemogenic endothelial cells in aorta-gonad-mesonephros (AGM) between the fourth and the fifth post-conception week in humans (Tavian et al. 1996; Oberlin et al. 2010). At this level, there is a precise transcriptional regulation and an upregulation of transcription factors such as *RUNX1*, *Sox17*, *Scl*, *Gfi1/Gfi1b*, and *Gata2* which is shown to induce EHT and the generation of HSC with robust long-term multilineage reconstitution potential (Ivanovs et al. 2017; 2011; North et al. 1999). In addition to that, HE adapts a specific phenotype characterized by specific markers such as CD31, CD309, CD34, and VE-cadherin (Labastie et al. 1998; Marshall et al. 1999; Oberlin et al. 2002; Tavian et al. 2001). Altogether, this step is accompanied by the action of regulator pathways such as Notch (Richard et al. 2013), c-KIT (Marcelo et al. 2013), and retinoic acid pathway (C. Yu et al. 2010) which will give rise to a mature HSC able to extravasate from the vascular wall into the circulation and to travel to the fetal liver. These HSCs that colonize the fetal liver, contribute to blood cell production making thus the liver become the main hematopoietic organ in the sixth and seventh post-conception week in human. The liver keeps this status until around birth when HSCs seed the bone marrow.

2.3. HSC generation from PSC

Hematopoietic differentiation from iPSC enables to recapitulate a part of the embryonic hematopoiesis *in utero*. Nevertheless, as mentioned above, generating *bona fide* HSC with an engraftment potential remains still a challenge. Most current protocols for HSC generation from iPSC only recapitulate primitive first wave similar to yolk sac hematopoiesis (Zambidis et al. 2005; Kaufman et al. 2001). Over the last several years, efforts are done to develop protocols for HSC generation, mostly using fetal stromal cells such as OP-9 as feeder cells for PSCs and by adjusting the concentrations and the type of growth factors. The use of OP-9 cells enables the generation of VE-cadherin+/KDR+ which differentiate later into endothelial cells with tight junctions (Claudin 5+) and finally into hematopoietic cells CD45+ CD41+ (Vodyanik et al. 2005). These protocols have enabled the generation of HSC with multipotent differentiation potential but without engraftment potential. T lymphocyte potential has been shown to track the onset of definitive hematopoiesis in human pluripotent cultures (Kennedy et al. 2012, 201; Ditadi et al. 2015).

Another important factor to be taken into account for the generation of HSCs with an engraftment potential is the microenvironment. Different physical factors have been described above but the

complexity of the ETH and of the bone marrow remains still the key to a successful HSC generation from PSC.

2.3.1. Current protocols for *in vitro* hematopoietic differentiation

During the last two decades, several protocols for *in vitro* hematopoietic differentiation have been established. Overall, they require the induction of mesoderm followed by the generation of cells belonging to different hematopoietic lineages under the influence of growth factors used. **Figure 25** shows the methods most commonly used for erythromyeloid hematopoietic differentiation from iPSC.

Generally, hematopoietic differentiation techniques from ESC/iPSC can be grouped into two different groups:

- **2D differentiation techniques** – which is the easiest and the shortest but does not necessarily represent the microenvironment and thus some crucial pathways such as Notch (Vargas-Valderrama et Mitjavila-Garcia 2019) or Wnt/ β -catenin (Mora-Roldan et al. 2021). This leads to a lesser number of CD34+ cells, and a lower CFC capacity potential compared to 3D techniques (Mora-Roldan et al. 2021). Nevertheless, 2D techniques remain more reproducible, cheaper, and easier to use (Tursky et al. 2020) thanks to their standardization done by companies such as STEMCELL Technologies through commercial kits (STEMdiff™ Hematopoietic Kit) (**Figure 24**). During my PhD, this standardized kit has been used for hematopoietic differentiation.

As described above, the principle of PSC into hematopoietic differentiation remains the same for 2D or 3D techniques namely by inducing mesoderm during the first steps and thereafter directing mesodermal cells into hematopoiesis.

For the 2D technique, PSC are plated at a defined density on a coated plate and the mesodermal induction is done through BMP-4, but also factors such as VEGF-A and b-FGF priming the cells to hematopoiesis (Medium A for the STEMDiff Kit). The second step consists properly of hematopoietic differentiation with factors such as b-FGF, BMP-4, VEGF-A, SCF, Flt3-L, and TPO (Medium B for the STEMDiff Kit). Hematopoietic cells (CD34+/CD45+) appear by day 12 of differentiation.

The lack of representativeness of the niche in 2D cultures is responsible for the generation of cells that lose very fast their self-renewing potential in favor of differentiation (Walasek, van Os, et de Haan 2012).

- **3D differentiation techniques** – To overcome the limitations of 2D cell culture, new approaches have been developed in 3D including also sophisticated biomaterials or bioreactors to mimic HSCs more closely (Rödling et al. 2017). 3D techniques aim to artificially reconstitute the hematopoietic niche even though its complexity makes that a very difficult task. Nevertheless, current techniques can take into account the paracrine function, the cellular heterogeneity to a certain extent, the mechanical environment, and also the hypoxia (Lee-Thedieck et Spatz 2014; Leisten et al. 2012; A et al. 2014). hPSCs spontaneously differentiate into embryoid bodies (EBs). EBs can be studied at this level or further differentiated into hematopoiesis. There are different methods for acquiring EBs, such as bioreactor cultures, hanging drop culture, or microwell technology (Höpfl, Gassmann, et Desbaillets 2004; Lim et al. 2013; Mohr, de Pablo, et Palecek 2006; Doetschman et al. 1985). In mice, the removal of mLIF induces systematic differentiation

of mPSC (Dang et al. 2002). Generated EBs could be differentiated in feeder-free conditions by adding hematopoietic factors (Cerdan, Hong, et Bhatia 2007) which increases the percentage of CD45+ cells as compared to cytokine-deprived conditions (feeder-free conditions). EBs can also be added to different mouse stromal cells (MS-5, OP-9, etc) that mimic the niche and this stroma can sometimes be modified to express specific transcription factors such as HOXB4, DLL1, DLL4, etc (Larbi et al. 2012; Nakano, Kodama, et Honjo 1994; T 1996; Mohtashami et al. 2010). OP-9 cells have been established from mouse bone marrow stromal cells (mesenchyme) and like MS-5, show hematopoietic supportive capacity (Gao et al. 2010). When used in co-culture with ESC/iPSC, these stromal cells can induce the differentiation of ESC/iPSC into blood cells (erythroid, myeloid, lymphoid lineages) (Nakano, Kodama, et Honjo 1994; T 1996; A. Suzuki et Nakano 2001). Lei et al. in 2009 used OP-9-DLL1 cells to generate T lymphopoieses (Lei et al. 2009). Delta-like proteins (DLL) 1 or 4 are shown to induce the Notch signaling pathway which supports lymphopoiesis (Mohtashami et al. 2010).

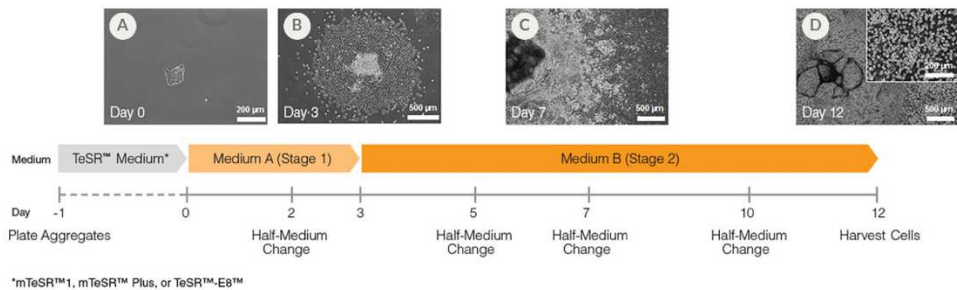


Figure 24: 2D differentiation protocol with STEMdiff™ from STEMCELL Technologies (STEMCELL Technologies Protocols).

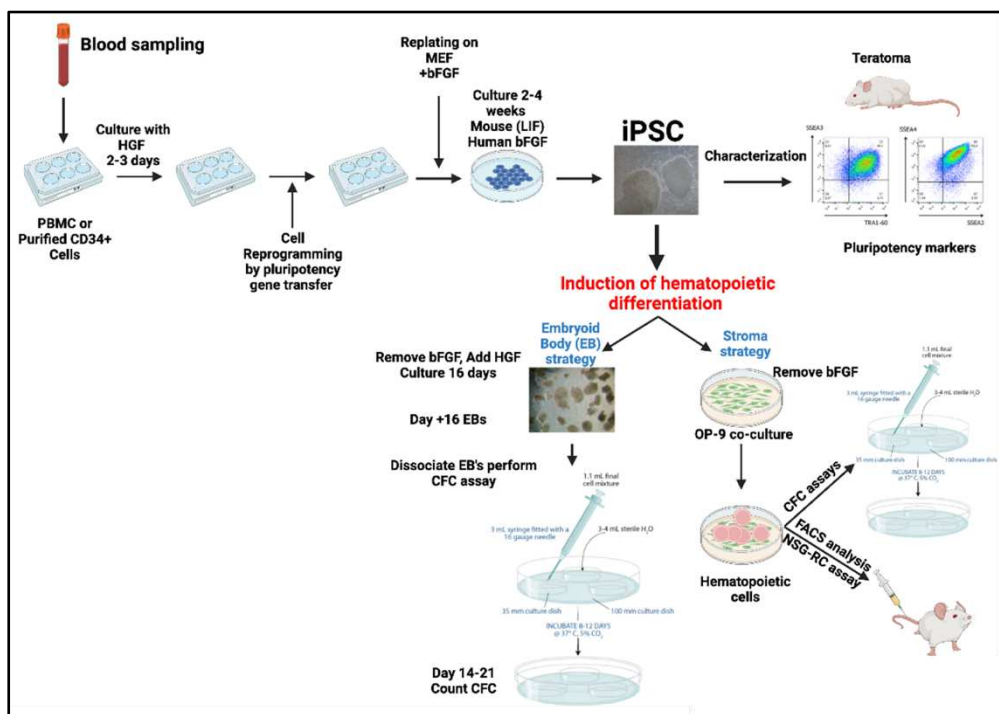


Figure 25: Schematic representation of iPSC generation through two different techniques of hematopoietic differentiation (feeder-free and OP-9 stroma based). Adapted from Turhan et al., 2020 – Williams Hematology Textbook.

3. Chimeric Antigen Receptor (CAR)-based therapies

3.1. General overview of CAR

The main role of the immune system is the defense against foreign organisms, with the destruction of damaged cells as well as permanent immunosurveillance against bacteria, viruses, and cancer (Bauer-Wu, S.M. 2005; Polovich, M., J.M. White, and L.O. Kelleher, 2005). In normal conditions, potentially malignant cells are eliminated by the immune system, but these cells can accumulate certain mutations, which make them undetectable to the immune system and this process is known as the “Immune escape” (Gonzalez, Hagerling, et Werb 2018). To overcome this problem, cancer immunotherapies aim to boost the patient’s immune system or to use the specific cytotoxic properties of immune cells by enabling them to destroy cancer cells. We will focus more on the potential strategies to enhance the cytotoxic properties of immune cells allowing them at the same time to target cancer cells expressing specific antigenic targets.

These strategies consist of the genetic modification of immune cells, mainly T and NK lymphocytes (but also macrophages), to make them express at their surface an artificial receptor designed under the term of chimeric antigen receptor (CAR). This CAR confers to the lymphocyte the ability to recognize specifically a cancer marker. This recognition activates the lymphocyte in an MHC-independent manner and induces specific cytotoxicity against the cancer cell (Sadelain, Rivière, et Riddell 2017).

Designed for the first time in the late 1980s by pioneers such as Gideon Gross and Zelig Eshhar (Gross, Waks, et Eshhar 1989), CAR therapies have now become a major therapeutic strategy in hematologic malignancies and more recently in solid tumors (June et Sadelain 2018).

CAR consists of an extracellular binding domain mostly derived from a monoclonal antibody fragment (single-chain variable fragment scFV), linked to intracellular binding domains of the T-cell receptor in the case of CAR-T. The other domains are the hinge domain, the transmembrane domain, and the costimulatory domains.

scFV – is a critical part of the CAR for its specificity. The general format of an scFV is two variable domains linked by a flexible peptide sequence (linker) either in the orientation VH-VL or VL-VH (**Figure 26**). This might affect the 3D structure of the scFV and consequently its affinity. The linker is usually a peptide of 6xGGGGS but the different lengths of the linker can be designed taking into account the fact that the linker can generate excessive signaling designed as tonic activity i.e., an antigen-independent activation (Singh et al. 2021).

scFV is generated from antibody clones and they recognize specifically an epitope on the antigen. Different CARs might exist for the same marker, they can target different epitopes and can have different affinities. An important factor to be taken into account is the glycosylation status of the proteins because it can change radically the recognition abilities of the scFV.

Hinge (spacer) – is the extracellular region of the CAR that separates the binding units from the transmembrane unit. They supply stability and flexibility to the scFV and its length might be critical for the access of the CAR to the epitope. Short hinges are adapted for membrane distal epitopes (Hudecek et al. 2013) whereas long hinges provide extra flexibility to the CAR and are adapted for membrane-proximal epitopes (Rd et al. 2005).

Hinge domains are designed with Ig-like domains CH2 and CH3, but this has been shown to induce non-specific activation because of the affinity of the CH2 domain to bind the Fc gamma receptor (Hombach, Hombach, et Abken 2010). Other sequences for hinge domains can be generated from extracellular domains lacking Fc γ R binding activity such as CD28 or CD8 α , which are also used in our lab.

The transmembrane domain (TD) – binds the extracellular domain responsible for the specific recognition of tumor cells and the intracellular domain responsible for lymphocyte activation. TD is a hydrophobic α helix and is involved also in CARs dimerization which confers it a functional role in addition to its structural role. They are mainly generated from CD4, CD8 α , or CD28 (Kochenderfer et al. 2009).

Co-stimulatory domains – are the second most important part of the CAR construct after the scFV. They enable the activation of the lymphocyte and according to their nature, they can induce different levels of activation and thus different risks of cell exhaustion. “First-generation” CAR (detailed below) incorporate a cytoplasmic domain containing one or more immunotyrosine-based activation motifs (ITAM) into a single chimeric receptor that uses a single scFV (Sadelain, Brentjens, et Rivière 2013). Co-stimulatory domains are usually derived from costimulatory molecules from the CD28 family, tumor necrosis factor receptor (TNF-R) including 4-1BB, OX40, or CD27 (Guedan et al. 2019). CD28 and 4-1BB are the most widely used costimulatory domains in CAR strategies. CD28 is shown to induce a rapid activation, proliferation, and glycolysis of the lymphocyte compared to 4-1BB but the persistence of CAR-T containing CD28 co-stimulatory domains is no longer found after 3 months (Park et al. 2018). On the other hand, 4-1BB-based CAR-T cells can persist in patients for several years after treatment (Fraieta et al. 2018). 4-1BB signaling induces a mild T response and maintains a sustained T cell persistence (Guedan et al. 2019). Regarding these specificities of co-stimulatory domains, it appears that CD28 is more adapted for the treatment of cancers requiring rapid tumor elimination and short-term persistence of the CAR cells whereas 4-1BB is more adapted against diseases in which a complete response requires sustained T-cell persistence (Fraieta et al. 2018).

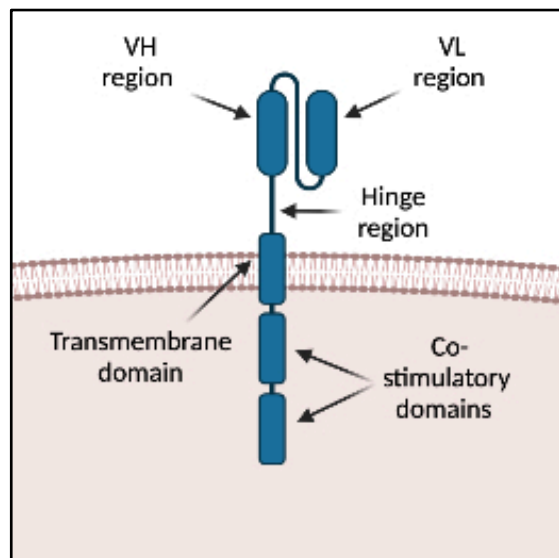


Figure 26: CAR structure with its extracellular domain containing VH, VL, and hinge region, the transmembrane domain, and the intracellular domain containing the co-stimulatory domains.

Similar to CAR-T cells, CAR-NK cells also harbor co-stimulatory domains which are important for their activation and proliferation. CD28 and 4-1BB has also been used for CAR-NK and are shown to induce NK activity (G. Xie et al. 2020). In addition to classical co-stimulatory domains, different signaling domains (CD3 ζ , DAP10 and DAP12) induce anti-tumor activity in NK cells and NK92 cell lineage (C. Imai, Iwamoto, et Campana 2005; Töpfer et al. 2015). In the case of tumors, there is a downregulation of HLA expression, exposing thus tumor cells to NK cytotoxicity. The absence of HLA class I expression in the case of virally infected or transfected tumor cells, dramatically decreases the threshold for NK cell activation (Kärre 1995). The activation of NK cells in an anti-tumor perspective, naturally or in CAR-NK context, implies different intracellular signals leading to cell activation. As mentioned before, most CAR-NK constructs were based on the intracellular proportion of the CD3 ζ signaling chain and conferred specific cytotoxicity to target cells. (T. Müller et al. 2008; J. Chu et al. 2014) In NK cells, the CD3 ζ transmits signals after binding the CD16 to IgG-opsonized targets which results in antibody-dependent cellular cytotoxicity (ADCC). However, NK92 cells are shown to lack CD16 and therefore ADCC (J. H. Gong, Maki, et Klingemann 1994). Alternative routes to NK activation are also the activation molecules DAP10 and DAP12. Despite their structural similarities, these two proteins have different signaling pathways. DAP12 activates the complex Syk/ZAP70 and DAP10 the phosphatidylinositol 3-kinase by the YxNM (Wu et al. 2000). In the immune system, DAP12 is found in cells of myeloid lineage (macrophages and granulocytes) and in the lymphoid lineage (NK cells). In NK cells DAP12 is associated with activating receptors such as NKG2C (L. L. Lanier et al. 1998), NKp44 (Campbell et al. 2004) and KIR3DS1 (Carr et al. 2007) . NKG2C is the dominant activating NK cell receptor for CMV infection in both human and mice (Gumá et al. 2004). For the CAR-NK construct used in this project, DAP12 has been used as activatory signal together with 4-1BB.

3.2. CAR vector transfer

As described above, CAR-based therapies generation consists of genetic modification by gene transferring of CAR sequence to a lymphocyte (T or NK). To achieve that, different techniques have been developed so far, among them; Viral, transposon, CRISPR/Cas9, electroporation, etc. In the case of clinical trials, viruses are the most used for their high gene transfection capacity, cost of production, and the number of cells able to transduce. Among these viruses, γ -retroviruses, Adeno-associated viruses (AAV), Adenoviruses (Ad), and Foamy Viruses (FV) are the most commonly used (Piscopo et al. 2018; Tumaini et al. 2013).

Recently, new techniques trying to avoid viruses begin to be used. Their aim is to reduce even more the cost of production, and the use of viruses for safety purposes. In that perspective, the use of Sleeping Beauty (SB) and PiggyBac transposon system has been demonstrated by Ramanayake et al. and Bishop et al (Ramanayake et al. 2015; Bishop et al. 2018).

Other techniques such as Zinc Finger (ZFNs) or nucleoside transcription activator-like effector nucleases (TALENs) were also used (Qasim et al. 2017).

During this PhD, only lentivectors have been used as gene transferring techniques for CAR generation.

Lentiviruses (LVs) are a member of the Retroviridae family. Lentiviruses can integrate a significant amount of viral complementary DNA into the genome of the host cell and can as well infect non-dividing cells (Cockrell et Kafri 2007). They contain the reverse transcriptase enzyme that converts RNA into DNA before the integration into the genome of the host cells (**Figure 27**).

LVs have a packaging capacity of up to 8 Kb, with the ability to confer long-term expression (Deng, Liang, et Cui 2022). However, there are some safety concerns related to the fact that they randomly integrate into the genome and can cause insertional mutagenesis by disrupting other genes, leading to severe adverse events (Priori et al. 2018). The availability of human genome sequence rendered possible the identification of preferentially LV integration sites which have been shown to be non-random and retrovirus-specific sites (Ciuffi 2008).

LVs production is based basely on the transfection of encapsidation cell lines (HEK-293) with plasmids containing the packaging system (Gag, Pol, Rev, and Tat genes) and the envelope protein (VSV-G) (2nd generation). Because of these safety concerns, 3rd generation lentiviruses have been designed by splitting the packaging system into two plasmids, one plasmid encoding Rev and the other one the Gag and Pol. Although safer, this system can be more cumbersome to use and result in lower viral titers due to the addition of one additional plasmid.

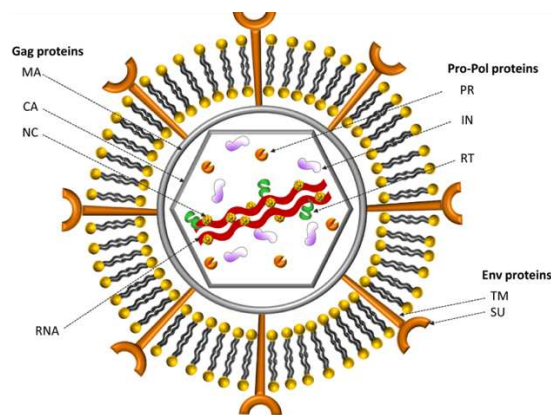


Figure 27: Structure of lentiviral viruses (Rodrigues, Alves, and Coroadinha et al., 2011).

3.3. Evolution of types of CARs

The design of CAR receptors has considerably changed over the years. Different molecular structures have been added to overcome challenges encountered during experimental results. The first generation of CARs contained only CD3 ζ and were unable to prime resting T cells and induce a sustained cytokine release (Brocker et Karjalainen 1995). For that reason, the costimulatory domain was added and it led to an improved activation and survival (2nd generation CAR-T cells) (Krause et al. 1998). More improved CAR-T cells appeared later on by combining the signaling potential of two costimulatory domains (both CD28 and 4-1BB) and this combination has been used for our project. The 4th generation CAR-T in addition to both co-stimulatory domains implies also further genetic modifications such as transgenes for cytokine release or costimulatory domains (Scarfò et Maus 2017) (Figure 28).

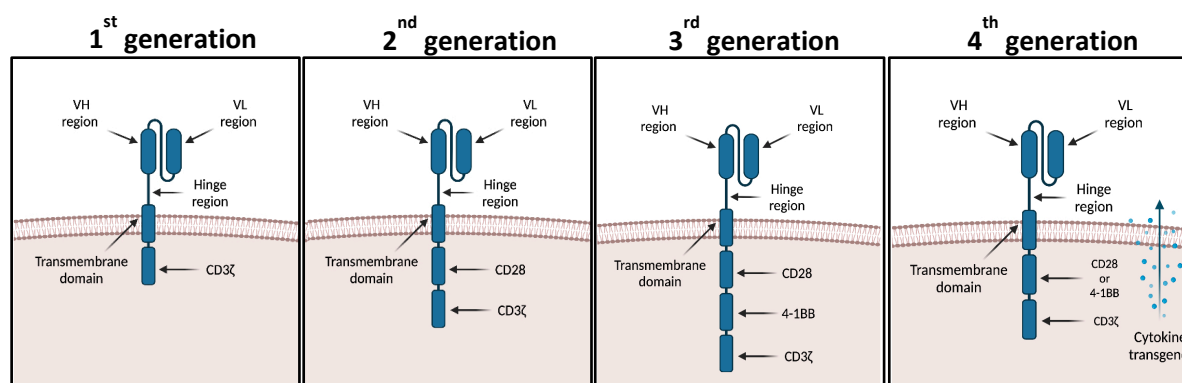


Figure 28: Evolution of CAR constructs – 1st generation CAR contained only one co-stimulatory domain and others were added in the following generations. The 4th generation contains also a cytokine transgene for maintaining cell activation

3.4. CAR-NK based therapies

As described above, NK cells represent 5-20% of human PBMC and play a crucial role in the innate immune system thanks to their rapidly expanding cytotoxic capacities. The role of NK cells is analogous to that of cytotoxic T cells in the adaptive immune response. They are characterized by the expression of CD56 but without the co-expression of CD3 and T cell receptors (TCR). In humans, NK cells are often classified according to the level of CD56 and CD16 expression. The CD56^{dim} CD16^{high} represents about 85-95% of PB-NK (the mature and cytotoxic fraction) whereas CD56^{bright}/CD16^{low} are the resting 5-15% and represent the immature NK cells with low cytotoxicity abilities (Caligiuri 2008). The specificity of NK cells compared to other immune cells is their ability to detect and kill stressed cells in the absence of antibodies and MHC, allowing thus a much faster immune reaction. Activation of NK cell cytotoxic activity is controlled by signals integrated from an array of germline-encoded activating and inhibitory signals (G. Xie et al. 2020) (**Figure 16**) (described in the paragraph “Immune effectors of CML” – NK cells and missing self).

In the same perspective as T cells, NK cells have also been used in adoptive CAR-based therapies by modifying them to confer specific recognition skills (E. Liu et al. 2020). Their unique biological characteristics make them an interesting tool for cancer immunotherapy.

3.4.1. Sources of NK cells for CAR-NK generation

NK cells have a particular interest in cell therapy compared to T cells because they represent a smaller risk for GvHD (detailed above). For that reason, different sources of NK cells have been used for clinical assays, including irradiated cell lines (NK92), UCB-NK cells, PBMCs, CD34+ hematopoietic cells, and more recently iPSC derived NK cells.

All of these sources have their advantages and disadvantages, e.g., the NK92 cell line, although the most used source of NK cells thanks to the illimited number of cells, they remain still a cell line with unpredictable behavior or phenotype and lack NKp44 and CD16 and so ADCC. They are systematically irradiated before being used *in vivo*.

PBMC-NK are a good source for NK cells and thanks to different techniques of isolation and expansion, this is an interesting source for further clinical applications. Moreover, the inability of NK cells to induce GvHD allows NK cells isolation from matched or mismatched HLA-donors (Miller et al. 2005), enlarging thus the choices for possible donors. Nevertheless, PBMC and UCB-NK have a major

common disadvantage which is the lack of homogeneity of the initial source inducing considerable issues of standardization.

iPSC-derived NK cells have revolutionized the way of the use of NK cells in clinics. The use of this technology implies many interesting assets, such as the illimited proliferation ability of iPSC (Valamehr et al. 2014), the ease of genetically engineered to stably express CAR, the clonogenicity, and consequently the homogeneity of the final production.

Clonogenicity is particularly important especially in gene therapy (such as CAR approaches) because it allows the identification of off-target genomic alterations via whole-genome sequencing. The use of iPSC-derived NK cells avoids also the requirement for cells to be collected from a donor and the cryo-conservation of iPSC-CAR is a real solution for future banking of these cells.

The first studies of NK differentiation from iPSC showed the feasibility of this concept and used different irradiated stromal cells to generate CD34+/CD45+ cells (Knorr et al. 2013). More recently, the group of Dan Kauffman showed the possibility of NK differentiation in feeder-free conditions and on a clinical scale (Zhu et Kaufman 2019).

Besides all these interesting features of iPSC-derived NK cells, these cells are characterized by an immature phenotype, with lower expression of markers such as KIR and CD16 and higher NKG2A compared to PBMC-NK cells (Knorr et al. 2013).

3.4.2. CAR-T vs. CAR-NK

As described previously, CAR-based therapies have been designed initially by using T cells. Although CAR-T cells have shown tremendous interest, especially in hematological malignancies (FDA-approved CAR-T cells in March 2022: Kymriah, Yescarta, Tecartus, Breyanzi, Abecma, Carvykti), some challenges remain about their use in clinic. One of the major problems observed has been severe neurotoxicity occurring in 25% of patients treated with anti-CD19-based CAR-T therapies (Park et al. 2018). Another issue related to CAR-T is their limited ability to migrate to and infiltrate the highly immunosuppressive tumor microenvironment. In addition to these problems, there is the fact that patients that have already received chemotherapies are not able to mobilize enough T cells needed for CAR-T manufacturing. To overcome these limitations, allogenic T cells approaches have been investigated by using healthy T cells nevertheless, these cells must be modified to avoid GvHD related to HLA class I and II, and invalidate the T cell receptor (TCR) (C. Graham et al. 2018). Finally, a common problem for all cell therapy and especially CAR-T cells is the high cost of manufacturing which can vary between 0.3-0.5 M\$ per patient making this therapy very expensive and highly inaccessible, especially in countries where it is not covered by social security.

According to their physiological features, NK cells have been seen as a solution for many problems related to CAR-T cells. The first interesting point is their independence from TCR for their anti-cancer effects, making them a real solution for allogeneic treatments. As mentioned previously, the other important advantage of NK cells is the fact that they do not need HLA matching and have therefore a safer therapeutic potential without the risk of GvHD in the allogeneic setting (Goldenson, Hor, et Kaufman 2022). In addition, NK cells have an intrinsic capacity to kill cancer cells (see chapter “NK cells in CML”) which confers them a double interest for their use in CAR-based therapies. **Table 4** summarizes and compares the characteristics of autologous and allogeneic CAR-T cells and CAR-NK cells.

Table 4: Comparison of characteristics between CAR-NK cells to autologous and allogeneic CAR-T therapies (Adapted from Gurney et al., 2021).

	Autologous CAR-T	Allogeneic CAR-T	CAR-NK
Efficacy	Established	Investigational	Investigational
Allogeneic sources	NA	Yes	Yes
Mechanism of Activation	CAR	CAR	CAR and Innate
CRS/ICANS	Established	Anticipated	Likely reduced
Cost of Product	0.37-0.47 M\$	Likely Reduced	Likely reduced
Cost of Care	Variable	Likely Equivalent	Potentially Reduced
Cryopreservation	Established	Established	Investigational
Viral Gene Delivery	Established, feasible	Established, feasible	Lower Efficiency
Non-Viral (Stable) Gene Delivery	Clinical trials		Described in iPSCs (Y. Li et al. 2018)

3.4.3. CAR-NK in cancer immunotherapy

CAR-NK remain still a freshly invented therapy needing thus a further comprehension of some phenomena and improving their activity. Nevertheless, nowadays they are a real tool against metastatic malignancies and the number of clinical trials is increasing every year (L. Zhang et al. 2022). **Figure 29** shows ongoing clinical trials of CAR-NK around the world in 2022.

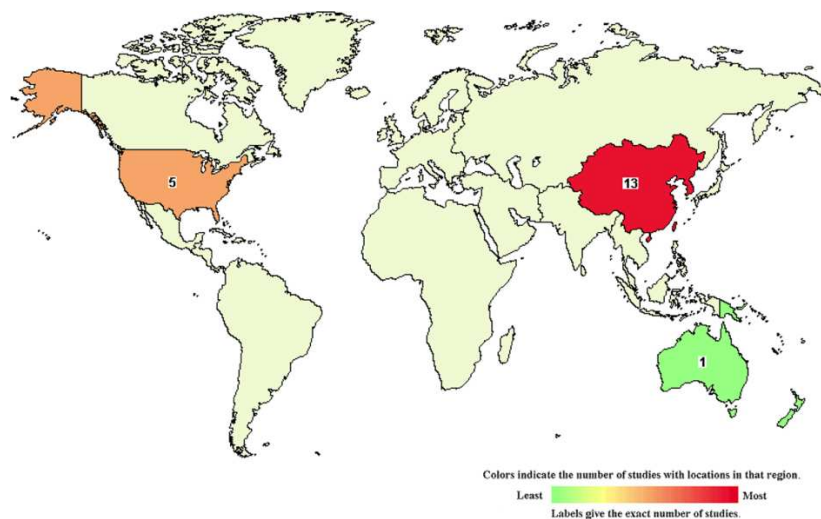


Figure 29: Distribution of CAR-NK cell-based clinical trials worldwide (Zhang et al., 2022).

Similar to CAR-T cells, CAR-NKs were initially aimed to be used against hematological malignancies and this is one of the reasons why CD19 CAR-NK is almost fully studied compared to other markers.

Among hematologic malignancies, an interesting disease where CAR-NK can be a therapeutic tool is AML. Nowadays, conventional therapies remain insufficient for the treatment of AML with the detection of residual blast cells leading often to relapse. While ASCT might be a solution for that, donor availability and transplant-associated toxicity remain still a challenge (Heuser et al. 2020). As described above, NK cells have a well-defined role in AML and BC-CML, they contribute to the GvL effect of ASCT and have an anti-leukemic effect.

Nevertheless, CAR-NK cells can also be a promising therapy for solid tumors if we take into account the characteristics described above such as their cytolytic ability, non-MHC restricted recognition, natural infiltration in TME, etc. Current studies have shown the use of CAR-NK in preclinical studies in breast cancer, ovarian cancer, pancreatic cancer, glioblastoma, colon cancer, hepatocellular carcinoma, and head and neck squamous cell carcinoma (B. Liu et al. 2019; Wrona, Borowiec, et Potemski 2021; Q. Zhang et al. 2018). Although these preliminary results are a real hope for the use of CAR-NK cells for the treatment of solid tumors, it should be taken into consideration that nowadays there are very limited clinical data existing on the potential of CAR-NK cells for solid tumor treatment. A combination of CAR-NK therapies with antitumor drugs is a promising approach for targeting solid cancers with this cell therapy (Wrona, Borowiec, et Potemski 2021). A new approach is the combination of CAR-NK cells with immune checkpoints or the combination of CAR-T cells with CAR-NK cells is also a real perspective for the future of the cell therapy because these cells kill the same target in different ways.

3.4.4. Application of CAR-NK cells in hematological malignancies (AML and MDS)

Myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) are two main myeloid malignancies characterized by an arrest of differentiation and uncontrolled proliferation. As described above, CML can convert to AML during the progression of the disease rendering thus these two diseases highly related and interesting for new therapies.

Hofmann et al in 2019 summarized a list of interesting targets in AML (Hofmann et al. 2019). Nevertheless, the use of CAR-based therapies for these diseases should be very careful because nowadays there are no specific markers present in leukemic cells (and absent from healthy tissues). A first clinical trial (NCT02944162) based on CAR-NK started in 2016 using an anti CD33 CAR-NK in Suzhou (China) in relapsed or refractory AML. Even though no conclusion regarding the clinical efficacy could be found, the safety of this therapy was promising.

Another interesting marker for CAR-NK therapies remains the NKG2D ligands which are highly expressed in AML and MDS but absent in healthy tissues. In 2020 a clinical trial (NCT04623944) led by Nkarta Inc. aims to study the safety of CAR-NK cells targeting NKG2D ligands in patients with relapsed/refractory AML or MDS. Primary data should be published by 2023.

Nowadays, clinical trials in AML/MDS based on CAR-NKs for several targets such as CD38, CD123, and FLT3 are ongoing.

Another interesting approach for NK cells in therapy remains their intrinsic ability to kill tumor cells in general and AML blasts in particular, in a CAR-independent manner. These mechanisms and their involvement in CML have been described in the chapter “Immune effector of CML”.

3.4.5. Challenges and perspectives of CAR-NK cells

Cryo-conservation

One of the technical challenges of working with CAR-NK cells it's their high sensitivity to the freezing and thawing process which impacts their anti-tumor capacity and survival rate (van Ostaijnten Dam et al. 2016; Klingemann 2015).

Immunosuppressive environment

As for other cells, the immunosuppressive environment of tumors is responsible for the decrease in the activity of NK cells inducing thus a loss of cytotoxicity. That is due to immunosuppressive cytokines such as TGF- β , adenosine, and indoleamine 2,3-dioxygenase which are known to have a negative impact on CAR-NK cells (Murray et Lundqvist 2016).

NK persistence

Among challenges that should be taken into consideration while using CAR-NK-based therapies, is also the limited expansion and persistence capacity of NK cells (Klingemann 2015) compared to CAR-T cells known to persist for years (Melenhorst et al. 2022). Liu et al showed the persistence of CAR-NK cells for at least one year (E. Liu et al. 2020), nevertheless only long-term follow-up of clinical trials can elucidate the persistence of CAR-NK in patients.

To circumvent the lack of persistence of CAR-NK cells, different groups have proposed genetical modifications of NK cells to introduce expressing genes for stimulating factors such as IL-15, IL-21, or IL-2 (Matosevic 2018; E. Liu et al. 2020), and others have proposed the generation of memory-like phenotype (with IL-12, IL-15, and IL-18) as a solution for the NK persistence (Romee et al. 2016; Ni et al. 2012; Gang et al. 2020).

Tumor infiltration

Tumor infiltration is a very important factor for efficient cell-based therapy. The rapid homing of NK cells has been controversial therefore different studies have tried to propose new ways to improve it. Among these studies, most are based on the overexpression of chemokines such as CCR7, CXCL10, CCL19, etc and by different techniques (viral, electroporation of mRNAs, etc.) (Wennerberg et al. 2015; Levy, Carlsten, et Childs 2016). The efficacy of these approaches needs to be validated in clinical trials.

Low lentivirus efficiency

Transduction of human NK cells has historically been challenging. As described in the previous chapter, retroviruses and lentiviruses are the transfer method of choice to introduce CAR sequences into cells. Amirache et al. described in 2014 the reason for this low efficiency. It is related to the lack of the low-density lipids (LDL) receptor on NK cells which is needed for the VSV-G-LV transduction (Amirache et al. 2014). Different techniques have been used to overcome this limitation such as the use of Rosuvastatin to increase the levels of LDL-R and consequently the transducibility (Y. Gong et al. 2020). Other molecules such as protamine sulfate or polymers (dextran or polybrene) are also used to modify the electrical charge on the cell surface and thus favor transduction (Yang et Hsieh 2001).

An interesting approach described to increase NK transducibility is the use of other envelopes than VSV-G such as the baboon envelope pseudo-type lentivectors (BaEV-LVs) (Colamartino et al. 2019). Bari and colleagues have shown that the use of modified baboon envelope glycoprotein has a 20-fold or higher transduction rate than that of a VSV-G pseudo-typed lentiviral vector (Bari et al. 2019).

OBJECTIVES OF THE THESIS

The first objective of my Ph.D. was to use CML patient derived-iPSCs in order to recapitulate the dynamic evolution of the CML *in vitro*.

In addition to the mechanistic point of view of CML evolution, generating relevant models of BC-CML remains very important in order to discover new potential therapeutic targets.

The second objective of my Ph.D. was to use the generated model based on mutagenized CML-iPSC for new target identification in CML. The most relevant targets were aimed to be validated in patients in order to pave the way for potential future therapeutic tools.

The third objective of my Ph.D. was to generate a CAR-NK therapy model targeting markers identified in the BC-CML model. This potential cell therapy targeting the IL2RA/CD25 was aimed to be tested in NK cell line (NK92) before generating iPSC derived CAR-NK.

RESULTS

1. Modelling global genomic instability in chronic myeloid leukemia (CML) using patient-derived induced pluripotent stem cells (iPSC)

This work was initiated by the previous PhD Student of Prof. A Turhan, to establish the basis of the modelling of progression of CML using patient specific iPSC. I have participated to this work by confirming the genetic instability patterns of iPSC treated with the mutagenic agent ENU. This work was initiated using a single iPSC line. I have then extended this work to other two iPSC lines and characterized the cells and searched for the identification and validation of novel targets in the article 2.

Article

Modelling Global Genomic Instability in Chronic Myeloid Leukemia (CML) using patient-derived induced pluripotent stem cells (iPSC)

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Simple Summary: Despite major progress obtained by the use of tyrosine kinase inhibitors (TKI) in the therapy of CML, progression towards blast crisis occurs, especially in patients resistant to TKIs, and due to genomic instability of the leukemic cells expressing BCR-ABL. We have asked whether we could reproduce this genomic instability by using induced pluripotent stem cells (iPSC) derived from leukemic cells expressing BCR-ABL. We show here the feasibility of enhancing this global genomic instability by in vitro mutagenesis and the validation of genomic events discovered using transcriptomic databases derived from primary leukemic cells.

Abstract: Purpose: To determine the feasibility of modeling genomic instability characterizing chronic myeloid leukemia (CML) progression. Methods: We used patient-specific induced pluripotent stem cell (iPSC) line treated with the mutagenic agent N-ethyl-N-nitrosourea (ENU). Genomic instability was validated using γ -H2AX and micronuclei assays and CGH array for genomic events. Results: An increased number of progenitors (x5-Fold) which proliferated in liquid cultures with a blast cell morphology was observed in the mutagenized condition as compared to the unmutagenized one. CGH array performed for both conditions in two different time points reveals several cancer genes in the ENU-treated condition, some known to be altered in leukemia (BLM, IKZF1, NCOA2, ALK, EP300, ERG, MKL1, PHF6 and TET1). Transcriptome geodataset GSE4170 (Radich et al. 2006) allowed us to associate 125 of 249 of the aberrations that we detected in CML-iPSC, with the CML progression genes already described during progression from chronic and AP to BC. Among these candidates, eleven of them have been described in CML and related to tyrosine kinase inhibitor resistance and genomic instability. Conclusion: These results demonstrated that we have generated for the first time to our knowledge, an in vitro genetic instability model, reproducing genomic events described in patients with BC.

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Keywords: CML, iPSC, Genomic instability

1. Introduction

Chronic myeloid leukemia (CML) is the prototype of a clonal malignancy of the hematopoietic stem cell (I. Sloma et al. 2010). The initiation of the disease by the appearance of the Ph1 chromosome in a very primitive hematopoietic stem cell is followed invariably by the progression of the clone by acquisition of novel abnormalities leading to clonal progression, accelerated and blast phases of the disease (Kantarjian 1987). This natural history has now been modified by the use of tyrosine kinase inhibitors (TKIs) as first line therapies ensuring, in patients with deep molecular response, prolongation of their survival on therapy (O'Brien et al. 2003). However, resistances to several lines of TKIs occur and accelerated and blast phases are observed potentially due to clonal selection by TKI therapies (Shah et al. 2002; Soverini et al. 2006).

Seminal and now classical work have established using serially collected patient samples, main cytogenetic and molecular events observed in patients during the progression of the disease towards accelerated and eventually blast phase. These include cytogenetic abnormalities identified as “major” and “minor” routes (Crisan et al. 2015; Mitelman 1993).

Several work performed using patients samples and models have also identified during the last two decades major molecular events involved in this progression such as, TP53 mutations (Perrotti et al. 2010a). BCR-ABL expressing cells are also prone to develop a mutator phenotype (Canitrot et al. 1999) which could be due to the presence of an increase oxidative stress (Koptyra et al. 2008) which leads to clonal selection under the influence of TKI therapies (Liu et Wang 2015). CML cells are finally doomed by the deficiency of several DNA repair mechanisms involving DNA-PKcs (Deutsch et al. 2001) BRCA1 (Deutsch et al. 2003) as well as abnormalities of specific DNA Repair genes such as Bloom (Slupianek et al. 2005) and other repair mechanisms such as NER (Canitrot 2003) MMR (Stoklosa et al. 2008).

Blast crisis is defined by a differentiation arrest decision of the leukemic progenitors and insensitivity to TKI. This alteration of the differentiation is due to this accumulation of genetic abnormalities.

iPSC technology offers the possibility of obtaining unlimited numbers of pluripotent cells which, despite difficulties of inducing definitive haematopoiesis (Lerou et Daley 2005), can be used for modelling some aspect of hematopoiesis, as it is possible to generate terminally differentiated progenitors and differentiated cells.

Blast crisis is characterized by the progressive occurrence of genomic abnormalities due to BCR-ABL-associated genetic instability inherent to leukemic cells. (Chereda et Melo 2015). The occurrence of these genomic events is difficult to predict in individual patients. CML patient-derived iPSC express BCR-ABL (G. Telliham et al. 2016), (Gladys Telliham et al. 2022) and can allow the possibility of enhancing global abnormalities by in vitro mutagenesis. In order to explore the possibility of generating such model from an iPSC derived from a CP-CML patient, we have chosen an in vitro mutagenesis approach using N-ethyl-N-nitrosourea (ENU). Here we show that CML patient derived iPSC can be used to generate an experimental model mimicking cytological and genomic instability patterns observed in primary blast crisis cells. Moreover, we show that these abnormalities induced by this technology are representative of those described in large database of genomic events described in primary CML patients in blast crisis.

2. Materials and Methods

2.1 Patient-derived iPSC and characterization.

Generation of CML-iPSC PB32 has been previously described (G. Telliham et al. 2016). Briefly, peripheral blood cells were obtained at diagnosis from a 14-year old CML patient with informed consents according to the declaration of Helsinki. This patient was treated with Imatinib mesylate as a first line therapy. At 12 months post-therapy there was no major molecular response and an allogenic bone marrow transplant was performed. The

patient remains in complete remission since the transplant performed in 2004. To generate iPSC, cryopreserved CD34+ cells were used as previously described (G. Telliam et al. 2016). All analyses were performed using a polyclonal stock of iPSC. Control cells used for these experiments included PB33, an iPSC cell line that was obtained from bone marrow CD34+ cells using Sendai virus. CML and control iPSC have been characterized for their pluripotency using cell surface markers as well as by generation of in vivo teratoma assays in immunodeficient mice (G. Telliam et al. 2016).

2.2. iPSC and ESC cultures.

iPS cell lines were maintained on the mitomycin-C-inactivated mouse embryonic fibroblast feeder cells with DMEM-F12 supplemented with 0.1mg/ml bFGF, with or without N-ethyl-N-nitrourea 10µg/ml or 50µg/ml and treated 2h with 1mg/ml Collagenase type IV (Gibco by life technologies ref 17104-019).

2.3. Embryoid body assays.

To induce embryoid body (EB) formation, ES and iPS cells at day 6–7 after cell passage, were treated with collagenase IV. Clumps were cultured in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 1% penicillin/streptomycin, 1 mM L-glutamine, 15% fetal calf serum (FCS, Invitrogen), 450 µM monothioglycerol, 50 µg/mL ascorbic acid (Sigma Aldrich) and 200 µg/L transferrin (Sigma Aldrich) and supplemented with hematopoietic cytokines: 100 ng/mL stem cell factor (SCF), 100 ng/mL fms-like tyrosine kinase 3 ligand (Flt-3L) and 50 ng/mL thrombopoietin (TPO) (all from Peprotech). ESC and IPSC derived EB were cultured in ultra-low attachment 6-well plates (Costar) for 16 days. Media was changed two or three times depending on EB proliferation. All cultures were incubated at 37°C in 5% CO₂.

2.4. Blast-colony forming assays.

Blast colony forming cell (BI-CFC) assays were performed using iPS cell pellets resuspended in Stemline II Hematopoietic Stem Cell Expansion medium (Sigma-Aldrich S0192) supplemented with 1% penicillin/streptomycin and L-glutamine, 50ng/mL rhVEGF (Peprotech) and 50ng/mL rhBMP4 (Peprotech). After 48h we added 20ng/mL of rhSCF (Peprotech), rhTPO (Miltenyi Biotec) and rhFLT3L (Peprotech). The embryoid bodies at day 3.5 were collected, dissociated with pre-heated stable trypsin replacement enzyme TrypLE Express (Gibco by Life technologies ref 12605-10) and filtered with 40µm Nylon Mesh sterile cell strainer (Fisher Scientific). Cells were resuspended in Stemline II Hematopoietic Stem Cell Expansion medium (Sigma-Aldrich S0192) supplemented with 1% penicillin/streptomycin and L-glutamine, 50ng/mL rhVEGF, rhBMP4, rhFLT3L and rhTPO, 20ng/mL FGfb (Peprotech) and 5 Units/mL EPO (Peprotech) and transferred in Methocult SF H4436 (Stem cell technologies) for 5 to 7 days.

2.5. Hematopoietic differentiation.

CFC assays from ES and iPS cells from EB or BL-CFC were plated at 10x10³ cells/mL into MethoCult GF (H4435 StemCell Technologies). Hematopoietic CFC was counted at day 19.

2.6. Western Blots.

Cells were lysed in ice with RIPA buffer containing (NaCl [200mM], Tris [pH 8; 50mM], Nonidet P40 [1%], acide deoxycholate [0.5%], SDS [0.05%], EDTA [2mM]) supplemented with 100µM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium fluoride (NaF), 1mM orthovanadate (Na₂VO₄). Separation of proteins was done by electrophoretic migration on a 3-8% polyacrylamide gel under denaturing conditions. The proteins were then

transferred in a semi-liquid condition on PVDF membrane pre-activated in methanol. After saturation with TBS Tween 5% BSA for 1h and hybridization of the membranes with primary (Anti-phosphoHistoneH2A.X (Ser139) clone JBW Millipore) and secondary antibodies coupled to HRP. Membranes were revealed by chemiluminescence with SuperSignal West Dura or Femto reagents and data acquired using G:BOX iChemi Chemiluminescence Image Capture system.

2.7. Evaluation of hematopoietic cells phenotypes.

At day + 19 of cultures, the methylcellulose was washed with PBS. Living cells were counted in trypan blue and stained with the following antibodies in PBS 4%BSA at 4°C 45 min: CD45Pe-Vio770 (Miltenyi), CD34Vioblue (Miltenyi), CD41aPE (BD), CD43FITC (Miltenyi), CD31FITC (Beckman Coulter), CD235aAPC (BD), IL1-RAP APC (R&D), CD38PE (Miltenyi), CD71PE (BD), CD14FITC (Beckman Coulter), CD33APC (BD), CD133APC (BD), BB9PE (BD), SSEA1PE (BD). After 1h, cells were washed and resuspended in PBS 4%BSA with viability staining reagent 1µg/mL (7-AAD) 7-aminoactinomycin D (Sigma Aldrich). Stained cells were analyzed with a MACSQuant 10 (Miltenyi Biotec) flow cytometer and Flowjo analysis software.

2.8. ENU experiments

CML-iPSC and control iPSC cells were cultured in the presence or in the absence of ENU at the concentration of 10 µg/mL with daily addition of ENU as described above. Experiments performed used iPSC cultured in ENU for either 40 days or 61 days. CGH array experiments were performed using EB's derived hematopoietic cells and BI-CFC generated from ENU-treated cultures as compared to cultures not treated with ENU.

For kinetic analysis, we recovered iPSC colonies on mouse embryonic fibroblasts (MEF). The "clumps" of colonies have been incubated in DMEM-F12 medium supplemented with 50µg/mL ENU at 37 °C for 2, 10 and 30 min. After treatment, the protein pellet was extracted from each condition in order to perform a kinetic analysis of the level of phospho γ-H2AX by Western blot.

2.9. Cytogenetic analyses

Caryotype analyses were performed using cell pellets collected at different time points using standard methods as previously described (G. Telliam et al. 2016).

2.10. Micronuclei analyses

The presence of micronuclei and anaphase bridges before and after exposure to ENU were analyzed as described previously (Zaguia et al. 2020). Briefly, cells were incubated in a humidified atmosphere of 5% (v/v) CO₂ in air at 37 °C until arrest and cell spread. The culture medium was discarded and hypotonic shock was induced by incubating the cells with 18mM KCl at room temperature. The cells were then fixed with acetic acid/ethanol (1:3, v/v), and the cell suspension was dropped onto the slides. The slides were stored at -20 °C until further use. Telomere and centromere staining was performed in order to detect the nature of micronuclei: with only telomere staining resulting mainly from acentric chromosomes or with telomere and centromere staining which predominantly contained lagging chromosomes.

Automatic scoring of MN was performed using MNScore software (version 3.8.101 MetaSystems, Althausen, Germany) with a Metafer 4 image analyser (MetaSystems, Althausen, Germany) comprised of a Zeiss Axioplan 2 imager to detect MN. An operator validated and excluded the false MN in cells. For each sample, 1000 cells were scored.

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2.11. DNA Extraction.	199
iPSC, EB's and BI-CFC obtained from either ENU-treated or not treated CML-iPSC as well as from control iPSC were used for DNA extraction using standard methods with DNeasy Kit (Qiagen).	200 201 202 203
2.12. Array Comparative Genomic Hybridization	204
To perform CGH arrays, we have used genomic DNA from ENU-treated iPSC derived EB's and BI-CFC's compared to CML-iPSC without ENU treatment. We followed Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis protocol. Briefly, we performed digestion from 500ng of DNA with SureTag DNA labeling kit (4-packs). After DNA amplification CML-iPSC was labeled with Cyanine 3 and the tested samples with Cyanine 5. Assembled chambers were loaded into the oven rotator rack, hybridization were performed at 67°C for 24 hours at 20 rpm.	205 206 207 208 209 210 211 212
2.13. Transcriptome dataset	213
Rosetta / Merck Human 25k v2.2.1 microarray Array data matrix of normalized log ratios from GSE4170 was downloaded on Gene Expression Omnibus website: (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4170), and annotated with annotation platform GPL2029 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL2029). In this dataset CD34+ cell samples belonging to the 3 experimental groups corresponding to the different evolution phases of chronic myeloid leukemia were analyzed: chronic phase, accelerate phase and blastic crisis were analyzed (Radich et al. 2006).	214 215 216 217 218 219 220 221 222
2.14. Bioinformatics	223
Boxplot, two-sided Student t.test with Welch correction were performed in R Software version 3.2.3. Heatplot was performed with made4 package (Culhane et al. 2005). Functional enrichment was performed with Go-Elite Standalone software version 1.2 on the Gene Ontology Biological Process, KEGG and CommonsPathway databases included in Homo sapiens EnsMart77Plus (Ensembl – Biomart) update (Zamboni et al. 2012). Functional interaction networks were performed with Cytoscape software version 3.2.1 (Cline et al. 2007). Unsupervised principal component analysis was performed on gene expression profile with R package FactoMiner; p-value was calculated by group discrimination on first principal component axis. Pubmed gene prioritization was realized with gene valorization web application (Brancotte et al. 2011). Circosplot on contingency table of Pubmed gene prioritization was realized with Circlize R package (Gu et al. 2014). Circosplot of genomic aberrations with HG19 coordinates were performed with OmicCircos R package (Hu et al. 2014). ROC curves were performed with ROCR R package. Genomic aberration gene candidates were also matched with important databases such as: database PLURINET for genes implicated in pluripotency (Müller et al., 2008), cancer genes COSMIC database: Catalogue of somatic mutations in Cancer (http://cancer.sanger.ac.uk/census) (Futreal et al. 2004) and transcription factors (Vaquerizas et al. 2009).	224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241

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3. Results

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3.1. Long term ENU exposure induces an enhancement of CML-iPSC derived hematopoiesis.

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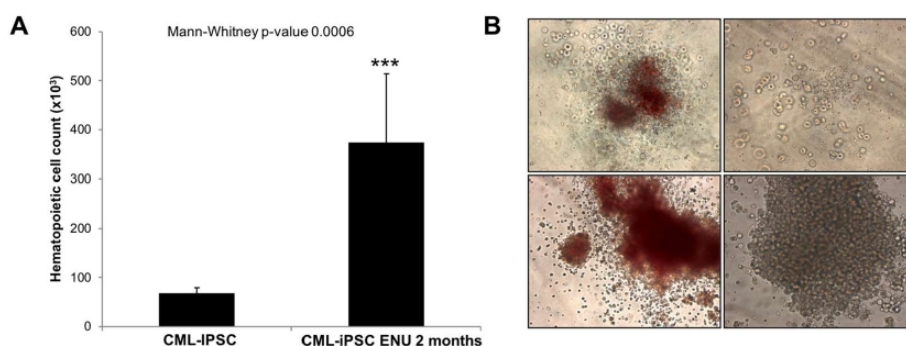
To this purpose we compared EB or BI-CFC and hematopoietic cells from CFC assay derived from iPSC cultured under normal culture conditions or after ENU exposure (**Figure 1B**). As we can see figure 1B, after 2 months of ENU exposure CML-iPSC showed a significant increase of hematopoietic cell potential (Mann-Whitney $p=0.0006$) (**Figure 1B**).

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Figure 1: Increased hematopoietic potential in CML-iPSC under ENU exposure. (A) Hematopoietic progenitor count from CML iPSC before or after exposure to 10µg/mL ENU during 2 months. Statistical Mann-Withney t-test was performed with PRISM software. (B) CFC generated in methycellulose cultures revealing large colonies with myeloid differentiation features associated with blast colonies.

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Interestingly, in CFC assays generated from PB32-ENU cells we have observed larger colonies which proliferated in methycellulose cultures (**Figure 1B**) as well as in liquid cultures in the presence of hematopoietic growth factors (SCF, TPO, Flt3L). Cytological features of these cells using May-Grunwald stain revealed the presence of myeloid cells with precursor and mature (myelocytes, metamyelocytes, neutrophils, monocytes/macrophages) cells as well as cells with undifferentiated blast cell morphology reminiscent of blast crisis cells (**Figure 2B**).

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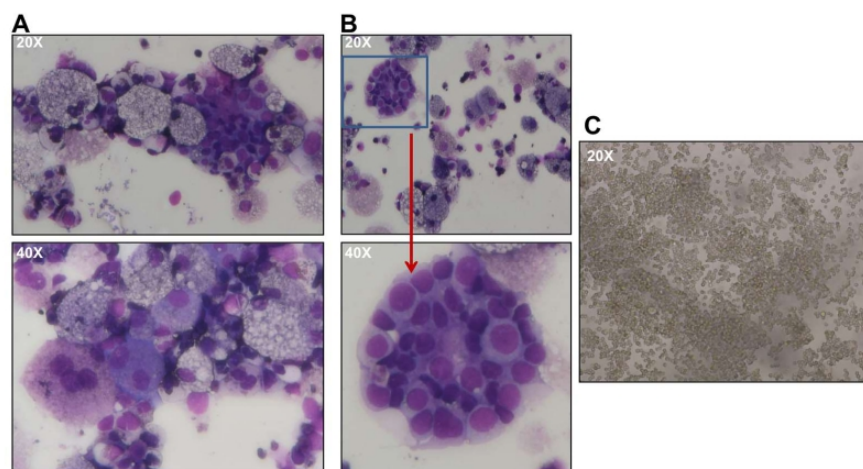


Figure 2: May-Grunewald staining of hematopoietic colonies. (A) Evidence of myeloid differentiation along with (B) blast colonies. (C) Proliferation of blast cells in liquid cultures in the presence of SCF, TPO and Flt3-ligand for > 2 weeks.

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FACS analysis of these cells showed that the majority of cells expressing CD45, with positivity of CD31, CD38, CD43 markers (**Figure 3**). Hemangioblast marker BB9, as well as CD133, CD26, SSEA1, CD13, CD33 and CD71-expressing cells were also detected (**Figure 3**).

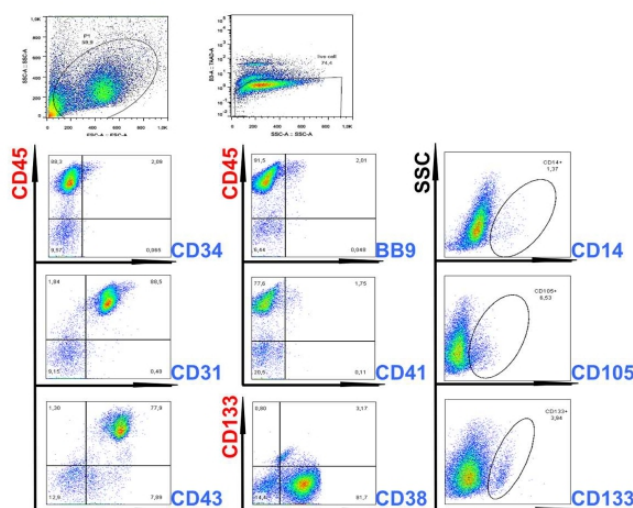


Figure 3: Flow cytometry evaluation of hematopoietic cells generated after from ENU-treated CML iPSC.

As can be seen in this Figure, double staining experiments showed the presence of CD45 + / CD43+ and CD45+/CD38+ cells demonstrating generation of hematopoietic cells with primitive characteristics

3.2. Genomic instability of CML-iPSC induced by BCR-ABL under ENU exposure.

Differences observed in the behavior of PB32 and PB32-ENU cells suggested that ENU could enhance the DNA repair abnormalities known to be inherent to CML cells. We have performed for this purpose a short-term exposure to ENU of our CML-iPSC and analyzed the kinetics of accumulation of phospho- γ H2AX in these cells.

We cultured iPSC colonies on mouse embryonic fibroblasts (MEF). The "clumps" of colonies have been incubated with ENU at 37°C for 2, 10 and 30 min. After treatment, the protein pellet was extracted from each condition in order to perform a kinetic analysis of the level of γ H2AX (**Figure 4A**). Kinetic analysis of an iPSC control cell line revealed an increase of γ H2AX expression level at 10 minutes of treatment with ENU with a progressive decrease over time. These observations suggest that ENU induces time dependent DNA breaks and the DNA repair system is set up around 10 minutes after detection of γ H2AX signal. However, CML-iPSC cell line showed a gradual accumulation of γ H2AX (**Figure 4B**) with a significant increase after 10 min and persistence of the phosphorylation after 30 min of ENU treatment (n=3, Two way ANOVA with Bonferroni's correction) (**Figure 4C**). These results suggested the presence of a baseline increase of double strand breaks in CML-iPSC which persisted after ENU exposure due potentially to a deficient DNA repair system.

The potential genomic instability of PB32-ENU cells was also evaluated by the micronucleus assays (Zaguia et al. 2020) which is a recognized marker of the chromosomal instability of the eukaryotic cells. As can be seen in the Supplementary Figure 3, the presence of spontaneous micronuclei was significantly higher in the mutagenized CML-iPSC lines (PB32-ENU) as compared to the non-mutagenized counterpart and to the control cell line (PB33), validating the genomic instability of the mutagenized CML-iPSC (**Supplementary Fig 3**).

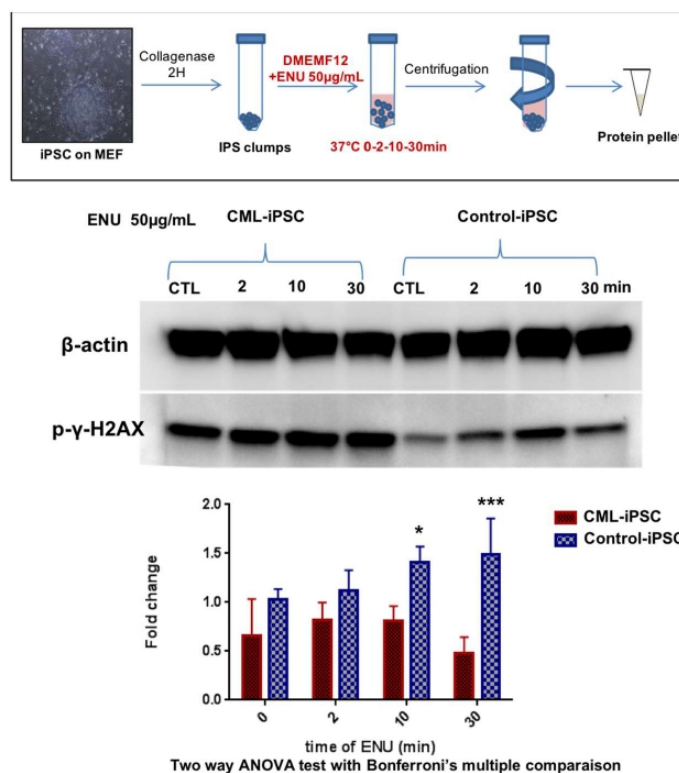


Figure 4: Evaluation of the phosphorylation of γ -H2AX in the CML iPSC after short-time ENU exposure. (A) CML iPSC cultured on murine embryonic fibroblasts were collected after collagenase treatment and the pellets subjected to a short time exposure to ENU followed by protein extractions. (B) Western blot analysis of the phosphorylation level of the histone variant gamma-H2AX (γ H2AX) on Ser-139 after exposure of the CML-iPS cell line and control iPSC to ENU at 50 μ g/mL for 0, 2, 10 and 30 minutes (30 μ g of protein per conditions). (C) Quantification of the γ H2AX as compared to β -Actin with ImageJ software, histograms represent means of 3 independent experiments. A Two-way ANOVA test with multiple comparison and Bonferroni's correction was made with PRISM software.

We then wished to evaluate the presence of other features of cytogenetic and genomic abnormalities in PB32-ENU cells as compared to PB32 not treated with ENU. To this purpose, we have performed cytogenetic analyses on CML-iPSC after long-term exposure to ENU (PB32-ENU) as compared to PB32 cultured without ENU. As can be seen in Table 1, several cytogenetic abnormalities were identified in addition to t(9;22) (Table 1). Among these abnormalities (64 mitosis analyzed), we have noted the deletion of der 9q (3 mitosis / 64) (5%) deletion of Chromosome 21 (5 mitosis / 64) (8%) (Table 1 and Supplementary Figure 1). Despite several passages, control iPS cells exposed to ENU (PB33) did not develop any detectable cytogenetic changes (Suppl Figure 2). To determine the genomic events at the molecular level, we have performed CGH array experiments by comparing EB derived CFC and BI-CFC DNA generated from PB32-ENU as compared to PB32 cultured without ENU.

Table 1: Evaluation of Additional Chromosomal Abnormalities in iPSCs before and After ENU-induced mutagenesis. PB32+ ENU: Ph1+ CML-iPSC line cultured with ENU. PB32 without ENU: Ph1+ CML-iPSC without ENU-mutagenesis. PB33+ENU: Control Ph1-negative cell cultured with ENU. PB33-Ph1- : Control Ph1-negative cell line.

iPSC Line	# of Chromosome Involved	Mitosis Ratio	%
PB32 + ENU (Ph1+)	21	5 loss / 64	8
	9	3 loss / 64	5
	17	1 loss / 64	>2
	19	1 loss / 64	>2
	20	1 loss / 64	>2
	13	1 loss / 64	>2
	1	1 loss / 64	>2
PB32 without ENU (Ph1+)	2	1 loss / 64	>2
PB33 + ENU (Ph1-neg)	None	/	/
PB33 without ENU (Ph1-neg)	None	/	/

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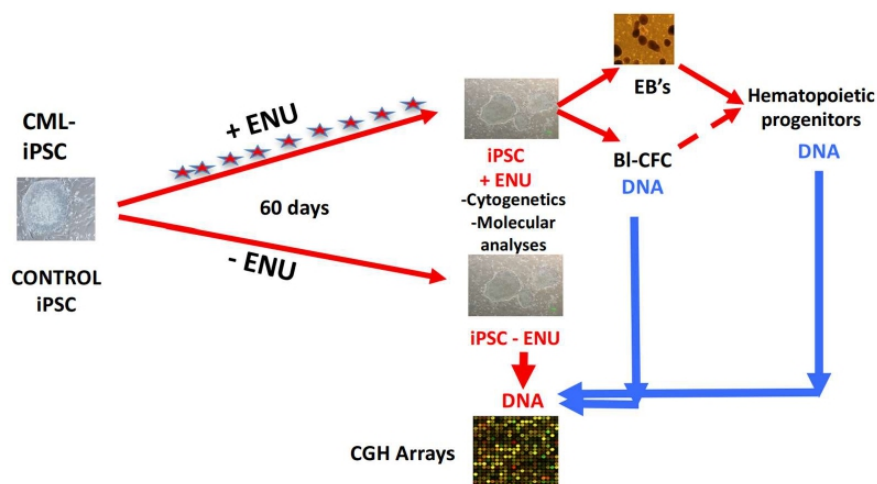


Figure 5: Experimental protocol used for aCGH experimental procedure. CML and control iPSC were cultured in murine embryonic fibroblast layers in the presence or in the absence of ENU for 60 days, with daily medium changes containing or not ENU. At day +60, DNA extracted from hematopoietic progenitors generated via EB's or Blast-CFC's were used in CHG arrays experiments using DNA extracted from iPSC cells without ENU exposure

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3.3. CGH array analysis of PB32-ENU-derived hematopoietic cells.

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Figure 5 shows the experimental strategy to perform this analysis. As can be seen in this figure, we have extracted DNA from either PB32-ENU EB-derived CFC or BI-CFC and compared the genomic abnormalities to that of PB32 cultured all along without ENU (Figure 5). Cytogenomics software from Agilent technologies with mosaic workflow allowed to detect 20 quantitative genomics aberrations: copy number variations CNVs which comprised 313 gene loci. After filtration on European Caucasian genomic polymorphism database still 295 gene loci are comprised in these genomic aberrations (Figure 6A and Supplementary Table 1). Majority of the gene loci events are affected by gain of genomic

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DNA (69%) versus loss (31%) (**Figure 6B and Supplementary Table 1**). Matching these genomic aberrations with transcription factor database, cancer gene database and pluripotency gene database allowed to observed that these important deregulated actors are principally affected on chromosomes 5, 7, 21, 22, and X (**Figure 6B**): majority of these genomic events implicated are transcription factors such as : zinc finger ZNF and IRX (**Figure 6B**). Some pluripotency genes were also identified as well as cancer genes such as TET1, ALK, EP300, ERG, MKL1, PHF6 already described as been involved in leukemia. Functional enrichment of genomic alterations of EB-derived CFC allowed to highlight perturbations in megakaryocytic development with HBD, HBE1, HBG1 and HBB molecules and HDAC enzymes including EP300 and SIRT1 (**Figure 6C and D**). Functional enrichment on Gene Ontology database allowed to identified SIRT1, EP300 and CDH13 as central molecules especially SIRT1 which is implicated in p53 dependent-DNA damage in response to hydrogen peroxide and cellular response to hypoxia. SIRT1 is also involved in negative regulation of NK-kappaB (**Figure 6E and 6F**).

When the same analysis was performed using DNA of BI-CFC, 22 quantitative genomics aberrations were detected. These included copy number variations (CNVs) which comprised 332 gene loci. After filtration on European Caucasian genomic polymorphism database still 255 gene loci were found to be comprised in these genomic aberrations (**Figure 7A and Supplementary Table 2**). Majority of the gene loci events represented loss of genomic DNA (71%) with loss of heterozygosity (23%) and 6% gains of DNA (**Figure 7B and Supplementary Table 2**). Matching these genomic aberrations with transcription factor database, cancer gene database and pluripotency gene database allowed to demonstrate that these important deregulated actors were principally involved on chromosomes 7, 8, 15, Y, and X (**Figure 7B**): circosplot analysis also allowed to show that majority of these events involved comprised transcription factors such as MESP1 implicated in mesodermal cell migration and IKZF1 (**Figure 7B and 7F**). Amongst the, few pluripotency and cancer genes we identified IDH2, NCOA2, IKZF1, BLM described as being involved in leukemia (**Figure 7B, 7F**). Functional enrichment of genomic alterations of PB32 iPSC on KEGG database allowed to highlight perturbations in hematopoietic lineage and cytokine-receptor interaction, affecting: TPO, CSF2RA, ILRA, PIK3RA, CRFL2 so cytokines and receptors allowing activation of JAK-STAT pathway (**Figure 7C and 7D**). Functional enrichment on Gene Ontology database allowed important implication of developmental molecules and especially sharing functionalities in mesodermal tissue (**Figure 7E and 7F**) such as IKZF1 a transcription factor of the mesodermal development and MESP1 et 2 two transcription factors implicated in migration of mesodermal cells; also important implication of molecules in relation with the extracellular matrix, cell fate specification and DNA repair.

3.4. Comparison of genomic aberrations identified by CGH in CML-iPSC as compared to primary leukemic blast crisis gene profiling.

Transcriptome of CML CD34+ cells in the 3 different phases of the disease: chronic phase (CP), accelerated phase (AP) and blast crisis (BC) were analyzed to extract information of genomic aberrations that we observed in CML PB32-ENU_derived hematopoietic progenitors. On 249 candidate genes, a supervised one-way ANOVA was performed between the 3 phases of the disease (with 1000 permutations and p-value threshold less than 1E-3): 125 genes were found significant and unsupervised principal component analyses performed with them allowed to have a very significant group discrimination (p-value of discrimination 9.43E-32, **Figure 8A and Supplementary Table 3**). Interestingly, among these genes some were identified in EB-derived CFC's and some in BI-CFC-derived cells (**Figure 8B**). Unsupervised classification performed with the gene candidates mostly correlated to the progression of the disease, allowed to obtain a perfect reclassification of the chronic phase samples as compared to the blast crisis samples (**Figure 8C**). NCBI gene valorization performed on these best candidates correlated to the progression disease allowed the identification of eleven genes (**Figure 8D**) which are well known in

CML pathology, genomic instability and tyrosine kinase inhibitors related keywords in published CML literature (Figure 8D). Majority of these candidates including a group of 7 genes (7 of 11) are predictive for the chronic phase (Figure 8D), whereas the other group are predictive for blast crisis (4 of 11) (Figure 8D). Among genes candidates not reported in CML pathophysiology, were found to be predictive of the blast crisis, such as DDX family such as DDX50 and DDX21, very well known in DNA repair and the implication of which is unknown in CML.

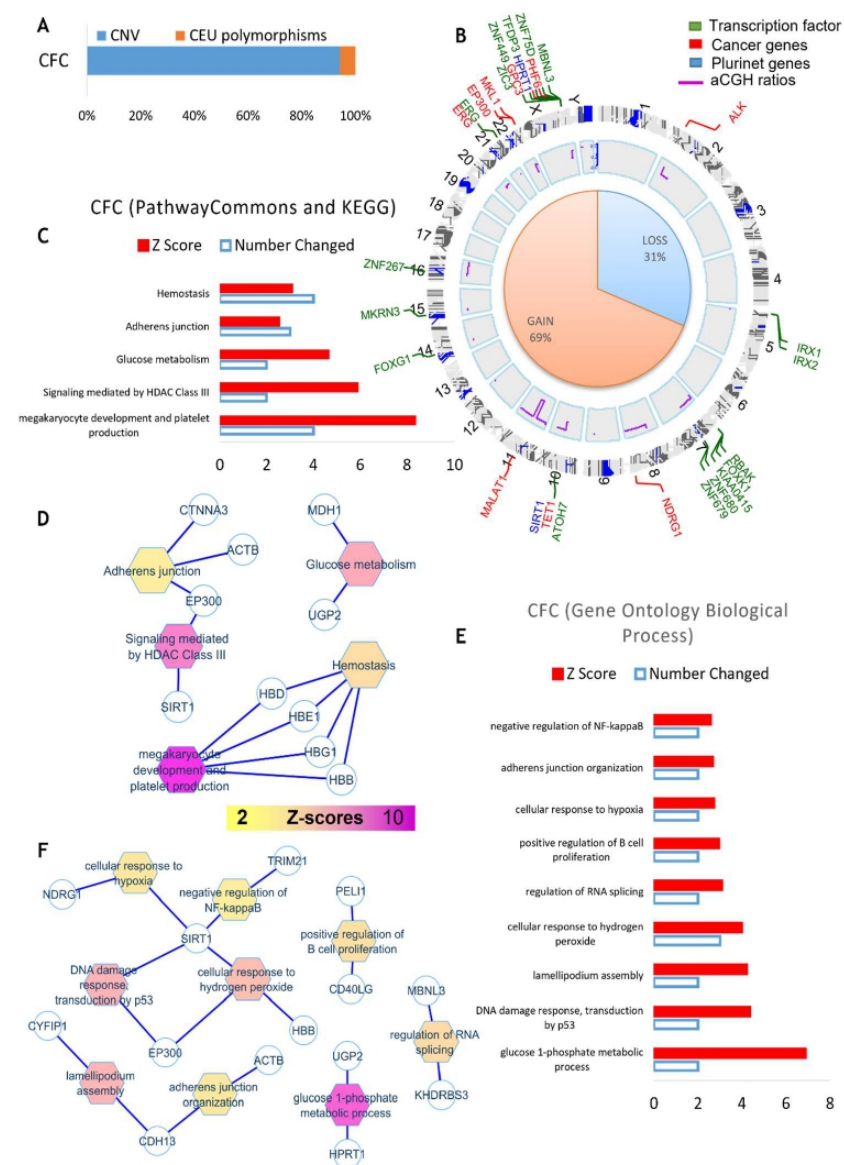


Figure 6: aCGH analysis of EB-derived CFC cells. (A) Quantitative genomic aberrations: copy number variations CNVs. (B) Matching of genomic aberrations with transcription factor database, cancer gene database and pluripotency gene database, circosplot show in green: 5 zinc finger ZNF genes and 2 IRX, in blue: pluripotency genes, in red: cancer genes. (C-D) Functional enrichment of genomic alterations of CFC on KEGG database. (E-F) Functional enrichment on Gene Ontology database.

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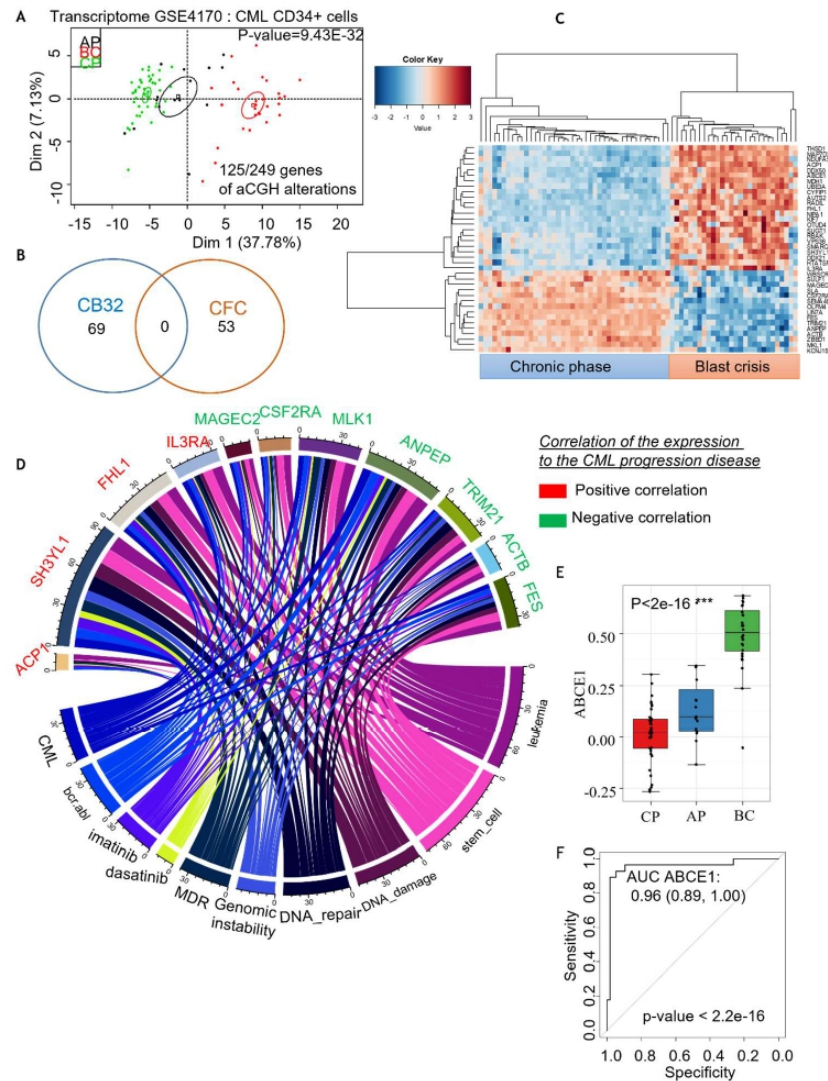


Figure 8: Comparative analysis of genomic aberrations discovered in CML-iPSc aCGH with the gene profiling described in primary CML CD34+ cells. (A) In silico analysis of genomic aberrations found in our study as compared to gene profiling of CML CD34+ cells described in Radish () in the 3 different phases of the disease: chronic phase (CP), accelerated phase (AP) and blast crisis (BC) were analyzed to extract information of genomic aberrations that we observed under ENU exposure, supervised with one-way ANOVA. (B) Genes related to CFC or BI-CFC aCGH analysis. (C) Classification of the chronic phase samples as compared to the blast crisis samples. (D) NCBI gene valorization performed one of the best candidates correlated to the progression disease and well known in CML pathology, genomic instability and tyrosine kinase inhibitors related keywords; in green: predictive candidates to chronic phase, in red: predictive candidates to blast crisis.

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4. Discussion

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In the current era of TKI therapies, the progression towards BC became rare but still occurs even after several years and correlates with resistance to several lines of therapies. Several CML cell lines have been generated from patients in established blats crisis, the first being K562 (Lozzio et Lozzio 1975). Mechanisms underlying the progression of CML towards have been extensively studied during the last four decades (Perrotti et al. 2010a) using serially collected patient samples or in vitro and in vivo models, essentially by analyzing blast crisis cell lines such as K562 or by overexpressing along with BCR-ABL, genes involved in blast crisis such as NUP98 (Ivan Sloma et al. 2021). Major cytogenetic events (Crisan et al. 2015) as well as molecular events contributing to the differentiation arrest have been identified (Melo et Barnes 2007) (Perrotti et al. 2010b) More recently, global genomic analyses performed in primary BC samples showed the enrichment of CML genome by the occurrence of mutations, essentially of the polycomb repressive complex (PRC) (Ko et al. 2020). However, these tools do not allow to study progressive changes occurring during the clonal evolution of the disease, which requires serial samplings. Similarly, blast crisis in CML can be modeled using murine CML models by using complementation assays using BCR-ABL and an oncogenic fusion protein such as NUP 98/HoxA9 (Dash et al. 2002; Neering et al. 2007). Although these studies can be a major interest to study the in vivo behavior of cells, they are not appropriate to study human leukemia in the context of a given genomic background of a patient.

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In this work we wished to evaluate the feasibility of generating a genetic instability model of CML using patient-specific induced pluripotent stem cells. An iPSC cell line has been established from a patient with TKI-resistant CML and previously has been characterized (G. Telliam et al. 2016). These cells exhibited all features of pluripotency including cell surface and in vivo teratoma generating abilities (G. Telliam et al. 2016) and have been cultured in a pluripotent state for several passages. We have shown that these cells express BCR-ABL and BCR-ABL-signaling which appears to be preserved during differentiation towards hematopoiesis generated via embryoid body or blast-cell colony assays (Gladys Telliam et al. 2022). Using clonogenic cell assays, we have shown that this iPSC cell line PB32 exhibits an increased hematopoietic potential as compared to standard ES cells such as H1 or control iPSC without BCR-ABL expression (G. Telliam et al. 2016).

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To mimic in vitro genetic instability, we have used ENU, a well-established mutagen and alkylating agent (Acevedo-Arozena et al. 2008). We have previously shown that ENU can be used in CML cell lines to generate ABL-kinase mutations that can be selected in vitro by TKI and allows in vitro production of clinically relevant mutations such as T315I substitution (Aggoune et al. 2014).

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After testing the appropriate dosing experiments, we have used ENU in vitro during each daily medium changes. PB32-ENU cells have thus been cultured for > 60 days with PB32 counterparts without ENU. This treatment did not appear to induce any toxicity and no morphological changes in iPSC (data not shown). However, in hematopoiesis induction experiments, we have observed a major increase of hematopoietic potential using either EB or BI-CFC-derived hematopoietic cells (**Figure 1**). Hematopoietic cells generated in these cultures could be expanded in short term cultures, revealing the presence of myeloid precursors and blast-like cell reminiscent of an accelerated phase of CML (**Figure 2**) whereas such pattern was not observed in iPSC cultured without ENU. Interestingly, cytogenetic analyses performed in PB32-ENU cells revealed the presence of several chromosomal abnormalities in addition to Ph1 chromosome (**Figure and Table 1**). These included the presence, in several mitoses (3 / 64 analyzed) deletion of the chromosome derq9, which has been shown to occur at diagnosis (Kreil et al. 2007) but also during progression towards blast crisis (Huntly et al. 2002; Reid et al. 2002). Another significant abnormality observed was the deletion of chromosome 21 in several mitoses in PB32-ENU cells (**Table**

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1). None of these abnormalities are representative of a major or minor route but they have been described in CML patients during blast crisis (Storlazzi et al. 2002). The analyses of PB32-ENU cells as compared to PB32 revealed also the presence of extensive micronuclei formation, suggesting the induction of significant genomic instabilities in these cells (**Supplementary Figure 3**). Micronuclei are characteristics of cells undergoing DNA damage especially in malignant cells (Terradas et al. 2010). They are formed during mitosis and can be due to the presence of double strand DNA breaks (Terradas et al. 2010). Increased micronucleus frequency has been shown to be a feature of myelodysplastic syndromes and has been shown to be higher in patients with refractory anemia with excess of blasts (Kuramoto et al. 2002). To analyze the chromosomal abnormalities at the genomic level, we performed CGH array analyses to compare PB32-ENU-derived hematopoietic cells or BI-CFC's as compared to PB32 without ENU treatment (**Figure 5**). This analysis revealed several major genomic aberrations (**Figure 6 and 7**). As can be seen in Figure 6, in hematopoietic cells we have observed several losses (31%) or gains (69%) interesting all chromosomes and some of major genes involved in oncogenesis such as TET1, ALK, ERG and MALAT1 (**Figure 6**). Several transcription factor genes were also involved such as ZNF or ERG family of transcription factors (**Figure 6**). Functional enrichment on Gene Ontology database showed the implication of SIRT1, EP300 and CDH13, SIRT1 being involved in TP53-dependent DNA damage.

The analysis performed using comparative hybridization of BI-CFC DNA, yielded additional information with losses (71%) and gains (6%), and matching these genomic aberrations with transcription factor database, cancer gene database and pluripotency gene database allowed us to confirm that these affecting essentially chromosomes 7, 8, 15, Y, and X (**Figure 7B**). Circosplot analysis showed that the majority of these abnormalities implicate transcription factors such as MESP (implicated in mesodermal cell migration) and IKZF1 (**Figure 7B and 7F**). Amongst pluripotency genes, we have identified cancer genes (IDH2, NCOA2, IKZF1, BLM) which are already described as been involved in leukemia. Functional enrichment of genomic alterations of PB32 iPSC on KEGG database allowed to highlight perturbations in hematopoietic lineage and cytokine-receptor interaction, affecting: TPO, CSF2RA, ILRA, PIK3RA, CRFL2, cytokines and receptors allowing activation of JAK-STAT pathway (**Figure 7C and 7D**).

We then asked whether the abnormalities identified using CGH arrays could be matched with genomic aberrations described in primary leukemic cells from blast crisis patients. Amongst 249 genes identified by aCGH in CML-iPSC, 125 have been found to be already described in CML progression (**Figure 8 and Supplementary Table 3**). These positively correlating genes include IL3RA, ACPI, FHL1, SH3YL1 (**Supplementary Figure 4**). It has been shown that IL3RA (IL3 receptor alpha) is highly expressed on AML cells (Ehninger et al. 2014) and can be targeted using an anti CD123 monoclonal antibody in CML blast cells (Nievergall et al. 2014). Genes correlating negatively with CML progression included CSF2RA (GM-CSF alpha receptor) which has been shown to be implicated in AML biology (Matsuura et al. 2012). ANPEP is the gene coding for CD13 antigen, which has been shown to be associated with monocytic/myeloid differentiation (Xu 2006). It is of interest to note that we have identified in this screen, this gene to be negatively correlated with CML progression. The relevance of some of the genes identified in the progression of CML in our screen will require further study.

5. Conclusion

In summary, we report in this work the possibility of inducing a genomic instability at the level of a leukemic, patient-derived iPSC. The genomic alterations were detected by the use of CGH arrays and interestingly, upon hematopoietic differentiation, we were able to show cytological and several genomic features of blast crisis in this patient specific genomic background. It remains to be determined if the patients that we analyzed for this purpose was prone to the development of such a tool due to the initial resistance of the leukemic cells to Imatinib. It remains also to be determined if the stem cell model that we

generated this way could lead to the generation of an in vivo model in immunodeficient mice. This first demonstration of the feasibility of using this approach to model blast crisis could open new perspectives for the future use of CML-iPSC for disease modeling and eventually for identification of novel targets if they are validated in primary patient samples.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: “Conceptualization: AGT, ABG, CD, GT, HA; methodology: GT, JL, NO, HA, MA, EB, RMK; software, CD; validation: GT, CD, JL; formal analysis: CD; investigation: AGT, ABG, HA; resources, AGT, ABG; data curation: CD; writing: GT, AGT, CD, ABG.”

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of INSERM.

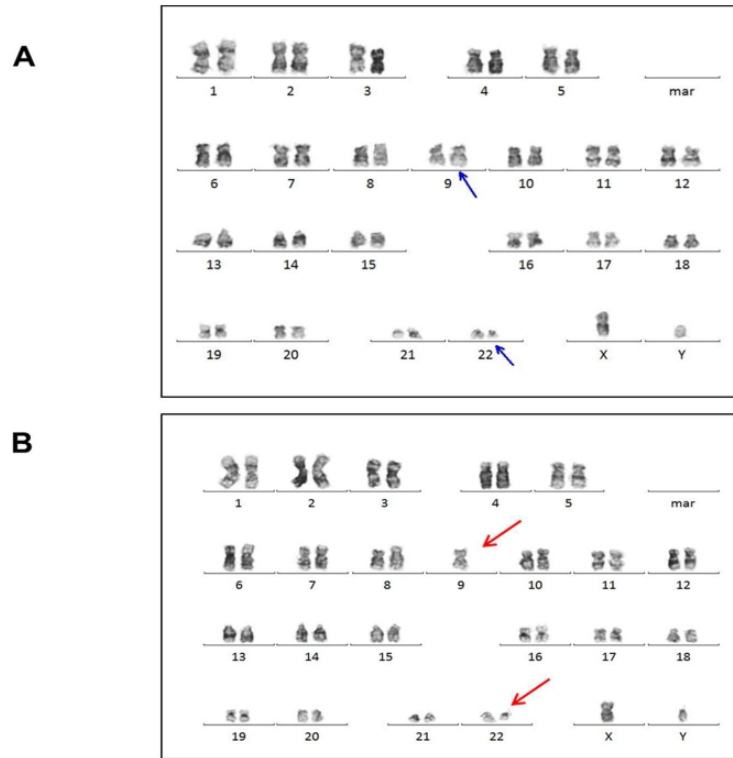
Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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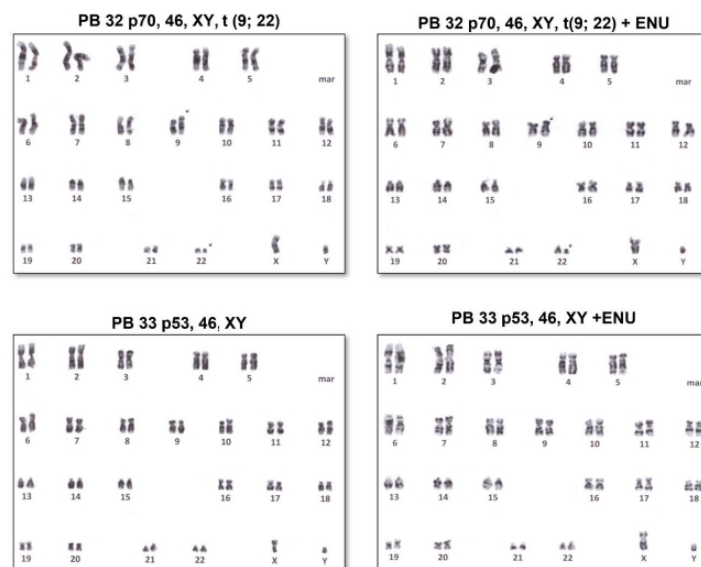
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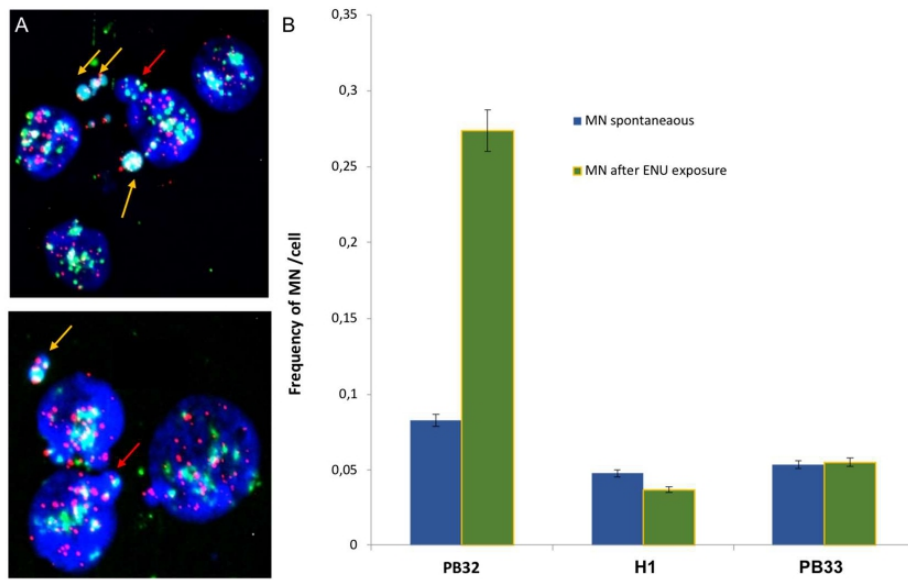
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Supplementary Figure 1: Evaluation of additional chromosome abnormalities in CML-iPSC after longterm ENU culture. (A) Karyotype of CML iPSC with Ph1 chromosome and 9q+. (B) CML iPSC karyotype after 2 months of ENU exposure showing loss of Chromosome 9.



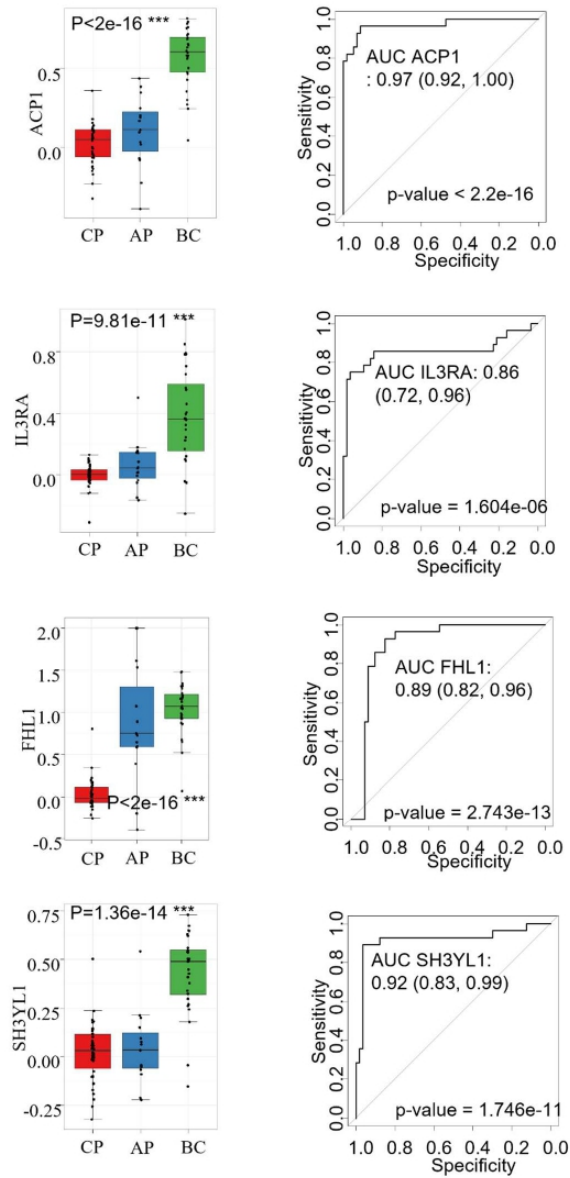
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Supplementary Figure 2: Representative cytogenetic analyses of PB32 (CML-IPC) and PB33 (control iPSC) showing with and without long term ENU culture. P53: passage 53, P46: passage 46.



Supplementary Figure 3: Evaluation of global genomic instability by micronucleus assay in CML-IPSC, control iPSC and human embryonic stem cell line H1 cell line before and after ENU exposure. (A) Photograph of CML-IPSC colonies after ENU exposure. Yellow Arrow show micronucleus and white arrow shows nucleus bud (NBUD's). **(B)** Micronucleus frequencies of CML-IPSC compared to control ESC and IPSC with and without long term ENU exposure.

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Supplementary Figure 4: Genes with negative correlation to CML progression.

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2. Modelling blast crisis using mutagenized chronic myeloid leukemia -derived induced pluripotent stem cells (iPSCs)

The article presented on the first part of my PhD established the basis of genomic instability of the iPSC subjected to mutagenesis. In this second article, the study was extended to two other CML patient-derived iPSCs and to the transcriptomic analysis of these three mutagenized iPSCs. I have also applied a methodology of induction of hematopoiesis to evaluate hematopoietic cell generation from these iPSCs.

In addition to the global tests of genetic instability, we have shown the realm of the mutagenesis by performing an exome analysis. We have then evaluated the hematopoietic potential of the mutagenized iPSC showing the generation of blast cells in vitro. These cells were highly similar to acute myeloid leukemia (AML) blasts and the transcriptome analysis showed a profile enriched in an AML signature. In two of the mutagenized cells, there was an increase of CFU-C as well as a long-term culture-initiating (LTC-IC) cell potential but the cells generated were essentially of CFU-blasts. To shed light into the potential mechanisms of mutagenesis-induced blast cell generation in vitro, I have performed a single-cell RNAseq analysis using 10X technology. This single-cell transcriptomics analysis performed at different time points of hematopoietic differentiation revealed a delay of differentiation for mutagenized cells which could contribute to the emergence of the blastic phenotype. Data analysis based on bulk transcriptomics showed a panel of potential clinically relevant targets and among them the *IL2RA* (*CD25*). In order to determine the validity of these findings in a real-life situation, we have analyzed by RT-qPCR the expression of *IL2RA* in a large cohort of CML patients at diagnosis (n=23) and in blast crisis (n=14) as compared to normal controls (n=15), patients with AML (n=15) and ALL (n=17). *IL2RA* expression was found to be very significantly increased only in CML patients in blast crisis. Interestingly, in seven patients, leukemic RNA was available both at diagnosis and during the progression of the disease towards an acute (blast) phase. In all these 7 patients, *IL2RA* expression was found to be increased.

Thus, this proof-of-principle study established the feasibility and relevance of this in vitro-mutagenesis technology using leukemic iPSC and could represent an “off-the-shelf” tool for discovery of novel targets.

Article

MODELLING BLAST CRISIS USING MUTAGENIZED CHRONIC MYELOID LEUKEMIA-DERIVED INDUCED PLURIPOTENT STEM CELLS (iPSCs)

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Simple Summary: Chronic myeloid leukemia (CML) is a myeloproliferative disease related to the occurrence of the chromosomal translocation t(9;22) in the leukemic stem cells. Although targeting therapies based on tyrosine kinase inhibitors (TKIs) have changed the evolution of CML, disease progression still occurs. Modelling CML progression remains an unmet need in order to understand the mechanisms involved as well as the identification of potential therapeutic targets. Our study aimed to model CML progression by using CML patient-derived induced pluripotent stem cells (iPSCs) mutagenized by the alkylating agent (ENU). Mutagenized CML-iPSC exhibited a monoblastic phenotype after hematopoietic differentiation, an increased hematopoietic potential as compared to their normal counterpart as well as an AML signature. Among different markers observed in this model, we identified CD25 as a progression marker of CML and verified its relevance by evaluating its expression in CML patients in chronic and advanced phases.

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Abstract: Purpose: To model CML progression *in vitro* and generate a blast crisis (BC-CML) model in *in vitro* in order to identify new targets. **Methods:** Three different CML-derived iPSCs lines were mutagenized with the alkylating agent ENU on a daily basis for 60 days. Cells were analyzed at D12 of hematopoietic differentiation for their phenotype, clonogenicity, and transcriptomic profile. Single-cell RNA-Seq analysis has been performed at three different time points during hematopoietic differentiation in ENU-treated and untreated cells. **Results:** One of the CML-iPSCs compared to non-mutagenized counterpart, generated myeloid blasts after hematopoietic differentiation, exhibiting monoblastic patterns and expression of cMPO, CD45, CD33, CD11b, and CD43. Single-cell transcriptomics revealed a delay of differentiation in the mutated condition as compared to the control with increased levels of *MSX1* (mesodermal marker) and a decrease of *CD45* and *CD41*. Bulk transcriptomics analyzed along with the GSE4170 GEO dataset reveal a significant overlap between ENU-treated cells and primary BC cells. Among overexpressed genes, *CD25* was identified and its relevance was confirmed in a cohort of CML patients. **Conclusion:** iPSCs are a valuable tool to model CML progression and to identify new targets. Here we show the relevance of *CD25* identified in the iPSC model as a marker of CML progression.

Keywords: iPSC, Blast crisis CML, CML modeling, Single-cell transcriptomics, CD25.

1. Introduction

Chronic myeloid leukemia (CML) is characterized by the presence of the *BCR::ABL1* fusion gene generated by the reciprocal translocation between chromosomes 9 and 22, t(9;22) (q34.1;q11.2). The *BCR::ABL* fusion protein generated from this translocation is highly oncogenic and characterized by an enhanced tyrosine kinase (TK) activity [1].

CML is a relatively rare disease with an incidence of 1/100 000 and a median age at diagnosis of 55 years. The incidence is higher in males as compared to females (ratio: 1.3-1.5/1) [2].

CML is the prototype of a clonal malignancy of the hematopoietic cell [3]. The disease is initiated by the appearance of the Ph1 chromosome in a hematopoietic stem cell which is followed by the acquisition of novel abnormalities leading to clonal progression, accelerated phase (AP), and blast crisis (BC) [4].

The frequency of this progression has now been considerably reduced since the introduction of tyrosine kinase inhibitors (TKIs) to therapy but BC-CML still occurs albeit at low frequency and remains of dismal prognosis even in the era of tyrosine kinase inhibitors (TKIs) [5], potentially due resistance to TKIs and to clonal selection [5,6]. Using serially collected patient samples, seminal and now classical work have established the main cytogenetic and molecular events observed during the progression of the disease towards AP-CML and eventually BC-CML. These include cytogenetic abnormalities identified as “major” and “minor” routes [7,8].

Several works performed using patient samples and models have also identified, during the last two decades, major molecular events involved in this progression such as TP53 mutations [9]. *BCR-ABL* expressing cells are also prone to develop a mutator phenotype [10] which could be due to the presence of an increased oxidative stress [11] which leads to clonal selection under therapy [12]. CML cells are finally doomed by the deficiency of several DNA repair mechanisms involving DNA-PKcs [13], and BRCA1 [14], as well as abnormalities of specific DNA repair genes such as BLM [15] and other repair mechanisms such as NER [16] or MMR [17].

It is now established that CML leukemic stem cells (CML-LSCs) are resistant to TKI [18–20]. These cells are difficult to isolate and despite recent work suggesting the expression of some markers predominantly on them [21], there are no specific biomarkers universally accepted for CML-LSCs [22,23]. Moreover, current *in vitro* and *in vivo* models do not recapitulate the evolution of human CML, especially with regard to the first stages of the disease and its natural evolution. We and others have shown previously the potential of the use of CML patient-derived induced pluripotent stem cells (iPSCs) for CML modeling [24,25]. These models can recapitulate some aspects of CML such as the myeloproliferative phenotype but they do not allow the generation of a dynamic *in vitro* model of CML progression. This progression occurs through several genomic events over several years in CML patients and the serial analysis of these events in individual cases is not possible. Patient-specific CML-iPSCs exhibit the potential of recapitulating this clonal progression as they can be expanded indefinitely at a pluripotent stage and induced for hematopoietic differentiation using *in vitro* techniques. We have previously shown that the genomic instability inherent to CML leukemic cells can be induced by *in vitro* mutagenesis by the use of N-ethyl-N-nitrosourea (ENU). This allows generation of a genomic instability and could therefore enable the dynamic modeling of CML evolution [26].

Here, we have designed a strategy to mimic AP/BP in CML patients by inducing *in vitro* mutagenesis. Applied to three CML-derived iPSCs, we show that this technology can be used to mimic cytological and genomic instability patterns observed in primary blast crisis cells including the overexpression of *IL2RA/CD25*, that have been validated in a cohort of primary CML patients in AP/BC-CML.

2. Materials and Methods

2.1. Patients

Table 1 shows the clinical characteristics of the three CML patients and their corresponding iPSCs included in the study. CD34+ cells from diagnostic (UPN-27 and UPN-32) and from AP-CML samples (UPN-34) have been used to generate iPSCs [27,28]. This non-clinical study has been approved by the INSERM Ethical committee. All patients and healthy donors gave their informed consent in accordance with the Declaration of Helsinki.

Table 1. Clinical Characteristics of the patients.

Patient #	Age / Sex	Status At Diagnosis	Reprogrammed cells / Origin
UPN27 (PB27)	56/M	CP-CML	CD34+ / Blood
UPN32 (PB32)	17/M	CP-CML	CD34+ / Blood
UPN34 (PB34)	70/M	CP-CML	CD34+ AP-CML/Blood

2.2. iPSC cultures

iPSCs were cultured either in feeder-free or in classical stroma-supported murine embryonic fibroblast (MEF) feeder conditions. Feeder-free cultures were performed in Geltrex coated dishes (ThermoFisher Scientific, A1413201) and fed daily with Essential 8 flex Medium (ThermoFisher Scientific, A2858501). iPSCs were passaged twice a week in aggregates with EDTA dissociation. Alternatively, they were maintained on mitomycin-C – inactivated (MEF) feeder cells with DMEM KnockOut (Gibco, 10829018) supplemented with 10% Knock-Out Serum Replacement (Gibco, 10828010), Glutamax (1X) (Gibco, 35050061) Penicillin/Streptomycin (100 U/mL) (Gibco, 15140122), β -mercaptoethanol and basic-FGF (0.1 mg/mL) (Miltenyi Biotec, 130-093-840). For passaging iPSCs cultured on MEFs, cells have been incubated with collagenase (10mg/mL) (Fisher Scientific, 10780004) for 5-7 minutes at 37°C, 5% CO₂, and the appropriate amount of aggregates was used to seed new MEF coated dishes.

2.3. Teratoma assays

Teratoma assays were performed by injecting 1.10⁶ iPSCs in NOD SCID gamma (NSG) mice. Cells were mixed with Matrigel (150 μ L of Matrigel for 1.10⁶ iPSCs) and injected in the right hind leg of the mouse. 10 weeks after injection, mice were sacrificed and pathological analysis of teratomas was performed.

2.4. N-ethyl-N-nitrosourea-(ENU) induced mutagenesis

CML-iPSCs were cultured in MEFs in the presence or absence of ENU (10 μ g/mL) for 60 days with daily addition of the drug in cultures. They then have been characterized using pluripotency and genomic instability markers and have been adapted to feeder-free conditions needed for further experiments.

2.5. Karyotyping

Karyotype analyses were performed using cell pellets collected at different time points using standard methods as described previously [25].

2.6. Hematopoietic differentiation from iPSCs before and after mutagenesis

Hematopoietic differentiation of iPSCs has been performed using a STEMDiff hematopoietic kit (STEMCELL Technologies, 05310) according to the manufacturer's recommendations. Briefly, iPSCs (cultured on Geltrex with E8-flex medium) have been dissociated in aggregates of 50-100 μm by using EDTA (0,5 mM). 50 aggregates have been seeded per well in a 12-well cell culture plate (Corning, 3513) coated with Geltrex. The medium was changed according to the manufacturer's instruction and cells were harvested on day 12 of hematopoietic differentiation using TrypLE select (ThermoFisher Scientific, 12604021). For single-cell transcriptomics assays, cells were harvested on day +5, day +9, and day +13 of hematopoietic differentiation.

2.7. Evaluation of cytological characteristics of cells before and after mutagenesis

Cells collected from the hematopoietic cultures on days 12-14 of differentiation were analyzed after May-Grunwald-Giemsa staining. Approximately 10^5 cells were washed twice with PBS. Cytospins were performed on slides using a Cytospin 4 centrifuge (ThermoFisher Scientific, USA). Thereafter, slides were air-dried for 25 minutes and stained with RAL Kit 555 (RAL Diagnostics, 361550-0000). The slides were analyzed on a Nikon Eclipse 90i microscope and images were taken with a Nikon camera DS-Fi1 and NIS-Elements V. 5.20 software.

2.8. Flow cytometry

Cells collected from day +12 of hematopoietic differentiation were counted in trypan blue to determine the viability and stained with the following antibodies (Table 2) in PBS at 4°C for 20 minutes.

Table 2. Antibody fluorophores and references

Antibody	Fluorophore	Reference
CD45	PC7	IM3548 Beckman Coulter
CD13	PE	A07762 Beckman Coulter
CD33	APC	IM2471 Beckman Coulter
CD117	PC5.5	B96754 Beckman Coulter
MPO	FITC	IM1874 Beckman Coulter
CD19	ECD	6604551 Beckman Coulter
CD43	PE	A32560 Beckman Coulter
CD3	APC-A750	B10823 Beckman Coulter
CD22	PC5.5	A80712 Beckman Coulter
CD79a	APC	B36287 Beckman Coulter
HLA-DR	ECD	B92438 Beckman Coulter
CD34	APC-A70	B92417 Beckman Coulter

Cells were thereafter washed and resuspended in PBS with 1 $\mu\text{g}/\text{mL}$ 7-Aminoactinomycin D (7-AAD) (Sigma Aldrich, 7240-37-1). Stained cells were analyzed with a BD LSRFortessa™ (BD Biosciences, USA) flow cytometer and FlowJo analysis software.

2.9. Clonogenic assays

Non-adherent cells collected at day +12 of hematopoietic differentiation were counted and plated in methylcellulose-based medium (MethoCult™ H4434, STEMCELL Technologies) at the concentration of 12500 cells/mL and incubated for 14 days in a 37°C incubator with 5% CO₂. On day +14, colonies were enumerated.

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2.10. Long-term culture initiating cell (LTC-IC) assays	179
LTC-IC assays have been performed according to previously reported techniques [29]. Briefly, cells collected at day +12 of hematopoietic differentiation were counted and started on long-term culture assays in triplicates using 4.5×10^4 cells/dish. Cultures were maintained at 33°C on MS-5 stromal cells with weekly half-medium changes (MyeloCult™ H5100, STEMCELL Technologies). At week +5, non-adherent and adherent cells were collected, counted, and plated in methylcellulose (MethoCult™ H4434, STEMCELL Technologies) at the concentration of 5×10^3 cells/dish in triplicates. The number of clonogenic growth was evaluated at days +14 and +21.	180 181 182 183 184 185 186 187 188
2.11. Evaluation of Ruxolitinib and Imatinib sensitivity of PB34 and PB34-ENU hematopoietic cells	189 190
Hematopoietic cells collected from day +12 of differentiation cultures were tested on clonogenic assays with and without TKIs. For each condition, 1×10^4 cells were plated in methylcellulose (MethoCult™ H4434, STEMCELL Technologies) with a TKI drug (alone or in combination) at the following concentrations: 1µM Imatinib (Sigma Aldrich, SML1027) and 1µM Ruxolitinib (Selleckchem, S1378). The number of clonogenic growth was evaluated at day +14. All experiments were done in triplicates.	191 192 193 194 195 196 197
2.12. Patients and healthy donors	198
CD25 mRNA expression was assessed by qRT-PCR on blood samples from 25 patients diagnosed with CP-CML and 14 patients with BC-CML. These experiments were simultaneously performed on the bone marrow of patients with acute myeloid leukemia (AML) and B-cell lymphoblastic leukemia (B-ALL) at diagnosis (Table 3). For acute leukemias, bone marrow aspirates were obtained from each patient at diagnosis. No additional samples were collected for this study. A cohort of 15 healthy donors was also used as a control. Seven patients were analyzed at diagnosis (CP-CML) and after the leukemic transformation (BC-CML). All patients and healthy donors provided informed consent in accordance with the declaration of Helsinki.	199 200 201 202 203 204 205 206 207 208
2.13. Quantitative RT-PCR assays	209
Total RNA from whole blood or bone marrow samples was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA), and qRT-PCR experiments were performed using the StepOnePlus real-time PCR system (Life Technologies). TaqMan pre-developed assays reagent (Life Technologies) were used to quantify CD25 (Hs00907777_m1) mRNA transcripts. CD25 expression was normalized by measuring ABL1 mRNA levels in the same cDNA samples (internal reference). PCR reactions were prepared in duplicates in a final volume of 25 µL using the TaqMan Universal PCR Master Mix (Life Technologies). The CD25/ABL1 percentage was established using the DCt method.	210 211 212 213 214 215 216 217 218 219
2.14. Single-cell RNA-Sequencing of mutagenized and unmutagenized CML-iPSC-derived hematopoietic cells	220 221
Non-adherent cells obtained from the hematopoietic induction cultures were used for this experiment performed on PB32 cells and their counterparts after ENU mutagenesis (PB32-ENU). Cells from day +5, day +9, and day +13 of hematopoietic differentiation were harvested. Three triplicate wells were independently collected for each time point, and cells were counted and further processed using the Chromium10x Genomics® protocol (V3.1). Single-cell Gel Bead-In-EMulsions (GEMs) were generated	222 223 224 225 226 227

using a Chromium Controller instrument (10x Genomics). Sequencing libraries were prepared using Chromium Single Cell 3' Reagent Kits (10x Genomics), according to the manufacturer's instructions. Briefly, GEM-RT was performed in a thermal cycler using a protocol including incubation at 53°C for 45 min and 85°C for 5 min. Post-GEM-RT Cleanup using DynaBeads MyOne Silane Beads was followed by cDNA amplification (98°C for 3 min, cycled 12x 98°C for 15 s, 67°C for 20 s, 72°C for 1 min, and 72°C 1 min). After a cleanup with SPRIselect Reagent Kit and fragment size estimation with High Sensitivity™ HS DNA kit run on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA), the libraries were constructed by performing the following steps: fragmentation, end-repair, A-tailing, SPRIselect cleanup, adaptor ligation, SPRIselect cleanup, sample index PCR, and SPRIselect size selection.

The fragment size estimation of the resulting libraries was assessed with High Sensitivity™ HS DNA kit run on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) and quantified using the Qubit™ dsDNA High Sensitivity HS assay (ThermoFisher Scientific, USA). Libraries were then sequenced by pair with a HighOutput flowcell using an Illumina Nextseq 500 with the following mode: 26 bp (10X Index + UMI), 8 bp (i7 Index), and 57 bp (Read 2).

The sequencing data were processed into transcript count tables with the Cell Ranger Single Cell Software Suite 1.3.1 by 10X Genomics (<http://10xgenomics.com/>). Raw base call files from the Nextseq 500 were demultiplexed with the cellranger mkfastq pipeline into library-specific FASTQ files. The FASTQ files for each library were then processed independently with the cellranger count pipeline. This pipeline used STAR21 to align cDNA reads to the *Mus musculus* transcriptome (Sequence: GRCm38, Annotation: Gencode v25). Once aligned, barcodes associated with these reads – cell identifiers and Unique Molecular Identifiers (UMIs), underwent filtering and correction. Reads associated with retained barcodes were quantified and used to build a transcript count table. The resulting data for each sample were then aggregated using the cellranger aggr pipeline, which performed a between-sample normalization step and concatenated the two transcript count tables.

For each of the 6 experimental conditions after sequencing, fastq files were mapped on GRCh38 human genome processed for demultiplexing with cellranger software version 4.0.0. h5 filtrated outfiles for each experiment were processed individually in R software environment version 4.2.1 with Seurat R-package version 4.1.1 [30]. Single-cell processing was done individually by filtrating cells expressing less than one hundred transcripts and also removing transcripts expressed in less than 3 cells. Before integration, the six experiments were individually log normalized and integration was based on searching common anchors on the twenty-first mathematical dimensions between experiments. After integration, the combined object composed of 63.462 cells and of 28.182 features was scaled. Successive dimension reductions by principal component analysis and t-distributed stochastic neighbor embedding were done to identify cell communities within a graph structure-for example, a K-nearest neighbor (KNN) graph approach [31]. Experiments/cell clusters proportion barplot was drawn with ggplot2 R-package version 3.3.6 [32].

2.15. Exome analysis of mutagenized CML-iPSCs

To determine genetic changes induced by ENU-induced mutagenesis and their stability, we have performed, in the PB32 and PB32-ENU cell line an exome sequencing analysis. Library preparation, exome capture, sequencing and data analysis have been done by IntegraGen SA (Evry, France). Genomic DNA is captured using Agilent in-solution enrichment methodology (SureSelect SureSelect XT Clinical Research Exome, Agilent Technologies, Santa Clara, USA) with their biotinylated oligonucleotides probes library (SureSelect XT Clinical Research Exome - 54 Mb, Agilent Technologies), followed by paired-end 75 bases massively parallel sequencing on Illumina HiSeq4000. Sequence

capture, enrichment, and elution are performed according to the manufacturer's instruction and protocols (SureSelect, Agilent) without modification except for library preparation performed with NEBNext® Ultra kit (New England Biolabs®). For library preparation, 600 ng of each genomic DNA are fragmented by sonication and purified to yield fragments of 150-200 bp. Paired-end adaptor oligonucleotides from the NEB kit are ligated on repaired, tailed fragments then purified and enriched by 8 PCR cycles. 1200 ng of these purified libraries are then hybridized to the SureSelect oligo probe capture library for 72hr. After hybridization, washing, and elution, the eluted fraction is PCR-amplified with 9 cycles, purified, and quantified by qPCR to obtain a sufficient DNA template for downstream applications. Each eluted-enriched DNA sample is then sequenced on an Illumina HiSeq4000 as paired-end 75b reads. Image analysis and base calling are performed using Illumina Real Time Analysis (2.7.7) with default parameters.

2.16. Bulk RNA transcriptomics of CML-iPSC-derived hematopoietic cells before and after mutagenesis

RNA was extracted from the hematopoietic cells derived from the three mutagenized and unmutagenized CML-iPSCs. Samples were processed in duplicate for performing hybridization of Clariom S assay human (ThermoFisher Scientific, USA). Robust microarray analysis (RMA) normalization [33] was performed in Transcriptome Analysis Console (TAC) software (ThermoFisher Scientific, USA) followed by differential expressed gene analysis with limma algorithm [34] between ENU and control conditions of the same individual iPSC. Gene signatures of the three distinct iPSCs were compared by barplot and Venn diagram. Upregulated genes by ENU in PB32 iPSCs were used as a predictive signature of the circulating hematopoietic progenitor of CML patients in blast crisis as compared with ones from patients in chronic phase (Geodataset GSE4170) [35]. This machine learning analysis was done with pamr R-package version 1.56.1 by leave one out cross-validation process [36]. ENU upregulated signature was also used as a geneset during geneset enrichment analysis (GSEA) [37]. Unsupervised principal component analysis was done with FactoMineR R-package version 2.5 [38]. Functional enrichment was done with the Toppgene web [39] application on Gene ontology cellular component database [40]. A functional enrichment network was drawn with Cytoscape standalone application version 3.6.0 [41]. Expression heatmap was drawn with pheatmap R-package version 1.0.12.

3. Results

3.1. Generation and characterization of mutagenized CML-iPS cells

CML-iPSCs from three patients were generated as previously described [27,42] (Table 1). Briefly, reprogramming was realized by using Sendai kit for either purified PB CD34+ cells for PB27 and PB32 or leukemic PBMC for PB34. To induce mutagenesis reprogrammed cells from similar passages were treated with ENU for 60 days (Supplementary Figure 1A). During this period of ENU treatment, there were no significant morphological differences detectable in the iPSC colonies. At day +60, ENU was removed from the cultures. Mutagenized iPSCs have been characterized for the induction of genetic instability markers as well as for their pluripotency. The genomic instability has been evaluated by staining by Western Blotting the phospho- γ H2AX in iPSCs after 30 and 60 days of ENU treatment. An increase of the phospho- γ H2AX was observed for all CML-iPSCs after ENU treatment suggesting that ENU induces genomic instability (Supplementary Figure 1B).

To determine the mutagenic events generated by ENU treatment of CML-iPSCs, we have performed exome sequencing in the PB32 cell line as compared to its ENU-mutagenized counterparts. The goal of this experiment was also to determine the stability of the mutagenic changes observed at the genomic level. As shown in Figure 1B, a large

unidisomy affecting the long arm of chromosome 19 was observed in the PB32-ENU condition (Figure 1B) as compared to the PB32 condition (Figure 1A). The calling of somatic variants was carried out for each of the conditions. After filtration of rare somatic variants (no synonymous missense and stop gained) no variants were found in the condition PB32 (Figure 1C) but 72 variants were identified in the PB32-ENU (Figure 1C). These 72 somatic single nucleotide variants were found to be localized in 57 gene loci across the whole exome (Figure 1C). Pubmed text mining performed on these mutated genes highlighted the importance of *MSH2*, *PEG3*, and *ING1* in genomic instability and *ING1*, *XPC*, and *CRP* in AML. However, for the remaining genes, there are no publications relating them to genomic instability, AML, CML, or LSC (Figure 1D).

The pluripotency of the mutagenized clones was then evaluated by using FACS analysis and teratoma assays. As can be seen in Supplementary Figure 2A-D, mutagenesis did not alter the expression of *SSEA4*, *Tra1-60*, and *OCT4* in all cell lines. In teratoma assays, all cell lines were able to generate *in vivo* the three embryonic layers (Supplementary Figure 2A-D). No additional cytogenetic abnormalities were detectable in PB34 and PB27, although some cytogenetic changes were documented previously in the PB32-ENU cell line [26] at a karyotypic level after ENU treatment. All analyzed clones were Ph1+ (Supplementary Figure 2A-D).

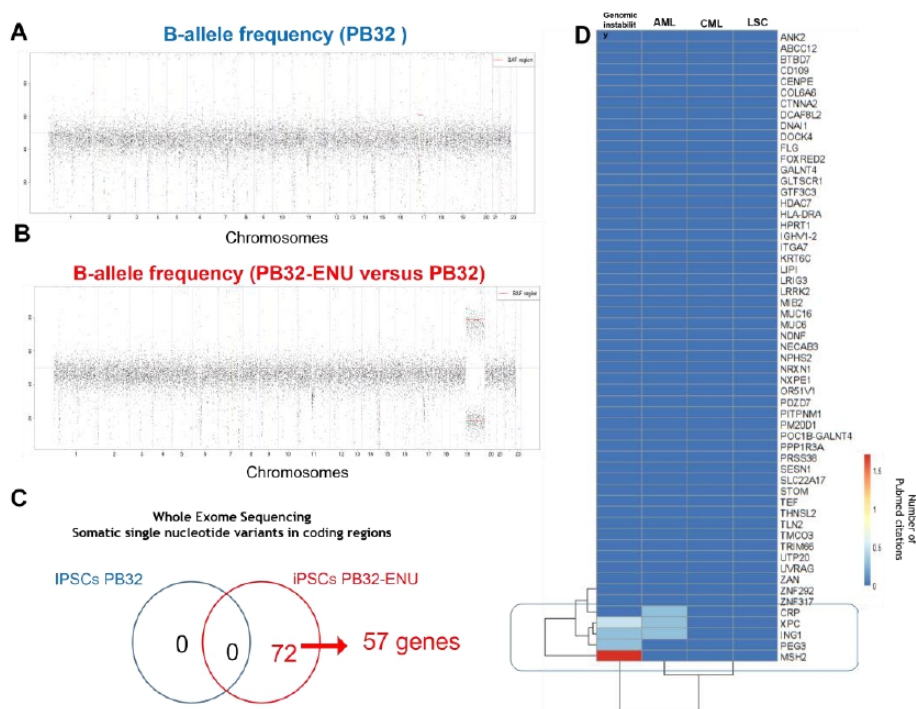


Figure 1. Whole exome sequencing of PB32 in the context of ENU treatment. (A) B-allele frequency plot all over human autosomes of PB32. (B) B-allele frequency plot all over human autosomes comparing PB32-ENU versus PB32. (C) Venn diagram of single nucleotide variations (SNV) found in PB32-ENU versus PB32. (D) Pubmed text mining citation heatmap highlighting genes affected by SNV and known to be implicated in genomic instability-related functions.

3.2. Induction of hematopoietic differentiation from ENU-mutagenized and unmutagenized iPSCs.

We induced hematopoietic differentiation of mutagenized and non-mutagenized iPSCs by using STEMdiff hematopoietic kit (STEMCELL Technologies) (Figure 2A).

Cultures from three CML-iPSCs as well as their mutagenized counterparts were evaluated daily for morphological changes. These cultures showed initially a complete adhesion of iPSC-derived cells to the dishes with no evidence of cells in the non-adherent fraction. Starting day +5, we observed the appearance of budding cells with expulsion to the non-adherent fraction of round cells which gradually increased in numbers (Figure 2B). Nonadherent cells obtained on days 12-14 were then analyzed by MGG staining. As can be seen in Figure 2C, there was a major difference in cytological features between cultures with and without ENU-induced mutagenesis as in ENU-treated cultures there was a clear arrest of differentiation. This difference was highly significant in PB32-ENU condition with a generation of blast cells with cytological features of monoblasts (Figure 2C) whereas hematopoietic cells from non-mutagenized cells exhibited a persistent differentiation with the presence of metamyelocytes and polynuclear neutrophils. Blast cells grew for several weeks in MS-5 stromal cultures in the presence of growth factors (IL-3, Flt-3L, TPO, and SCF) but did not give rise to permanently growing cell lines.

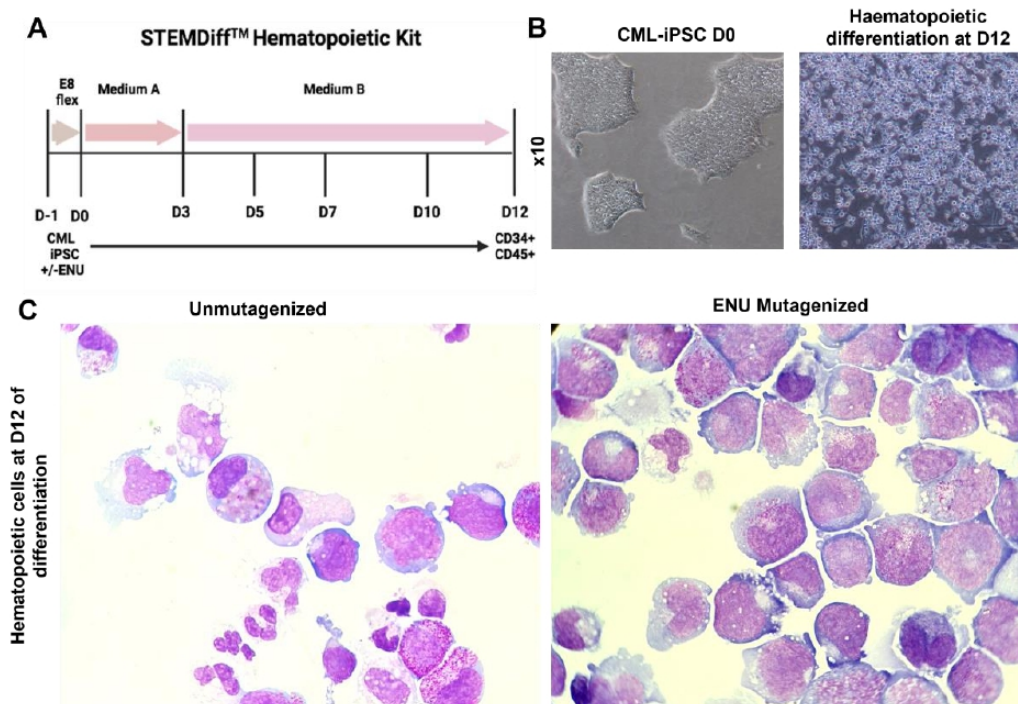


Figure 2: Hematopoietic differentiation of mutagenized and unmutagenized CML-iPSC. (A) Hematopoietic differentiation protocol of mutagenized and unmutagenized CML-iPSCs. During the first three days, the generation of mesoderm is followed by hematopoietic induction from D3 to D12. (B) Morphology of CML-iPSCs at their pluripotent stage (left) and at D12 of differentiation (right). (C) Microscope pictures (magnification x60) of May-Grunwald and Giemsa staining at day 12 of floating hematopoietic cells differentiated from unmutagenized CML-iPSC (left) and mutagenized CML-iPSC (right).

The induction of blast phenotype was highly prominent in the PB32 cell line but in the PB34 cells after mutagenesis, some arrest of differentiation has also been documented cytologically (Supplementary Figure 3). Despite the fact that there was a clear difference between PB27 cells with or without mutagenesis, mutagenized PB27 cells (See below) did not generate a cytologically detectable blast cell phenotype (data not shown).

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3.3. Characterization of mutagenized hematopoietic cells using flow cytometry.

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At day +13 of hematopoietic differentiation, cells were characterized by flow cytometry for different surface markers. Their phenotype analysis showed a typical myeloid profile with an expression of CD45, cMPO+, CD43+, CD33+, CD13+ with the absence of CD19, CD3, and CD79a confirming the myeloid engagement of cells (Figure. 3A-B). Only about 2% of PB32-ENU cells were CD34+, while 90% of PB34-ENU were positive for this marker. PB34-ENU-derived cells were highly positive for c-Kit (CD117). Both cell lines were negative for HLA-DR (Figure. 3A-B).

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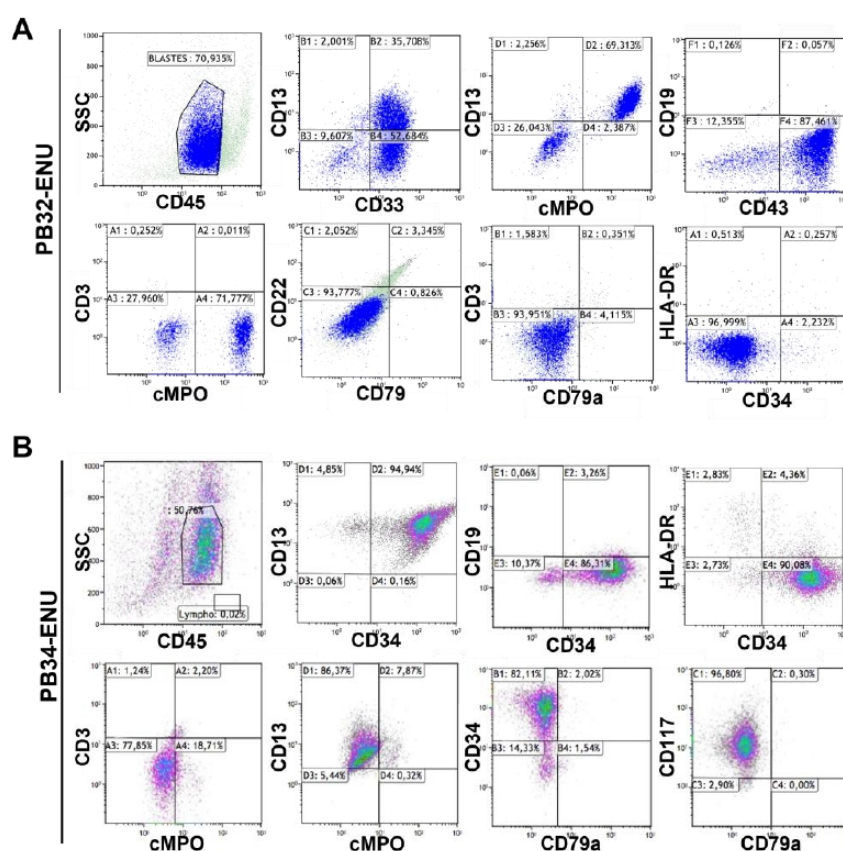
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Figure 3: Phenotypic characterization of in vitro generated blasts. FACS staining for myeloid and lymphoid markers of PB32-ENU (A) and PB34-ENU (B) cells at day 13 of hematopoietic differentiation.

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3.4. Long-term ENU exposure induces an enhancement of CML-iPSC hematopoiesis as compared to normal iPSC-derived clonogenic activity

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Colony-forming cell (CFC) assays have been performed for PB32, PB34, their mutagenized counterparts, and PB33 (control iPSC), after hematopoietic differentiation. At day +14 of hematopoietic differentiation, non-adherent cells were plated in methylcellulose. Colonies were enumerated 14 days after. A major increase in the number of colonies was observed in the PB32-ENU condition as compared to the PB32 and to the control condition (Figure 4A). As can be seen in this figure, non-mutagenized PB32 generated almost no clonogenic activity whereas, in its mutagenized counterpart, the majority of colonies included CFU-GM but also blast cell colonies and large numbers of

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BFU-E. The number of colonies of PB34-ENU was also superior to the control (PB33) but no significant differences were observed with its unmutagenized counterparts (PB34) (Figure 4B). Interestingly, no BFU-E were observed in the PB34 nor in the PB34-ENU condition, colonies were mainly of blast type.

Cells collected on day +14 of hematopoietic differentiation, were then assayed in long-term culture-initiating cell (LTC-IC) experiments to determine their self-renewal potential. As can be seen in Figure 4, only PB32-ENU-derived hematopoietic cells generated a long-term culture potential with essentially blast cell colonies whereas no LTC-IC potential could be obtained in their non-mutagenized counterparts (PB32), and in the control PB33-derived cells. (Figure 4C). All colonies generated from PB32-ENU were CFU-GMSs and blast colonies. PB34 and PB34-ENU also showed colonies with a blastic and CFU-GM morphology and the number of colonies from PB34-ENU show no significant difference compared to PB34 (Figure 4D).

In addition to *BCR::ABL1*, PB34 carry also the mutation *JAK2 V617F* in the same clone as previously reported [28]. Progenitors derived from PB34 and its mutated counterparts were therefore tested for their sensitivity to JAK inhibitor Ruxolitinib, alone or in combination with Imatinib. For both cell lines, a decreased number of colonies was observed while treated with both Imatinib and Ruxolitinib meaning that the ENU treatment did not modify the activity of Ruxolitinib (Supplementary Figure 4).

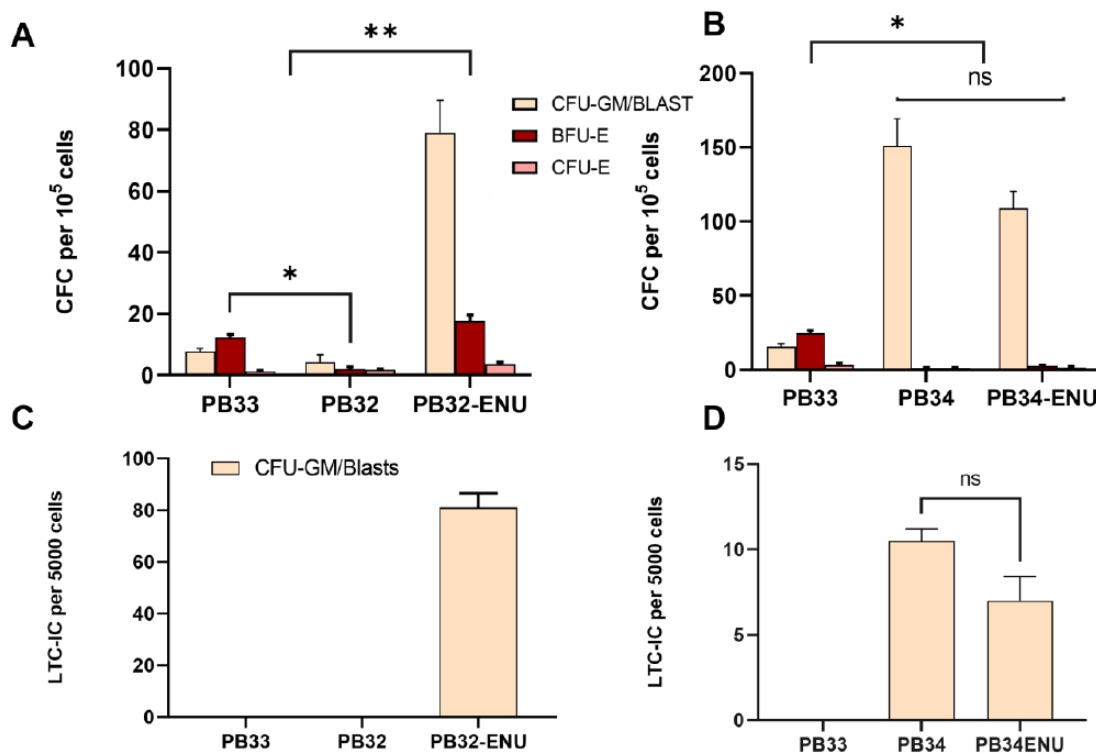


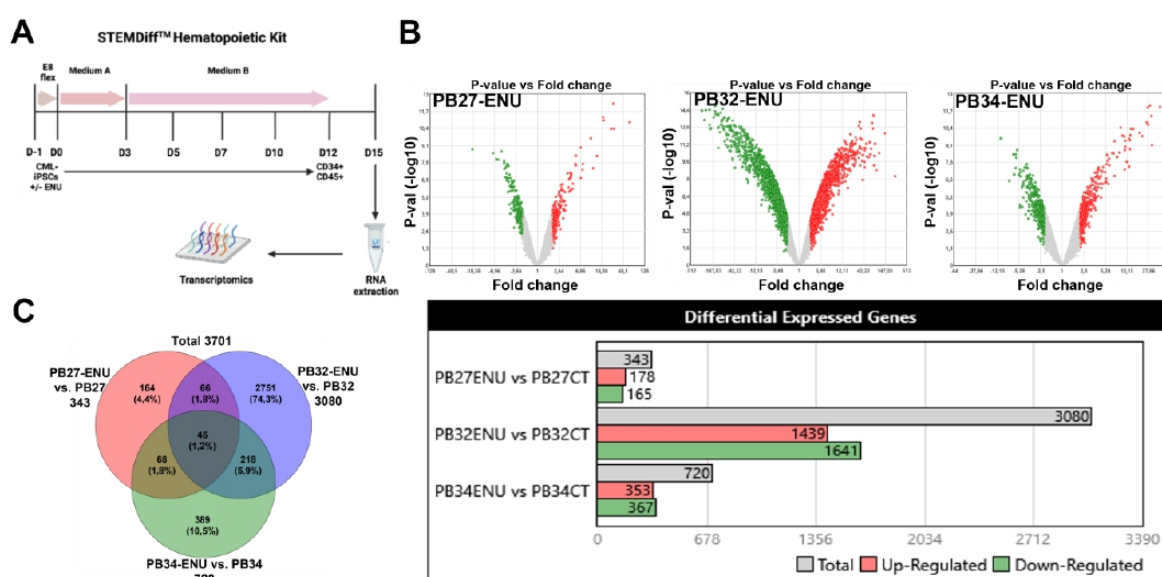
Figure 4: CFC and LTC-IC assays for mutated and unmutated cells at D13 of hematopoietic differentiation. (A) CFC assays for PB33 (WT, no *BCR::ABL*), PB32, and PB32-ENU according to the number of colonies per 10⁵ cells. (B) LTC-IC for PB33, PB32, and PB32-ENU according to the number of colonies per 5000 cells. (C) CFC assays for PB33, PB34, and PB34-ENU according to the number of colonies per 10⁶ cells. (D) LTC-IC for PB33, PB34, and PB34-ENU according to the number of colonies per 5000 cells. All experiments have been performed in triplicates. The total number of

colonies was compared and P-values were calculated using a one-tailed Student's t-test. ns, not significant; *, $P < 0.032$; **, $P < 0.0021$.

3.5. Transcriptomic analysis of hematopoietic cells derived from mutagenized and non-mutagenized CML-iPSCs

Bulk transcriptomics analysis was performed from hematopoietic cells obtained at day +15 of the three distinct mutagenized or unmutagenized CML-derived iPSCs (Figure 5A). A differential gene expression was found between ENU and control condition for the individual three iPSCs allowing us to observe distinct expression profiles. Volcano plots (Figure 5B) after limma analysis highlighted a more important gene signature differentially expressed for the PB32 as compared to PB27 and PB34. These results are shown in Barplot and Venn diagram analyses (Figure 5B and C). Interestingly, we observed an enrichment of PB32-ENU signature with AML profiles and especially with AML5, this enrichment was observed only for PB32-ENU cells and not for their unmutated counterpart (Figures 6A and B). Genes upregulated of PB32 transcriptomics were used as a predictive signature for stratification of hematopoietic progenitors from BC-CML patients as compared with CML patients in CP. This learning machine process allowed us to clearly discriminate patient cells from the two distinct disease phases (Figure 6C) with null misclassification error (Figure 6D). These results suggest that ENU treatment on PB32 cells upregulated genes that reflect blast transformation of the advanced disease stage. GSEA analysis confirmed a significant enrichment of genes upregulated in PB32-ENU cells as compared to their non-mutagenized counterparts (Figure 6E). Unsupervised principal component analysis performed with PB32-ENU upregulated genes on HP of the distinct CML phase: CP: chronic phase, AP: accelerated phase, BC: blast crisis, showed a significant progressive separation of disease phases on the first principal axis (p -value= $1.8e-28$, Figure 6F).

Functional enrichment performed with PB32-ENU upregulated genes on Gene Ontology cellular component analysis was then allowed to identify some membrane markers present in this signature (Figure 6G) confirming the upregulation of these membrane markers in blast crisis as compared with chronic phase by unsupervised clustering (Figure 6G). Among these markers upregulated in BC-CML, *CD25* (alias *IL2RA*) was found to be of interest.



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Figure 5: Transcriptomic profile comparison between three mutated CML-iPSCs and their normal counterparts after hematopoietic differentiation. (A) Hematopoietic differentiation protocol. Transcriptomic analysis was performed at D15 of differentiation. (B) Differential expression analysis (DEG) between ENU and control condition of each CML-iPSCs. (C) Venn diagram comparing the 3 DEG.

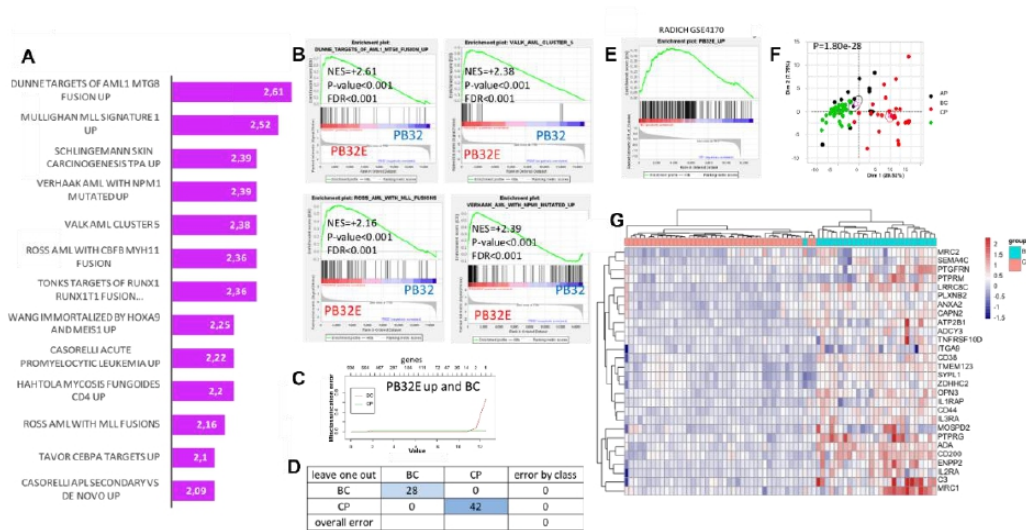


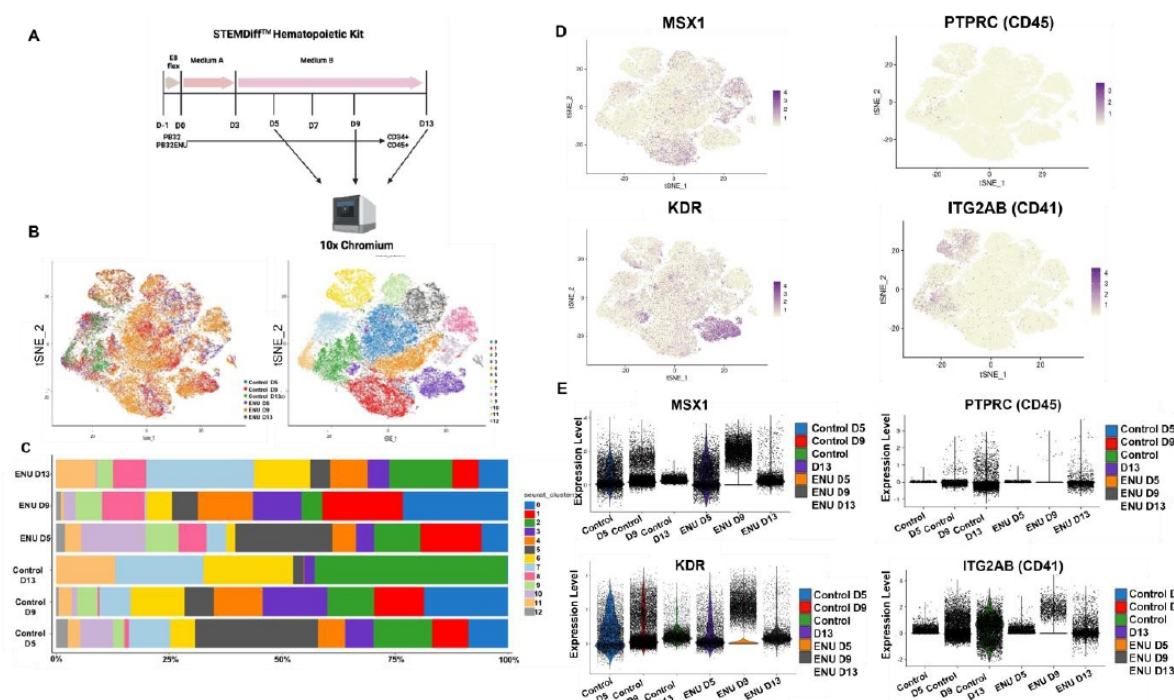
Figure 6: Blast-enriched signatures by ENU on PB32 CML-iPSCs differentiated into hematopoiesis (A) Bar plot of GSE analysis between ENU and control condition for PB32 performed on C MSigDb database. (B) Representation of AML GSE plot enriched signature on PB32 and PB32-ENU (PB32E). (C) Predictive signature for stratification of hematopoietic progenitors from CML patients in blast crisis as compared with ones from CML patients in the chronic phase. (D) Misclassification error. (E) Integration of PB32-ENU signature on GSE4170. (F) Unsupervised principal component analysis was performed with PB32-ENU upregulated genes on HP of the distinct CML disease phase. (G) Upregulated receptors highlighted by integrative analysis during Blast Crisis.

3.6. Single-cell transcriptomics during hematopoietic differentiation of ENU-mutagenized iPSCs as compared to their non-mutagenized counterparts

To unravel the potential molecular events during the hematopoietic differentiation of mutagenized CML-iPSCs, we have chosen the PB32-ENU cell line to perform single-cell transcriptomics. Single-cell transcriptomics was processed by 10x genomics 3' RNA technology on PB32 cell line treated or not with ENU at day +5, day +9, and day +13 of hematopoietic differentiation (Figure 7A). The six individual experiments were integrated into a single-cell object composed of 63.432 cells after preprocessing. After tSNE dimension reduction, it could be observed distinct cell repartition between cells of the distinct experimental conditions corresponds to 13 cell communities (Figure 7B). Evaluation of cell community proportions through the distinct experiments highlighted a reduction in the proportion of cell community number 2 (in green) in condition ENU D13 as compared with control day +13 cells (Figure 7C) corresponding to cells expressing *PTPRC* (alias *CD45*, adult hematopoietic marker) and *ITGA2B* (alias *CD41*, adult megakaryocyte marker) (Figure 7D) suggesting that a less proportion of differentiated hematopoietic cells in conditions treated by ENU as compared to controls. Indeed, *KDR* endothelial marker and *MSX1* mesodermal marker were found to be highly upregulated in the ENU day +9 condition as compared with the control condition (Figure 7E). *KDR* was found to be particularly expressed by cell community number 3 (Figure 7B and D) which is more represented by the ENU day +9 condition than by the control day +9 condition (Figure 7E). *MSX1* mesodermal marker is particularly expressed by clusters 1

and 8 (Figure 7B) and Cluster 8 is mainly present in ENU conditions at distinct times (pink cluster, Figure 7D). In cluster 1 (red cluster) also expressing *MSX1*, this cluster is still present at D13 in the ENU condition but absent in D13 of the control condition (Figure 7E). Altogether, these results suggested that ENU treatment delayed hematopoietic differentiation of CML-derived iPSCs with a still accumulation of mesodermal precursor at day +13 of differentiation.

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Figure 7: RNA Single-cell transcriptomics analysis of PB32 and PB32-ENU at D5, D9, and D13 of hematopoietic differentiation. (A) STEMDiff Hematopoietic differentiation protocol. Cells were analyzed at D5, D9, and D13. (B) tSNE analysis stratified on experimental conditions and cell population. (C) Bar plot of the percentage of cell population in each experimental condition. (D) tSNE analysis of relevant markers of mesoderm (*MSX1*), endothelium (*KDR*), and hematopoietic (*CD45* and *CD41*).

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3.7. *CD25* is overexpressed in BC-CML as compared to CP-CML

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Based on its important clinical relevance, we wished to evaluate the level of *CD25* expression in patients at different phases of CML. A cohort of 22 patients at CP-CML and 14 patients at BC-CML was analyzed for *CD25* expression using qRT-PCR as compared to *ABL* control (Table 3). For the experiments, we have also used blood RNA from normal controls (n=15), CP-CML patients (n = 22), BC-CML (n = 14), AML (n = 15), and Acute lymphoblastic leukemia (ALL) (n = 17). As can be seen in Figure 8A, as compared to the expression found in normal blood, CP-CML, and ALL, the expression of *CD25* was found to be increased in BC-CML (percentage of *CD25/ABL*=19.3 in CP-CML and 75 in BC-CML) and AML.

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CD25 mRNA expression was also evaluated comparatively in seven CML patients both in CP and after their evolution to BC-CML. *CD25* upregulation was clearly observed in all patients during BC-CML, with a 5- to 10-fold increase in 4 out of 7 patients. (Figure 8B).

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Table 3. Characteristics of the different cohorts tested for CD25 mRNA expression. CP-CML, chronic phase CML; AP-CML, acute phase CML; AML, acute myeloid leukemia; B-ALL, B-cell lymphoblastic leukemia); N/A, anonymized healthy donors.

	Controls	CP-CML	BC-CML	AML	B-ALL
n	15	23	14	15	17
Gender					
Male	N/A	13	11	9	5
Female	N/A	10	3	6	12
Age, range (years)	N/A	58.1 [22.7-81.1]	47.4 [22.9-75.2]	67.6 [26.0-83.6]	37.8 [4.6-82.5]
<i>Molecular rearrangements</i>					
<i>BCR::ABL1</i> <i>M-BCR (e13a2 or e14a2)</i>		23	14		
<i>BCR::ABL1</i> <i>m-BCR (e1a2)</i>					5

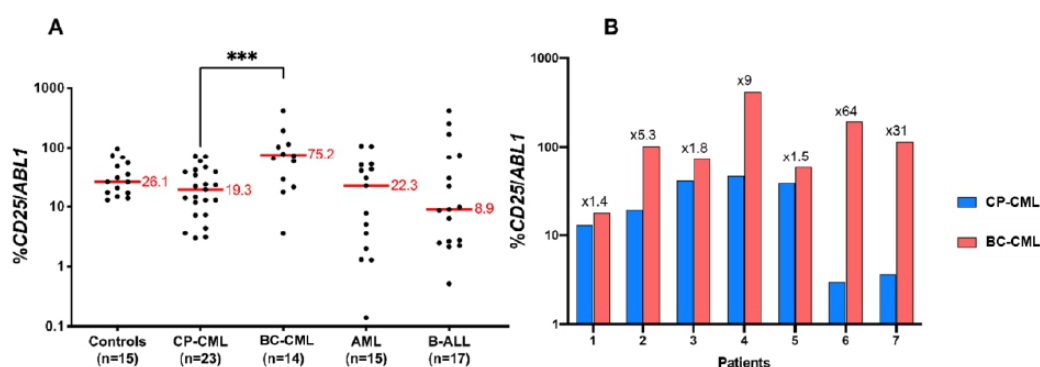


Figure 8: CD25 expression in CP-CML, BC-CML, AML and ALL patients

(A) CD25 is overexpressed in BC-CML patients. The scatter dot plot shows the CD25 mRNA expression in CML patients in the chronic phase at diagnosis and patients in the acute phase compared with healthy donors and patients with acute leukemias. For each group, the median is shown in red. P-value was calculated by the two-sided Mann-Whitney test. The three (***) asterisks indicate a P value < 0.001. (B) CD25 mRNA levels are increased in all CML patients who progressed to an acute phase. The interleaved bar diagram shows the evolution of the CD25 mRNA expression in 7 patients who were analyzed at the chronic and blast phases. CD25 expression is shown in a logarithmic (Log10) scale, and the multiplication coefficient between the two phases is shown. Two data points are outside the axis limits (>1000).

4. Discussion

BC-CML remains a life-threatening complication of CML and still occurs in the TKI era, especially in patients with TKI resistance. Modelling BC-CML is therefore an unmet need for developing new biomarkers in order to predict BC progression and discover new therapies. The goal of this work was to take advantage of the theoretically unlimited proliferation potential of iPSCs, to create a progression model of CML in vitro, using patient-derived iPSCs. We have previously shown the feasibility of generating CML-patient-derived iPSC using leukemic cells [27,42]. To model the blast crisis which is due to the accumulation of genetic events in the context of *BCR::ABL1* expression, we have chosen the use of the alkylating agent ENU. We have previously shown that this

procedure gives rise to one of the PB32 cell lines used here, a global genomic instability [26]. In this work, we have further analyzed this event in three CML-iPSCs by generating two additional CML-iPSCs mutagenized by ENU treatment. CML-iPSCs treated with ENU, exhibited increased levels of γ -H2AX phosphorylation, suggesting a DNA damage response (**Supplementary Figure 1**). To further evaluate the ENU-induced mutagenesis we performed exome sequencing to uncover the mutations and their stability at the genetic level. Mutations identified in the ENU-treated condition confirm the mutagenic effect of ENU and some of the affected genes are shown to be involved in the genomic instability in cancer. We then performed hematopoietic differentiation assays from ENU-mutagenized iPSCs as compared to their unmutagenized counterparts. In these experiments, the PB32-ENU cell line showed major and reproducible morphological changes, with *in vitro* generation of large numbers of blast cells indistinguishable from AML of monoblastic phenotype (AML-5). These experiments were highly reproducible using this cell line (n = 10 experiments). These cells proliferated also in the MS-5 cell line but did not generate growth-independent cell lines and did not give rise to leukemia growth in NSG mice (data not shown). Between the other two iPSCs lines, the “blast” type of transformation was not observed but in the PB34-ENU cell line (expressing also *JAK2 V617F* mutation), we have observed cytological changes with increased numbers of undifferentiated cells at day +12 and +14 of differentiation. In the PB27 cell line, although there was a clear transcriptomic difference between the ENU-mutagenized iPSC and its unmutagenized counterpart, we have not observed a cytological difference at day +12 of the cultures. It is most likely that these differences highlight not only patient-related genomic sensitivity to mutagenesis but also disease-related differences.

In the PB32-ENU-derived hematopoietic cells, we observed phenotypically exhibited blast cell surface markers such as MPO, CD34, CD45, CD33, CD43, CD13, and CD33 (**Figure 3**). A difference in the level of expression is an interesting indicator of the type of AML generated, for instance, low levels of CD34 for PB32 correspond to AML [43] which has also been found in the transcriptomic results (**Figure 6**). On the other hand, PB34-ENU exhibit high levels of CD34+ related to another type of AML (M0-M1) [44]. Meantime both cell lines PB32-ENU and PB34-ENU are CD3-, CD79-, and CD19- showing definitely their myeloid characteristics. In clonogenic assays, there was an increase in the number of colonies for the PB32-ENU compared to unmutagenized PB32 (more than ten fold) and PB33 (**Figure 4A**). This increased number of colonies could reflect the undifferentiated status of mutated cells observed morphologically and phenotypically. The majority of colonies exhibited blast morphology reinforcing the hypothesis that the increased number of colonies is directly related to the arrest of differentiation. PB34 and its mutated counterpart showed also an increased number of colonies compared to the control but no significant difference was observed between the ENU-treated and untreated PB34. This might be related to the clinical background of the patient UPN-PB34 who was already in AP-CML and developed later a BC-CML (**Table 1**). Interestingly, no BFU-E were observed among the PB34 nor in PB34-ENU, there were all heterogenous with a CFU-GM and blast cell colony morphology. Another interesting characteristic was the important increase in primitive hematopoietic stem cells in PB32-ENU assessed by LTC-IC (**Figure 4C**) which is consistent with the arrest of differentiation seen morphologically and consequently with the increase of the stemness of the blast cells. As for the CFC assays, LTC-IC assays of PB34 and PB34-ENU show no significant differences (**Figure 4D**). The effect of Ruxolitinib alone or in combination with Imatinib on PB34 and PB34-ENU cells shows that ENU mutation does not affect the effectiveness of these drugs on the *JAK2 V617F* mutation (**Supplementary Figure 4**).

The transcriptional analysis confirmed that PB32-ENU exhibited the highest number of mutated genes followed by PB34-ENU and PB27-ENU (**Figure 5B**) and that was consistent with the potent BC-CML phenotype showed by PB32-ENU when compared to

BC datasets (**Figure 5B**). PB32-ENU enrichment signatures showed an AML profile whereas it is not the case for PB32. This shows the effect of the ENU on the *in vitro* blastic transformation of the disease. Comparing GSEA4170, which contains expression profiles of CML patients in all three different stages of CML, and PB32-ENU shows the relevance of our model through its high correlation with transcription profiles of patients in BC-CML (**Figure 6E**). This correlation with BC-CML patients allowed us also to discover the expression in PB32-ENU of a group of genes expressed also in patients in BC-CML (**Figure 6G**) and among which some are potential targets such as *IL2RA* (*CD25*). *CD25* has already been described on AML blasts, BC-CML, and LSCs [45,46] as an aberrant marker and related to bad prognosis, it is also a potential target for new therapies in BC-CML and AML.

To uncover the mechanisms of the generation of mutagenized hematopoietic cells from iPSCs, we have performed single-cell transcriptomics in the most 'performant' CML-iPSC line, PB32, during hematopoietic differentiation. For this experiment, we analyzed single cells at three different time points in order to better understand the effect of ENU during differentiation and the cell populations affected by the mutagenesis. Single-cell data reinforced the results we had obtained previously by confirming the hypothesis for a delay of differentiation due to ENU treatment. We could observe a change in different communities of cells between mutated and unmutated cohorts, as well as different genes. Among these genes, the most striking was the reduction of *CD45* (a marker of differentiated hematopoietic cells) and *CD41* (a marker of megakaryocytes) in the ENU-treated cells compared to untreated one, suggesting strongly the arrest of hematopoietic differentiation observed morphologically and phenotypically. Moreover, the increase of *MSX1* (a marker of mesoderm) and *KDR* (a marker of hemangioblast), in ENU-treated cells but not in untreated cells reveals the accumulation of mesodermal and primitive markers and consequently the arrest of differentiation.

After the identification of *CD25* expression in PB32-ENU and the correlation with the cohort of CML patients, we wished to evaluate the real amount of its expression during the progression of CML toward an AP/BC phase. Using a large number of CP-CML (n = 22) progressing toward BC-CML (n = 14) and AML cases, we have shown that the expression of *CD25* is increased during the progression of CML and this could be a potential therapeutic target (**Figure 8A and B**). Other targets that we have identified are currently under study.

Overall, these experiments validate the relevance of our model for analysis of CML progression using iPSCs due to their major proliferation potential and their potential of use as "off-the-shelf" tools for individual CML patients but also in other progressive hematological malignancies such as myelodysplastic syndromes (MDS) and AML.

The clinical background of the patients and individual differences are certainly a limit and these experiments need to be extended to other CML-iPSCs, which might generate an interesting tool to predict the probability of CML progression in patients. The validation of discovered markers in patients is proof of the representativeness of our model and it can be used for drug screening or novel drug discovery.

5. Conclusions

CML-iPSCs represent a novel and disruptive technology for modelling pathophysiology of CML and for discovering novel targets. ENU-induced *in vitro* mutagenesis allows the generation of a dynamic, *in vitro* model of CML progression allowing discovery of relevant biomarkers and novel targets. Several novel targets identified are now under study.

Supplementary Materials: The following supporting information can be downloaded at: 665
www.mdpi.com/xxx/s1, 666
Supplementary Figure 1: Generation of ENU mutagenized CML-iPSC. **Supplementary Figure 2:** 667
 Pluripotency confirmation of mutagenized and unmutagenized CML-iPSC with ENU. 668
Supplementary Figure 3: Morphological changes observed during hematopoietic differentiation of 669
 ENU mutagenized and unmutagenized CML iPSC and MGG staining at D12 of differentiation. 670
Supplementary Figure 4: Imatinib and Ruxolitinib sensitivity assay. 671

Author Contributions: Conceptualization: J.I., P.M., C.D., A.T.; methodology: J.I., P.M., C.D., A.T.; 672
 software: C.D.; validation: J.I., A.B-G., A.T.; formal analysis: J.I., Y.E., A.T.; investigation: J.I., P.M., 673
 A.T.; resources: J.I., P.M., T.L., Y.E., P.H., S.S., N.S., G.T., E.C., J.C.C.; A.T., data curation: J.I., P.M., 674
 A.T., writing-original draft preparation: J.I., A.T.; writing-review and editing: J.I., P.M., C.D., A.T.; 675
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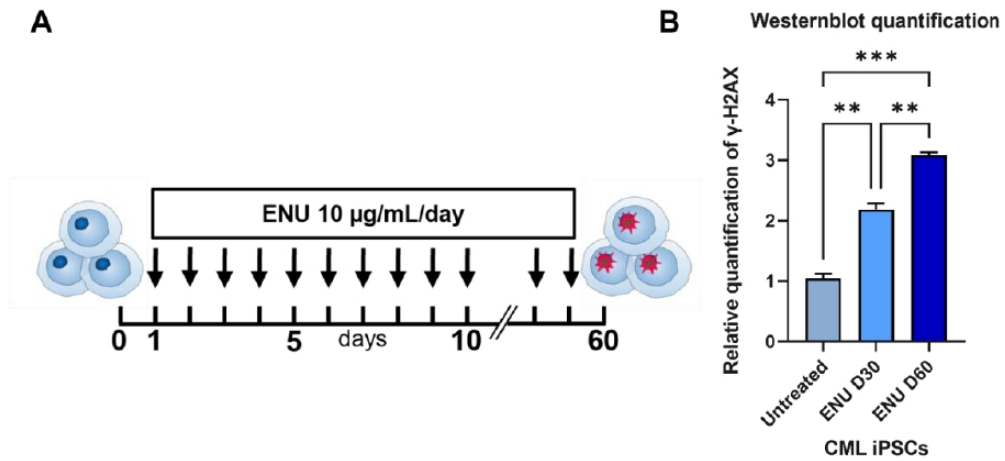
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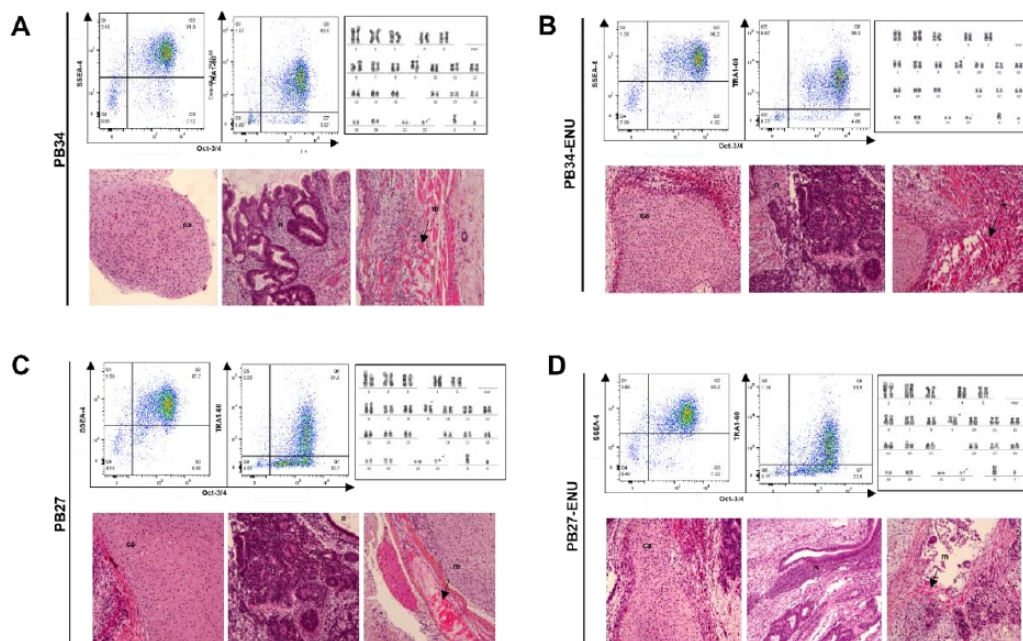
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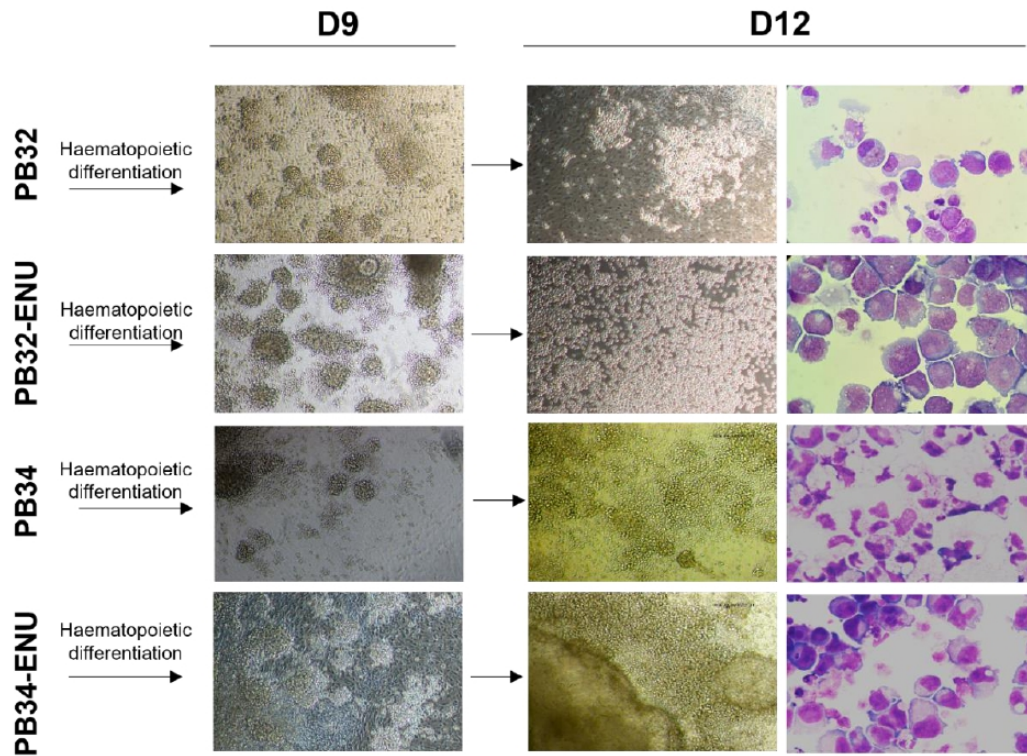
Supplementary Figure 1: Generation of ENU mutagenized CML-iPSCs.

(A) Schematic representation of CML-iPSC mutagenesis with ENU. Arrows show the daily treatment with ENU for 60 days. (B) Relative quantification of γ -H2AX in ENU-treated CML-iPSC at day 30 and day 60 using western blot analysis. Phospho γ -H2AX was normalized to b-actin and control. Mean of 3 CML iPSCs treated with ENU were compared and P-values were calculated using one-tailed Student's t-test. **, $P < 0.0021$; ***, $P < 0.0002$.



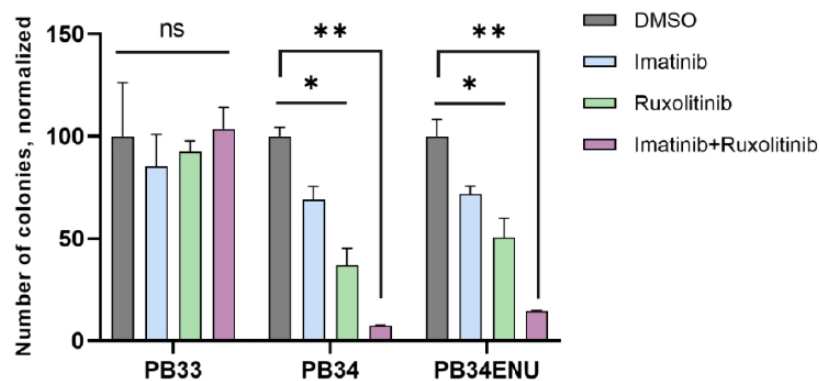
Supplementary Figure 2: Pluripotency confirmation of mutagenized and unmutagenized CML-iPSCs with ENU.

Pluripotency was tested by flow cytometry, teratoma induction, karyotype checking for absence of chromosomal aberrations and presence of Ph⁺ chromosome for PB34 (A), PB34-ENU (B), PB27 (C), PB27-ENU (D).



Supplementary Figure 3: Morphological changes observed during hematopoietic differentiation of ENU mutagenized and unmutagenized CML-iPSCs and MGG staining at D12 of hematopoietic differentiation.

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Supplementary Figure 4: Imatinib and Ruxolitinib sensitivity assays in PB34 CML iPSC expressing both BCR::ABL and JAK2V617F mutation.

Number of colonies normalized to control (PB33) after incubation either with Imatinib, Ruxolitinib or combined for PB34 and PB34-ENU hematopoietic derived cells. Mean number of colonies treated with TKIs were compared to control DMSO for each CML-iPSC and P-values were calculated using one-tailed Student's t-test. ns, not significant; *, $P < 0.032$. **, $P < 0.0021$.

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3. Chimeric antigen-receptor engineered (CAR) natural killer (NK) cells in a chronic myeloid leukemia (CML) blast crisis model

In this article, I followed the results obtained in the previous study which showed the relevance of the overexpression of CD25 expression in CML progression. Our laboratory has described several years ago a technology for generation of functional NK cells from human embryonic stem cells and is now developing NK-cell differentiation technologies from iPSCs for future cell therapy applications. From this regard, the use of a CD25 appeared to be an interesting target.

I have therefore, in this part of my PhD project, set up the experimental tools to evaluate the feasibility of targeting CD25/IL2RA in the experimental setting using NK cells as tool and CD25/IL2RA as a target, with the goal of generating CD25 CAR-NK cells. For this purpose, we have designed two CD25 CAR lentiviral vectors, either under the control of a constitutive promoter (EF1- α) or TRE promoter (Doxycycline inducible). In order to have an experimental cellular tool prior to translate this technique to iPSC-derived NK cells, we have used the NK92 cell line which exhibits a high cytotoxic activity against leukemic cell targets.

In order to achieve that we transduced the NK92 cell line with the CD25 CAR vector under the control of the constitutive promoter. To obtain an appropriate leukemic cell target, I have engineered a K562 cell line expressing CD25 (K562-CD25) using a lentiviral gene transfer strategy.

Preliminary data *in vitro* showed a higher cytotoxic ability of CD25 CAR-NK92 cells against the K562-CD25 as compared to NK92 WT. Preliminary *in vivo* results have been performed in a series of 10 mice generated promising results but novel *in vivo* experiments are underway in larger cohort of mice. Finally, as it is described in this article in preparation, I have transduced an iPSC line generated in our laboratory with both CD25 CAR constructs (constitutive and inducible) and showed the expression of CD25 CAR in the transduced iPSCs. Thus, all tools for future generation of iPSC-derived CD25 CAR-NK cells have been established and work for their differentiation into NK is in progress.

Article 3 (In preparation)

CHIMERIC ANTIGEN-RECEPTOR ENGINEERED (CAR) NATURAL KILLER (NK) CELLS IN A CHRONIC MYELOID LEUKEMIA (CML) BLAST CRISIS MODEL

Authors

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SUMMARY

During the last two decades, the introduction of tyrosine kinase inhibitors (TKIs) to the therapy changed the natural history of CML but progression into accelerated and blast crisis (AP/BC) remains still an unmet medical need to be addressed. Indeed, in TKI-refractory patients in advanced phases of their disease, the only curative option is the stem cell transplantation. We and others have shown the relevance of IL2RA/CD25 as a biomarker of CML progression suggesting its potential use as a therapeutic target for CAR-based therapies. Here we show the development of a CAR-NK therapy based on the scFV of the clinically approved anti-CD25 monoclonal antibody (Basiliximab) using both a constitutive and Doxycycline (DOX) inducible strategies. We report the efficacy of both strategies to target engineered K562-cells expressing CD25 *in vitro* and *in vivo* in NSG mice. We also show that CD25 CAR expression can be successfully introduced in iPSC lines, with the goal of generating iPSC-derived CD25 CAR-NK cells in future experiments. These proof-of concept of this study could represent a first step for the further development of this technology in refractory/relapsed (R/R) CML patients in BC as well as in R/R acute myeloblastic leukemias (AML).

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the t(9;22) reciprocal translocation leading to the generation of the Philadelphia (Ph1) chromosome in a very primitive hematopoietic stem cell [1]. Ph chromosome is responsible for the generation of a fusion protein, BCR::ABL. Therapies targeting the tyrosine kinase (TK) activity of BCR::ABL have changed the prognosis of this leukemia with prolongation of the survival. However, the majority of the patients fail to achieve deep molecular response which is required for TKI-cessations attempts, leading to molecular relapses. The highly quiescent leukemic stem cells (LSCs) are known to be resistant to TKIs [2,3] and responsible for relapse after TKI treatment cessation [4–7]. Therefore the majority of patients require the administration of life-long TKI therapies and a large fraction of these develop resistances to these drugs. TKI resistance and disease progression into an AP/BC remain problematic especially in this last group of patients as the only curative approach remains stem cell transplantation which cannot be applied especially on patients lacking donors.

New therapies targeting new targets expressed in advanced phases of CML and blast crisis remains an unmet medical need even in the era of TKI therapies.

CD25 (also known as the α subunit of IL-2 receptor) is one of the three subunits that constitute the receptor to the IL-2, the two others are the β and the γ subunit or CD122 and CD132 respectively [8]. In normal conditions, CD25 is highly expressed on activated T cells and regulatory T cells, which plays an important role in the homeostasis of T cell activity and immune tolerance [9,10]. CD25 is also shown to be expressed in, BC-CML, BC-CML transformed into acute myeloid leukemia AML but also *de novo* AML with dismal prognosis [11–17]. These studies have shown the CD25 expression by flow cytometry (proteic level) proposing thus its detection as prognosis marker. More interestingly, CD25 is shown to be aberrantly expressed in the LSC of CML rendering it a very interesting target for the eradication of the LSC and the CML treatment [18].

We have previously shown the discovery of the CD25 as an overexpressed marker in a BC-CML model generated by the use of patient-specific iPSC and confirmed the increase of its expression during CML progression (Imeri et al., 2022, Submitted). These findings prompted us to evaluate the possibility of targeting this receptor using chimeric antigen receptors (CAR)-based therapies in the setting of CML.

CARs are fusion proteins introduced into lymphocytes (T and NK) and recently into macrophages to confer them specific recognition skills. CAR proteins contain an extracellular domain composed of the single chain variable fragment (scFV) which allows the specific recognition of the target and intracellular domains responsible for the lymphocyte activation [19]. First-generation CARs contained only CD3 ζ (for the cell activation), while second-generation CARs possess also a costimulatory

endodomain (CD28 or 4-1BB) whereas third-generation CARs have two costimulatory domains [19]. During the last decade, cell therapies based on CAR technology have shown groundbreaking results in R/R hematological malignancies leading to their clinical licensing in acute lymphoblastic leukemias (ALL) and lymphomas, by essentially targeting the B-cell marker CD19. The efficacy of CAR-T cells against solid tumors has been more limited due to tumor site accessibility, immunomodulatory properties of the tumor microenvironment and the lack of identification of targets suitable for clinical translation [20,21]. The use of CAR-T cells involves systematic autologous use of T cells generating major technical issues from their generation up to their clinical use, including the possibility of collecting sufficient numbers of functional T cells [22]. It has been shown that, due to their high efficiencies against target cells, CAR-T cells can provoke life-threatening cytokine-releasing syndromes as well as severe neural cytotoxicity [23]. In order to circumvent these limitations, recently, NK cells have been used for CAR therapies. NK cells have the innate ability to kill cancer cells or virally-infected cells [24]. Additionally, they have the advantage to induce cytotoxicity in an HLA-independent manner and do not give rise to graft-versus-host-disease (GvHD) which is the major therapeutic problem in the setting of allogeneic stem cell transplantations. The activation of NK cells is a stochastic balance between activating and inhibiting receptors expressed on the surface of NK cells [24,25]. Other advantages of NK cells over T cells include the absence of cytokine release syndrome or neurotoxicity [26,27], their antibody-dependent cellular cytotoxicity (ADCC) and their ability to kill tumor cells in a CAR-independent manner [28]. NK cells have been shown to have an antileukemic effect against CML cells but this effect is reduced with the progression of the disease and TKI long-term treatment through the increase of inhibitory ligands on cancer cells and the decrease of activating receptors on NK cells [29].

Different sources of NK cells have been used so far in CAR therapies such as NK cells from PBMCs, cord-blood, NK cell lineages (such as NK92), CD34+ HPSC, or iPSC generated NK cells [28]. All of these sources have their own advantages and limitations mostly based on their accessibility, cryo-preservation, time of generation, expansion, survival time and the type of cancer. NK92 cell line may address some of these limitations thanks to their activated profile and unlimited proliferation ability. Clinical trials have demonstrated their safety as allogeneic cell therapies in patients with advanced malignancies [30,31]. Nevertheless, their use requires a complete inactivation via irradiation prior to their administration.

Here we show the experimental development of a third-generation CAR-NK therapy strategy against the IL2RA/CD25 which we have previously reported as being overexpressed in advanced CML. This strategy is based on the use of the scFV of the clinically approved monoclonal humanized antibody, Basiliximab recognizing specifically CD25 and targeting K562 cells that we have genetically

engineered to overexpress CD25. We therefore use the intrinsic antileukemic potential of NK92 cells and the increased specificity and activation through the CAR constructs to target K562-CD25 cells. We demonstrate an increased cytotoxic activity of CD25 CAR-NK92 cells against leukemic cells expressing CD25 and their efficiency *in vivo*. We also report the feasibility of generating iPSC cells expressing our CAR constructs using both constitutive and DOX-inducible vectors.

MATERIALS AND METHODS

Cell culture

The Lenti-X 293T cell line (a kind gift from Dr. Lucas Eduardo Botelho de Souza) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, 11560636) and 100 U/mL of Penicillin-Streptomycin (PenStrep) solution (Gibco, 11548876). K562 were cultured in RPMI 1640 (Gibco Ref, 11875093) supplemented with 10% of FBS and 100 U/mL of PenStrep. NK92 were cultured in RPMI 1640 supplemented with 20% of FBS, 100U/mL of PenStrep, and 150U/mL of IL-2 (Miltenyi Biotec, 130-097-745). NK92 and K562 cells were passaged every 3 to 4 days at a dilution of 3×10^5 cells/mL and Lentix were plated at $3,5 \times 10^4$ cells/cm².

iPS cell culture

iPSC lines were generated by reprogramming cord blood CD34+ cells with Cyto-Tune 2.0 Sendai Reprogramming Kit (A16517, ThermoFisher Scientific). Pluripotency was characterized by immunofluorescent staining of pluripotent markers (Tra1-60, Sox2, Nanog and SSEA-4) as well as by teratoma induction in NOD-SCID mice. iPSC culture was performed in feeder-free conditions in Geltrex coated dishes (A1413201, ThermoFisher Scientific) and fed daily with Essential 8 flex Medium (A2858501, ThermoFisher Scientific). iPSCs were passaged twice a week in aggregates by using EDTA.

Production of lentiviruses and viral transduction

In order to produce IL2RA expressing lentiviruses, we used Lenti-X 293T as a packaging cell line and ps-PAX2.2, and pMD2.G as packaging vector and envelope vector, respectively. Briefly, the Lentix-293T cell line was cultured on a T150 mm dish as described above and co-transfected by lipofectamine 3000 reagent (ThermoFisher, L3000015) with 20 µg of packaging vector ps-PAX2.2 (Addgene, USA), 10 µg envelope vector of pMD2.G (Addgene, USA) and 30 µg transfer vector (EF1α-hIL2RA). The supernatant was collected at 24h and 48h. K562 cells were transduced by the use of unconcentrated virus at three different dilutions. Transduced cells were spinoculated at 1200 rpm for 90 minutes at RT and 8 µg/mL of Polybrene was added. After 48 hours, the virus was removed and cells were stained for anti-CD25 as described above and analyzed by flow cytometry.

For CD25 CAR lentivirus production, Lentix-293T cells have been transfected with 3rd generation lentiviral system using lipofectamine 3000 with the following plasmids: pLP1 (HIV-1 gag and pol), pLP2

(HIV-1 rev), pLP VSV-G (vesicular stomatitis virus G glycoprotein) and the plasmid coding for the CAR at a ratio of 5: 5: 3: 7.

Transduction of NK92 was performed using a co-culture procedure. To this end, 24 hours after transfection of Lentix-293T cells, wild type NK92 (NK92 WT) cells were added at the concentration of 5×10^5 cells/mL to the transfected Lentix-293T cell culture, with a total period of co-culture of 48 hours. Thereafter, NK92 cells remaining in the supernatant, were isolated and cultured in their appropriate culture medium as described above for 48 hours before selection with Puromycin or GFP-sorting as described below.

Transduction of iPSC with CAR lentiviruses was performed by using freshly passaged iPSC. Unconcentrated lentivirus was used at a proportion of $\frac{1}{4}$ with E8 flex media during 48 hours. Thereafter media has been changed and replaced with fresh E8 flex. Puromycin selection was performed by using 1 μ g/mL of Puromycin (12122530, Fisher Scientific).

For LV EF1-Tet3G-9 lentiviral transduction of iPSC, an MOI of 10 was used during 24h after what media was replaced with fresh E8 flex media.

For DOX induction of the CAR, Doxycycline (D9891, Sigma Aldrich) was used at a concentration of 1 μ g/mL.

Molecular constructs

The hIL2RA lentivirus plasmid was purchased from VectorBuilder (Guangzhou, China). hIL2RA was under the EF1 α promoter and Blastidicin S resistance was under mPGK promoter.

CD25 CAR lentivirus expression plasmids were purchased from Creative Biolabs (New York, USA). In this construct, Basiliximab scFV was used for the CAR specificity under either EF1 α or TRE promoter, the last construct allowing an inducible system. In both constructs, the scFV was followed with the CD8a hinge domain, CD28 transmembrane domain, 4-1BB co-stimulatory domain, and DAP12 for NK cell activation. In both plasmids, we added GFP and Puromycin resistance as selection markers. The Tet-ON lentivirus was purchased from Vectalys (Toulouse, France) and was used under the EF1 α promoter.

Flow cytometry

For extracellular staining, cells were washed with DPBS and incubated with antibodies in a volume of 100 μ L of DPBS with an adequate amount of antibody (see list below). For intracellular staining, cells were thereafter fixed with BD Cytifix™ (BD, 554655) for 10 minutes at room temperature, protected from light, washed, and permeabilized with BD Perm/Wash™ (BD, 555028) for 20 minutes at 4°C.

Intracellular antibodies were added during the permeabilization step. Flow cytometry was performed with a BD FACS LSRFortessa™ and data were analyzed using FlowJo.

<i>Antibody</i>	<i>Reference</i>
AffiniPure F(AB) Anti mouse IgG	115-606-072 Jackson Immuno Reserach
CD56	555518 – Beckton Dickinson
CD107a	130-111-662 - Miltenyi Biotec
Granzyme B	563389 - Beckton Dickinson
KIRDL2/3	130-116-835 - Miltenyi Biotec
NKp30	130-112-432 - Miltenyi Biotec
NKp46	130-112-123 - Miltenyi Biotec
Perforin	563393 - Beckton Dickinson
TIGIT	130-116-817 – Miltenyi Biotec

Cell sorting

Cells were stained with extracellular antibodies as described above and were resuspended in their culture media at a concentration of 5×10^6 cells/mL. They were sorted with a FACS Aria SORP (BD) and sorted cells were immediately resuspended at the adequate concentration in the appropriate medium and cultured at 37°C, 5% CO₂.

CD107a expression

NK92 WT and CD25 CAR-NK92 cells were co-cultured with target cells in a 96 well plate. CD107a Antibody was added to each well at a dilution of 1:100 and incubated for 1 hour. Golgistop and GolgiPlug were thereafter added for 2 additional hours. After the incubation cells were washed with FACS buffer and stained with CD56 surface marker for 25 minutes at 4°C protected from light. Following this last incubation, cells were washed with FACS buffer and analyzed by FACS.

Annexin V Apoptosis assay

Apoptosis induced by NK cells was evaluated by staining the Annexin V on the surface of target cells. After 4h of co-culture as previously described, cells were stained for Annexin V according to the manufacturer's instruction. Briefly, cells were washed with 1X binding buffer, centrifuged and resuspended in binding buffer containing Annexin V-PE (88-8102-72, ThermoFisher Scientific). After 15 minutes incubation, cells were washed again and analyzed by FACS.

In vivo assays

2.5×10^5 K562-Luc cells were injected intraperitoneally in 8 weeks old NOD/SCID mice, previously treated with busulfan (20 mg/kg) once a day for 2 days. NK92 WT and CD25 CAR-NK92 were inactivated during 24h with 1µg/mL of Mitomycin (Sigma-Aldrich M4287). 3 Days after K562-Luc injection, 15.10^6 mitomycin-inactivated NK92 WT or CD25 CAR-NK92 cells were injected intra peritoneally and the evolution of transplanted leukemic cells was followed weekly by luminescence (IVIS 200, Life Sciences).

RESULTS

Generation of K562 cells overexpressing IL2RA/CD25

As the native K562 cells do not express the CD25, we first generated a cell line that could be used as a proof-of-concept of our CD25 CAR strategy. K562-Luciferase (Luc) leukemic cells were lentivirally transduced with the CD25 transgene in order to generate a CML model expressing this marker. The CD25 gene was under the control of CMV promoter (**Figure 1A**). As shown in this Figure 1, K562 do not express CD25 at their native state as shown by FACS analysis (**Figure 1B**). 48h after transduction, the level of CD25 was found to be very high, close to 100%. K562-CD25-positive cells were further enriched by sorting cells with the highest level of CD25 expression (**Figure 1B**). These cells represented therefore the optimal targets to test the efficiency of CD25 CAR-NK cells.

Constitutive CAR expression on NK92 cells

The constitutive construct of CD25 CAR-NK92 was a polycistronic construct that included the scFv of the clinically approved monoclonal antibody, Basiliximab under the strong EF1 α promoter. CD8a was used as signal peptide to address the CAR protein to the cell membrane. Two costimulatory domains, 4-1BB as well as the NK characteristic DAP12 were used, followed by puromycin resistance and GFP, those two being under the PGK promoter (**Figure 2A**). After lentiviral transduction of NK92 WT cells, the level of CAR expression at the surface was evaluated with an anti-Fab antibody and 90% of cells were found to be double-positive (GFP and CAR) (**Figure 2B**). The level of CAR expression was also evaluated by RT-PCR and we observed a relative expression of 150 times more of CAR mRNA in CD25-CAR-NK92 as compared to the NK92 WT which was negative (**Figure 2C**). From a phenotypical point of a view, we observed no difference on the expression of NK activatory and inhibitory signals between CD25 CAR-NK92 and NK92 WT (**Figure 2D and E**).

CD25 CAR-NK92 cells specifically target K562-CD25-positive leukemic cells *in vitro*.

In order to assess the functionality of the CD25 CAR-NK92 cells, we evaluated the level of Granzyme B and Perforin expression. We observed a remarkable increase of Granzyme B expression in CD25 CAR-NK92 cells as compared to NK92 WT cells (**Figure 3A**). In the same perspective, we assessed also the level of Perforin which was found to be significantly higher in CD25 CAR-NK92 cells as compared to NK92 WT (**Figure 3B**).

The degranulation was assessed by staining the CD107a of unstimulated and stimulated cells with K562-CD25 or PMA (positive control). As can be observed in **Figure 3C**, CD25 CAR-NK92 cells show a much higher degranulation profile as compared to NK92 when stimulated with K562 WT or K562-CD25.

In order to evaluate the cytotoxic capacity of CD25 CAR-NK92 cells, we evaluated the number of apoptotic cells after co-culture between NK92 WT or CD25 CAR-NK92 with K562-CD25 at different ratios. We observed a much higher percentage of double positive (Annexin V/PI) cells in the CD25 CAR-NK92 condition as compared to NK92 WT (**Figure 3D**).

***In vivo* assays of CD25 CAR-NK92 targeting K562-CD25-Luc cells.**

Preliminary *in vivo* assays were performed in NSG (NOD-Prkdc^{scid} IL2rg^{tm1}/Bcgen) mice. As shown in **Figure 4A**, ten mice were divided in three groups, two groups containing four mice each and the group control containing two mice (**Figure 4A**). At D-2 and D-1 before leukemia injection, mice were treated with busulfan and at D0, 2.5x10⁵ K562-CD25-Luc cells were injected interperitoneally (IP) in each mouse. At D3 after leukemia injection, mitomycin inactivated NK92 WT or CD25 CAR-NK92 cells were injected IP (at a ratio of 1:50). The evolution of the leukemia was followed weekly by bioluminescence imaging (BLI) (**Figure 4B**). These preliminary results show a potentially better response of CD25 CAR-NK92 cells against K562-CD25-Luc *in vivo* as compared to NK92 WT cells. Among mice that engrafted with the tumor, in the CD25 CAR-NK92 treated condition, two of them appeared free of tumor whereas in the NK92 WT condition, two mice developed the tumor until day 40. (**Figure 4C**). This experience will be repeated with a higher number of mice per group.

Inducible CD25 CAR expression on NK92 cells (CD25-iCAR-NK92)

The same CAR construct used in the constitutive vector was used for a Tet-ON inducible system. The CAR construct was under the control of the inducible TRE promoter and similar to the constitutive one, CD8a was used as signal peptide for directing the CAR to the cell membrane. 4-1BB and DAP12 were used as co-stimulatory domains and puromycin resistance and GFP for cell selection (**Figure 5A**). NK92 cells transfected with this lentivector were selected by puromycin and transduced for a second time with the rLV.EF1.Tet3G-9 vector to enable the Dox inducible (Tet-ON) expression. CD25 CAR expression was evaluated by RT-PCR and we have observed an increase of 3000 folds of the CAR transcript after DOX induction as compared to the DOX- condition (**Figure 5B**). The phenotype of selected cells was characterized by FACS. We observed a slight increase in CD56 expression in CD25 iCAR-NK92 cells as compared to NK92 WT (**Figure 5C**). We observed also an increase of NKp30 in CD25 iCAR-NK92 cells as compared to NK92 WT and a similar expression to the control for other NK markers such as KIR2DL2-3, NKp46, DNAM, and TIGIT (**Figure 5C**).

Generation of iPSC expressing CAR-NK constructs

In order to evaluate the feasibility of generation of iPSC-derived NK cells using our CD25 CAR constructs, the first step was the evaluation of the efficient CD25 CAR expression on the iPSC lines. To this purpose we have used a cord-blood-derived iPSC line generated in our INSERM Unit. Both CAR

constructs (the constitutive and the inducible) were also introduced into this iPSC cell line (PB68) using lentivirus-mediated gene transfer. The constitutive CAR (as described above) is a 3rd generation CAR under the control of EF1- α (**Figure 6A**). 48h after transduction, GFP-expressing colonies were identified in iPSC cultures (**Figure 6B**). We started the puromycin selection of the iPSC cells at day +3 and CAR expression was evaluated by FACS in puromycin resistant cells using an anti-Fab antibody. As it can be seen in **Figure 6B**, 61,6% of cells were found to be positive for CAR expression and 23,5% were double-positive for CAR and GFP expression. Only 1,27% of the cell fraction were found to express GFP alone (**Figure 6C**).

We then generated iPSC expressing the inducible CAR. To this end, cells were lentivirally transduced with the vector described above (**Figure 7A**). After puromycin selection and transduction with the rLV.EF1.Tet3G-9, GFP expression was assessed by microscopy in puromycin resistant cells. As can be observed in **Figure 7B**, GFP-expressing iPSC colonies were clearly distinguishable. After 3 days of DOX treatment, the CAR expression was assessed by FACS. This experiment showed the presence of 75 % of double-positive (CAR/GFP) cells (**Figure 7C**). To evaluate the inducible pattern by DOX, cells were deprived for doxycycline for 72h and the CAR expression was evaluated. As can be seen in **Figure 7C**, we observed a decrease in the level of double positive CAR/GFP cells upon DOX deprivation (45,3%). These results established that our CAR constructs had been successfully expressed in the iPSC line, allowing the experiments of NK cell generation.

Discussion

Although the use of TKI therapies against CML has shown tremendous results during the past twenty years, the development of TKI resistance and CML progression remain still challenging issues because of limited therapeutic solutions at this stage of the disease. Developing new therapeutic tools against AP/BC-CML is therefore an unmet therapeutic need for this life-threatening step of CML. We and others have shown previously the involvement of CD25 as a biomarker detected on primary CML blast crisis [12,18] as well as its expression in *in vitro* BC-CML models (Imeri et al., 2022 in submission).

We have also previously shown, using an iPSC-derived blast crisis model that *CD25* is overexpressed in blast cells and this finding is confirmed in CML-CP patients progressing towards blast crisis (Imeri et al, in submission, 2022). These findings prompted us to design a procedure of iPSC-derived NK cells engineered to target CD25 as an off—the-shelf immunotherapy for CML blast crisis.

iPSC-derived NK cells have recognized major developments during the last several years, with the possibility of generifying major gene edited iPSC giving rise to NK cells with non-cleavable CD16 [32]. These cells are currently being used in clinical trials which have been approved by the FDA in lymphomas and in solid tumors (NCT05182073).

Our laboratory has previously shown the possibility of generating NK cells from human embryonic stem cells [33] and experimental work is under way to develop strategies of NK cell differentiation from human iPSC. In order to test the feasibility and the potential translation of the findings using CD25 in the context of CML, we first aimed to use the NK92 cell model. NK92 is a lymphoma cell line with cytotoxic activity against several cancer cells and is currently being used in the clinical setting either by itself or after CAR-engineering (NCT00900809, NCT03940833).

We first transduced NK92 cell line with two different lentivectors, one expressing constitutively the CAR and a Tet inducible CAR expression for the 2nd one. No loss of the main NK markers was observed, moreover the slight increase of CD56 and NKp30 in the inducible one showed a potential further activation of the CD25 CAR transduced NK92 cell line. We also observed around 90% of CAR/GFP expressing cells. Preliminary data for the constitutive CAR show an increase of the Granzyme B and Perforin (**Figure 3A and B**). Degranulation assays assessed by CD107a staining shows a higher level of degranulation in CD25 CAR-NK92 cells as compared to the NK92 WT after co-culture with K562 and K562-CD25.

Evaluation of apoptosis by Annexin V showed clearly an increased cytotoxic potential of the CD25 CAR-NK92 cells as compared to NK92 WT cells against K562-CD25. Altogether, these data allowed us to perform preliminary results *in vivo*. Preliminary *in vivo* analysis included only 10 mice (**Figure 4A**). As can be seen in **Figure 4B**, ten busulfan pre-conditioned mice were injected with K562-CD25-Luc and the CAR treatment tested in four of them. The fact that not all mice engrafted with the leukemic cells is probably related to the low number of injected cells. That led to a reduced cohort of mice for statistical analysis. Nevertheless, these preliminary results *in vivo* indicate a potentially better *in vivo* antileukemic activity of CD25 CAR-NK92 cells as compared to NK92 WT cells. Currently, experiments with a larger cohort of mice are under progress.

Although CAR-NK therapies exhibit a major clinical potential due to their safer profile in term of GvHD compared to CAR-T cells as well as to their HLA independence and allogenic utility, some questions remain still to be raised. One of them is the source of NK cells for therapy. Heterogeneity and batch to batch differences for peripheral blood NK (PB-NK) and umbilical cord blood NK (UCB-NK) as well as the availability suggest the need for new NK sources. NK92 cell line, even though largely used in therapy, remain a cell tumorigenic cell line with potential risks for the patients. The use of iPSC derived NK cells have revolutionized the way of the use of NK cells in clinic thanks to their homogeneity and large-scale production ability. Moreover, their clonogenicity allows to identify clones without genomic alterations via whole-genome sequencing, but also their cryo-conservation ability renders them a very interesting tool for CAR-NK therapies. The final aim of our work was to generate iPSC

derived CAR-NK cells with previously validated constructs in NK92 cell line. Here we demonstrate the generation of two models, a constitutive and a Tet-ON inducible CAR expression system in a UCB derived iPSC. In these preliminary results, we could observe the expression of CAR at the surface in both systems as well as the sensitivity of the iCAR to doxycycline 3 days after DOX treatment and 3 days after DOX deprivation. Ongoing experiments aim to assess the level of CAR expression in the iCAR model later than three days after deprivation in order to evaluate the time needed to have the lower CAR expression in absence of DOX. Moreover, ongoing experiments also aim to differentiate CD25 CAR-iPSC into NK cells.

Targeting BC-CML blasts with an CD25 CAR-NK cell seems to be a potential tool against this disease, moreover, if we take into consideration the intrinsic and CAR-independent cytotoxic capacity of NK cells to leukemic cells. This could reinforce further the anti-leukemic potential of this treatment. In addition, the use of this potential therapy could target at the same time the LSC as well as the Tregs which are highly increased during CML and responsible for immune escape [34,35]. Altogether, these results pave the way for a new potential way of fighting BC-CML by targeting together the leukemic blast cell mass as well as the LSC, while targeting by the same approach the immune escape of the leukemic cells.

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FIGURE LEGENDS

Figure 1: Generation of K562 cells expressing CD25

(A) Human CD25 (hIL2RA/CD25) expression vector under the control of CMV promoter and followed by blasticidin S resistance.

(B) CD25 expression on K562 cell line after lentiviral transduction (FACS). The gate shows the sorted population (left). CD25 expression on sorted cells (in blue) as compared to K562 WT (in pink) (right).

Figure 2: Generation of CD25 CAR-NK92 cells from NK92 WT cells

(A) Constitutive CAR expression construct under EF1 α promoter followed by puromycin resistance and GFP under PGK promoter. DAP12 and 4-1BB as co-stimulatory signals. CD8a as peptide signal, and the transmembrane (TM) sequence of CD28. Hinge domain is based on CD8.

(B) CAR and GFP expression level of CD25 CAR-NK92 cells gated on NK92 WT.

(C) Relative expression of CAR mRNA measured by RT-PCR.

(D) FACS histograms of CD56 and NKp46 expression of NK92 WT (blue) and CD25 CAR-NK92 (pink)

(E) Main NK markers expression. P-values were calculated using a one-tailed Student's t-test. ns, not significant.

Figure 3: Functional and cytotoxic assessment of CD25 CAR-NK92

(A) and (B) Granzyme B and Perforin expression levels of CD25 CAR-NK92 and NK92 WT cells at their basal level.

(C) Degranulation assay of NK92 WT and CD25 CAR-NK92 cells after co-culture with K562 +/- CD25.

(D) Apoptosis assessed by annexin V (in Y axis) and PI (in X axis) staining of NK92 WT and CD25 CAR-NK92 cells after co-culture with K562-CD25 (left). Data shown in barplot (right).

P-values were calculated using a one-tailed Student's t-test. **, P < 0.0021; ***, P < 0.0002; ****, P < 0.0001

Figure 4: In vivo assay comparing the antileukemic effect of CD25 CAR-NK92 vs. NK92 WT

(A) Schematic representation of three experimental groups of mice used to assess the CD25 CAR-NK anti-cancer activity.

(B) Schematic representation of the timeline and the experimental design for the *in vivo* assay.

(C) BLI of K562-CD25-Luc injected in IP with NK92 WT and CD25 CAR-NK92.

Figure 5: Generation of CD25 iCAR-NK92 from NK92 WT cells

(A) Inducible CD25 CAR under the TRE promoter followed by the puromycin resistance and GFP. DAP12 and 4-1BB as co-stimulatory signals. CD8a as peptide signal, and the transmembrane sequence of CD28. Hinge domain is based on CD8.

(B) Relative expression of CAR mRNA with and without DOX measured by RT-PCR.

(C) Assessment of main NK markers by FACS after transduction of NK92 WT cells with iCAR.

Figure 6: Generation of CD25 CAR-iPSC (constitutive)

(A) Constitutive CAR expression construct under the EF1 α promoter followed by puromycin resistance and GFP under the PGK promoter.

(B) PB68 iPSC after lentiviral transduction expressing GFP.

(C) CAR and GFP expression of PB68 iPSC.

Figure 7: Generation of CD25 iCAR-iPSC (inducible).

(A) Inducible CAR construct under the TRE promoter followed by the puromycin resistance and GFP.

(B) PB68 iPSC cell line after lentiviral transduction expressing GFP.

(C) CAR and GFP expression after lentiviral transduction gated on PB68 WT. DOX treated CD25 iCAR-iPSC during 3 days (in the middle) and DOX deprived CD25 iCAR-iPSC during 3 days (at the right).

Figure 1: Generation of K562 cells expressing CD25

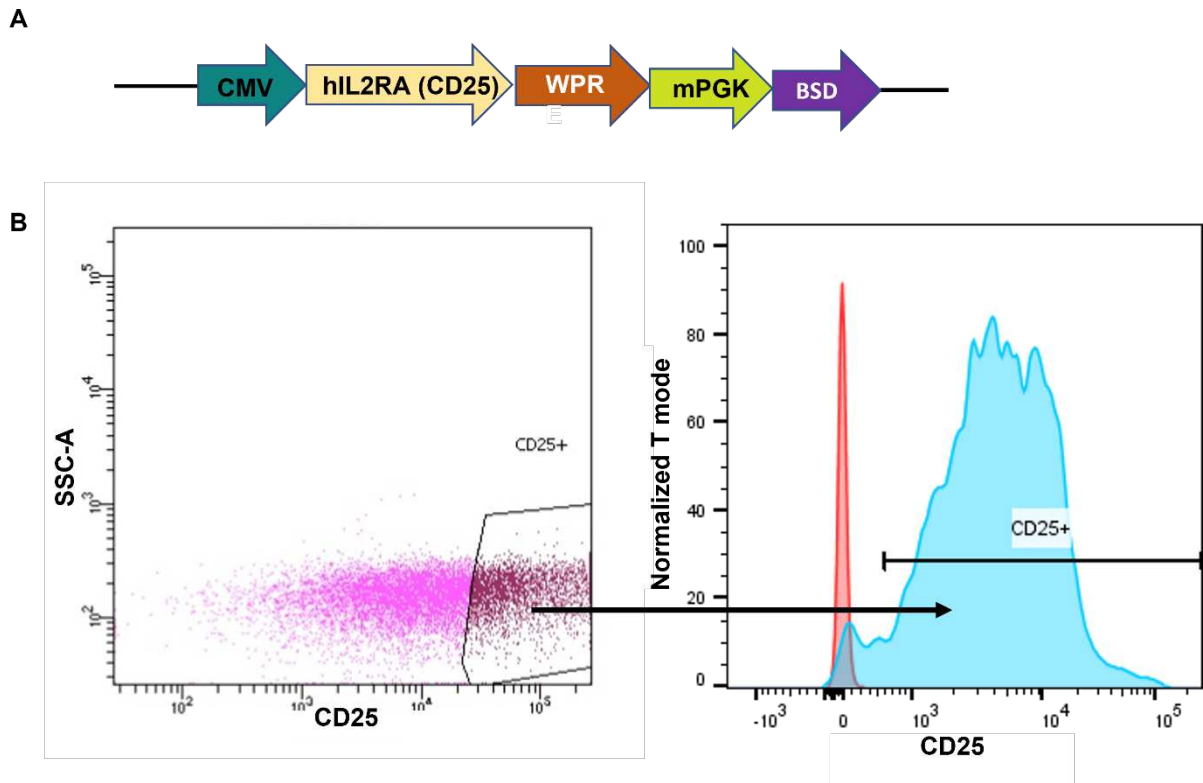


Figure 2: Generation of CD25 CAR-NK92 cells from NK92 WT cells

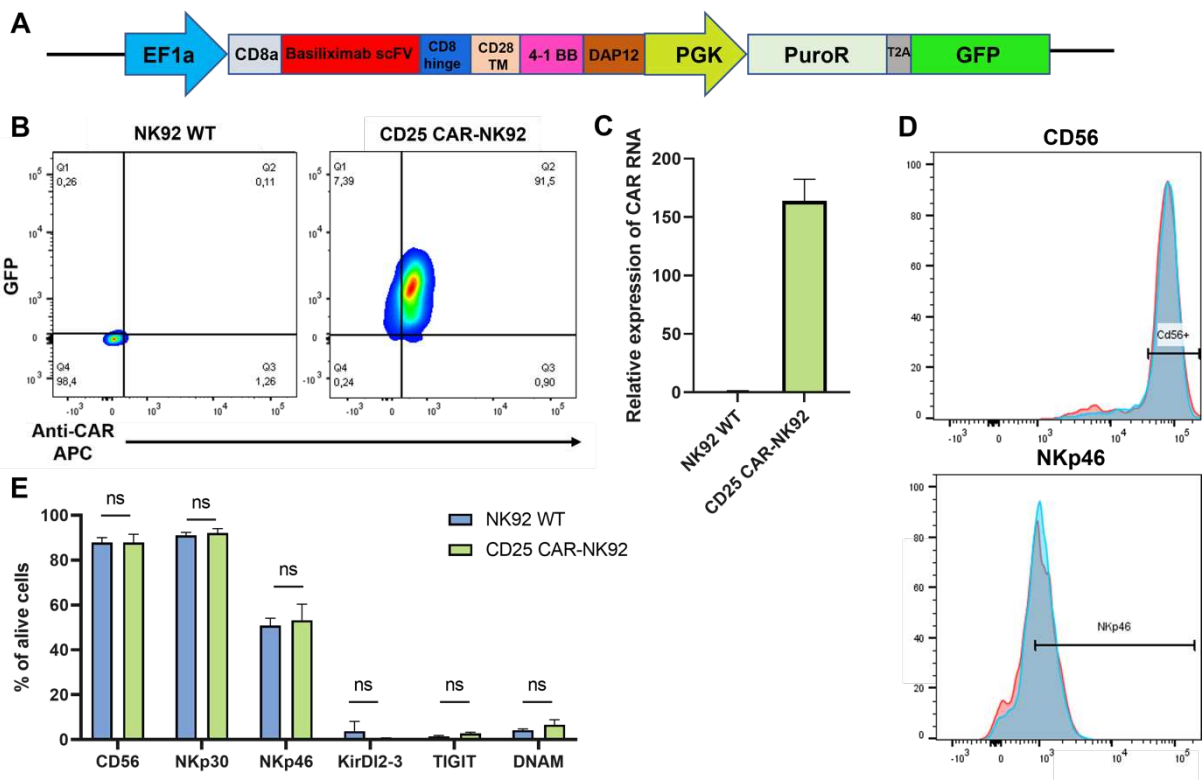


Figure 3: Functional and cytotoxic assessment of CD25 CAR-NK92

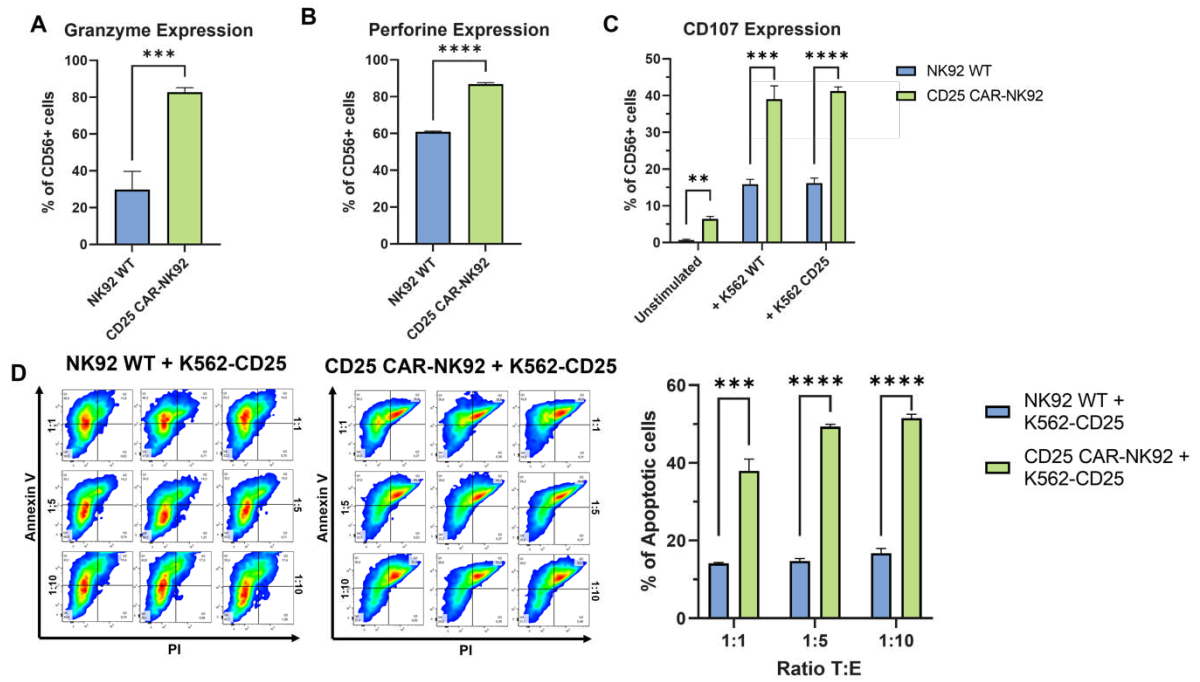


Figure 4: In vivo assay comparing the antileukemic effect of CD25 CAR-NK92 vs. NK92 WT

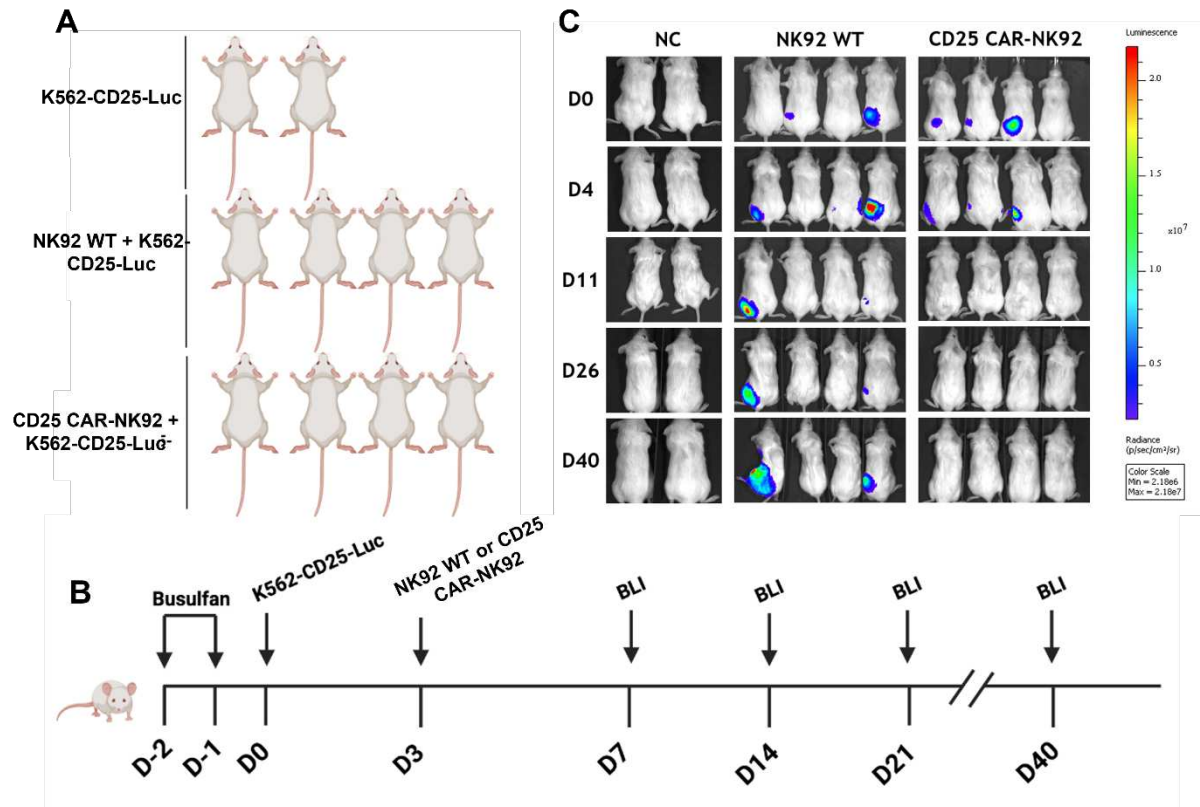


Figure 5: Generation of CD25 iCAR-NK92 from NK92 WT cells

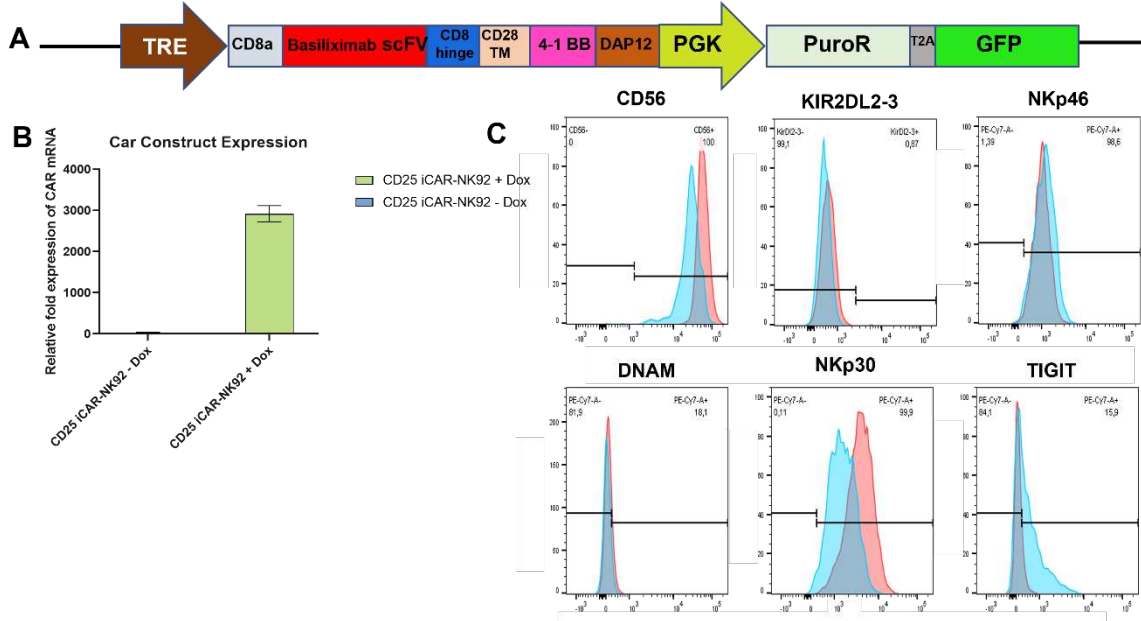


Figure 6: Generation of CD25 CAR-iPSC (constitutive)

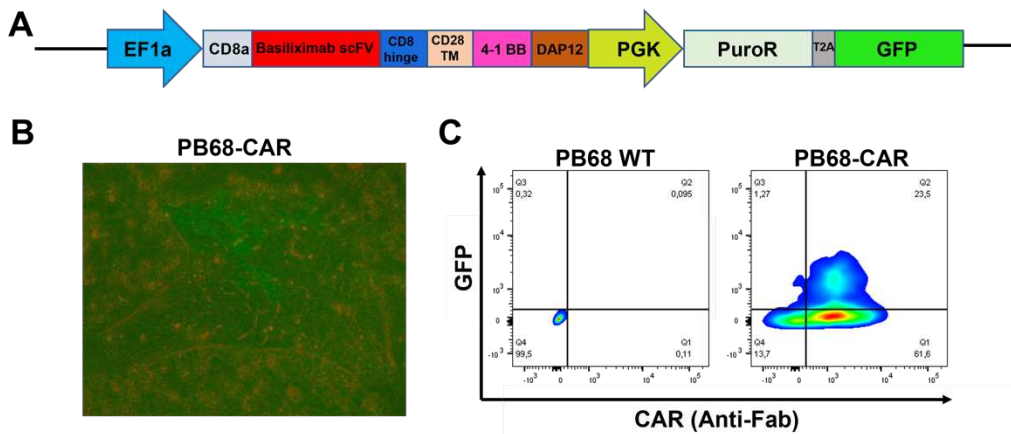
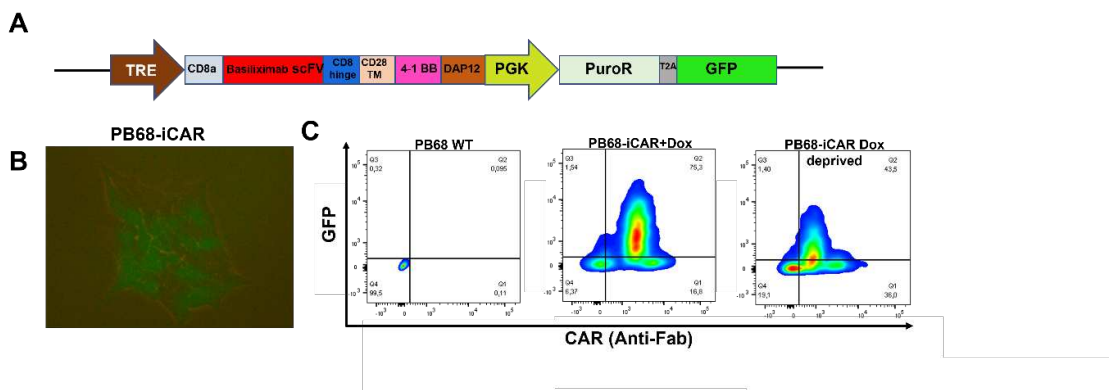


Figure 7: Generation of CD25 iCAR-iPSC (inducible).



DISCUSSION AND PERSPECTIVES

Since the generation of the first cell line from a CML patient in blast crisis in 1975 (C. B. Lozzio et al. 1975), different *in vitro* and *in vivo* models of CML have been developed, aiming to recapitulate different features of the disease and to generate novel tools for new therapies. These efforts have led to the ability to test the efficacy of targeted therapies, allowing the rapid translation of these findings to the generation of the tyrosine kinase inhibitors (TKI) (Mauro et al. 2001). As discussed in the work presented in the Articles, these therapies have modified the natural history of this deadly leukemia with prolongation of survival in the majority of the patients. However, despite the major success of TKIs, two major problems are the resistance of the most primitive HSC to TKIs and the progression of the disease to blast crisis which still occurs, albeit much less frequently, in patients developing TKI resistance. The current experimental tools do not allow to study the progressive changes occurring during the clonal evolution of the disease, which requires serial samplings which are rarely available. Blast crisis in CML can be modeled using murine CML models by using complementation assays using BCR::ABL and an oncogenic fusion protein such as NUP98/ HoxA9 (Dash et al. 2002; Sjö et al. 2007). Although these studies can be of major interest to study the *in vivo* behavior of cells, they are not appropriate to study human leukemia in the context of a given genomic background of a patient.

The generation of ES cell lines from mice and humans (Thomson et al. 1998; Evans et al. 1981) opened novel possibilities for CML modelling. These models enabled to understand crucial pathways dysregulated by BCR::ABL such as STAT3 and its specific activation by the oncogene (Coppo et al. 2003) in mouse ES cell lines and later on in BC-CML (Coppo et al. 2006). Our group (Melkus et al. 2013) and others (Perlingeiro, Kyba, et al. 2001) have shown that the overexpression of BCR::ABL in murine ES cells can confer to these cells the long term hematopoietic potential which is not possible to generate otherwise. The goal of generating long-term HSC potential from ESC and iPSC is a major objective for future therapeutic purposes and which has not been achieved so far.

The discovery of human induced pluripotent stem cells in 2007 (Takahashi et al. 2007), opened a new era for modelling human diseases in particular human malignancies (Turhan et al. 2019). Indeed, very shortly after the Takahashi paper, Ph⁺ cells such as K562 cell line and later those isolated from CML patients were reprogrammed in many labs including ours, opening thus new perspectives for modelling CML. These perspectives are mainly due to the potential ability of CML-iPSC to induce long-term hematopoietic and hemangioblastic potential (Gladys Telliam, Desterke, et al. 2022).

Our INSERM Unit has generated during the last 20 years, expertise on the development of hematopoietic potential from murine and human ESC. From this regard, ESC and later iPSC have been extensively used for disease modelling (Yamanaka 2020) (including in the field of CML (K. Hu et al. 2011; Turhan et al. 2019)). Amongst the patient-specific iPSC cell lines generated in our Unit, a cell line was generated and used for characterization by the previous Ph.D. student of the laboratory (G. Telliam et al. 2016) This cell line was generated from the CD34⁺ cells of a patient who developed a clinical resistance to TKI, requiring a bone marrow transplantation (G. Telliam et al. 2016). This cell line has been shown to exhibit an enhanced hemangioblastic and hematopoietic potential (Gladys Telliam, Féraud, et al. 2022) and represented therefore an important tool to generate a progression model of CML. This work was initiated by the previous student G Telliam, included essentially testing the effects of an *in vitro* mutagenesis using ENU in this iPSC cell line. I have participated to the final steps of this work confirming the genomic instability pattern and the leukemic transformation potential of this cell

line (Gladys Telliam, Desterke, et al. 2022), Article 1). The first steps of my own project consisted on the extension of this work to other CML-iPSCs and test their potential to model leukemic progression through *in vitro* mutagenesis. To this purpose, I have used two other CML-iPSCs designed as the PB27 and the PB34. The first one was generated from a CP-CML patient with a good response to TKI and the second one is from the cells of a patient in accelerated phase of CML. These cell lines had both been characterized and one of them (PB34) expressing JAK2 V617F mutation in the BCR::ABL+ clone, was previously published (Sloma et al. 2017) but their ability to be used for modelling CML progression had not been established. For these two cell lines, I have also used the alkylating agent N-Ethyl-N-nitrosourea (ENU), a well-established mutagen (Acevedo-Arozena et al. 2008) Our Unit had previously shown that ENU could be used to induce ABL-kinase mutations that can be selected *in vitro* by TKI and allow *in vitro* production of clinically relevant mutations such as T315I substitution (Aggoune et al. 2014).

As mentioned, the previous work in which our Unit has shown the effect of the ENU on the PB32 cell line aimed essentially to test the establishment of global genomic instability by this *in vitro* mutagenesis, using DNA repair tests and CGH arrays (Gladys Telliam, Desterke, et al. 2022). This study showed an increase of genetic instability after ENU treatment seen at the level of phospho- γ -H2AX as well as an increase in the number of micronuclei evaluated in the PB32 cell line (Gladys Telliam, Desterke, et al. 2022). Several losses and gains interesting all chromosomes were identified and among them different oncogenes. More interestingly, when comparing the affected genes by the mutagenesis with genomic abnormalities in primary leukemic cells, 125 genes were found in common, showing thus the relevant impact of ENU at the DNA level.

I have extended this study by using two other CML-iPSCs and treated them the same way as the PB32, namely during 60 days with daily doses of ENU addition to the cultures. After this period of ENU-induced mutagenesis, it was important to evaluate their persistent pluripotency. I have analysed these cells by using classical pluripotency test including FACS analysis and teratoma assays in NSG Mice (Article 2). I have demonstrated their pluripotency status and then evaluated their genomic instability. I have performed comparatively phospho- γ -H2AX status of these cell lines and showed an increase of this marker in the cell lines subjected to ENU-mutagenesis. We have also performed in one of the cell lines (PB32) a whole exome sequencing showing the establishment of genetic mutations after mutagenesis (Article 2). Several of these mutations have been found to be highly relevant in the field of oncogenesis and in particular in leukaemia.

I have then set up in the laboratory the methodology using the StemDiff technology allowing the generation of hematopoietic cells from ESC and iPSC without the intermediate of hematopoietic embryoid body generation (STEMCELL Technologies). Using this technology, I have shown that PB32-ENU cells generate hematopoietic cells with blastic transformation features (Article 2). Morphological and phenotypical analysis of PB32-ENU revealed typical blast crisis features with undifferentiated cells with big nuclei exhibiting monoblastic patterns and expression of MPO, CD45, CD33, CD13, and CD43. This blast potential was also shown in the increase of the hematopoietic potential of PB32-ENU (CFC and LTC-IC). I have then tested comparatively, the two other iPSC cell lines which underwent ENU-induced mutagenesis. As compared to PB32-ENU, these two cell lines exhibited less identifiable cytological features of a blast crisis but at the genomic level, the effects of mutagenesis were identified (see below).

Indeed these three mutagenized cell lines were characterized at the transcriptomic level. All three mutated CML-iPSCs and their unmutated counterpart were differentiated into hematopoietic tissue and at day 13 of differentiation, RNA was analyzed by Affymetrix MicroArray. In total 3701 genes were differentially expressed between the mutated and the wild-type cell lines. Interestingly, the highest impact of ENU was seen on PB32-ENU versus PB32 with 3080 different genes followed by the PB34-ENU and the PB27-ENU. Most interestingly, the ENU-mutagenized CML-iPSC PB32, as compared to PB32 without mutagenesis, exhibited a pattern of enrichment of acute myeloid leukemia (AML) (Article 2, Figure 7A and B)

The comparison of transcriptomics analysis of PB32 and PB32-ENU with Geodataset GSE4170 (J. P. Radich et al. 2006) which collects transcriptomic results from a cohort of patients at different stages of CML, revealed the relevance of our model through the correlation of our data with patients in BC-CML (NES=1.84). Interestingly, among the concordant genes, we found the *IL2RA* (*CD25*) which is already shown to be related to CML and AML LSC as well as to bad prognosis AML (Sadovnik et al. 2016; 2017; Kobayashi et al. 2014; Kuhn et Dou 2005).

To check the involvement of *CD25* in the progression of CML from CP-CML to BC-CML, we evaluated the level of expression of this gene in a cohort of 22 patients in CP-CML, 14 patients in AP-CML, and 15 healthy donors. The clear increase in CD25 expression between CP-CML and BC-CML (75% versus 19.3%) validated together the relevance of our model and the involvement of *CD25* in CML progression. The analysis of transcriptomic changes induced by ENU-mutagenesis in the other two CML-iPSCs lines is currently under progress.

One interesting question was the molecular mechanisms by which ENU operates during the iPSC-derived hematopoiesis using the StemDiff technology. As it can be seen in the Article 2, during this technology undifferentiated iPS cells are seeded in the culture and after mesoderm induction, one can observe generation of non-adherent hematopoietic cells.

In order to further investigate the molecular events occurring in the ENU-induced mutagenesis in this model, we performed single-cell sequencing by using 10X Genomics technology. We sequenced PB32-ENU and PB32 cells at different time points of hematopoietic differentiation (D5, D9, and D13) with the objective to better understand the effect of the ENU during the hematopoietic differentiation. The increase of the mesodermic *MSX1* gene and the decrease of hematopoietic genes such as *CD45* and *CD41* in the ENU-treated condition versus its unmutated counterpart showed the delay in differentiation suggesting that this event allowed accumulation of less differentiated blast cells (Article 2). However, this technology has been used only in the PB32 cell line and in future work it would be interesting to extend these finding to other CML-iPSCs.

As we had identified CD25 as a clinically relevant target in advances phase CML, the second part of my work aimed to develop a strategy of targeting CD25-expressing leukemic cells using iPSC-derived NK cells.

In normal conditions, CD25 is expressed in regulatory T-cells (Tregs) and plays an important role in the homeostasis of T cell activity and immune tolerance (Malek et Bayer 2004). Tregs have been shown to be of interest in the pathophysiology of CML as their level is significantly increased with the progression of the disease and decreases when patients are in CMR (Zahran, Badrawy, et Ibrahim 2014; Rojas et al. 2010). Moreover, Tregs are shown to inhibit the activity of CD8⁺ CTL and thus provoking potentially a direct immune escape of the LSC (Hinterbrandner et al. 2021). In some TKI-cessation trials,

leukemia relapse has been shown to be correlated with high levels of Tregs and low levels of NK cells (Hughes et al. 2017). In the context of immunotherapy, eliminating Tregs by targeting CD25 has already been shown to induce CD8 T cell-mediated systemic antitumor immunity (Onda, Kobayashi, et Pastan 2019).

As described in the introduction, in addition to the context of Tregs and its overexpression in patients in AP/BC-CML, CD25 is also a relevant marker of CML stem cells. Indeed, in the majority of patients with CML, LSC with CD34+CD38- phenotype express CD25 (IL2RA) (at gene and protein level) whereas the expression of CD25 is either absent or very low in CD34+CD38- cells from normal donors (Sadovnik et al. 2017). Thus, these findings prompted us to develop a strategy targeting CD25 in CML which could be of interest not only in advanced CML but also for eradicating leukemic stem cells. In addition, leukemic stem cells from AML patients have also been shown to express aberrantly CD25 and a CAR-T strategy has been proposed in an experimental setting (Itoh-Nakadai et al. 2020)(Saito et al. 2010).

As discussed previously, CAR-T cells have been shown to generate immense success in the treatment of hematopoietic malignancies and many of them are already approved by the FDA in ALL and lymphoma. However, our goal was to apply this leukemic cell targeting strategy to the field of iPSC and from this regard, the T-cell differentiation from pluripotent cells (ESC or iPSC) is still challenging. Moreover, NK cells have been shown to target CML cells in different cell contexts. The natural cytotoxicity of NK cells against CML cells has been shown *in vitro* as well as *in vivo* more than twenty years ago (Yan et al. 1998). Our group has also shown in 2002 that NK cell activity is highly correlated to the BCR::ABL expression levels in experimental leukemia models (Baron et al. 2002) suggesting their potential activity in patients in blast crisis.

Thus, despite the fact that CML targeting strategies using CARs were not invented in these early studies, they paved the way for their future use in the therapeutic setting. The advantage of using iPSC-derived NK cells as compared to autologous therapies, is obviously the major expansion potential inherent to iPSC and the availability of NK cell differentiation methods which are currently in more advanced stages (Fate Therapeutics). Before undertaking such an approach, using iPSC, we have tested in this work the possibility of using the CD25 CAR targeting in the context of the NK92 cell line. As discussed previously, this cell line derived from a patient with lymphoma displays a major NK cell cytotoxicity (J. H. Gong, Maki, et Klingemann 1994). More interestingly, it has also been accepted for clinical use by FDA in clinical trials after inactivation via irradiation (Arai et al. 2008; Tonn et al. 2013).

Thus, this cell line represented an ideal target for testing the feasibility of expression CAR constructs and evaluation of their effects in a leukemia cell line expressing BCR::ABL. For these experiments, we have established a collaboration with the group of Dr. Lucas E. Botelho De Souza (Sao Paulo, Brazil) where I have spent 3 months to develop experimental tools.

As leukemic K562 cell do not express CD25, we designed an overexpression strategy. In the laboratory of Dr. de Souza, I have overexpressed the CD25 in the K562 cell line via lentiviral transduction. K562 is a blast crisis cell line and its use was therefore because of its proliferation potential and its aggressiveness in mice.

To generate the CD25 CAR we have used a CAR based on the scFV of a clinically approved monoclonal antibody (Basiliximab) either under a constitutive promoter or an inducible one. The

constitutive promoter was designed for a fast and intense response and the inducible one for a controlled expression of the CD25 CAR.

Transducing NK cells with lentiviruses is a difficult task and the efficiency of transduction of NK92 cells is approximately 15% (Savan, Chan, et Young 2010). In order to overcome this difficulty, we co-cultured NK92 cells with Lentix293T cells (lentivirus-producing cells) transfected with CAR constructs.

Interestingly, NK92 cells transduced by CD25 CAR were shown to express higher levels of CD56 as compared to NK92 WT, possibly related to a more active status of these cells. They also expressed high levels of activating receptors such as NKp30, but no inhibitory receptors (KirDL2-3).

Functional assays showed a higher level of Granzyme B and Perforin expression in CAR-NK cells as compared to NK92 WT even without target cells showing their highly active phenotype.

Degranulation was assessed by staining CD107a and an increased degranulation of CAR-NK compared to NK92 WT was found. Moreover, we have found a higher degranulation of CAR-NK against K562-CD25 compared to K562 WT showing the specific activation of the effector cells.

In preliminary *in vivo* experiments using NOD/SCID mice, I have also tested the activity of CAR-NK92 cells against the target K562-CD25 cells. These preliminary results showed an increased antileukemic effect against K562-CD25 compared to NK92 WT but the absence of transplant of K562 cells in two mice renders the interpretation difficult. We are now planning to repeat this experiment with larger number of mice.

Finally, we have also tested the feasibility of generating an iPSC line expressing CD25 CAR constructs. To this purpose, I have used lentivirus supernatants that I have generated using both constitutive and inducible CAR vectors. These supernatants were used to transduce an iPSC line (PB68) generated in our laboratory from a cord blood sample. After selection in the presence of puromycin, iPSC lines were analyzed for the expression of CAR CD25. As can be seen in the Figures 6 and 7 of the Article 3, both iPSC lines express CD25 CAR (**Figure 6 and 7**).

After further characterization, these cell lines will be used to generate NK cells using the protocols established in our laboratory. Preliminary results have shown that the iPSC lines that we have generated with the CD25 CAR express all pluripotency markers and are able to undergo successful differentiation towards NK cells.

As the immediate perspective of this work, we would like to evaluate the possibility of using these iPSC-derived NK cells against CD25-expressing K562 cells and primary human blast cells.

The use of the inducible system will be very interesting for iPSC-derived CAR-NK experiments because we would be able to induce the CAR expression at different steps of NK differentiation. The use of iPSC-derived CAR-NK will also pave the way for a big number of cells with an interesting homogeneity. Overall, the major advantage of the use of iPSC-derived NK cell therapy will be their potential use as allogeneic therapies instead of current autologous CAR-T cell therapies.

I hope these experimental tools generated in this work will be of interest for the validation and further development of novel iPSC-derived cGMP grade therapeutic tools that could be applied to the clinic, in refractory CML in acute phase as well as in patients with AML.

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Titre : Les cellules souches induites à la pluripotence (CSIP) dans la Leucémie Myéloïde Chronique (LMC) : Modélisation de la crise blastique et design d'un modèle de thérapie à base de cellules CAR

Mots clés : LMC ; CAR ; crise blastique ; CSIP ; Cancer ; Modélisation

Résumé : L'histoire naturelle de la leucémie myéloïde chronique (LMC) qui était une affection constamment mortelle il y a 20 ans en raison d'une évolution vers une transformation en leucémie aiguë (ou crise blastique, CB) a été modifiée par l'utilisation des inhibiteurs tyrosine kinase (ITK). Malgré le succès de ces traitements, la résistance aux ITK et la progression clonale vers une crise blastique (CB) restent des problèmes majeurs dus à l'instabilité génétique inhérente à l'oncogène BCR::ABL. Pour modéliser les événements impliqués dans cette progression, nous avons utilisé des cellules souches pluripotentes induites (CSPI) dérivées des cellules leucémiques Ph1+ de patients atteints de LMC. Nous avons montré la possibilité de générer une instabilité globale *in vitro* par une stratégie de mutagenèse (Article 1). Les expériences de différenciation hématopoïétique ont montré la possibilité de générer en 12 jours, des cellules ayant des caractéristiques de leucémie aiguë, mimant la CB.

Les analyses de transcriptome ont permis de mettre en évidence la surexpression du gène *CD25 (IL2RA)* dans ces cellules leucémiques, confirmé dans les cellules primaires de patients en CB (Article 2). La possibilité d'utiliser une stratégie de CD25 CAR utilisant des cellules NK a ensuite été développée. Nous avons utilisé dans ce but la lignée NK92 que nous avons génétiquement modifiée par des lentivirus CD25 CAR. Nous avons montré la possibilité de cibler les cellules leucémiques surexprimant CD25 dans des modèles *in vitro* et *in vivo*. Enfin, nous avons appliqué la même stratégie de transduction à des cellules CSPI et détecté l'expression du CD25 CAR sous contrôle d'un promoteur constitutif ou inductible. (Article 3). Ces expériences de preuve de concept représentent une étape importante pouvant conduire aux thérapies CD25 CAR-NK dérivées des CSPI dans le futur.

Title : Induced Pluripotent Stem Cells (iPSC) in Chronic Myeloid Leukemia (CML): Modelling Blast Crisis and Designing a CAR-Cell Therapy Model

Keywords : CML ; CAR ; Blast Crisis; iPSC; Cancer; Modelling

Abstract : The natural history of chronic myeloid leukemia (CML) which was a consistently fatal condition 20 years ago due to progression into acute leukemia (or blast crisis, BC), has been changed by the use of tyrosine kinase inhibitors (TKIs). Despite the success of these treatments, resistance to TKIs and clonal progression to BC remain major problems, due to inherent genetic instability of the BCR::ABL oncogene. To model the events involved in this progression, we used induced pluripotent stem cells (iPSC) derived from Ph1+ leukemic cells of CML patients. We have shown the possibility of generating global instability *in vitro* by a mutagenesis strategy (Article 1). Hematopoietic differentiation experiments have shown the possibility of generating, in 12 days, cells with characteristics of acute leukemia, mimicking BC.

Transcriptomic analysis revealed the overexpression of *CD25 (IL2RA)* gene in these leukemic cells, confirmed in the primary cells of BC patients (Article 2). The possibility of using a CD25 CAR strategy based on NK cells was then developed. For this purpose, we used the NK92 cell line that we genetically modified with CD25 CAR lentiviruses. We have shown the possibility of targeting leukemic cells overexpressing CD25 in *in vitro* and *in vivo* models. Thereafter, we transduced iPSC with the CAR lentiviruses and showed the expression of CD25 CAR via the constitutive or inducible promoters (Article 3). These proof-of-concept experiments represent an important step that could lead in the future to iPSC-derived CD25 CAR-NK.