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ARMING NK CELLS WITH CHIMERIC ANTIGEN RECEPTORS (CARs) FOR TREATMENT OF REFRACTORY AND/OR RELAPSED HEMATOLOGICAL CANCERS: PRECLINICAL STUDY TO DETERMINE THE BEST CELL SOURCE

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ABBREVIATIONS

AB: Adult peripheral blood

ALL: Acute Lymphoblastic Leukemia

BCRs: B Cells Receptors

CAR: Chimeric Antigen Receptor

CB: Umbilical cord blood

CFU: Colony Forming Unit

CLL: Chronic Lymphocytic Leukemia

FBS: Fetal Bovine Serum

GvHD: Graft versus Host Disease

GvL: Graft versus Leukemia

hESCs: human Embryonic Stem Cells

hiPSCs: human induced Pluripotent Stem Cells

HLA: Human Leukocyte Antigens

HSCs: Hematopoietic Stem Cells

HSCT: Hematopoietic Stem Cell Transplant

IL: interleukin

IMC: Inner Cell Mass

iPSC: induced Pluripotent Stem Cell

ITAM: tyrosine-based activatory motif

ITIM: tyrosine-based inhibitory motif

KIR: immunoglobulin-like receptor

mEpiSCs: mouse Epiblast Stem Cells

mESCs: mouse Embryonic Stem Cells

MHC: Major Histocompatibility Complex

MOI: Multiplicity of Infection

NK: Natural Killer

PBMCs: Peripheral Blood Mononuclear Cells

rpm: revolutions per minute

TCRs: T Cells Receptors

RESUMEN

RESUMEN

El cáncer es un crecimiento anormal de las células las cuales tienden a proliferar de una manera incontrolada, y en algunas ocasiones, hacen metástasis. El cáncer no es una única enfermedad; de hecho, es un grupo de más de 100 enfermedades diferentes. Puede producirse en cualquier tejido y tiene muchas formas diferentes dependiendo de cada parte del cuerpo donde se produzca. La mayoría de los cánceres reciben su nomenclatura dependiendo del tipo de célula u órgano en el cual se origine. La leucemia es un tipo de cáncer que se origina en el tejido sanguíneo. En un paciente con leucemia, la médula ósea produce células blancas aberrantes, y estas no mueren cuando es debido. Estas células pueden desplazar a las células blancas normales, los eritrocitos y las plaquetas. Esto hace que las células sanas de la sangre no puedan realizar su trabajo correctamente. Respecto a los linfomas de tipo B, el más común en el linfoma non-Hodgkin (LNH), con un 85% de prevalencia. Otros de los tipos más frecuentes de leucemias de tipo B son la Leucemia Linfoblástica Aguda (LLA), siendo la más común en pacientes pediátricos; y la Leucemia Linfocítica Crónica (LLC), siendo más prevalente en pacientes de avanzada edad.

En pacientes con LLA, muchos de los progenitores hematopoyéticos se convierten en células linfocitos B muy rápidamente, las cuales no funcionan de la manera adecuada y no son capaces de luchar contra las infecciones de manera eficaz. Por otro lado, el rápido crecimiento de células neoplásicas hace que apenas quede espacio para las células blancas sanas, los eritrocitos y las plaquetas; por lo que esto lleva a infecciones, anemias y propensión al sangrado. Hoy en día, el diagnóstico y clasificación de la leucemia pasa por una combinación de métodos como son la morfología, citoquímica, citogenética, genética molecular, y especialmente, inmunofenotipado. En las células linfoblásticas de tipo B observamos la presencia del marcador de superficie CD19, junto con la expresión total o parcial de otros marcadores como CD20, CD22 y CD33. La media de presencia de blastos es del 82%. LLA es el cáncer más común en niños, con una prevalencia del 20-25% entre todos los casos. Esta enfermedad en niños ha conseguido tasas de supervivencia bastante altas en 5 años (90%). Sin embargo, los resultados son muy diferentes en adultos, los cuales tienen una tasa de supervivencia a 5 años del 40%.

LLC es una enfermedad de la sangre y la médula ósea que va empeorando lentamente. Es uno de los tipos de leucemia más comunes en adultos, y las células neoplásicas de tipo B expresan CLL CD5, CD19, CD20 dim y CD23. Esta se da normalmente en personas de avanzada edad, siendo muy rara en niños. En LLC los linfocitos B son aberrantes, por lo que no funcionan de la manera adecuada y no son capaces de luchar contra las infecciones de manera eficaz. Normalmente no causa sintomatología y esta se detecta en análisis de sangre rutinarios. La tasa de supervivencia a 5 años de estos pacientes es del 79,2%, pero aún hay muchos pacientes incurables.

Los tratamientos para leucemias varían mucho dependiendo del tipo de cáncer detectado y del estado de la enfermedad. Algunos de estos tratamientos son terapia dirigida, quimioterapia e inmunoterapia, los cuales puedes darse solos o combinados. La quimioterapia es el principal tratamiento en LLA, la cual normalmente se divide en tres partes: inducción, consolidación y mantenimiento. A veces, la quimioterapia no es suficientemente efectiva para curar esta

enfermedad, por lo que habría que recurrir a otro tipo de abordajes, como el trasplante de progenitores hematopoyéticos. Hay dos tipos de trasplantes: autólogo, donde las células provienen del mismo paciente; y alogénico, donde las células provienen de un donante. Esta última es la más recomendada para pacientes con LLA. Las células para el trasplante alogénico pueden provenir de la médula ósea de un donante histocompatible o de la sangre de cordones umbilicales almacenados en los bancos de sangre a nivel mundial. Las opciones de tratamiento para pacientes con LLC son muy variadas, y dependen de la edad del paciente, el grupo de riesgo y el motivo de tratamiento (que síntomas presente el paciente). Muchos pacientes viven muchos años con LLC, pero es muy difícil de curar.

Los pacientes con LLA o LLC refractarios o en recaída necesitan de nuevas terapias alternativas para hacer frente a la enfermedad. Estas nuevas terapias, especialmente inmunoterapias, están emergiendo para tratar este tipo de cánceres hematológicos. La terapia basada en CARs (del inglés, Chimeric Antigen Receptor) se está posicionando como una de las terapias más prometedoras para luchar contra este tipo de células malignas.

Un CAR es un receptor recombinante compuesto por un fragmento variable extracelular de una sola cadena, derivado de un anticuerpo; una región bisagra, un dominio transmembrana y motivos intracelulares. Dependiendo del número de motivos intracelulares, los CARs se clasifican en primera generación (1 motivo), segunda generación (2 motivos), tercera generación, (3 motivos) y cuarta generación (2 o 3 motivos además de otro dominio transmembrana que interactúa con el CAR). Hoy en día los más utilizados son los de segunda generación. Típicamente, el uso de la terapia CAR está asociada a los linfocitos T autólogos, formando los CAR-T, los cuales han demostrado ser muy eficaces en clínica. Sin embargo, hay ciertos efectos secundarios de gran riesgo asociados a ellos, como puede ser la tormenta de citoquinas y la neurotoxicidad. Además, algunos pacientes puede que no estén en condiciones que aportar sus propios linfocitos T para el tratamiento, y el tiempo de preparado del producto es de 5 semanas. Esto lleva a tantear un producto alogénico para este tipo de tratamientos. Los linfocitos T alogénicos causan la enfermedad injerto contra huésped (GvHD), por lo que para ser utilizados en terapias CAR, deberían ser primero genéticamente modificados. En la búsqueda de otro tipo celular del sistema inmune susceptible a ser el sustituto de los linfocitos T para terapias CAR alogénicas, nos encontramos con las células Natural Killer (NK), las cuales no causan GvHD.

Las células NK son grandes células granulares del sistema inmune, que constituyen la tercera mayor población de linfocitos en la sangre circulante (10-15%). Las células NK son capaces de matar células tumorales y células infectadas por virus de manera “natural”, es decir, sin la necesidad de presentación de antígeno. El fenotipo tradicional de las células NK es la ausencia de CD3 en su superficie (presente el linfocitos T) y la presencia de CD56 en la superficie de su membrana (ausente en linfocitos T). Las células NK se derivan de los progenitores hematopoyéticos en la médula ósea. Dependiendo del grado de maduración de las células NK, se han descrito distintos estadios en base a la presencia y/o ausencia de marcadores clave: estadio 1 (CD34+, CD45RA+, CD117−, CD94−, CD56−, CD16−), estadio 2 (CD34+, CD45RA+, CD117+, CD94−, CD56−, CD16−), estadio 3 (CD34− CD117+, CD94−, CD56−, CD16−), estadio 4 (CD34−, CD94+, CD117+/−, CD56bright, CD16+/−) y estadio 5 (CD34−, CD94+/−, CD117−, CD56dim, CD16+). Estos dos últimos estadios, el 4 y 5, son propios de células NK maduras. Como se ha mencionado con anterioridad, las células NK tienen un papel fundamental en la

lucha contra células cancerígenas. El reconocimiento por parte de las células NK implica una unión inicial a células Diana potenciales; y las interacciones entre receptores activadores e inhibidores con los ligandos de la célula diana. La integración de las señales transmitidas por estos receptores determina si las células NK atacan o no a esa célula diana. Si se produjese una activación de las células NK, estas contienen gránulos en su interior con moléculas como la perforina o la granzima-B, que son liberados al medio lisando la célula diana.

Hoy en día las células NK ya se usan en numerosos ensayos clínicos para combatir distintos tipos de cáncer, especialmente tumor sólido. Debido a la experiencia en la clínica con células NK y la ausencia de GvHD en tratamientos alogénicos, proponemos las células NK como vehículo de terapias basadas en la tecnología CAR. Para ello, en primer lugar obtenemos células NK de distintas fuentes celulares como son sangre periférica de adulto (AB), sangre de cordón umbilical (CB), progenitores hematopoyéticos de sangre de cordón umbilical y células pluripotentes inducidas (hiPS) o células embrionarias (hES). A continuación, probamos la eficacia de la tecnología CAR con las células NK derivadas de cada una de las cuatro fuentes celulares.

En primer lugar se obtuvieron células NK tanto de AB como de CB siguiendo el mismo procedimiento: se separan las células mononucleadas del restos de las células sanguíneas median un gradiente de ficoll; y mediante inmunoprecipitación magnéticas de consiguen aislar con gran eficacia (92.68±2.90 en AB y 91.46±5.14 en CB). Estas células se cultivan con el cóctel de citoquinas IL-2+IL-15, previamente testadas junto a otras citoquinas, durante 1 semana antes de la infección con el CD19-CAR ARI-0001, cedido por el Hospital Clinic. Una vez que las células son transducidas con este Car, se cultivan por una semana más antes de realizar los ensayos de funcionalidad. Como control, siempre se dejan parte de las células sin transducir. A pesar de que está descrito que las células NK no son fáciles de infectar, obtenemos unos porcentajes de infección del 47.46 % en AB NKs y un 46.8% en CB NKs. Al realizar los ensayos de funcionalidad, observamos que tanto las células no transducidas como aquellas transducidas de ambas fuentes celulares están en buen estado, ya que no altera su capacidad citotóxica y matan manera similar a las células control K562, que no expresan el CD19 en su superficie. Si nos centramos en el comportamiento frente a aquellas células que presentan el CD19 en su membrana, vemos que aquellas que están infectadas con el CAR ARI-0001 matan mejor que aquellas que no están infectadas; observándose un efecto significativo de la presencia del CAR. Las células NK de ambas fuentes celulares se comportan de manera similar, observándose un ligero incremento en la capacidad de matar en las células NK de AB. Otro aspecto importante de cara a la clínica es el mantenimiento de la expresión de CAR en las células NK, por lo que las dejamos en cultivo hasta 28 días después de la transducción. Observamos que el CAR se mantiene presente en NKs de ambas fuentes celulares durante 21, sin embargo, tras 28 días de cultivo, baja. Esto se correlaciona con la viabilidad de las células durante estos días en cultivo. No obstante, la presencia y viabilidad del CAR baja un poco más rápidamente en NKs de AB. Sobre las células NK de estas dos fuentes celulares, podemos concluir que serían candidatas a tener en cuenta para futuros ensayos clínicos CAR-NK alogénicos.

A continuación, nuestro siguiente objetivo fue la obtención de células NK funcionales y maduras a partir de progenitores hematopoyéticos de sangre de cordón umbilical. Para ello el primer paso es purificar las células CD34+ de la sangre del cordón. Primero se separan las

células mononucleadas del restos de las células sanguíneas mediante un gradiente de ficoll; y mediante inmunoprecipitación magnéticade consiguen aislar las células CD34+ con gran eficacia (>90%). A continuación estas células se cultivan durante 42 días con un medio que contiene un cóctel de citoquinas y sobre células soporte: OP9 o M2-10B4. A partir del día 14 de cultivo, se chequea semanalmente el estado de maduración de las células NK viendo la presencia y/o ausencia de marcadores clave (CD117, CD94, CD56 y CD16) mediante citometría de flujo. Además, para determinar su funcionalidad, a los días 28, 35 y 42 de cultivo se realizan ensayos de citotoxicidad y degranulación. Con ambas fuentes celulares observamos un patrón de maduración *in vitro* que se correlaciona con el que se da *in vivo.* A día 28 de cultivo observamos en punto óptimo de células CD56+ maduras, siendo significativamente mayor el número de células NK maduras generadas con las células soporte OP9, llegando a doblar 700 veces el número inicial de células. Durante los días 28, 35 y 42 también observamos la presencia de moléculas granulocíticas como perforina y granzima-B. Además, estas células NK generadas *in vitro* son totalmente funcionales. Teniendo estos datos en cuenta, se concluyó que las células NK diferenciadas *in vitro* a partir de progenitores hematopoyéticos de CB más adecuadas para la terapia CAR serían aquellas cultivadas durante 28 días sobre OP9. Aunque estas células tuvieron valores de transducción satisfactorios con el CAR ARI-0001 (38.85±5.71%), no funcionan frente a células que expresan el CD19 en su superficie como se esperaba.

Seguidamente, la siguiente fuente celular a tratar fueron hiPS e hES. Para obtener células NK a partir de células pluripotentes se debe realizar un protocolo en dos pasos, diferenciando primeramente hacia progenitores hematopoyéticos CD34+. En un principio trabajamos con hiPS y hES adaptadas a células soporte, las cuales posteriormente fueron adaptadas a un cultivo estas células soporte. En estas condiciones, las hiPS y las hES se encuentran en un estadío de pluripotencia llamado “primed”, el cual está más comprometido que el estadío “naïve”. Este estadío es más parecido morfológica y potencialmente a las células embrionarias de ratón. Aunque ambos corresponden a estadíos de pluripotencia, está descrito que aquellas células en estadío naïve son más flexible a la hora de ser diferenciadas a cualquier tipo celular. Es por ello que convertimos nuestras células primed a células naïve. Realizamos experimentos de diferenciación con todas ellas y observamos que obteníamos un mayor porcentaje de células CD34+ funcionales con aquellas hiPS o hES en estadío primed sin células soporte. Una vez obtenidos los progenitores hematopoyéticos, procedemos a realizar la diferenciación *in vitro* hacia célula NK con el protocolo anteriormente descrito. Sin embargo, no obtenemos un buen número de células NK, éstas no representan en patrón de diferenciación *in vivo* y no son funcionales. Es por ello que se necesita realizar más trabajo con esta fuente celular.

Por último, el CAR-CD19 utilizado en todos estos experimentos (ARI-0001) está adaptado para células T. Es por ello que secuenciamos siete nuevas variantes con motifs adaptados a células NK (2B4, NKG2D, DAP10 y DAP12). En primer lugar se probó la eficacia de la transducción de las siete nuevas variantes en células NK de AB. Las variantes de CAR tienen una eficacia similar al CAR ARI-0001, con la salvedad de la variante NK1, muy por debajo de CAR ARI-0001; y la variante NK2, por encima del CAR ARI-0001. Siguiendo la literatura, primeramente se testó en células NK de AB la funcionalidad de la variante NK5, la cual combina NKG2D y 2B4, junto con el CAR-ARI0001 como control de referencia. Cuando enfrentamos las células NK infectadas con el CAR-CD19 NK5 contra células diana que expresan CD19 en su membrana, observamos una mayor actividad por parte de las células infectadas comparándolas con aquellas que no lo

están. Cabe destacar que, aunque el CAR-CD19 NK5 funcione correctamente, el CAR-CD19 ARI-0001 es ligeramente superior. Posiblemente esto sea debido a que el porcentaje de infección es algo menor con el CAR NK5. Además, no solo probamos las células NK de AB, sino que también probamos la variante NK5 con células NK diferenciadas in vitro a partir de progenitores hematopoyéticos de CB. En esta ocasión, estas células NK transducidas con el NK5 sí que responden mejor frente a las células diana que presentan CD19 en su superficie. Aunque se han de realizar más experimentos, parece ser que esta variante del CAR podría ser la alternativa para estas células NK diferenciadas in vitro a partir de CD34+ de CB.

En conclusión, las células NK de AB y CB podrían ser buenos vehículos para terapias basadas en tecnología CAR, gracias a su fácil accesibilidad y su buen funcionamiento. Sin embargo, hay mejoras a realizar, como la expansión ex vivo de estas células. Por otro lado, las células NK diferenciadas in vitro a partir de progenitores hematopoyéticos de CB podrían solventar este problema, ya que obtenemos grandes cantidades de células NK, las cuales podrían ser almacenadas como producto "off the shelf". Hemos observado que el CAR ARI-0001 parece no funcionar con ellas, pero las nuevas variantes de CAR adaptadas a células NK, como en CAR NK5, parecen ser una buena alternativa. Respecto a la última fuente celular de NKs, desde nuestro punto de vista no podemos proponerlas como candidatas para este tipo de terapias, ya que primero necesitamos mejorar los protocolos de diferenciación. Por último, las nuevas variantes de CAR-CD19 necesitan ser testadas para concluir si hay alguna de ellas que destaque, para ser incluida en un futuro en un ensayo clínico.

SUMMARY

SUMMARY

Cancer is an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metastasize (spread). Cancer is not one disease; in fact, it is a group of more than 100 different and distinctive diseases. It can involve any tissue of the body and have many different forms in each body area. Most cancers are named for the type of cell or organ in which they start. Leukemia is a cancer type that starts in the tissue that forms blood. In a patient with leukemia, the bone marrow makes abnormal white blood cells. The aberrant cells are leukemia cells. Unlike normal blood cells, leukemia cells do not die when they should. They may crowd out normal white blood cells, red blood cells, and platelets. This makes it hard for normal blood cells to do their work. Regarding B cell lymphomas, non-Hodgkin Lymphomas (NHL) make up most (about 85%) of these kind or lymphomas. Other two of the main types of leukemia involving B cells are Acute Lymphoblastic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL).

ALL represents a biologically and clinically heterogeneous group of diseases. In a patient with ALL, too many stem cells become lymphoblasts, B lymphocytes very quickly. The cells do not work like normal lymphocytes and are not able to fight infection very well. Also, as the number of leukemia cells increases in the blood and bone marrow, there is less room for healthy white blood cells, red blood cells, and platelets. This may lead to infection, anemia, and easy bleeding.The current gold standard in the diagnosis and classification of leukemia involves a combination of methods that include morphology, cytochemistry, immunophenotype, cytogenetics, and molecular genetics. In particular, immunophenotype is an essential component of the initial diagnostic work-up of ALL. In all B-lineage ALL, total expression of CD19 surface marker is present, along with the total or partial expression of other surface markers such as CD20, CD22 and CD33. The median content of blast cells is around 82%. ALL is the most common cancer among children with a prevalence of 20-25% of all cases. This disease has become highly curable with 5 years survival rates above 90%. In contrast, these results are very different in adults, where the 5 years survival rate falls to 40% with the conventional treatments.

CLL is a blood and bone marrow disease that usually gets worse slowly. It is one of the most common types of leukemia in adults. It is a low-grade lymphoproliferative neoplasm with $\geq 5 \times 10^9$/L clonal B-cells in the peripheral circulation that express CD5, CD19, dim CD20, and CD23. It often occurs during or after middle age, being quite rare in children. In CLL, too many blood stem cells become abnormal lymphocytes and do not become healthy white blood cells. As a consequence, the lymphocytes are not able to fight infection very well. Usually CLL does not cause any signs or symptoms and is found during a routine blood test. The survival rate for these patients at 5 years is 79.2%, but it is still an incurable disease in many patients.

Treatments for leukemia vary widely depending on the type of cancer diagnosed and the stage of the disease. Some treatments, such as targeted therapy, chemotherapy and immunotherapy, may be recommended either alone or in combination. Chemotherapy is the main treatment for ALL. Treatment is usually divided into 3 phases: induction, consolidation and maintenance. Sometimes standard doses of chemotherapy are not always able to cure ALL. Due to this, other treatments must be used, as hematopoietic stems cell transplant

(HSCT). There are two main types of stem cell transplants: allogeneic stem cell transplant, in which the stem cells come from someone else. This is the preferred type of transplant when treating ALL; and autologous stem cell transplant, in which the patient gets back his or hers own cells. For an allogenic transplant,the cells may come from an HLA-matched, unrelated donor or umbilical cord blood units storage in blood banks worldwide.

Treatment options for CLL patients vary largely, depending on the person's age, the disease risk group, and the reason for treating (for example, which symptoms it is causing). Many people live a long time with CLL, but in general it is very difficult to cure, and early treatment hasn't been shown to help people live longer. Chimeric antigen receptor (CAR) based therapy is becoming one of the most promising therapies to fight against these refractory and relapsed hematological cancers.

A CAR is a recombinant receptor construct composed of an extracellular single-chain variable fragment (scFv) derived from an antibody or a full-length antibody, linked to a hinge region, a transmembrane domain, and an intracellular tyrosine-based activation motif comprised of either a region of CD3 T-cell receptor (TCR) complex, the CD3 ζ chain, or FcR receptor γ. Depending on the number of intracellular motifs, CARs are classified in first generation (1 motif), second generation (2 motifs), third generation (3 motifs) and fourth generation (2 or 3 motifs and a transmembrane motifs which interacts with the CAR). Nowadays the most used ones are second generation CARs. Typically, CAR therapy is associated with autologous T lymphocytes, forming CAR-T cells, which are very efficient in clinic. However, there are some high risk side effects associated to them, like cytokine storm and neurotoxicity. Moreover, some patients may not meet the conditions to contribute with their own lymphocytes for the treatment; and the standard preparing time for the product is 5 weeks. This leads us to explore a allogenic product for these kind of treatments. Allogenic T lymphocytes cause Graft versus Host Disease (GvHD), so that, in order to use them in CAR therapies, they must be genetically modified. Looking for another cell type of the immune system susceptible of being a substitute of T cells for allogenic CAR therapies, we found Natural Killer (NK) cells, which do not cause GvHD.

NK cells are large granular cells of the immune system that constitute the third major lymphocyte subset and represent approximately 10–15% of circulating lymphocytes in blood. NK cells are able to kill tumor cells and virally infected cells "naturally," that is, in a spontaneous fashion that do not require any priming and is not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules. The traditional cell surface phenotype defining human NK cells within the lymphocyte population shows a lack of CD3 (by that excluding T cells) and expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) found on NK cells and a minority of T cells. Depending on the maturation stage of the NK cells, different stages have been described based on the presence or absence of key markers: *stage 1* (CD34+, CD45RA+, CD117−, CD94−, CD56−, CD16−), *stage 2* (CD34+, CD45RA+, CD117+, CD94−, CD56−, CD16−), and *stage 3* (CD34− CD117+, CD94−, CD56−, CD16−). Once they reach a mature stage, NK cells are phenotypically described by their surface markers as *stage 4* (CD34−, CD94+, CD117+/−, CD56bright, CD16+/−) and *stage 5* (CD34−, CD94+/−, CD117−, CD56dim, CD16+). Stage 4 and 5 are owned of mature NK cells. As previously mentioned, NK cells have a key role against cancer cells. NK cell recognition involves the initial binding to potential target cells, interactions between activating and inhibitory

receptors with ligands available on the target, and the integration of signals transmitted by these receptors, which determines whether the NK cell detaches and moves on or stays and responds. NK cells respond by reorganizing and releasing cytotoxic granules (perforin and granzyme-B) and by transcribing and secreting cytokines.

Nowadays, NK cell are being used in numerous clinical trials to fight different types of cancer, especially solid tumors. Due to the clinical experience with NK cells and the absence of GvHD in allogenic treatments, we propose NK cells as a vehicle for CAR based therapies. Firstly, we obtain NK cells from different cells sources, such as adult peripheral blood (AB), umbilical cord blood (CB), umbilical cord blood hematopoietic progenitors and human pluripotent stem cells (hiPS) or embryonic stem cells (hES). Next, we prove the efficacy of CAR technology with NK cells of each cell source.

Firstly, we obtain NK cells from AB and CB following the same protocol: mononuclear cells are separated from the rest of the blood by density gradient; and NK cells are isolated by inmunomagnetic precipitation with great efficacy (92.68±2.90 in AB y 91.46±5.14 in CB). These cells are culture with a cytokine cocktail of IL-2+IL-15, previously testes along with other cytokines, for a week before being transduced with CD19-CAR ARI-0001 kindly provided by Hospital Clinic. Once these cells are transduced with this CAR, they are cultivated for one more week before performing the functional assays. As a control, we always keep non-transduced NK cells. In spite of being described that NK cells are not easy to transduced, we obtain infection percentages of 47.46 % in AB NK cells and 46.8% in CB NK cells. When performing the functional assays, we observe, that non-transduces and transduced NK cells from both sources are in good shape, meaning that their cytotoxic capability is not compromised and they kill K562 target cells, which do not express CD19 in their surface in the same manner. If we focus on the behavior of NK cells when facing CD19 expressing target cells, we observe that those which are infected with the CAR ARI-0001 kills better than those which are not infected; due to a positive effect of the presence of the CAR. NK cells from both sources behave in a similar way, although we observe a little increase in the killing capability of AB NK cells. Another important point is the maintenance of the CAR expression in the NK cells, so that we let the cells in culture for 28 days after transduction. We observe that CAR is still present in the NK cells from both sources for 21 days, however, after 28 days in culture it drops. This correlates with the viability of the NK cells during culture. Nonetheless, the presence and viability of the CAR drops slightly quicker in AB NK cells. WE can conclude that NK cells from both cells sources are good candidates for future allogenic CAR-NK cell clinical trials.

Then, our next goal was obtaining functional and mature NK cells from umbilical cord blood hematopoietic progenitors. For that, the first step is purifying CD34+ cells from cord blood. Firstly, mononuclear cells are separated by a density gradient; and CD34+ cells are isolated by inmunomagnetic precipitation with great efficacy (>90%). Next, these cells are cultured for 42 days in a medium with a cytokine cocktail and feeder cells: OP9 or M2-10B4. From 14 days of culture on, we check the state of maturation of the NK cells, taking into account the presence or absence of key markers (CD117, CD94, CD56 y CD16) by flow cytometry. Besides, to determine their functionality, at 28, 35 and 42 days of culture we perform cytotoxicity assays

and degranulation assays. We observe with both feeder cells an *in vitro* maturation patron that correlates with the one *in vivo*. At day 28 of culture, we achieve the optimal point of CD56+ mature cells, with a significantly higher number of mature NK cells generated onto OP9, multiplying by 700 times the initial number of cells. During 28, 35 and 42 days of culture, we also observe the presence of perforin and granzyme-B. Moreover, these in vitro generated NK cells are totally functional. Taking this data into account, we conclude that the more appropriated culture conditions for NK cells in a CAR therapy would be the *in vitro* differentiated NK cells onto OP9 feeder layer at 28 days of culture. Although these NK cells have a satisfactory transduction rates with CAR ARI-0001 (38.85±5.71%), they do no kill as expected, when confronting them against CD19 expressing target cells.

Afterwards, the next cell source to dig in was hiPS and hES. In order to obtain NK cells from pluripotent stem cells, we need to perform a two-step protocol, firstly obtaining CD34+ cells. At first, we worked with hiPS and hES in a feeder system, but alter, they were adapted to a feeder-free system. In these conditions, hiPS and hES are in a pluripotent staged called primed, which is more compromised than naïve stage. This last stage is similar morphologically and potentially to mouse embryonic stem cells. Though both stages are pluripotent stages, it has been described that naïve cells are more prompt to differentiate to any cells type. Due to this, we convert our primed cells to naïve cells. We perform experiments of differentiation with all the pluripotent and culture conditions and we observe that we achieve a higher percentage of CD34+ cell with primed hiPS or hES in a feeder-free system. Once we obtain the hematopoietic progenitors, we follow the previously described protocol to obtain *in vitro* generated NK cells from these CD34+ cells. However, we do not obtain a good number of NK cells, and these are not mature and functional. As a consequence, we need to improve this protocol.

Lastly, CD19-CAR used in all our experiments (ARI-0001) is adapted to T cells. Because of this, we sequenced seven new variants with motifs adapted to NK cells (2B4, NKG2D, DAP10 and DAP12). Firstly, we check the transduction efficacy of all the new variants in AB NK cells. These CAR variants have very similar transduction efficiency, except for NK1 variant, with is much lower than ARI-0001 CD19-CAR; and NK2 variant efficiency is higher that ARI-0001 CD19-CAR. According to the literature, we firstly tried NK5 variant with AB NK cells, which combines NKG2D and 2B4; and we tested along with ARI-0001 CD19-CAR, as a control. When facing AB NK cells CD19 expressing target cells, we observe that those which are infected with the CAR ARI-0001 kills better than those which are not infected. Although NK5-CAR variant works properly, we must say ARI-0001 CD19-CAR works slightly better. This could occur due to the lower transduction percentage of NK5-CAR. Moreover, we not only tried NK5-CAR with AB NK cells, but also with CB CD34+ *in vitro* generated NK cells. This time, NK cells transduced with NK5-CAR kill better D19 expressing target cells than the ones that are not transduced. Even though we need to perform more experiments, this CAR variant could be an alternative for the use of these CB CD34+ *in vitro* generated NK cells in a CAR based therapy.

To sum up, AB and CB NK cells could be good vehicles for CAR based therapy, due to their accessibility and their good functioning. Yet, there is room for improvement, as we could improve the ex vivo expansion of these cells. What is more, CB CD34+ *in vitro* generated NK

cells could solve the expansion problem, as we achieve huge amount of NK cells, which could be storage as an “off the shelf” product. We have observe that ARI-0001 CD19-CAR seems to not work with them; but the new CAR variants, like NK5-CAR could be a good alternative for them. Regarding the last cell source, hiPS and hES, we cannot propose them as candidates for these kinds of therapies, as we need to improve our differentiation protocols first. Lastly, all the new CD19-CAR variant need to be tested in order to check which one could be the best for a future clinical trial.

INTRODUCTION

INTRODUCTION

Human person: a physical being, a knower, a responsible agent, a person in relation to other persons, to society, and to the end, or purpose, of human life. Biologically, human beings are known as *Homo sapiens* (from Latin “wise man”), they are one of several species grouped into the genus *Homo*, but it is the only one that is not extinct. Human beings are anatomically similar and related to the great apes but are distinguished by a more highly developed brain and a resultant capacity for articulate speech and abstract reasoning. In addition, human beings display a marked erectness of body carriage that frees the hands for use as manipulative members (1). We like to think about human beings as unique and somewhat separated from animal kingdom, but truth is human beings are still animals, mammals precisely. As complex multicellular organisms, human beings are made up by billions of cells, which are organized into tissues (2).

Tissues are groups of similar cells that perform a particular function within an organism. There are four basic types of animal tissues: epithelial tissue, muscle tissue, nervous tissue and connective tissue (3).

Epithelial tissue has four main functions: protection of the underlying tissues, absorption, secretion, and reception of sensory stimuli. There are different types of epithelial tissue depending on their function in a particular location. These tissues are classified based on the number of cell layers or based on the shape of the cells (4).

Muscle tissue is a soft tissue that composes muscles in animal bodies, and gives rise to muscles' ability to contract. This tissue varies with function and location in the body. In mammals the three types are: skeletal or striated muscle; smooth or non-striated muscle; and cardiac muscle (5).

Nervous tissue is the main tissue component of the nervous system. The nervous system regulates and controls bodily functions and activity and consists of two parts: the central nervous system (CNS) comprising the brain and spinal cord, and the peripheral nervous system (PNS) is comprising the branching peripheral nerves. Functions of the nervous system are sensory input integration, control of muscles and glands, homeostasis, and mental activity (6).

Connective tissue is found in between other tissues everywhere along the body and made up of many different types of cells that are all involved in structure and support of the body. Connective tissue can be broadly subdivided into proper connective tissue, and special connective tissue. Connective tissue proper consists of loose connective and dense connective tissues. Loose and dense connective tissues are distinguished by the ratio of ground substance to fibrous tissue. (7). Special connective tissue consists of reticular connective tissue, adipose tissue, cartilage, bone, and blood (8).

Blood

Definition

Blood is a fluid connective tissue in humans and other animals that delivers necessary substances such as nutrients and oxygen to the cells and transports metabolic waste products away from those same cells (9). It also delivers immune cells to fight infections and tumor cells; and contains platelets that can form a plug in a damaged blood vessel to prevent blood loss. The average human adult has 4- 5 liters of blood in his or her body (10). In vertebrates, it is composed of blood cells suspended in blood plasma. Plasma, which constitutes 55% of blood fluid, is mostly water (92% by volume), and contains proteins, glucose, mineral ions, hormones, carbon dioxide (plasma being the main medium for excretory product transportation), and blood cells themselves. Albumin is the main protein in plasma, and it functions to regulate the colloidal osmotic pressure of blood. The blood cells are mainly red blood cells (also called RBCs or erythrocytes), white blood cells (also called WBCs or leukocytes) and platelets (also called thrombocytes).

Hematopoiesis

All these cellular components are derived from hematopoietic stem cells (HSCs). HSCs reside in the medulla of the bone (bone marrow) and have the unique ability to give rise to all of the different mature blood cell types and tissues (11). The ability of stem cells to divide asymmetrically to produce one stem and one non-stem daughter cell is often considered to be one of the defining characteristics of stemness. On the other hand, there is ample evidence suggesting that adult stem cell can and do divide symmetrically, giving 2 stem cells or two differentiated cells (**Figure 1**) (12). HSCs are self-renewing cells and these cells divide asymmetrically: when they differentiate, at least some of their daughter cells remain as HSCs, so the pool of stem cells is not depleted (13). The other daughters of HSCs (myeloid and lymphoid progenitor cells) can follow any of the other differentiation pathways that lead to the production of one or more specific types of blood cell, but cannot renew themselves. The pool of progenitors is heterogeneous and can be divided into two groups; long-term self-renewing HSC and only transiently self-renewing HSC, also called short-terms (14). This is one of the main vital processes in the body. This process is called hematopoiesis (**Figure 2**).

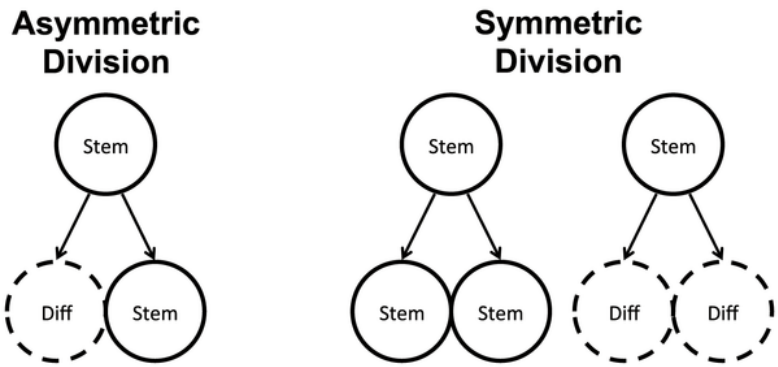

Figure 1: Differences between asymetric division, where the stem cell divides into another stem cell and a differentiated cell; and symmetric division, where the stem cell divides into two identical cells. Shahriyari L et al, Plos One.2013

Blood cells

There are several types of different cells in blood, all derived from HSCs. These cells can be gathered in three main groups: red blood cells, platelets and white blood cells.

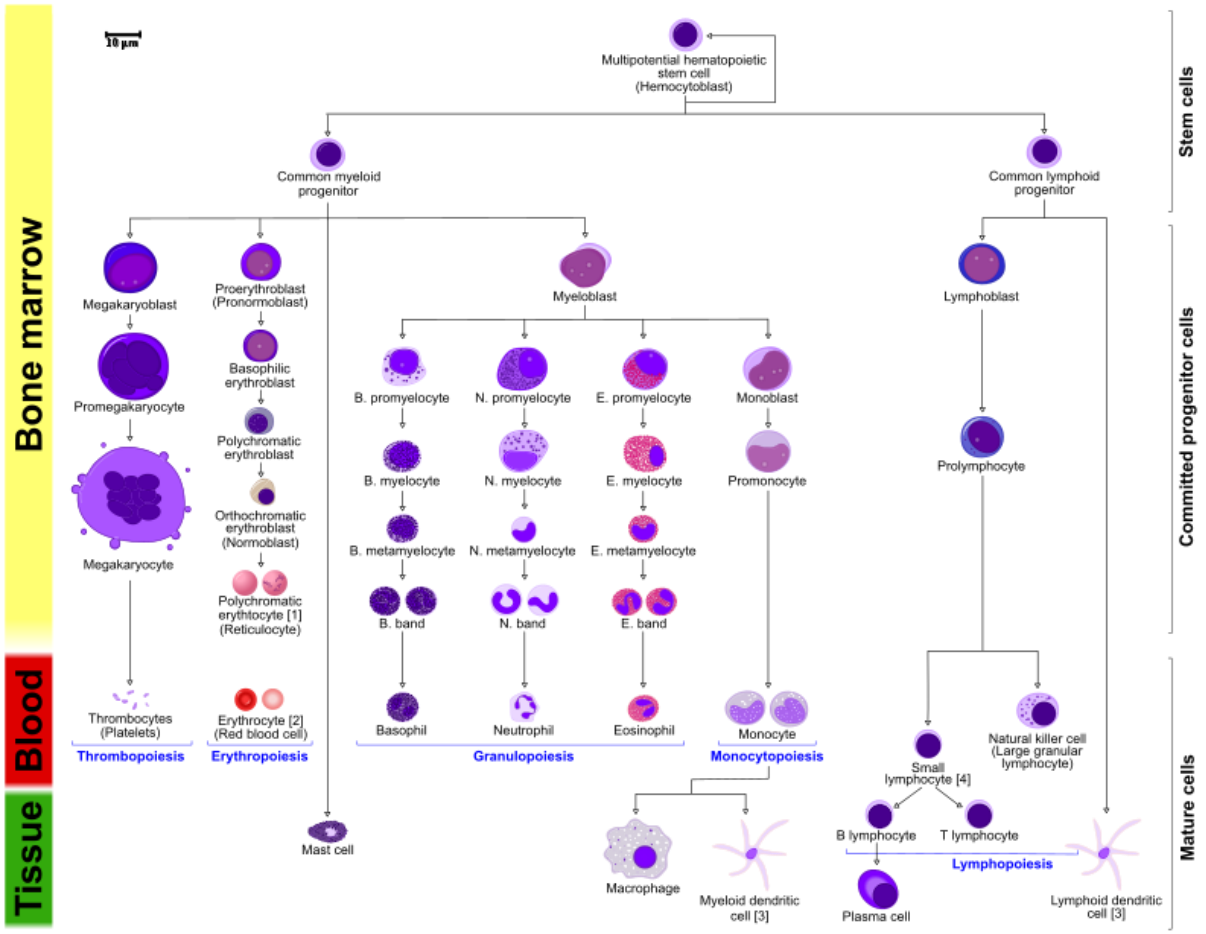

Figure 2: Human hematopoiesis diagram, from the hematopoietic stem cells, to committed progenitor cells and, finally, mature blood cells . Hematopoietic stem cells are essential to hematopoiesis, the formation of the cells within blood. Hematopoietic stem cells can replenish all blood cell types and self-renew, and this occurs in the bone marrow. There are two main lineage of the blood cells: myeloid and lymphoid. The myeloid and lymphoid progenitors will suffer several transformations to become a mature blood cell.

Red blood cells

The most abundant cells in vertebrate blood are red blood cells, also known as RBCs, red cells, red blood corpuscles, haematids, erythroid cells or erythrocytes. These cells are derived from myeloid progenitors. These are anucleated cells which contain hemoglobin, an iron-containing protein, that facilitates oxygen transport by reversibly binding to this respiratory gas and greatly increasing its solubility in blood. In contrast, carbon dioxide is mostly transported extracellularly as bicarbonate ion transported in plasma (15) (**Figure 2**).

Platelets

Platelets, also called thrombocytes , are a cellular component of blood whose function (along with the coagulation factors) is to react to bleeding from blood vessel injury by clumping, thereby initiating a blood clot (16). These cells are derived from myeloid progenitors. Platelets have no cell nucleus: they are fragments of cytoplasm that are derived from the megakaryocytes of the bone marrow, and then enter into circulation (17). Circulating inactivated platelets are biconvex discoid (lens-shaped) structures, while activated platelets have cell membrane projections covering their surface (18). Platelets are found only in mammals. (**Figure 2**)

White blood cells

White blood cells (also called leukocytes or leucocytes and abbreviated as WBCs) are the cells of the immune system that are involved in protecting the body against infectious disease, foreign invaders and tumor cells. All white blood cells are produced and derived from multipotent cells in the bone marrow known as hematopoietic stem cells (**Figure 2**). Leukocytes are found throughout the body, including the blood and lymphatic system (19). The name "white blood cell" derives from the physical appearance of a blood sample after centrifugation. White cells are found in the buffy coat, a thin, typically white layer of nucleated cells between the sediment red blood cells and the blood plasma. The scientific term “leukocyte” directly reflects its description. It is derived from the Greek roots *leuk*- meaning "white" and *cyt*- meaning "cell". All white blood cells are nucleated, which distinguishes them from the other blood cells, the enucleated red blood cells (RBCs) and platelets. Types of white blood cells can be classified in standard ways. Two pairs of broadest categories classify them either by structure (granulocytes or agranulocytes) or by cell lineage (myeloid cells or lymphoid cells). These broadest categories can be further divided into the five main types: neutrophils, eosinophils, basophils, lymphocytes, and monocytes (20). These types are distinguished by their physical and functional characteristics. Monocytes and neutrophils are phagocytic cells. Monocytes can differentiate into macrophages and myeloid lineage dendritic cells. Further subtypes can be classified; for example, among lymphocytes, there are B cells, T cells, and Natural Killer cells.

B cells, also known as B lymphocytes, function in the humoral immunity component of the adaptive immune system by secreting antibodies. Additionally, B cells present antigen (they are also classified as professional antigen-presenting cells (APCs)) and secrete cytokines. B cells, unlike the other two classes of lymphocytes, T cells and NK

cells, express B cell receptors (BCRs) on their cell membrane. BCRs allow the B cell to bind to a specific antigen, against which it will initiate an antibody response (21). Immature B cells express CD19, CD20, CD34, CD38, and CD45R in the surface membrane, but not IgM. For most mature B cells the key markers include IgM and CD19, a protein receptor for antigens (22). Naïve B cells are immature B cells that have not been exposed to an antigen. Once exposed to an antigen, the naïve B cell either becomes a memory B cell or a plasma cell that secretes antibodies specific to the antigen that was originally bound. During an initial infection (or primary immune response) involving a T-dependent antigen, naïve follicular B cells are activated in the presence of T follicular helper cells within the follicles of secondary lymphoid organs (i.e. spleen and lymph nodes) and undergo clonal expansion to produce a clonal colony of B cells that are specific for the antigen. Most of these clones differentiate into the plasma cells, also called effector B cells which produce a first wave of protective antibodies and help clear the infection, but a fraction persist as dormant memory cells that survive in the body on a long-term basis after having gone through a highly mutative and selective germinal center reaction (23) (**Figure 3**).

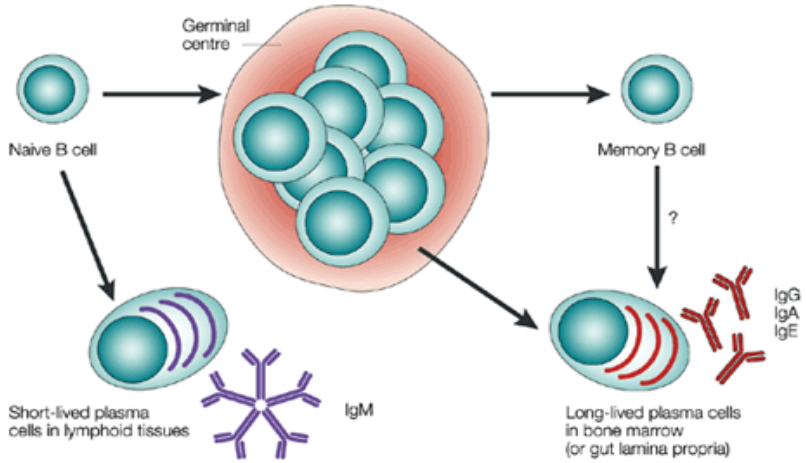

Figure 3: In vivo maturation and activation of B cells. Naïve B cells can become short-lived plasma cells in lymphoid tissues or go to a germinal center in where they will become long-lived plasma cells or memory B cells, which eventually will become long-lived plasma cells. Adapted from David Gray. 2002

A **T cell** is a CD3+ lymphocyte which develops in the thymus gland and plays a central role in the immune response. T cells can be distinguished from other lymphocytes by the presence of a T-cell receptor (TCR) on the cell surface; which is responsible for recognizing fragments of antigen as peptides bound to major histocompatibility complex (MHC) molecules. These immune cells originate as precursor cells, derived from bone marrow, and develop into several distinct types of T cells once they have migrated in to the thymus gland - for which these cells are named. T cell differentiation continues even after they have left the thymus. Groups of specific, differentiated T cells have an important role in controlling and shaping the immune response by providing a variety of immune-related functions. One of these functions is immune-mediated cell death, and it is carried out by T cells in several ways: *CD8+ T cells*, also known as "Killer cells", are cytotoxic - this means that they are able to directly kill virus-infected cells as well as cancer cells. CD8+ T cells are also able to utilize small signalling proteins, known as cytokines, to recruit other cells when mounting an

immune response. A different population of T cells, the *CD4+ T cells*, functions as "Helper cells". Unlike CD8+ Killer T cells, these CD4+ Helper T cells function by indirectly killing cells identified as foreign: they determine if and how other parts of the immune system responds to a specific, perceived threat. Helper T cells also use cytokine signalling to influence regulatory B cells directly, and other cell populations indirectly (24). The regulatory T cells, formerly known as suppressor T cells, are a subpopulation of T cells that modulate the immune system, maintain tolerance to self-antigens, and prevent autoimmune disease. *Tregs* are immunosuppressive and generally suppress or downregulate induction and proliferation of effector T cells. Tregs express the biomarkers CD4, FOXP3, and CD25 and are thought to be derived from the same lineage as naïve CD4 cells (25). Finally, *Natural killer T* (NKT) cells are a heterogeneous group of T cells that share properties of both T cells and natural killer cells. Many of these cells recognize the non-polymorphic CD1d molecule, an antigen-presenting molecule that binds self and foreign lipids and glycolipids. They constitute only approximately 0.1% of all peripheral blood T cells. Natural killer T cells should not be confused with natural killer cells. Upon activation, NKT cells are able to produce large quantities of interferon gamma, IL-4, and granulocyte-macrophage colony-stimulating factor, as well as multiple other cytokines and chemokines (such as IL-2, IL-13, IL-17, IL-21, and TNF-alpha). Natural Killer T (NKT) cells recognize protected microbial lipid agents which are presented by CD1d-expressing antigen presenting cells. This serves as a pathway for NKT cells to fight against infections and enhance the humoral immunity (26,27).

Natural Killer cells, or NK cells, are a type of cytotoxic lymphocyte critical to the innate immune system. The role NK cells play is analogous to that of cytotoxic T cells in the vertebrate adaptive immune response. NK cells provide rapid responses to virus-infected cells, acting at around 3 days after infection, and respond to tumor formation. Typically, immune cells detect the major histocompatibility complex (MHC) presented on infected cell surfaces, triggering cytokine release, causing lysis or apoptosis. NK cells are unique, however, as they have the ability to recognize stressed cells in the absence of antibodies and MHC, allowing for a much faster immune reaction. They were named "natural killers" because of the initial notion that they do not require activation to kill cells that are missing "self" markers of MHC class 1. This role is especially important because harmful cells that are missing MHC I markers cannot be detected and destroyed by other immune cells, such as T lymphocyte cells (28).

Natural Killer Cells

Description

Natural Killer (NK) cells and their functions were already described more than 30 years ago. For the first time, in 1975, these cells were described as bigger lymphocytes than B cells and T cells, which contained distinctive cytoplasmic granules. NK cells were characterized as cells which showed a co-stimulatory independent spontaneous cytotoxic capacity. This fact differentiates functionally NK cells from other lymphocytes such as B cells and T cells. (29,30).

NK cells are large granular cells of the immune system that constitute the third major lymphocyte subset and represent approximately 10–15% of circulating lymphocytes in blood (31). NK cells are able to kill tumor cells and virally infected cells "naturally," that is, in a spontaneous fashion that do not require any priming and is not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules (32,33). The traditional cell surface phenotype defining human NK cells within the lymphocyte population shows a lack of CD3 (by that excluding T cells) and expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) found on NK cells and a minority of T cells (34).

Development

It is clear that NK cells are part of the hematopoietic system and are derived from CD34+ hematopoietic progenitor cells or stem cells (HSCs) (35); but while human T cells develop in the thymus and human B cells develop in the bone marrow, characterization of full NK-cell developmental pathway from CD34+ HSCs within the bone marrow (or in the thymus) have not been fruitful (36). We could know that NK development might not occur wholly in the bone marrow from the observation that a subgroup of NK cells, designated $CD56^{bright}$ for their high-density surface expression of CD56, could be isolated from lymph nodes and tonsils (secondary lymphoid tissue or SLT). $CD56^{bright}$ NK cells are relatively dominant in SLT compared with their more abundant $CD56^{dim}$ NK analogue found in bone marrow, blood, and spleen (37). This is an important fact that supports this hypothesis, as $CD56^{bright}$ NK cells are described to be less mature than $CD56^{dim}$ NK cells (38). The rich presence of CD34+CD45RA+ pre-NK cells and $CD56^{bright}$ NK cells within SLT in comparison to bone marrow or blood, along with an abundance of dendritic cells (DCs) and other antigen presenting cells (APCs) that express membrane-bound IL-15 (39), which is required for NK-cell maturation,(40) suggested that SLT may be a site for NK-cell development *in vivo*. Before reaching a mature stage, they acquire progressively and orderly different surface markers, being classified into *stage 1* (CD34+, CD45RA+, CD117−, CD94−, CD56−, CD16−), *stage 2* (CD34+, CD45RA+, CD117+, CD94−, CD56−, CD16−), and *stage 3* (CD34− CD117+, CD94−, CD56−, CD16−). Once they reach a mature stage, NK cells are phenotypically described by their surface markers as *stage 4* (CD34−, CD94+, CD117+/−, CD56bright, CD16+/−) and *stage 5* (CD34−, CD94+/−, CD117−, CD56dim, CD16+) (41) (**Figure 4**).

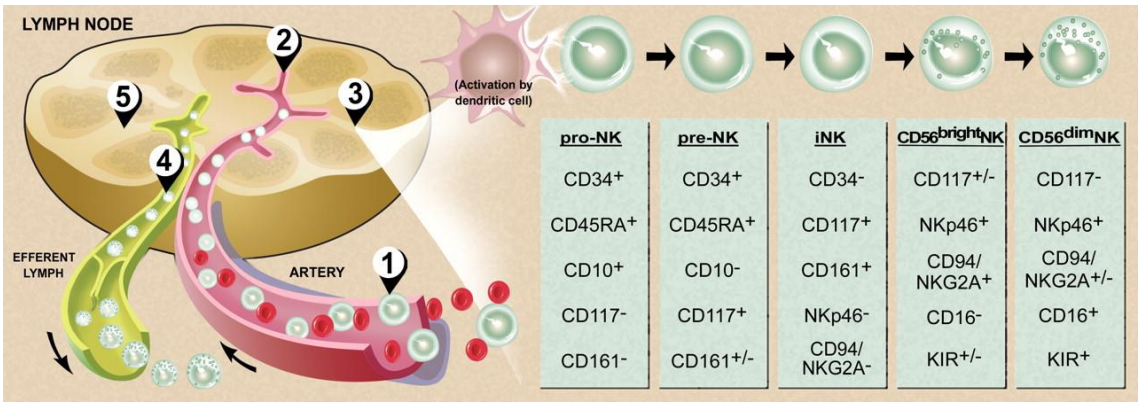

Figure 4: Different surface markers during NK cell development from pro-NK cells until mature stages. Pro-NK correlates with NK cell maturation stage 1, pre-NK with stage 2, iNK with stage 3, CD56bright NK with stage 4 and CD56dim with stage 5. (36).

Function

NK cells are part of the innate immune system. NK cells trigger a spontaneous response since the first contact with the antigen, without prior sensitization. These cells have an important and well characterized role in the immune response against infection and malignant transformations. In particular, NK cells have a main role in cytomegalovirus (42), herpes virus (43) and influenza (44) virus infection. NK cells have specific receptors for the most common protein viral motifs (like hemagglutinin). Moreover, NK cells are recruited to the infection site by chemokines, and ones there, NK cells perform a direct cytotoxicity by the secretion of perforin and IFN-γ. These innate lymphocytes are essential for controlling virus proliferation and generating a needed inflammatory microenvironment to trigger the T cell mediated adaptive response (45).

NK cells also have a relevant role in the immunity against tumor cells, using the same mechanisms described for the immunity against viral infections. NK cells exercise a direct cytotoxicity over the tumor cell, produce cytokines which activate T cells, dendritic cells and promote B cell maturation (46). Tumor cells, generally, lose or reduce the expression of human leukocyte antigens (HLA) as an immune evasion mechanism; but NK cells are capable of detecting by their Ig-like (KIR) receptors the absence of HLA and therefore, destroy the tumor cell. The infiltration of NK cells in tumors has been associated with a good prognosis in gastric cancer (47), colorectal cancer (48) or in lung carcinoma (49).

Among their functions, NK cells maintain the homeostasis of the lymphoid system, controlling the inflammation associated to the immune response by destroying the antigen presenting cells and activated T cells, which could lead into a protective mechanism in autoimmune diseases. In reproduction, NK cells constitute the majority of the lymphocytes present in the uterus. NK cells interact with the trophoblastic cells until the implantation in the uterus and they contribute to the maternal vascularity remodeling (50).

Recently, NK cells have been characterized with responses which were only associated to adaptive immunity, like regulatory functions, in addition to show the adaptive a memory capacity against viral infections (51).In this regard, the study of the role of NK cells in cytomegalovirus infection has allowed to understand the adaptive function of the NK cells in infectious viral process (52). NK cells have been detected to have a memory-like phenotype, expansion capability, long-term activation and a higher protection against infection or re-infections (53, 54)

Receptors

NK cell recognition involves the initial binding to potential target cells, interactions between activating and inhibitory receptors with ligands available on the target, and the integration of signals transmitted by these receptors, which determines whether the NK cell detaches and moves on or stays and responds. NK cells respond by reorganizing and releasing cytotoxic granules and by transcribing and secreting cytokines. The inhibitory MHC class I receptors on NK cells serve as a controller regulating and dampening signals transduced through activating receptors. Experimental evidence suggests that the MHC class I inhibitory receptors may serve only to dampen, rather than completely terminate, NK cell effector function and that the amount of MHC class I on the surface of the target is proportional to the degree of inhibition. In some circumstances, inhibitory receptors recognizing ligands other than MHC class I proteins may suppress NK cell responses. When interacting with target cells expressing ligands for both inhibitory and activating receptors, the outcome is determined by the summation of the strength of signals. The amount of activating and inhibitory receptors on the NK cells and the amount of ligands on the target cell, as well as the qualitative differences in the signals transduced, determine the extent of the NK cell response (**Figure 5**) (55).

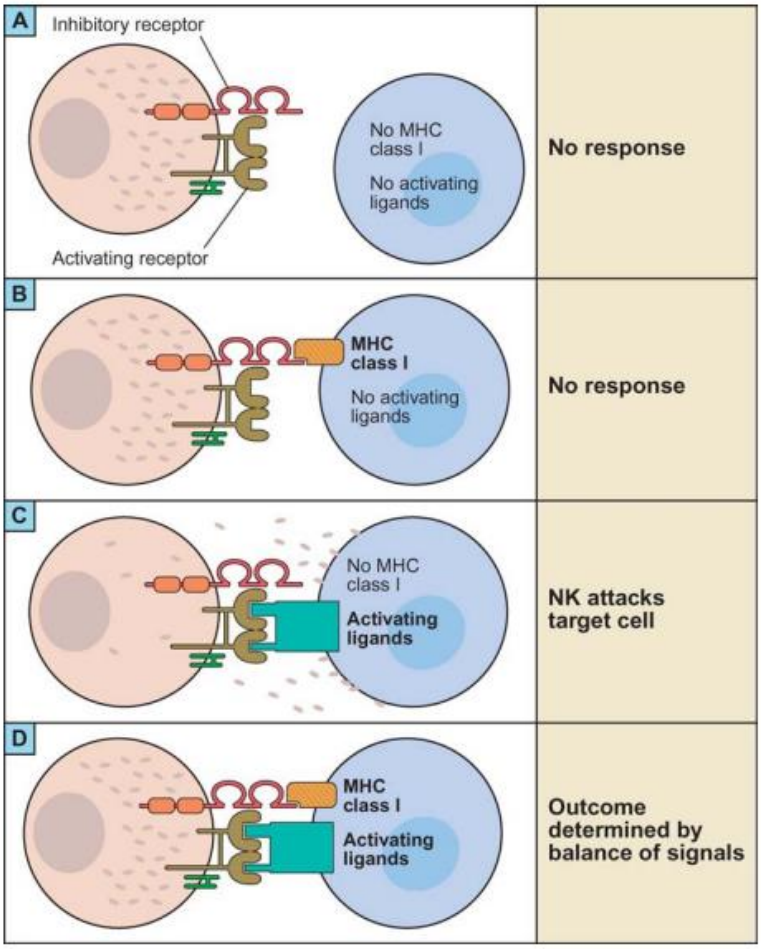

Figure 5: *Outcome of the NK cell response depending on the activating and inhibitory signals. In A situation, there is no response of the NK cell due to the lack of ligands of the target cells for inhibitory and activating receptors. In B situation, there is no response of the NK cell due to the recognition of MHC class I by the inhibitory receptors and the absence of activating ligands. In C situation, NK cell attacks target cell due to the presence of activating ligands and the absence of MHC class I. In D situation, the outcome will be determined by the balance of the signals, as inhibitory and activating receptors are linked to ligands.From Lanier, L. L. 2005*

Inhibitory receptors

NK cells express clonally distributed inhibitor receptors named killer cell immunoglobulin-like receptors (KIRs), that recognize allotypic determinants (KIR ligands) shared by particular groups of HLA class I alleles. The regulatory mechanism mediated by these receptors is thought to protect normal cells from autologous NK cell attack, while rendering cells for which class I expression is compromised (e.g. by tumor transformation or viral infection) susceptible to NK-mediated killing (56). KIRs are glycoproteins of the superfamily of Igs. They are named based on their structure and their function. The names “2D” and “3D” refer to the number of Ig extracellular domains, while letters “L” or “S” distinguish if the cytoplasmic domain is long or short. Inhibitory KIR receptors have a long intracellular domain associated with tyrosine-based motifs (ITIM). There are 15 functional KIR genes in the genome, and they can be inherited in multiple different combinations, which determined two haplotypes in human beings. Haplotype A mainly has in NK cells inhibitory KIRs, while haplotype B

has a larger number of KIR activating receptors in NK cells (57). Each of them recognizes specific class I HLA molecules in the surface of target cells. The mechanism through which the recognition is done is known as missing-self (58).When a tumor cell presents low or none expression of HLA-I, KIR receptors of NK cells do not bind to any HLA molecule, so that, the inhibitory signal is not triggered, and as a consequence, an activation is produced. The absence of a ligand for the KIR receptor is the triggering of the activation (59). Thus, when haploidentical KIR ligand–mismatch occurs, NK cells play a major role as antileukemia effector cells, correlating with better responses to NK therapy (60, 61). Several clinical trials to treat cancer infused autologous and allogeneic NK cells isolated and expanded from peripheral blood (NCT02271711, NCT03420963) or cord blood (NCT03019640). While autologous NK cells represented the first choice since they are safe and immediately available, their clinical impact against tumors is poor due to the inhibitory signal received by self-HLA molecules (62). Hence, the latest approaches are now focusing their attention on the infusion of allogeneic NK cells that gave much better results in terms of alloreactivity against cancer cells thanks to the mismatch between HLA and KIRs (63). Besides, allogenic NK cells do not induce graft versus host disease (GvHD) while they enhance graft-versus-leukemia (GvL) (64).

In the C-type lectine or NKG2 family, inhibitory receptor NKG2A is found. This receptor forms heterodimers with CD94 and recognized HLA-E as its ligand. NKG2A has its own ITIM motifs in its cytoplasmic domain. When binding with HLA-E, the lysis of the tumor cells is inhibited (65) (**Figure 6**).

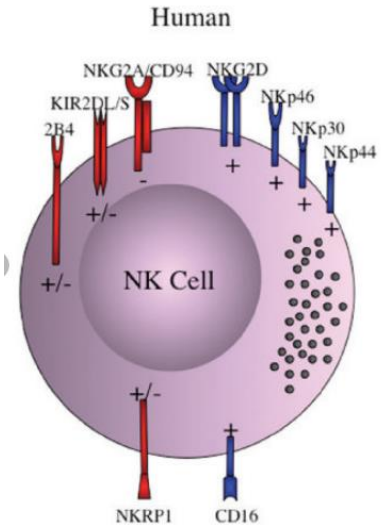

Figure 6: *Main activating (blue), inhibitory (red) and dual (red) surface receptors of NK cells. Adapted from Peter D Krueger et al .*

Activating receptors

The absence of HLA molecules in the membrane is not enough to trigger a response in NK cells. A larger number of activating signal are needed, as inhibitory signal usually has a main role to avoid an excessive cytotoxic response.

Among the principal activating receptors, C-type lectine family is found. NKG2C and NKG2E form heterodimers with CD94 and also recognize HLA-E. In their intracellular domain, they interact with the adaptive molecule DAP12, which have tyrosine-based activatory motifs (ITAM) (66).

NKG2D (CD314) is the only homodimer protein of this family, and the one which has a more relevant role in activation. Once it is stimulated, NKG2D binds to DAP10 or DAP12, which also form homodimers and they get autofosforilated in their ITAMs to transduce the signal, as NKG2D has not its own intracellular motifs. Unlike the rest of the NKG2 members, NKG2D detects several ligands induced in the stressed cell or DNA damaged cells (67).

Another dominant activating receptor family is NCR, practically exclusive in NK cells. This is form mainly by NKp30 (CD337), NKp44 (CD336) and NKp46 (CD335). NKp30 has no homology with NKp44 or NKp46, but they are grouped based on their function and their profile expression (68). While NKp30 and NKp46 are associated with adaptive molecules CD3ζ and FcRγ, which have ITAM motifs, NKp44 binds to DAP12.

SLAM family receptors are also very well characterized in the activation of NK cells. NK cells express all the members of this family except for SAMF1. These receptors interact with other cells through a homofilic union, except for 2B4 (CD244), which binds to CD48. SLAM family receptors have tyrosine-based activatory motifs called ITSM, which transmit the activating signal through SLAM associated protein (SAP) (69).

As it has been mentioned before, there is KIR activating receptors, which have a short intracellular motif associated to ITAM motifs. The main KIR activating receptors are KIR2DS1 and KIR3DS1, which binds to HLA-B and C respectively.

NK cells also perform antibody dependent cytotoxicity (ADCC), which is triggered through CD16 receptor (FcγRIIIa). Via the union to the antibodies constant fraction FC, CD16 produces a very powerful activating signal n the NK cells, with surpasses the predominant inhibitory signal, without needing additional signals, to destroy the target cells covered in antibodies (50) (**Figure 6**).

Cytotoxic mechanism

NK cells use a combination of several mechanisms to lyse different target cell. The first step that activates the NK cells is the **union ligand-receptor** which produces the grouping of the receptors. These receptors suffer a conformational change due to the reorganization of the actine, and subsequently, lead to the transduction of signals which are initiated in the phosphorylation of ITAM or ITIM motifs. This process is family Src kinases dependent, which assemble in the group areas of the receptors (70). Rapidly, after the ligand-receptor interaction, the signal cascade in the NK cells produces the activation of VaV proteins, which are essential for the polarization of the granules toward the tumor cell, and also, for the phosphorylation of the PLC-γ2 protein and the subsequent mobilization of the calcium from the endoplasmic reticulum in order to produce the degranulation to the intercellular space. As degranulation occurs,

secretory lysosomes are released, and the lysosome-associated membrane protein-1, also known as LAMP-1 or CD107a, is transported to the surface of NK cell (71). Perforine literally drills the cell membrane of the target cell, allowing the entrance of serine proteases known as granzymes (**Figure 7A**). Granzymes A and B are the most abundant ones, and they produce apoptosis through caspase dependent and non-dependent mechanisms (72).

The cell-surface Fas receptor (Fas), also termed Apo-1 or CD95, is a member of the tumor necrosis factor (TNF) which is typically present in cells with features of apoptotic cells. Apoptotic cell death induced by the engagement of Fas by FasL (**Fas/FasL killing pathway**) plays a major role in modulation of immune function. Trimerization of the Fas receptor by Fas ligand results in activation of caspase 8 , mediated by the adapter protein Fas-associated death domain (FADD)/mediator of receptor-induced toxicity (MORT1). Active caspase 8 is then responsible for activation of downstream caspases and cell death (**Figure 7B**) (73).

NK cells, especially early in viral infections, release antiviral **cytokines**, such as IFN-γ and TNF-α (TNF), as immunodefensive agents that additionally serve to activate resident inflammatory cells and recruit other cells. These NK cell-sourced cytokines also regulate dendritic cells, T cells, and B cells and regulate TNF-mediated apoptosis of the NK cells and neighboring cells to delimit immune responses (**Figure 7C**). Thus, cytokine production by NK cells influences both innate and adaptive immune responses (74).

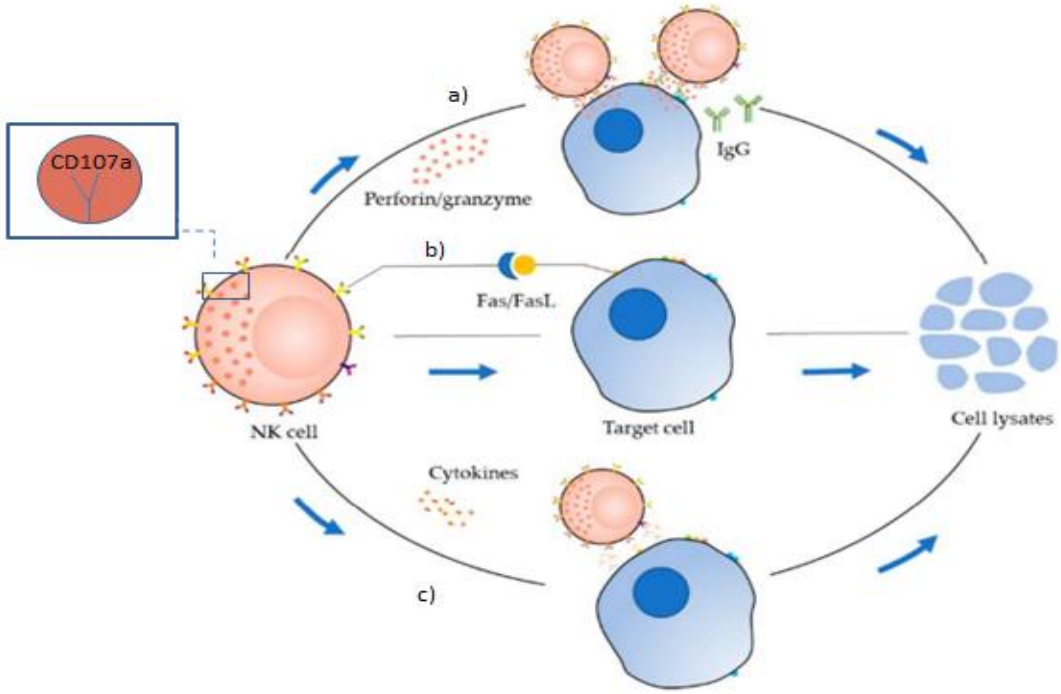

Figure 7: Different pathways of target lysis by NK cells. a) NK cells can use granulocytes, as perforin and granzymes in order to make holes in the surface of the target cells and lyse them.; b) Engagement of the cell death surface receptor Fas by Fas ligand (FasL) results in apoptotic cell death, mediated by caspase activation. cytokines and Fas/FasL. c)Cytokines recruit other cells from the immune system in order to attack the target cells and lyse them. Adapted from Zhang et al 2019.

Clinical uses

There are several clinical trials taking place in order to treat different types of cancer; combination of cryosurgery and NK immunotherapy for advanced kidney cancer (NCT02843607), NK cell-based immunotherapy as maintenance therapy for small-cell lung cancer (NCT03410368) or NK cells plus IL-2 following chemotherapy to treat advanced melanoma or kidney cancer (NCT00328861). iPSC-derived Natural Killer (NK) cells, named FT500 are also being used in combination with Immune Checkpoint Inhibitors (ICI) in a clinical trial to treat subjects with advanced solid tumors (NCT03841110) (**Table 1**). In April 2019, Fate Therapeutics, a clinical-stage biopharmaceutical company dedicated to the development of programmed cellular immunotherapies for cancer and immune disorders, announced that the first patient treated with FT500 successfully completed an initial safety assessment. The patient received three once weekly doses of FT500, and the treatment cycle was well-tolerated with no dose-limiting toxicities or serious adverse events reported during the initial 28-day observation period. The universal, off-the-shelf natural killer (NK) cell product candidate is the first-ever cell therapy derived from an induced pluripotent stem cell (iPSC) administered to a patient in the U.S. FT596 is also iPSC-derived NK cells derived product which is also combined with CAR technology. This product is still in preclinical phase. Not only are NK cells able to treat solid tumors, but they play a key role in immunotherapies against hematological cancer like acute myeloid leukemia (AML), by using high doses of these cells (75). Nonetheless, expanded and stimulated NK cells or high-dose NK cell therapy are not the only options when treating patients. Although T cells have been typically used in CAR technology-based therapy, NK cells are also emerging as one of the new promises in this field (76). Due to their low infecting rate, poorly *in vivo* expansion and short life span, NK cells were not taking into account since the beginning for

this kind of therapy. Nevertheless, newer protocols that enhance viral transduction efficiency and prosperous expansion of these cells have made NK cells a space in the CAR therapy field (77). Furthermore, allogenic NK cells have a major advantage over allogenic T cells, that is, they could be used as a “universal” product as they do not cause GvHD (78).

Table 1: Relevant clinical trials in which NK cells are used as immunotherapy.

Cell source	Treated disease	Clinical trial
Autologus peripheral blood NK cells	Recurrent/Refractory Brain Tumors	NCT02271711
	Small cell lung cancer	NCT03410368
Autologus peripheral blood NK cells + IL-2	Advanced Melanoma/ Kidney Cancer	NCT00328861
Allogenic peripheral blood NK cells	Pediatric solid tumors	NCT03420963
	High Risk Myeloid Malignancies	NCT01823198
	Adult Acute Myeloid Leukemia in Remission	NCT03955848
Peripheral blood NK cells	Advanced kidney cancer	NCT02843607
Umbilical cord blood NK cells	B-Cell Non-Hodgkin's Lymphoma	NCT03019640
Umbilical cord blood NK cells	Leukemia	NCT02280525
iPSCs derived NK cells	Advanced solid tumours	NCT03841110

Hematological cancers

Cancer is an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metastasize (spread). Cancer is not one disease; in fact, it is a group of more than 100 different and distinctive diseases (**Figure 8**). It can involve any tissue of the body and have many different forms in each body area. Most cancers are named for the type of cell or organ in which they start (79). Leukemia is a cancer type that starts in the tissue that forms blood. In a patient with leukemia, the bone marrow makes abnormal white blood cells. The aberrant cells are leukemia cells. Unlike normal blood cells, leukemia cells do not die when they should. They may crowd out normal white blood cells, red blood cells, and platelets. This makes it hard for normal blood cells to do their work. Regarding B cell lymphomas, non-Hodgkin Lymphomas (NHL) make up most (about 85%) of these kind or lymphomas. Other two of the main types of leukemia involving B cells are Acute Lymphoblastic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL) (80).

Non-Hodgkin lymphoma (NHL) is a group of blood cancers that includes all types of lymphoma except Hodgkin's lymphomas. (81) Lymphomas are types of cancer that develop from lymphocytes, a type of white blood, mostly B cells. Subtypes of non-Hodgkin's lymphoma that involve B cells include diffuse large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma and Burkitt lymphoma. The prognosis of NHL can be good but depends on the type of lymphoma, the extent of spread (staging), and response to therapy. A health care provider will discuss the prognosis with the patient. The overall five-year survival rate for people with NHL is 71%, while the overall 10-year survival rate is 60% (82).

ALL represents a biologically and clinically heterogeneous group of diseases. In a patient with ALL, too many stem cells become lymphoblasts, B lymphocytes very quickly. The cells do not work like normal lymphocytes and are not able to fight infection very well. Also, as the number of leukemia cells increases in the blood and bone marrow, there is less room for healthy white blood cells, red blood cells, and platelets. This may lead to infection, anemia, and easy bleeding. (83).The current gold standard in the diagnosis and classification of leukemia involves a combination of methods that include morphology, cytochemistry, immunophenotype, cytogenetics, and molecular genetics (84). In particular, immunophenotype is an essential component of the initial diagnostic work-up of ALL. In all B-lineage ALL, total expression of CD19 surface marker is present, along with the total or partial expression of other surface markers such as CD20, CD22 and CD33. The median content of blast cells is around 82% (85). ALL is the most common cancer among children with a prevalence of 20-25% of all cases (86). This disease has become highly curable with 5 years survival rates above 90% (87). In contrast, these results are very different in adults, where the 5 years survival rate falls to 40% with the conventional treatments (88).

CLL is a blood and bone marrow disease that usually gets worse slowly. It is one of the most common types of leukemia in adults. It is a low-grade lymphoproliferative neoplasm with $\geq 5 \times 10^9$/L clonal B-cells in the peripheral circulation that express CD5, CD19, dim CD20, and CD23 (89). It often occurs during or after middle age, being quite rare in children. In CLL, too many blood stem cells become abnormal lymphocytes and do not become healthy white blood cells. As a consequence, the lymphocytes are not able to fight infection very well. Usually CLL does not cause any signs or symptoms and is found during a routine blood test (90). The survival rate for these patients at 5 years is 79.2%, but it is still an incurable disease in many

patients (91). One of the most serious complications of CLL is a change (transformation) of the leukemia to a high-grade or aggressive type of non-Hodgkin Lymphoma (NHL) called diffuse large B-cell lymphoma (DLBCL) or to Hodgkin lymphoma (HL). This happens in 2% to 10% of CLL cases, and is known as Richter's transformation. In rare patients with CLL, the leukemia transforms into ALL. If this happens, treatment is likely to be similar to that used for patients with ALL (92).

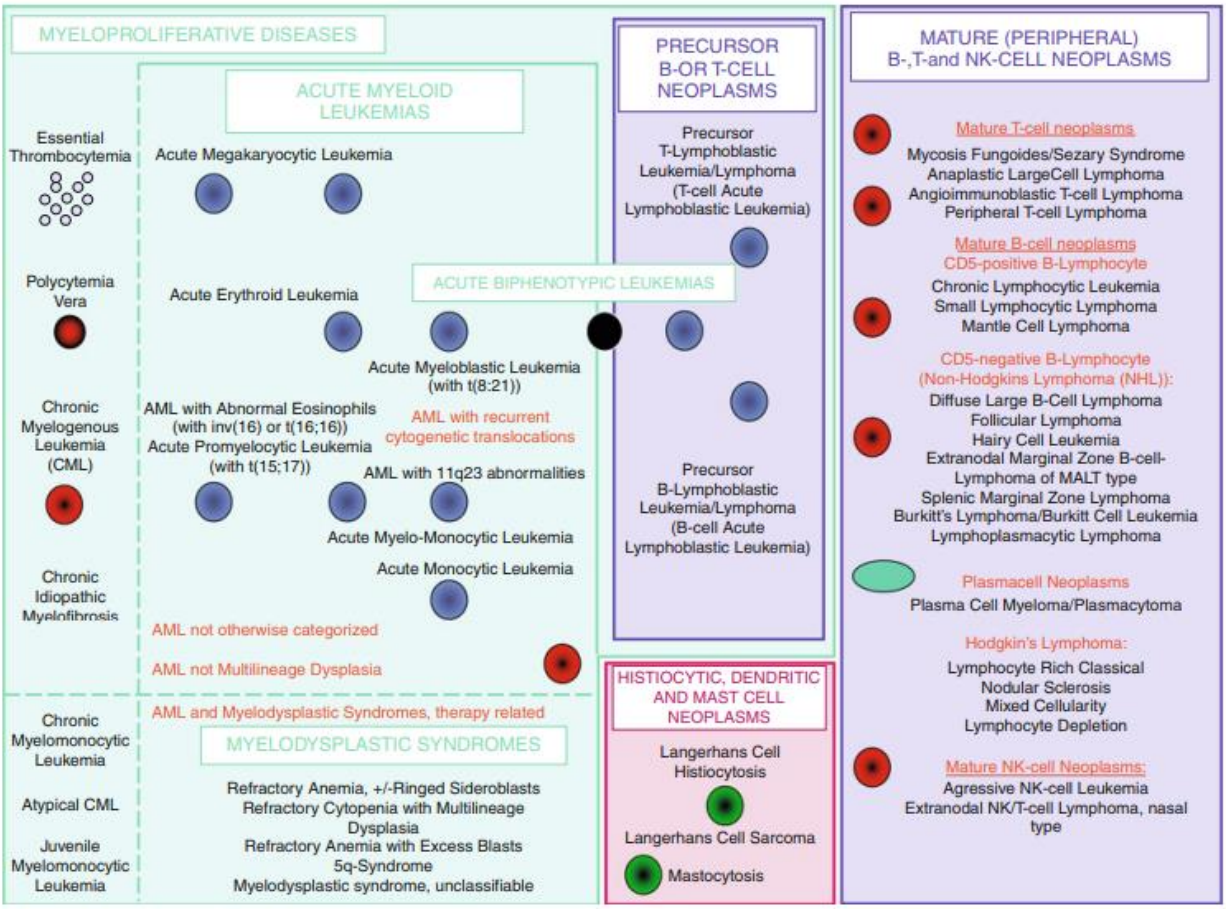

Figure 8: Diagram of the main hematological malignances regarding the immune system. From Jurlander, J. 2011.

Standard therapies and treatment improvements against hematological cancers

Leukemia is the one of the most common diagnosed cancer. But leukemia is not just one disease—many types of leukemia originate in different parts of the bloodstream and affect different types of blood cells. Also, some leukemias are regarded as acute, meaning they may require aggressive treatments. Others are considered chronic, which means they are slow to develop and may require a less aggressive treatment plan. Treatments for leukemia vary widely depending on the type of cancer diagnosed and the stage of the disease. Some treatments, such as targeted therapy, chemotherapy and immunotherapy, may be recommended either alone or in combination (93).

ALL against therapies

Over the last 40 years, multiple effective chemotherapy regimens have been developed to successfully treat acute lymphoblastic leukemia (ALL). Advances in the field of ALL treatment have primarily been demonstrated in the pediatric and adolescent populations, with a more limited progress reported in adults. Generally, children with ALL are better able to tolerate chemotherapy and experience improved outcomes compared with adults treated with similar chemotherapy programs (94,95). Chemotherapy is the main treatment for ALL. Treatment is usually divided into 3 phases: induction, consolidation and maintenance. The goal of induction chemotherapy is to get the leukemia into remission (complete remission). This means that leukemia cells are no longer found in bone marrow samples (in a bone marrow biopsy), the normal bone marrow cells return, and the blood counts return to normal levels. But a remission is not necessarily a cure, as leukemia cells may still be hiding somewhere in the body. This phase of the treatment lasts about a month. Different combinations of chemotherapy drugs might be used, but they typically include: Vincristine, dexamethasone or prednisone, and an anthracycline drug such as doxorubicin (Adriamycin) or daunorubicin, and L-aspaginase. Next, the aim of the consolidation phase is to destroy any leukemia cells that may still be in the blood or bone marrow but cannot be picked up on tests. It reduces the risk of the leukemia coming back. It often consists of another fairly short course of chemotherapy, using methotrexate (MTX) and cytarabine (ARA-C). This typically lasts for a few months. Usually the drugs are given in high doses so that the treatment is still reasonably intense. Lastly, after consolidation, the patient is generally put on a maintenance chemotherapy program of methotrexate and 6-mercaptopurine (6-MP). In some cases, this may be combined with other drugs such as vincristine and prednisone. The goal of this phase is to help keep the leukemia in remission. Chemotherapy is given in lower doses than in the other phases of treatment, but it is the longest phase, with an estimated duration of two years. If the leukemia is refractory – that is, if it doesn't go away with the first treatment (which happens in about 10% to 20% of patients) – then newer or more intensive doses of chemotherapy drugs may be tried, although they are less likely to work (96-99). Monoclonal antibodies such as blinatumomab (Blincyto) (100), inotuzumab ozogamicin (Besponsa) or rituximab may be an option for patients with B-cell ALL (101). A Hematopoietic Stem Cell Transplant (HSCT) may be tried if the leukemia can be put into at least partial remission (102). If leukemia goes into remission with the initial treatment but then comes back (relapses or recurs), it will most often do so in the bone marrow and blood. Occasionally, the brain or spinal fluid will be the first place it recurs. In these cases, it is sometimes possible to put the leukemia into remission again with more chemotherapy, although this remission is not likely to last. The approach to treatment may depend on how soon the leukemia returns after the first treatment. If the relapse occurs after a long interval, the same or similar treatment may be used to try for a second remission. If the time interval is shorter, more aggressive chemotherapy with other drugs may be needed (103,104).

Sometimes standard doses of chemotherapy are not always able to cure ALL. Even though higher doses of chemotherapy drugs might be more effective, they cannot be given because they could severely damage the bone marrow, which is where new

blood cells are formed. This could lead to life-threatening infections, bleeding, and other problems due to low blood cell counts. A HSCT allows doctors to use higher doses of chemotherapy (sometimes along with radiation) to kill the cancer cells. After this conditioning is finished, the patient gets an infusion (transplant) of blood-forming stem cells to restore their bone marrow. Blood-forming stem cells used for a transplant are obtained either from the blood, from the bone marrow, or from umbilical cord blood (CB).

There are two main types of stem cell transplants: allogeneic stem cell transplant, in which the stem cells come from someone else. This is the preferred type of transplant when treating ALL; and autologous stem cell transplant, in which the patient gets back his or hers own cells.

- **Allogeneic transplant**: A donor tissue type (also known as the HLA type) needs to closely match the patient's tissue type to help prevent the risk of major problems with the transplant. The best donor is often a close relative, such as a brother or sister, if they have the same tissue type as the patient. If there are no siblings with a good match, the cells may come from an HLA-matched, unrelated donor – a stranger who has volunteered to donate their cells (**Figure 9**). In most European centers the gold standard is to look for an HLA-A, -B, -C, -DRB1, and -DQB1-matched donor, a so-called 10/10 match. An alternative matching algorithms to look for an HLA-A, -B, -C, and -DRB1-compatible donor (8/8 match) (105). Unrelated donor umbilical cord blood (CB) has the potential to resolve these limitations for many patients. CB is capable of reconstituting hematopoiesis after myeloablative or reduced-intensity/non-myeloablative therapy. Unrelated donor public CB banking programs have been initiated in many countries around the world. In Spain there are around 65,000 units banked for public use and an estimated 1600 unrelated donor CB transplantations performed to date. Multiple studies in patients with hematologic malignancies have demonstrated that cryopreserved CB from 4-6/6 HLA-A,B antigen and DRB1 allele matched unrelated donors contains sufficient numbers of HSC to engraft most pediatric patients. While CB has both potential benefits and limitations, two clinical benefits deserve emphasis. Firstly, CB is a cryopreserved HSC source that is rapidly available without the risk of donor unavailability, and transplantation can be scheduled almost entirely according to patient needs rather than donor availability. This can be highly advantageous for some patients. Perhaps more important, for a given degree of HLA match there is a decreased incidence of GvHD compared with that seen after transplantation of unrelated volunteer HSCT yet a graft-versus-leukemia (GvL) effect is retained (106). Some patients cannot have this kind of transplant because a matching donor isn't available. The use of allogeneic transplant is also limited by its side effects, which are often too severe for people who are older or who have other health problems, like graft-versus-host-disease (GvHD). One option that may help patients who cannot have an allogeneic transplant because of age or health issues is to use lower doses of chemo and radiation that don't completely destroy the cells in their bone marrow. This is known as a non-myeloablative or reduced-intensity transplant (**Figure 10**).

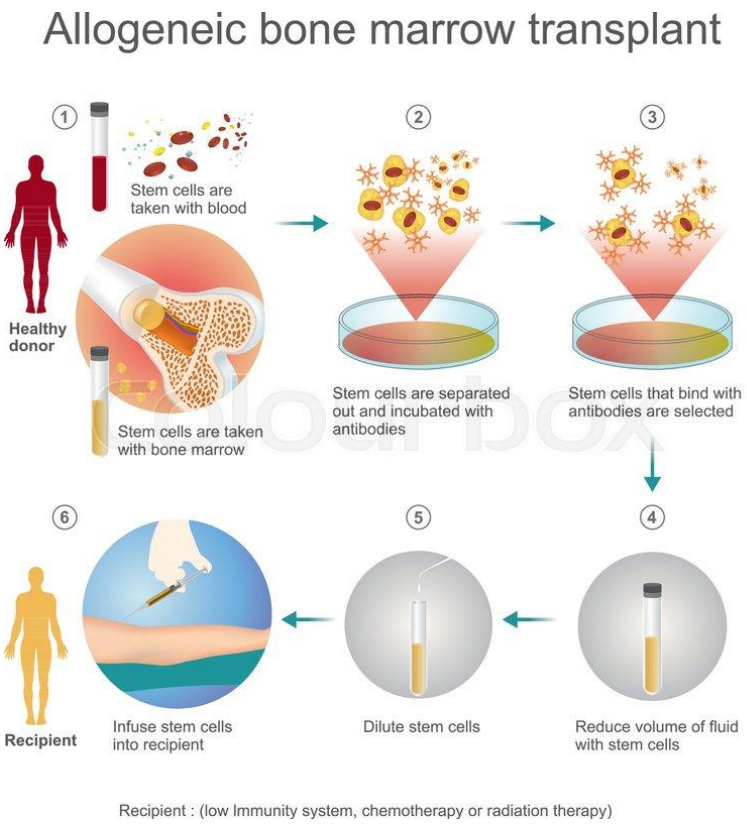

Figure 9: Diagram of the procedure of an allogeneic bone marrow transplant. Firstly, stem cells from a healthy donor are taken with blood or bone marrow (1). Secondly the stem cells are culture with antibodies (2) and the ones that are binded to the antibodies are selected (3). These stem cells are in a reduced volume of fluid (4), and they get diluted (5) in order to transplant them to the patient (6). From Chompipat P

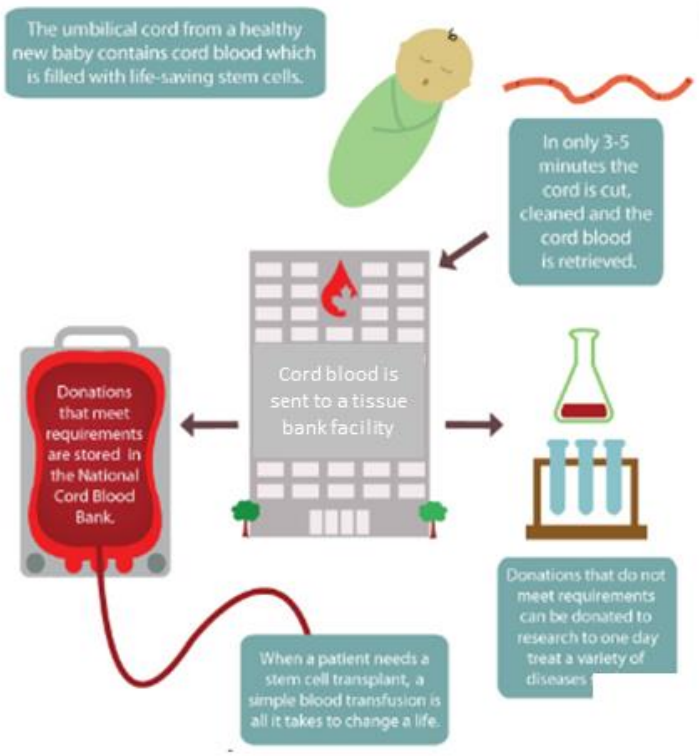

Figure 10: Diagram of the procedure of umbilical cord blood collection, processing and cryopreservation for allogeneic hematopoietic stem cell transplantation. The umbilical cord blood of a healthy newborn is collected. This cord blood is sent to a to a tissue bank facility, where is processed to its storage. Cord blood that does not accomplish the requirements can be used for research purposes. Adapted from Brampton Civic's Public Cord Blood Bank

- **Autologous transplant**: A patient's own stem cells are removed from his or her bone marrow or blood. They are frozen and stored while the person gets treatment (high-dose chemotherapy and/or radiation). A process called purging may be used in the lab to try to remove any leukemia cells in the samples. The stem cells are then put back (reinfused) into the patient's blood after treatment. An autologous transplant may be an option for patients who cannot have an allogeneic transplant because they don't have a matched donor, or for some other reason. One problem with autologous transplants is that leukemia is a disease of the bone marrow and blood, so even after purging, there is a danger of giving the patient back leukemia cells together with the stem cells (**Figure 11**).

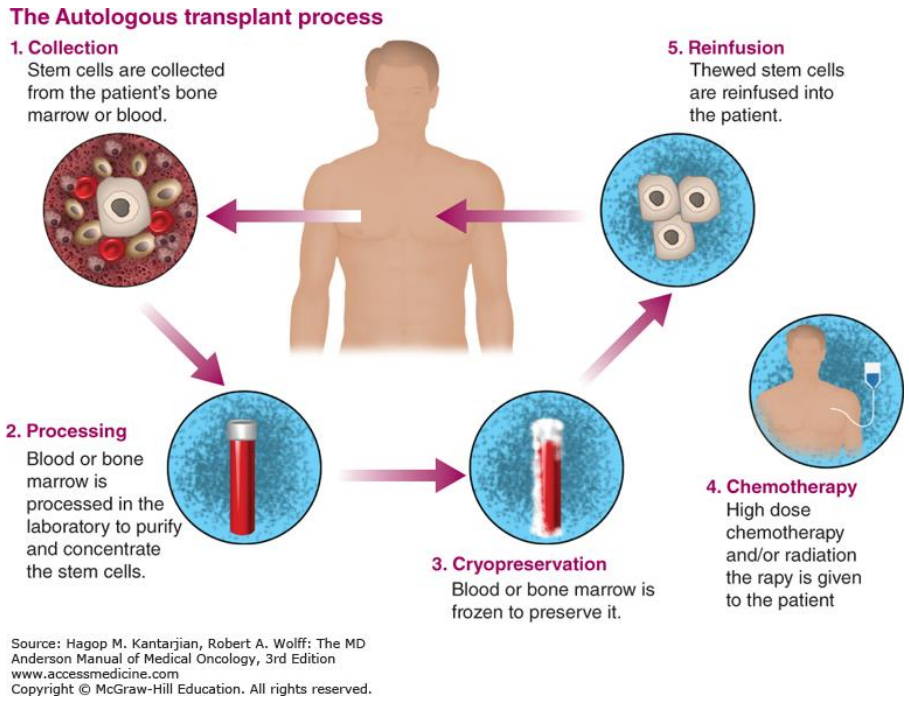

Figure 11: Diagram of the procedure of an autologous bone marrow transplantation. Firstly, stem cells from the patient are taken with blood or bone marrow (1). Secondly, stem cells are purified and concentrated (2) and cryopreserved (3). In the meantime, the patient gets conditioned with chemotherapy and/or radiation (4). Finally, the stem cells and thawed and reinfused to the patient.

CLL against therapies

The great majority of CLL patients have early-stage asymptomatic disease at diagnosis. All patients should undergo risk stratification at the time of diagnosis. Patients in the low- and intermediate-risk category (~75% patients, median time to first therapy ~7 years) should be monitored for disease progression every 6–12 months. Patients in the high- and very high-risk group (~25% patients, median time to first therapy ~2 years) should be monitored for disease progression every 3–6 months (107). Only those patients who meet the 2018 International Workshop on CLL (IWCLL) criteria for initiation of therapy should be offered treatment. For the initiation of a treatment against CLL, patients must present any one of the following symptoms such as progressive marrow failure, progressive lymphocytosis with an increase of ≥50% over a 2-month period or lymphocyte doubling time of <6 months, Massive (≥10 cm in longest

diameter) or progressive or symptomatic lymphadenopathy or Autoimmune complications of CLL (108). Patients could also present clinical symptoms such as fever, asthenia, or night sweats. Treatment options for Chronic Lymphocytic Leukemia (CLL) vary largely, depending on the person's age, the disease risk group, and the reason for treating (for example, which symptoms it is causing). Many people live a long time with CLL, but in general it is very difficult to cure, and early treatment hasn't been shown to help people live longer. Patients without significant comorbidity are treated with rituximab, fluradabine and cyclophosphamide; patients with comorbidity are treated with obinutuzumab-chlorambucil or rituximab-chlorambucil. Finally, patients who present deletions in 17p or alterations of p53 are treated with ibrutinib, idelalisib or ofatumumab (109).

Patients with relapsed or refractory ALL or CLL after 2 or more treatment lines may need alternative therapies to treat their disease. New therapies, especially immunotherapies, are emerging to treat these kind and other hematological cancers. Chimeric Antigen Receptor (CAR) based therapy is positioning as one of the most promising therapies in order to get rid of the maleficent cells.

Immunotherapy

Immunotherapy is a type of therapy that uses substances to stimulate or suppress the immune system to help the body fight cancer, infection, and other diseases. The 2018 Nobel Prize in Physiology or Medicine awarded Prof. James P. Allison and Prof. Tasuku Honjo for their discovery of cancer immunotherapy by inhibition of negative immune regulation. Prof. James P. Allison studied a known protein that functions as a brake on the immune system. He realized the potential of releasing the brake and thereby unleashing our immune cells to attack tumors. Prof. Allison then developed this concept into a brand new approach for treating patients. In parallel, Prof. Tasuku Honjo discovered a protein on immune cells and, after careful exploration of its function, eventually revealed that it also operates as a brake, but with a different mechanism of action. Immunotherapies based on his discovery proved to be strikingly effective in the fight against cancer.

Several types of immunotherapy are used to treat cancer (**Figure 12**). These treatments can either help the immune system attack the cancer directly or stimulate the immune system in a more general way. Types of immunotherapy that help the immune system act directly against the cancer include: **checkpoint inhibitors**, which are drugs that help the immune system respond more strongly to a tumor; **adoptive cell transfer**, which is a treatment that attempts to boost the natural ability of your T cells to fight cancer; **monoclonal antibodies**, also known as therapeutic antibodies, are immune system proteins produced in the lab. These antibodies are designed to attach to specific targets found on cancer cells; and **Treatment vaccines**, which work against cancer by boosting your immune system's response to cancer cells. The types of immunotherapy that enhance the body's immune response to fight the cancer include: **cytokines**, which are proteins made by your body's cells. They play important roles in the body's normal immune responses and also in the immune system's ability to respond to cancer; and **BCG**, which stands for Bacillus Calmette-Guérin, is an immunotherapy that is used to treat bladder cancer. It is a weakened form of the bacteria that causes tuberculosis. When inserted directly into the bladder with a catheter, BCG causes an immune response against cancer cells (110). There are also modified versions of viruses have been designed to target and attack tumors that have already formed. These are known as **oncolytic viruses** (111). The field of immunotherapy in cancer treatments has been accelerating over recent years and has entered the forefront as a leading area of ongoing research and promising therapies that have changed the treatment landscape for a variety of solid malignancies. Prior to its designation as the Science Breakthrough of the Year in 2013, cancer immunotherapy was active in the treatment of hematologic malignancies (112).

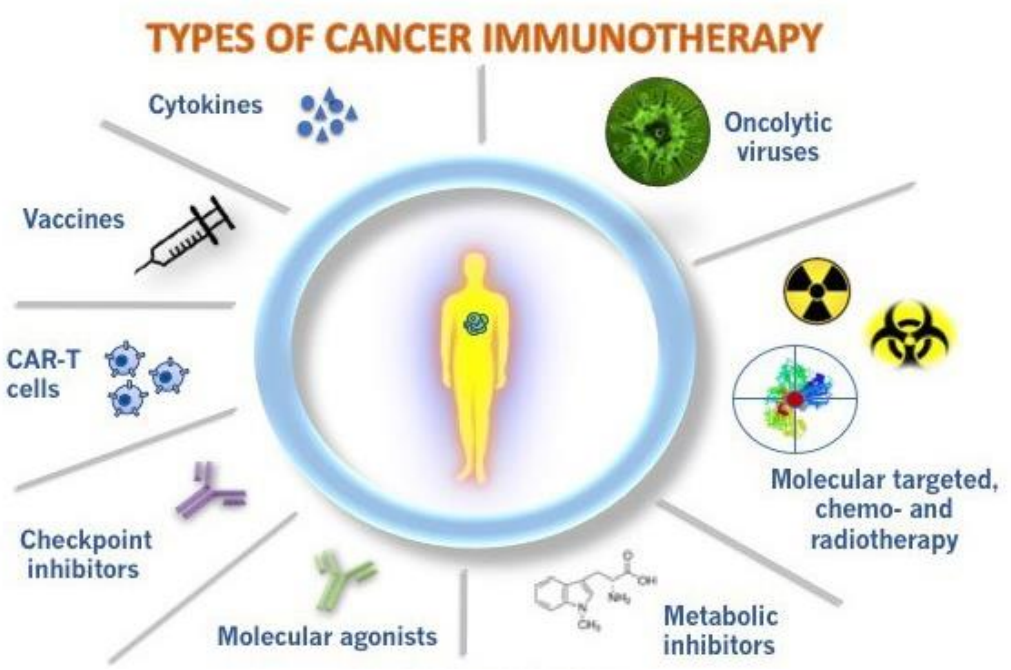

Figure 12: Diagram of different types of immunotherapy treatments against cancer. From cancer.net editorial board

Immunotherapies against ALL and CLL

In ALL, complete remission (CR) rate after chemotherapy are low and ranges between 25 and 45% (113), with most of these patients conclusively dying, which lead into a lot of room for improvement. Generally, four types of immunotherapies have been developed to date, including naked monoclonal antibodies (mAbs), conjugated monoclonal, bispecific T cell engager (BiTE), and chimeric antigen receptor (CAR) T cell therapy (114). These therapies can target different antigens present in the surface of B lymphoblasts (**Table 2**).

CD20 is expressed on approximately 30 to 50% of precursor B cell lymphoblasts and is associated with a higher relapse rate and lower overall survival (115,116). Several drugs that target this receptor are used in the clinic, such as rituximab, a chimeric anti-CD20 monoclonal antibody with a wide ranging spectrum of activity that has been incorporated into chemotherapy regimens for precursor B cell ALL (pre-B ALL) (117); ofatumumab, a second-generation anti-CD20 monoclonal antibody that binds to a different epitope on the CD20 molecule than rituximab, inducing higher levels of CDC and ADCC compared with rituximab (118); Obinutuzumab is another novel CD20 monoclonal antibody that was engineered to have enhanced ADCC as compared to rituximab and ofatumumab (119) . It has been FDA approved for the first-line treatment of CLL, but has not yet been studied in the clinical setting of pre-B ALL.

CD22 is expressed on leukemic blasts in > 90% of patients with ALL (120). Mainly, two drugs are used for ALL targeting CD22 antigen. Epratuzumab is an unconjugated humanized monoclonal antibody against CD22 (121), and inotuzumab ozogamicin (InO) is a conjugated anti-CD22 monoclonal antibody that is linked to calicheamicin, a potent cytotoxic compound that induces double-strand DNA breaks and apoptosis (122).

CD19 is present in 90% of pre-B and mature ALL lymphoblasts, making it an attractive target for immunotherapy. Blinatumomab is the first of a newer class of antibody constructs labeled BiTEs which binds CD19-positive B cells and CD3-positive cytotoxic T cells. Upon binding to CD19, the cytotoxic T cells become activated and induce cell death via direct tumor lysis (123). Regarding this receptor, one of the most promising cellular therapy-based treatments was recently approved by the Food and Drug Administration (FDA) in October 2017: Tisagenlecleucel (Kyriam from Novartis) for relapsed B-cell Acute Lymphoblastic Leukemia (ALL). Shortly after, the FDA approved Axicabtagene Ciloleucel (Yescarta from Kite-Gilead) for relapsed or refractory large B cell lymphoma Both drugs are Chimeric Antigen Receptor (CAR) T-cell based therapies (124). More recently (June 2018), the European Medicines Agency (EMA) approved these products in Europe.

Regarding CLL, in most cases several immunotherapies are applied to the patients as a first-line therapy. These antibodies target the CD20 pathway, such as Rituximab, Ofatumumab or Obinutuzumab (107). Alemtuzumab is a monoclonal antibody that binds to CD52, a protein present on the surface of mature lymphocytes. These CD52-bearing lymphocytes are targeted for destruction. This immunotherapy is usually used in patients who have been treated with alkylating agents and who have failed fludarabine therapy (125).

Table 2: Several immunotherapies that target different antigens present in the surface of B lymphoblasts, as CD20, CD19 and CD22.

Targeted antigen	Drug name	Type of drug
CD20	rituximab	Chimeric monoclonal antibody
	ofatumumab	second-generation monoclonal antibody
	Obinutuzumab	monoclonal antibody
CD22	Epratuzumab	unconjugated humanized monoclonal antibody
	inotuzumab ozogamicin (InO)	conjugated monoclonal antibody
CD19	Blinatumomab	BiTES
	Tisagenlecleucel	CAR-NK cell
	Axicabtagene Ciloleucel	CAR-NK cell

Chimeric Antigen Receptor (CAR)

The hypothesis of CAR T-cell treatment dates to the 1980s, when Eshhar et al. expressed chimeric TcR genes including the TcR constant domains united to the variable domain from an antibody (126). Therefore, the first CAR-T cells were developed in 1989 by Gideon Gross and Zelig Eshhar at Weizmann Institute, Israel. Some years later, Prof. Carl H. June from University of Pennsylvania tested the ability to culture genetically modified CAR-Ts in humans for the treatment of cancer and its clinical use. His work led to the development and commercialization of Tisagenlecleucel (Novartis), the first FDA-approved gene therapy.

A CAR is a recombinant receptor construct composed of an extracellular single-chain variable fragment (scFv) derived from an antibody (127) or a full-length antibody (128), linked to a hinge region, a transmembrane domain, and an intracellular tyrosine-based activation motif comprised of either a region of CD3 T-cell receptor (TCR) complex, the CD3 ζ chain, or FcR receptor γ. This was the structure of the first generation CARs (1G) (**Figure 13A**). The hinge region acts a "spacer" between the "chimeric" antigen-recognizing domain and the transmembrane domain, thus increasing conformational flexibility for antigen binding (129). TCR ligation of host antigen could lead to T-cell activation, autoimmunity, and even death.

To minimize this potential, T cells require at least two signals to fully activate (130). The first signal is through the TCR, but the co- stimulation, is mediated through ligation of CD28 by CD80 or CD86, which are normally expressed on antigen-presenting cells (APC). Therefore, when a T cell encounters a cross-reactive peptide expressed on a normal (non-APC) cell, it is unable to provide co-stimulation and T-cell activation is unsuccessful. However, when APCs are activated, as during inflammation or infection, they upregulate CD80 and CD86 and can induce both signals 1 and 2, thereby supporting full T-cell activation, target killing, and long-term persistence (130,131).

Researchers therefore incorporated the two-signal model of T-cell activation by modifying CARs to include a CD28 co-stimulatory domain in tandem with CD3ζ ITAM domains (132). It has also been demonstrated that co-stimulatory domains other than CD28, such as 4-1BB, provide similar *in vivo* enhancements to CAR T-cell function and persistence (133). These kinds of constructs constitute the second generation CAR (2G) (**Figure 13B**). However, the two domains determine different functional properties of CAR-T cells; for example, CD28-based CARs direct an immediate antitumor potency, whereas 4-1BB-based CARs have the capacity for long-term persistence (134). Accordingly, third generation (3G) (**Figure 13C**) CARs that contain two co-stimulatory elements, for example from both the CD28 and 4-1BB intracellular portions, have been developed (135). CARs containing 4-1BB or both CD28 and 4-1BB have also showed superior *in vivo* expansion and anti-tumor efficacy compared to CARs carrying CD28 (136).

Due to the broad heterogeneity of cancer cells in solid tumors, a fourth generation CAR (4G) (**Figure 13D**), known as TRUCKs or armored CARs, where developed. These CARs are additionally modified with a constitutive or inducible expression cassette for a transgenic protein, for instance a cytokine, which is released by the CAR T cell to modulate the T-cell response; for example NFAT-responsive expression cassette for the inducible expression of IL-12. IL-12 improved T-cell activation, modulated the immunological and vascular tumor environment and recruited additional immune cells for the fight against those cancer cells that are not recognized by CAR T cells (137).

On the one hand, the most common and dangerous side effect of CAR T-cell therapy is cytokine-release syndrome and its most severe manifestation, “cytokine storm”, in which massive T-cell activation triggers a cascade of released pro-inflammatory cytokines causing fever, flushing, and dyspnea. Severe cytokine storm can be potentially life threatening, however it can be effectively treated with the anti-interleukin-6 receptor antibody tocilizumab, along with hydration and supportive therapy (138). On the other hand, expansion and manufacturing of autologous modified T-cells from lymphocytes of heavily treated patients is not always easy and successful due to low lymphocyte counts and poor health condition of the cells. Thus, off-the-shelf allogeneic CAR T-cells, manufactured from lymphocytes of healthy donors, seem attractive in many ways, yet there are still many issues that hamper their wider use in clinical trials (139). Allogeneic T cells may suffer from the human leucocyte antigen (HLA) mismatch between donor and recipient, which in worst case can lead to severe, even life threatening graft-versus-host disease (GvHD) (140). This leads to a new source of cells, and Natural Killers (NK) cells are good candidates as they suppress GvHD without causing GvHD themselves (78).

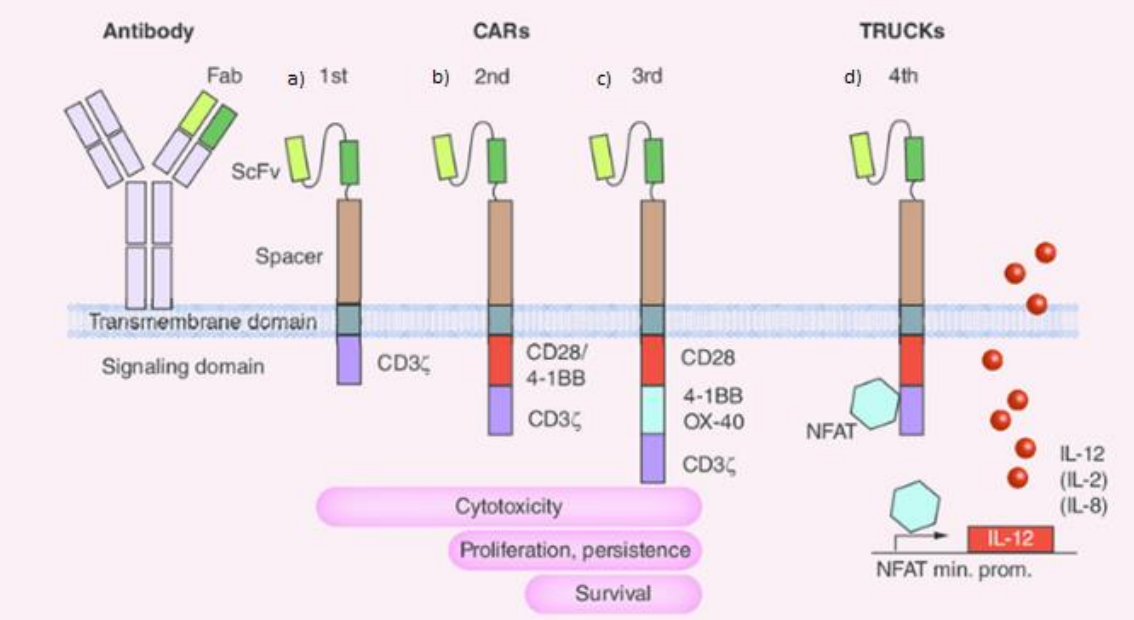

Figure 13: CAR generation according to their intracellular motifs and functions. a) First generation CARs have an intracellular tyrosine-based activation motif, such as a CD3 ζ chain. b) Second generation CARs (2G) include a co-stimulatory domain (CD28 or 4-1BB) in tandem with CD3ζ. c) Third generation CARs (3G) contain two co-stimulatory elements (CD28 and 4-1BB/OX40) in tandem with CD3ζ. d) Fourth generation CARs (4G or TRUCKs) are additionally modified with a constitutive or inducible expression cassette for a transgenic protein. Modified from Kalaitsidou M et al 2015

As CARs seem to be a newer and more effective way to treat cancers in relapse or refractoriness, especially hematological cancers, there are several clinical trials going on. Most of them use T cells as a vehicle for the CARs, being more than 800 clinical trials worldwide, and mostly CD19 is used as the CAR antigen. However, due to the success of the CD19-CART clinical trials, new targets are being carried into the field, as BCMA for multiple myeloma (NCT03338972), dual CD19/CD22 for ALL, CLL and non-Hodgkin's lymphoma (NCT04029038), CD38 for multiple myeloma (NCT03464916) or HER-2 for brain or leptomeninges metastasis (NCT03696030). In comparison CAR-NK based clinical trials are a minority, as the number of them is under 20. NK cells from different sources are used in these trials, as cord blood NK cell,

adult peripheral blood NK cells and NK-92 cell line (**Table 3**). FT596 is iPSC-derived NK cells derived product which is also combined with CAR technology. Fate Therapeutics presented new preclinical data for this product. FT596 is derived from a clonal master iPSC line engineered to express a proprietary CAR targeting CD19, a hnCD16 Fc receptor, and a novel IL-15 receptor fusion. In a mixed co-culture assay, the Company showed that the concurrent activation of the CAR and hnCD16 targeting modalities of FT596 exert synergistic anti-tumor activity. Increased degranulation (CD107a) and cytokine release (INF-$^{\gamma}$ and TNF-α) were observed upon concurrent activation of both the CAR and CD16 receptors in CD19+CD20+ Raji cancer cells with rituximab as compared to activation of each receptor alone, suggestive that dual antigen engagement may elicit a deeper and more durable response.

Table 3: Most relevant CAR-NK clinical trials using different sources of NK cells and different antigens, depending of the cancer

NK source	Antigen	Treated disease	Clinical trial
Umbilical cord blood	CD19	B-malignancies	NCT03056339
Adult peripheral blood	CD19/CD22	Refractory B-Cell Lymphoma	NCT03824964
	CD19	Refractory B-Cell Lymphoma	NCT03690310
	CD22	Refractory B-Cell Lymphoma	NCT03692767
	NKG2D	Metastatic solid tumors	NCT03415100
	Mesothelin	Ovarian cancer	NCT03692637
	PSMA	Castration-resistant prostate cancer	NCT03692663
	ROBO1	Pancreatic cancer	NCT03941457
NK-92	BCMA	Multiple myeloma	NCT03940833

Stem Cells

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. In addition, in many tissues they serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell or a brain cell (141). The type of cells that stem cell will produce depend on their cell potency, that is, the ability of the cells to differentiate into other cell types. The more cell types a cell can differentiate into, the greater its potency. It begins with totipotency to designate a cell with the most differentiation potential, pluripotency, multipotency, and finally unipotency (142,143).

Cell potency

Totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism together with the extraembryonic tissues. Zygote is an example of totipotent cells. In the spectrum of cell potency, totipotency represents the cell with the greatest differentiation potential, being able to differentiate into any embryonic cell, as well as extraembryonic cells (144). Pluripotency refers to a stem cell that has the potential to differentiate into any of the three germ layers: endoderm (interior stomach lining, gastrointestinal tract, and the lungs), mesoderm (muscle, bone, blood, urogenital), ectoderm (epidermal tissues and nervous system) or germ cells. However, cell pluripotency is a continuum, ranging from the completely pluripotent cell that can form every cell of the embryo proper (totipotency), to the incompletely or partially pluripotent cell that can form cells of all three germ layers and germ cells but that may not exhibit all the characteristics of totipotent stem cells (145). Multipotency describes progenitor cells which have the gene activation potential to differentiate into some cell types but not all. Multipotent cells are found in many, but not all human cell types. Multipotent cells have been found in cord blood, adipose tissue and bone marrow, among others (146). A unipotent cell is the concept that one stem cell has the capacity to differentiate into only one cell type, for example muscle cells (**Figure 14**).

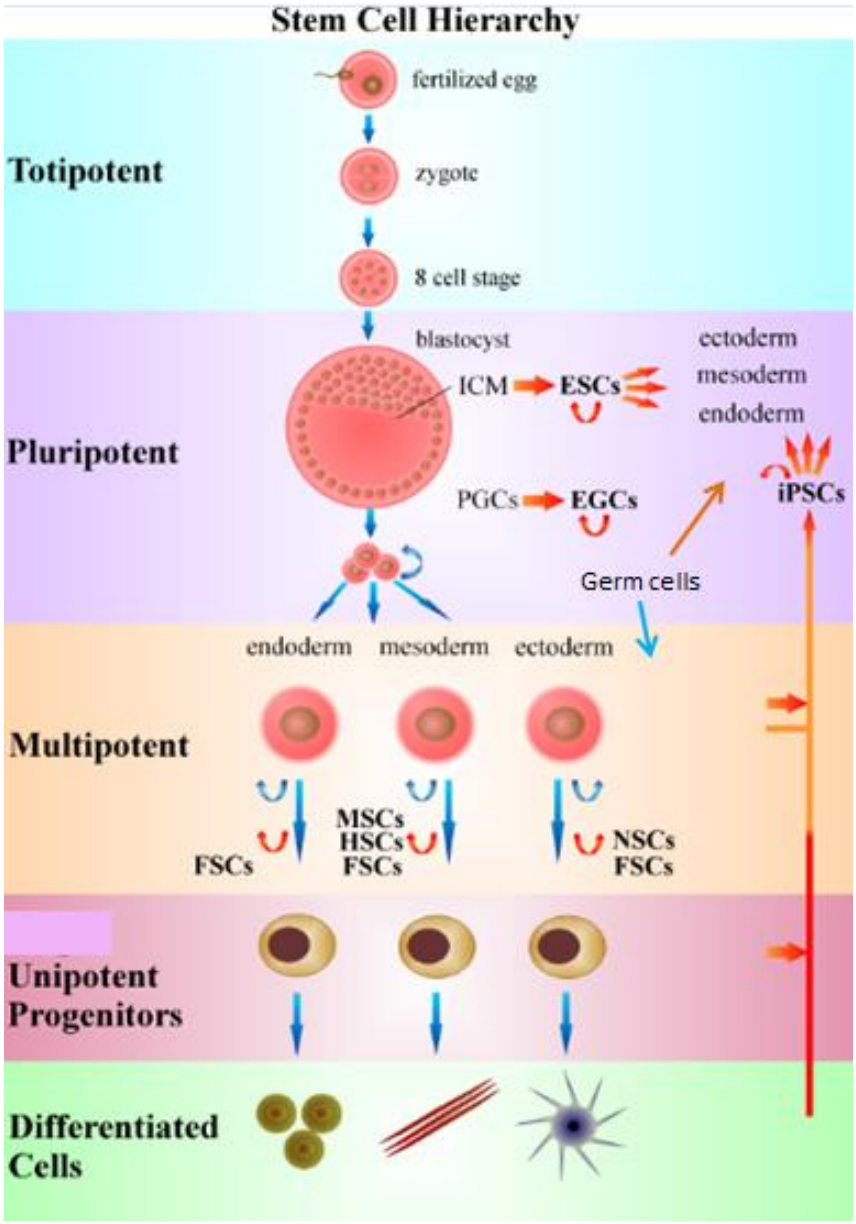

Figure 14: Stem cells hierarchy scheme regarding their potency to differentiate towards other cells. Totipotent stem cells can differentiate towards all cell types, and can develop a whole organism. Pluripotent stem cells can differentiate towards all cell types of the three germ layers: mesoderm, endoderm and ectoderm and germ cells, and they can also self-renew. Multipotent stem cells will differentiate only towards a unique lineage, such as hematopoietic lineage. Unipotent progenitors can only differentiate towards one type of cell, such as neurons. Adapted from Forostyak O et al

History

In 1998, a report published in the scientific journal Science marked the beginning of the modern era of regenerative medicine (147). For the first time, scientists were able to derive stem cells from a human embryo and show that these cells could be maintained in the so-called pluripotent state. Human embryonic stem cells (hESCs) are pluripotent, and their derivation sparked new possibilities, from the production of damage tissue to treating a numerous of degenerative conditions, the study of early embryonic development, to revolutionizing drug screening and development and broadening the spectrum of human toxicology research. In 2004 Clark et al documented the transcriptional and translational events of early human germ cell development *in vitro* during ES cell differentiation; and also demonstrated that hESCs can differentiate into ectoderm, endoderm and mesoderm, but also towards the germ line lineage.

In 2007, Prof. Shinya Yamanaka reprogrammed adult human dermal fibroblasts cells with the introduction of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) under ES culture conditions creating the human induced pluripotent stem cells (hiPSCs). The iPS cells could differentiate into cell types of the three germ layers and germ cells *in vitro* and in teratomas (148, 149). This discovery opened a new door to autologous regenerative medicine, providing an alternative source of human pluripotent stem cells (hiPSCs) without the need to use human embryos, thus alleviating some of the ethical concerns associated with hESCs (150).For this breakthrough, Prof. Yamanaka, along with Prof. John Gurdon been awarded with the 2012 Nobel Prize in Physiology or Medicine for the somatic reprogramming discovery.

iPSCs

The inherently low efficiency of iPSC derivation benefits from selection approaches that distinguish successfully reprogrammed clones from partially reprogrammed or simply transformed colonies. The reactivation of endogenous pluripotency-associated gene such as Nanog or Oct4 (151) linked to drug selection cassettes has been successfully employed for this purpose. A general limitation of any drug selection approach is that it requires genetic engineering of cells. To circumvent this problem, lentiviral vector systems have been developed that carry promoter fragments of pluripotency genes whose activity can be selected for, and that, in principle, can be applied to a wide range of human cell types (152). Importantly, high-quality iPSCs can be derived from unmodified somatic cells without drug selection or fluorescent reporters at all by simply using morphological criteria (153), although this approach requires careful characterization of the resultant cell lines.

The derivation of iPSCs from most studied somatic cells is extremely inefficient (0.01%–0.1%) and occurs at a slow speed (~2 wk) (154). Even in the context of fully differentiated tissues, in which somatic cells homogenously express the factors, the efficiency of fibroblast reprogramming generally does not exceed 1%–5% (155), although one report documented an efficiency of up to 20% (156). The so-called “elite” or “deterministic” model proposes that the efficiency of iPSC derivation is low because

only a few cells in a somatic cell culture are susceptible to reprogramming. Somatic stem or progenitor cells, present in most adult tissues and possibly also in explanted cell populations, are the most obvious candidate cells, as they are rare and developmentally closer to pluripotent cells than differentiated cells. In contrast, the "stochastic" model poses that all somatic cells are equally amenable to factor-mediated reprogramming, but have to go through a series of stochastic epigenetic events to acquire pluripotency. Only a few cells may pass all of these roadblocks, resulting in the overall low efficiency. Mature cells, such as lymphocytes, reprogram into iPSCs at lower efficiencies than immature cells, such as hematopoietic stem cells. This may be due to a lower number of stochastic epigenetic events that are required in immature cells to acquire pluripotency. The precise number and nature of such changes is unclear. Reprogramming of progenitor or stem cells may occur in a shorter period of time, which is consistent with the observed fast up-regulation of pluripotency markers upon factor expression (157).

A related issue is the question of whether iPSCs retain an "epigenetic memory" of their cell type of origin, which is defined here as remaining epigenetic marks originating from the starting cell that influence transcription in resultant iPSCs. Previous experiments using Somatic Cell Nuclear Transfer (SCNT) suggested that cloned embryos exhibit gene expression patterns reflective of their cell type of origin (158). A recent study found gene expression differences indicative of a transcriptional memory in human iPSCs derived from fibroblasts, adipose tissue, and keratinocytes (159). While the analyzed cell lines were derived in independent laboratories and with different technologies, which can confound gene expression analyses (160), another study confirmed and extended this finding by comparing genetically matched iPSCs derived from granulocytes, muscle progenitors, fibroblasts, and lymphocytes (161). iPSCs derived from these cell types exhibited discernible gene expression and DNA methylation patterns as well as differentiation biases into hematopoietic cells *in vitro*, some of which could be attributed to their cell type of origin.

These epigenetic marks present in iPSCs may be linked to their primed pluripotency status, which are blocking a proper differentiation to certain cell types and depending of their of the reprogrammed cell origin. Naïve pluripotent stem cells, it has been described that have much less epigenetic marks than primed pluripotent stem cells, so that, the differentiation protocols with these kind of cells could be more efficient. Depending to the pluripotency stage could be also been taking into account for immunotherapy purposes, as one of our aims in this study.

Naïve vs primed pluripotency state

In the past two decades, insights into early embryo development have broadened our perception of pluripotency. As such, pluripotency is no longer viewed as a fixed state but rather a highly dynamic, malleable signaling network (162). Unraveling the complete potency spectrum and its transitions will remain central to our understanding of lineage commitment. Mouse ESCs (mESCs) are one of the earliest and better characterized models of pluripotency (163). Derived from the Inner Cell

Mass (ICM) of mouse blastocysts, mESCs demonstrated characteristic features of pluripotency, including long-term self-renewal, ability to differentiate toward all germ layers, high single-cell clonogenicity and efficient contribution to chimeras (164). hESCs derived from human pre-implantation embryos, however, were markedly different from mESCs; hESCs had an epithelial morphology, could not be propagated efficiently as single cells and had different growth requirements (147). It soon became evident that hESCs rely on different signaling pathways to maintain pluripotency (165). Some years later, mouse epiblast stem cells (mEpiSCs) were isolated from post-implantation embryos and were found to share many similarities with hESCs. Their transcriptome was similar to that of the post-implantation epiblast (166), indicating that hESCs were more representative of later stages of embryo development. Subsequently, two states of pluripotency were proposed: naïve and primed (165). Accordingly, mESCs exist in a naïve state, which constitutes the functional *in vitro* equivalent of the pre-implantation epiblast, while hESCs are in a primed state (**Figure 15**). The naïve or ground state of pluripotency is characterized by a seemingly unbiased differentiation potential, low variability in pluripotency linked gene expression, global DNA hypo-methylation and two active X-chromosomes in female cells. Conversely, cells in the primed state display distinct pluripotency associated gene patterns, DNA hyper-methylation, X-chromosome inactivation and inefficiency in forming chimeras; this state corresponds to the transition of naïve epiblast cells toward a more committed state *in vivo* (165,167).

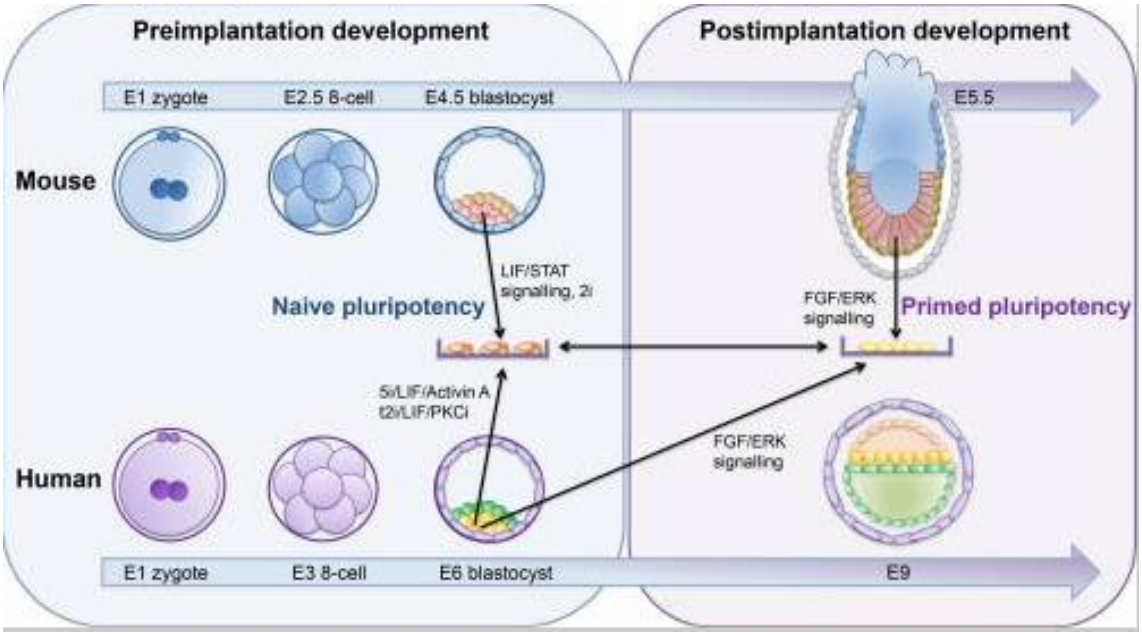

Figure 15: States of pluripotency: primed vs naive. A comparison between potency states of cells in the developing embryo from mice (top) and human (bottom) and the in vitro manipulation necessary to maintain/convert each stage to the other. LIF/STAT, leukemia inhibitory factor/signal transducer and activator of transcription 3; PKCi, protein kinase C inhibitor; E, day of embryonic development; FGF, fibroblast growth factor; ERK, extracellular regulated kinase. From Eguizabal et al. 2019

Conventional reprogramming protocols produce developmentally advanced or primed hiPS, restricting their use to post-implantation human development modeling (**Figure 16**) (168). hiPS also appear to lack many of the defining hallmarks of naïve mESCs. These include important features of the naïve ground state murine epiblast, such as an open epigenetic architecture, reduced lineage-primed gene expression, and chimera and germline competence following injection into a recipient blastocyst-stage embryo (169). Several groups have aimed to capture naïve pluripotency in humans and to establish culture conditions closely recapitulating the signature of human ICM cells. These studies attempted to induce a naïve state in hiPS by reprogramming primed hiPS with cytokines or small molecules or by directly culturing hiPS isolated from pre-implantation ICM cells under conditions that favor naïve stemness (170). Several surface membrane markers are used to distinguish between naïve and primed pluripontent stages. CD75 and CD130 are present in the surface of naïve stem cells, but not in primed stem cells; CD24 and CD57 are exclusively present in primed stem cells (171).

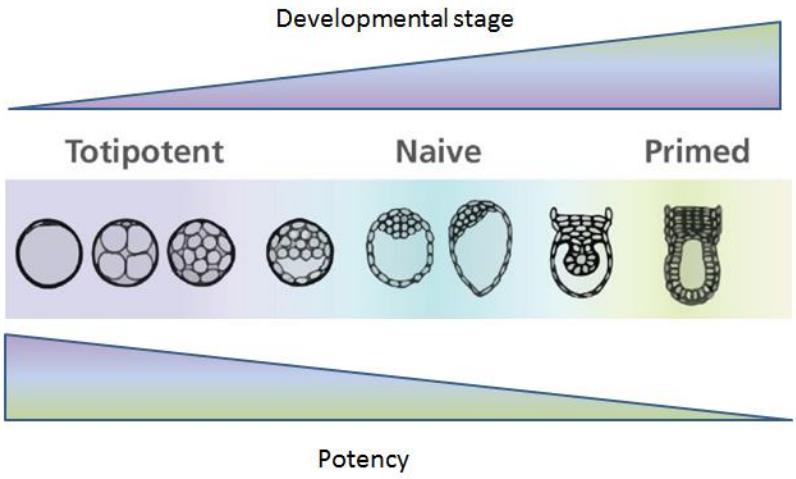

Figure 16: Grade of developmental stage and cell potency from totipotent stem cells to primed stem cells. Adapted from Stem Cell Company.

hESCs and hiPSCs differentiation

The discovery of induced pluripotent stem cells (iPSCs) by Prof. Shinya Yamanaka was heralded as a major breakthrough of the decade in stem cell research. The ability to reprogram human somatic cells to a pluripotent embryonic stem cell-like state through the ectopic expression of a combination of embryonic transcription factors was greeted with great excitement by scientists and bioethicists. The reprogramming technology offers the opportunity to generate patient-specific stem cells for modeling human diseases, drug development and screening, and individualized regenerative cell and gene therapy (**Figure 17**).

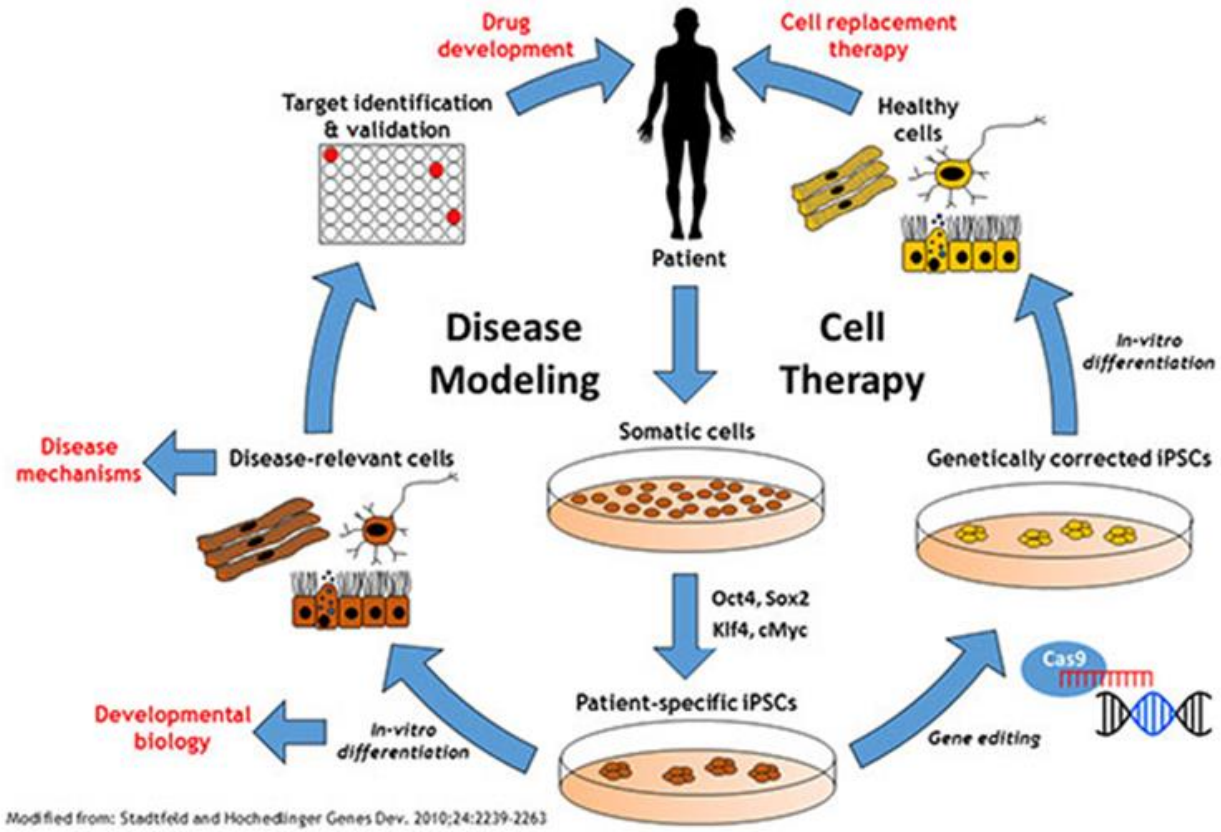

Figure 17: Generation of patient-specific stem cells for modeling human diseases, drug development and screening, and individualized regenerative cell and gene therapy.

As a consequence, researchers have focused in the differentiation from hiPSCs and hESCs into a whole variety of different cells types (**Figure 18**). Cardiomyocytes and human engineering heart tissue are a promising regenerative therapy for heart damage (172, 173). Differentiated neural cells from hiPSCs are being used as a model for treating and understanding neural diseases as Alzheimer (174), Parkinson's disease (175) or Neuropsychiatric brain disorders (176). Retinal cells can also be obtained from hiPSCs for a possible treatment of retinal degenerative diseases (177). Not only single differentiated cells can be obtained from hiPSCs, but organoids are newer approach to the use these stem cells in regenerative medicine, as kidney organoids could do (178).

Among all the uses could be attributing to hiPSCs, the generation of hematopoietic stem cells is one of the most studied ones and several protocols have been proposed in order to get *in vitro* CD34+ cells (179-181). Generating CD34+ hematopoietic precursors is the first important step in the specific hematopoietic lineage differentiating protocols from hESCs and hiPSCs. There are a variety of stromal cell lines employed for co-culture systems with hES/hiPS cells; of these, OP9 cells are most widely used (182). The initial protocols achieved to obtain up to 20% of CD34+ cells by coculturing the hESCs with the OP9 mouse bone marrow stromal cells. Choi et al. induced hematopoietic differentiation in seven hiPS cell lines by coculture on mouse stromal cells (OP9) and evaluated hematopoietic potential by generation of colony forming cells (183). Not only OP9 co-culture is used, but M210-B4 is another cell line which can help with the differentiation towards CD34+ hematopoietic progenitors

(184). These *in vitro* generated hematopoietic stem cells could be used to obtain different cell from the hematopoietic lineage such as T cells (185), platelets (186), red blood cells (187) and NK cells. hiPS could became a new source for immunotherapies involving NK cells, as several groups have achieved a method to produce clinical scale NK cells from hiPSCs (184,188). Moreover, as it was previously mentioned in the Natural Killer cells clinical trials section, a hiPSCs derived NK cell pharmacology product has been developed for treating several solid tumors (FT500) in 2019. Not only did they create this product, but they also believed in hiPSCs derived NK cells for CAR based treatments (FT596).

Not only hiPS constitute a source for NK cells, but CD34+ cells from umbilical cord blood themselves too, as these cells could have therapeutically used apart from hematopoietic stem cells transplantation (189, 190). These are being considered a source for the production of a large number of NK cells (191,192). Obtaining NK cells from CB CD34+ hematopoietic progenitors has been extensively described (193).

However, further research is needed to obtain even larger numbers of mature and functional NK cells from stem cells ready to use in cancer immunotherapy.

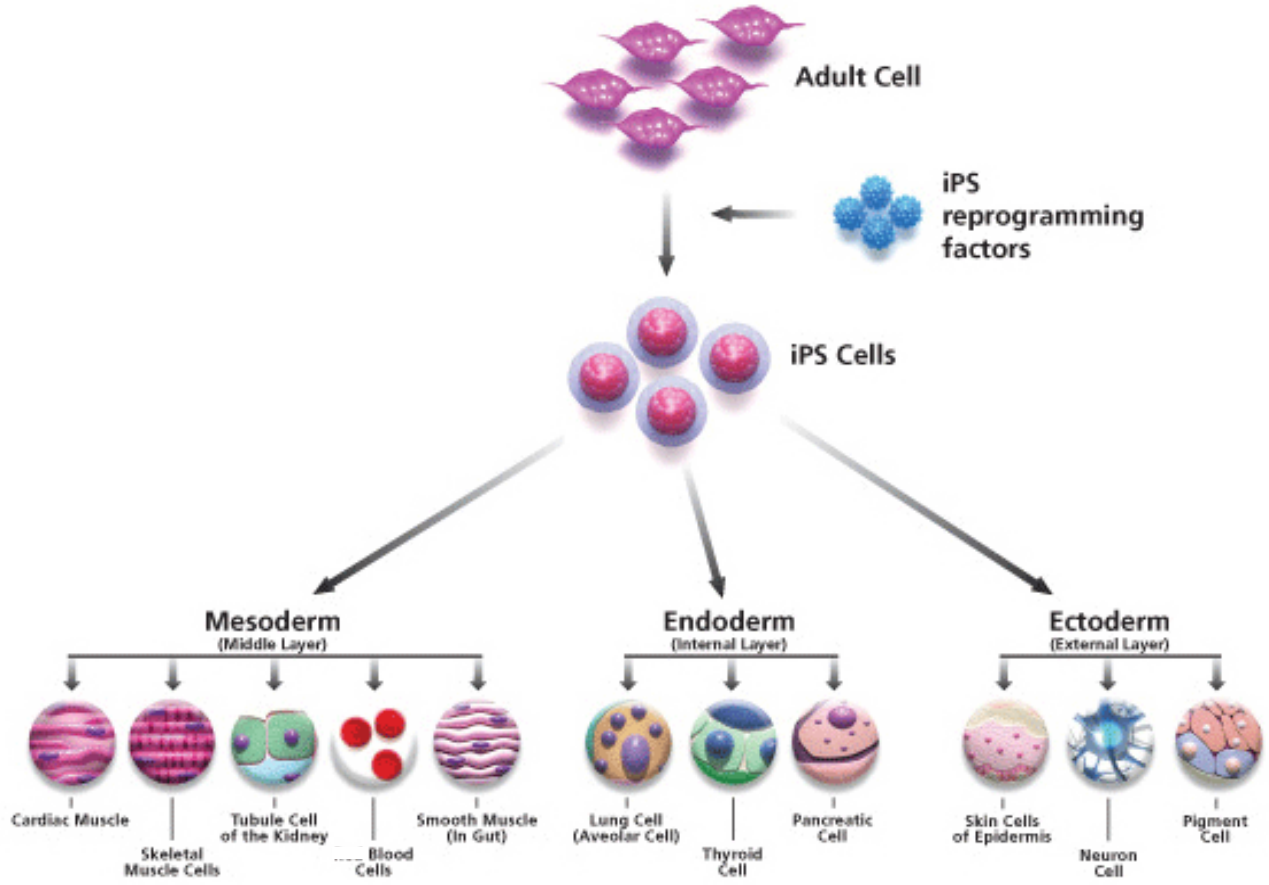

Figure 18: Reprogramming of adult cells into iPS and their in vitro differentiation towards severalcells of the three primary germ layers: mesoderm, endoderm and ectoderm, including blood

In summary, although numbers of patients are benefit from chemotherapy treatments, there is still an important amount of them that do not respond to these kinds of treatments. CAR based therapy with T cell has been prove to work satisfactorily, in spite of certain obstacles such as cytokine storm, neurotoxicity or GvHD in allogenic treatments. For these reason, NK cells are believed to be good candidates for allogenic CAR based immunotherapy therapy, as they are prompt to attack neoplastic cells and do not cause GvHD. In this thesis, we propose four different sources of NK cells (AB, CB, CD34+ from CB and iPS) to examine which one is the most accurate to be used in a future clinical treatment with CAR immunotherapy.

HYPHOTESIS AND OBJECTIVES

HYPHOTESIS AND OBJECTIVES

Hypothesis

Allogenic NK cells from several sources are a good alternative for “off the self” CAR-based treatments against refractory or relapsed hematological malignances involving CD19 antigen positive expression.

Objectives

1. Obtaining, culture and expansion of NK cells from four different cell sources: Adult peripheral blood, Umbilical Cord Blood, *in vitro* differentiated NK cells from Umbilical Cord Blood hematopoietic progenitors and *in vitro* differentiated NK cells from hES or hiPS.
2. Arming NK cells from different sources with a CD19-CAR in order to determine the best cell source of NK cells for treating CD19 expressing hematological cancers in relapsed or refractory patients using CAR technology.

MATERIALS AND METHODS

OBJECTIVE 1

MATERIALS AND METHODS

Cell Lines

OP9, M2-10B4, HFF1 and K562 cell lines were purchased from ATCC (CRL-2749, CRL-1972, SCRC-1041 and CCL-243, respectively). OP9 cells were cultured with α-MEM (Gibco), 20% fetal bovine serum (FBS) (Hyclone), 1% penicillin/streptomycin, and 1% Glutamax. M2-10B4 cells were culture with RPMI, 10% FBS (Hyclone), 1% penicillin/streptomycin, and 1% Glutamax. HFF1 were cultured with IMDM (Gibco), 10% FBS (Hyclone), 1% penicillin/streptomycin, and 1% Glutamax. Finally, K562 cells were cultured with RPMI, 10% FBS (Hyclone), 1% penicillin/streptomycin, 1% Glutamax, 1% NEAA, and 1% sodium pyruvate. Human ES and iPS cells were obtained from the Spanish National Bank of Cell Lines (BNCL). ES and iPS were cultured onto HFF1 feeder layer with the following medium: Knock out DMEM (Gibco), 20% Knockout Serum Replacement (KSR) (Gibco), MEM NEAA 1x (Gibco), 50 µM 2-Mercaptoethanol (Gibco), 0,5% P/S (Gibco), 1% Glutamax (Gibco) and 8ng/ml bFGF (Miltenyi Biotec). When iPS and ES cells were cultured in a feeder free system, cells were culture over vitronectin (VTN-N) Recombinant Human Protein, Truncated (Thermofisher, A31804) along with Essential 8 medium (Thermofisher, A1517001).

Isolation and culture of Adult Peripheral Blood NK cells

Adult Peripheral Blood (AB) samples were obtained from blood donors with prior signed informed consent and ethical committee approval from the Basque Ethics Committee for Clinical Research [Comité Ético de Investigación Clinica de Euskadi-CEIC-E (PI2014138)]. AB mononuclear cells were obtained by density gradient using Ficoll-Paque™ PLUS (GE Healthcare). 10 ml of Ficoll were placed in a 50 ml tube. Buffy coat blood was diluted four times with PBS, obtaining a total of 40 ml of blood per buffy coat unit. 20 ml of blood were carefully placed in each tube on top of the Ficoll, without mixing. Sample tubes were centrifuged at 2500 rpm for 20 minutes, with soft acceleration and brake. After the centrifugation, mononuclear cells were collected with a Pasteur pipette and placed in a new tube. Tubes were filled up with PBS and two different centrifugations were performed in order to clean up the cells (1100 rpm, 10 minutes for the first one; 1500 rpm 5 minutes for the second one). Once cells were clean, they were counted with a Neubauer chamber and trypan blue staining. NK cells from $150*10^6$ adult healthy donors' PBMCS were isolated with the NK Cell Isolation Kit from Miltenyi Biotec (catalog number 130-092-657) (**Figure 19**). These cells were cultured with RPMI, 10% AB serum (Innovative Research, Inc), 1% penicillin/streptomycin and 1% Glutamax. For the first two days, 500 U/ml of IL-2 were added to the culture medium. From this point on, 20 ng/ml of IL-15 was also added to the culture medium. AB NK cells were cultured for a week before the viral transduction and for another week before the AB CAR-NK functional assays.

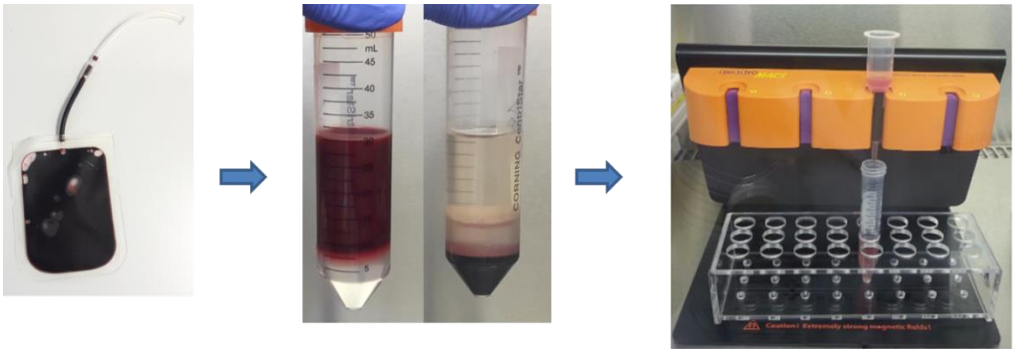

Figure 19: Purification process of NK cells from adult peripheral blood.

Isolation and culture of Umbilical Cord Blood NK cells

Umbilical Cord Blood (CB) samples were obtained with prior signed informed consent and ethical committee approval from the Basque Ethics Committee for Clinical Research [Comité Ético de Investigación Clinica de Euskadi-CEIC-E (PI2014138)]. Fully signed written informed consent was obtained from the pregnant mothers. CB units that contain between 1.5×10^9 and 8×10^8 mononuclear cells were used for investigation purposes. A fresh CB unit (less than 30 h between the extraction and the processing) was used to perform a set of experiments. CB mononuclear cells were obtained by density gradient using Ficoll-Paque™ PLUS (GE Healthcare). 10 ml of Ficoll were place in a 50 ml tube. Cord blood was diluted two times with PBS, obtaining approximately between 180 and 200 ml of blood per cord blood unit. 30 ml of blood were carefully placed in each tube on top of the Ficoll, without mixing. Sample tubes were centrifuged at 2500 rpm for 20 minutes, with soft acceleration and brake. After the centrifugation, mononuclear cells were recollected with a Pasteur pipette and placed in a new tube. Tubes were filled up with PBS and two different centrifugations were performed in order to clean up the cells (1100 rpm, 10 minutes for the first one; 1500 rpm 5 minutes for the second one). Once cells were clean, they were counted with a Neubauer chamber and trypan blue staining. NK cells from $150*10^6$ umbilical cord blood´s PBMCS were isolated with the NK Cell Isolation Kit from Miltenyi Biotec (catalog number 130-092-657) (**Figure 20**). These cells were cultured with RPMI, 10% AB serum (Innovative Research, Inc), 1% penicillin/streptomycin and 1% Glutamax. For the first two days, 500 U/ml of IL-2 were added to the culture medium. From this point on, 20 ng/ml of IL-15 was also added to the culture medium. CB NK cells were cultured for a week before the viral transduction and for another week before the CB CAR-NK functional assays.

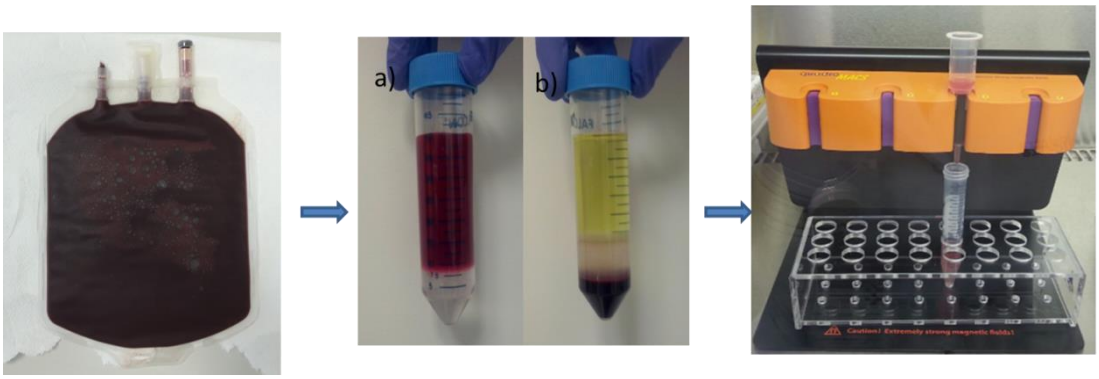

Figure 20: Purification process of NK cells from umbilical cord blood.

Hematopoietic Stem Cell (HSC) differentiation protocol into NK Cells

Umbilical cord blood mononuclear cells were obtained by density gradient using Ficoll-Paque™ PLUS (GE Healthcare). Cord blood was diluted two times with PBS, obtaining approximately between 180 and 200 ml of blood per cord blood unit. 30 ml of blood were carefully placed in each tube on top of the Ficoll, without mixing. Sample tubes were centrifuged at 2500 rpm for 20 minutes, with soft acceleration and brake. After the centrifugation, mononuclear cells were collected with a Pasteur pipette and placed in a new tube. Tubes were filled up with PBS and two different centrifugations were performed in order to clean up the cells (1100 rpm, 10 minutes for the first one; 1500 rpm 5 minutes for the second one). Once cells were clean, they were counted with a Neubauer chamber and trypan blue staining. Then, HSCs (CD34+ cells) were isolated by MACS sorting, using the CD34 MicroBead kit from Miltenyi Biotec (**Figure 21**) and 2 columns per each sample. CD34+ cells (5,000 cells/well) were plated onto 6-well plates pre-plated with OP9 or M2-10B4 cells mitotically inactivated with Mitomycin C (10 µg/ml) (Sigma) and also plated in feeder-free system and cultured with the media described by Ni et al. (194): Ham F12+ DMEM (1:2), 20% human serum (AB serum-Invitrogen, Life Technologies), 1% penicillin/streptomycin, 2-mercaptoethanol (25 µM), ascorbic acid (20 µg/ml), and sodium selenite (5 ng/ml). At the beginning of the differentiation process, IL-3 (5 ng/ml), IL-7 (20 ng/ml), IL-15 (10 ng/ml), SCF (20 ng/ml), and FLT3 ligand (10 ng/ml) (Miltenyi Biotec) were added to the medium. Half of the medium was changed every week. From the second week of differentiation, IL-3 was no longer added to the medium as was described by Cichocki and Miller (195) and Grzywacz et al. (196). The differentiation protocol that we carried out consisted of plating purified CD34+ cells over two different culture conditions using three feeder cells layers, AFT024 OP9 and M2-10B4; and a feeder free culture system, and cultured for 42 days with the differentiation medium previously described. From day 14 up to day 42 of differentiation, immunophenotyping analyses were performed, along with cytotoxicity and degranulation assays at 28, 35, and 42 days of differentiation (**Figure 22**). Characteristics of the CB units used for these experiments are summarized in **Table 4**.

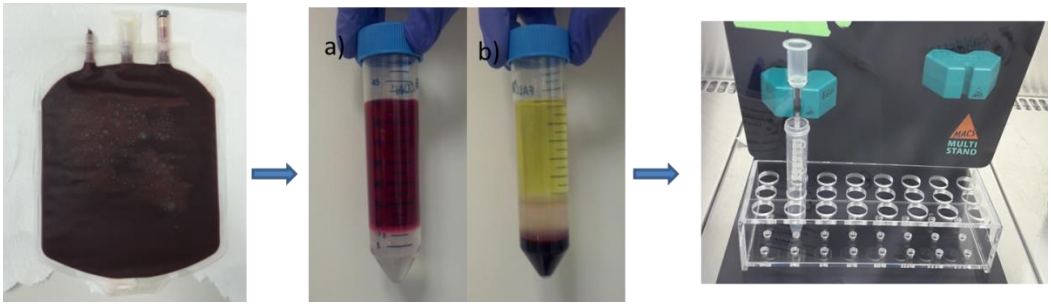

Figure 21: Purification of CD34+ hematopoietic progenitors from umbilical cord blood.

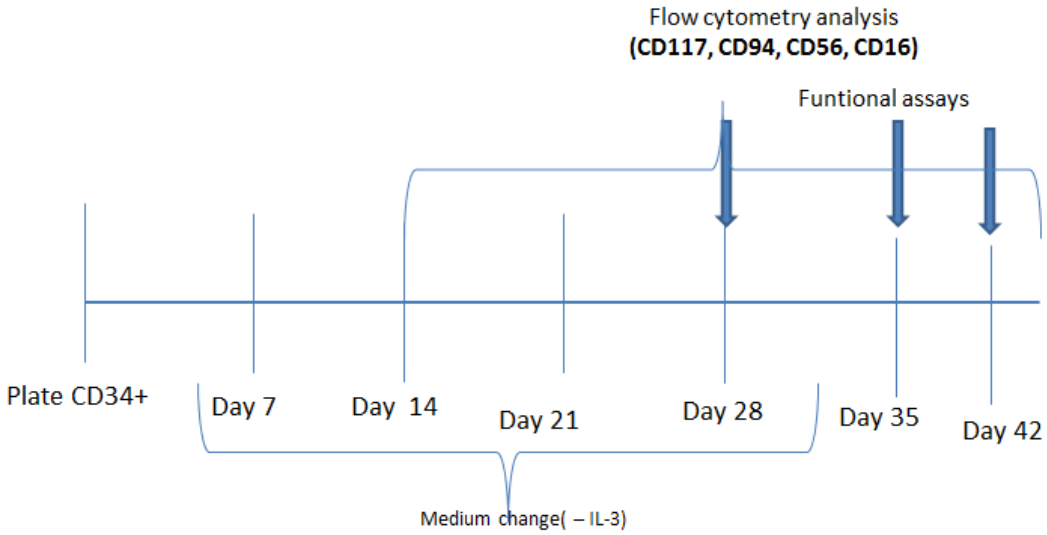

Figure 22: Protocol and time course of the in vitro NK cell differentiation from UCB CD34+ cells up to 42 days in culture.

Table 4: Number of fresh umbilical cord blood (UCB) units, volume in milliliters, number of mononuclear cells per UCB, and CD34+ cells remaining after purification for each experiment. UCB were fresh, being not longer than 30 h after the extraction.

Experiment Nº	UCB units	Volume (ml)	Mononuclear cells ($x10^6$)	CD34+ cells after purification
1	1	105.0	1136.1	325000
2	1	111.4	1366.878	315000
3	1	111.4	1340.142	350000
4	1	144.5	1473.9	287000

Flow cytometry

Purity of CD34+ sorted cells from CB samples was analyzed with CD34-PE antibody (BD Biosciences, clone 581) in a FACS Canto II (BD Biosciences). Purity of the CD34+ cells isolated had to be higher than 80% in order to perform our protocol of differentiation. The number of remaining CD56+ cells in the purified sample was not significant.

Different populations and maturation stages of *in vitro* differentiated NK cells were analyzed by flow cytometry at 14, 21, 28, 35, and 42 days in culture. Cells were washed with PBS/10% FBS and incubated for 30 min at 4°C for labeling with anti-CD94-FITC (BD Biosciences, clone HP-3D9), anti-CD117-PE (Miltenyi Biotec, clone A3C6E2), anti-CD56-APC (Biolegend, clone MEM-188), and anti-CD16-BV421 (BD Biosciences, clone 3G8). Next, cells were fixed and permeabilized with BD Cytofix/Cytoperm™ Plus in order to label them with anti-Perforin-PerCP-eF710 (BD Biosciences, clone δG9) and anti-Granzyme B-BV510 (BD Biosciences, clone GB11) (**Table 5**). 50,000–100,000 events were acquired for analyses. Populations were analyzed using FlowJo v.X.0.7 (TreeStar Inc.). The gating strategy in all experiments was: Lymphocytes were selected by size and internal complexity (FCS/SSC). Then, duplets were

discarded and NK cells were analyzed by the different stages of maturation. Firstly, CD117 and CD56 were confronted and next, the populations resulting from this gating were faced to CD94 and CD16. Stage <3 population was CD56-, CD94-, CD117low; stage 3 population was CD56+, CD94-, CD16- and CD117high; stage 4 population was CD56+, CD94+, CD16-, CD117low and finally, stage 5 population was CD56+, CD94+, CD16+, CD117 low. Perforin and granzyme B were analyzed regarding CD56+ NK cells (**Figure 23**).

Table 5: Flow cytometry panel for determining the differentiation stages of the in vitro generated NK cells.

NK cells differentiation panel							
488 nm laser				635 nm laser		405 nm laser	
FITC	PE	PerCP-Cy5-5	PE-Cy7	APC	APC-Cy7-A	BV421	BV510
CD94	CD117	Perforine		CD56		CD16	Granzyme B

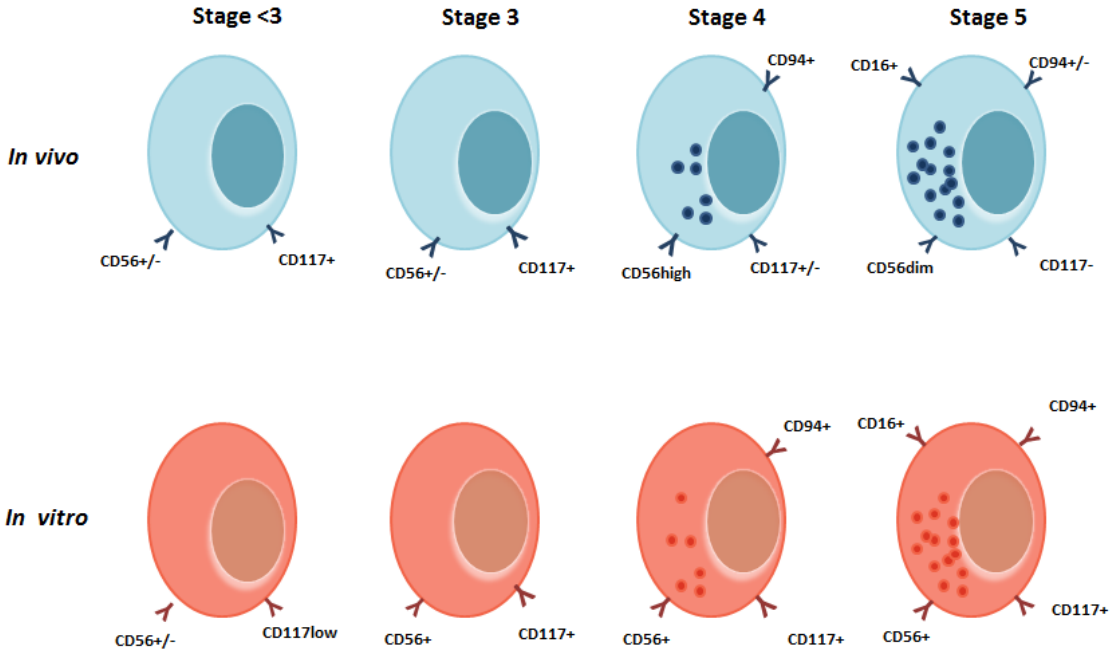

Figure 23: Key markers expressed at different stages through natural killer cell differentiation/maturation *in vivo* and *in vitro*. There are some differences between the *in vivo* and *in vitro* expression of these markers. In *stage <3,* CD117 marker is positive form *in vivo* NK cells, while *in vitro* NK cell present it in low proportion. In *stage 3*, *in vivo* NK cell can or cannot express CD56 in their surface, while *in vitro* NK cells express it. In *stage 4*, *in vivo* expression of CD56 marker is high, while *in vitro* is considered as positive; moreover, CD117 marker can or cannot be express in *in vivo* NK cells, while *in vitro* NK cells maintain it. In *stage 5*, CD56 markers is expressed as dim in *in vivo* NK cells while *in vitro* is considered as positive; CD117 marker is still maintained in *in vitro* NK cells but not in *in vivo* NK cells. Finally CD94 marker may be present or not in *in vivo* NK cells, while *in vitro* NK cells express it.

Cytotoxicity assay

In order to check the *in vitro* lytic activity of the differentiated NK cell against the K562 target cell line, we performed a calcein-AM-based cytotoxicity assay (197). Briefly, target cells used in this assay interiorize the calcein-AM in their cytoplasm. When these cells get in contact with the effector cells, they will release the calcein-AM to the medium as a consequence of being lysed by the effector cells (**Figure 24**). K562 cell line was used as target cells. 10^6 cells were incubated for 30 min at 37°C with 15 µM of calcein-AM (Life technologies C3099). These cells were washed twice after incubation. Calcein-AM-labeled K562 cells were co-cultured with NK cells differentiated from CD34+ progenitors from CB in a U-bottom 96-well plate for 4 h at 37°C at different ratios (25:1, 12.5:1, 6.25:1, and 3.125:1). As a control, we used NK cells from adult healthy donors' blood (AB) isolated with the NK Cell Isolation Kit from Miltenyi Biotec (catalog number 130-092-657). These adult PB-NK cells were stimulated overnight under the same culture conditions as CB CD34+ cells following the *in vitro* differentiation protocol (IL-7, IL-15, SCF, and FLT3). Adult PB-NK cells were purified by MACS sorting, using the NK Cell Isolation Kit from Miltenyi Biotec (130-092-657). For measurement of spontaneous release, K562 target cells were incubated with no NK cells. Total released was achieved by adding 4% Triton™ X-100 (Sigma-Aldrich) to the target cells. Each condition was performed in triplicates. After the incubation, 100 µl of supernatant was collected and transferred to a black 96-well plate to measure the calcein-AM release in a Fluoroskan Ascent (Thermo Fisher) (excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). The percentage of specific lysis is calculated according to the following formula: [(Test release) − (Medium fluorescence)] − [(Spontaneous release) − (Medium fluorescence)]/[(Total release) − (Triton fluorescence)] − [(Spontaneous release) − (Medium fluorescence)] × 100.

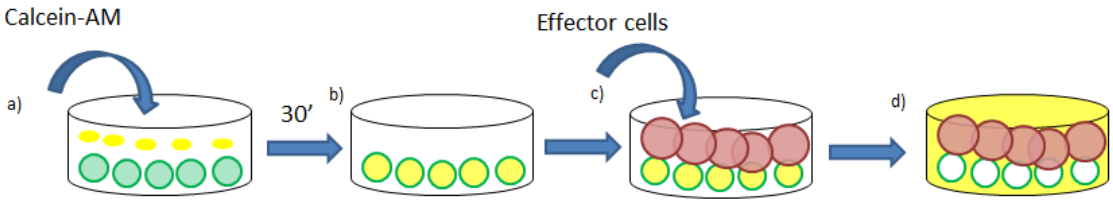

Figure 24: Description of the mechanism of calcein-AM based citotoxicity assay. a) target cells (green) are incubated with 15µM of calcein-AM for 30 minutes at 37°C. b) During this incubation, target cells absorb the calcein – AM inside the cytoplasm. Target cells are washed to get rid of the remained calcein-AM on the medium. c) The target cells are co-cultured with the effector cells (red) at different ratios for 4 hours at 37°C. d) Target cells that have been lysed by the effector cells will release the calcein-AM to the medium as a consequence of the breaking of the surface membrane. The amount of calcein-AM released to the medium is measured in order to establish a percentage of lysis made by the effector cells against the target cells.

Degranulation assay

In this assay, CD107a present in the granulocytes of NK cells is measured. CD107a is found in the inside of the NK cells, but when these cells are presented to target cells, these cellsliberate their granules, as perforin or granzyme B, exposing the CD107a marker in the surface membrane. CD107a antibody present in the medium joins to the exposed CD107a protein of NK cells (**Figure 25**). Natural killer cells were co-cultured with K562 target cells at ratio 1:1 in a 24-well plate for 6 h at 37°C. At the beginning of the assay, anti-CD107a BV421 (BD Biosciences, clone H4A3) was added in order to detect the degranulation activity of the effector cells against the target cells. Golgi Stop™ (BD Biosciences) (monensin) was added following the manufacturer's protocol. After the incubation, cells were collected, washed, and labeled with anti-CD94-FITC and anti-CD56-APC. Degranulating NK cells (CD107a+) were determined in the CD56+ cells, both on stage 3 (CD56+CD94−) and stages 4–5 (CD56+CD94+) cells. The gating strategy was: Lymphocytes were selected by size and internal complexity (FCS/SSC). Then, duplets were discarded and NK cells were analyzed by facing CD94 and CD56. Populations of stage 3 and stage 4-5 were faced to CD107a marker.

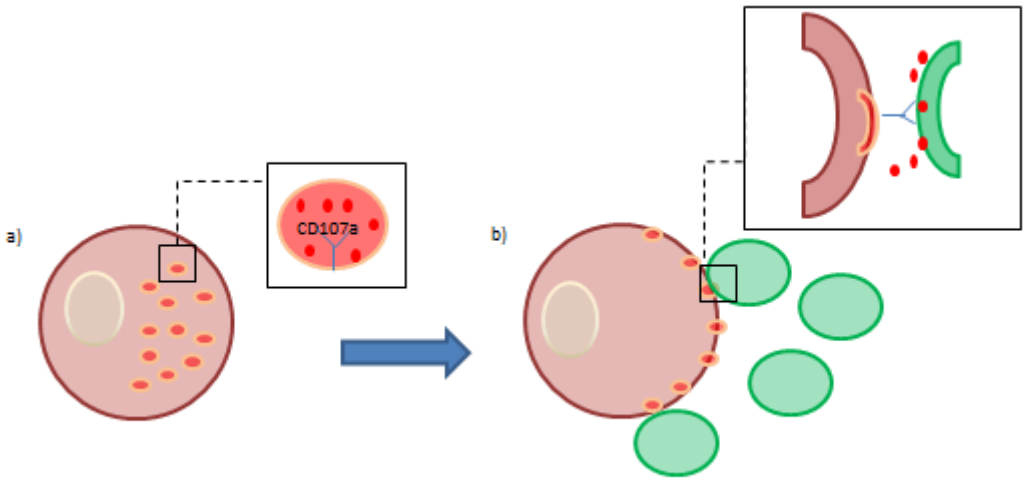

Figure 25: Description of the mechanism of the degranulation assay. NK cells contains with cytotoxic molecules inside. a) These granules present CD107a marker in the inner part of their surface. b) When NK cells degranulate, they release molecules like perforin and granzymes by a process of directed exocytosis; which exposes the CD107a markers to the NK cell surface.

NK cells differentiation from human induced pluripotent stem cells (hiPS) and human embryonic stem cells (hES)

CD34+ hematopoietic stem cell obtaining from hiPS and hES

Primed hiPS and hES cell lines

hiPS (KiPS 4F-1, KiPS 3F-7, KiPS 4F-8 and CBiPS 30 4F-3) and hES (ES-2) were cultured onto inactivated HFF1 feeder layer with the medium described above. When the cells were 80% confluent, colonies were manually picked up. These small fragments were placed in an ultra-low attachment p60 plate with DMEM and 10% FBS for 48 hours in order to form the embriod bodies (EBs). After this time, EBs were placed onto overgrown OP9 feeder cells on 6 well plate for 8 more days with the following

medium: StemSpan Serum free (Miltenyi Biotec), 10 ng/ml BMP4 (Miltenyi Biotec), 10ng/ml VEGF (Miltenyi Biotec), 25 ng/ml SCF (Miltenyi Biotec), 10 ng/ml FGF (Miltenyi Biotec), 20 ng/ml TPO (Miltenyi Biotec) and 10 ng/ml Flt 3 Lingand (Miltenyi Biotec). At day 10, cells were disgregated with TrypLE Express for 10 minutes. Then, cells were analyzed by flow cytometry and CD34+ resulting cells were isolated by MACS sorting, using the CD34 MicroBead kit from Miltenyi Biotec. (**Figure 26A**) CD34+ resulting cells were frozen in FBS + 10% DMSO. Colony Forming Unit (CFU) assays was performed for every CD34+ batch. Moreover, another alternative method to obtain CD34+ cells was performed. Firstly, hiPS or hES were manually picked up. Then, the fragments of hiPS or hES colonies were passed onto Mytomicin c inactivated M2-10B4 cells with the following medium: RPMI, 15% FBS, 2mM l-glutamina, 1% NEAA, 1% P/S and 0,1 mM β-mercaptoetanol. Cells were culture for 11-13 days. Cells were analyzed by flow cytometry and CD34+ resulting cells were isolated by MACS sorting, using the CD34 MicroBead kit from Miltenyi Biotec. CD34+ resulting cells were frozen in FBS + 10% DMSO. Colony Forming Unit (CFU) assays was performed for every CD34+ batch and checked with StemVision (Stem Cell Technologies)(**Figure 27**).

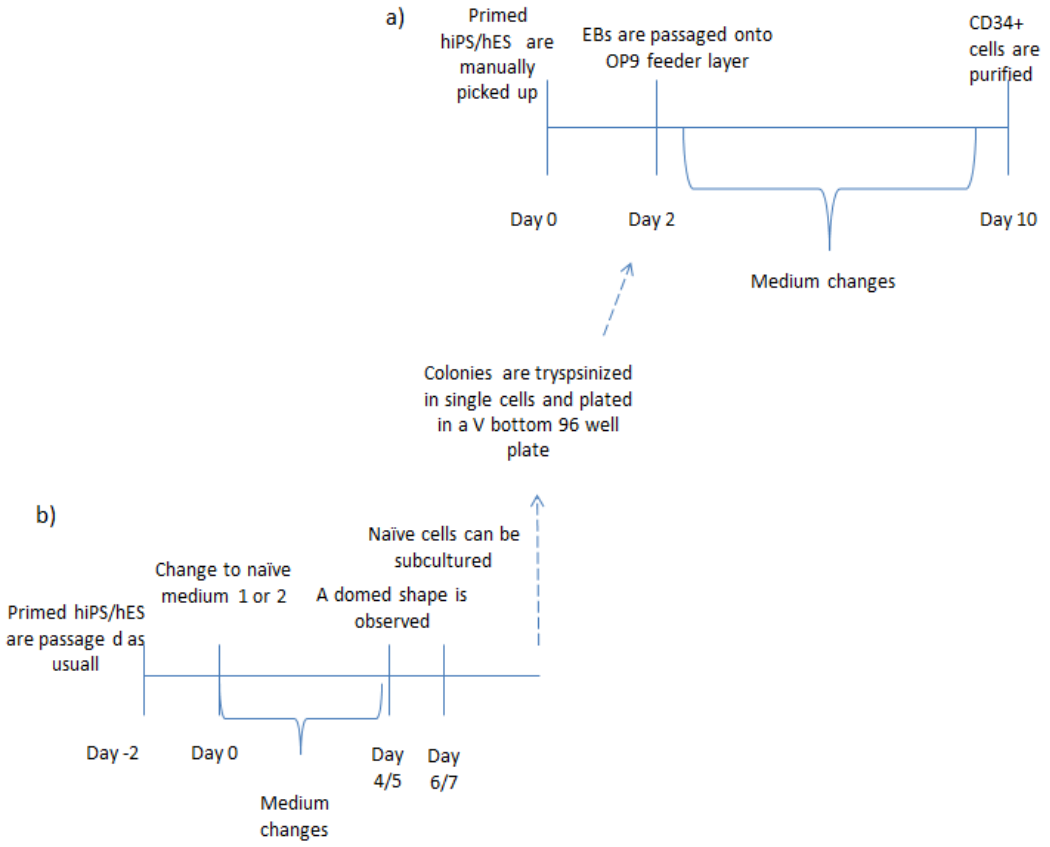

Figure 26: Protocol of differentiation of CD34+ cells from hiPS/hES. a) Obtaining of CD34+ cells from primed hiPS/hES. Cell are manually picked, and culture to form EBs for 48 hours. These EBS are picked and passaged onto OP9 feeder layer up to 10 days, when CD34+ can be purified. b) Primed hiPS/hES are converted to naïve hiPS/hES. Two days prior conversion primed hiPS/hES are passaged as usual. At day 0, medium is change into naïve medium 1 or 2. After 4/5 days, a domed morphology can be appreciated in the cells; and after 6/7 days, cells can be passaged. In order to obtain CD34+ cells from naïve hiPS/hES, cells are trypsinized and plate in 96 well plate to form EBs. These EBS are picked and passaged onto OP9 feeder layer up to 10 days, when CD34+ can be purified.

Some of the hiPS (KiPS 4F-1 and KiPS 4F-8) and hES (ES-2) cell lines were adapted to a feeder free condition with the intention of increasing the efficiency of differentiation towards CD34+ cells. Firstly, cells were passaged on top of 1/6 of the feeder cells and Matrigel (Corning) with KO DMEM based medium. On the second day, medium was changed to half KO DMEM based medium, half mTeSR 1 medium (StemCell). When cells were grown, they were passaged with EDTA 0,5 mM (Gibco) and seeded onto matrigel with full mTers1 medium. After 1 or 2 passages, medium was gradually changed to Essential 8 (Gibco). On the next passaged, cells were plated onto of vitronectine (Thermofisher) and cultured with E8 medium.

The protocol used for obtaining CD34+ cells was the same applied for hiPS and hES onto feeder layer cells (**Figure 26A**). Besides, an alternative method was used in order to obtain EBs from these feeder free adapted hiPS and hES. These cells were disaggregated into single cells with 0.5mM of EDTA (Thermofisher). Single cells were counted and adjusted to a 50,000 cells per milliliter concentration in DMEM + 10% FBS medium. Then, in a 96 conical well plate, the whole sample was plate adding 5,000 cells per well. Plate or plates were centrifuged at 2000 rpm for 5 minutes. Cells were incubated for 48 hours in order to form the EBs. After this time, EBs were placed onto overgrown OP9 feeder cells on 6 well plate for 8 more days with the medium described above.

Naïve hiPS and hES

HFF1 feeder dependent primed hiPS (KiPS 4F-1, KiPS 4F-8 and CBiPS 30 4F-3) and hES (ES-2) were transformed into naïve pluripotent stem cells using two different medium. Medium #1 consists of Knock out DMEM (Gibco), 20% Knockout Serum Replacement (KSR) (Gibco), MEM NEAA 1x (Gibco), 50 µM 2-Mercaptoethanol (Gibco), 0,5% P/S (Gibco), 1% Glutamax (Gibco), 12ng/ml bFGF (Miltenyi Biotec), 1000U/ml hLIF (Miltenyi Biotec), 1 µM PD0325901(Cayman Chemical Company), 3µM CHIR99021 (Axon Medchem), 10µM Forskolin (PKAag) (Sigma) and 50 ng/ml ascorbic acid (Sigma) (198). Medium #2 consists of Knock out DMEM (Gibco), 20% Knockout Serum Replacement (KSR) (Gibco), MEM NEAA 1x (Gibco), 50 µM 2-Mercaptoethanol (Gibco), 0,5% P/S (Gibco), 1% Glutamax (Gibco), 8ng/ml bFGF (Miltenyi Biotec), 1000U/ml hLIF (Miltenyi Biotec), 1 µM PD0325901(Cayman Chemical Company), 3µM CHIR99021 (Axon Medchem), 10mg/ml Albumax (Invitrogen), 10 µl/ml N2 (Invitrogen), 1ng/ml TGFβ (Miltenyi Biotec), 10µM SP600125 (Tocris) and 10µM SB203580 (Tocris) (199). At day -2, primed hiPS and hES were passaged as usual, over HFF1 feeder layer. At day 0 medium was change to medium #1 or medium #2. The following days, medium was changed every day. At day 4/5 a more domed shaped colonies were observed. At day 6/7 of culture, cells were subcultured using 0.25% Trypine (Thermofisher) and passage onto a HFF1 feeder layer (**Figure 26B**). In order to guarantee the conversion of the cells to a naïve stage, flow cytometry analysis was performed to check primed and naïve expression markers: anti-CD75-FITC (clone LN1, BD Bioscience), anti-CD24-PE (clone ML4, Biolegend), anti-CD57-APC (clone NK-1, BD Bioscience) and anti-CD130-BV421 (clone AM64, BD Bioscience). Other markers were used in order to make a clearer analysis of the populations: LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen),

anti-TRA-1-81- PerCP-Cy5-5 (clone TRA-1-81, BD Bioscience) and anti-CD90- PE-Cy7 (clone 5E10, BD Bioscience) (**Table 6**).

Once primed hiPS and hES were transformed into naïve cells, these colonies were tryspsinized in single cells in a concentration of 50.000 cell per ml in DMEM + 10% FBS medium. 100 µl of cell solution was plated per well in a V bottom 96 well plate, and was centrifuged at 1500 rpm 4'. After 48 hours, EBs were picked up with a pasteur pippete and were placed onto top of overgrown OP9 feeder cells on 6 well plate for 8 more days with the following medium: StemSpan Serum free (Miltenyi Biotec), 10 ng/ml BMP4 (Miltenyi Biotec), 10ng/ml VEGF (Miltenyi Biotec), 25 ng/ml SCF (Miltenyi Biotec), 10 ng/ml FGF (Miltenyi Biotec), 20 ng/ml TPO (Miltenyi Biotec) and 10 ng/ml Flt 3 Ligand (Miltenyi Biotec). At day 10, cells were disgregated with TrypLE Express for 10 minutes. Then, cells were analyzed byflow cytometry and CD34+ resulting cells were isolated by MACS sorting, using the CD34 MicroBead kit from Miltenyi Biotec. CD34+ resulting cells were frozen in FBS + 10% DMSO (**Figure 26B**). Colony Forming Unit (CFU) assays was performed for every CD34+ batch (**Figure 27**).

Table 6: Flow cytometry panel for determining the transformation of primed hiPS and hES into naïve pluripotent stem cells.

NAÏVE cells panel							
488 nm laser				635 nm laser		405 nm laser	
FITC	PE	PerCP-Cy5-5	PE-Cy7	APC	APC-Cy7-A	BV421	BV510
CD75	CD24	TRA-1-81	CD90	CD57	Viability	CD130	

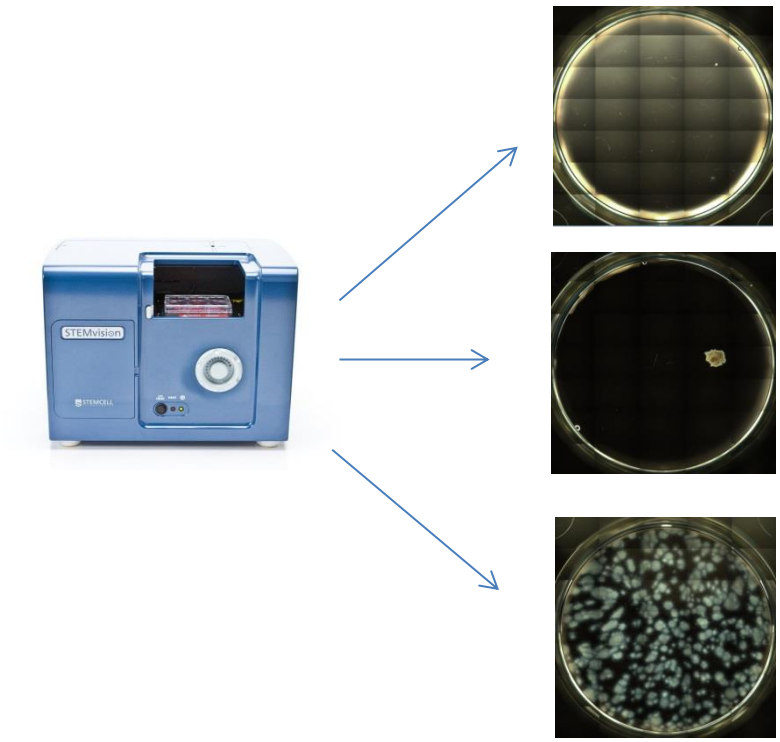

Figure 27: Analysis by stemVision of CFU assay after 14 days in culture. The picture above corresponds to a negative result; the pictures below and in the middle correspond to positive results.

<u>NK cells differentiation from CD34+ cells derived from hiPS and hES.</u>

CD34+ cells were thawed and plated onto mytomicin inactivated OP9 cells in a 6 well plate. 10,000 CD34+ cells were plated per well. Same culture medium as describe above in the *Hematopoietic Stem Cell (HSC) differentiation protocol into NK Cells* section was used in order to culture and differentiate these cells. Cells were cultured for 28 days; from day 14 to day 28 of the differentiation immunophenotyping flow cytometry analysis were performed, and at day 21 degranulation assay was performed in order to check their functionality (**Figure 28**).

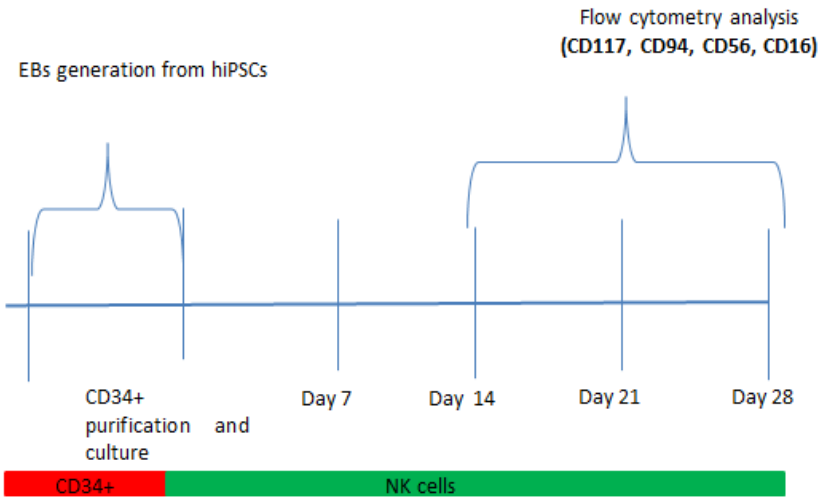

Figure 28: Protocol and time course of the in vitro NK cell differentiation from hiPS and hES cells. This is a two-step protocol in which, firstly hiPS and hES are differentiated towards CD34+ cells; and then, obtained CD34+ cells are differentiated towards NK cells.

Data analysis

Differences between groups were evaluated using paired Student's t-test. p-Values <0.05 were considered significant. Statistical calculations were done using GraphPad Prism 6 (GraphPad Software, Inc.) Bars represent the mean and error bars represent the SEM.

RESULTS

OBJECTIVE 1

RESULTS

Isolation and culture of Adult Peripheral Blood NK cells and Umbilical Cord Blood NK cells

NK cells were isolated from AB or CB PBMCs by negative selection after magnetic cell isolation (**Figure 29**). Percentage of the purity of the CD56+ NK cell population was 92.68±2.90 in AB and 91.46±5.14 in CB. CD3+ cells still present in the culture were less than 0.5%. After that, we tried different culture conditions in order to determine the best one for culturing the primary NK cells from AB and CB. NK cells were cultured with the cultured described in Materials and Methods, and different cocktails of cytokines (IL-2, IL-2+IL-15, IL15+IL-7). We obtained a higher percentage of viability with the combination of IL2 and IL-15 (**Figure 30**). Once we determined the best culture condition, the NK cells were cultured with IL-2 and IL-15 for 8 days, followed by transduction with CD19 CAR, as it has been described in the second objective. NKp46 levels during the culture increase significantly from day 0 to day 7 in both cell sources. In fact, CB NK cells were significantly more stimulated than AB NK cells (**Figure 31**). After that, the transduced and non-transduced NK cells from both sources were cultured one more week before performing the functional assays (**Figure 32**). We obtained more cells from the isolation from CB than from AB (**Figure 33**).

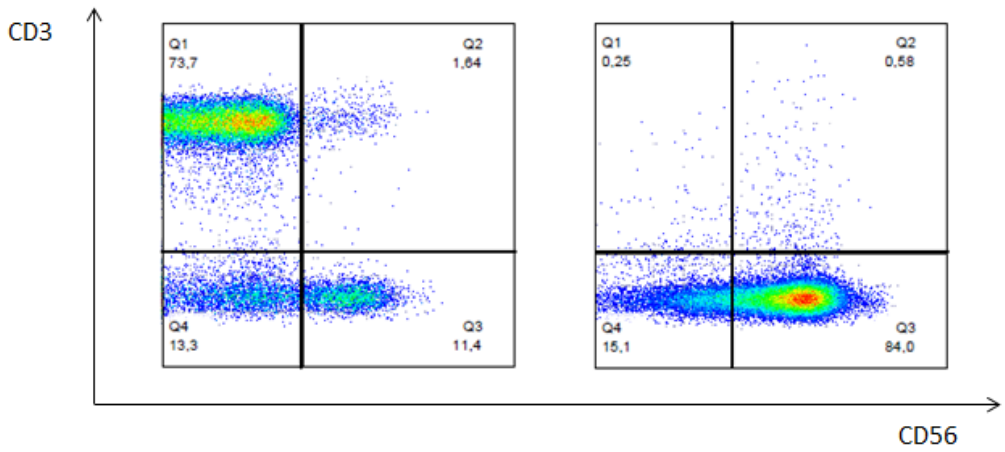

Figure 29: Remaining percentage of CD56+ cells after purification of CD34+ cells from AB or CB.

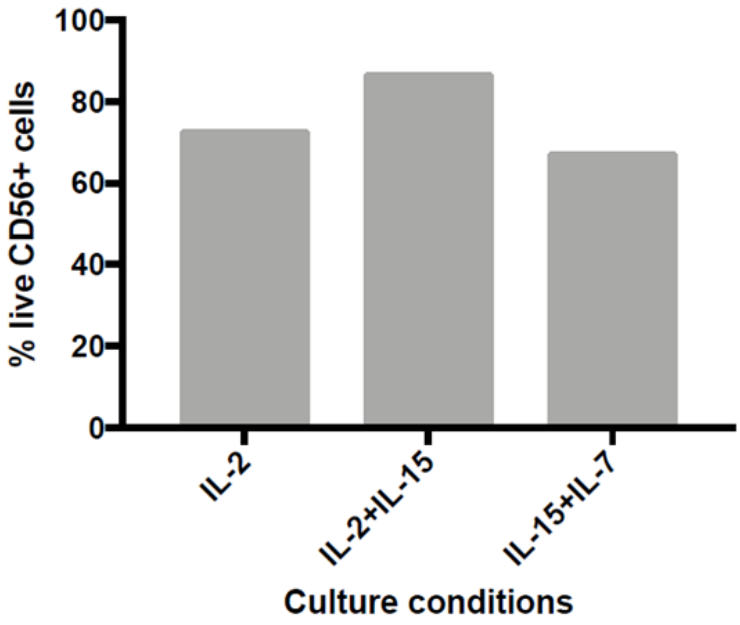

Figure 30: Percentage of the viability of primary NK cells when culturing them with different cytokine cocktails: IL-2, IL-2 + IL-15 and IL-15 + IL-7.

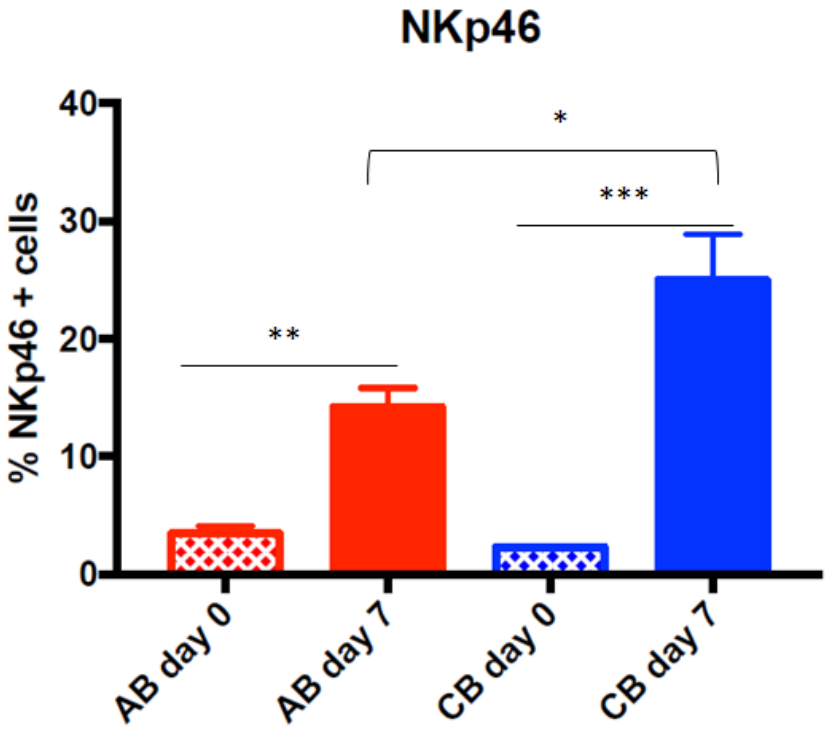

Figure 31: Percentage of NKp46 positive cells from AB and CB at different time points during the culture. Day 0 AB (n=4), CB (n=4); day 7 AB (n=4), CB (n=4). The symbols represent the mean and error bars represent SEM. Student's t-test was used to analyze the data. p-Value: *p < 0.05, **p < 0.005, ***p < 0.001, **** p<0.0001.

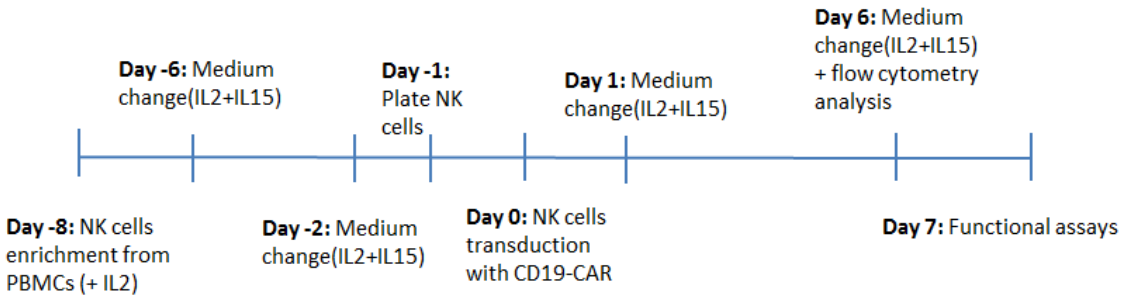

Figure 32: Time course of the followed protocol from the isolation of the NK from PBMCs of AB and CB until performing of the functional assays, degranulation or cytotoxicity assay.

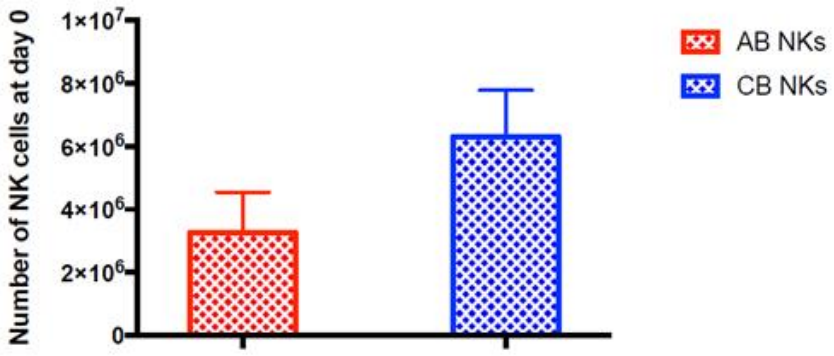

Figure 33: NK cell number and fold expansion in vitro. Total number of NK cells obtained from the AB and CB PBMCs isolation starting from 150x106 PBMCs. The bars represent the mean and error bars represent SEM.

Hematopoietic Stem Cell (HSC) differentiation protocol into NK Cells

Successful extraction of CD34+ HSC from Umbilical Cord Blood

PBMCs were separated from the rest of the CB by density gradient. Percentage of CD34+ cells presented in the PBMCs was checked. Then CD34+ cells were separated by MACS sorting doing only one round by using one column. Purity of the sample was not acceptable at this point, so that a second round was performed in order to increase the purity of the sample (**Figure 34**). For reaching an acceptable (>90%) of CD34+ cells for the CB sample, two columns were used in each experiment.

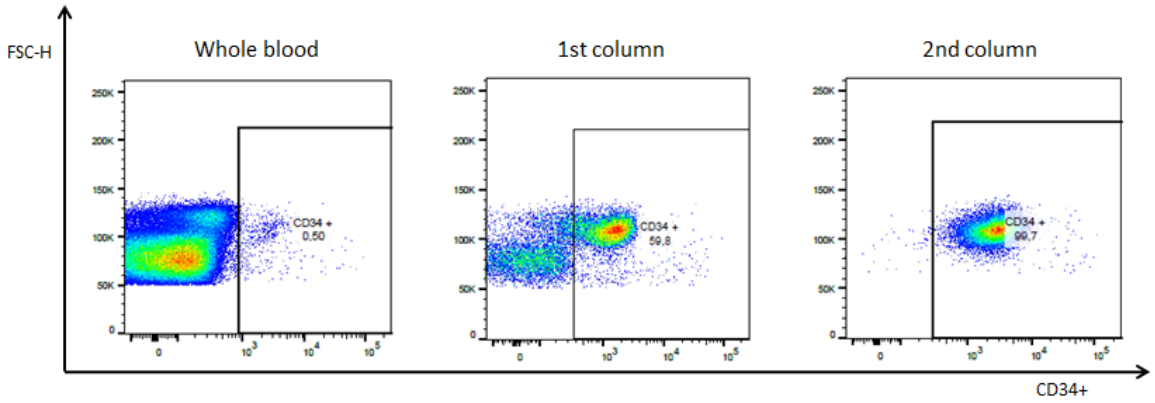

Figure 34: Purity of CD34+ cell population from umbilical cord blood (UCB) obtained by immunomagnetic isolation after passing the cells for one column and two columns.

OP9 cell-based coculture system generates higher numbers of CD56+ NK cells than M2-10B4 cell-based coculture system

CD34+ CB cells were cultured up to 42 days using the protocol described in Materials and Methods section. Cell number and percentage of CD56+ NK cells were checked weekly. CD34+ CB co-cultured with OP9 cells feeder layer exhibited a better proliferative capacity as compared with CD34+ CB co-cultured with M2-10B4 cells feeder layer at day 21. In addition, we found a higher number of CD56+ NK cells with OP9 cells co-culture at 28 days of differentiation ($p<0.05$), reaching $2x10^7$ NK cells on average, while this number dropped in the next days. Meanwhile, the number of CD56+ cells in the M2-10B4 cells co-culture condition increased gradually until 35 days of differentiation, reaching $1x10^7$ NK cells, and dropped slightly at 42 days of differentiation (**Figure 35A**). Similarly, we observed a higher frequency of CD56+ cells in the OP9 cells co-culture than in the M2-10B4 cells co-culture, with significant differences at day 21 ($p<0.001$) and day 28 ($p<0.01$) (**Figure 35B**). Likewise, we observed a higher fold expansion of CD56+ cells in the OP9 cells co-culture condition, especially at day 28 of differentiation ($p<0.05$) which correlates with the pattern followed by the number of CD56+ cells obtained (**Figure 35C**). Also we perform a feeder free culture condition. Our results showed that a very small number of NK cells were obtained in this culture condition (**Figure 36**).We believe that the presence of the feeder layer is crucial for the correct differentiation of NK cells from CD34 + cells.

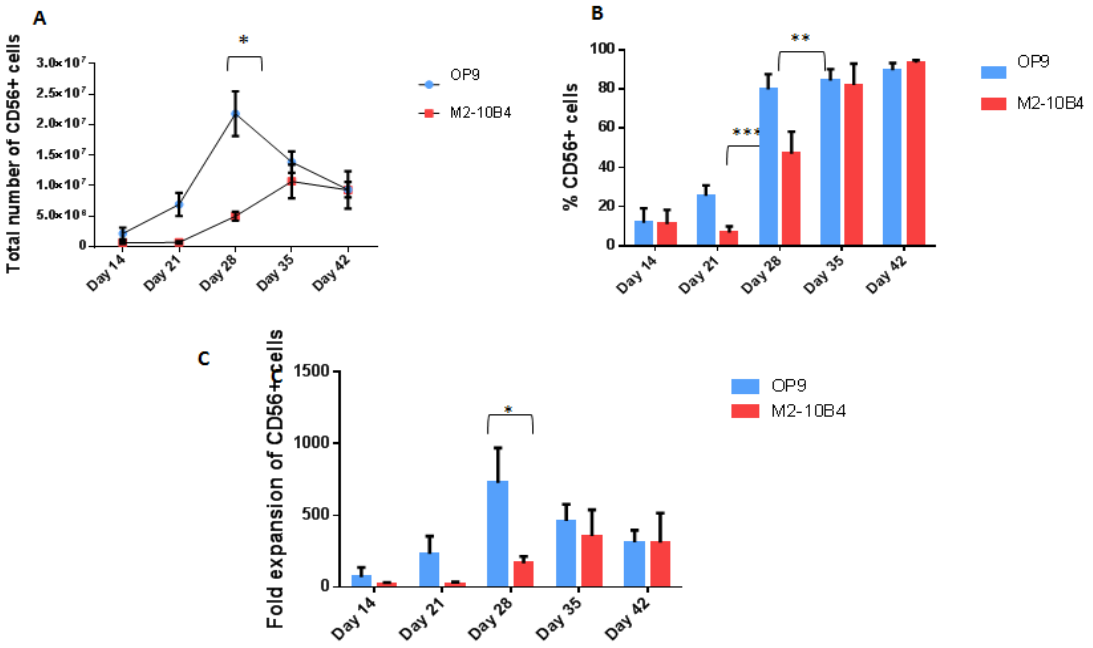

Figure 35: Number of CD56+ NK cells and fold expansion. a) Total number of CD56+ cells at different time points during the in vitro differentiation protocol with the two different culture conditions: OP9 and M2-10B4 cells co-culture. b) Percentage of CD56+ cells at different time points of the differentiation protocol with the two different culture conditions: OP9 and M2-10B4 cells co-culture. c) Fold expansion of the CD56+ cells regarding the initial number of CD34+ cells plated in each experiment. (n=4). The bars represent the mean and error bars represent SEM. P-value: * <p 0.05, **p <0.005, *** p <0.001.

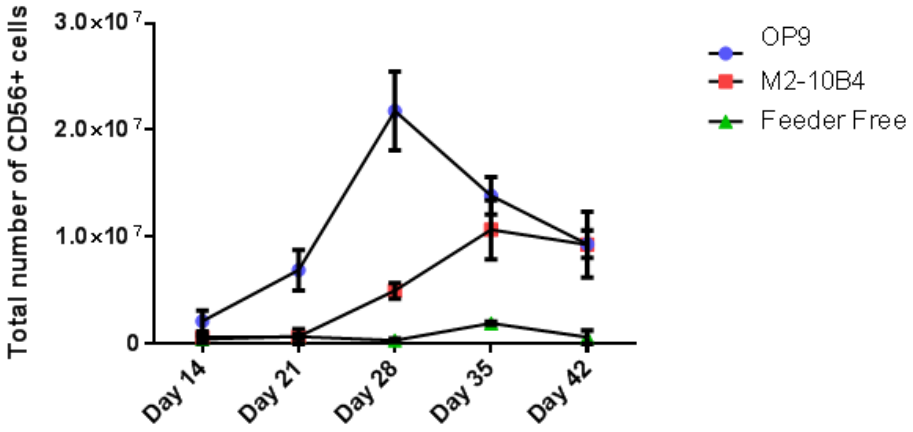

Figure 36: Total number of CD56+ cells at different time points during the in vitro differentiation protocol with three different culture conditions: OP9 and M2-10B4 cells coculture (N:4) and feeder-free (N:2).

The *in vitro* differentiation pattern of NK cells resembles the *in vivo* differentiation pattern

According to the differentiation pattern from CD34+ CB to mature CD56+CD3- NK cells *in vivo* (**Figure 23** and **Figure 37**), key markers were selected in order to determine the *in vitro* differentiation pattern obtained under our differentiation protocols (200). Depending on the presence or absence of different markers, NK cells were classified in different stages (*Stage <3*: CD56-, CD94-, $CD117^{low}$; *Stage 3*: CD56+, CD94-, CD16-, $CD117^{high}$; *Stage 4:* CD56+, CD94+, CD16-, $CD117^{low}$ and *Stage 5*: CD56+, CD94+, CD16+, $CD117^{low}$), which were determined according to the expression of the NK cell markers CD56 and CD94. The correct analysis of the stages was assured by the expression of the CD117 marker on NK cells. CD56+/CD94+ population was divided in stage 4 and 5 based on the presence or absence of the CD16 marker. In this manuscript, stages 4 and 5 were represented as stages 4-5 in order to include mature NK cells in a single population.

The percentage of more mature NK cells increased over time during the *in vitro* differentiation protocol (**Figure 38A**), with higher percentage of more mature stages in culture conditions in the presence of OP9 cells in comparison with cultures in the presence of M2-10B4 cells. On one hand, these more mature stages are reached earlier by the cells co-cultured with the OP9 cell line than with the M2-10B4 cell line and the percentage of cells in the stage 4-5 is maintained over time with the OP9 cell line, while it gets higher with the M2-10B4 (**Figure 38B**). On the other hand, the total number of cells at stages 4-5 of maturation equalizes between the two culture conditions at the end of the differentiation protocol, having more cells at day 28 with the culture condition using OP9 cells (**Figure 38C**).

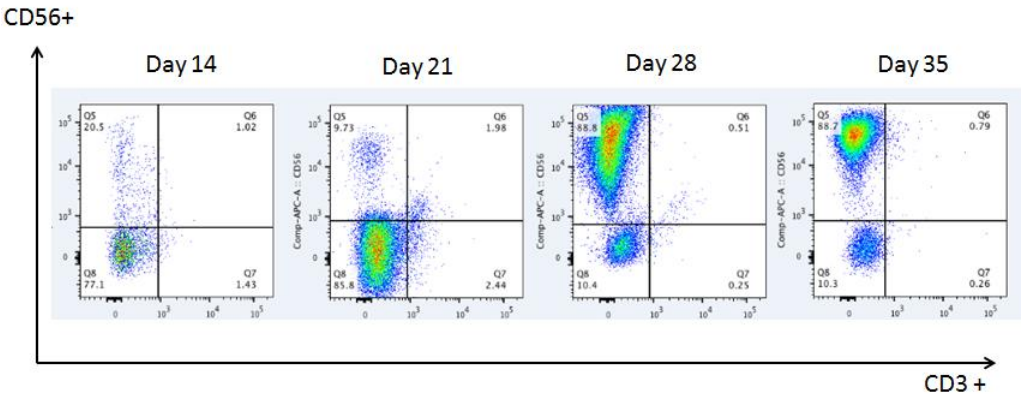

Figure 37: Dot-plots of the expression of CD3 at different stages of natural killer cells during the differentiation protocol. During in vitro NK cells differentiation residual CD3+ cells are checked in order to assure there are no other lymphocytes in our culture but NK cells.

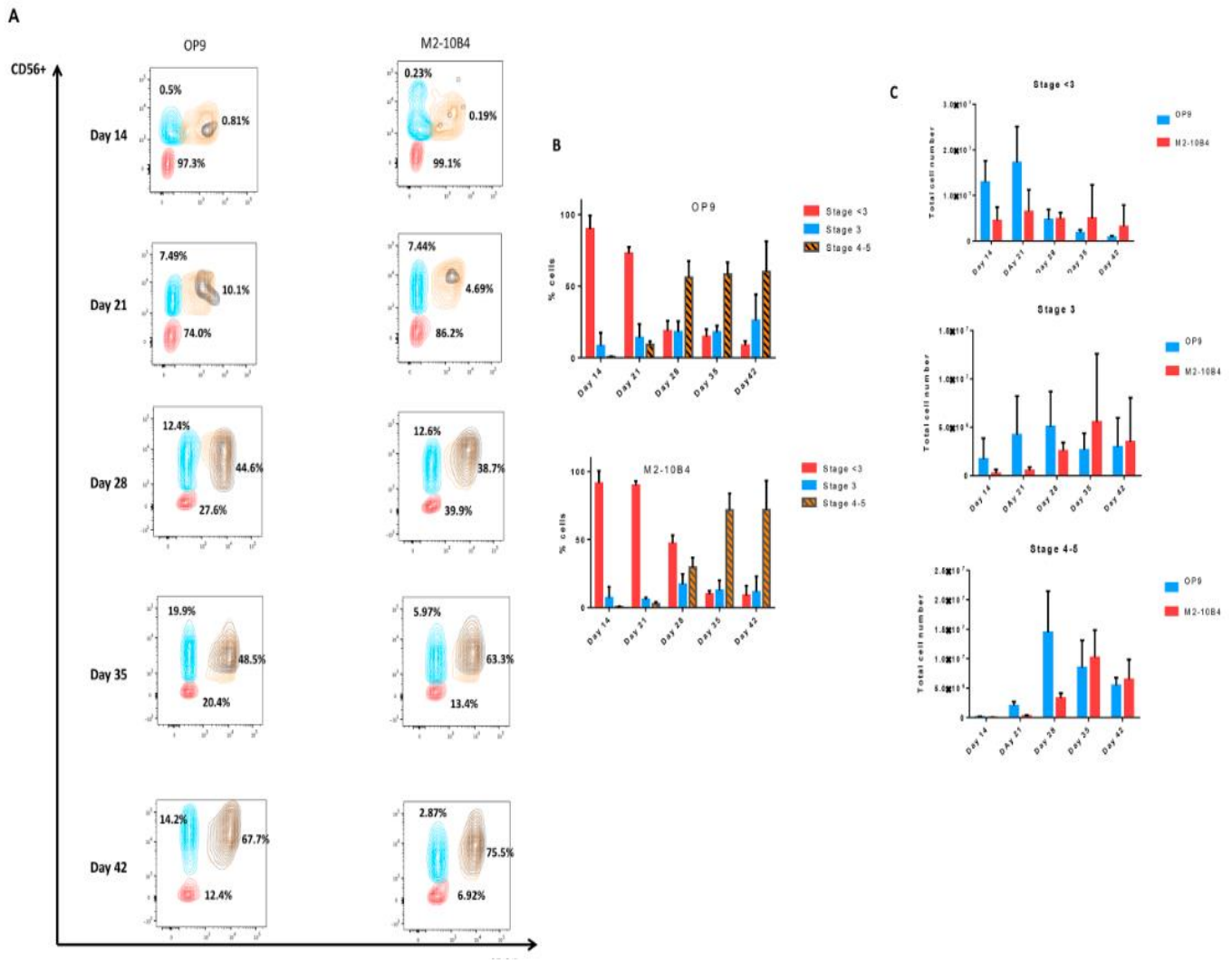

Figure 38: Flow cytometry analysis of the maturation stages of NK cells in vitro. a) Contour plots representing the different NK cell maturation stages during the differentiation protocol using OP9 and M2-10B4 cells as feeder layers. CD94, CD56 and CD16 markers were analyzed to determine the maturation stage as described in the text. Numbers represent the percentage of cells in stage <3 (red), stage 3 (turquoise) and stages 4-5 (orange and black). b) Percentage of NK cells in the different stages of maturation (<3, 3 and 4-5) along the differentiation time course. c) Total number of cells in the different stages (<3, 3, 4-5) cultured with the two conditions: OP9 and M2-10B4 cells co-culture. The bars represent the mean and error bars represent SEM. (Stage <3: CD56-, CD94-, CD117low; Stage 3: CD56+, CD94-, CD16-, CD117high; Stage 4: CD56+, CD94+, CD16-, CD117low and Stage 5: CD56+, CD94+, CD16+, CD117low)

In vitro generated NK cells exhibit cytotoxic activity

Mature NK cells (stages 4 and 5) express cytolytic granules containing perforin and granzyme (201). We determined the expression of perforin and granzyme B in developing NK cells at day 28, 35 and 42 of the differentiation protocol. No significant differences in the expression of these two cytolytic markers were found between cells cultured with the OP9 cells layer in comparison with cells cultured with the M2-10B4 feeder cells. In both culture conditions the percentage and the intensity of perforin expression in positive cells was higher than the expression of granzyme-B (**Figure 39A and 39B**). Moreover, the frequency of cells expressing perforin at days 28, 35 and 42 with OP9 cells feeder layer was very similar to the percentage of CD56+ NK cells, as well as to the condition with M2-10B4 cells feeder layer at days 35 and 42. We also

found that *in vitro* differentiated NK cells did not show a significant difference in the expression of perforin and granzyme B between stage 3 and stages 4-5 in both culture conditions (data not shown).

Next, we determined the degranulation capacity of the *in vitro* generated NK cells. To do that, we measured the expression of CD107a on NK cells after being stimulated with K562 target cells, according to the protocol described in Material and Methods. Activated NK cells from peripheral blood of healthy adult donors were used as a control. At days 28, 35 and 42, the degranulation tended to be higher in NK cells generated with M2-10B4 feeder cells than in the NK cells generated in the presence of OP9 feeder cells, although there were no significant differences (Figure **40A**). We also analyzed the degranulation in the stage 3 and stages 4-5 of the *in vitro* generated NK cells. The degranulation in both culture conditions exhibited a very significant difference between stage 3 and stages 4-5 (**Figure 40B**). Finally, we tested the cytolytic activity of NK cells against the K562 target cells. NK cells purified and activated from adult peripheral blood were used as a control. At day 28, NK cells differentiated in the presence of OP9 cells were more cytotoxic than NK cells differentiated in the presence of M2-10B4 cells (**Figure 40C**). At days 35 and 42, the cytotoxic activity was similar in both culture conditions at several effector:target ratios (**Figure 40C**). However, at day 42, at ratio 25:1, NK cells differentiated in the presence of M2-10B4 cells were significantly more cytotoxic than the NK cells differentiated in the presence of OP9 cells.

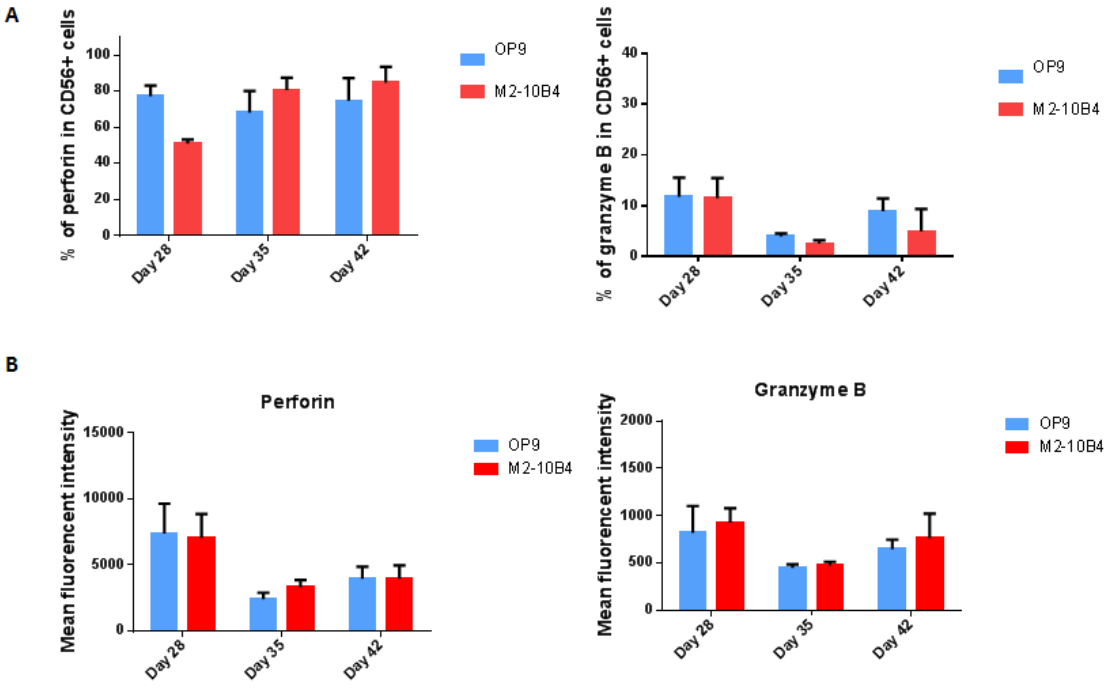

Figure 39: Measurement of perforin and granzyme B in in vitro generated NK cells. a) Percentage of CD56+ NK cells expressing perforin and granzyme B cultured with OP9 and M2-10B4 feeder cells at day 28, 35 and 42 of the differentiation protocol, b) Mean fluorescence intensity of perforin and granzyme B of CD56+ NK cells generated in the presence of OP9 and M2-10B4 feeder cells at day 28, 35 and 42 of differentiation. P-value: * <p 0.05, **p <0.005, *** p <0.001.

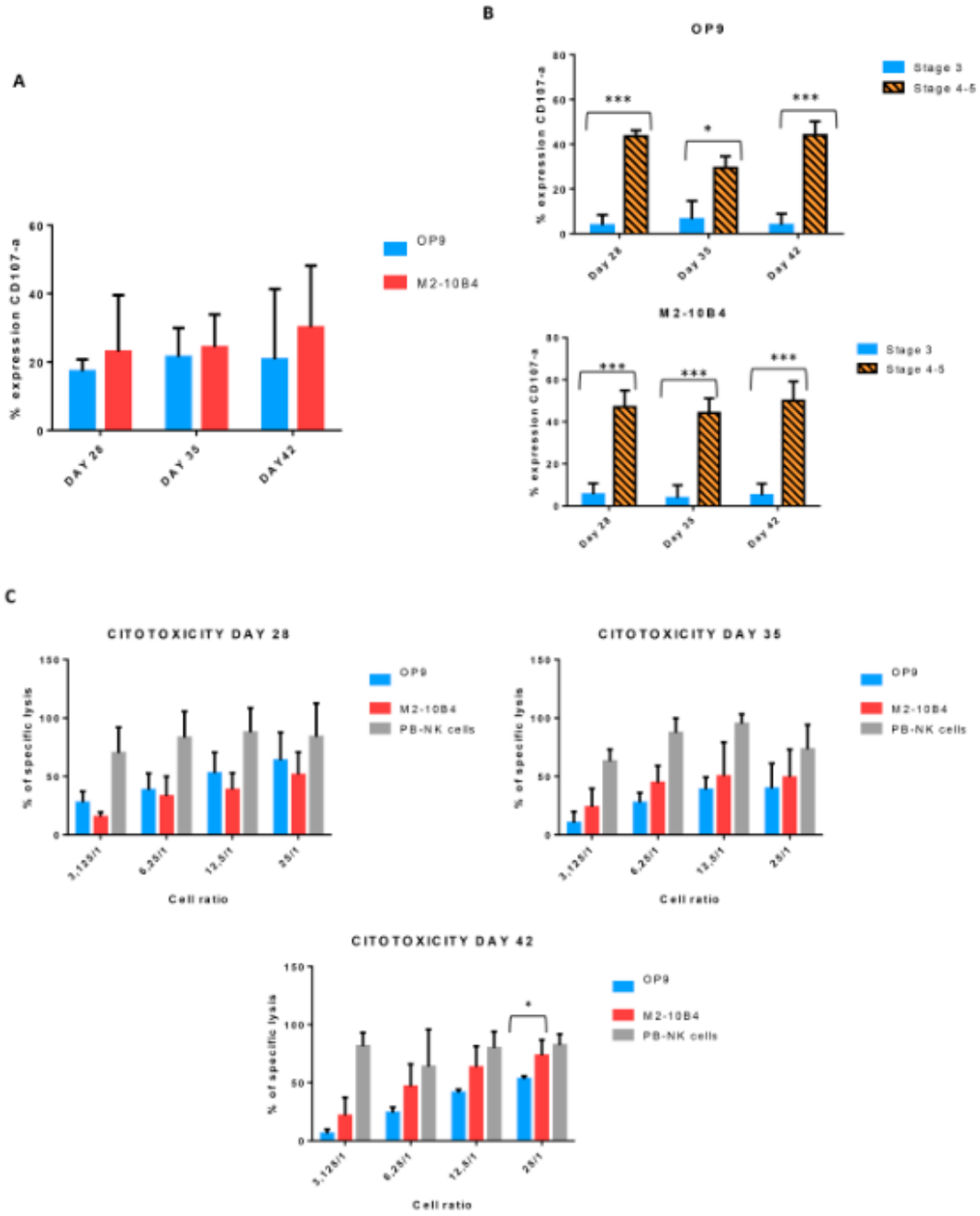

Figure 40: Functional assays of in vitro generated NK cells. a) Expression of CD107a, a marker of degranulation, in the in vitro generated NK cells (CD56+) at different time points of the differentiation protocol in response to the stimulation with K562 target cells, b) Expression of CD107a in both conditions (with OP9 and M2-10B4 feeder cell layers) at stage 3 and stages 4-5 at different times of the differentiation protocol, c) Cytotoxicity activity of NK cells against K562 target cells at day 28, 35 and 42 of the differentiation protocol. Overnight cultured NK cells obtained from adult peripheral blood stimulated with the same cytokines as our in vitro generated NK cells were used as a control. The bars represent the mean and error bars represent SEM. P-value: * <p 0.05. The absence of any asterisk indication means there are not significant differences. (Stage <3: CD56-, CD94-, CD117low; Stage 3: CD56+, CD94-, CD16-, CD117high; Stage 4: CD56+, CD94+, CD16-, CD117low and Stage 5: CD56+, CD94+, CD16+, CD117low)

NK cells differentiation from human induced pluripotent stem cells (hiPS) and human embryonic stem cells (hES)

CD34+ Hematopoietic Stem Cell obtaining from hiPS and hES

- Primed

A total of 44 experiments were performed in order to obtain CD34+ hematopoietic stem cells from hiPS and hES cultured onto inactivated HFF1 feeder layer. From that total number, a total of 31 experiments (70.46%) resulted in a positive population for CD34+ marker. When performing CFU assay, 51.61% of the CD34+ experiments developed colonies, meaning the obtained CD34+ cells are functional (**Figure 41**). There are no significant differences between the percentage of CD34+ cells between the experiment that was negative for CFUs (4.53±0.7) and the ones that were positive for CFUs (6.71±0.8). The other alternative method to obtain CD34+ mentioned in Materials and Methods did not work with these cells.

hiPS and hES cultured onto inactivated HFF1 feeder layer were successfully adapted to feeder free condition. A total of 25 experiments were performed in order to obtain CD34+ hematopoietic stem cells. From that total number, a total of 20 experiments (80%) resulted in a positive population for CD34+ marker. When performing CFU assay, 50% of the CD34+ experiments developed colonies, meaning the obtained CD34+ cells are functional (**Figure 41**). There are no significant differences between the percentage of CD34+ cells between the experiment that was negative for CFUs (7.24±1.11) and the ones that were positive for CFUs (8.71±1.18). A total of 9 experiments where performed using the alternative method previously mentioned in Materials and Methods, with 96 conical well plates, and they failed at differentiated towards CD34+ cells.

When comparing the effect of culturing the hiPS and hES cells with or without a feeder layer, we observed that hiPS and hES cultured in feeder free condition differentiated towards CD34+ in a significantly higher percentage. When separating the obtained CD34+ in positive for CFU or negative for CFU,, there were not significant differences between percentages obtained from the ones that form CFU; however, feeder free hiPS and hES showed a significantly higher percentage of differentiation in CD34+ cells that did no form CFUs (**Figure 41**).

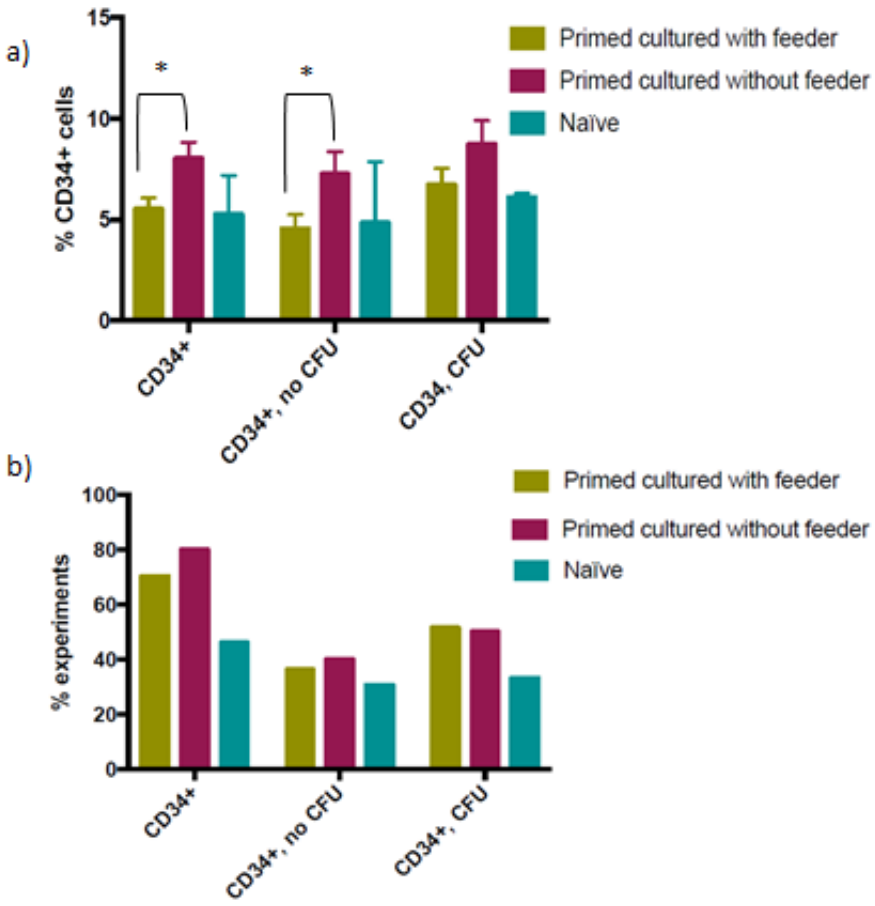

Figure 41: CD34+ in vitro differentiation from hiPS and/or hES. a) Percentage of CD34+ cells obtained from the differentiation of primed hiPS or hES cultured onto feeders, primed hiPS or hES onto feeder free culture and naïve hiPS or hES. b) Percentage of experiments of the differentiation of CD34+ from primed hiPS or hES cultured onto feeders, primed hiPS or hES onto feeder free culture and naïve hiPS or hES resulting in generally obtaining CD34+ cells, obtaining CD34, but negative CFU results and obtaining CD34+ cells with positive CFU result. The bars represent the mean and error bars represent SEM. P-value: * <p 0.05.

- Naïve

Primed hiPS and hES were successfully transformed into naïve cells with the medium 1 and the medium 2 (**Figure 42**). We obtained a domed morphology with both media. When we checked the characteristic markers of the naïve stage (CD75, CD130) and the primed stage (CD57, CD24) by using flow cytometry, we observed that in both culture conditions, the presence of naïve surface markers were increased and the presence of primed surface markers were decreased. Nevertheless, cells cultured with medium 1 were increased the expression of naïve markers at day 10; and cells culture with medium 2 were decreased quicker the expression of primed markers (**Figure 43**). Moreover, naïve cells cultured with medium 1 were maintained up to 40 days before they started to differentiate, while naïve cells cultured with medium 2 were maintained up to 95 days before losing their pluripotent properties.

Once we assured hiPS and hES were in a naïve stage, a total of 13 experiments were performed in order to obtain CD34+ cells. 6 experiments out of 13 presented a CD34+ population (46.16%). From these experiments, 33.33% of them were capable of creating CFUs (**Figure 41**). There are no significant differences between the percentage

of CD34+ cells between the experiment that ended not forming CFUs (4.82±3.33) and the ones that did (6.1±0.2).

When comparing the primed and naïve stages, we observed that there are not significant differences with any of the primed hiPS and hES culture conditions (**Figure 41**). The lower number of experiments performed with naïve hiPS and hES may lead to a non-conclusive situation. Due to this, new experiments need to be performed in the future.

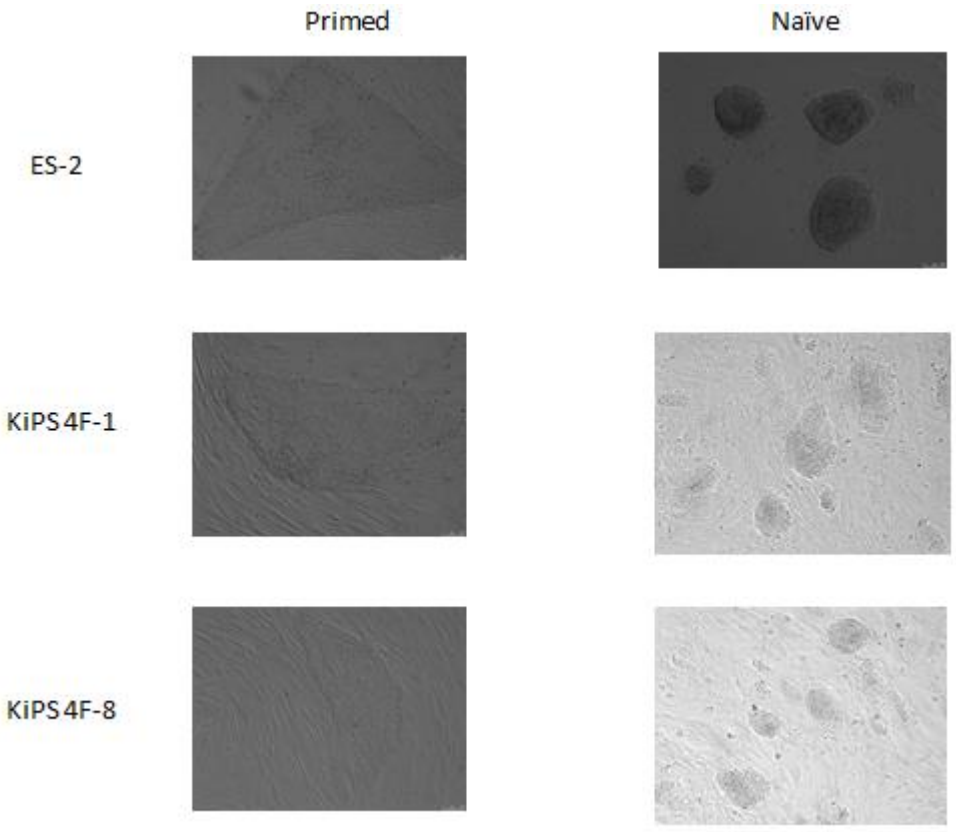

Figure 42: Morphology of primed cells vs naïve cells of different hES and hiPS cells lines cultured onto feeder layers.

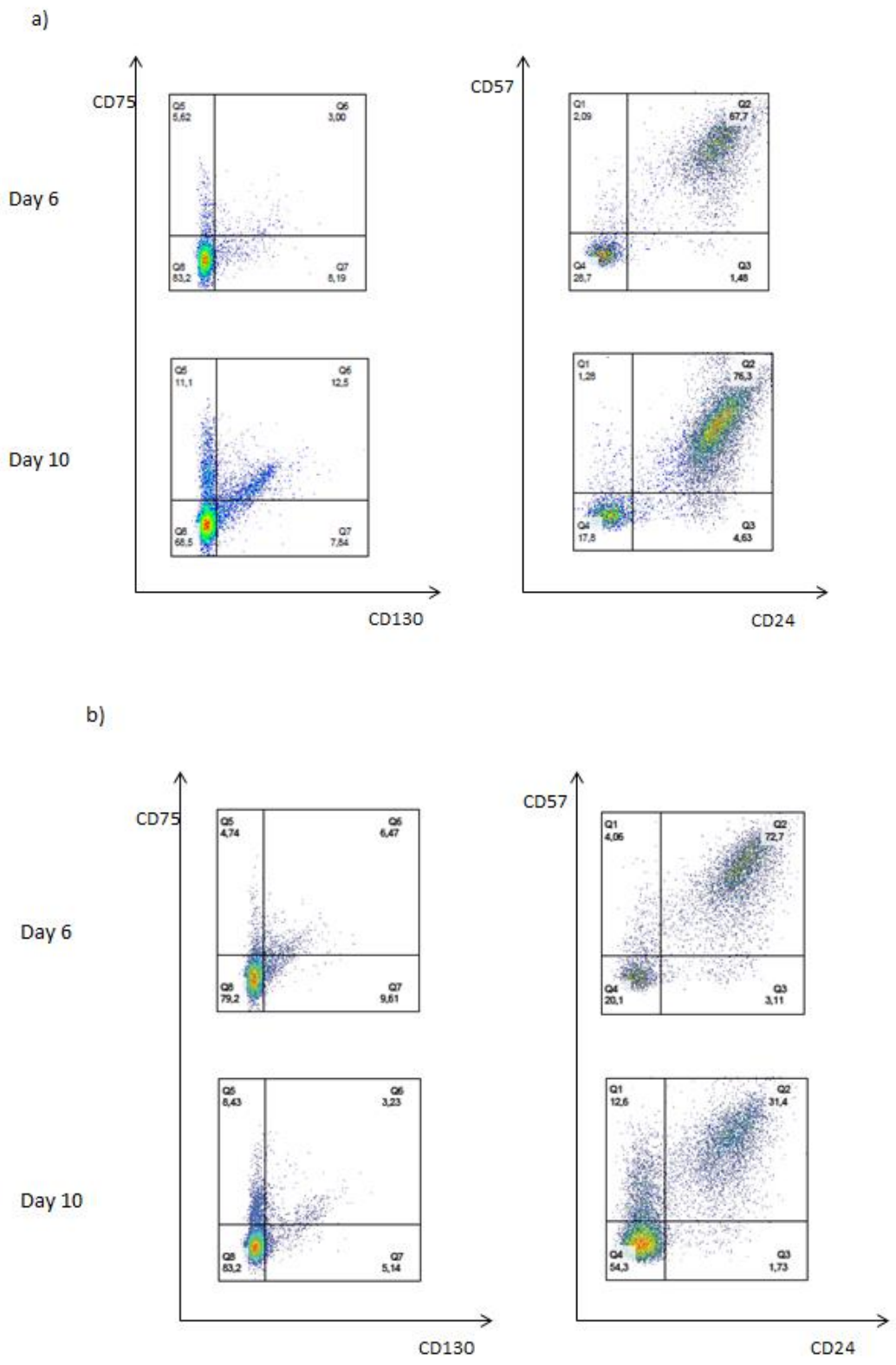

Figure 43: Flow cytometry measurement of naïve and primed surface markers. a) acquiring of naïve markers (CD75, CD130) and loss of primed markers (CD57, CD24) of hiPS or hES cells at day 6 and day 10 post-transformation with naïve medium 1. KiPS 4F-1 cell line was used for this experiment. b) Acquiring of naïve markers (CD75, CD130) and loss of primed markers (CD57, CD24) of hiPS or hES cells at day 6 and day 10 post- transformation with naïve medium 2. KiPS 4F-1 cell line was used for this experiment.

NK cells differentiation from CD34+ cells derived from hiPS and hES.

From a total number of 13 experiments, only 5 of them were able to be maintained in cultured and differentiated towards NK cells. Phenotypically, CD16+ marker was present quicker and in higher percentage than CD56+ (**Figure 44**); and other markers like CD94+ and CD117+ were not presented along 28 days in culture. Degranulation assay was performed in two of the experiments, but the number of NK cells was low and as well as the viability. We could not make any conclusion out of these assays. Further studies needs to be done in this section.

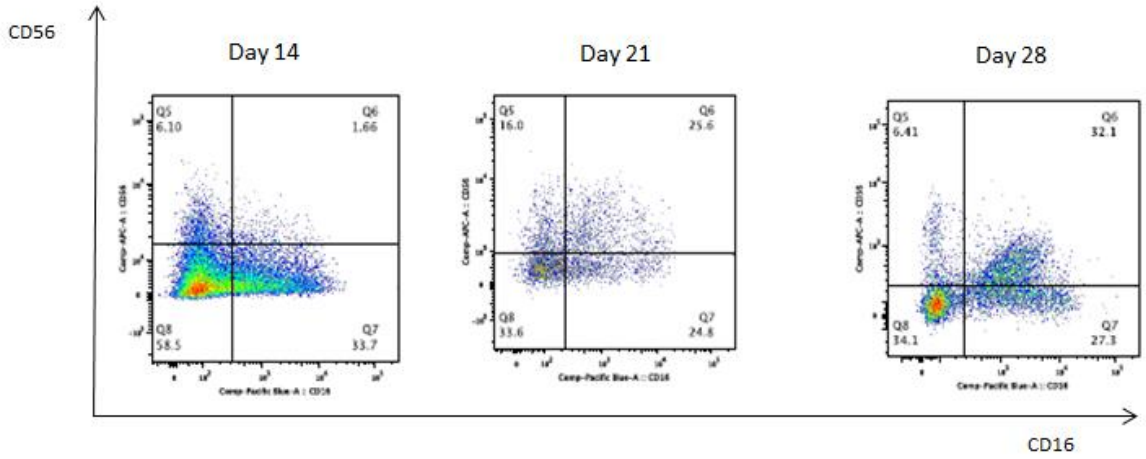

Figure 44: Flow cytometry analysis representing the CD56+ and CD16+ markers in NK cells differentiated from hiPS or hES at different time points of the differentiation protocol.

MATERIALS AND METHODS

OBJECTIVE 2

MATERIALS AND METHODS

Cell lines and patient samples

K562 cell line was purchased from the ATCC (CCL-243); HEK293T and Nalm 6 cell lines were kindly provided by Immunotherapy Department of the Hospital Clinic-IDIBAPS, Barcelona, thanks to our collaboration with Dr. Manel Juan; Acute Lymphoblastic Leukemia (ALL) cells (GM20390 and GM16726) were purchased from Coriell Company. K562, Nalm6 and ALL cells were cultured with RPMI, 10% FBS (Hyclone), 1% penicillin/streptomycin, 1% Glutamax, 1% NEAA, and 1% sodium pyruvate. HEK293T were cultured with DMEM+ 10% FBS + 1% P/S + 1% Glutamax.

Primary Chronic Lymphocytic Leukemia (CLL) cells from nine patients were used for *in vitro* studies of NK-CAR functionality, thank to our collaboration with Dr. Tomás Carrascosa from the Hematology Department of the Hospital of Galdakao. Samples were obtained with prior signed informed consent and ethical committee approval from the Basque Ethics Committee for Clinical Research [Comité Ético de Investigación Clinica de Euskadi-CEIC-E (PI2014138)]. Patient characteristics are summarized in Table 7.

Table 7: Characteristics of CLL patients. All of the patients present CD19 positive cells. 5 ml peripheral blood samples from the patients were processed in order to obtain PBMCs as target cells. Patients were not treated for CLL before the collection of the sample.

Patient	Sex	Age	Diagnose	Leukocytes/ ml	Lymphocytes /ml	CD5	CD10	CD19	CD23	FMC	CD38	Surface chain	Cytogenetic /FISH	Prior treatment
1	F	82	CLL-B	70.100	63.300	+	-	+	+	-	-	Kappa	13q	Untreated
2	M	66	CLL-B	57.940	52.800	+	-	+	+		-	Lambda		Untreated
3	F	69	CLL-B	40.390	34.700	+	-	+			-	Negative	13q	Untreated
4	M	73	CLL-B	9.930	3.400	+	-	+			-	Kappa		Untreated
5	M	61	CLL-B	18.740	15.500	+	-	+	+		-	Kappa	No mutation	Untreated
6	M	84	CLL-B	28.050	22.000	+	-	+	+	-	+	Kappa		Untreated

CD19-CAR lentivirus production

a) Plasmids

CD19 CAR plasmid, also known as ARI-0001 was provided by the Immunotherapy Department of the Hospital Clinic-IDIBAPS, Barcelona, thanks to our collaboration with Dr. Manel Juan. ScFv of anti-CD19 A3B1 antibody (an antibody against CD19) was cloned in frame with the rest of the CAR signaling domains (4-1BB and CD3z) in a lentiviral vector (pCCL) (**Figure 45**) (202). Transfer and envelope plasmids were also provided by the Immunotherapy Department of the Hospital Clinic-IDIBAPS, Barcelona, thanks to our collaboration with Dr. Manel Juan. Rev is a small regulatory protein of HIV-1 that is essential for virus replication; Rev binds to a highly structured RNA region, the Rev Response Element (RRE), where it forms an oligomeric ribonucleoprotein complex. VSV-G is the envelope plasmid used for lentivirus production.

Figure 45: Diagram of A3B1 CAR19 construct (ARI-0001). From Castella et al. 2019

b) Virus

HEK293T cell line (kindly provided by Immunotherapy Department of the Hospital Clinic-IDIBAPS, Barcelona) was used in order to pack CD19-CAR containing lentivirus. HEK293T cells were thawed and after 3-4 days they were harvested and plated $8*10^6$ in each p150 dish. 10-15 p150 dishes were plated for each batch of viral supernatant. The following day, HEK293T medium was change one hour prior transfection. Meanwhile, DNA mix was prepared. In a sterile tube, 35 µg of total DNA (17 µg vector trans, 8.53 µg RRE MOL, 4.25 µg REV, 5.2 µg VSV-G) were mixed with 1 ml of DMEM. DNA concentration was 1 µg/µl and this quantity was used for each p150. Hereafter, 102.5 ul of PEI (1 µg/µl) was added to the mixture and this was incubated at room temperature for 20 minutes. After that, 1 ml of the mixture was added to each p150, drop by drop; up to a final volume of 14-15 ml. Cells along with the mixture were incubated for 4 hours at 37C. Afterwards, medium was changed and cells were cultured for 48-72 hours. Next, viral supernatant was harvested and spined at 2000 rpm for 5 minutes. Supernatant was filtered with 45 µm filters (Millex-HV, SLHV033RB) and centrifuged in an ultracentrifuge (Optima L-90K Ultracentrifuge, Beckman Coulter) at 19600 rpm for 2 hours and 30 minutes. Once centrifuge program is done, supernatant was discarded and pellet was resuspended in medium without DMSO. Concentrated virus was stored in aliquots at -80C (**Figure 46**).

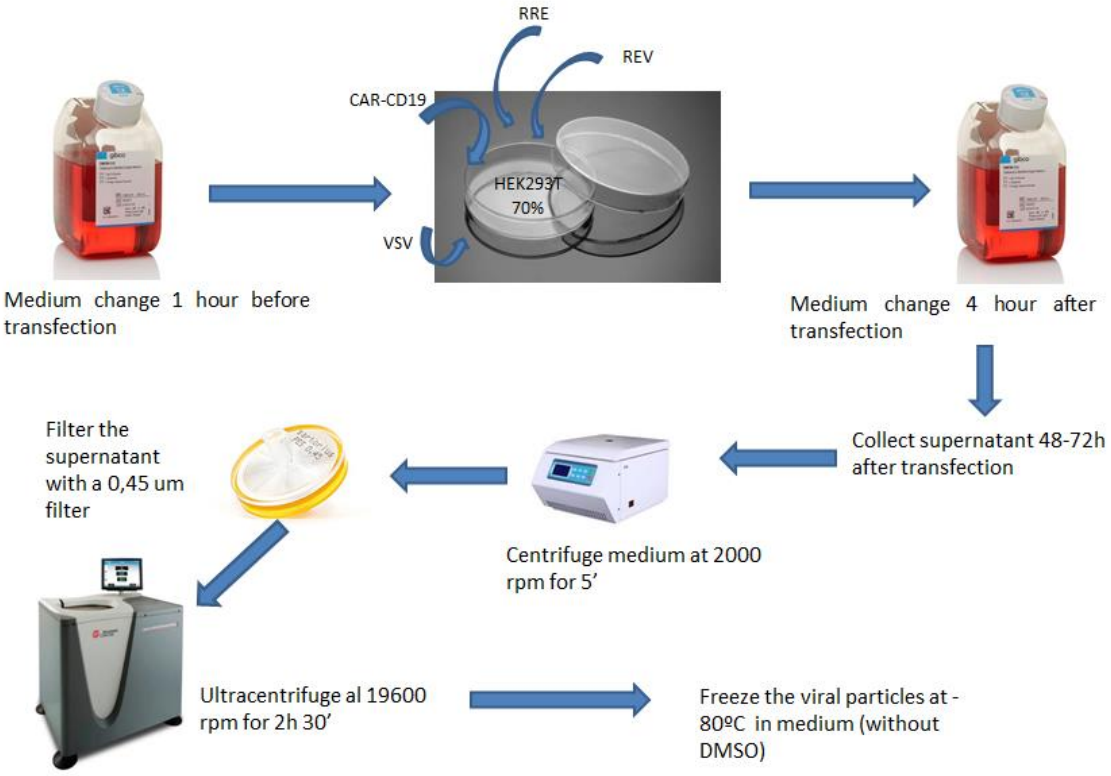

Figure 46: Diagram of lentiviral production and storage. Medium of HEK293T cells is changed 1 hour prior transfection. Plasmids are added to the cells and cells are incubated for four hours. After that, medium is changed again and cells are cultivated for 48-72 hours before collecting the supernatant. Once supernatant is collected, it is centrifuged and filtered. Then, the supernatant is ultracentrifuged and resulting viral particles are frozen in medium without DMSO at -80ºC

c) Viral titration

HEK293T cells were used in order to titrate the virus. 200,000 cells were plate per well in a 24 well plate. The next day, medium was changed adding 500 µl to each well. Cells were infected with serial dilutions of the virus (1 µl, 0.1 µl, 0.01 µl and 0.001 µl). Polybrene was added in order to help with cell infection (8 µg/ml). Cells were incubated overnight and the next day, first thing in the morning, medium was changed in order to remove polybrene. 2 days after the infection, cells were collected mechanically and placed in cytometer tubes. Cells were washed twice with PBS and stained with APC CAR Fab/Fab'2 antibody (Jackson Immunoresearch) and incubated for 15-20 minutes at room temperature. After this step, cells were washed twice again with PBS and checked by flow cytometry.

d) Infection protocol

<u>NK cells from adult healthy donors' blood and umbilical cord blood</u>

NK cells from $150*10^6$ adult healthy donors' blood and umbilical cord blood PBMCS were isolated with the NK Cell Isolation Kit from Miltenyi Biotec (catalog number 130-092-657). These cells were cultured with RPMI, 10% AB serum (Innovative Research, Inc), 1% penicillin/streptomycin and 1% Glutamax. The addition of several cytokines at different

combinations was tried for the optimization of the viability and infection of the NK cells. The combinations were the following ones: 500U/ml of IL-2; 500 U/ml of IL-2 and 10 ng/ml of IL-15; 500 U/ml of IL-2 and 20 ng/ml of IL-7; and 10 ng/ml of IL-15 and 20 ng/ml of IL-7. For the first two days, 500 U/ml of IL-2 were added to the culture medium. From this point on, 10 ng/ml of IL-15 was also added to the culture medium. On day +7, cells were harvested and counted in order to plate 400,000 cells per well in a 24 well plate. On day +8, NK cells were transduced with lentiviral vector containing supernatant in the presence of 6 µg/ml polybrene (sc-134220, Santa Cruz Biotechnology) or in the presence of 10 µg/ml of vectofusin-1 (130-111-163 , Miltenyi Biotec) and MOI 10. Cells were centrifuged at 2000 rpm for 1 hour at 37°C. At the same time, a mock transduction was performed by following the same steps without the addition of lentivirus supernatant. The next day, NK cells were washed to get rid of the polibrene or vectofusin-1. Viability was checked by trypan blue. CD19-CAR transduction efficiency was checked by flow cytometry following the upcoming protocol. Cells were harvested and washed with PBS+ 10% FBS twice. Then, cells were stained with Fab/Fab´2 antibody, which detects CD19-CAR presence on the surface membrane. Cells were incubated for 30 minutes at 4°C and darkness. Subsequently, cells were washed twice with PBS and stained with anti-CD56- PE/Cy7 antibody (clone MEM-188, BioLegend) and anti-CD3-PerCP/Cy5.5antibody (clone SK7, BioLegend). Cells were washed twice with PBS, resuspended in 400 µl of PBS and checked by flow cytometry (**Figure 32**).

In vitro generated NK cells from CB derived CD34+ cells

CB CD34+ cells were co-cultured with OP9 cell line feeder layer with the culture medium previously described in Materials and Methods of the 1st objective. At day 28 of the differentiation protocol, cells were harvested, counted and plated in a 24 well plate, 400,000 cells per well with the same medium used in the differentiation protocol. Same medium used for adult healthy donors' blood and umbilical cord blood NK cells was try prior infection. The following day, cells were transduced with vector containing supernatant in the presence of 6 µg/ml polybrene or 10 µg/ml of vectofusin-1 (130-111-163 , Miltenyi Biotec) and MOI 10 and 20, depending on the viral batch. Cells were centrifuged at 2000 rpm for 1 hour at 37°C. At the same time, a mock transduction was performed by following the same steps without the addition of lentivirus supernatant. The next day, NK cells were washed to get rid of the polybrene or the vectofusin-1. Viability was checked by tripan blue. CD19-CAR transduction efficiency was check by flow cytometry following the upcoming protocol. Cells were harvested and washed with PBS+ 10% FBS twice. Then, cells were stained with Fab/Fab´2 antibody, which detects CD19-CAR presence on the membrane. Cells were incubated for 30 minutes at 4°C and dark. Subsequently, cells were washed twice with PBS and stained with CD56 antibody and CD3 antibody. Cells were washed twice with PBS, resuspended in 400 µl of PBS and checked by flow cytometry.

Functionality assays

Cytotoxicity assay

In order to check the *in vitro* lytic activity of CAR NK cells against CD19 expressing target cell lines (Nalm 6, ALL and CLL patient cells) we performed a calcein-AM-based cytotoxicity assay. Mechanism of the assay was detailed in the cytotoxic assay section of "Material and Methods 1st objective". K562 cell line, which is negative for CD19

marker, was used as control target cell. 500.000 cells were incubated for 30 min at 37°C with 15 µM of calcein-AM (Life technologies C3099). The CAR-NK cells were washed twice after incubation. Calcein-AM-labeled cell lines were co-cultured with transduced and non-transduced NK cells U-bottom 96-well plate for 4 h at 37°C at different ratios (10:1, 5:1 and 1:1). For measurement of spontaneous release, all target cells were incubated with no NK cells. Total released was achieved by adding 4% Triton™ X-100 (Sigma-Aldrich) to the target cells. Each condition was performed in triplicates. After the incubation, 100 µl of supernatant was collected and transferred to a black 96-well plate to measure the calcein-AM release in a Fluoroskan Ascent (Thermo Fisher) (excitation filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). The percentage of specific lysis is calculated according to the following formula: [(Test release) − (Medium fluorescence)] − [(Spontaneous release) − (Medium fluorescence)]/[(Total release) − (Triton fluorescence)] − [(Spontaneous release) − (Medium fluorescence)] × 100

Degranulation assay

Mechanism of the assay was detailed in the degranulation assay section of "Material and Methods 1st objective". Transduced and non-transduced NK cells were cocultured with previously mentioned target cells at ratio 1:1 in a 24-well plate for 4 h at 37°C. Concentration of the cells was 500,000 cells per milliliter. At the beginning of the assay, anti-CD107a BV421 (BD Biosciences, clone H4A3) was added in order to detect the degranulation activity of the effector cells against the target cells. Golgi Stop™ (BD Biosciences) (monensin) was added following the manufacturer's protocol. After the incubation, cells were collected, washed, and labeled with anti-CD3-PerCP/Cy5.5, anti-CD56-APC and anti-CD19 BV510 (BD Biosciences, clone HIB19). Degranulating NK cells (CD107a+) were determined in the CD56+/CD3- cells. Target cells with a similar size and granularity (FSC-SSC) to NK cells were discarded by a negative selection of CD19 marker. The gating strategy was: Lymphocytes were selected by size and internal complexity (FCS/SSC). Then, duplets were discarded and target cells were discarded from the analysis by a negative selection of CD19 marker. Then, remaining non NK cells lymphocytes were discarded by the negative selection of CD3 marker. Next CD56+ cells were faced to CD107a marker.

CAR19 Cloning and Lentivirus Production

The sequence corresponding to the variable light (VL) and variable heavy (VH) regions of A3B1 antibody was extracted from A3B1 hybridoma cells using Mouse Ig-Primer Set (Novagen, 69831-3) (kindly provided by Dr. Manel Juan; Hospital Clinic in Barcelona, Spain).

The complete CAR19 sequence (including signal peptide, A3B1 scFv, CD8 hinge, and transmembrane regions 4-1BB and CD3z) was synthesized by GenScript and cloned into the third-generation lentiviral vector pCCL (kindly provided by Dr. Manel Juan; Hospital Clinic in Barcelona, Spain), under the control of EF1α promoter (**Figure 45**).

The seven pCCL-CAR19 variants (CAR19-NK1 to 7) were produced by replacing the intracellular domain of 4-1BB from the original CAR19 by different combinations of transmembrane or intracellular domains of the following genes: 2B4, CD28, DAP10, DAP12 and NKG2D, as indicated in **Table 8**. For generation of the lentiviral vector variants, the gene fragments

cassettes were first synthesized by GeneArt (Invitrogen, Thermo Fisher Scientific, city, country) and cloned into the pMS-RQ (SpcR/StrR) backbone (Table 8). Subsequent subcloning of each MreI/BstBI-digested cassette was performed into MreI/BstBI-digested pCCl-CAR19 resulting in pCLL-CAR19-NK1 to pCLL-CAR19-NK7. Correct assembly of the expression cassettes was confirmed by restriction digest mapping and by direct sequence of the ORFs. Final DNA concentration was adjusted to 1 µg/µl in miliQ water.

HEK293T cell line (kindly provided by Immunotherapy Department of the Hospital Clinic-IDIBAPS, Barcelona) was used in order to pack CD19-CAR containing lentivirus. HEK293T cells were thawed and after 3-4 days they were harvested and plated $8 \cdot 10^6$ in each p150 dish. 10-15 p150 dishes were plated for each batch of viral supernatant. The following day, HEK293T medium was change one hour prior transfection. Meanwhile, DNA mix was prepared as follow. In a sterile tube, 35 µg of total DNA (17 µg vector trans, 8.53 µg RRE MOL, 4.25 µg REV, 5.2 µg VSV-G) were mixed in 1 ml of DMEM for each p150. Hereafter, 102.5 µl of PEI (1 µg/µl) was added to the mixture and this was incubated at room temperature for 20 minutes. After that, 1 ml of the mixture was added to each p150, drop by drop; up to a final volume of 14-15 ml. Cells along with the mixture were incubated for 4 hours at 37ºC. Afterwards, medium was changed and cells were cultured for 48-72 hours. Next, viral supernatant was harvested and spin down at 2000 rpm for 5 minutes. Supernatant was filtered with 45 µm filters (Millex-HV, SLHV033RB) and centrifuged in an ultracentrifuge (Optima L-90K Ultracentrifuge, Beckman Coulter) at 19600 rpm for 2 hours and 30 minutes. Once centrifuge program is done, supernatant was discarded and pellet was resuspended in medium without DMSO. Concentrated virus was stored in aliquots at -80Cº. Viral particles were titrated following the protocol described in CD19-CAR lentivirus production section, part "c".

Table 8: Design of the new CAR constructs. In bold we have the target sequence of the restriction enzyme in 5´ (MreI) and 3´ (BstBI). The sequences underlined in colors correlate with the different added domains: in yellow 2B4, in grey CD28, in blue NKG2D, in green DAP10 and in pink DAP12.

pMS-RQ constructs	Cassette Size (bp)	Sequence		Ref.
CAR-NK1	908	Intracellular domain of 2B4 (UniProt Q9BZW8; aa. 246-365)	**cgccggcg**ccgagaccacctacacctgcaccaactattgcc tctcagccactgagtctgcgccccgaggcatgtcgacctgccg ctggcggggctgtgcacaccaggggcctagacttcgcctgcg atatctatatttgggctccactggcaggaacctgtggcgtgctgc tgctgtctctggtcatcacactgtactgctggaggagaaagag gaaggagaagcagtcagagaccagtcccaaggaatttttga caatttacgaagatgtcaaggatctgaaaaccaggagaaatc acgagcaggagcagacttttcctggaggggggagcaccatc tactctatgatccagtcccagtcttctgctcccacgtcacaagaa cctgcatatacattatattcattaattcagccttccaggaagtctg gatccaggaagaggaaccacagcccttccttcaatagcacta tctatgaagtgattggaaagagtcaacctaaagcccagaacc ctgctcgattgagccgcaaagagctggagaactttgatgtttatt ccgcgtgaaattttctcggagtgcagatgccccagcttacca gcagggccagaaccagctgtataacgagctgaatctggggc ggagagaggaatacgacgtgctggataagaggcgcgggcg	Li Y et al Cell Stem Cells 2018

			agatccagaaatgggaggaaaaccccagcgacggaagaa ccctcaggagggactgtacaatgaactgcagaaggacaaa atggcagaggcctattccgaaatcgggatgaaaggagaaag aaggcgcggcaaggggcatgatggcctgtatcagggactgt caaccgcaacaaaagatacttatgatgctctgcacatgcagg ctctgcccccgcggtaa**ttcgaa**	
CAR-NK2	758	Transmembrane and intracellular domain of CD28 (UniProt P10747; aa. 150-220)	**cgccggcg**ccgagaccacctacacctgcaccaactattgcc tctcagccactgagtctgcgccccgaggcatgtcgacctgccg ctggcggggctgtgcacaccaggggcctagacttcgcctgcg atatctatatttgggctccactggcaggaacctgtggcgtgctgc tgctgtctctggtcatcacactgtactgcaagccctttgggtgct ggtggtggttggtggagtcctggcttgctatagcttgctagtaac agtggcctttattattttctgggtgaggagtaagaggagcaggc tcctgcacagtgactacatgaacatgactccccgccgccccg ggcccacccgcaagcattaccagccctatgccccaccacgc gacttcgcagcctatcgctcccgcgtgaaattttctcggagtgc agatgccccagcttaccagcagggccagaaccagctgtata acgagctgaatctggggcggagagaggaatacgacgtgctg gataagaggcgcgggcgagatccagaaatgggaggaaaa ccccagcgacggaagaaccctcaggagggactgtacaatg aactgcagaaggacaaaatggcagaggcctattccgaaatc gggatgaaaggagaaagaaggcgcggcaaggggcatgat ggcctgtatcagggactgtcaaccgcaacaaaagatacttat gatgctctgcacatgcaggctctgcccccgcggtaa**ttcgaa**	Oelsner S et al Cytotherapy 2017
CAR-NK3	1043	Transmembrane domain of NKG2D (UniProt P26718; aa. 52-72) + Intracellular domain of 2B4 (UniProt Q9BZW8; aa. 246-365) + Intracellular domain of DAP10 (UniProt Q9UBK5; aa. 70-93)	**cgccggcg**ccgagaccacctacacctgcaccaactattgcc tctcagccactgagtctgcgccccgaggcatgtcgacctgccg ctggcggggctgtgcacaccaggggcctagacttcgcctgcg atatctatatttgggctccactggcaggaacctgtggcgtgctgc tgctgtctctggtcatcacactgtactgcccatttttttctgctgctt catcgctgtagccatgggaatccgtttcattattatggtaacatg gaggagaaagaggaaggagaagcagtcagagaccagtcc caaggaatttttgacaatttacgaagatgtcaaggatctgaaa accaggagaaatcacgagcaggagcagacttttcctggagg ggggagcaccatctactctatgatccagtcccagtcttctgctcc cacgtcacaagaacctgcatatacattatattcattaattcagcc ttccaggaagtctggatccaggaagaggaaccacagcccttc cttcaatagcactatctatgaagtgattggaaagagtcaaccta aagcccagaaccctgctcgattgagccgcaaagagctggag aactttgatgtttattccctgtgcgcacgcccacgccgcagccc cgcccaagaagatggcaaagtctacatcaacatgccaggca ggggccgcgtgaaattttctcggagtgcagatgccccagctta ccagcagggccagaaccagctgtataacgagctgaatctgg ggcggagagaggaatacgacgtgctggataagaggcgcgg gcgagatccagaaatgggaggaaaaccccagcgacggaa gaaccctcaggagggactgtacaatgaactgcagaaggac aaaatggcagaggcctattccgaaatcgggatgaaaggaga aagaaggcgcggcaaggggcatgatggcctgtatcaggga ctgtcaaccgcaacaaaagatacttatgatgctctgcacatgc aggctctgcccccgcggtaa**ttcgaa**	Li Y et al Cell Stem Cells 2018
CAR-NK4	1127	Transmembrane domain of NKG2D (UniProt P26718; aa. 52-72) + Intracellular domain of 2B4 (UniProt	**cgccggcg**ccgagaccacctacacctgcaccaactattgcc tctcagccactgagtctgcgccccgaggcatgtcgacctgccg ctggcggggctgtgcacaccaggggcctagacttcgcctgcg atatctatatttgggctccactggcaggaacctgtggcgtgctgc tgctgtctctggtcatcacactgtactgcccatttttttctgctgctt catcgctgtagccatgggaatccgtttcattattatggtaacatg gaggagaaagaggaaggagaagcagtcagagaccagtcc caaggaatttttgacaatttacgaagatgtcaaggatctgaaa accaggagaaatcacgagcaggagcagacttttcctggagg ggggagcaccatctactctatgatccagtcccagtcttctgctcc cacgtcacaagaacctgcatatacattatattcattaattcagcc ttccaggaagtctggatccaggaagaggaaccacagcccttc	Li Y et al Cell Stem Cells 2018

		Q9BZW8; aa. 246-365) + Intracellular domain of DAP12 (UniProt O43914; aa. 61-113)	cttcaatagcactatctatgaagtgattggaaagagtcaacctaaagcccagaaccctgctcgattgagccgcaaagagctggagaactttgatgtttattcctacttcctgggccggctggtccctcgggggcgaggggctgcggaggcagcgacccggaaacagcgtatcactgagaccgagtcgccttatcaggagctccagggtcagaggtcggatgtctacagcgacctcaacacacagaggccgtattacaaacgcgtgaaattttctcggagtgcagatgccccagcttaccagcagggccagaaccagctgtataacgagctgaatctgggcggagagaggaatacgacgtgctggataagaggcgcgggcgagatccagaaatgggaggaaaaccccagcgacggaagaaccctcaggagggactgtacaatgaactgcagaaggacaaaatggcagaggcctattccgaaatcgggatgaaaggagaaagaaggcgcggcaaggggcatgatggcctgtatcagggactgtcaaccgcaacaaaagatacttatgatgctctgcacatgcaggctctgcccccgcggtaa**ttcgaa**	
CAR-NK5	971	Transmembrane domain of NKG2D (UniProt P26718; aa. 52-72) + Intracellular domain of 2B4 (UniProt Q9BZW8; aa. 246-365)	**cgccggcg**ccgagaccacctacacctgcaccaactattgcctctcagccactgagtctgcgccccgaggcatgtcgacctgccgctggcggggctgtgcacaccaggggcctagacttcgcctgcgatatctatatttgggctccactggcaggaacctgtggcgtgctgctgctgtctctggtcatcacactgtactgcccatttttttctgctgcttcatcgctgtagccatgggaatccgtttcattattatggtaacatggaggagaaagaggaaggagaagcagtcagagaccagtccaaggaatttttgacaatttacgaagatgtcaaggatctgaaaaccaggagaaatcacgagcaggagcagacttttcctggaggggggagcaccatctactctatgatccagtcccagtcttctgctccccacgtcacaagaacctgcatatacattatattcattaattcagccttccaggaagtctggatccaggaagaggaaccacagcccttccttcaatagcactatctatgaagtgattggaaagagtcaacctaaagcccagaaccctgctcgattgagccgcaaagagctggagaactttgatgtttattccccgcgtgaaattttctcggagtgcagatgccccagcttaccagcagggccagaaccagctgtataacgagctgaatctggggcggagagaggaatacgacgtgctggataagaggcgcgggcgagatccagaaatgggaggaaaaccccagcgacggaagaaccctcaggagggactgtacaatgaactgcagaaggacaaaatggcagaggcctattccgaaatcgggatgaaaggagaaagaaggcgcggcaaggggcatgatggcctgtatcagggactgtcaaccgcaacaaaagatacttatgatgctctgcacatgcaggctctgcccccgcggtaa**ttcgaa**	Li Y et al Cell Stem Cells 2018
CAR-NK6	602	Transmembrane domain of NKG2D (UniProt P26718; aa. 52-72)	**cgccggcg**ccgagaccacctacacctgcaccaactattgcctctcagccactgagtctgcgccccgaggcatgtcgacctgccgctggcggggctgtgcacaccaggggcctagacttcgcctgcgatatctatatttgggctccactggcaggaacctgtggcgtgctgctgctgtctctggtcatcacactgtactgccccatttttttctgctgcttcatcgctgtagccatgggaatccgtttcattattatggtaacacgcgtgaaattttctcggagtgcagatgccccagcttaccagcaggggccagaaccagctgtataacgagctgaatctggggcggagagaggaatacgacgtgctggataagaggcgcgggcgagatccagaaatgggaggaaaaccccagcgacggaagaaccctcaggagggactgtacaatgaactgcagaaggacaaaatggcagaggcctattccgaaatcgggatgaaaggagaaagaagaggcgcggcaaggggcatgatggcctgtatcagggactgtcaaccgcaacaaaagatacttatgatgctctgcacatgcaggctctgcccccgcggtaa**ttcgaa**	Li Y et al Cell Stem Cells 2018
CAR-NK7	980	Intracellular domain of 2B4 (UniProt Q9BZW8; aa. 246-365) +	**cgccggcg**ccgagaccacctacacctgcaccaactattgcctctcagccactgagtctgcgccccgaggcatgtcgacctgccgctggcggggctgtgcacaccaggggcctagacttcgcctgcgatatctatatttgggctccactggcaggaacctgtggcgtgctgctgctgtctctggtcatcacactgtactgctggaggagaaagaggaaggagaagcagtcagagaccagtcccaaggaatttttgacaatttacgaagatgtcaaggatctgaaaaccaggagaaatcacgagcaggagcagacttttcctggaggggggagcaccatc	Li Y et al Cell Stem Cells 2018

		Intracellular domain of DAP10 (UniProt Q9UBK5; aa. 70-93)	tactctatgatccagtcccagtcttctgctcccacgtcacaagaa cctgcatatacattatattcattaattcagccttccaggaagtctg gatccaggaagaggaaccacagcccttccttcaatagcacta tctatgaagtgattggaaagagtcaacctaaagcccagaacc ctgctcgattgagccgcaaagagctggagaactttgatgtttatt ccctgtgcgcacgcccacgccgcagccccgcccaagaaga tggcaaagtctacatcaacatgccaggcaggggccgcgtga aattttctcggagtgcagatgccccagcttaccagcagggcca gaaccagctgtataacgagctgaatctggggcggagagagg aatacgacgtgctggataagaggcgcgggcgagatccaga aatgggaggaaaaccccagcgacggaagaaccctcagga gggactgtacaatgaactgcagaaggacaaaatggcagag gcctattccgaaatcgggatgaaaggagaaagaaggcgcg gcaaggggcatgatggcctgtatcagggactgtcaaccgca acaaaagatacttatgatgctctgcacatgcaggctctgcccc cgcggtaa**ttcgaa**	

Data analysis

Differences between transduced and non-transduced NK cells were evaluated using Two-Way ANOVA with multiple comparisons. Differences between AB and CB NK cells were evaluating using non-paired Student's t-test or Two-Way ANOVA. p-Values < 0.05 were considered significant. Statistical calculations were done using GraphPad Prism 6 (GraphPad Software, Inc.) Bars or symbols represent the mean and error bars represent the SEM.

RESULTS

OBJECTIVE 2

DISCUSSION

DISCUSSION

CARs are chimeric antigen receptors that alter the specificity and function of immune cells, commonly T cells (203). CAR-T cell therapy is a successful immunotherapy for treating hematological cancer targeting CD19+ malignancies, such as ALL and CLL used in nearly 200 clinical trials worldwide. Patients with ALL have shown complete response (CR) rates of ≈ 90% in single center trials and ≈ 70–80% in multicenter trials after being treated with CAR-T cells (138,204,205). Despite the success of the therapy, a significant problem is the toxicity generated by the overwhelming cytokine response that causes what is called a cytokine release syndrome (CRS) (206,207), as well as the potential development of graft versus host disease (GvHD) in allogenic therapies (208,209). In fact, to overcome these side effects, there is a unique CD19 CAR CB-NK cells clinical trial on going (NCT03056339) in USA for the time being. Regarding the objective 1, we studied NK cells from different cell sources: adult peripheral cord blood, umbilical cord blood, umbilical cord blood hematopoietic stem cells and human pluripotent stem cells.

In this study, we surmised that NK cells from different sources could be promising candidates for CAR therapy, due to the lack of risk of GvHD development as a consequence of their non-MHC restricted recognition (210). Moreover, NK cells persist less time in circulation as a consequence of their shorter lifespan, which could lead to a softer B cell depletion of the patient (211). In term of cell numbers, NK cells constitute up to 10% of AB and up to 30% of CB (212) in both sources, yielding enough number of NK cells for this immunotherapy to be practical. In both cell sources CD56bright and CD56dim NK cells, are present at the same proportions (213) and contain equivalent levels of perforin and granzyme B, which have been associated with NK maturation, resulting in functional NK cells in both cases (214,215). As expected (216), higher number of CB NK cells than AB NK cells are obtained, with less variability in the numbers obtained from CB. In fact, when leukapheresis from adult peripheral blood are performed in order to obtain NK cells, the variability is very high (217).

Although resting CB NK cells have shown in previous studies less cytotoxicity than AB NK cells (218), the stimulation of these cells with some cytokines results in the improvement on their killing activity, being equivalent to equally treated AB NK cells (219). As a consequence, we used IL-2 and IL-15 to stimulate NK cells from both sources. IL-2 increases the NK cell population (220), while IL-15 helps with survival, proliferation and higher cytotoxicity (221). Viability of the NK cells from both sources drops slightly every week until day 28, when it is reduced considerably. This could be due to the short life span on NK cells (222). Although viability is very similar between AB and CB NK cells, a slight increase was observed in CB NK cells at every check-point. Regarding fold expansion, it is well known the difficulties for an acceptable expansion of NK cells in a feeder free scenario (223). In our case, fold expansion is still an issue, as we are not culturing them with feeder cells as other studies do (224,225). Despite the low fold expansion number, we observed a slightly higher fold expansion of CB NK cells over AB NK cells, with no statistical differences between them, at 2 weeks of culture in presence of IL-2 and IL-15.

We also established another NK cells source. For this purpose, we used and compared the effect of two different culture conditions during the generation of *in vitro* functional and mature NK cells from HSC precursors from CB. We showed that by using OP9 cells as a feeder

layer we obtained higher number of CD56+ mature NK cells in comparison with M2-10B4 cells as a feeder layer. To date, several articles have described different protocols for *in vitro* NK cell differentiation from hematopoietic progenitors from CB (CD34+ cells) including the usage of different feeder cells, cytokine cocktails, and time of culture (191-193,195,196,226-236). However, we believe that our study for the first time describes in detail the maturation stages of NK cells during the *in vitro* differentiation process in which a high number of functional NK cells are achieved with the possibility for using them in future immunotherapies against cancer.

We have described a new culture condition, using two cell lines as feeder layers, i.e., OP9 cells and M2-10B4 cells, to generate NK cells from CB CD34+ hematopoietic precursors.

Other authors have described the use of these two cell lines to differentiate CD34+ cells from pluripotent stem cells, such as ESCs and induced pluripotent stem cells (237-240) and also to support and maintain CD34+ cells in a long-term culture (241,242).

The use of feeder cell lines with the aim of maintenance and differentiation of stem cells towards blood lineage cells is an usual practice. First, the cell line AFT024 was described by Moore et al., immortalized with SV-40 T antigen, and derived from murine fetal liver stromal cells (243). Specifically, to study human NK cell ontogeny, Miller and McCullar suggested that NK cell differentiation from CD34+ cells and receptor acquisition was contact dependent with the feeder layer AFT024 (244). Other groups investigated the AFT024 and EL08-1D2 potential to generate *in vitro* NK cells and found that EL08-1D2 is significantly better at recapitulating NK cell development (235).

M2-10B4 is the other feeder cell line commonly used, and it derives from murine bone marrow stromal cells. M2-10B4 has been used earlier for NK cell expansion (245, 246) and others determined its differentiation potential for NK cell generation from hESCs (247). Remarkably, these findings suggest the need of stromal cell microenvironment, and the importance of direct contact with the feeder layer.

Finally, few reports have used the OP9 cell line (234, 236) to differentiate NK cells from bone marrow CD34+ cells. Also, OP9-DL1 (OP9 modified with Notch ligand delta-like 1) is used to develop T lymphocytes (248) and NK cells from hematopoietic precursors (249). To summarize, the majority of published data indicate the need of a microenvironment supported by stromal cells. This microenvironment provides necessary factors for the correct maturation of NK cells. Therefore, we wanted to explore new culture conditions to obtain high number of mature and functional NK cells from CB CD34+ cells, to improve the *in vitro* differentiation protocols previously published, and also to in detail characterize there *in vitro* development in comparison with the maturation stages described *in vivo*.

To do this, we have compared both cell lines (OP9 and M2-10B4) in terms of differentiation capacity, number of CD56+ cells and NK fold expansion from the first week up to 6 weeks of differentiation. OP9 have been described to secrete numerous proteins to the medium like Npc2 or Fstl1, among others (250). In general, we have obtained higher number, fold expansion, and frequencies of CD56+ NK cells, specifically when they are generated in the presence of OP9 feeder cells, than other authors who have used similar protocols. For example, with the EL08.1D2 cell line as feeder cells, several groups have obtained a similar fold of expansion (225) or a lower number and frequency of CD56+ NK cells (198,230,235) than us. Others have also obtained lower number or lower fold expansion than us when the AFT024 cell line (235) or Stro-1+ cell line (231) were used as feeder layers. In our study, using both cell lines (OP9 and M2-10B4), we obtain a higher number of NK cells with OP9 cell line. Importantly, we have also obtained higher number and frequencies of CD56+ NK cells than others who have also used OP9 cells as feeder layer (234,236).

Currently, the vast majority of researchers accept a linear model of differentiation of human NK cells with five stages of maturation, each characterized by a pattern of expression of surface receptors, functional capabilities, and differentiation potential (200). In stage 3, cells have variable expression of the markers CD161 and CD56, typical of mature NK cells, but they do not express inhibitory receptors for MHC class I molecules, i.e., KIR and CD94/NKG2A, which they are characteristic of mature NK cells. In addition, cells in stage 3 have the two signs of functional identity of mature NK cells: IFN-γ production and perforin-dependent cytotoxic activity. CD94 expression marks the transition to stage 4 in the development of human NK cells. Cells at this stage are characterized by high CD56 expression. The acquisition of CD16 in some CD94+ cells is considered a marker of cells in stage 5, the group defined by the phenotype CD94highCD56dimCD16+ cells. Therefore, CD56dim and CD56bright cells in PB represent the two terminal stages of differentiation of human NK cells (251).

We have observed that our *in vitro* differentiation and maturation process of NK cells follows a similar pattern regarding the surface markers acquisition, which are observed *in vivo*. For example, CD117 expression in our *in vitro* NK cells is always present in late stages (36,252), whereas is down-regulated in *in vivo* differentiated NK cells at stage 5. We believe this is due to the presence of SCF in our culture system, as it has been reported that the presence of this cytokine up-regulates the expression of CD117 in CD56+ NK cells and significantly increases the capacity of CD56bright NK cells to degranulate (253). In our cultures, we cannot distinguish between CD56dim and CD56bright NK cells, which probably are due to the fact that this *in vitro* phenotype is slightly different to the *in vivo* due to the culture conditions.

We also observed differences in maturation stages between the two cell lines used as feeder layers. More mature stages are reached earlier by using OP9 cell line than with the M2-10B4 cell line. Otherwise, the total number of cells at stages 4–5 of maturation equalizes between

the two conditions at the end of the differentiation protocol, having more NK cells at day 28 when the culture conditions include the OP9 feeder cells, which also may be responsible for the observed higher cytotoxicity activity. We think that these differences in timing of maturation and number is due to the NK cell differentiation potential properties among stromal cell lines used in this study, because it has been reported that depending of the origin of the stromal cell lines used in hematopoietic differentiation, the features of maturation, and functionality of the terminal cell type could be different (254).

Few groups have described the *in vitro* developed NK cells from CB in the presence of OP9 feeder cells. First, it has been reported that using OP9 cell line (234) during the *in vitro* development of NK cells from bone marrow or CB CD34+ cells, the levels of TGF-β may influence the developmental progression and subset formation of NK cells, like CD56brightCD16− subset, but there are no studies in the progression of NK stages *in vitro*. Second, other group (236) also using OP9 feeder cells, obtained 80% of CD56+ cells at 28 days, but they did not distinguish between different stages of maturation. In our case, we are able to achieve 70–80% of CD56+ cells at the same day of differentiation but we studied in detail that around 38–45% of CD56+ cells are already in stages 4–5, showing the typical markers of mature NK cells. Besides, other groups using other culture conditions achieved a minor percentage of CD56+ cells in stages more immature than we did (226,232).

In general, we have also obtained better or similar results than others when we look at the expression of cytolytic markers, degranulation properties, and killing activity of *in vitro*-generated NK cells from CB-CD34+ cells. For example, in feeder-free systems, *in vitro*-generated NK cells expressed lower levels of perforin (192,232), lower (232) or equal (193) cytotoxic activity, and equal levels of granzyme B (232) and degranulation potential (228,229). When NK cells were generated in the presence of the EL08.1D2, they exhibited similar levels of perforin and granzyme B (226), similar degranulation levels (191, 226), and lower cytotoxic activity (196). Finally, in a study where OP9 feeder cells were used for the differentiation of NK cells, the authors found that they expressed lower levels of perforin (236) compared with the NK cells we obtained in our study.

In conclusion, we found that the use of OP9 and M2-10B4 cell lines to generate NK cells *in vitro* from CB CD34+ is a feasible option that offers the advantage of obtaining higher functional and mature NK cell numbers with enhanced killing capacity. To the best of our knowledge, this is the first and the most comprehensive study comparing these two culture conditions for the generation of NK cells from fresh CB CD34+ cells, being OP9 cells culture condition better than M2-10B4 cells, highlighting the great potential for CB CD34+ for future NK cell-based immunotherapy due to their acquired NK cell phenotype and functionality, and the high amount of obtained NK cells. Our next goal will be to translate these results into GMP conditions for future use in clinic.

For the purpose of finding the best NK cell source to be used in CAR-based therapies, we explored a fourth source: pluripotent stem cells (hiPS and hES). In order to achieve a successful differentiation towards NK cells, firstly, obtaining good quality and functional hematopoietic stem cells is crucial. The generation of hematopoietic stem cells is one of the most studied ones and several protocols have been proposed in order to get *in vitro* CD34+ cells (179-181). There are a variety of stromal cell lines employed for co-culture systems with hES/hiPS cells; of these, OP9 cells are most widely used (182). However, pluripotency is no longer viewed as a fixed state but rather a highly dynamic, malleable signaling network (162). Laboratory generated hiPS and cultured hES are known to be in a primed stage. These cells are typically cultured over feeder layer cells, but they can be converted into non-feeder dependent hiPS or hES. Moreover, primed hiPS and hES can be converted into a more ductile pluripotent stage: naïve stage (165).

In this study, we differentiated CD34+ cells from primed hiPS and hES with feeder layer, primed hiPS and hES feeder-free and naïve hiPS and hES. 70.46% of the performed experiments with primed hiPS and hES with feeder layer resulted in CD34+ cell population, while 80% of the performed experiments with primed hiPS and hES feeder-free resulted in CD34+ cell population. Although primed hiPS and hES feeder-free were slightly better, there were not significant differences between these two methods. Making into account the functional CD34+ obtained from these experiments, we did not found significant differences between using the primed hiPS and hES with or without feeder layer. Nevertheless, primed hiPS and hES without feeder layer system seems to more efficient for the obtaining of CD34+, as we achive significantly higher percentage of CD34+ population than using primed hiPS and hES with feeder layer. While we obtained and average of 5.5% and 8.5% of CD34+ cells depending on the culture condition of the hiPS and hES source, other authors have similar results (180,255) than our results. We do not observe any significant difference between hiPS and hES to obtain CD34+ cells in an efficient manner.

We also successfully converted primed hiPS and hES into naïve hiPS and hES, as we obtained the characteristic phenotype of naïve cells, and also we observed the acquirement of surface markers described in naïve cells when primed surface markers (171) decreased. When comparing the two different media used for this purpose, we observed that, despite the good result obtained with both of them, cells cultured with medium 1 got a higher expression of naïve markers at day 10; and cells cultured with medium 2 lost quicker the expression of primed markers. In order to decide which medium is more appropriate, we took into account the time that cells remained in naïve stage before starting to be differentiated. We concluded that medium 2 was better for long term culture. Next, we differentiated CD34+ cells from naïve hiPS and hES. 46.16% of the performed experiments with these cells resulted in CD34+ population, which is much lower than the result obtained with any of the primed hiPS or hES. In contrast, some authors claim that naïve stage has higher differentiation capacity that primed state (256,257). So that, taking into account that, the difference is not significantand due to the lower number of performed experiments with naïve cells,more experiments are necessary in order to obtain a stronger conclusion.

Taking into account all the experiments performed in order to obtained CD34+ population from any hiPS and hES, we saw a common denominator: the number of the percentage of the CD34+ obtained population is not an indicator of the functionality of these cells.

Once we had functional CD34+ cells, we differentiated them towards NK cells. Although some groups had described successful differentiation of NK from hiPS or hES (184, 258), and some of their products are in clinical trial (FT500) or in preclinical studies for a future clinical trial (FT596) we were not very successful and efficient in this point. However, in these studies mentioned the cells line H9 and H1 hES were used in order to obtain NK cells, so that, the kind of hES of hiPS used for this purpose might be crucial.

Once we completed the first objective, we proceeded with the second objective: Arming the NK cells from different sources with a CD19-CAR, as preclinical studies.

NK cells have been reported to be challenging to infect, with <10% transduction efficiencies (259). Nonetheless, the improvement of viral infection protocols has led to better results infecting AB and CB NK cells. Here, the mean AB CAR-NK transduction efficiency was 47.46 (range 62.6–20.2%) while CB CAR-NK transduction efficiency was 46.8 (range 79.7–18.1%). Several research groups prefer the use of the NK-92 cell line to AB NK cells in order to be transduced because of the better infection rates (260-263). However, CB NK cells have been use as a reliable source for CAR therapy with similar viral infection rates than us (41.6% ± 8.9) (259) or even higher 66.6% (range, 47.8–87.4%)(77).

Comparing the degranulation and cytotoxicity activity of the non-transduced and CD19-CAR transduced cells, we observed a significant increase with the CD19-CAR NK cells, as expected (264-266). Focusing on AB NK cells, we noted significant differences at degranulating with CD19-CAR with all the target cells expressing CD19, and a better killing with CAR-CD19 NK cells than with non-transduced NK cells, these being non-significantly better. CB NK cells are also significantly better at degranulating with CD19-CAR with all the target cells; however, as for their killing activity, they are not only better than non-transduced NK cells, but significantly better in most conditions. As mentioned previously, AB CD19-CAR NK cells and CB CD19-CAR NK cells do not present a significant difference in their degranulation activity, but AB CD19-CAR NK cells perform slightly better at killing CD19 expressing target cells. Since we used a T-cell designed CAR, we obtained better results in both degranulation and killing activity by AB NK cells than other published works using the same CAR structure (267).

Due to their great fold expansion, CB CD34+ *in vitro* generated NK cells would constitute another great source for CAR therapy purpose. As mentioned before, we are capable of obtaining higher functional and mature NK cell numbers with enhanced killing capacity with this differentiation method. CB CD19-CAR-CD34+ differentiated NK cells transduction efficiency was 38.85%. When comparing these NK cells with AB NK cells and CB NK cells no statistical differences were found regarding the transduction efficiency. So that, we still get an acceptable transduction efficiency to work with. However, when facing these CB CD19-CAR-CD34+ differentiated NK cells against CD19 expressing target cells we do not see an increasing killing activity mediated by the presence of the CD19-CAR in their surface. This inconvenience could occur due to a lack of signaling by the CAR motifs inside the cell, which could lead to a suppression of the CD19-CAR activity (267). The phosphorylation levels of the proteins of different activation pathways, such as Fyn-PLC pathway or Syk-Vav1-Erk pathway need to be checked in order to confirm this hypothesis.

The fact that CB CD19-CAR-CD34+ differentiated NK cells do not work at better killing CD19 expressing target cells make us, for the time being, not consider them for future clinical

applications in CAR therapy. Nonetheless, the high fold expansion achieved with this NK cell source make us want to explore GMP conditions for expanding these cells, as well as, improving the CAR functioning.

Still, while most studies have evaluated T cells adapted CARs; here we also wanted to evaluate different CAR constructs that could improve Natural Killer (NK) cell-mediated killing. Some suitable NK cell motifs were included, such as 2B4, NKG2D, DAP10 and DAP12, and they were combined among them to a total of seven different new variants. We were capable of transducing AB NK cells with all the new variants, allowing us to try new potential weapons to be used in CAR-based therapies with allogenic NK cells as their main vehicle. According to the literature, a CAR containing the transmembrane domain of NKG2D, the 2B4 co-stimulatory domain, and the CD3ζ signaling domain mediates strong antigen-specific NK cell signaling (267), which corresponds with the structure of our NK5 CD19-CAR. Due to this reason, we decided to start the pre-clinical experiments with this CAR variant, following with the rest of variants.

Following the protocols that have been used with the CD19-CAR ARI-0001 (T-cell adapted CAR), we tried our NK5 CD19-CAR in AB NK cells. As we can observe in the results, NK5 CD19-CAR AB NK cells kill better CD19 expressing target cells than the ones that are now transduced. It is true that more experiments need to be run, as significance does not shown in most cases due to the low number of replicas. When we compare the effect of NK5 CD19-CAR with ARI-0001, we observe that ARI-0001 works slightly better than NK5. However, this could occur due to the lower percentage of transduction of NK5 CD19-CAR AB NK cells. Improving the transduction efficacy, we are positive that we could improve the killing as well.

Taking into account the undesirable results of CB CD19-CAR-CD34+ differentiated NK cells transduced with ARI-0001, we decided to try NK5 CD19-CAR in these cells as well. As we can notice, this time, CB CD19-CAR-CD34+ differentiated NK cells transduced with NK5 CD19-CAR were able to perform a better killing that the ones that were non- transduced. Once again, this is a very preliminary data, as more experiments need to be run as significance does not shown in most cases due to the low number of replicas. Checking the phosphorylation levels of the proteins of different activation pathways (such us….) mentioned before could be very interesting, especially, if we could detect any differences between the effect of ARI-0001 and NK5 in these cells.

This work is open to many more improvements and future steps. Our main goal is to bring these kinds of CAR-NK therapies to the clinic to treat relapse or refractory hematological cancers in adult and pediatric patients. In order to do that, *in vivo* assays need to be performed with all the different NK cell sources together with the new CAR variants. In consequence, although we obtain enough number of NK cells of AB and CB to treat the patients with the same criteria as the existing clinical trial (NCT03056339), there is room in order to enhance the NK cells expansion, which could lead to the creation of many “off the shelf” product from one single donor. If we wanted to use CB CD34+ cells as the NK cell source, we would need to adapt it to GMP conditions, and work deeper in the correct function of the CARs in these cells. Moreover, the protocols for obtaining NK cells from our fourth source (hiPS and hES) need to be upgrade. We possibly could achieve functional NK cells from hiPS and hES by exploring in depth the different stages of these cells, like naïve stage.

In addition to the foregoing, all the new variants of the CD19-CAR need to be tested, as we believe we could boost the performance of CAR-NK cells by supplying them with NK cell adequate motifs.

CONCLUSIONS

CONCLUSIONS

1. Adult peripheral blood and umbilical cord blood are easy and reachable cells sources for obtaining NK cells. Regarding AB NK cells, buffy coats from blood donors are nowadays discarded in the blood banks. These CAR therapies could give these blood products a new life, making the most of the donors´ blood. Respecting CB NK cells, there are many cord blood units storage worldwide which are never expected to be transplant. For these reasons, we believe in new uses of AB and CB NK cells from blood banks as immunotherapies against cancers.
2. Umbilical cord blood NK cells present a more stable number of cells per unit than adult peripheral blood NK cells, and they can be stimulated with different interleukins in order to enhance the *in vitro* expansion, their killing activity and survival.
3. Umbilical cord blood NK cells present a higher expression of Nkp46 activating marker than adult peripheral blood in their surface after two week in culture.
4. After trying different cytokine cocktails, we observe than the combination of IL-2 and IL-15 is the best culture condition for freshly isolated adult peripheral blood and umbilical cord blood NK cells.
5. We obtain a large number of mature and functional NK cells fromumbilical cord blood hematopoietic progenitors at 28 days of *in vitro* culture with OP9 co-culture system. This source is very efficient in order to get a huge amount of functional NK cells, that could be storage as an “off the self” product for future clinical uses.
6. We developed an *in vitro* differentiation protocol for obtaining NK cells from umbilical cord blood hematopoietic progenitors thanks to which we were able to the differentiation patron of the NK cells.
7. In our case, we are able to achieve 70–80% of CD56+ cells at 28 days of differentiation but studying in detail the different maturation stages of NK cells, we obtain around 38–45% of CD56+ cells are already in stages 4–5, showing the typical markers of mature NK cells.
8. We obtain higher number and frequencies of CD56+ NK cells than others who have also used OP9 cells as feeder layer.
9. We obtain better or similar results than others when we look at the expression of cytolytic markers, degranulation properties, and killing activity of *in vitro*-generated NK cells from umbilical cord blood hematopoietic stem cells.
10. Comparing the CD19-CAR NK cell clinical trials ongoing up to date, we obtain enough number of allogenic NK cells from adult peripheral blood, umbilical cord blood and umbilical cord blood CD34+ cells to treat patients in the future.
11. In order to obtain new NK cells sources, we differentiate human pluripotent stem cells (hiPS/hES) achieving functional CD34+ cells as a first step in our differentiating protocol.
12. Among all the culture conditions and pluripotency stages, primed hiPS and hES in a feeder-free system are a little bit more efficient at obtaining functional CD34+, than primed hIPS and hES in a feeder system and naïve hiPS and hES. However, we believe that new experiments need to be performed with naïve hiPS and hES, as this pluripotent stage is supposed to be more promp to differetiate.
13. When differentiating CD34+ cells from hiPS and hES into NK cells, we do not obtain great results, in terms of cell numbers, maturity and functionality. Therefore, this protocol must be improved performing new changes on it.

14. Despite the difficulty of infecting NK cells, in our hands we obtain around 40-50% of infection with adult peripheral blood, umbilical cord blood NK cells and *in vitro* generated NK cells from CB CD34+ cells with ARI-0001 CD19-CAR.
15. CD19 expressing target cells are killed more efficiently by adult peripheral blood and umbilical cord blood NK cells transduced with ARI-0001 CD19-CAR than by non-transduced adult peripheral blood and umbilical cord blood NK cells.
16. CD19-CAR infected NK cells from adult peripheral blood and umbilical cord blood kill similarly CD19 expressing target cells. However, we observe a significantly higher degranulation of the adult peripheral blood NK cells against Nalm 6 target cell; and significantly higher killing of CLL target cells at 1:1 ratio.
17. In spite of *in vitro* generated NK cells from CB CD34+ cells being functional against target cells like K562, it seems that when infecting with CD19-CAR ARI-0001, these NK cells do not kill better CD19 expressing target cells.
18. As we believe on introducing to the clinic CAR-NK based treatments, we desired to adaptour CAR more suitable for NK cell signalling. All the new seven variants adapted to NK cells motifs seem to be transduced efficiently into AB NK cells.
19. CAR-NK5 variant kills very similar to ARI-0001 CAR in AB NK cells. Further experiments will determine the best new CD19-CAR variant to kill CD19+ cells from patients in order to translate these preclinical data into the clinic.
20. Even though more experiments need to be performed, we already observe an increase of these NK cells activity with the CD19-CAR NK5 compared to the non-transduced NK cells. This leads us to suggest that CB CD34+ differentiated NK cells could be good candidates to be used as vehicles in CAR based therapies.

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