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Expanded Natural Killer cell-based therapy for hematological cancers. Inmunoterapia basada en células Natural Killer expandidas para canceres hematológicos

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EXPANDED NATURAL KILLER CELL-BASED THERAPY FOR HEMATOLOGICAL CANCERS. INMUNOTERAPIA BASADA EN CÉLULAS NATURAL KILLER EXPANDIDAS PARA CANCERES HEMATOLÓGICOS

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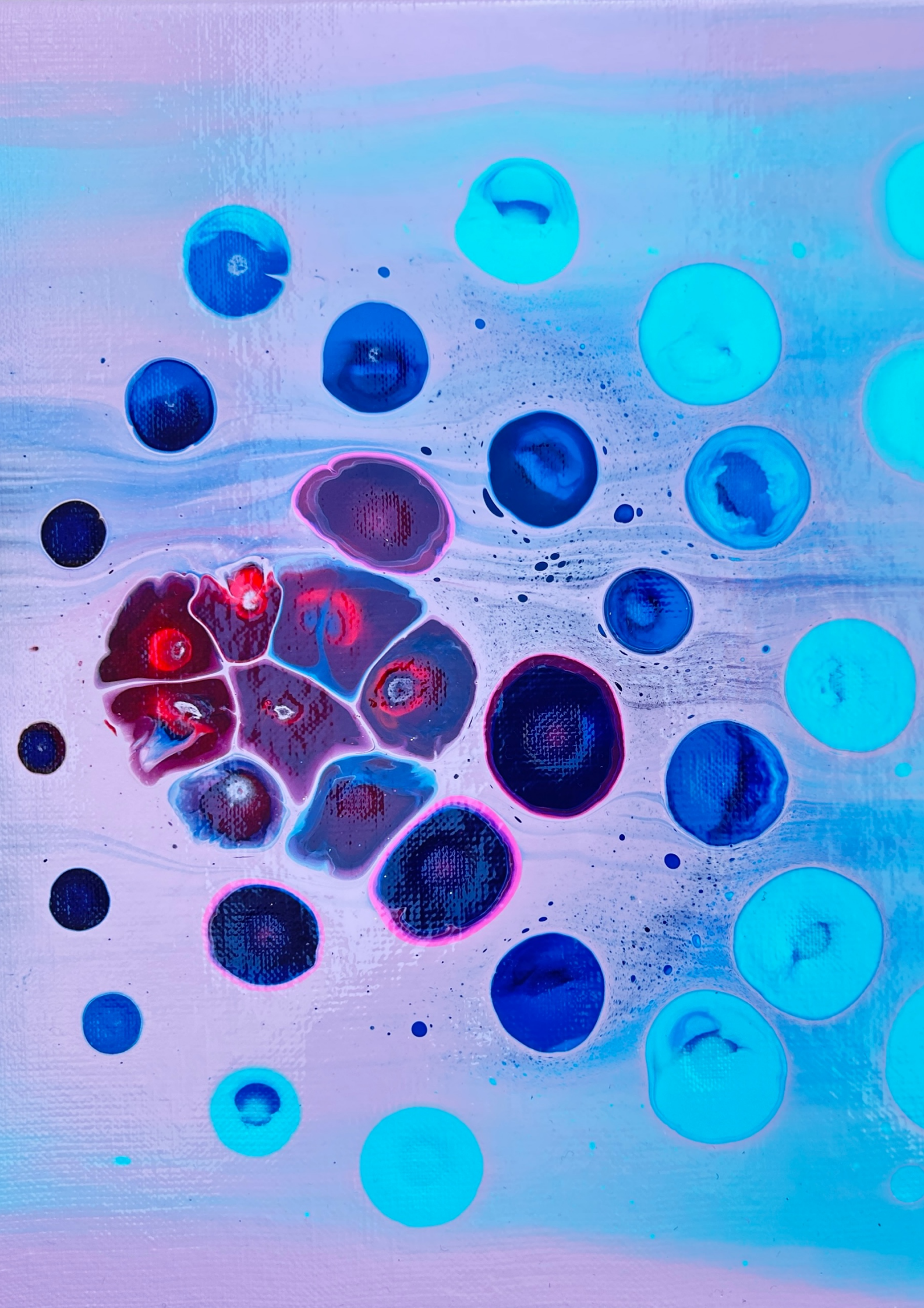

Expanded Natural Killer cell-based therapy for hematological cancers.

Inmunoterapia basada en células Natural Killer expandidas para canceres hematológicos.

Chantal Reina-Ortiz, MSc.

Presents a Doctoral Thesis in the Field of Biochemistry

for the Degree of Doctor of Science in Biochemistry and Molecular Biology

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2023

Certificado

D. LUIS ALBERTO ANEL BERNAL, Catedrático de Biología Celular, adscrito al Departamento de Bioquímica y Biología Molecular y Celular de la Universidad de Zaragoza,

CERTIFICA

Que la memoria de la Tesis Doctoral titulada: “Inmunoterapia basada en células Natural Killer expandidas para canceres hematológicos.”, presentada por Dña. Chantal Reina-Ortiz, ha sido realizada en el Departamento de Bioquímica y Biología Molecular y Celular de la Facultad de Ciencias bajo su dirección y que reúne, a su juicio, las condiciones requeridas para que su autor pueda optar al Grado de Doctor en Ciencias por la Universidad de Zaragoza.

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Reina-Ortiz C, Mozas MP, Ovelleiro D, Gao F, Villalba M, Anel A. **Dynamic Changes in miRNA Expression during the Generation of Expanded and Activated NK Cells.** Int J Mol Sci. 2023 Aug 31;24(17):13556. doi: 10.3390/ijms241713556.

Reina-Ortiz C, Giraldos D, Azaceta G, Palomera L, Marzo I, Naval J, Villalba M, Anel A. **Harnessing the Potential of NK Cell-Based Immunotherapies against Multiple Myeloma.** Cells. 2022 Jan 24;11(3):392. doi: 10.3390/cells11030392.

Dedication

This Dissertation is dedicated to my parents

Luis Reina, MD

and

Desiree Ortiz-Cruz, MD JD

whose ardent faith, boundless love, and unconditional devotion humble and inspire me to ever greater height

Abstract

Natural Killer (NK) cells are an integral member of the immune system capable of mediating the anti-tumor response in several cancers. NK cell cytotoxicity against cancer and other transformed cells involves secretion perforin, granzyme B and pro-inflammatory cytokines such as interferon-γ. However, cancer cells have developed multiple mechanisms, such as down-regulation of activating receptors and recruitment of immunosuppressive cell populations, which enables tumor escape from immunosurveillance. This results in impeded NK cell function and the abrogation of antitumor immune response. NK-cell based immunotherapies have been developed to overcome this immune suppression. In this thesis, we examine the promising therapeutic potential of expanded and activated NK cells and their implications in hematological cancers.

In **paper I**, we began by optimizing our protocol for expansion and activation of healthy human donor NK cells. Healthy human donor NK cells were expanded and activated over the course of 20 day through cultivation with a cytokine cocktail of IL-2 and IL-15 alongside the presence of feeder cells. To verify the effectiveness our expanded NK (eNK) cells, we performed cytotoxicity assays on B-cell chronic lymphocytic leukemia (B-CLL) patient samples ex vivo. We found that B-CLL patients were highly suspectable to NK cell mediated cell death. *De novo* resistance to our eNK cells was observed in samples from two patient taken months later. We concluded lack of cell death was due to increased expression of PD-L1 in these patients, not KIR match inhibition. One of these patients was subsequently treated with idelalisib, a PI3Kδ inhibitor, resulting in loss PD-L1 expression and re-acquiring sensitivity to eNK cell mediated cell death. Resistance to eNK cell lysis due to PD-L1 expression, and abrogation of this resistance with idelalisib treatment was verified in a B-

CLL cell line. Our optimized eNK cells are thus an excellent treatment option for B-CLL patients and should be explored in clinical trials.

Given the success of eNK treatment in B-CLL, we widened our use of eNK cell therapy to multiple myeloma (MM), a clonal plasma cell cancer within the bone marrow in **paper II**. Although introduction of other immunotherapies such as monoclonal antibodies (mAbs) and immune checkpoint inhibitors (ICI) has improved rates of overall survival, MM remains a fatal disease with a high rate of recurrence. Daratumumab, a CD38 mAb, has had great success in clinic. However, it depends on NK cells to participate in antibody dependent cellular cytotoxicity (ADCC) as a main avenue of target cell death. Due to the immunosuppressive tumor microenvironment, NK cells are repressed and have limited ability to carry out ADCC. Using both our previously established peripheral blood eNK (PB eNK) cells and umbilical cord blood eNK (UCB eNK) cells, we performed cytotoxicity assays on bone marrow aspirates of MM patients. The assays were performed alone and in combination with daratumumab to test the potential for a potent combination therapy. We found UCB eNK cells were effective against MM cells as single therapy. However, expression of PD-1 reduced the induction of cell death dramatically, suggesting PD-1 expression is a marker for limited cytotoxic potential in UCB eNK cells. PB eNKs were highly cytotoxic against MM cells when used in combination with daratumumab, presenting an exciting path for future treatment options.

To understand the molecular underpinnings that promote the production of our highly cytotoxic eNK cells, we examined the transcriptomic changes that occurred between the start and end of our expansion and activation protocol. In **paper III**, we compared the changes in

miRNA profiles of eNK cells at day 0 and day 20 of the protocol using RT-qPCR. Of the 64 miRNAs differentially expressed at day 20, 7 were especially significant. MiRs-146a, -124, -34a, and -10a were up-regulated at day 20 and are key in the regulation of cell survival, controlling the post-transcriptional activity of pro-apoptotic genes such as PUMA. The 3 down-regulated miRNAs are miRs-199a, -223, and -340. These perform important functions in the promotion of NK cell cytotoxicity mechanisms, of particular importance when producing effective eNK cell therapies. Identifying vital miRNAs that are involved in the longevity and cytotoxicity of our eNK cells creates additional targets for optimizing production of future eNK cell-based therapies.

Having established the potency of our eNK cells in treatment of hematological cancers, we performed a comprehensive review of the current NK cell-based treatments in clinical trials against MM. In **paper IV**, we examine the wide variety of sources of therapeutic NK cells including autologous NK cells, UCB NK cells, off-the-shelf PB NK cells, NK cell lines. The clinical trials are compared based on their NK cell of origin, side effect profile, and the known clinical benefits, if reported. Due to MM's high rate of recurrence, there is potential use for several NK cell-based therapies based on a patient's treatment history and cytogenetic profile. Taken together, we analyzed the increasing number of NK cell-based therapies being trialed for use against MM and their success against a heterogenous cancer like MM.

In this thesis, we presented our eNK cells and their significance in hematological cancer therapy. NK cells in patients with B-CLL, MM, and other cancers are unable to mount an effective immune response. Our objective was to produce an eNK cell-based

immunotherapy apt for use alone or in combination with therapies, identify the molecular changes that cause their increased cytotoxicity, and eradicate hematological cancer cells by driving a new, potent immune response.

Resumen

Las células Natural Killer (NK) son miembros esenciales del sistema inmune capaces de mediar la respuesta anti-tumoral en varios canceres. La citotoxicidad de células NK contra el cáncer y otras células transformadas involucra la secreción de perforina, granzima B, y citoquinas pro-inflamatorias como el interferón-γ. Sin embargo, células cancerosas han desarrollado múltiples mecanismos, como la disminución de receptores activadores y el reclutamiento de poblaciones inmunosupresoras, que permite el escape tumoral de la inmunovigilancia. Esto impide la función de células NK y la abrogación la respuesta inmune antitumoral. Inmunoterapias basadas en células NK han sido desarrolladas para sobreponer esta inmunosupresión. En esta tesis doctoral, examinamos el potencial terapéutico prometedor de células NK expandidas y activadas y su implicación en canceres hematológicos.

En el **artículo I**, empezamos por optimar nuestro protocolo para la expansión y activación de células NK humanas de donantes sanos. Estas células fueron expandidas y activadas en el transcurso de 20 días a través de cultivación con un coctel de citoquinas de IL-2 y IL-15 junto a la presencia de células 'feeder'. Para verificar la efectividad de nuestras células NK expandidas (eNKs), realizamos ensayos de citotoxicidad en células de pacientes *ex vivo* de leucemia linfocítica crónica de célula B (B-CLL). Encontramos que pacientes de CLL son altamente susceptible a muerte celular mediad por células NK. Resistencias *de novo* a nuestras células eNKs se observó en muestras de dos pacientes adquiridas meses después de la muestra inicial. Nosotros llegamos a la conclusión que falta de muerte celular fue causado por el incremento en expresión de PD-L1 en estos pacientes, no por inhibición debida a 'KIR match'. Uno de estos pacientes luego fue tratado con idelalisib, un inhibidor del PI3Kδ,

resultando en la perdida de la expresión de PD-L1 y readquiriendo sensibilidad a muerte celular inducida por eNKs. Resistencia a lisis celular mediada por eNKs por la expresión de PD-L1 y la abrogación de esta resistencia con tratamiento con idelalisib fue verificado en líneas celulares de CLL. Nuestras células eNK optimizadas son una opción excelente para tratamiento de CLL y debe ser explorada en ensayos clínicos.

Dado el éxito de tratamiento de CLL con células eNKs, ampliamos nuestro uso de terapias eNK a mieloma múltiple (MM), un cáncer de células clonales plasmáticas en la medula ósea en el **artículo II**. Aunque la introducción de otras inmunoterapias como anticuerpos monoclonales (mAbs) e inhibidores de puntos de control inmunológicos (ICI) ha mejorado tasas de sobrevivencia general, MM sigue siendo una enfermedad mortal con alta tasas de recurrencia. Daratumumab, un anticuerpo monoclonal contra CD38, ha tenido gran éxito en la clínica. Sin embargo, daratumumab y otros anticuerpos monoclonales dependen de células NK para participar en citotoxicidad celular dependiente en anticuerpos (ADCC) como avenida central para efectuar muerte celular en la célula diana. A causa del entorno inmunosupresor tumoral, NKs son inhibidas y tiene su habilidad de ejercer a través de ADCC limitada. Usando nuestras células de eNKs proveniente de sangre periférica (PB eNKs) y células eNKs proveniente de sangre de cordón umbilical (UCB eNKs), realizamos ensayos de citotoxicidad en aspirados medulares de pacientes de MM. Los ensayos se realizaron solo y en combinación con daratumumab para verificar el potencial para una terapia combinado potente. Encontramos que UCB eNKs fueron efectivas contra células de MM como terapia única. Sin embargo, la expresión de PD-1 bajo la inducción de muerte celular dramáticamente, sugiriendo que la expresión de PD-1 es un marcador para potencial de citotoxicidad limitada en UCB eNKs. PB eNKs fueron altamente citotóxicas contra células de

MM en combinación con daratumumab, presentando un camino prometedor para futuros tratamientos.

Para entender los fundamentos moleculares que promueven la producción de nuestras eNKs altamente citotóxicas, examinamos los cambios en la transcriptoma que ocurrieron entre el inicio y el final de nuestro protocolo de expansión y activación. En el **artículo III**, comparamos los cambios en el perfil de miRNAs de células eNK a día 0 y día 20 del protocolo usando RT-qPCR. De los 64 miRNA expresados diferencialmente a día 20, 7 fueron significativo. MiRs-146a, -124, -34a, y -10a tuvieron una expresión más alta a día 20 y son claves en la regulación de sobrevivencia celular, controlando la actividad post-transcripcional de genes pro-apoptóticos como *PUMA*. Los 3 miRNAs que bajaron en expresión fueron miRs-199a, -223, y -340. Estos realizan funciones importantes en la promoción de los mecanismos de citotoxicidad de células NK. Es de importancia particular para producir terapias basadas en eNKs efectivas. Identificando miRNAs implicados en la longevidad y citotoxicidad de nuestras eNKs crea dianas adicionales para optimizar la producción de futuros terapias basadas en eNKs.

Habiendo establecido la potencia de nuestras eNKs para el tratamiento de canceres hematológicas, realizamos una revisión exhaustiva de los tratamientos basados en células NKs para el tratamiento MM en ensayos clínicos. En el **artículo IV**, examinamos una amplia variedad de fuentes de NKs terapéuticas incluyendo células NK autólogas, UCB NKs, PB NKs listos para usar, y NKs de líneas celulares. Los ensayos clínicos fueron comparados basados en el tipo de NK usado, el perfil de efectos secundarios, y el beneficio clínico. A causa de la alta tasa de recurrencia en MM, existe la posibilidad de usar varias NKs

terapéuticas utilizando el historial de tratamiento del paciente y su perfil citogenético. En total, analizamos el número creciente de terapias basadas en células NKs en ensayos clínicos para el tratamiento de MM y sus éxitos contra un cáncer heterogéneo como es la MM.

En esta tesis, presentamos nuestras células eNKs y su importancia en terapia contra canceres hematológicos. Células NKs en pacientes con CLL, MM, y otros canceres tienen dificultad en montar una respuesta inmune efectiva. Nuestro objetivo fue producir una inmunoterapia basada en células eNKs apto para uso solo o en combinación con otras terapias, identificar los cambios moleculares que causaron el aumento en capacidad citotóxicas de las eNKs, e erradicar las células de canceres hematológicos, impulsando una nueva y potente respuesta inmunitaria.

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Chapter I

Introduction

Chapter I.

Introduction

Thucydides, the Athenian general and historian, noted during the Peloponnesian War in 430 B.C. "…it was with those who had recovered from the disease that the sick and dying found most compassion… for the same man was never attacked twice." This is considered, by many, the first reflections on a possible system of immunity. Based on his accounts and modern epidemiological analysis, we can be sufficiently certain smallpox caused the Plague of Athens to which Thucydides referred [1]. Countless observations of the same ilk have been made throughout history culminating in the emergence of immunology and the concept of the immune system. Edward Jenner laid the foundation for it in the late 18th century with development of the smallpox vaccine. A century later, groundbreaking work such as germ theory of disease by Louis Pasteur and Robert Koch and cellular theory of immunity by Elie Metchnikoff led to the creation of modern immunology as its own branch of science.

1.1 The Immune System

From these works, the immune system has emerged as an intricate and finely tuned network of tissues, cells, and molecules tasked with safeguarding the host organism against threats. Mounting an effective response to each threat requires a bipartite collaboration between the innate and adaptive immune systems. The innate immune system serves as the body's initial line of defense, providing immediate, non-specific response to invading pathogens. It relies on an array of evolutionarily conserved pattern recognition receptors (PRRs) on innate immune cells to detect pathogen-associated molecular patterns (PAMPs) or self-derived danger-associated molecular patterns (DAMPs) [2-6]. Detection induces the

secretion of pro-inflammatory cytokines and chemokines leading to recruitment of immune cells into the affected area. These innate immune cells are comprised of phagocytic granulocytes and macrophages, antigen-presenting dendritic cells (DCs), and, importantly to this thesis, Natural Killer (NK) cells.

Antigen presentation by antigen presenting cells (APCs) such as DCs is pivotal for the initiation of the adaptive immune response and bridges the gap between it and the innate immune system. In the days that follow initial pathogen detection and the innate immune system response, the activated adaptive immune system mounts an educated and tailored response. It relies primarily on T and B cells. T cells, through their T-cell receptors (TCRs), recognize specific antigens presented by APCs and orchestrate a cellular immune response: cytotoxic T cells directly killing infected cells and helper T cells coordinate immune reactions. B cells, on the other hand, produce antibodies with specific binding sites that recognize antigens with high precision, crucial in humoral immunity. As a lasting line of defense, adaptive immune cells have the ability to generate immunological memory. Upon re-encounter with the same pathogen, memory T and B cells are able to mount a more rapid and potent immune response. This provides specific and long-lasting protection against certain pathogen. It was through research into T and B cells that NK cells were discovered.

1.2 Natural Killer cells, origins

The earliest reports of NK cells are thanks to the study of T cells in leukemia patient samples. Observations were made about lymphocytes exhibiting non-specific reactivity differing from that of known antigen dependent T cells[7, 8]. Previously, any anti-tumor activity of unstimulated lymphocytes was attributed to *in vitro* artifact [9, 10]. These findings

marked the launch of numerous research efforts into defining the role of this novel cell group in relation to cancer and, subsequently, to virally infected cells.

The 1970s marked a critical decade in the discovery and understanding of NK cells. Dr. Ronald Herberman's group at the National Cancer Institute in the United States and Dr. Rolf Kiessling's group at the Karolinska Institute in Sweden concurrently published seminal works defining the existence of NK cells in 1975. Herberman published two papers focused on first identifying the population of "N" cells by their lack of T and B lymphocyte associated cell-surface markers [11]. In the second, they focused on the lytic ability of these cells in mice using *in vitro* cytolytic assays against cancer cells with no prior sensitization [12]. Kiessling's group published similar murine results of spontaneous lysis using Moloney leukemia and lymphoma cells[13, 14]. While other groups termed these lymphocytes "Null", "K", or "N" cells, it was Kiessling's group who coined the broadly accepted phrase "natural killer" [13, 15-17]. These cells were also found in humans and were able to target not only cancer cells, but also virally infected cells [18-22].

The 1980s and 90s continued to yield NK cell studies focused on NK cell cytokine production and Karre's "missing self" hypothesis[23-27]. Research in the following decades continued Herberman and Kiessling's goal to discover more about these naturally killer cells. Below I will present important concepts on NK cells and immunotherapy relevant to my doctoral thesis.

1.3 NK Cell Development

NK cells are generated in the bone marrow (BM) and mature both within the BM and secondary lymphoid organs[28, 29]. They begin as CD34+ hematopoietic stem cells (HSCs) that later transition into the common lymphoid progenitor (CLP)[30, 31]. CLPs are able to differentiate into pre- B, T, NK or Innate Lymphoid Cells (ILCs)[32]. Although the both originate from the CLP, NK cells lack T-cell receptor and CD3 expression on their surface. In order for CLPs to commit to the NK cell lineage and become immature NK cells (iNK), they must express IL-2Rβ (CD122). IL-2Rβ is the shared β receptor chain for binding both IL-2 and IL-15, cytokines critical for NK cell maturation and function[33, 34]. IL-15 is of particular importance during development as NK cell maturation occurs by the expression and upregulation of CD56. Stages of NK cell development are pictured in Figure 1.

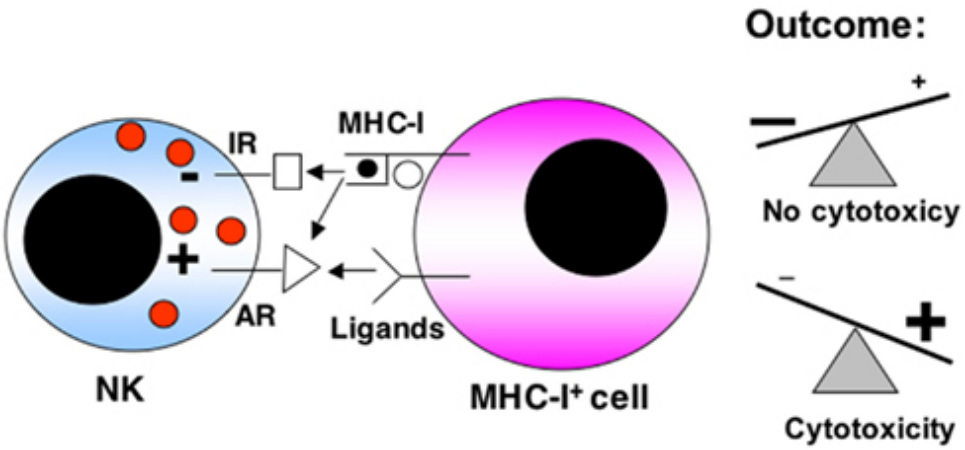

Figure 1. Representation of the balance between activating and inhibitory receptors in NK cells. Anel, 2012

In order to have functional, discriminate NK cells, they must be able to distinguish between self and non-self. The mechanism was proposed by Karre and Ljunggren as the "missing self" hypothesis[27]. It was developed though the observation that murine NK cells were able lyse lymphoma cells from a cell line deficient in major histocompatibility complex-class I (MHC-I) molecules. Conversely, the lymphoma cells which retained their MHC-I

expression were resistant to NK cell lysis. NK cells undergo an "education" process during development whereby they engage with self MHC-I molecules becoming "licensed" and tolerant to self MHC-I[35]. On NK cells, there are two types of inhibitory receptors that engage MHC-I molecules for the purpose of educating NK cells on "self"[36]. During early NK cell development, the inhibitory receptor NKG2A/CD94 heterodimer binds to its ligand, the non-classical MHC-I molecule Human Leukocyte Antigen (HLA)-E[37]. However, mature NK cells heavily rely on engagement through inhibitory Killer Cell Immunoglobulin-like Receptors (iKIRs)[35, 38]. iKIRs bind to classical MHC-I molecules HLA-A, HLA-B, or HLA-C allotypes. Lack of expression of either an NKG2A or iKIR receptor specific for self-MHC-I renders NK cells hyporesponsive and anergic [39, 40]. Only educated NK cells are able to respond competently to target cell interactions, whether it be tolerance to self or activation of NK cell cytolytic functions.

1.4 NK Cell Receptors

To stimulate or inhibit activation, a complex group of inhibitory and activating receptors are present on the surface of mature NK cells. The receptors recognize their respective ligands on the surface of target cells, be they stressed, transformed, or infected cells. NK cell receptors are divided by their functional ability to activate or inhibit NK cell function and according to their structure into either the immunoglobulin superfamily (Ig-SF) or C-type lectin superfamily (CL-SF). When the target cell presents both MHC-I molecules and activating ligands and the NK cell presents both inhibitory and activating receptors, it is the cumulative balance of all signals that determines the fate of the target cell [41]. Figure 2 shows the inhibitory and activating receptors of each family alongside their corresponding ligands. Relevant receptors are detailed below.

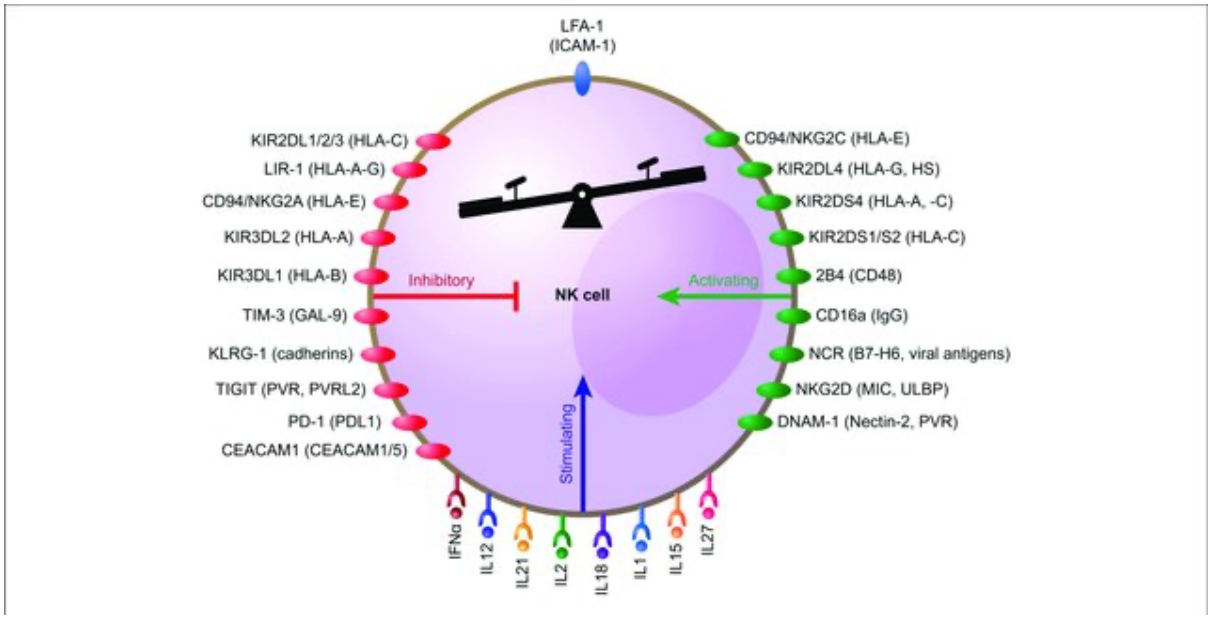

Figure 2. Inhibitory and activating NK receptors with their corresponding ligands. Carlsten, 2019.

1.4.1 Inhibitory Receptors

The first members of the Ig-SF are the aforementioned KIRs. While the "I" in their name initially stood for "Inhibitory", it has since been found that KIRs can be either inhibitory or activating [42]. KIR nomenclature is based first on the number of immunoglobulin (Ig)-like domains in their extracellular region: '2D' for two domains or '3D' for three domains. Second, they are classified by the length of their cytoplasmic tail: either S for short or L for long. **Inhibitory KIRs** (**iKIRs**) have long intracellular tails carrying an inhibitory motif called the immunoreceptor tyrosine-based inhibition motif (ITIM)[43]. When an iKIR recognizes its corresponding ligand (HLA-A,B,C), ITIM phosphorylation is mediated by Src-family kinase (SFK)[44]. Once phosphorylated, ITIMs activate two main protein tyrosine phosphatases: Src homology region 2-containing protein tyrosine

phosphatase-1 (SHP-1) and Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2)[45, 46]. SHP-1 dephosphorylates activating signal molecules thus downregulating activation signals [47, 48]. The substrates targeted by SHP-1 and SHP-2 that have been proposed are Vav1, LAT. Crk is also a target, but it is instead phosphorylated. Further downstream targets that lead to the transient repression of NK cell activation are still being identified. As seen in Figure 2, iKIR receptors include **KIR2DL1-3**, **KIR3DL1** and **KIR3DL2**. Their ligands being HLA-C, HLA-B, or HLA-A haplotypes, respectively.

The central inhibitory receptor family from CL-SF is the NKG2 family which heterodimerizes with CD94. The **CD94/NKG2A** receptor was previously described as integral in NK cell education. As an inhibitory receptor it uses the same signaling mechanism as iKIRs; transducing signals through an ITIM and subsequent dephosphorylation. CD94/NKG2A binds with great affinity to its non-classical HLA-E ligand[49-51]. A CD94/NKG2A antibody has been developed to block its inhibitory signal in hematological malignancies as HLA-E is overexpressed[52]. LIR-1, leukocyte Ig-like receptor 1, also known as ILT2 or CD85j, is an inhibitory receptor from the Ig-SF that binds to the non-classical HLA-G with greatest affinity while also binding to the majority of classical HLA class I molecules with weaker affinity [53]. LIR-1, alongside CD94/NKG2A, is highly expressed on NK cells from maternal decidua [54]. It has also been implicated in the education of NK cells after they undergo expansion [55].

There are a series of non-HLA-specific NK inhibitory receptors that contain ITIMs. T cell immunoglobulin and ITIM domain (**TIGIT**) and **CD96** are inhibitory receptors expressed on T and NK cells[56, 57]. While they share CD155, each have individual ligands. TIGIT ligands also include CD112 and CD113, all part of the nectin and nectin-like protein

family. TIGIT and CD96 act as co-inhibitory receptors competing for binding with DNAM-1, an activating receptor discussed below[58]. Both receptors are being targeted as immune checkpoints to decrease immune escape in several cancers including gliomas and multiple myeloma[59, 60].

1.4.2 Activating Receptors

As with inhibitor receptors, activating receptors on NK cells can be either MHC-dependent or independent. **Activating KIRs** (**aKIRs**) are MHC-dependent and are designated "S" due to their short cytoplasmic tail[61]. Their intracellular region does not contain ITIMs. A charged residue (Lys) is present in their transmembrane region that associates with adaptor proteins containing immunoreceptor tyrosine-based activating motif (ITAM) sequences. KIR2DS1,2 and 4 recognize HLA-C while KIR3DS1 recognizes HLA-B[62]. The adaptor protein for aKIRs is DAP12 which contains a single intracellular ITAM. When an aKIR ligand is bound, ITAM is phosphorylated and tyrosine kinases (Syk or ZAP70) are recruited, leading to a signal cascade, which can result in cytotoxicity and cytokine secretion[63, 64]. Stimulation of a single activating receptor is insufficient to overcome inhibitory signaling (Figure 3). Within the KIR family there exists one outlier: KIR2DL4. While KIR2DL4, by its 'L' designation has a long cytoplasmic tail and carries a single ITIM, it also contains a charged residue in its transmembrane region. Due to these elements, KIR2DL4 is able to deliver both inhibitory and activating signals when bound to its ligand, the non-classical HLA-G[65, 66].

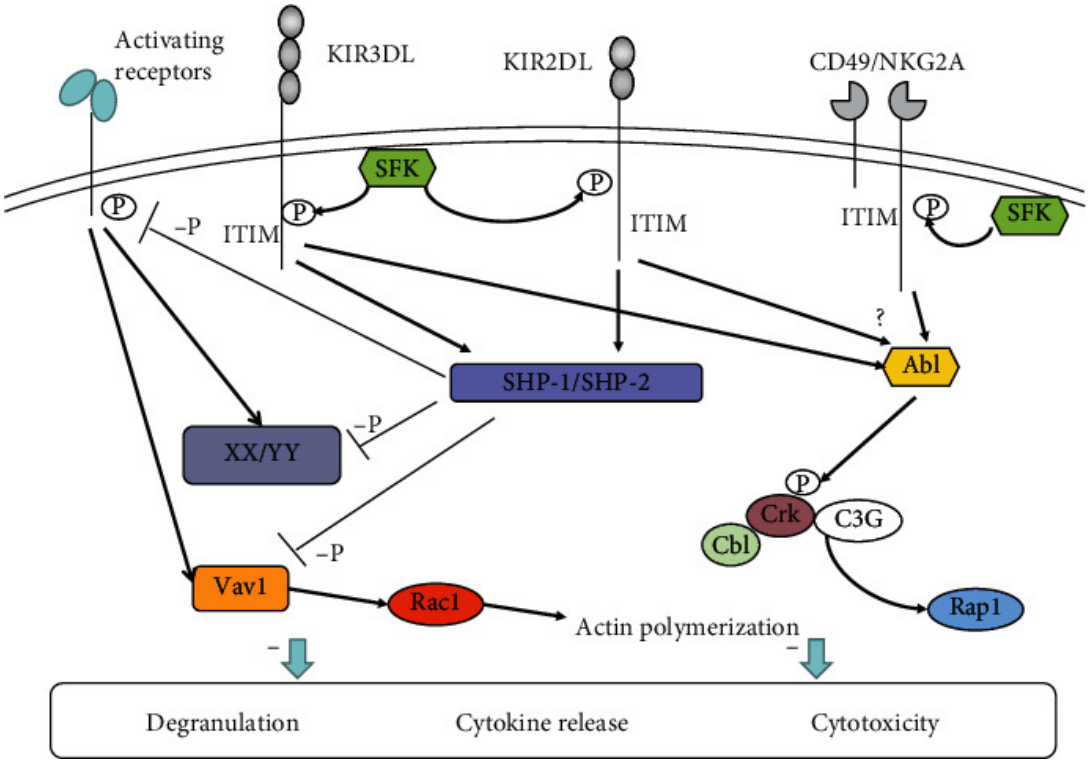

Figure 3. Signaling pathways of inhibitory receptors in NK cells. Chen, 2020

The activating arm of the CL-SF is composed of NKG2 receptors – **NKG2C** and **NKG2D**. NKG2C dimerizes with CD94 and has as its ligand the non-classical HLA molecule HLA-E. As with aKIRs, NKG2C does not contain ITIM sequences but signals through the recruitment of the adaptor protein DAP12. Under stressed conditions, HLA-E binds preferentially with NKG2C, instead of the inhibitory NKG2A, and induces NK cell activation[50, 67]. However, of greater interest in NK-cell control of cancers is the distantly related NKG2D receptor. Expressed as a homodimer, NKG2D noncovalently binds to two adaptor protein DAP10 homodimers forming a hexamer receptor complex[68]. It can recognize multiple ligands such as the stress-inducible MHC class I chain-related gene (MIC)-A and MICB, and ULBP1, ULBP2, ULBP3 and ULBP4[69, 70]. Ligation leads to the

phosphorylation of YINM motif. It is currently the only known signaling motif in the cytoplasmic tail of DAP10[71]. When phosphorylated YINM promotes recruitment of PI3K and the Grb2-Vav1-SOS1 complex[72, 73]. Ca^{2+} influx, cellular degranulation, and secretion of cytokine is eventfully promoted[74]. On their ligands, the cellular stress ligands MICA and MICB are induced upon malignant transformation[75]. MICA can cause both the upregulation of NKG2D and the inhibition of inhibitory receptors[76]. Yet, soluble forms of MICA and ULBPs in the tumor microenvironment suppress NKG2D mediated cytotoxicity[77-79]. Indeed, there needs to be careful balance of NKG2D activation as sustained activation effects the reaction of other activating receptors CD16 and NKp44[80, 81]. This occurs due to the need of NK cell activating receptors to coordinate with each other and form a synergistic activation signal to overcome inhibitory signaling[82].

Alongside KIRs, natural cytotoxicity receptors (NCRs) also belong to the Ig-SF and play a pivotal role in NK cell cytotoxicity. These activating receptors include **NKp30**, **NKp40**, **NKp46** which are named according to their corresponding molecular weight[83-86]. They recognize a wide range of viral, bacterial, parasitic and stress protein ligands. While each has its own set of specific ligands, they all recognize heparan sulfate glycosaminoglycans (HS-GAGs)[87, 88]. The most important cancer related ligands for NKp30 are B7-H6, BAG6, and BAT3[89-91]. NKp44 tumor ligands are proliferating cell nuclear antigen (PCNA), NKp44L, and platelet-derived growth factor DD (PDGF-DD)[92-94]. While known to exist, NKp46 tumor ligands have not been fully described.

NCRs transmit activating signals through ITAM-related receptors, similar to the mechanism used by aKIRs. NKp30 and NKp46 have ITAM containing adaptor proteins FcεRIγ and CD3ζ, while NKp44 contains DAP12[86, 95, 96]. Once phosphorylated by either

Lck or Fyn of the Src family, the ITAM recruits the tyrosine kinases Syk and/or ZAP70 in the cytosol as do ITIMs in inhibitory receptor signaling [97-100]. The activated Syk and ZAP70 leads to downstream phosphorylation and activation of phosphatidylinositol-3-OH kinase (PI3K) complex, PCL-γ and VAV-1/2/3[99, 101, 102]. These are the same molecules dephosphorylated during NK cell inhibition. NKp30 and NKp46 are constitutively expressed by resting and activated NK cells[103]. However, NKp44 is upregulated on activated NK cells, induced by IL-2 and IL-15 stimulation [104]. While most NCR ligands are activating, new evidence has emerged on the role of soluble ligands in the tumor microenvironment that result in the inhibition of these receptors. These include soluble forms of NKp30 ligand BAG6 and BAT3[105, 106].

As previously discussed, **DNAM-1** competes with the inhibitory coreceptors TIGIT and CD96 to bind to CD155 and CD112, particularly in cancers [107-109]. It translates its signal through lymphocyte function associated antigen-1 (LFA-1)[110, 111]. When DNAM-1 binds to its ligand, the tyrosine kinases Fyn and PKC phosphorylate its tyrosine residues. This leads to its association with LFA-1 and binding of DNAM-1 to Grb2[110]. Downstream, PI3K, SLP76, PCLγ1/2, and Vav1 are recruited[111]. It is at this point where inhibitory receptor led dephosphorylation and inhibition would occur[47]. When these downstream targets remain phosphorylated, the AKT and ERK signaling pathways are activated and NK cell cytotoxicity is triggered[112]. DNAM-1 works in tandem with other activating receptors, such as 2B4 and NKG2D, to produce an NK cell response. **2B4** (CD224), is an activating receptor expressed on NK cells, CD8+ T cells and other immune cells. It is part of the CD2-related SLAM family of receptors, as is its ligand CD48[113]. In order to activate degranulation, 2B4 requires a coreceptor, NKG2D or DNAM-1 for example, to then synergize when CD48 is bound[103]. ITSM is then phosphorylated, and SAP is recruited

leading to downstream NK cell activation. However, if SAP is absent, 2B4 produces an inhibitory signal through SHP-1/-2 and downstream phosphorylation of Vav1[114].

Of great importance to this dissertation is **CD16**, the Fcγ III activating receptor[115, 116]. It exists in two forms: CD16A and CD16B. CD16 will be used to refer to CD16A as it is expressed on 90% NK cells while CD16B is only expressed on neutrophils[117]. CD16 binds to the Fc portion of IgG antibodies enabling their role as mediators of antibody dependent cellular cytotoxicity (ADCC)[118, 119]. Upon ligation, CD16 associates with homo- or hetero-dimers of FcRγ and/or CD3ζ adaptor proteins containing ITAMs in their intracellular region[120]. As previously described in other Tyr motif based activating receptors, cross-linking of CD16 leads to phosphorylation of the ITAMs by Src kinase family members, generating docking sites for Syk and ZAP70[121]. Downstream phosphorylation occurs of Vav1, PCL-γ and ERK ultimately resulting in the release of Ca^{2+} and NK cell activation[122, 123]. CD16 can and does work in synergistic pairs with other activating receptors such as LFA-1 to produce a cytotoxic response. However, CD16 is unique in that it does not require co-stimulation to induce a potent series of signals resulting in degranulation[103]. ADCC is able to occur with sole engagement of CD16, making CD16 an important mechanism behind monoclonal antibody (mAb)-based immunotherapies, discussed further [124]. Aside from degranulation, CD16 is involved in survival and proliferation of NK cells and the recruitment of immune cells through the release of cytokines and chemokines[125, 126].

Depicted in Figure 4 are the multiple activating receptors are involved in NK cell activation. While CD16 can function alone, receptor synergy is essential for activating receptors in need of additional stimulation. Neither DNAM-1, NKG2D, nor NCRs are able to

singularly trigger NK cell cytotoxic or cytolytic functions. Activation requires a minimum of 2 receptor types to be activated.

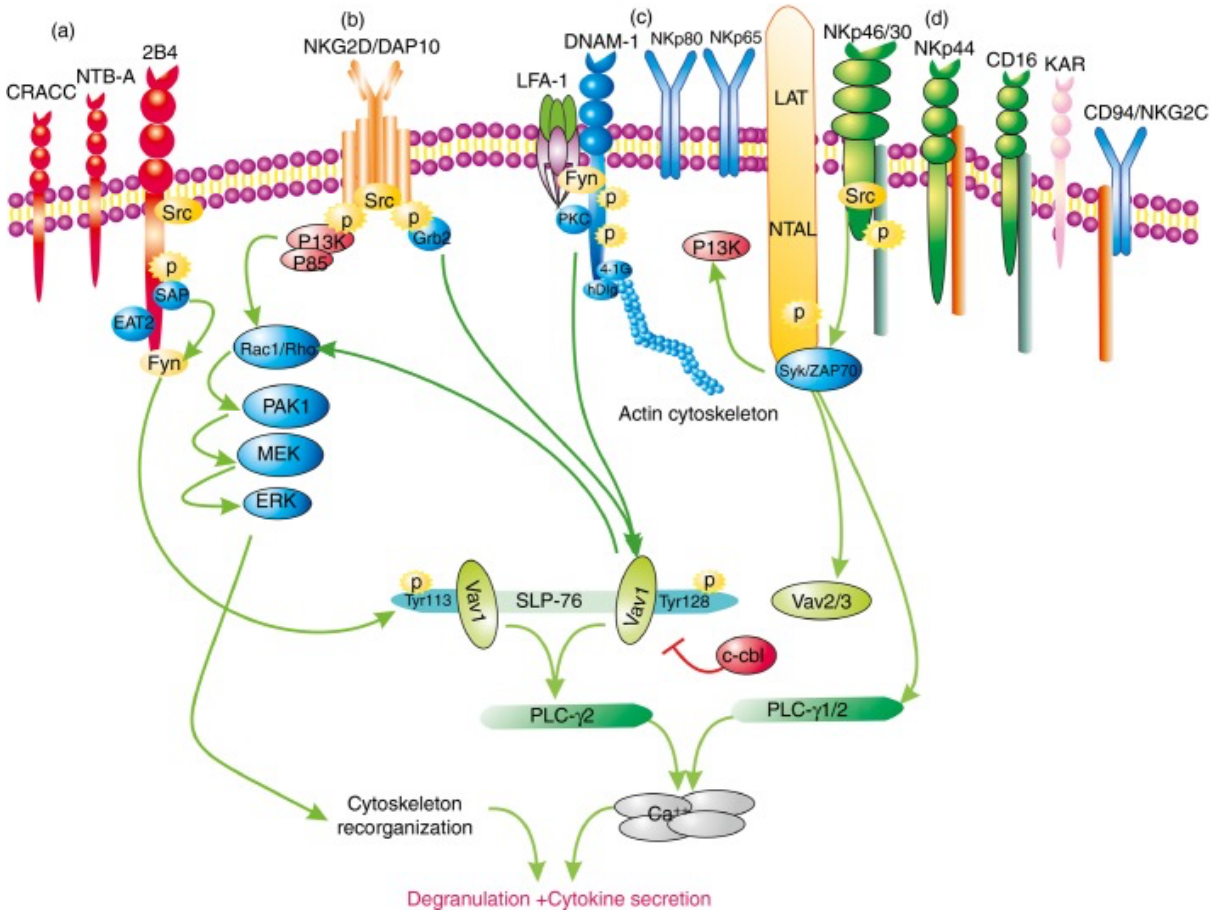

Figure 4. Example of activating receptors in NK cells and their representative signaling pathways. Xiong, 2015.

1.5 NK Cell Subsets

The receptor repertoire expressed on each NK cell varies. NK cells can be typified by their degree of cell surface molecule CD56 expression: $CD56^{bright}$ or $CD56^{dim}$. As the fundamental marker for NK cells, CD56 is universally used to differentiate NK cell

populations. NK cells represent 5-15% of peripheral blood (PB) lymphocytes. Of those, 10% are $CD56^{bright}$ and the remaining 90% are $CD56^{dim}$ [127]. $CD56^{bright}$ NK cells are predominantly found in secondary lymphoid tissue [128]. They both express similar concentrations of common IL-2 and IL-15 receptors, chemokine receptor CXCR4, and activating receptors DNAM-1, NKG2D, and NKp30[33, 75, 89, 103, 129]. These shared aspects allow both subsets to mediate cytotoxicity and produce cytokines but in distinct forms. There is growing debate over the significance of CD56 expression during NK cell development. Although established that NK cells originate from CD34+ HSCs, it has not been categorically confirmed if $CD56^{bright}$ NK cells are premature $CD56^{dim}$ NK cells or an independent branch [130]. Whatever their order, profound functional differences exist between the two populations[131].

1.5.1 $CD56^{dim}$ NK cells

$CD56^{dim}$ NK cells are considered cytotoxic effectors. On their surface, they express high levels of CD16 making them the central mediators of ADCC [132]. They also display a high variety of KIRs. This is coupled with low to variable expression of inhibitory CD94/NKG2A. Within $CD56^{dim}$ NK cells are large quantities of perforin and granzymes A & B enabling their cytotoxic function. Stimulation of $CD56^{dim}$ NK cells with IL-2, IL-15, and IL-18 results in low amounts of cytokine production, when compared to $CD56^{bright}$ cells [133, 134]. Though, when $CD56^{dim}$ NK cells are activated through direct target engagement, they produce copious amounts of cytokines[135, 136]. As the central cytotoxic effector arm of NK cells, they are labelled as the final stage of NK cell maturation.

1.5.2 CD56^{bright} NK cells

CD56^{bright} NK cells are considered the immature, immunomodulatory arm of NK cells. Unlike CD56^{dim} NK cells, they have little to no expression of CD16 and have limited expression of KIRs and activating receptors, making them poor mediators of ADCC. However, their location in secondary lymphoid tissues allows them to interact with dendritic cells and T cells. This leads to NK cell stimulation by cytokines (IL-1β, -2, -15, -18) produced by these cell types[133, 137]. CD56^{bright} NK cells respond by producing large amounts of immunomodulatory cytokines and chemokines such as IFN-γ and tumor necrosis factor (TNF)-α. They constitutively express the high affinity IL-2 receptor and preferentially bind IL-2, inducing rapid activation and proliferation. Priming by IL-2 and IL-15 produces the greatest effect upon CD56^{bright} cells. Of note to this dissertation is the increase in cytotoxic abilities of CD56^{bright} NK cells after IL-2 and/or IL-15 stimulation[138, 139]. This population not only has increased proliferative ability, but also gains the typically CD56^{dim} functions of cellular cytotoxicity including CD16 expression[140, 141].

1.6 NK cell cytotoxicity pathways

NK cells contain two major pathways to induce cell death in a target cell. Once sufficient activating signals are received to overcome inhibitory signaling, a cascade of events occurs. The first and most immediate is the lytic granule dependent cytotoxicity pathway. The second is the death ligand cytotoxicity pathway.

1.6.1 Granule dependent cytotoxicity pathway

In the granule-dependent cytotoxicity pathway, lytic granules containing granzymes and perforin are released from the NK cell into the target cell triggering target cell death. It begins with the formation of the immunological synapse (IS) at the point of contact between the NK cell and the target cell[142]. LFA-1, briefly mentioned in Section 1.5.2 in relation to DNAM-1, is the central adhesion molecule responsible for the firm and stable adhesion of the target cell to the NK cell[110, 143]. Through its association with both the adhesion molecule ICAM-1 on the target cell and activating receptors, it mediates the reorganization of the actin cytoskeleton[144]. The IS is composed of an outer part known as the peripheral supramolecular activation cluster (pSMAC) where LFA-1 and other integrins are located. The cytoskeleton is reorganized in the pSMAC through the accumulation of filamentous actin to create a structure capable of transporting secretory lysosomes to the cell membrane[145, 146]. The inner ring is known as the central activation cluster (cSMAC) and is the area of exocytosis for the secretory lysosomes as it contains little actin. Amassed in the cSMAC are receptors that co-engage and produce synergic signaling as they bind with their ligands on the target cell[147].

Once the IS has been formed and the actin cytoskeleton reorganized, the lytic granules converge on the microtubule organizing center (MTOC) using dynein motor proteins along the microtubules[148, 149]. Both the MTOC and the lytic granules are then polarized towards the IS. Upon their arrival to the IS, the lytic granules dock and tether to the cell membrane with the help of small GTPase Rab27a and Munc13-4 proteins [150-152]. Munc13-4 also helps prime lytic granules for fusion through the promotion of the N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex formation. The SNARE complex allows a fusion pore between the lytic granule and NK cell membrane

to form[153]. The contents of the lytic granule are released into the synaptic cleft, completing the degranulation process.

Lytic granules contain granzymes, perforin, granulysin, and small peptides. Perforin is, as the name suggests, a pore forming protein that is able to bind membranes in a calcium dependent manner[154]. Once released into the synaptic cleft, the neutral pH and physiological levels of Ca2+ allow perforin to form pores in the target cell plasma membrane and allow granzymes entry into the cytosol[155, 156]. This manner of allowing granzyme entry into the target cell is highly debated. The other pathway supports perforin and granzymes entry into the target cell through endocytosis, and perforin-dependent granzyme delivery from endosomes to the cytoplasm [157]. Within the cytosol, granzymes begin to induce the cascade of cell death through different mechanisms.

Granzymes are soluble proteins with a globular structure that belong to a family of serine proteases. Humans have granzymes A, B, H, K, and M. They are synthesized as proenzymes and are inactive until the cysteine proteases cathepsin C or H remove their inhibitory dipeptide while localized in the lytic granule. Cathepsin C has been shown to be key in **granzyme B** (**gzmB**) activation within NK cells[158]. Once within the target cell, granzymes cleave intracellular substrates leading to different types of cell death [159]. GzmB has the highest cytotoxic potential. It cleaves several intracellular substrates such as caspase-3 and caspase-7 resulting in their activation and induction of caspase-dependent cell death. GzmB also induces cell death by cleaving the BH3-only protein Bid. The truncated form of Bid (tBid) relocates to the mitochondria where it disrupts the mitochondrial membrane through interaction with proapoptotic proteins Bax and/or Bak [160]. In turn, cleavage of Mcl-1 leads to the release of the pro-apoptotic Bcl-2 family member Bim[161]. This

culminates in the release of cytochrome c from the intermembrane space of mitochondria, formation of the apoptosome, and caspase-9-activation of executor caspase-3 [162]. This pathway has also been demonstrated by using CTL from granzyme A (gzmA) knockout (KO) mice [163, 164]. GzmB can also activate other forms of caspase-independent cell death through direct cleavage of the inhibitor of the caspase activated DNase (ICAD), or through cleavage of gasdermin E, causing pyroptosis [165, 166].

Granulysin is part of the saposin-protein family. It exists in two forms – 9kDa and 15kDa[167]. The smaller form is contained within lytic granules while the larger form, the originally synthesized form, is secreted by cytotoxic cells such as NKs and CTLs[168]. It preferentially disrupts membranes lacking in cholesterol such as those found in microbes. It participates in NK cell killing of mycobacteria, alongside perforin[169]. Several studies have shown the cytotoxic ability against tumor cells of recombinant granulysin and have the mitochondrial apoptotic pathway as the main cell death mechanism [170-172].

1.6.2 Death ligand cytotoxicity pathway

NK cells are also able to exert cytotoxicity through death ligand expression [173]. Upon activation, NK cells express Fas ligand (FasL), TNF-α, and TNF-related apoptosis-inducing ligand (TRAIL) – all members of the TNF superfamily[174]. They induce apoptosis through engagement with their target cell ligands - CD95 (APO-1/Fas), TNF-R1/-R2, and TRAIL-RI/-R2, respectively[175]. FasL is a type II transmembrane protein that is synthesized in the endoplasmic reticulum (ER) and, in NK cells, is stored in lytic granules similar but separate to those containing perforin and granzymes[176, 177]. During NK cell degranulation, FasL is either expressed on the surface of NK cells, cleaved by

metalloproteases or secreted in association with exosomes [178-180]. Soluble FasL can inhibit the activation of membrane bound FasL[181]. Membrane bound FasL engages with its ligand CD95 on the target cell and recruit the adaptor protein Fas-associated death domain (FADD). FADD alongside the death-inducing signaling complex (DISC) binds procaspase 8. From there, two apoptotic pathways open. Either DISC activates sufficient quantitates of caspase 8 to directly activate caspase 3 or insufficient quantities of caspase 8 were activated and are unable to directly activate caspase 3 and must cleave Bid and go through the mitochondrial apoptotic pathway.

TRAIL has similar properties to FasL and can bind to 4 distinct membrane receptors although only the above-mentioned TRAIL-R1/R2 induce apoptosis[182]. Soluble TRAIL is effective only on a limited number of cancer cell lines while the bioreactivity of membrane bound is significantly higher as TRAILs activity is increased if bound to the membrane[183-185]. TRAIL is expressed on activated NK cells and mediate apoptosis through DISC's recruitment of caspase 8, like FasL[186, 187]. Under specific conditions, both FasL and TRAIL can induce necroptosis [171]. TNF-α also forms multi-protein complexes – Complex I, IIa, and IIb – and induces apoptosis on sensitive tumor cells through caspase-8 activation[186, 188]. An important difference between granule cytosis and death ligand pathways is the timing. Lytic granule-based apoptosis occurs within minutes of contact with the target cell while death ligand-based apoptosis occurs within 1-2 hours[177]. In serial killing of target cells, the first target cells encountered by an NK cell are lysed using granule mediated killing through granzyme B. The same NK cell switches to death-receptor mediated killing on later target cells[189]. This is of particular importance in NK cell-based therapies and their lasting activity against target cells.

1.7 MicroRNAs in NK cells

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding RNAs that post-transcriptionally modify gene expression. A single 22 nucleotide (nt) long miRNA is capable of targeting hundreds of genes simultaneously[190, 191]. miRNAs begin in the nucleus as pri-miRNAs that are cleaved into pre-miRNAs and exported into the cytoplasm[192, 193]. In the cytoplasm, pre-miRNAs are processed into double-stranded RNA within the Dicer complex, resulting in a guide strand and a passenger strand[194]. The guide strand is incorporated into a miRNA-induced Silencing Complex (miRISC) along with Dicer and Argonaute (Ago) proteins[195, 196]. The mature miRNA engages with the target mRNA and repress the translation or degrade the transcript of their target mRNA by binding to the four 3' untranslated regions (UTRs). Formation of the miRNA:mRNA complex silences expression of targeted genes[197].

miRNAs vary across cell types and development stages. In NK cells, miRNAs mediate cell development, proliferation, survival, and cytotoxic effector functions. NK cells deficient in miRNAs exhibit impaired proliferation and reduced survival, however they have increased production of IFN-γ[198, 199].

Of the thousands of known miRNAs, there are several that have proved essential to NK cells. Figure 5 summarizes some of the currently known miRNAs found to influence NK cell development and function (it also includes murine miRNAs). MiR-181 inhibits Notch signaling through targeting of Nemo-like Kinase (NLK), promoting differentiation of lymphoid progenitors to mature NK [200]. During the terminal stages of maturation, miR-

146a targets KIR2DL1 and 2, downregulating these inhibitory receptors and modulating the NK cell receptor repertoire[201].

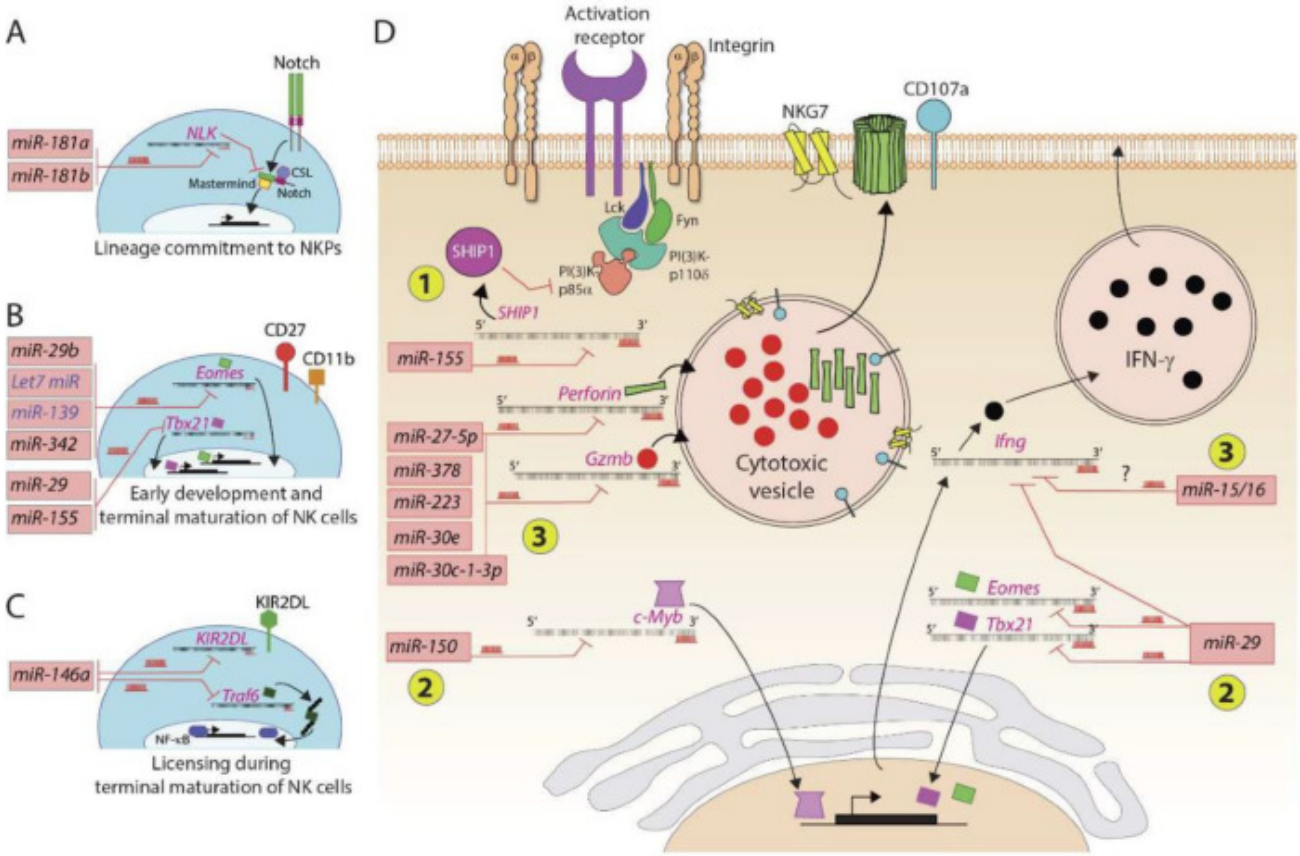

Figure 5. Human and murine miRNAs that regulate the development and function of NK cells. Nanbakhsh, 2021.

Once matured, the effector functions of NK cells are also affected by miRNAs. miR-155, induced by IL-2, IL-15, and/or IL-18, targets SHIP-1, increasing both NK cell cytotoxicity by downregulating an essential part of inhibitory signaling and increases production of IFN-γ[202]. Several miRs can also have the same target. GzmB is negatively regulated by miR-27a-5p and miR-378, inhibiting NK cell cytotoxic activity in resting NK

cells[203, 204]. The activity of perforin (Prf1) is also negatively regulated in resting cells by miR-27a, miR-30e, and miR-150[203-205]. As importantly, miR-23a downmodulates the expression oof cathepsin C in NK cells, inhibiting gzmB maturation and NK cell cytotoxicity [158]. Different types of NK cells also have different miRNA signatures. Pesce et al identified 108 miRNA signatures able to discriminate $CD56^{bright}$ from $CD56^{dim}$ NK cells [201].

1.8 Hematological cancers

Hematological cancers are a diverse group of malignancies that originate from the lymphohematopoietic system. They are characterized by the clonal proliferation of blood-forming cells within the blood, bone marrow, and lymph nodes. These cancers, aptly referred to as blood cancers, can be categorized by their cell of origin: either lymphoid, from lymphocytes and lymphoid progenitors, or myeloid, from myeloid cells within the bone marrow. Each has distinct characteristics and subtypes. Lymphoid neoplasms are further subdivided into precursor lymphoid neoplasms (B & T cell lymphoblastic leukemia/lymphoma), mature B-cell neoplasms (e.g., follicular lymphoma, chronic lymphocytic leukemia, and multiple myeloma), and Hodgkin lymphomas. Myeloid neoplasms have extensive subclassifications including acute myeloid leukemia, chronic myeloid leukemia and myeloproliferative syndromes.

Genetic mutations, environmental factors, and immune dysregulation contribute to onset and progression of these cancers. According to the 2023 American Cancer Society report, hematological cancers represent an estimated 9.4% of all cancer deaths[206]. The average age of sufferers ranges from 65 to 70 years old and afflicts a higher proportion of

men. With more than 180,000 new diagnoses per year, effective therapies are needed. Due to the highly heterogeneous nature of these cancers, traditional treatments have neither cured nor prevented relapse and resistance. The introduction of immunotherapies such as monoclonal antibodies (mAbs) and cellular therapies have given rise to promising outcomes in clinic.

The history of cancer immunotherapy is deeply entwined with hematological cancers (summarized in Figure 6). The first allogeneic hematopoietic stem cell transplant (HSCT) was done in 1957 on patients with hematological cancers ranging from chronic myelogenous leukemia to multiple myeloma[207]. Performed by E. Donnall Thomas, this began a life-long exploration into cell transplantation therapies, for which he earned the Nobel Prize in Physiology or Medicine in 1990. Rituximab, an anti-CD20 mAb, was the first mAb approved by the FDA for follicular lymphoma [208]. Chimeric antigen receptor therapies were also first used in B-cell lymphomas [209]. The unique features of these cancers such as direct access to target cells through easy tissue access and immune responsiveness make them ideal candidates in which to trial immunotherapies. This dissertation carries on the tradition of developing immunotherapies for hematological cancers – focusing on B-cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM).

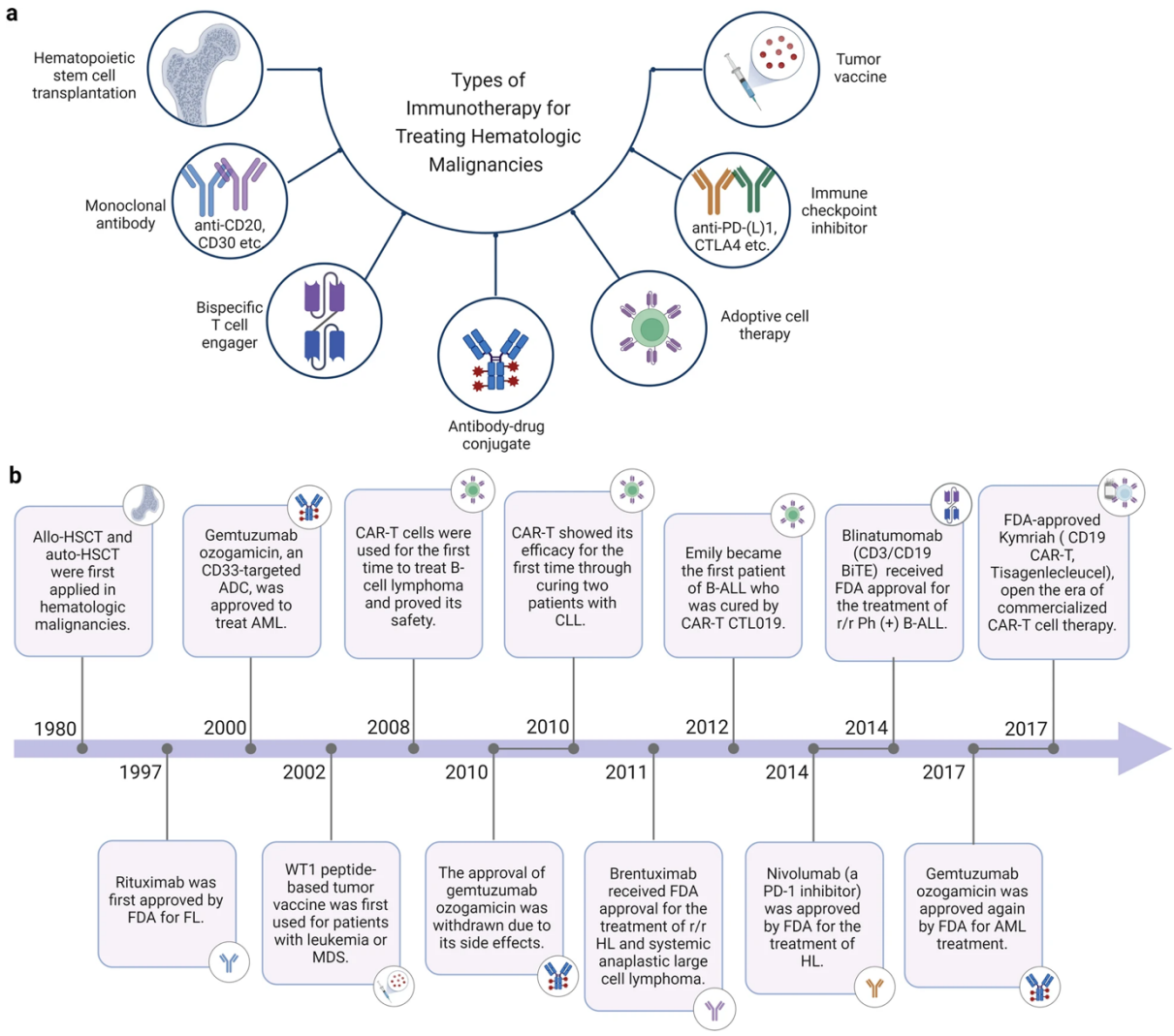

Figure 6. Timeline of immunotherapies for the treatment of hematological cancers. Zhang 2020.

1.8.1 B-cell chronic lymphocytic leukemia

B-cell chronic lymphocytic leukemia (interchangeably abbreviated B-CLL or CLL) is a clonal proliferation and accumulation of mature, antigen-experienced B cells within the blood, bone marrow, lymph nodes, and spleen. CLL is the most frequent type of adult leukemia in the western world, representing 37.9% of all leukemias and 1.3% of all newly diagnosed cancers according to the latest data in the SEER Explorer report. The median age

of diagnosis is 70 years old with men affected at almost twice the rate as women (1.9:1)[210]. Although CLL is generally diagnosed in older populations, there has been an increase in asymptotic diagnoses in younger populations due to increase in blood screenings [211]. The 5-year survival rate went from 68% between 1975-1977 to 91% between 2012-2018 [212]. This statistically significant increase in 5-year survival rates indicates progress has been made in treatment options. However, with over 4,000 deaths estimated this year in the United States, a definitive cure has yet to be developed [211].

1.8.1.1 Diagnosis

The majority of CLL patients are asymptomatic at time of diagnosis. As such, CLL is usually diagnosed through routine blood tests, requiring the sustained presence of $\geq 5 \times 10^9$/L B lymphocytes in the peripheral blood for at least 3 months[213]. In blood smears, the leukemia cells are small, mature lymphocytes characterized by a dense nucleus, no discernable nucleoli, surrounded by a narrow strip of cytoplasm. These morphologically typical CLL cells can also contain larger atypical cells mixed within whose presence may indicate a more aggressive form of CLL. However, there must be fewer than 55% of these atypical cells, also known as prolymphocytes, or the diagnosis may change from CLL to prolymphocytic leukemia[214]. It can also be diagnosed by the presence of small lymphocytic lymphoma (SLL), its non-leukemic variant [213]. Of the 5-10% of patients who present with symptoms (fever, unintentional weight loss, extreme fatigue, and no evidence of infection), more than half are found with localized/generalized lymphadenopathy and splenomegaly upon physical examination[213]. Presence of skin lesions (leukemia cutis) are indicative of disease as the skin is the most common non-lymphoid tissue affected in CLL[215].

1.8.1.2 Origins and classification

Currently, there is no consensus on the normal cell counterpart of CLL B-cells or whether they are derived from a single or multiple B-cell types. Data suggest CLL may originate in the early stages of hematopoiesis due to presence of the same genetic characterizations in mature CLL B-cells as CD34+ HSCs [216, 217]. Accordingly, CLL cells express the B-cell associated antigens CD19, CD20, and CD23, to varying extents. The mature B and T cell antigen CD5 is also co-expressed along with either κ or $\boldsymbol{\lambda}$ immunoglobulin light chains[218]. Altogether, CLL cells can be identified through phenotyping peripheral blood lymphocytes using flow cytometry, looking for the expression of CD5, CD19, CD20, CD23, and κ or $\boldsymbol{\lambda}$[219].

Not only do CLL cells express B-cell associated antigens, but they can also be divided on the basis of the presence or absence of mutations in the immunoglobulin heavy chain variable region (*IGHV*) genes[220-222]. *IGHV* genes encode part of the clonotypic B-cell receptor (BCR) and remain stable throughout the life of each CLL clone, regardless of disease progression[223]. CLL with no or limited somatic hypermutations (SHM) in IGHV genes are considered unmutated (*IGHV*-UM) while CLL with significant SHM load is considered mutated (*IGHV*-M). *IGHV*-M CLLs come from antigen-experienced B cells that have passed through the germinal center of secondary lymphoid organs. The source for *IGHV*-U CLLs has not been so clearly defined. It has been proposed they come from either naïve B-cells that have yet to go through the germinal center or antigen experienced B-cells independent of the germinal center. BCR signaling also differs as *IGHV*-M cells have strong autonomous BCR-BCR signaling resulting in low levels of proliferation and anergy[224]. Conversely, *IGHV*-UM, due to different antigenic pressures when selecting the leukemic

clone, have weak autonomous BCR-BCR signaling, high BCR responsiveness, and increased proliferation[225, 226]. As such, *IGHV* mutational status is an excellent predictor of the clinical course of CLL and its response to chemoimmunotherapy regimens. *IGHV*-M patients have longer time till first treatment, higher probability of indolent disease, and longer progression free survival (PFS) after chemotherapy and/or immunotherapy[227]. Conversely, *IGHV*-UM patients are associated with aggressive CLL, high probability of Richter's syndrome development, shorter time till first treatment, and worse PFS when treated with chemoimmunotherapy[228]. *IGHV* mutational status, coupled with other cytogenic mutations, described below in section 1.8.1.3, create multiple subsets of CLL classification with distinct clinical and biological associations[229]. Although useful in predicting the PFS after chemotherapy/immunotherapy, *IGHV* mutational status has limited prognostic impact when BCR/BCL2 inhibitors are used, necessitating other predictors[230, 231].

1.8.1.3 Genetic lesions

Beyond cell surface markers, CLL can also be defined through several genetic aberrations that have been found in over 80% of untreated CLL patients. These include both copy number variations and recurrent gene mutations. Fluorescence *in situ* hybridization (FISH) assay is used to detect chromosomal abnormalities in peripheral blood smears[232]. Patients usually exhibit one of four chromosomal aberrations[233]. The most common is 13q14 deletion which is found in 55% of cases. Next is chromosome 12 trisomy in 15%, 11q22-23 deletions in 12%, and 8% of cases have 17p12 deletions[233]. These cytogenic abnormalities can correspond to clinical outlook. Although cases with 13q14 deletion have a heterogeneous clinical course, it is associated with a good prognosis. The minimal deleted region contains the genes for *miR-15a* and *miR-16a* miRNAs which regulate apoptosis and

cell cycle[234, 235]. Deletion of these in CLL leads to post-transcriptional negative regulation of BCL2, an anti-apoptotic protein, resulting in BCL2 overexpression [236, 237]. Trisomy 12 is associated with atypical morphology, high rates of proliferation and has a mid-range OS[238]. Patients with trisomy 12 usually die of secondary cancers or Richter transformation[239]. Richter transformation is when CLL or SLL transforms into a more aggressively malignant disease, typically diffuse large B-cell lymphoma (DLBCL). Although genes and miRNAs relevant to its pathology are still being explored, NFAT signaling pathway and NT5E (CD73) have been found to be possible targets[240, 241].

The two high-risk cytogenetic abnormalities are 11q deletions and 17p deletions. The 11q chromosome contains the ataxia-telangiectasia mutated (*ATM*) gene and occurs increasingly after first treatment and upon relapse. 11q deletion is associated with intermediate-risk disease and a short time till progression[242]. *ATM* is a member of the PIKK PI3K-like protein kinase(PIKK) family and mobilizes the response to DNA damage, allowing for cell repair of double stranded breaks through the phosphorylation of tumor suppressor p53 protein (p53)[243]. p53 plays a key role in preventing damaged cell accumulation, cell cycle control, and apoptosis. CLL, a cancer founded upon damaged cell accumulation, is associated with the functional loss of p53. Deletion of 11q and thus *ATM* loss abrogates p53 phosphorylation resulting in compromised proapoptotic response to DNA damage and prevents cell cycle arrest[244, 245].

17p deletion also disrupts the p53 pathway. Deletion of 17p typically involves loss of the whole short arm of chromosome 17, including the locus for *TP53* gene[246]. Patients with this chromosomal aberration have significantly shorter overall survival (OS) than those with the preceding 3 aberrations[233]. It is the most important prognostic marker, alongside

TP53 status and is used for treatment decisions.[247] Standard chemotherapies are not used when a patient presents with 17p deletion as it conveys resistance and results in short PFS, and OS. Thus, first line treatment for these patients would not include the standard regimen.[248]

Another lesion of note is *NOTCH1* lesions increase in frequency as disease stage progresses and in patients with trisomy 12 culminating in a negative association with OS[249]. It is also used as an independent predictor of survival, particularly in *IGHV*-UM CLL[250]. Of note, patients with *NOTCH1*- mutated CLL cells show limited benefit from anti-CD20 therapies as these cells have decreased expression of CD20 on their surface[251, 252].

1.8.1.4 Disease progression

CLL pathogenesis is preceded by $CD5^+$ B cells being continuously activated due to onset of genetic mutations. This is defined as monoclonal B-cell lymphocytosis (MBL), a CLL precursor. During the course of MBL, the genetic mutations accumulate in mature B cells within the lymph nodes. It is defined by an absolute lymphocyte count of less than 5,000/mcL. Patients typically present no symptoms and are monitored for any changes in lymphocyte count. Once sufficient number of mature B cells accumulate with mutations, and the criteria in either Binet or Rai systems are met, MBL is considered to have transformed into CLL. Standard practice uses either the Binet, Rai, or the novel CLL-IPI staging systems to classify CLL and dictates treatment based on factors such as genetic risk stratification, progressive lymphocytosis, and physical symptoms. In the late or advanced stages of the disease, 5% of CLL cases develop into DLBCL, an incidence known as Richter's

transformation[253]. This indicates a worse prognosis and requires a different treatment regiment, as it is classified as new onset of a different cancer[254].

1.8.1.5 Treatment

CLL treatment has substantially progressed in recent decades. The majority of patients are asymptomatic at diagnosis. They receive no active treatment and are maintained under vigilant observation. Of those, one third will never require treatment. The remaining two-thirds, when clinical symptoms develop, are treated based on cytogenetic status. Mutations in *IGHV* genes and the above-mentioned disruption in *TP53* gene are two molecular somatic changes of greatest relevance in CLL treatment selection. Patients with *TP53* disruption are associated with chemotherapy failure and are thus treated through alternate methods. Conversely, patients with the *IGHV*-M have been identified as better responders to combination therapy with fludarabine, cyclophosphamide, and rituximab. This is due to *IGHV* mutation status, whether unmutated or mutated, influencing BCR signaling.

Classic standard treatment for symptomatic patients is chemoimmunotherapy. It is a combination of chemotherapeutic agents fludarabine and cyclophosphamide with the immunotherapeutic agent rituximab – all together known as FCR regime[255]. Rituximab is a chimeric monoclonal antibody (mAb) that binds to CD20 and is used in most B cell malignancies. As a mAb, it is able to trigger ADCC and the complement system, inducing elimination of the target cell ($CD20^+$ CLL cells in this case). Alternative anti-CD20 mAbs now include obinutuzumab and ofatumumab. Patients with *IGHV*-M benefit from FCR while those with *IGHV*-UM, 17p deletion, 11q deletion, and mutated *TP53* did not[256]. It is

understood they are better suited to therapies which do not require functional DNA repair machinery, rendered useless with the above mentioned p53 related mutations.

For patients with these high-risk mutations, ibrutinib is used as first line monotherapy treatment. Ibrutinib is a Bruton tyrosine kinase (BTK) inhibitor meaning it binds to BTK with high affinity resulting in the inhibition of BCR signaling as BTK is located downstream of the BCR. Inhibition also leads to decrease in B-cell activation and induction of apoptosis, circumventing the proapoptotic mechanism activated by CLL high-risk mutations. Ibrutinib is also approved for use in relapsed/refractory CLL post FCR[257]. Also approved for R/R CLL patients are idelalisib and duvelisib, phosphoinositide 3-kinase (PI3K) inhibitors. Idelalisib inhibits the δ-isoform of PI3K while duvelisib inhibits both the δ- and γ-isoforms. Idelalisib is also approved in combination with rituximab. PI3K pathway is essential for cell growth survival and metabolism and its inhibition can induce apoptosis and/or cell cycle arrest[258]. It is also implicated in the maturation and activation of immune cells such as T regulatory (Tregs) cells. PI3K inhibition through idelalisib treatment can suppress Tregs and promote activation of cytotoxic lymphocytes.

BCL2 protein is overexpressed in CLL, particularly in patients with 13q14 deletion, increasing the cell's resistance to apoptosis[259]. A series of BH3 mimetics have been developed that antagonize BCL2[260]. One such approved BH3 mimetic for use in CLL as a first line monotherapy or in combination with rituximab in R/R is venetoclax[261]. It has shown to cause deep remission in especially high-risk groups such as 17p deletions as it induces apoptosis of CLL cells in a *TP53*-independent manner[262]. Due to the high degree of heterogeneity in CLL and the multiple combinations of genetic lesions, treatment regimens can seem complex.

CAR-T cell therapy has shown great promise in several hematological malignancies, particularly for $CD19^+$ B cell cancers[263]. There are several ongoing clinical trials with CD1 CAR-T cells in CLL, although none have been approved for use by the EMA or FDA[264]. Phase1/2 trials have shown these CAR-Ts to be effective in high-risk patients with 100% PFS in certain subsets of patients at the 6-month follow-up[265]. Due to a better side-effect profile, CAR-NK based therapy is also gaining traction [266].

In order for cancer to grow and evolve, it must first develop mechanism by which to escape immunosurveillance. One such mechanism is the activation of immune checkpoint pathways that abrogate the immune response. Within the tumor microenvironment (TME) infiltrated immune cells are suppressed, including NK cells, by way of inhibitory immunoreceptors on their surface. Named immune checkpoints (IC), they include PD-1, CTLA-4, LAG3, TIM3, TIGIT, and BTLA. They rely on ITIM and ITSM to deliver inhibitory signals to the immune cell. In order to block these ICs, immune checkpoint inhibitors (ICIs) have been developed. Their development caused a paradigm shift in immunotherapy of such impact that the 2018 Nobel Prize in Physiology or Medicine was awarded to James P. Allison and Tasuku Honjo for their discovery of cancer therapy by inhibition of negative immune regulation[267].

ICIs are blocking antibodies that prevent ligand-receptor engagement. This can then allow for immune cell activation and, in turn, inhibit immune evasion and suppression. MAbs directed against PD-1 include pembrolizumab and nivolumab. Those directed against PD-1's ligand (PD-L1, present on transformed cells and certain immune cells, are atezolizumab, avelumab, and durvalumab. Both types of mAbs have been employed in several cancers such

as triple-negative breast cancer, cervical cancer, and cutaneous squamous cell carcinoma[268-270]. In CLL, PD-1 has been found to be overexpressed on T cells while PD-L1 is expressed by CLL cells. PD-1/PD-L1 crosstalk contributes to immunosuppression through several mechanism including blocking T cell secretion of IFN-gamma, skewing T cell response. In cases of Richter's transformation, blocking PD-1 seems helpful in a variable set of patients[271]. Given the success of both PD-1 and PD-L1 mAbs in other cancers, they continue to be an avenue of interest in CLL. However, there is limited data on the effects of PD-1/PD-L1 on NK cell function in the TME, besides NK cell cytolytic dysfunction[272].

1.8.2 Multiple Myeloma

Multiple myeloma (MM) is an incurable malignancy characterized by the neoplastic proliferation of clonal plasma cells within the bone marrow (BM). These post-germinal center B cells acquire and accumulate genetic mutations over time leading to growth advantages in certain subclones. Progression of MM is due to both the clonal evolution of subclones and the ever-changing tumor microenvironment[273]. The clonal plasma cells accumulate within the BM eventually migrating to extramedullary sites as disease progresses[274]. MM is characterized by the continuous secretion of a monoclonal proteins designated M proteins, found in serum and urine. MM cells can be identified based on their expression of CD38 on their surface and, to a more variable extent, CD138.

Clinically, patients can present with anemia, elevated levels of creatinine, fatigue, and weight loss. Almost a third of patients have hypercalcemia caused by bone demineralization resulting in increased urination, thirst, and abdominal pain. Bone pain can stem from osteolytic lesions leading to spinal cord compression and kyphosis [275].

After leukemia, MM is the second most common hematological cancer representing 1.8% of all newly diagnosed cancers in 2023 [206]. Demographically, MM is slightly more prevalent in men than in women while being diagnosed twice as often in the African American population than the Caucasian population[276]. The median age of diagnosis is 65 years. Although MM has a 5-year survival rate of 59.8%, it remains fatal, representing 2.1% of all cancer deaths in the United States[211].

1.8.2.1 Diagnosis

The International Myeloma Working Group (IMWG) has established a standardized diagnostic criterion encompassing all plasma cell disorders[277]. A bone marrow aspirate or biopsy is used to determine the percentage of abnormal plasma cells in each patient as is critical to diagnose the clinical stage, accompanied by urine and serum analysis[278].

MM is consistently preceded by a pre-malignant, asymptomatic stage known as monoclonal gammopathy of undetermined significance (MGUS)[279]. MGUS is defined by the presence of less than 3 g/dL serum M protein and less than 10% clonal BM plasma cells. It can be subdivided as either non-IgM MGUS or IgM MGUS. Non-IgM MGUS is diagnosed through the presence of serum M proteins (IgG, IgA, or IgD) while IgM MGUS has serum IgM M proteins at the above-mentioned concentrations[280]. They make up 85 and 15% of the MGUS cases, respectively, with rare exceptions featuring abnormal ratios[281]. Both require the absence of myeloma defining events (MDE), described below.

Smoldering myeloma (SMM) is the intermediate pre-malignant stage, as asymptomatic as MGUS. It is defined by the presence of monoclonal protein greater than or equal to 3g/dL and clonal BM plasma cells ranging from 10 – 59%[282]. Importantly, patients must present with no evidence of MDE. Several studies have been completed to evaluate risk stratification in SMM so as to better predict which patients will be part of the 10% that progress annually to MM during the first 5 years after diagnosis[283]. Of note is the decrease in rate of progression as the patient ages. Years 5-10 post-SMM diagnosis sees the rate drop to 5% per year. Upon attaining 10 years of stable SMM, the rate of progression to MM is halved and remains at 1.5% in perpetuity[284].

In order for MM to be diagnosed, one or more MDEs must be present. MDEs are described as end-organ damage according to the classic CRAB criteria: hypercalcemia, renal failure, anemia, or lytic bone lesions[278]. There are also 3 criteria apart from MDE than can be used to diagnose MM: bone marrow plasma cells equal to 60% of the population, an involved/uninvolved serum free light chain ratio greater than or equal to 100, or one or more focal lesions greater than 5 mm on an MRI. Exhibiting any of the CRAB or additional criteria (jointly named SLiM CRAB) is sufficient for a MM diagnosis and treatment commencement[278].

1.8.2.2 Genetic lesions

Multiple myeloma has a highly complex, heterogenous genomic landscape. Both the initiation of MM and its progression are dependent on genetic lesions[285]. Next-generation sequencing (NGS), FISH analysis, and cytogenetics are used to detect these genetic aberrations, allowing for better diagnostic and treatment models. These genetic lesions

include chromosomal translocations, copy number abnormalities (CNAs), and point mutations.

1.8.2.2.1 Primary cytogenetic abnormalities

Primary cytogenetic abnormalities begin early in MM pathogenesis, meaning at the MGUS stage. The primary genomic events involved are the acquisition of hyperdiploidy or translocations affecting the immunoglobulin heavy chain (*IgH*) gene[286]. Hyperdiploidy is present in approximately 50% of MGUS cases and is the copy gains of odd-numbered chromosomes (3, 5, 7, 9, 11, 15, 19, and/or 21)[287]. Although it is associated with formation of lytic bone disease, hyperdiploidy is the cytogenetic abnormality with the best overall prognosis. This is partially due to the finding that patients with hyperdiploidy are more likely to respond to lenalidomide treatment and autologous stem cell transplant[288, 289].

There are 5 common IgH translocations which are characterized by an oncogene from a partner chromosome translocating to the IgH region on chromosome 14q32 in MM cells. All 5 are involved in the dysregulation of a D-group cyclin[290]. Overexpression of D-group cyclin is an early molecular abnormality instrumental in MM cell longevity as it deregulates of the G1/S transition. The IgH translocation t(6;14) affects the gene *CCND3* which is the gene for cyclin D3. It has a frequency less than 5% and represents a low to standard risk. The translocation t(11;14), associated with the cyclin D1 gene *CCND1*, has a greater frequency ranging from 15-20% of cases. Of intermediate risk, t(11;14) is characterized by high rates of free light chain only and IgM/IgD myeloma[291]. Together with t(6;14), MM patients with both these translocations present more often with bone disease at their initial MDE than those with t(4;14) or t(14;16)[292].

The following 3 IgH translocations are high-risk markers. The most frequent of the high-risk markers, present in 10-15% of cases, is t(4;14) which is involved with *MMSET*[293]. It frequently co-occurs with +1q, a secondary abnormality of high risk[294]. It is associated with markedly high free light chain ratios. SMM patients with this translocation are considered high risk SMM with a greater probability of progression to full MM and should be considered for early treatment. Treatment that includes bortezomib and autologous stem cell transplantation in patients with t(4;14) increase their OS to patients with standard risk cytogenetic markers[295, 296].

Translocations t(14;16) and t(14;20) are both present in less than 6% of cases and are associated with *MAF* and *MAFB*, respectively. *MAF* is linked to the increased expression of cyclin D2 leading to increased adhesion to BM stromal cells through integrin B7 and accelerated DNA synthesis and cell division[297]. *MAFB*, also associated with cyclin D2, protects cells from drug-induced apoptosis and induces proliferation[298, 299].

1.8.2.2.2 Secondary cytogenetic abnormalities

Secondary cytogenetic events can occur in any of the primary stages of MM. They often appear concurrently and are associated with high risk of progression from SMM to MM or an adverse prognosis if found in established MM patients. These do not usually involve the IgH locus as do primary cytogenetic abnormalities. Secondary genetic events include copy number variations, epigenetic changes, and acquired mutations. The most frequent is deletion 13q which occurs in 45% of cases and affects genes including RB1, DIS3, and MIR15A/MIR16.

The next most frequent secondary abnormality, found in approximately 40% of new cases, is the gain of the long arm of chromosome 1 (+ 1q) [300]. Its frequency increases as disease progresses. As stated above, it can co-occur with t(4;14) and is associated with other abnormalities such as del(17p) and del(13q). While impact of +1q is still being considered, presence of more than 4 copies is associated with shorter PFS and OS[301]. The other abnormality in chromosome 1 is del(1p) which has 3 commonly deleted regions: 1p12 (*FAM46C*), 1p22.1 (*RPL5*), and 1p32.3 (*CDKN2C*)[302]. Deletion of 1p12 and 1p32.3 was associated with reduced OS in MM patients receiving ASCT. These deletions can be found in 30% of newly diagnosed MM and is a poor prognostic marker[303].

Of highest risk in both time to progression and overall survival is hemizygous deletion of 17p del(17p). It is found in 8-10% of newly diagnosed MM cases and increases to 25% of relapsed/refractory MM (RRMM) cases. Deletion of 17p covers *TP53* leading to deletion, mutation, or biallelic inactivation. and is associated with even worse outcomes. The relation between mono and biallelic del(17p) and TP53 mutational status is still being defined, biallelic inactivation is associated with critically short OS. Use of these and other cytogenetic abnormalities are valuable in MM stage stratification as they are relevant to disease progression and treatment response.

1.8.2.3 Disease staging

MM disease staging is composed of several systems. Those include the Durie-Salmon Staging (DSS) and the Revised - International Staging Systems (R-ISS). The DSS was the standard guide that factored tumor burden, hemoglobin, Ig levels, urine M protein levels and

bone damage. However, due to the subjectivity of these parameters, reproducibility was problematic. Consequently, the R-ISS was developed and updated. It is used for its simplicity, inclusion of cytogenetic risk, and greater reproducibility. It is divided into 3 stages (I, II, III)[304]. The conditions for each stage are described in Table XYC.

The R-ISS is divided into 3 stages. The 5-year OS for patients in each stage is approximately 82%, 62%, and 40% respectively. The R-ISS incorporates the previous ISS criteria with the novel addition of chromosomal abnormalities and lactate dehydrogenase[305]. The chromosomal abnormalities considered by FISH assay were del(17p), t(4;14), and t(14;16), previously described in section XUCK . Together, primary and secondary cytogenetic abnormalities are important factors in the progression of MM, its response to treatment, and overall prognosis.

1.8.2.3 Treatment

The past few decades have seen important improvements in survival rates for MM. The decision of when to begin treatment has been key in improving these statistics. As was discussed previously, patients with MGUS or SMM are not treated as long as they remain asymptomatic. When 1 or more of the diagnostic criteria appear, disease is considered symptomatic, and treatment commences. There are several clinical trials underway trying to determine whether earlier treatment, i.e. treatment onset based on high-risk cytogenetic profile vs CRAB symptoms, is beneficial to PFS and OS in patients with SMM[306]. We will review the currently approved treatments for MM and then discuss standard treatment regimens for both newly diagnosed MM (NDMM) and RRMM.

Proteasome inhibitors (PIs), as the name suggests, inhibit proteasomes from degrading or processing intracellular proteins. The proteasome is made of multiple catalytic proteases that work within the ubiquitin proteasome pathway (UPP) and is essential for removing misfolded or unfolded proteins from the endoplasmic reticulum (ER)[307]. Cancer cells, generally, have an accumulation of proteins in response to abnormal gene transcription making them more dependent on the UPP than normal cells. In MM, the excessive production of M protein is a signature of the disease, thus making inhibition of the proteasome a promising target. The first PI, bortezomib, works by binding to the catalytic site of the 26S proteasome, leading to inhibition of the proteasome's enzymatic activity[308]. Bortezomib has an inhibitory effect on the activation pathway of nuclear factor-κB (NF-κB), resulting in the activation of both the intrinsic and extrinsic caspase cascade, ultimately leading to apoptosis. Use of bortezomib thus leads to apoptosis of the MM cell, inhibition of myelomagenesis, and inhibition of MM cell adherence to bone marrow stromal cells. It is a central component of induction in NDMM and RRMM treatment.

Carfilzomib is a second generation PI that is only approved for use in RRMM although clinical trials for use in NDMM are underway. While it binds irreversibly to the proteasome and inhibits chymotrypsin-like activity, it is associated with cardiovascular adverse effects (AE)[309]. The most common AE for bortezomib is peripheral neuropathy. Due to its irreversible binding to 20S proteasome, it has a prolong inhibitory effect, even after the free drug has metabolized and cleared the system. As a treatment for RRMM, carfilzomib can overcome PI resistance in several bortezomib-resistant MM cell lines[310]. Ixazomib is also a second-generation PI that binds reversibly to the β5 subunit of the 20S proteasome and inhibits the chymotrypsin-like activity. It is a bioavailable PI, that induces caspase-mediated

apoptosis in MM cells. It is currently approved for use alongside lenalidomide and dexamethasone in RRMM[311].

Immunomodulatory agents (IMiDs) are used in both NDMM and RRMM. They employ a unique mechanism of action by promoting the cereblon (CRBN)-dependent ubiquitination and degradation of a subset of zinc-finger transcription factors, Ikaros (IKZF1) and Aiolos (IKZF3). This results in both antiproliferative and immunomodulatory effects. They are able to enhance T and NK cell cytotoxicity through ZAP-70/CRBN dependent and independent mechanisms. The 3 central IMiDs in use for MM are thalidomide, lenalidomide, pomalidomide. Thalidomide was the first approved for use in MM and continues to be used despite its extensive AEs[312]. Lenalidomide is a second generation IMiD that is key to both induction regimens and post autologous stem cell transplant (ASCT) maintenance. It has an enhanced safety profile compared to thalidomide[313]. Pomalidomide, a third generation IMiD, has been shown to have a greater inhibitory effect on targeted cytokines compared to previous generations[314]. As such, it is currently approved for use in RRMM, even those refractory to either thalidomide or lenalidomide[315].

Immunotherapeutic approaches are now part of standard MM treatments. Monoclonal antibodies (mAbs) are specific antibodies from a single clone that target neoplastic cells and activate the immune system, ultimately leading to target cell death. The have 4 central mechanisms to produce this cell death: antibody-dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and apoptosis upon secondary cross-linking. In the last decade, 3 mAbs have been approved for the treatment of MM: daratumumab (anti-CD38), isatuximab (anti-CD38), and elotuzumab (anti-SLAMF7).

Daratumumab is a humanized IgG1k mAb that targets the cell surface protein CD38. CD38 is a 46-kDa type II transmembrane glycoprotein expressed on multiple types of hematologic cells including plasma cells, committed hematopoietic progenitor cells, NK cells, B and T lymphocytes. Daratumumab targets a unique CD38 epitope and functions through multiple mechanisms[316]. ADCC, ADCP, and direct crosslinking depend on the interaction of the Fc region of the antibody with the Fcγ receptors expressed on immune cells. In ADCC, the Fcγ receptors (FcγRs) on, mainly, NK cells, bind to the Fc tail of daratumumab which is bound to the target MM cell. Once bound, NK cells are able to kill the target cell through the mechanisms described previously. Daratumumab acts as an activating bridge between the NK cell and MM cell. Monocytes and macrophages are the effector cells for ADCP. Phagocytosis occurs when the antibody-opsonized MM cells bind to the FcγRs on monocytes and macrophages, efficiently killing the MM cell. CDC occurs when C1q binds to the Fc tail of daratumumab and initiates the complement cascade[317]. This ultimately generates the membrane attack complex and leads to the permeabilization of the cell membrane. Complement components are also deposited on the surface of the MM cell which results in the phagocytosis of the tumor cell. Daratumumab is the CD38 mAb which most effectively induces CDC[318]. Daratumumab also has immunomodulatory effects as CD38 is expressed on immune cells. In practice, daratumumab can cause NK cell fratricide due to NK cell expressing variable concentrations of CD38[319]. Daratumumab has proved incredibly successful in patients. It is currently approved as both a single agent and in combination with other standards of care.

Isatuximab is also a mAb directed against CD38. However, it is directed at a different epitope on CD38. While the main mechanism used by daratumumab is CDC, isatuximab

relies heavily of ADCC. As opposed to daratumumab, can induce direct cytotoxicity via caspase-dependent apoptosis and lysosome-mediated nonapoptotic cell killing[320]. Isatuximab has been approved for use in RRMM in combination with pomalidomide and dexamethasone or carfilzomib and dexamethasone.

The current standard of care model for NDMM is dictated by a patient's transplant eligibility status and risk stratification. ASCT has been the standard treatment since the 1990s and [321]continues to be produce superior response rates, PFS, and OS especially with the introduction of PIs and IMiDs as part of the induction treatment. In eligible, standard-risk NDMM, ASCT is preceded by several rounds of induction therapy to achieve adequate disease control[277]. This induction therapy consists of bortezomib, lenalidomide, dexamethasone (VRd). However, in high-risk NDMM, induction therapy can consist of VRd or VRd plus daratumumab. Maintenance (treatment till relapse) is either lenalidomide or bortezomib plus lenalidomide in high-risk patients. For ASCT ineligible patients, VRd is customary for both standard and high-risk disease although daratumumab can replace bortezomib.

In RRMM, addition of mAbs isatuximab and elotuzumab are considered, alongside switch to PIs and IMiDs with which the patient has yet to be refractory. After the second or subsequent relapses, CAR-T and bispecific antibodies can be trialed as emerging options[322]. Clinical trials are ongoing for NK cell-based immunotherapies in MM. They are being tested as both monotherapies and in conjunction with mAbs that augment their cytotoxic effects. This shall be explored in detail throughout this doctoral thesis.

The BM microenvironment (BMME) favors immunotolerance and tumor escape from immunosurveillance. To re-establish the immunological balance, immune checkpoints have become a target in MM. Increased levels of PD-1 on T cells are correlated with disease progression[323, 324]. NK cells in MM present with impaired function due to reduction in the expression of activating receptors and the up-regulation of PD-1 receptors which bind to PD-L1 expressed by MM cells[324]. PD-L1 is expressed on MM cells and the expression increases as disease progresses, especially in patients with RRMM. Both nivolumab and pembrolizumab, in combination with pomalidomide and dexamethasone had some success in stable MM patients[325]. However, the clinical trial combining IMiDs and anti-PD-1/PD-L1 mAbs was discontinued due to excessive immune response[326]. Due to the suppressive force of PD-1/PD-L1 on NK cells, use of ICIs in combination with NK cell-based therapies presents an opportunity to restore the immunological balance.

Chapter II

Scientific Premise and Objective

Scientific Premise

Cancer immunotherapy is a continuously growing field that has given rise to novel therapeutic strategies capitalizing on the power of the immune system. Excellent clinical results have been obtained with the introduction of monoclonal antibodies (mAbs) and chimeric antigen receptor (CAR) T cells. However, it is evident that other immunotherapeutic strategies are needed to produce more ample and effective responses that overcome the many mechanisms of tumor escape.

NK cell-based immunotherapies are being avidly pursued through several avenues. Currently, therapeutic NK cells include healthy human donor NK cells, umbilical cord blood NK cells, chimeric antigen receptor NK cells (CAR NK), and NK cell lines. The efficacy of these methods are being explored both in pre-clinical and clinical trials. Due to the suppressive tumor microenvironment (TME) that leads to NK cell dysfunction within different cancers, using allogenic NK cells for treatment seems an easily accessible way to employ NK cell cytotoxicity while avoiding inhibitory aspects of this environment[327-329].

MAbs are a cancer therapy based on the recognition of specific antibodies on the surface of cells. Certain mAbs act by directing NK cells to produce cell death through antibody dependent cellular cytotoxicity (ADCC)[319]. Having had great impact in hematological malignancies is daratumumab. It is a IgG1κ mAb directed against CD38, expressed on different cell types including multiple myeloma (MM) cells. It exerts its cytotoxic function through several mechanisms including ADCC and complement dependent cytotoxicity (CDC)[330]. Highly expressed on MM cells, CD38 is a type II transmembrane glycoprotein with low expression on normal myeloid and lymphoid cells [331]. mAbs

directed against CD38 was thus elected as viable therapeutic target in MM, however its mechanism of action depends on functional NK cells to carry out ADCC[318, 332].

In patients with B-cell chronic lymphocytic leukemia (CLL) and MM, the activation and cytotoxicity of their NK cells is deteriorated. This mechanism of immune escape is possible thanks to increase in NK inhibitory receptors and immune checkpoint inhibitors[333, 334]. High levels of TGF-β are secreted by immunosuppressive regulatory T cells (Tregs) and plasma cells. It has been demonstrated that this immunosuppressive cytokine causes reduction in various NK activating receptors and alter their cytotoxic abilities[335]. IL-6, another cytokine that inhibits NK cell function, is also abundantly produced in the MM TME[336, 337]. Other soluble factors inhibit the activating signals transduced by NCR and NKG2D. These are some of the factors within the TME that contribute to NK cell dysfunction.

The use of allogenic NK cell therapy is a viable way to re-initiate the cytotoxicity mediated by NK cells in patients with hematological malignancies[338]. Our research group has participated in several studies to produce a protocol for the activation of healthy human donor NK cells. This was a 5-day protocol that relied on Epstein Barr virus transformed lymphoblastoid cells (EBV+) as feeder cells. In those studies, it was demonstrated that NK cells are capable of eliminating cells from mutant hematological cancer cell lines that were resistant to apoptosis[339]. The cytotoxicity of these activated NK cells were also trialed on ex vivo samples from CLL patients. Their success in eliminating these cells proved the capacity of activated NK cells against cells with several lines of prior treatment[340]. Subsequently, the research group led by Martin Villalba developed a protocol to expand NK cells sourced from umbilical cord blood (UCB). His group used these expanded and activated

UCB NK cells in antibody dependent cytotoxicity (ADCC) experiments in a variety of tumor types[341]. Their protocol based on both activating and expanding NK cells was the basis for the NK cell protocol utilized in this doctoral thesis.

Also of great interest globally is the inhibition of immune checkpoints including the PD-1/PD-L1 axis. Studies by Ardolino et al. present mechanistic evidence that blocking PD-1 can cause an antitumoral response mediated by NK cells. They also argue that PD-1 is an important checkpoint for the activation of NK cells. When PD-1, expressed on NK cells in suppressive TMEs, binds to its ligand, the activation of NK cells is encumbered, and its cytotoxic ability is inhibited[342]. To adequately see the effect of immune checkpoint inhibitors on NK cell function and cytotoxicity, PD-1 inhibitors such as pembrolizumab should be employed. As a humanized IgG4 antibody, pembrolizumab has been approved for use in several cancers such as metastatic melanoma, refractory Hodgkin's lymphoma, and certain metastatic solid tumors that present with genetic abnormalities[342].

Submitting NK cells from any source to an expansion and activation protocol could lead to changes within their transcriptomic signature. To elucidate the changes that occur in NK cells, analysis of their miRNA are necessary. It has been demonstrated that miRNAs regulate the fundamental processes of NK cells. Not only do they effect NK cell activation in terms of cytokine production, cytotoxicity, and proliferation, but also NK cell development and maturation[199, 343, 344]. Previous work from our collaborators showed NK cells, having undergone a 5-day activation protocol, highly differentially expressed miRNAs signaling the dramatic change that can occur in NK cell while preparing NK cell-based therapies[158].

NK cells are potent effectors of cytotoxicity that provide a first line of defense against virally infected and cancerous cells. However, they have great difficulty in exercising their cytotoxic function against cancer cells due to the suppressive tumor microenvironment. NK-cell based therapy using healthy human donor cells expanded and activated in vitro can circumvent this immune suppression in hematological malignancies. They will be able to successfully eliminate target cancer cells as a single or combination therapy with the addition of monoclonal antibodies.

Objectives

Based on the previous works described above, the central objective of this doctoral thesis was to define the role of expanded and activated NK cells as an effective cellular therapy for the treatment of hematological cancers. This main objective has been fractioned into the following aims:

- Optimize the expansion and activation protocol for healthy human donor peripheral blood eNK cells (Article I).
- Characterize the cytotoxic ability of eNK cells against ex vivo B-CLL patient samples (Article I).
- Ascertain differences between eNK cells originating from peripheral blood versus umbilical cord blood (Article II).
- Characterize the cytotoxic ability of eNK cells against ex vivo MM patient samples, alone and in combination with mAbs (Article II).
- Identify and interpret the transcriptomic changes which occur in NK cells before and after their expansion and activation (Article III).

- Evaluate the state of current clinical trials involving NK cell-based therapies in MM (Article IV).

Chapter III

Published Articles

Paper I

Expanded and activated allogeneic NK cells are cytotoxic against B-chronic lymphocytic leukemia (B-CLL) cells with sporadic cases of resistance.

Tania Calvo* Chantal Reina-Ortiz*, David Giraldos, María Gascón, Daniel Woods, Judit Asenjo, Joaquín Marco-Brualla, Gemma Azaceta, Isabel Izquierdo, Luis Palomera, Diego Sánchez-Martínez, Isabel Marzo, Javier Naval, Carlos Vilches, Martín Villalba, and Alberto Anel

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Expanded and activated allogeneic NK cells are cytotoxic against B-chronic lymphocytic leukemia (B-CLL) cells with sporadic cases of resistance

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Adoptive transfer of allogeneic natural killer (NK) cells is becoming a credible immunotherapy for hematological malignancies. In the present work, using an optimized expansion/activation protocol of human NK cells, we generate expanded NK cells (eNK) with increased expression of CD56 and NKp44, while maintaining that of CD16. These eNK cells exerted significant cytotoxicity against cells from 34 B-CLL patients, with only 1 sample exhibiting resistance. This sporadic resistance did not correlate with match between KIR ligands expressed by the eNK cells and the leukemic cells, while cells with match resulted sensitive to eNK cells. This suggests that KIR mismatch is not relevant when expanded NK cells are used as effectors. In addition, we found two examples of de novo resistance to eNK cell cytotoxicity during the clinical course of the disease. Resistance correlated with KIR-ligand match in one of the patients, but not in the other, and was associated with a significant increase in PD-L1 expression in the cells from both patients. Treatment of one of these patients with idelalisib correlated with the loss of PD-L1 expression and with re-sensitization to eNK cytotoxicity. We confirmed the idelalisib-induced decrease in PD-L1 expression in the B-CLL cell line Mec1 and in cultured cells from B-CLL patients. As a main conclusion, our results reinforce the feasibility of using expanded and activated allogeneic NK cells in the treatment of B-CLL.

Abbreviations

NK	Natural killer
eNK	Expanded NK cells
KIR	Killer-cell immunoglobulin-like receptors
ADCC	Antibody-dependent cell-mediated cytotoxicity
AML	Acute myeloid leukemia
B-CLL	B-cell chronic lymphocytic leukemia
CTLA-4	Cytotoxic T lymphocyte antigen 4
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
EBV	Epstein Barr virus

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LCL	Lymphoblastoid cell line
HLA-I	Human leukocyte antigen class I
IL-2	Interleukin 2
IL-15	Interleukin 15
IFN-α	Interferon-α
IL-4	Interleukin 4
NKG2D	NK cell receptor group 2D
NKG2A	NK cell receptor group 2A
NKp30, NKp44, NKp46	NK cell activating receptors p30, p44 and p46, respectively
NCR	NK cell activating receptors
DNAM-1	DNAX accessory molecule 1
IL-T2	Ig-like transcript 2
R-COP	Rituximab plus cyclophosphamide, vincristine and prednisone
R-Benda	Rituximab plus bendamustine
MFI	Mean fluorescence intensity
PBMC	Peripheral blood mononuclear cells
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
APC	Allophycocyanin
7-AAD	7-Amino actinomycin D
CTG	Cell tracker green

Although the immune system can prevent the development of tumors, through a process known as anti-tumor immune surveillance, many cancers are able to evade this surveillance. This is achieved through multiple pathways such as the production of immunosuppressive cytokines, the induction of regulatory T cells and the interference with tumor antigen presentation to cytotoxic T lymphocytes (CTL)[1]. One of the advantages of T cell responses, their specificity, can also be a limitation if tumor cells develop strategies to hide tumor-specific antigen expression[2]. This limitation is not shared by NK cell responses, as they are not antigen-specific. Certain immune evasion mechanisms used by tumor cells to avoid attack by CTL are ineffective against NK cells[3]. Although they differ in antigen specificity, NK cells and CTLs share the same effector mechanisms to efficiently kill tumor cells: perforin-mediated granzyme B delivery inside target cells and death ligand-induced apoptosis, namely FasL and TRAIL[4]. Therefore, advances in the understanding of NK cell biology and function make them a powerful tool for new immunotherapies[5], some of which are currently in clinical trials[6].

NK cells have been tested in selected patients with aggressive or high-risk hematological cancers. The incompatibility between HLA-I molecules expressed by the tumor and the inhibitory receptors (KIR) of the donor's NK cells (mismatch) improves clinical results[7,8] and the allogeneic NK response observed in pioneering studies is beneficial and seemed safe[8–10]. Additionally, the combination of the NK cell response with use of anti-tumor antibodies, through antibody-dependent cellular cytotoxicity (ADCC), offers therapeutic opportunities yet to be explored[11]. In previous clinical studies, NK cells transferred to patients, always respecting the incompatibility between their KIR and the HLA-I expressed by the tumors, were neither activated nor expanded[9,10,12–14]. However, recently, phase I/II clinical studies have been performed using NK cells expanded through different approaches, on multiple myeloma and acute myeloid leukemia (AML), including pediatric patients[15–18]. Other pertinent clinical trials are currently ongoing[19]. The importance of KIR mismatch in expanded NK cells is yet unknown.

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in adults in the Western world and is characterized by the accumulation of mature B-lymphocytes in peripheral blood, bone marrow and secondary lymphoid organs. The leukemia cells express a variety of proteins of the Bcl-2 family that favor the inhibition of apoptosis, which, together with the interaction with the cellular microenvironment and the release of cytokines, results in the accumulation of B-CLL cells in several organs[20]. Alkylating drug-based therapies, alone or in association with corticosteroids or anti-CD20 antibodies, such as rituximab, have been the first line of B-CLL treatment for decades, as well as purine analogs. However, chemotherapeutic treatments may impair antitumor immune responses due to their immunosuppressive side effects[20,21]. Owing to the intrinsic heterogeneity of B-CLL, there is still a substantial percentage of patients with unfavorable evolution, particularly those that present mutated p53 or 17p deletion[22]. Despite advances in treatment, the 5-year mortality rate in B-CLL patients is highly variable, and patients with high-risk features still show low rates of survival[22]. Hence, new and more efficient treatments are needed.

In patients with B-CLL, the total number of NK cells in peripheral blood is increased, but they exhibit defective cytotoxic activity. Culturing NK cells with cytokines such as IL-2 and IL-15 can stimulate this activity[23]. The introduction of properly activated and expanded allogeneic NK cells, as indicated above, for the adoptive therapy of B-CLL is worth further exploration.

The immune checkpoints refer to inhibitory pathways that modulate the duration and amplitude of the physiological immune response. One of the mechanisms of immune suppression developed in cancer is the induction of these control points on the surface of activated T cells, CTLA-4 and PD-1 being the two most studied[24]. PD-1 is a receptor member of the immunoglobulin superfamily present in activated T cells. Together with its ligands, PD-L1 and PD-L2, it has an important function in the regulation of immune responses[25]. Therefore, blocking this receptor is among the most promising approaches to therapeutic anti-tumor immunity[26]. The use of anti-PD1 blocking antibodies such as pembrolizumab and nivolumab has become a first-line treatment in tumors with poor prognosis[27]. Some reports indicate that PD-L1 expression in B-CLL patients could be a negative prognostic marker, related to an exhausted phenotype in T cells[28–30]. Moreover, PD-L1 expression in tumor cells results in

functional NK cell impairment[31,32]. However, no information is available on the regulation of NK cell function by PD-1 in B-CLL patients.

In this work, using an optimized protocol for expansion and activation of NK cells from healthy adult donors, we tested the expanded NK cells on samples from 35 B-CLL patients. The initial 30-patient cohort included patients at different stages of the disease, either previously treated or untreated (see Suppl. Table I). In some cases, we obtained samples from the same patients at different times during the course of the disease. This follow-up allowed for the detection of de novo resistances to eNK treatment. We undertook studies to determine the molecular basis for, and possible treatments to reverse, these resistances.

Materials and methods

NK cells and cells from B-CLL patients. NK cells were generated from PBMCs of healthy donors obtained from leukopaks provided by the Blood and Tissue Bank of Aragón. Cells from B-CLL patients were obtained by the hematologists involved in the study.

NK cell expansion protocols. PBMC were isolated from leukopaks by Ficoll-Paque (Sigma) density centrifugation. Partial T cell depletion was then performed by using anti-CD3 mAb bound to magnetic beads and MACS immunomagnetic negative isolation (Miltenyi Biotec). Then, 50×10^6 cells were cultured at 2×10^6 cells/ml in the presence of 25 IU/ml of IL-15 and 100 IU/ml of IL-2; or with 25 IU/ml of IL-15, 100 IU/ml of IL-2 and 100 IU/ml of IFN-α. In both protocols, cells were cultured in the presence of the HLA-I negative, EBV^+ lymphoblastoid B cell line 721.221[33] at a 10:1 ratio, previously treated with mitomycin C to prevent their proliferation. These cultures were maintained for 20 days, with changes of medium to add fresh cytokines, and with the addition of feeder cells every 5 days. Culture viability and NK cell expansion, defined as $CD3^-$ $CD56^+$ by flow cytometry, was also determined. At the end of the expansion period, NK cells were isolated by positive selection using anti-CD56 magnetic beads (Miltenyi Biotec). Purity and viability of isolated NK was always 95% or higher.

Phenotyping of expanded NK cells (eNK cells). Expression levels of the most relevant activating and inhibitory NK cell receptors were determined in NK cells at day 0 and day 20 of expansion by flow cytometry using PE-labelled mAb. The expression of activating NCR NKp30, NKp44 and NKp46 was determined using mAb from Beckman-Coulter, clones Z25, Z231 and BAB281, respectively. NKG2D expression was determined with the clone 1D11 mAb from BD, CD16 with the clone VEP13 mAb from Miltenyi Biotec, and DNAM-1 using the clone #102511 mAb from R&D Systems. The expression of the inhibitory receptors NKG2A and ILT2 was determined using mAb from Beckman-Coulter, clones Z199 and A07408, respectively. PD-1 expression was also analyzed using an anti-PD1 mAb conjugated with FITC from Biolegend (clone EH12.2H7).

Study of the match or mismatch bewteen eNK cells and B-CLL cells. The extraction of genomic DNA was carried out using DNAzol (MRC). The analysis of the KIR epitopes in HLA class I genes in NK cells and cells from B-CLL patients was carried out by PCR with sequence-specific primers as indicated in Sánchez-Martínez et al.[34].

Cytotoxic assays of eNK cells on cells from B-CLL patients. eNK cells were isolated between day 15 and 22 of expansion by positive selection using anti-CD56 magnetic beads (Miltenyi Biotec) and used for cytotoxicity assays against cell lines or cells from B-CLL patients. Cells from B-CLL patients were obtained by Ficoll-Paque density centrifugation and cultured for 2 h in complete medium with 100 IU/ml IL-4 to improve viability. All patients exhibited more than 85% of leukemic blasts in blood, so they were not separated. Purified eNK cells were labeled with 1 μM Cell Tracker Green (CTG) (Invitrogen) and mixed with target cells at a 5:1 effector to target ratio. After incubating for 4 h at 37 °C, DNA damage was measured in the target population (CTG negative cells) by flow cytometry using 7AAD (Immunostep) labeling. See Supplemental Fig. I for an example of the method using the B-CLL cell line Mec-1 as target.

Analysis of PDL-1 expression in B-CLL cells. PD-L1 expression was analyzed on the surface of B-CLL cells from patients or on the B-CLL cell line Mec-1 using the anti-PDL1 mAb conjugated with PE (clone 10F.9G2, BioLegend) and flow cytometry analysis.

Statistical analysis. Differences between the percentages of NK cells expressing specific surface receptors upon expansion were assessed using the Student's t test. In the case of cytotoxicity assays, we used the one-Way ANOVA Tukey test.

Differences were considered statistically significant at $P < 0.05$.

Ethical statement. All NK cells used were generated from PBMC of healthy donors obtained from leukopaks provided by the Blood and Tissue Bank of Aragón, under the permission of the Clinical Research Ethical Committee from Aragón (CEICA) (Ref. PI16/0129). Cells from B-CLL patients were obtained by the hematologists involved in the study, with the corresponding permission of the CEICA, reference number PI13/0146, and all patients signed an authorized informed consent. The involvement with human subjects complies with the Declaration of Helsinki.

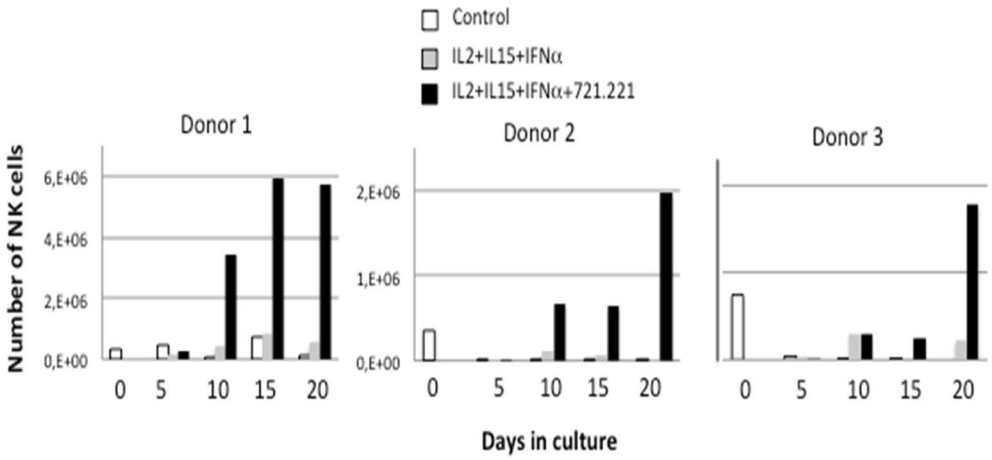

Figure 1. Expansion of NK cells is dependent on the presence of feeder cells. Total PBMC from three different donors were placed in culture at 2×10^6 cells/ml in complete medium (Control, white bars), or in medium supplemented with 100 IU/ml IL-2, 25 IU/ml IL-15 and 100 IU/ml of IFN-α in the presence (black bars) or absence (grey bars) of 721.221 feeder cells previously treated with mitomycin C. At the times indicated, the percentage of $CD3^-CD56^+$ cells was determined by flow cytometry, viable cells counted by Trypan blue exclusion and the total number of NK cells calculated. At day 5, 10 and 15, fresh medium and cytokines and new feeder cells were added and total cell density adjusted around 2×10^6 cells/ml.

Results

Expansion protocols and phenotype of expanded NK cells (eNK). In previous works, we developed a protocol for human NK cell activation using a 5-day stimulation of PBMC in the presence of lymphoblastoid cell lines transformed with the Epstein Bar virus (EBV)[34,35], following the pioneering work of Perussia et al.[36]. Subsequently, we developed NK cell expansion protocols from umbilical cord blood[11], which were similar to a previously reported protocol[37]. A further optimization of these expansion protocols was undertaken using NK cells from healthy donors and the HLA-I negative EBV^+ lymphoblastoid cell line (LCL) 721.221[33] as feeder, to avoid any inhibitory KIR signaling during expansion. Two cytokine cocktails were compared during the expansion protocol, always in the presence of the feeder cells: IL-2 + IL-15 or IL-2 + IL-15 + IFN-α. The inclusion of IFN-α is justified as this stimulatory cytokine increases cytotoxic potential in mouse NK cells in comparison with IL-15 that was instead implicated in the maintenance of viability[38]. Figure 1 shows how the presence of the feeder cells was necessary for the efficient expansion of NK cells. As expansion rates were reduced by the presence of T cells, they were partially depleted from PBMC before beginning the cultures. This allowed for a consistently greater expansion rate of NK cells, which is indicated in Supplemental Table II for the 10 donors whose cells were used in the following cytotoxicity tests. The inclusion of IFN-α did not improve the expansion rate, being the combination of IL2 + IL15 enough to support NK cell expansion.

After the 20-day expansion, the NK cell population increased in percentage and in total number, along with upregulated levels of surface CD56 expression (see Supplemental Fig. 2). The high percentage of NK cells expressing CD16, NKG2D, DNAM-1 and NKp46 in unstimulated cells was maintained in eNK cells (Supplemental Fig. 3). The low percentage of unstimulated NK cells expressing NKp44 was significantly increased in eNK cells. An average increase in the percentage of NKp30-expressing cells was observed, but it was not statistically significant. Regarding inhibitory receptor expression, the percentage of NKG2A- or ILT2-expressing cells was variable in unstimulated cells, and, although a tendency to increase was observed upon expansion, it was not statistically significant. A significant effect on the final phenotype whether IFN-α was included in the expansion protocol or not was not observed.

Cytotoxicity of eNK cells. As shown in Suppl. Fig. 1, we first tested eNK cells in 4 h assays on the B-CLL-like cell line Mec1 and obtained 60% of specific cytotoxicity. We also tested eNK cells on the HLA-I negative erythroleukemia K562 and on the HLA-I positive acute lymphocytic leukemia cell line Jurkat, reaching specific cytotoxicity nearing 70% at the 9:1 E.T ratio in both cases (Suppl. Fig. 4A). We compared the cytotoxicity of NK cells activated for 5 or 20 days on K562 cells and found that, although cells activated for 5 days were cytotoxic, the level of cytotoxicity exerted by eNK cells was higher, especially at 3:1 or 9:1 effector to target ratios (Suppl. Fig. 4B). Then, we tested non activated NK cells (Control) or eNK cells obtained in the presence of IL2 + IL15 and in the absence (IL) or presence of IFN-α (IL + IFN) on Jurkat cells overexpressing Bcl-x_L (Jurkat-Bcl-x_L). We observed that non-activated NK cells exerted less than 10% of specific cytotoxicity, while eNK cells induced between 35 and 60% depending on the donor (Suppl. Fig. 4C). Again, IFN-α did not significantly increase cytotoxicity.

Once the cytotoxic potential of eNK cells was ascertained, we were able to test them against cells from 30 B-CLL patients. The clinical data at the time of sampling are depicted in Suppl. Table I. This cohort included patients at different stages of the disease, either previously treated or untreated. Standard diagnosis protocols

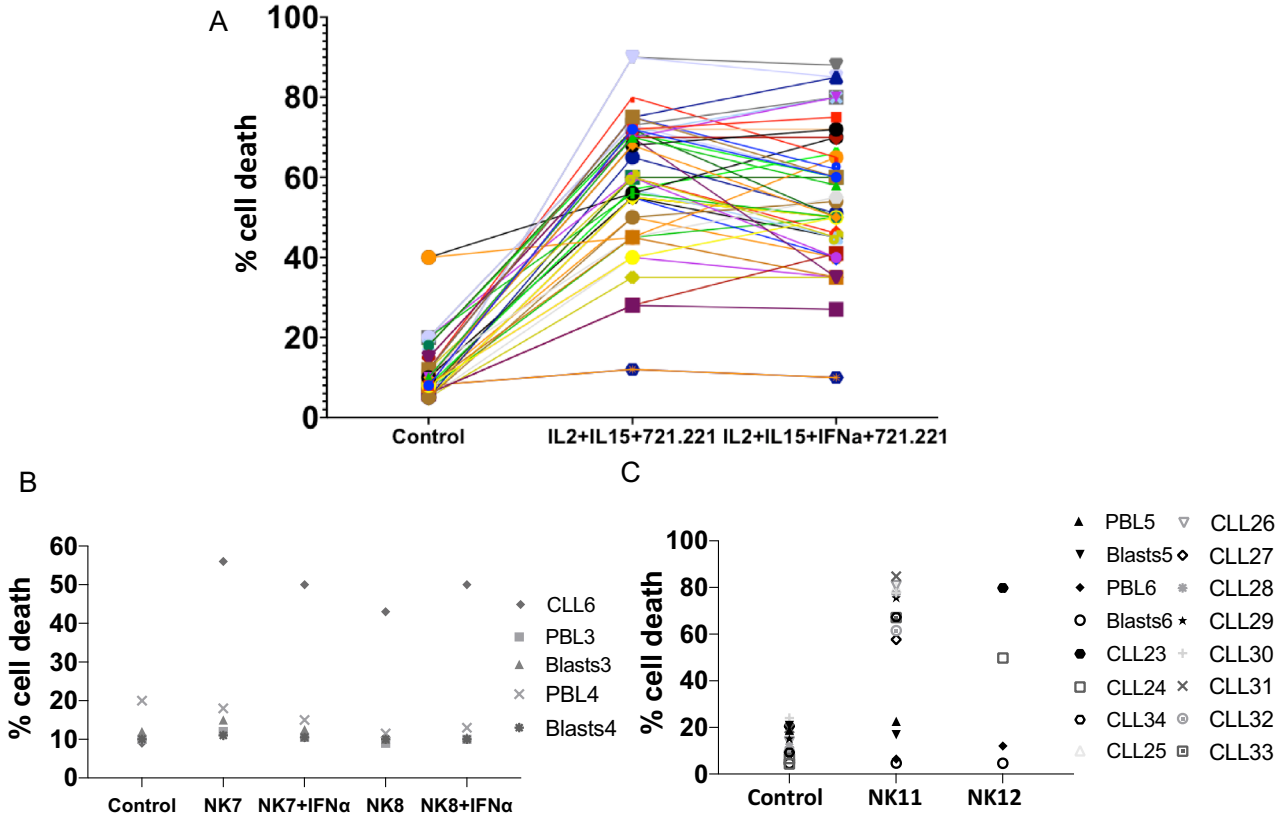

Figure 2. Cytotoxicity of eNK cells on cells from B-CLL patients. (**A**) Cytotoxicity of eNK cells from the 10 donors shown in Suppl. Table II and expanded in the presence (IL2 + IL15 + IFNα + 721.221) or in the absence of IFN-α(IL2 + IL15 + 721.221) on cells from 23 patients of B-CLL. Cytotoxicity tests were conducted during 4 h at a 5:1 E:T ratio. Data show the percentage of cell death in the target population estimated by 7-ADD labeling. The Control points show 7-AAD labeling of leukemic cells alone after the 4 h incubation in complete medium. (**B**) Cytotoxicity of NK7 and NK8 eNK cells, expanded in the presence (+ IFNα) or in the absence of IFN-α on cells from B-CLL patient 6 or on PBL or T cell blasts from two unrelated donors. (**C**) Cytotoxicity of NK11 and NK12 eNK cells, expanded in the presence of IL2 + IL15, but in the absence of IFN-α, on cells from B-CLL patients CLL23 to CLL34, or on PBL or T cell blasts from two additional unrelated donors. Cytotoxicity tests were conducted during 4 h at a 5:1 E:T ratio and data show the percentage of cell death in the target population estimated by 7-ADD labeling. The "Control" points show 7-ADD labeling of target cells alone after the 4 h incubation in complete medium.

were followed in all the patients. In certain patients, genetic analysis of specific risk factors was also performed (see Suppl. Table I).

In 7 samples, basal cell death was higher than 50%, precluding their use in the cytotoxicity experiments. Data presented in Fig. 2A shows how eNK cells exerted significant cytotoxicity against cells from 22 B-CLL patients, whereas only 1 were resistant, patient 18 (see Table 2). Patient 18 cells' were the only ones that showed less than 25% specific cell death induced by eNK cells. This result was obtained when testing eNK cells from the 10 donors which expansion rates are depicted in Supplemental Table II. Basal cell death averaged 11% and cytotoxicity exerted by eNK cells on leukemic cells was variable, in some cases more than 80%. On average, cell death was 58%, significantly higher than basal values, and represented a mean of 47% specific cell death induced by eNK. Addition of IFN-α during expansion did not substantially affect the cytotoxic potential of eNK cells on cells from B-CLL patients, averaging 54% (Fig. 2A). The increase in cytotoxicity was statistically significant in both types of expansion, using a Tukey non-parametric analysis, with $P = 0.001$ in both cases.

In order to ascertain their specificity against tumor cells, we also tested the cytotoxicity of 2 eNK cells (NK7 and NK8) used in the cytotoxicity assays shown in Fig. 2A and two additional donors (NK11 and NK12), on freshly isolated PBMC or T cell blasts from 4 unrelated healthy donors (Fig. 2B,C). The T cell blasts were obtained through PHA stimulation in the presence of IL-2 during 5 days. The cytotoxicity of the eNK cells on normal PBMC and on T-cell blasts was low (Fig. 2B,C). Importantly, the eNK cells exerted substantial cytotoxicity against cells from B-CLL patient 6 (CLL6; Fig. 2B) and on cells from 12 additional B-CLL patients (from CLL23 to CLL34; Fig. 2C). This clearly shows that eNK cytotoxicity mainly targets transformed cells.

Analysis of the KIR-epitope match between eNK and B-CLL cells. The sporadic resistance observed in leukemic cells from patient 18 could be due to the match between KIRs expressed by eNK cells and HLA-I

Sample	C1	C2	Bw4	A3/A11
CLL1	+	−	+	−
CLL2	+	−	+	−
CLL3	+	+	+	N.D
CLL4	+	+	+	−
CLL5	+	+	+	−
CLL6	+	−	+	−
CLL7	+	+	+	−
CLL8	+	−	+	−
CLL9	+	−	+	+
CLL10	+	−	−	+
CLL11	+	−	+	−
CLL12	+	−	+	−
CLL13	−	+	+	+
CLL14	+	−	+	−
CLL15	+	−	+	−
CLL16	+	−	+	−
CLL17	+	−	N.D	−
CLL18	+	−	+	+
CLL19	+	−	+	−
CLL20	+	−	−	−
CLL21	N.D	N.D	N.D	−
CLL22	+	+	+	−
NK1; NK2; NK8	N.D	N.D	N.D	N.D
NK3	+	+	−	−
NK4	+	+	+	−
NK5	+	+	−	+
NK6	+	+	N.D	N.D
NK7	+	−	+	+
NK9	+	+	+	−
NK10	+	+	+	−

Table 1. Expression of the C1, C2, Bw4 and A3/A11 HLA class I epitopes in B-CLL patients and in NK cells used in the cytotoxicity assays shown in Fig. 2A.

expressed by the leukemic cells. The inhibitory KIRs 2DL2/3, 2DL1, 3DL1 and 3DL2 recognize the HLA class I epitopes C1, C2, Bw4 and the A3/A11 alleles, respectively[39,40]. When a target cell lacks one or more of the allotypes present in an NK-cell donor ('KIR-ligand mismatch'), allogeneic NK-cell reactivity can be expected. KIR ligands in DNA from 22 of the B-CLL patients and from 7 of the 10 eNK with which cytotoxicity was assayed in Figs. 2A,B were genotyped. Unfortunately, we could not obtain enough genomic DNA from NK1, NK2 and NK8, indicated as N.D in Tables 1 and 2. In most of the cases, there was a mismatch between eNK cells and cells from B-CLL patients, as shown in Table 2, and those B-CLL were sensitive to eNK cytotoxicity. However, although leukemic cells from patient 18 were also mismatched with the effector cell ligands, they were resistant to cytotoxicity exerted by NK9 and NK10. Conversely, cells from patients 3 and 5 had matched KIR epitopes with their effector cells and were also sensitive to cytotoxicity exerted by NK3 and NK4 (Table 2).

Sporadic development of resistances correlates with high PD-L1 expression. In two patients (CLL5 and CLL8), samples were obtained at different stages of the disease, separated temporally by several months. CLL5 cells were sensitive to NK3 and NK4 at the time of the 1st sample acquisition, but some months later, they showed resistance to NK9 and NK10 (Fig. 3, upper panels). CLL8 cells were sensitive to NK1 and NK2, but again showed almost complete resistance to NK9 and NK10 some months later (Fig. 3, lower panels). This was not due to a deficient activation of NK9 and NK10 as these eNK cells were effective against leukemic cells from patients 19, 20 and 21 (44%, 45% and 35% of specific cytotoxicity, respectively; see Table 2). Unfortunately, experiments could not be repeated with eNK cells from NK1, NK2, NK3 and NK4 on patient samples at that moment of disease progression as the entire expanded population was spent in the experiments performed on the different B-CLL patients tested in the first assays and shown in Fig. 2. In addition, the law protects the identity of the volunteer donors of the Blood Bank and obtaining a second identical sample was impossible.

The clinical data of CLL5 and CLL8 was analyzed to identify possible common features. CLL5 was 75 years-old at first sampling and, after having undergone 6 R-COP cycles and one R-Benda cycle was in partial remission (Suppl. Table III). The patient exhibited a 13q deletion in heterozygosis, a factor that in principle is associated with relative low risk[41], 70% positivity for CD38, and 10% positivity for ZAP70. From the first to the second

Combination	Match	Resistance
NK1 or NK2/CLL1	N.D	No
NK1 or NK2/CLL2	N.D	No
NK1 or NK2/CLL4	N.D	No
NK1 or NK2/CLL7	N.D	No
NK1 or NK2/CLL8	N.D	No
NK1 or NK2/CLL9	N.D	No
NK1 or NK2/CLL10	N.D	No
NK3 or NK4/CLL1	No	No
NK3 or NK4/CLL3	**YES**	No
NK3 or NK4/CLL5	**YES**	No
NK3 or NK4/CLL11	No	No
NK3 or NK4/CLL12	No	No
NK3 or NK4/CLL13	No	No
NK5 or NK6/CLL14	No	No
NK5 or NK6/CLL15	No	No
NK5 or NK6/CLL16	No	No
NK5 or NK6/CLL17	No	No
NK7/CLL6	No	No
NK9 or NK10/CLL19	No	No
NK9 or NK10/CLL20	No	No
NK9 or NK10/CLL21	No	No
NK9 or NK10/2nd CLL5	**YES**	**YES**
NK9 or NK10/2nd CLL8	No	**YES**
NK9 or NK10/CLL18	No	**YES**

Table 2. Determination of the match or mismatch between HLA class I epitopes in B-CLL patients and in NK cells used in the cytotoxicity assays shown in Fig. 2A. Bold indicates the situations in which a match or a resistance could be detected. *N.D.* not determined.

analysis, CLL5 had progressed from stage IA to IV-C and was under treatment with R-Benda at the time of the second sample. CLL8 was 89-years old at the time the first sample was obtained. This patient had an indolent disease, which did not evolve from stage 0, and was without any treatment, conditions that continued at the time of the second sample (Suppl. Table III). From this, eNK resistance development could be associated with a disease worsening in CLL5 but not in the case of CLL8. Common clinical features were not found between these patients.

Regarding a possible match between eNK cells and leukemic cells, KIR epitopes expressed by cells from CLL5 were indeed in match with the ligands expressed by NK9 and NK10, and, in this case, the match correlated with the resistance to cytotoxicity. For CLL8, the leukemic cells were sensitive to cytotoxicity exerted by NK1 and NK2, although the HLA-I haplotypes of NK cell donors could not be analyzed. However, cells from this patient were resistant to cytotoxicity exerted by NK9 and NK10, and no match was observed in that case (Table 2). Resistance to eNK cell cytotoxicity correlated with match in one of the patients, but not in the other, indicating that, although match could contribute to the final outcome, other intrinsic factors of leukemic cells should also account for resistance to eNK cells.

Remarkably, both the percentage of cells positive for PDL-1 expression and the mean expression level (MFI) increased in the two resistant samples as compared to the sensitive patient samples (Fig. 4A).

Effect of idelalisib treatment on PD-L1 expression and on sensitivity to eNK cells. A third sample from CLL5 was obtained after the resistance to eNK treatment was developed. Between the second and third sampling, the patient was treated in clinic with the PI3Kδ inhibitor idelalisib, which is used in B-CLL treatment[42]. Remarkably, PD-L1 expression was lost in the leukemic cells of the patient after treatment with idelalisib (compare Fig. 4B with Fig. 4A, upper panels). Unfortunately, none of the eNKs used on the 1st and 2nd samples from this patient were available, as the 3rd sample was taken almost 2 years later.

eNK cells were generated from 3 new donors (NK13, NK14 and NK15), also obtained from the Blood and Tissue Bank of Aragón, and tested against the third sample of CLL5. This was perfromed in the presence of the blocking anti-PD-1 mAb pembrolizumab. As shown in Fig. 4C, B-CLL cells from CLL5 recovered the sensitivity to cytotoxicity exerted by the three eNK cells, reaching specific cytotoxicity levels of 80%, contrasting with the resistance observed in 2nd sample from this patient (see Fig. 3). The anti-PD-1 blocking mAb pembrolizumab did not further increase these high cytotoxicity levels, as expected since target cells did not express PD-L1. We also analyzed the PD-1 expression in the eNK cells from these donors, observing that they were rather negative (Fig. 5), correlating with the lack of effect of pembrolizumab. We analyzed PD-1 expression in a total of 14 eNK cell productions and we can conclude that our expansion protocol generates eNK cells with a small percentage

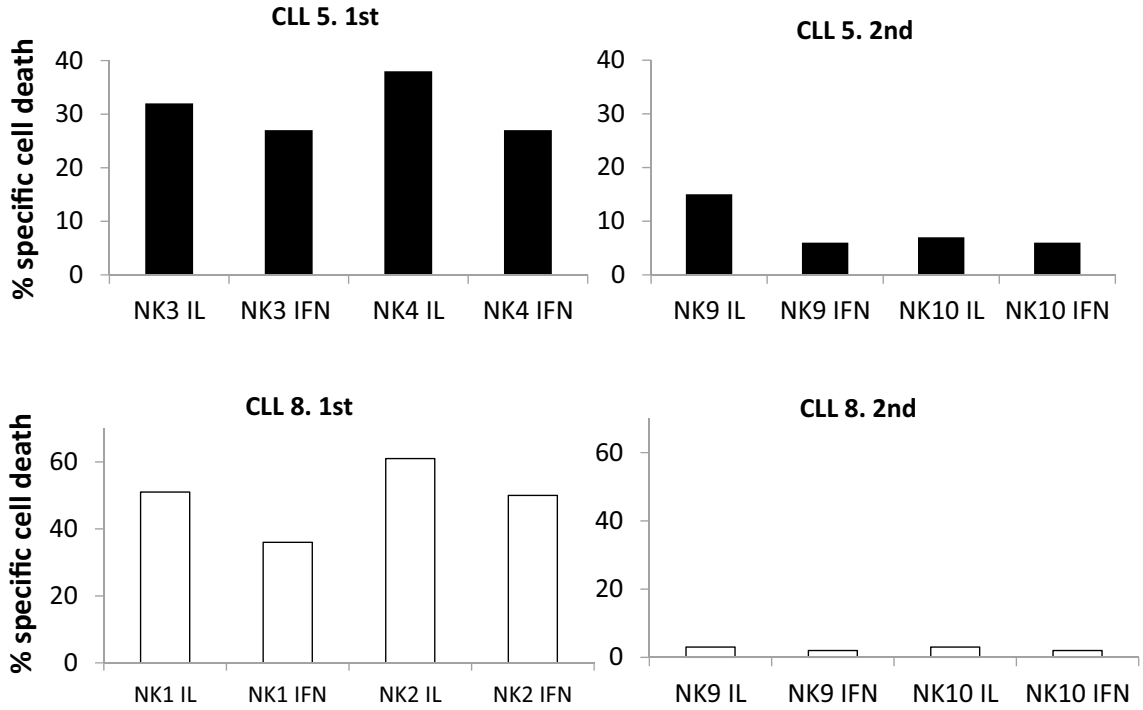

Figure 3. Development of resistance to eNK cells in two B-CLL patients. Upper panels: NK3 and NK4 cells expanded in the presence (IFN) or in the absence of IFN-α were tested at a 5:1 E:T ratio against cells from B-CLL patient 5 at the first time of sample acquisition. Some months later, cells from the same patient were obtained for a 2nd time and confronted with NK9 and NK10. Lower panels: NK1 and NK2 cells expanded in the presence (IFN) or in the absence of IFN-α were tested at a 5:1 E:T ratio against cells from B-CLL patient 8 at the first time of sample acquisition. Some months later, cells from the same patient were obtained for a 2nd time and confronted with NK9 and NK10. Cell death was tested by 7-AAD labeling. Results are shown as the percentage of specific cell death induced, after subtracting basal leukemic cell death, which was never higher than 15%.

of $PD1^+$ cells in most cases: only in 4 of those 14 donors was there a population of $PD1^+$ cells representing more than 30% of eNK cells.

As the observation on the effect of idelalisib on PD-L1 expression could be interesting from a clinical perspective, we further studied this effect and its impact on sensitivity to eNK cells in both the B-CLL cell line Mec-1 and on cells from B-CLL patients. Mec-1 cells are positive for PD-L1 expression at the basal level (see Fig. 6A). First, we performed a dose–response between 1 and 20 μM of idelalisib on this cell line, and, although it partially inhibits cell growth (50% inhibition at the highest dose), it was not able to induce cell death in this concentration range. Next, we analyzed the effect of those doses of idelalisib on PD-L1 expression. We observed a clear reduction in PD-L1 surface expression, with a 40% reduction at 1 μM, 24% at 10 μM and peaking at 60% reduction with 20 μM (Fig. 6A). Lastly, eNK cytotoxicity was tested in either the presence of the blocking anti-PD1 mAb pembrolizumab or after 48 h supplementation with 10 μM idelalisib, arriving at a similar increase in cytotoxicity on Mec-1 cells in both cases (Fig. 6B). These experiments were performed with eNK cells from two donors (NK16 and NK17) that expressed PD-1 in at least a 30% of their population (see the insets in Fig. 6B). The tests were also performed with eNK cells with low or null PD-1 expression from other donors, and, in those cases, no effect of idelalisib or of pembrolizumab was observed (data not shown).

PD-L1 expression and the effect of idelalisib in cells from 12 additional B-CLL patients was tested: 6 were positive for PD-L1 expression and 6 were negative. In those patients with higher PD-L1 expression, we observed how incubation with a non-toxic dose of idelalisib (10 μM) in the presence of IL-4 for 48 h resulted in a net reduction of specific PD-L1 expression of around 30% (Fig. 7, left panels). We tested the cytotoxicity of eNK cells from one additional donor (NK18) on those leukemic cells and observed that it was high at the basal level. Cytotoxicity was slightly increased by incubation with idelalisib, but this effect was not reproduced by treatment with pembrolizumab (Fig. 7, right panels). This correlates again with the almost null expression of PD-1 in eNK cells from this donor (see the inset).

Discussion

The present work demonstrates how activation and expansion of NK cells in the presence of IL-2, IL-15 and the EBV^+, HLA-I negative 721.221 cells clearly increases their cytotoxicity on cells from B-CLL patients. The average expansion rates obtained should allow for the treatment of one leukemic patient using eNK cells from one donor. We observed cytotoxicity in vitro in 92% of the patients' samples tested. Meanwhile, eNK cells were not cytotoxic against PBMC or T cell blasts obtained from healthy donors. Altogether, these data reinforce the feasibility of using expanded NK cells in the treatment of B-CLL. This is especially relevant, as this approach

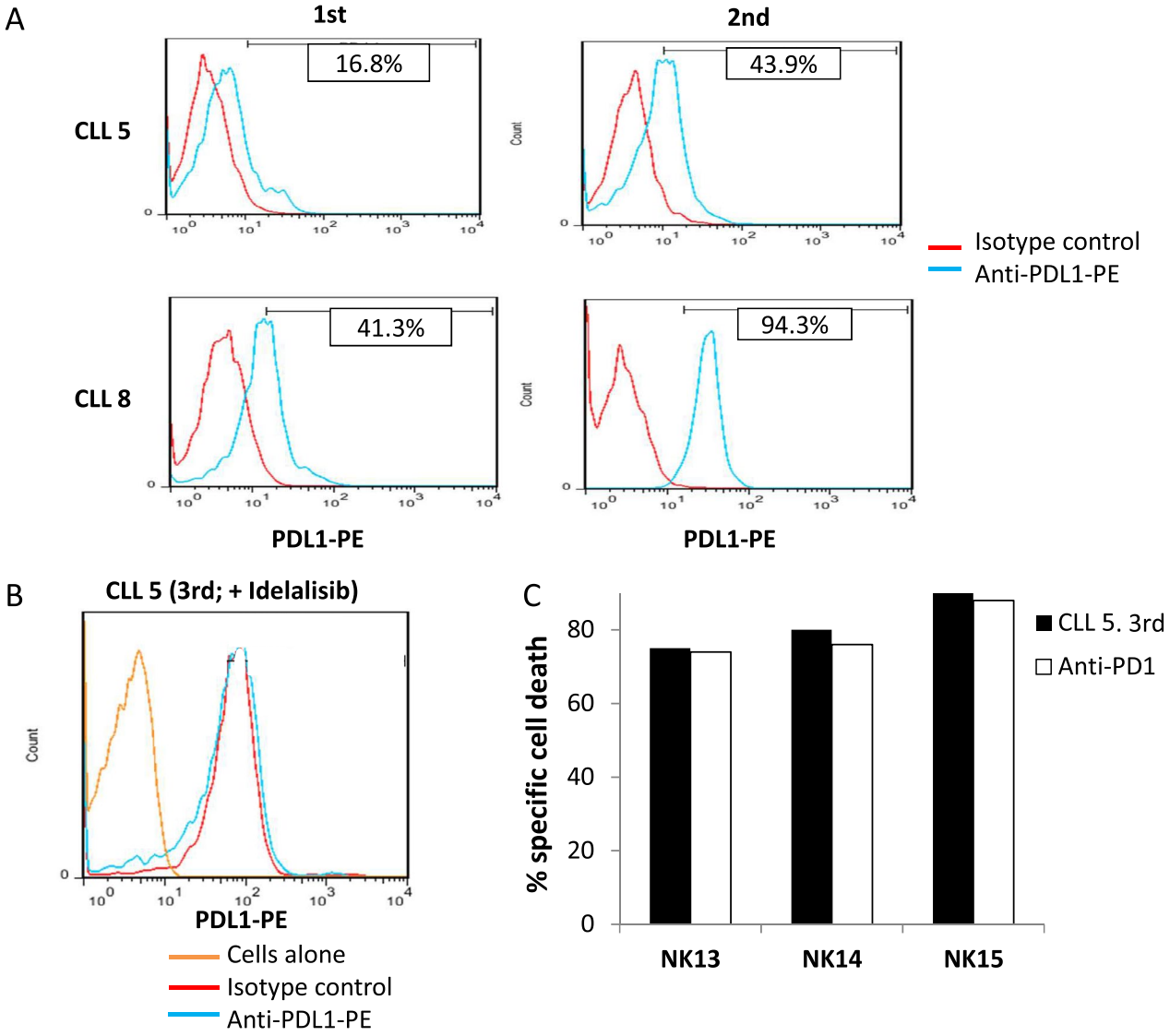

Figure 4. Expression of PDL-1 in leukemic cells sensitive and resistant to eNK cells. Effect of idelalisib treatment. (**A**) The expression of PDL-1 on the surface of cells from B-CLL patients 5 and 8 at the time when they were first tested against eNK cells, or when they showed resistance towards eNK cells at the 2nd time of sample acquisition, was analyzed by flow cytometry using an specific mAb conjugated with PE (blue histograms). Red histograms show the labeling on the same cells using an irrelevant mouse antibody of the same isotype and labeled with the same fluorophore. Numbers on the histograms show the percentage of cells positive for the labeling in each case. (**B**) PDL-1 expression on the surface of cells from B-CLL patient 5 at the 3rd time of sample acquisition, after treatment with idelalisib, was analyzed by flow cytometry using a specific mAb conjugated with PE (blue histogram). Red histogram shows the labeling on the same cells using an irrelevant antibody of the same isotype and labeled with the same fluorophore. Orange histogram shows the signal obtained with the same cells without labeling. (**C**) NK13, NK14 and NK15 cells were expanded using 721.221 cells as feeders in the presence of IL2 + IL15 and tested in 4 h assays at a 5:1 E:T ratio against cells from B-CLL patient 5 at the 3rd time of sample acquisition, after treatment with idelalisib, in the absence (black bars) or in the presence of 10 μg/ml of the anti-PD-1 mAb pembrolizumab (white bars). Leukemic cell death was tested by 7-AAD labelling. Results are shown as the percentage of cell death, after subtracting basal leukemic cell death, which was never higher than 10%.

based on NK cell activation, either alone or in combination with antibodies, is still not approved as a B-CLL treatment. Selected patients with aggressive refractory disease would especially benefit from eNK cell therapy. In some clinical contexts, clinicians used CAR T cells. This approach, however, is more expensive and has secondary effects. NK cells, in view of their low toxicity[5], will probably have fewer undesirable effects.

The sporadic resistance observed initially in one patient did not correlate with match between the KIR ligands expressed by eNK cells and HLA-I expressed by leukemic cells. In fact, we observed a match in four cases in which eNK cells were cytotoxic. Previously we have stimulated NK cells for 5 days with LCL in the absence of cytokines[34], in which NK cell expansion was more limited. In that study, B-CLL susceptibility significantly correlated with HLA mismatch between NK cell donor and B-CLL patient. In our present study we expanded NK

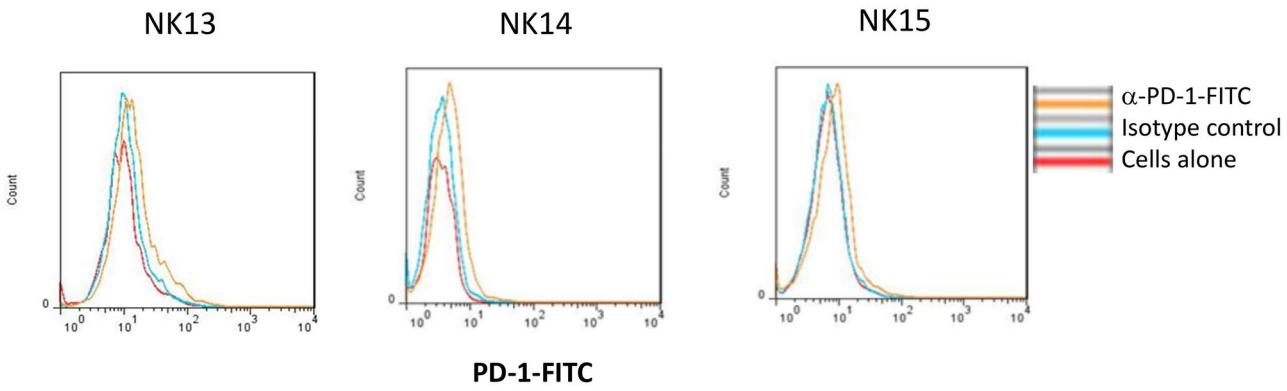

Figure 5. PD-1 expression in eNK cells. PD-1 expression on NK cells after 20 days of expansion was analyzed by flow cytometry on $CD56^+$ cells using a specific anti-PD-1 mAb on the indicated 3 different donors, NK13, NK14 and NK15. Red histograms show the labeling of cells alone, blue histograms the labelling obtained using an irrelevant antibody of the same isotype and labeled with the same fluorophore and orange histograms the specific PD-1 labeling.

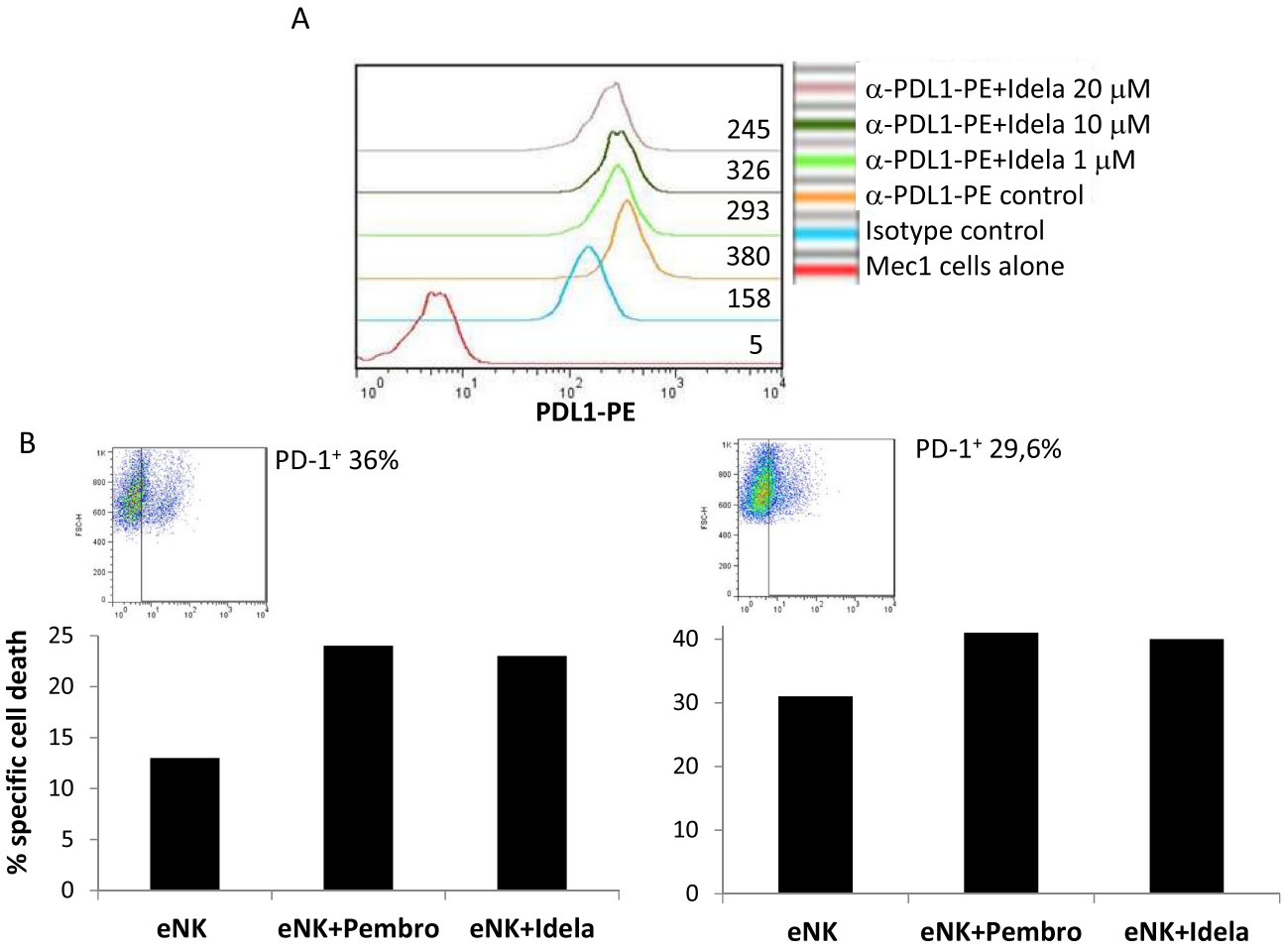

Figure 6. Effect of idelalisib on PDL-1 expression and on eNK cytotoxicity in the B-CLL cell line Mec-1. (**A**) The expression of PDL-1 on the surface of the B-CLL cell line Mec-1 was analyzed in the absence of idelalisib treatment (orange histogram) or after 48 h incubation with 1 μM (green histogram), 10 μM (black histogram) or 20 μM idelalisib (purple histogram). Red histogram corresponds to the labeling of the cells alone, and blue histogram shows the labeling using an irrelevant mouse antibody of the same isotype and labeled with the same fluorophore. Numbers on the histograms correspond to MFI values in each sample. (**B**) NK cells from donors 16 and 17 were expanded using 721.221 cells as feeders in the presence of IL-2 + IL-15 and tested overnight at a 1:1 E:T ratio against Mec-1 cells in the absence (eNK) or in the presence of 10 μg/ml of the anti-PD1 blocking mAb pembrolizumab (eNK + pembro), or after 48 h supplementation with 10 μM idelalisib (eNK + Idela). Mec-1 cell death was tested by annexin-V-APC labelling. The expression of PD-1 in the eNK cells used in each case is shown in the insets.

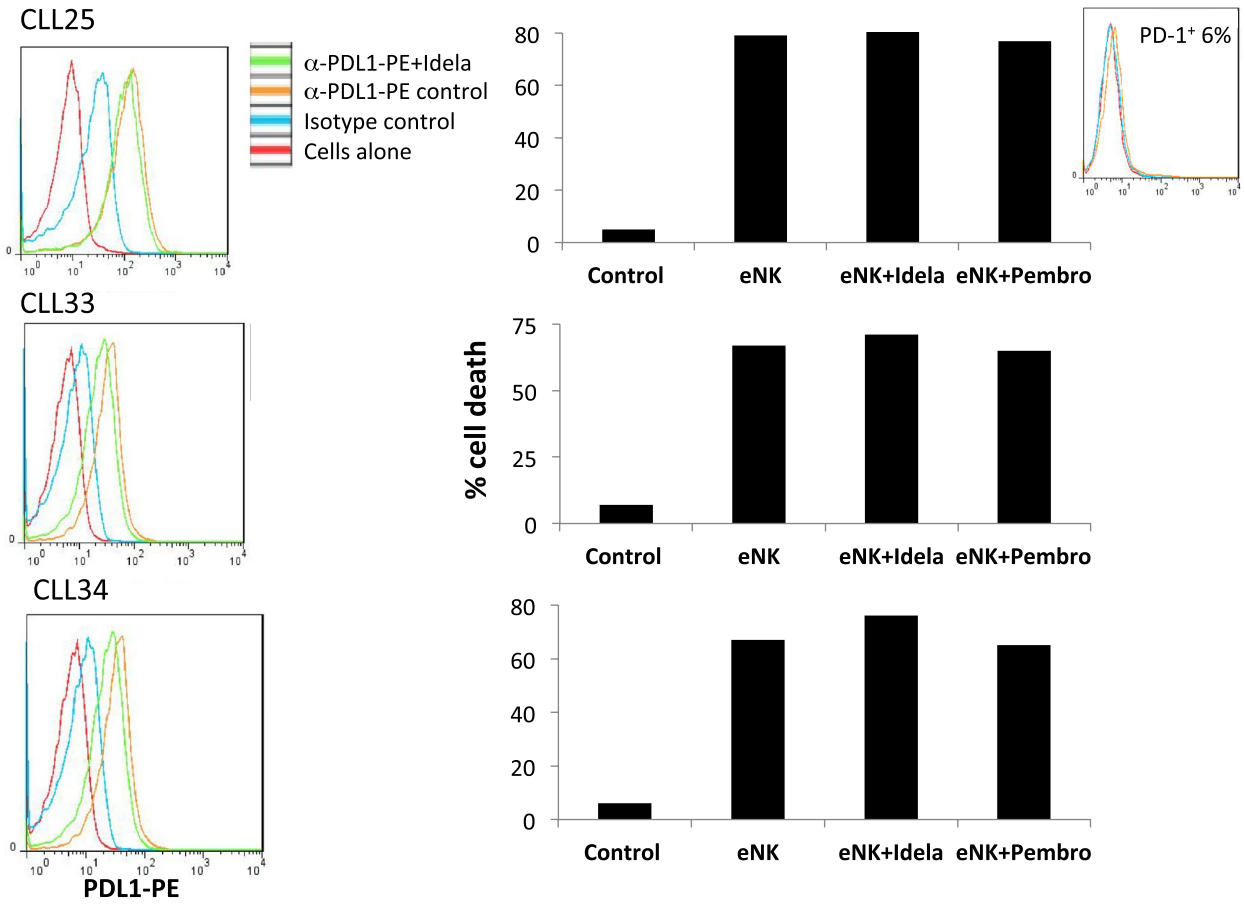

Figure 7. Effect of idelalisib on PDL-1 expression and on eNK cytotoxicity in cells from B-CLL patients. Left panels: The expression of PDL-1 on the surface of cells from three B-CLL patients positive for PD-L1 was analyzed in the absence of idelalisib treatment (orange histogram) or after 48 h incubation with 20 μM idelalisib (green histogram). Red histograms correspond to the labeling of the cells alone, and blue histograms show the labeling using an irrelevant mouse antibody of the same isotype and labeled with the same fluorophore. Right panels: NK18 cells were expanded using 721.221 cells as feeders in the presence of IL-2 + IL-15 and tested at a 5:1 E:T ratio for 4 h against cells from the patients after 48 h supplementation with 100 IU/ml IL-4 in the absence (eNK) or in the presence of 20 μM idelalisib (eNK + Idela). Tests were also performed in the presence of 10 μg/ml of the anti-PD1 blocking mAb pembrolizumab (eNK + pembro). Cell death was tested by 7-AAD labeling on target cells. The expression of PD-1 in the eNK cells used is shown in the inset.

cells that reached a more active status against cells from B-CLL patients and data support that, in most instances, the activation and expansion of NK cells can significantly overcome KIR match limitation.

Phenotypically, eNK cells substantially increased CD56 expression and maintained that of CD16, indicating that eNK cells could combine with therapeutic anti-tumor antibodies. The population of NKp44$^+$ cells increased in eNK cells. A similar study demonstrated that the main change justifying the increase in cytotoxicity was the net increase in the level of granzyme B expression in activated NK cells. In that study, using a 5-day activation protocol in the absence of cytokines, the most significant increase in receptor expression was, as in our case, that of NKp44[35].

We also observed two sporadic cases of de novo resistance to eNK cells during the clinical course of the disease. Both patients did not possess any common feature in either the state of the disease or in the treatment received that may have caused their resistance. A KIR match could explain resistance, but this was found in only one of the two cases. Remarkably, PD-L1 expression increased substantially in the two resistant samples from the first to the second testing.

PD-1 is an inhibitory receptor present in NK cells and activated CD4$^+$ and CD8$^+$ T lymphocytes involved in immunosuppression by binding to its ligands PD-L1 and PD-L2, the former showing a broader expression[25]. T cells from B-CLL patients presented defects in the formation of the immunological synapse that correlated with increased expression of PD-L1 in leukemic cells and of PD-1 in T lymphocytes[30]. B-CLL patients with the Richter transformation benefit from anti-PD-1 treatment[43]. The immunomodulatory drug lenalidomide decreases PD-L1 expression in leukemic cells and PD-1 in T cells[30] and does not increase PD-1 in NK cells[44]. Lenalidomide and the anti-CD20 mAb obinutuzumab enhance NK cell activation markers in B cell lymphoma patients[44]. The expression of PD-1 and CTLA-4 was higher in T lymphocytes of B-CLL patients than in healthy

donors of the same age[28,29]. When NK cells were expanded in the presence of an anti-PD-1 blocking antibody, NK cells had greater cytotoxicity, although this expansion protocol used an anti-CD16 antibody and IL-2, and the anti-PD1 blocking antibody was not used in cytotoxicity assays[45]. PD-L1 expression in tumor cells decreases NK cell cytotoxicity in vitro[32]. PD-1$^+$ NK cells exist in humans, showing a semi-exhausted phenotype, and this population increases in patients with ovarian carcinoma[46]. Moreover, PD-1 expression mediates NK cell functional exhaustion in patients with Kaposi's sarcoma[31].

Patient follow-up allowed recovery of cells from one patient some months after the observed resistance. During this time, the patient had been treated with the PI3Kδ inhibitor idelalisib, which has been recently introduced in the treatment of B-CLL[42]. Remarkably, the expression of PD-L1 was lost and leukemic cells were again highly sensitive to eNK cells cytotoxicity from three different donors. This observation could have clinical interest, and although it was limited to one patient and should be interpreted with caution, we confirmed the reduction of PD-L1 expression and the increase in sensitivity to eNK cytotoxicity mediated by idelalisib in the B-CLL cell line Mec-1. The partial reduction in PD-L1 expression induced by idelalisib in cells from B-CLL patients that were initially positive for its expression was also confirmed. These data indicate that PD-L1 expression is dependent on PI3Kδ activity in B-CLL cells. Remarkably, a recent study has shown a dependence of PD-L1 expression on the PI3K/Akt signaling pathway in anaplastic large cell lymphoma[47].

Our expansion protocol generates eNK cells negative for PD-1 expression in most cases. However, in those donors in whom a population of PD-1$^+$ cells is present, it could contribute to a diminished activity on PD-L1$^+$ targets.

Our present data reinforce the feasibility of using eNK cells in the treatment of B-CLL, in view of their high cytotoxicity against leukemic cells from most B-CLL patients. We propose the clinical use of eNK cells on B-CLL patients, alone or in combination with therapeutic anti-CD20 antibodies, as suggested in our recent study[11]. Additionally, although limited to two patients, our data suggest that anti-PD-1 blocking mAbs or idelalisib could improve adoptive immunotherapy with allogeneic eNK against B-CLL in especially refractory patients.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

T.C., C.R.O., D.G., M.G., D.W., J.A. and J.M.B. performed experimental work. T.C. prepared Figs. 1, 2A,B, 3, Suppl. Figs. 2, 3 and 4. C.R.O. prepared Figs. 2C, 5 and 7. M.G. and J.M.B. prepared Fig. 4. D.G. prepared Fig. 6. D.W. and J.A. prepared Tables 1 and 2. J.M.B. prepared Suppl. Fig. 1. I.M., D.S.M. and J.N. provided resources and methodology. G.A., I.I. and L.P. provided samples from patients, clinical data and patient evolution II prepared Suppl. Tables II and III. A.A., C.V. and M.V. supervised the study and the experimental results and acquired funding. A.A. wrote the original draft of the manuscript. C.V. and M.V. reviewed and edited the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Paper II

Expanded NK cells from umbilical cord blood and adult peripheral blood combined with daratumumab are effective against tumor cells from multiple myeloma patients.

Chantal Reina-Ortiz, Michael Constantinides, Alexis Fayd-Herbe-de-Maudave, Jessy Présumey, Javier Hernandez, Guillaume Cartron, David Giraldos, Rosana Díez, Isabel Izquierdo, Gemma Azaceta, Luis Palomera, Isabel Marzo, Javier Naval, Alberto Anel, Martín Villalba

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ORIGINAL RESEARCH

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Expanded NK cells from umbilical cord blood and adult peripheral blood combined with daratumumab are effective against tumor cells from multiple myeloma patients

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ABSTRACT

In this study we evaluated the potential of expanded NK cells (eNKs) from two sources combined with the mAbs daratumumab and pembrolizumab to target primary multiple myeloma (MM) cells *ex vivo*. In order to ascertain the best source of NK cells, we expanded and activated NK cells from peripheral blood (PB) of healthy adult donors and from umbilical cord blood (UCB). The resulting expanded NK (eNK) cells express CD16, necessary for carrying out antibody-dependent cellular cytotoxicity (ADCC). Cytotoxicity assays were performed on bone marrow aspirates of 18 MM patients and 4 patients with monoclonal gammopathy of undetermined significance (MGUS). Expression levels of PD-1 on eNKs and PD-L1 on MM and MGUS cells were also quantified. Results indicate that most eNKs obtained using our expansion protocol express a low percentage of PD-1$^+$ cells. UCB eNKs were highly cytotoxic against MM cells and addition of daratumumab or pembrolizumab did not further increase their cytotoxicity. PB eNKs, while effective against MM cells, were significantly more cytotoxic when combined with daratumumab. In a minority of cases, eNK cells showed a detectable population of PD1$^+$ cells. This correlated with low cytotoxic activity, particularly in UCB eNKs. Addition of pembrolizumab did not restore their activity. Results indicate that UCB eNKs are to be preferentially used against MM in the absence of daratumumab while PB eNKs have significant cytotoxic advantage when combined with this mAb.

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Multiple Myeloma; cell Therapy and Immunotherapy; NK cells; daratumumab; cytotoxicity; PD-1

1. Introduction

The immune system largely prevents the development of tumors but clinical cancers evade this immune surveillance. Multiple pathways help create an immunosuppressive tumor environment, interfering with tumor antigen presentation to cytotoxic T cells.[1] Tumor cells hide the expression of tumor-specific antigens, limiting the specific T cell anti-tumor response.[2] NK cells, which are not antigen specific, provide a potent antitumor response and are a source of powerful immunotherapies.[3] NK cells are used in the clinic, especially in hematological cancers with poor prognosis, and can safely be used in allogeneic settings.[4–6] Their combination with anti-tumor antibodies, through antibody-dependent cellular cytotoxicity (ADCC), offers interesting therapeutic opportunities.[7,8]

The immune checkpoints refer to molecules that inhibit or modulate immune responses, being CTLA-4 and PD-1 the two most studied, especially in the context of T cell activation.[9–11] The use of anti-PD1 blocking antibodies such as pembrolizumab and nivolumab has become a first-line treatment in tumors with poor prognosis.[12,13] Some reports indicate that PD-L1 expression in tumor cells results in functional impairment of PD-1$^+$ NK cells.[14–16] While certain reports show that NK cell function can be partially restored using blocking mAbs,[15,16] others show a more acute impairment that requires cytokines such as IL-2 and IL-15 for functional restoration.[14] Regarding NK cells, additional molecules act as checkpoint inhibitors, such as the inhibitory NK cell receptor NKG2A, which inhibits NK cell activity when ligated by HLA-E, expressed on the surface of tumor cells. The use of a NKG2A blocking mAb, monalizumab, offers promise as a new tumor immunotherapy.[17] Other NK cell checkpoint mechanisms include blocking the activating receptor NKG2D by tumor shedding of their MIC ligands,[18] or the negative action of LAG-3 and TIM-3.[19]

Multiple myeloma (MM) arises from uncontrolled proliferation of abnormal plasma cells and accounts for 10–20% of all hematological neoplasms and 0.9% of all newly diagnosed cancer cases worldwide.[20] MM is normally preceded by a premalignant phase, termed monoclonal gammopathy of undetermined significance (MGUS). MGUS is found in 3% of

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the population above the age of 50, but the rate of progression from MGUS to MM is very low.[21] Over the past two decades, treatment regimens and survival rates of MM patients have witnessed a radical improvement, with proteasome inhibitors and monoclonal antibodies (mAbs) as the main contributors.[22] However, although overall survival and patient outcomes have considerably improved, drug resistance is still a major concern,[23] mainly in high-risk patients, such as patients with t(4;14) or del17p. For this reason, novel and more efficient therapeutic approaches are needed.

NK cell-based immunotherapy could have a positive impact in MM treatment. One obstacle is the immunosuppressive tumor microenvironment present in MM patients, in whom almost all arms of the immune system are subverted.[24] For example, NK cells from MM patients are ineffective against disease progression.[25] Hence, allogeneic activated and expanded NK cells could be an interesting approach. In fact, NK-based treatments have been recently optimized for its use in MM,[26–30] including a combination of a tumor NK cell line with the anti-CD38 mAb daratumumab.[31]

CD38 is highly expressed in aberrant MM cells while its expression on normal lymphoid cells, including plasma cells, is relatively low. Daratumumab shows efficacy in relapsed and refractory MM patients treated with at least two prior lines of therapy.[32–34] NK cell-mediated cytotoxicity seems to be one of the main mechanisms for its anti-MM activity. Since patient NK cell status is variable, this could explain difference in responses among patients.[35]

Certain reports indicate PD-L1 expression in MM cells which correlates with disease progression from the MGUS stage.[36,37] However, clinical trials using PD-1 or PD-L1 blockers, alone or in combination with other treatments have failed in MM.[38]

The potent cytotoxicity of activated NK cells from PB[39–41] or UCB cells[7,42] against a variety of malignancies has previously been published by our laboratories. In one study, the expansion of these UCB NK cells was also reported.[7] In the present work, we compare the cytotoxic capacity of PB and UCB eNKs against MM patient samples.

2. Results

2.1. Peripheral and umbilical cord blood NK expansion

NK cells were expanded using two different protocols. UCB NK cells, due to their need for both KIR and KAR signals to reach a mature phenotype, were cultured with PLH, an EBV-transformed HLA-I$^+$ B lymphoblastoid cell line which works as an accessory cell with both required signals.[7] Conversely, PB NKs are fully mature and need only activating signals thus the EBV+ HLA-I negative cell line 721.221 was employed.[43] As a first step, T cells and NKT cells were depleted from the cultures using anti-CD3 mAb, to favor NK cell expansion (see Figure 1(a), d 0). Each protocol required different ratios of accessory cells, while IL-2 and IL-15 were added at the same concentrations. UCB NKs were treated with accessory cells and cytokines every 3 d and benefited from little manipulation. PB

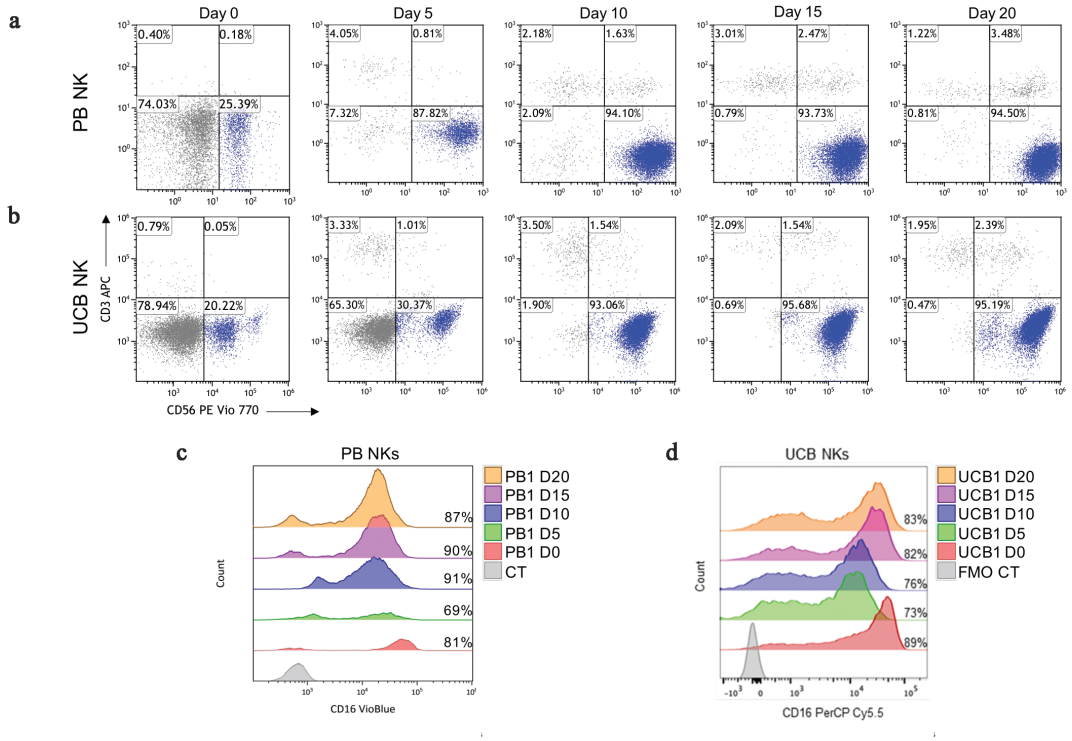

Figure 1. Progression of NK phenotypes through the expansion protocol. Flow cytometry dot plots showing CD3 APC (y-axis) and CD56 PE Vio 770 (x-axis) staining of PB (a) and UCB (b) NKs from d 0 through d 20 of expansion protocol indicated in materials and methods. Histograms depict the progression of PB (c) and UCB (d) NK cells expressing CD16 throughout the 20-d expansion. Numbers indicate the percentage of CD16$^+$ NK cells. Cells were stained with CD16 VioBlue or PerCP, as indicated. Lines correlate to testing every 5 d.

NKs were sustained for 5–6 d before culture renewal and withstood daily manipulation seen in Figure 1. By d 20, almost all cells from both sources present in the culture were $CD56^{+}CD3^{-}$ NK cells (see Figure 1(a,b)). The purity of NK cells was 94.28 ± 2.08% for UCB and 95.8 ± 1.46% as an average (see Suppl. Figure 1 and Suppl. Table I). The $CD3^{+}$ fraction, which was successfully depleted at d 0, did not overtake the $CD56^{+}$ population, allowing for successful expansion of NK cells (Figure 1(a,b)). Beginning with 1E6 NKs in each expansion, PB NKs reached an average of 240E6 cells by d 20 and UCB NKs averaged a 700-fold expansion (Suppl. Fig 2).

The initial characterization of the phenotype of NK cells expanded using EBV-transformed LCL as feeders has been performed in previous studies of our laboratories. A detailed description of all genes and miRNA expressed upon a 5-d activation is found in.[44] The expression of specific activating and inhibitory NK cell receptors was studied following this 5-d activation protocol, showing increases in the percentage of $NKp30^{+}$ and especially $NKp44^{+}$ populations.[40] The phenotype of eNK cells following these expansion and activation protocols as compared to freshly isolated NK cells has also been studied in previous work from our groups. eNK cells from UCB show increased expression of activation markers such as CD69 and reduced that of CD45RA, becoming $CD45RA^{dim}$ cells, keeping high CD16 expression.[7] Regarding eNK cells obtained from PB, the significant increase in the $NKp44^{+}$ population previously described in Sanchez-Martinez et al.[40] was clearly confirmed in our latest study.[45] Consequently, we have determined the expression of NKp44 ligands on the surface of patient MM cells and found faint levels of expression (Suppl. Fig 3). Their scant expression level does not justify the increase in cytotoxicity of eNKs. Rather, this increase in cytotoxicity could more closely be related to the previously demonstrated increase in granzyme B expression observed in activated NK cells.[40]

Regarding CD16 expression, PB NKs presented high levels at d 0, which was maintained throughout the expansion protocol (see a representative expansion in Figure 1(c) and a summary of data on all donors in Suppl. Table II). In general, fresh UCB NKs also showed CD16 expression, and this level of expression was also maintained during the expansions (see a representative expansion in Figure 1(d) and a summary of data on all donors in Suppl. Table II). The maintenance of CD16 expression in the expanded NK cells is important in order to combine them with therapeutic antibodies.

As the average purity of NK cells at d 20 averaged 95%, we did not proceed with further NK cell isolation before the cytotoxicity experiments. We estimated that both types of eNK cells could be used in cytotoxicity experiments from d 10 on, though expansion continued until d 20 to obtain a maximum number of eNK cells.

2.2. Limited fratricide of eNKs in the presence of daratumumab

As NK cells can express CD38, we have analyzed this point in our expanded NK cells. We show in Figure 2(a) the CD38 expression in the 10 expansions performed. All expansions expressed CD38 to varying degrees. While most expansions had CD38 expression in the majority of their NK cell population, a few expansions from both PB and UCB expressed CD38 in only a minority of the eNK cell population. As daratumumab binds to CD38, when $CD38^{+}$ NKs are in the presence of daratumumab, cross-linking can occur between NK cells

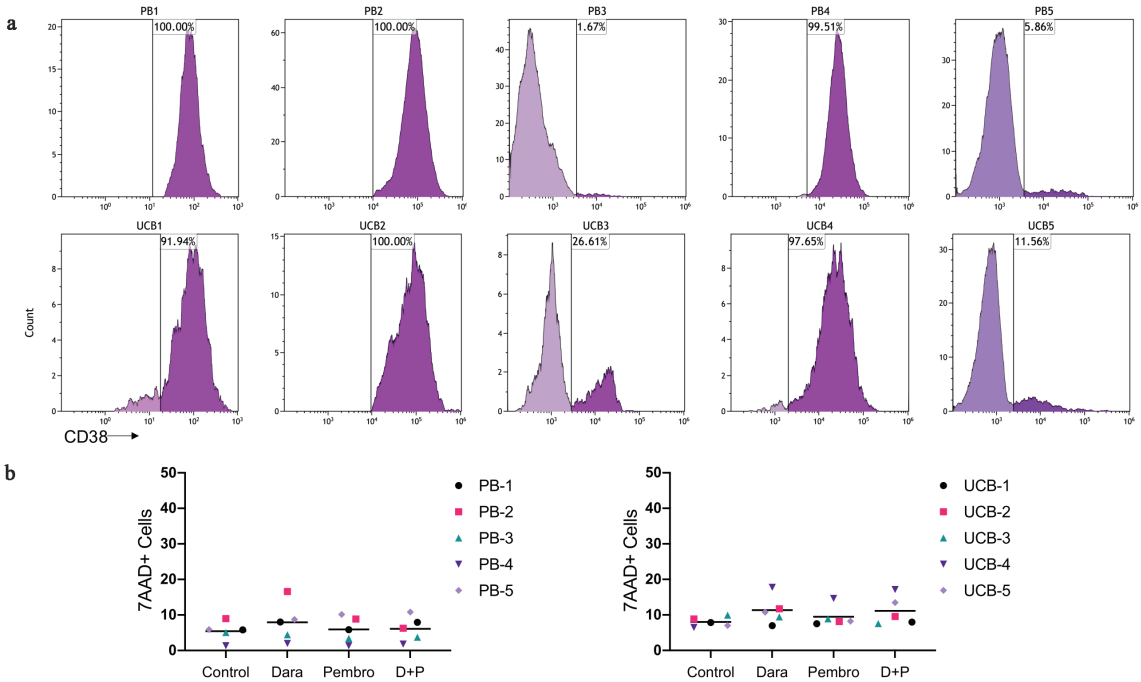

Figure 2. A. CD38 expression determined by flow cytometry on d-20 expanded NK cells (eNK), obtained from PB or from UCB; B, D-20 expanded NK cells (eNK) obtained from PB (left panel) or from UCB (right panel) were left untreated (Control), or they were incubated for 4 h with 5 µg/ml of daratumumab and/or 10 µg/ml of pembrolizumab, as indicated. After the incubations, eNK cell death was estimated by 7-ADD labeling. Horizontal lines indicate the mean cell death in each experimental condition.

leading to cell killing via ADCC.[46] The daratumumab-induced fratricide among NK cells could deplete the number of functional eNK cells available to act against MM cells. To examine the extent of fratricide in our eNK cells, we cultured the eNKs from PB or UCB with daratumumab and/or pembrolizumab, as a secondary control. Daratumumab exhibited a limited cytotoxic effect, averaging less than 5% of specific cell death (Figure 2(b)). Higher levels of CD38 expression on NK cells did not correlate with increased fratricide. Pembrolizumab did not have a cytotoxic effect alone or when combined with daratumumab. We conclude that daratumumab-induced fratricide would not significantly impair eNK cytotoxic potential, although this should be tested in each eNK preparation before use.

2.3. PD-1 expression on eNK cells

Next, we tested PD-1 expression in the 5 PB and 5 UCB eNK used in our study. As shown in Figure 3, most eNK cells obtained (4 out of 5 both in the case of PB and UCB), showed a very limited population positive for PD-1 expression: between 3.9% and 8.3% of the total eNK population in the case of eNK derived from PB and between a 6.5% and a 12.7% in eNK cells derived from UCB. However, eNK cells from PB2 and UCB1 showed the presence of a defined and significant population positive for PD-1 expression, which accounted for 30.9% in PB2 and 23.13% in UCB1. The MFI of the whole eNK population in UCB1 was 4146 while the average MFI in the other 4 UCBs was 1615. In the PB expansions, the MFI value for PB2 was 1481, while the average of the other 4 donors was 766. The population of eNK cells positive for PD-1 expression was never higher than 31%, remaining a minor subset of the whole eNK population. This result is in agreement with the reported low PD-1 expression in most eNK cells obtained from PB in our previous study.[45]

2.4. eNK cytotoxicity assays against MM and MGUS patient samples

We first obtained bone marrow aspirates of MM and MGUS from 22 patients at different stages of disease. Half of the MM patients had undergone at least one previous treatment and had experienced recurrence. Patient 1 (MM1) underwent six previous treatments and had again relapsed. MGUS patients were monitored but had received no treatment at the time of the bone marrow biopsies. Their clinical data are presented in Table 1. All samples were frozen in liquid nitrogen at the moment of extraction and thawed before being used in the cytotoxicity assays.

To analyze the therapeutic potential of both PB and UCB eNKs, we tested them against the same patient samples in the presence or in the absence of the mAbs. In order to better understand the effect of PD-1 expression on the cytotoxic ability of eNKs, results were stratified attending to PD-1 expression in eNKs.

We first focused on results obtained using PD-1 negative eNK cells on MM patient samples (Figure 4). Neither daratumumab nor pembrolizumab, alone or in combination, had a significant cytotoxic effect on MM samples (Figure 4(a)).

On average, UCB eNKs were more cytotoxic on MM cells than PB eNKs when used as lone treatment (Figure 4(a,b)). UCB eNKs cytotoxicity was not significantly increased with the addition of daratumumab. This was not due to low CD16 expression, which was observed in more than 80% of the different UCB eNKs population (see Figure 1(d) and Suppl.

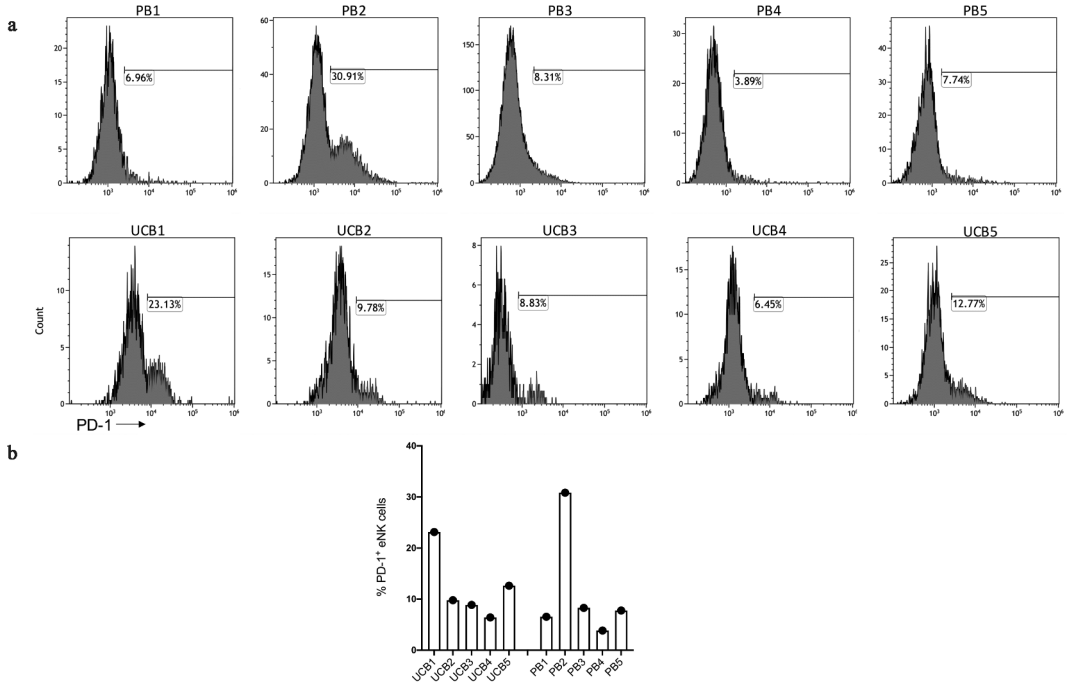

Figure 3. A. Pattern of PD-1 expression on all d-20 expanded NK cells (eNK) used in the study, obtained from PB (upper panels) or from UCB (lower panels). B, Summary of the percentages of PD-1^{+} eNK cells obtained from UCB or from PB, as indicated.

Table 1. MM and MGUS patient clinical data. (A) Age and previous treatment data were calculated based on the date of patient bone marrow biopsy. For MGUS patients, no treatment was given. The Durie-Salmon score is not applicable (NA) to MGUS patients. PD-L1 expression is based on our data. Patients with 0 previous treatments indicate newly diagnosed MM. (B) Numbers of MM patients treated with each of the UCB or PB NK cell expansions. (C) Numbers of MGUS patients treated with each of the UCB or PB NK cell expansions.

A		
	MM 18 (82%)	MGUS 4 (18%)
Age (at time of sample)		
Mean, years (range)	68 (45–83)	59 (49–77)
Gender		
Male	12 (67%)	3 (75%)
Female	6 (33%)	1 (25%)
Durie Salmon score		
I	5 (28%)	NA
II	4 (22%)	NA
III	9 (50%)	NA
Previous treatments (at time of sample)		
0	9 (50%)	NA
1	4 (22%)	NA
2	2 (11%)	NA
3+	3 (17%)	NA
PD-L1 Expression		
+ (>50% cells+)	9 (50%)	0 (0%)
– (<50% cells+)	9 (50%)	4 (100%)
B		
MM PTS	UCB eNK	PB eNK
1,2,3,4	1	1
8,9,10	2	2
11,13,14,15	3	3
16,17,18,19	4	4
20,21,22	5	5
C		
MGUS PTS	UCB eNK	PB eNK
5	–	1
6,7	2	–
12	3	3

Table II). In contrast, PB eNKs increased their specific cytotoxicity when combined with daratumumab from 25% to 38% on average (Figure 4(b)), close to the level obtained by using UCB eNKs alone. Finally, as expected, pembrolizumab had no significant effect on these PD-1–negative eNKs (Figure 4(a,b)).

Neither daratumumab nor pembrolizumab exhibited appreciable cytotoxicity when used alone or in combination on MGUS samples (Figure 5(a)). When eNKs were tested on MGUS cells, we observed again a significantly higher cytotoxicity of UCB eNKs than PB eNKs on the same samples (Figure 5 (a,b)). Cytotoxicity of UCB eNKs was slightly higher on MGUS cells than on MM cells. Again, the combination with daratumumab did not further increase the high level of UCB eNK cytotoxicity. Although we did not find any effect of pembrolizumab on MM cells, we observed a small average increase of cytotoxicity of PB eNKs on MGUS samples, but it was not statistically significant. The increase in cytotoxicity when combining PB eNKs with daratumumab, although observed, was also not statistically significant. Pembrolizumab had no further effect. Cytotoxicity of PB eNKs was also higher on MGUS than on MM cells. These data, obtained with eNKs from both PB and UCB, could indicate that MGUS patients may benefit from eNK treatment prior to disease progression.

2.5. Cytotoxicity of eNK cells with a significant PD-1$^+$ population

Figure 6(a,c) show results obtained with samples from the three patients treated with the only eNK obtained from PB that contained a significant PD-1$^+$ population (PB2). Curiously, the eNK from PB2 showed a higher overall average cytotoxicity than that observed using PD-1-negative PB eNKs, around 40% of specific cell death (Figure 6(c)). However, the increase in cytotoxicity when PB eNKs were combined with daratumumab, and previously observed in Figure 4, was lost.

For the four patient samples treated with the only eNK obtained from UCB that contained a significant PD-1$^+$ population (UCB1), we observed a dramatic decrease in cytotoxicity when compared to results obtained from PD-1-negative UCB eNKs (Figure 6(b,d)). The specific cell death average dropped from 42% (see Figure 4(b)) to 20%. This low cytotoxicity level was not due to resistance of the patient samples themselves, as cells from these same patients were sensitive to PD-1-negative PB1 eNKs, as shown in Figure 4. Addition of daratumumab did not increase the cytotoxicity of these eNKs, even though CD16

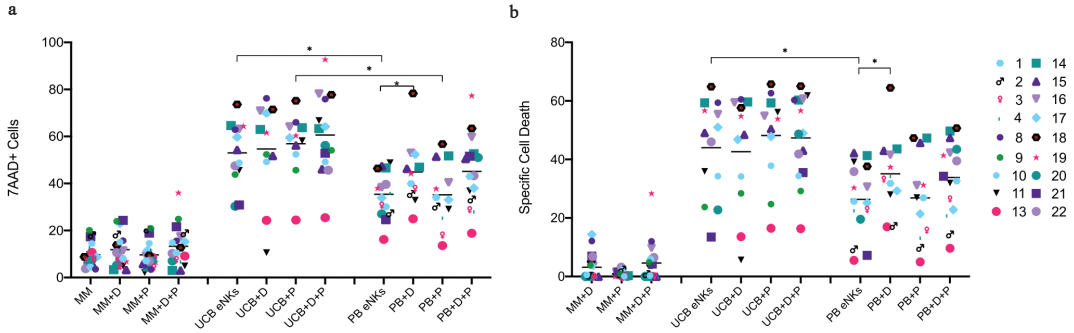

Figure 4. eNK cytotoxicity assays on samples from MM patients. Cells from MM patients were left untreated (MM), or they treated with daratumumab at 5 μg/ml (d), with pembrolizumab at 10 μg/ml (p), or with their combination (D + P), or incubated with PD-1-negative UCB or PB eNKs for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination. After the incubations, cell death was estimated by 7-AAD labeling in gated target cells (a), as indicated in Material and Methods. In (b) results are shown as the percentage of specific cell death induced, after subtracting basal cell death in each sample. Horizontal lines indicate the mean cell death in each experimental condition. One way ANOVA, ANOVA post hoc Tukey's analysis were applied for multiple comparisons among various groups. * indicates *p* value less than 0.05 and considered to be significant.

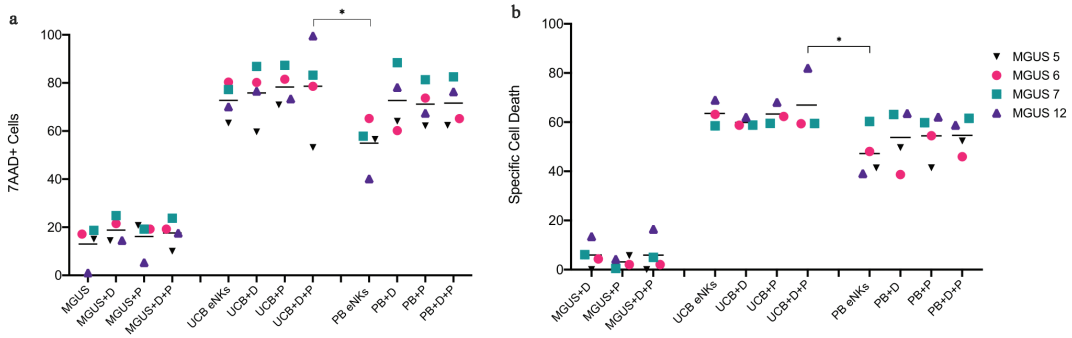

Figure 5. eNK cytotoxicity assays on samples from MGUS patients. Cells from MGUS patients were left untreated (MGUS), or they were treated with daratumumab at 5 µg/ml (d), with pembrolizumab at 10 µg/ml (p), or with their combination (D + P), or incubated with PD-1-negative UCB or PB eNKs for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination. After the incubations, cell death was estimated by 7-AAD labeling in gated target cells (a), as indicated in Material and Methods. In (b) results are shown as the percentage of specific cell death induced, after subtracting basal cell death in each sample. Horizontal lines indicate the mean cell death in each experimental condition. One way ANOVA, ANOVA post hoc Tukey's analysis were applied for multiple comparisons among various groups. * indicates *p* value less than 0.05 and considered to be significant.

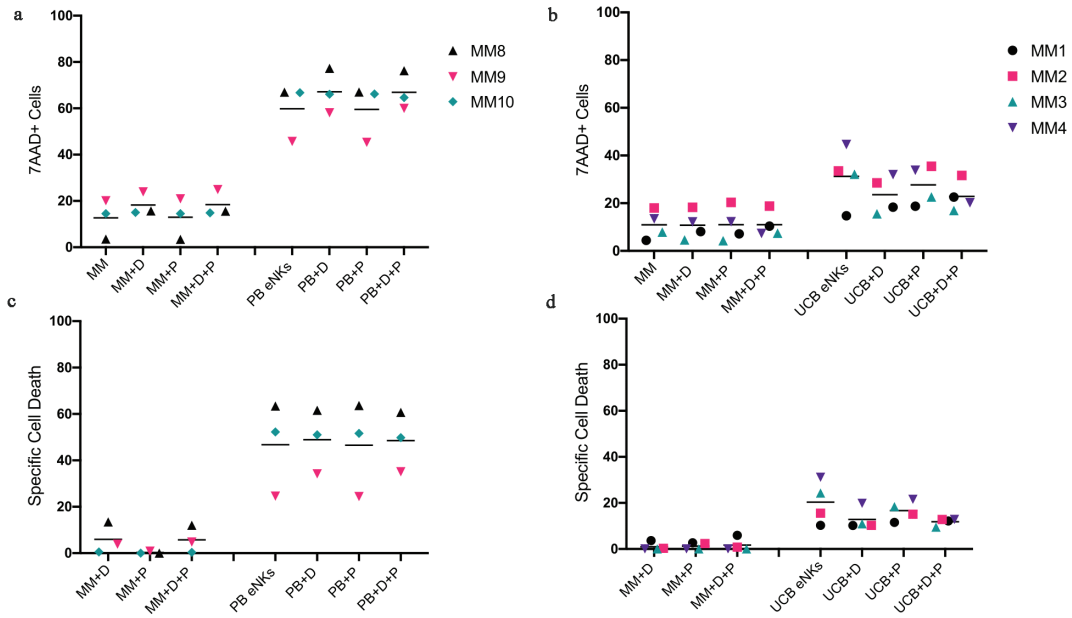

Figure 6. Cytotoxicity of eNK cells that contained a PD-1^{+} population. (a, c) Cells from the three indicated MM patients were left untreated (MM), or they were treated with daratumumab at 5 µg/ml (d), with pembrolizumab at 10 µg/ml (p), or with their combination (D + P), or incubated with eNK cells from PB2 for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination. (b, d) Cells from the four indicated MM patients were left untreated (MM), or they were treated with daratumumab at 5 µg/ml (D), with pembrolizumab at 10 µg/ml (P), or with their combination (D + P), or incubated with eNK cells from UCB1 for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination After the incubations, cell death was estimated by 7-AAD labeling in gated target cells (a, b), as indicated in Material and Methods. In (c, d) results are shown as the percentage of specific cell death induced, after subtracting basal cell death in each sample. Horizontal lines indicate the mean cell death in each experimental condition.

expression was high. This decrease in cytotoxicity was not due to the inhibitory effect of PD-1 because pembrolizumab did not increase cytotoxicity on any of the four patients tested. Interestingly, UCB1 was the only expansion that stopped proliferating at d 15 (see Suppl. Fig 2).

To complete the information on the cytotoxicity experiments, we include as Supplementary Figure 4 the individual cytotoxicity data obtained from the 22 patient samples, each treated with the eNK cells delineated in Table 1(b,c).

2.6. Pattern of PD-L1 expression in MM cells

In order to better understand the possible role of the PD-1/PD-L1 axis in the regulation of eNK cytotoxicity, we analyzed the PD-L1 levels of each MM sample (Figure 7(a)). In most of the samples, a population of PD-L1^{+} cells could be detected among MM cells, 40% being the average value. Only MM3 could be considered negative for PD-L1 expression. Thus, we divided MM samples as PD-L1^{high} or PD-L1^{low} depending on whether

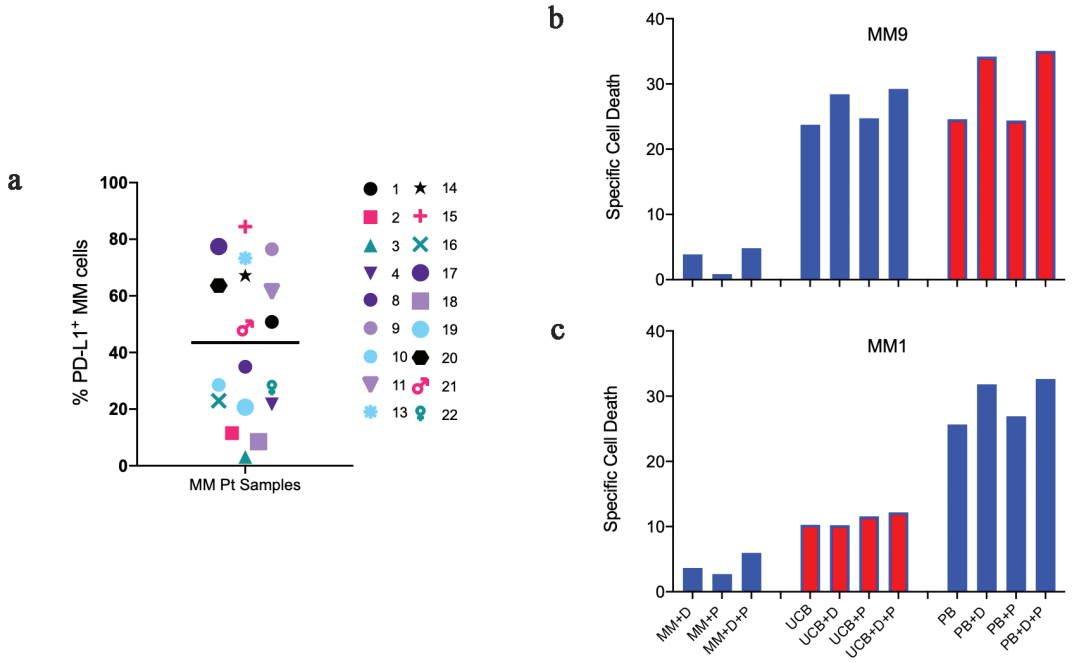

Figure 7. (a) Expression of PDL-1 on the surface of MM samples. The black line corresponds to the average value and is used to divide samples as PD-L1^{high} and PD-L1^{low}. Only cells from MM3 can be considered negative for PD-L1 expression. (b, c) Cytotoxicity assays using eNK cells that contained a PD-1^{+} population on PD-L1^{high} MM samples. B, MM9, which is PD-L1^{high}, was treated with PB2 eNK cells (red bars) or with PD-1 negative UCB2 eNK (blue bars). C, MM1, which is PD-L1^{high}, was treated with UCB1 eNKs (red bars) or with PD-1 negative PB1 (blue bars). Cell death was tested by 7-AAD labeling, as indicated in the legends of Figures 4, 5 and 6. Results are shown as the percentage of specific cell death induced, after subtracting basal cell death, which was never higher than 15%.

this population accounted for more or less than 40%, respectively. Nine patients fell into each category, although within each group there was high variability in expression levels. The average MFI value in the patients considered PD-L1^{high} was 10819, while in the patients considered PD-L1^{low} was 5720. The PD-L1 labeling for each individual MM and MGUS patient is shown in Suppl. Fig 5. We confirm previous data indicating that MGUS cells are negative for PD-L1 expression, while most MM samples showed PD-L1 expression to some degree.[35]

Taking into account that only few NK cell expansions contained a significant PD-1^{+} population (PB2 and UCB1), we were able to study the effect of pairing these eNK cells with PD-L1^{high} MM cells only in one patient for each UCB and PB eNKs. Patient 9 (MM9), in which 79% of the cells expressed PD-L1, was one of the patients treated with the PB2 eNKs. The cytotoxicity of PB2 eNKs on MM9 cells was intermediate (Figure 7(b), red bars). Of the MM samples treated with these eNKs, MM9 exhibited one of the lowest levels of cytotoxicity (see Figure 6(a,b)). In this respect, the interaction of PD-1 on eNKs with PD-L1 on MM cells correlates with rather low cytotoxicity levels. A slight increase in cytotoxicity was observed when PB2 eNKs were combined with daratumumab. However, the expected increase in cytotoxicity due to PD-1 blocking by pembrolizumab was not observed. MM9 cells were also treated with eNKs from the PD-1 negative UCB2. A similar level of cytotoxicity was observed, slightly increased by the combination with daratumumab and unaffected by incubation with pembrolizumab (Figure 7(b), blue bars).

Patient 1 (MM1) showed 54% of cells expressing PD-L1, and was one of the patients treated with the UCB1 eNKs. In agreement with results obtained from the other patients treated with this UCB, cytotoxicity was markedly low with no more than 10% of specific cell death induction (compare Figure 7(c), red bars, with Figure 6(c,d)). Neither daratumumab nor PD-1 blocking with pembrolizumab increased the cytotoxicity of UCB1 eNKs on cells from this patient. Of note, the same result was observed using the same UCB eNKs against MM cells from patients 2, 3 and 4 (Figure 6(c,d)) that were PD-L1^{low} or negative (see Figure 7(a)). MM1 cells did not exhibit an intrinsic resistance to NK cell cytotoxicity, since when treated with eNKs from PB1, that were PD-1 negative, showed an intermediate level of cytotoxicity, somewhat increased by combination with daratumumab (Figure 7(c), blue bars).

Pembrolizumab was active in the experimental conditions used in this study. When used in combination with two PB eNKs that exhibited PD-1 expression in a significant percentage of cells, pembrolizumab was able to increase cytotoxicity on the PD-L1^{+} B-CLL cell line Mec-1 (Supplemental Fig. 6). When this assay was performed using another 5 PB eNKs that did not exhibit PD-1 expression, pembrolizumab was without effect (data not shown).

3. Discussion

The present work demonstrates how the activation and expansion of allogeneic NK cells generates eNK cells that are active against MM cells. The activation and expansion were achieved in UCB and PB Nks by using LCL lymphoblasts as feeders in combination with IL-2 and IL-15. In both cases, the final eNK product exhibited CD16 expression on an important fraction of their population (more than 80%), which would allow for

their combination with a variety of therapeutic mAbs directed against tumor antigens.[7] This increase in cytotoxicity could be associated with increased expression of activation markers,[7] and of the activating receptor NKp44.[45] Moreover, in a similar study using a 5-d activation protocol, it was shown that the main change justifying the increase in cytotoxicity was the net increase in the level of granzyme B expression in activated NK cells.[40]

We observed how UCB eNKs showed a higher average cytotoxicity on MM cells than PB eNKs. The combination of PB eNKs with the anti-CD38 mAb daratumumab, which is already an effective treatment in a fraction of MM patients, increased their cytotoxicity to the levels observed for UCB eNKs.

This is especially relevant as allogeneic NK cell activation-based therapy, alone or in combination with antibodies, is yet to be approved for treatment of MM. While CAR T cell technology is at the forefront of current studies, it is expensive and has detrimental side effects, such as humoral immunity inhibition that needs to be treated over long periods of time. eNK cell treatments do not generate a durable memory response and would not have this side effect. Given the wide variety of previous treatments undergone by the patients in this study, relapsed and refractory patients would benefit from and respond to eNK cell therapy.

MM patients present an immunosuppressive tumor microenvironment, in which almost all arms of the immune system are subverted.[22–24] Specifically, NK cell activity in MM patients is compromised and patient NK cells are ineffective against the development of the disease.[25] However, clinical data indicate that NK cells could be a therapeutic strategy in MM. In a pioneering work, Shi used haplo-identical mismatched NK cells treated with IL-2 for 2 d (no expansion) resulting in positive outcomes in 5 out of 10 relapsed MM patients.[29] Another study used expanded NK cells from patients as a therapy, with response in 2 out of 7 patients.[30] A similar study used expanded patient NK cells in combination with anti-myeloma drugs in five relapsed MM patients, showing partial but durable responses.[26] Nevertheless, no studies have been performed using allogeneic expanded NK cells obtained from healthy donors. More recently, an increase in cytotoxicity has been demonstrated on *ex-vivo* cells obtained from MM patients by combining expanded NK cells with carfilzomib[27] and also by combining daratumumab with the transformed NK cell line KHYG1 transiently expressing CD16.[31] Additionally, preclinical studies have demonstrated the efficiency of anti-CS1 (SLAMF7, CD319) NK-CAR cells on MM cell lines.[47] A recent study using a humanized mice model demonstrated a positive effect of combining NK cells with the anti-CD137 mAb urelumab, but not with daratumumab.[48]

PD-1 is an inhibitory receptor present on NK cells and activated $CD4^+$ and $CD8^+$ T lymphocytes. It is involved in immunosuppression by binding to its ligands PD-L1 and PD-L2, the former showing a broader expression.[9] MM is characterized by augmented PD-L1 expression[49,50] and some reports indicate that PD-L1 expression correlates with disease progression from the MGUS stage.[36,37] Because of that, several phase III clinical trials were conducted with checkpoint inhibitors in combination with established MM treatments:[51] pembrolizumab plus lenalidomide and dexamethasone (Dex) (KEYNOTE-185, NTC02579863); pembrolizumab plus pomalidomide (Poma) and dexamethasone (KEYNOTE-183, NTC02576977); and another study testing three different combination regimens (Poma and Dex vs. nivolumab, Poma and Dex vs. nivolumab, elotuzumab, Poma and Dex; CheckMate 602, NCT02726581). However, these studies were discontinued due to an increase in deaths in the pembrolizumab group as well as no objective responses. Indeed, the FDA has put on hold another clinical trial combining the anti-PD-L1 mAb atezolizumab with daratumumab for similar reasons (NCT02431208, see[38]).

Our data indicate that most eNK cells obtained using the protocol described showed a small population of $PD\text{-}1^+$ cells, suggesting that this inhibitory pathway would have a low relevance in the treatment of MM patients by these eNKs. However, in a minority of the cases, eNK cells showed a significant population of $PD1^+$ cells. Particularly in the case of UCB eNKs that contained this $PD\text{-}1^+$ population, cell death induction on MM cells decreased dramatically and addition of pembrolizumab did not restore cytotoxicity. This result was observed on MM cells positive or negative for PD-L1 expression. This suggests that these eNK cells from UCB are intrinsically nonfunctional, independent of the PD-1 signaling pathway. This result indicates that the status of PD-1 expression in the final cellular UCB eNK product should be determined as a marker of lower cytotoxic activity.

The present data could be supported by studies in 3D cultures or in *in vivo* MM models. However, 3D cultures require relatively long-term periods *in vitro*. During this time, tumor cells express ligands that were not expressed *ex vivo* and down-modulate the expression of other molecules. Moreover, there is a selection bias of certain clones that grow better in *in vitro* culture. In contrast, we examined tumor cell sensitivity to eNK cells directly *ex vivo*, without culturing them *in vitro*. We chose to test tumor cell sensitivity to eNK cells quickly after obtaining the cells from the patient, avoiding culturing the tumor cells, a process that can affect their sensitivity to allogeneic NK cells. Likewise, similar problems can be envisaged in human MM cell engraftment in NSG mice, as the human microenvironment is not reproduced.

UCB, rather than PB, contains different NK cell progenitor populations with the capacity to differentiate into NK cells[52] and this could originate NK cells with higher cytolytic activity against primary MM cells. Interestingly, UCB NK cells, compared to PB NK, have a higher expression of the bone marrow homing receptor CXCR4[52] and somehow this could help to recognize primary MM cells that niche in the bone marrow.

The present data indicate that UCB eNKs are to be preferentially used against MM in the absence of daratumumab while PB eNKs have significant cytotoxic advantage when combined with this mAb. Our present data, obtained with MM patients ranging from newly diagnosed to relapsed/refractory, reinforce the feasibility of using allogeneic eNK cells in the treatment of MM, mainly in view of their high cytotoxicity against tumor cells in combination with daratumumab.

4. Materials and Methods

4.1. Ethical statement

The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All

methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient or donor prior to surgery.

4.2. Bone marrow aspirates from multiple myeloma patients

Data and samples from patients were collected at the Clinical Hematology Department of the CHU Montpellier, France, after patient's written consent and following French regulations. Patients were enrolled in the HEMODIAG_2020 (ID-RCB: 2011-A00924-37) clinical program approved by the "Comités de Protection des Personnes Sud Méditerranée I" with the reference 1324. Samples were collected at diagnosis and kept by the CHU Montpellier. MM and MGUS samples were processed to isolate the mononuclear cell fraction from the bone marrow aspirate by density gradient centrifugation. Cells were then washed and frozen in a 10% DMSO solution in liquid nitrogen until use.

4.3. Feeder cell lines

721.221 and PLH are EBV+ transformed, human B-cell lymphoblastoid cell lines. Both were cultured in RPMI (Invitrogen) with 10% FBS at 37 C. Before use in the expansion protocol, cells were inactivated with γ-irradiation. PCR was carried out on cell lines periodically to test for mycoplasm contamination.

4.4. NK cell isolation and expansion

Peripheral blood was obtained from five individual donors of the "Etablissement Français du Sang (EFS)". This work also benefited from umbilical cord blood units (UCBs) and the expertise of Prof. John De Vos, in charge of the Biological Resource Center Collection of the University Hospital of Montpellier – http://www.chu-montpellier.fr/en/platforms (BIOBANQUES Identifier – BB-0033-00031).

PBMCs and UCBMCs were isolated through density gradient centrifugation using Histopaque-1077 (Sigma). Blood samples were diluted at 1:1 ratio with RPMI then layered above 10 mL Histopaque in a 50 mL conical tube. Once centrifuged for 30 minutes at 400xg, the white layers of mononuclear cells (MCs) were collected and washed.

Using EasySepTM Human CD3 Positive Isolation kit (StemCell Technologies), the $CD3^+$ cell fraction (T and NKT cells) of the MCs was depleted in each sample to better culture the NK cells. Once depletion was verified through flow cytometry, cells were cultured for 20 d. UCB NKs were cultured with Υ-irradiated PLH at a 1:4 (NK cell: feeder cell) ratio. PB NKs were cultured with Υ -irradiated 721.221 cells at a 5:1 (NK cell: feeder cell) ratio. The same concentration of IL-2 (100IU/mL) and IL-15 (5 ng/mL) were added to both NKs. Feeder cells and cytokines were refreshed every 3–4 d for UCB NKs and every 5 d for PB NKs. To monitor expansions, NK cells were stained with APC-labeled anti-CD3 mAb and PE or Vio770-labeled anti CD56 mAb (both from BD Biosciences) and the percentage of $CD3^-CD56^+$ cells estimated at each time point. At d 20, NK cell purity was 95% as average (see Suppl. Table I). Culture viability was determined at regular intervals through flow cytometry analysis.

4.5. FACS analysis

The expression of CD16 during the expansions was also estimated by flow cytometry using VioBlue or PerCP. Cy5.5-labeled anti-CD16 mAb (BD Biosciences). The expression of PD-1 in the final eNK product was analyzed with PE-labeled anti-PD-1 mAb (BD Biosciences). PD-L1 expression in gated MM cells from patients (positive for CD38) was tested with an APC-labeled anti-PD-L1 mAb (BD Biosciences). Flow cytometry analysis was performed with Beckman Coulter Gallios and data analyzed using BC Kaluza Analysis Software.

4.6. Cytotoxicity assays of patient samples with eNKs and mAbs

The cytotoxic potential of eNKs and mAbs against target cells was determined through a flow cytometry cytotoxicity assay. Patient samples were removed from liquid nitrogen storage, washed and incubated at 37°C for 30 minutes with relevant antibodies (daratumumab at 5 µg/ml, and pembrolizumab at 10 µg/ml) prior to use in the assays. eNK cells were co-cultured with target cells at a 5:1 effector:target ratio in the presence or absence of the indicated mAbs and incubated for 4 hours at 37°C. Subsequently, cells were marked with an APC-labeled anti-CD38 mAb or with a PE-labeled anti-CD138 mAb (all from Beckman) and with 7-AAD. NK cells were excluded from the gating by their smaller size and lower CD38 expression than MM cells, or by the absence of CD138 expression, respectively. MM cells present in the sample were gated using the CD38 or CD138 labeling and cell death estimated in the gated population by 7AAD positivity. In the samples where daratumumab is used, there is a competition between the therapeutic mAb and the anti-CD38 mAb used for flow cytometry, so only a CD138-based gating was used. Basal cell death was calculated from control samples and values were subtracted from those obtained for treated cells to obtain specific cell death. It is possible that this flow cytometry method would underestimate cytotoxicity, since cell debris coming from dead cells cannot be included in the analysis.

4.7. Statistical analysis

Statistical data were obtained using GraphPad Prism (5.0). The statistical relevance of each study was first evaluated via ANOVA, analysis variance. For statistical significance ($p < .05$), Post-hoc Tukey's analysis was employed.

5. Conclusions

NK cells are a promising source for cancer immunotherapy against hematological malignancies. Both UCB and PB are potential sources of NK cells. Our data present the optimal conditions for each eNK to produce maximal cytotoxicity against all types of MM – new, relapsed/refractory and MGUS. UCB eNKs were highly cytotoxic when used as monotherapy,

while PB eNKs combined with daratumumab had the greatest cytotoxic effect. These results indicate a potent future for eNK cell adoptive immunotherapy against MM and a need to tailor each eNK cell treatment based on treatment regimen.

Conflicts of interest

The authors report no conflict of interest.

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Paper III

Dynamic Changes in miRNA Expression during the Generation of Expanded and Activated NK Cells.

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Dynamic Changes in miRNA Expression during the Generation of Expanded and Activated NK Cells

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Abstract: Therapies based on allogenic Natural Killer (NK) cells are becoming increasingly relevant, and our laboratory has produced expanded and activated NK (eNK) cells that are highly cytotoxic against several hematological cancers when used alone or in combination with currently approved therapeutic monoclonal antibodies. In order to produce eNK cells, healthy human donor NK cells undergo a 20-day expansion protocol with IL-2, IL-15 and Epstein–Barr virus (EBV)-transformed lymphoblastoid feeder cells. In order to produce an even more potent eNK-based therapy, we must elucidate the changes our protocol produces within healthy NK cells. To understand the post-transcriptional changes responsible for the increased cytolytic abilities of eNK cells, we performed microRNA (miRNA) expression analysis on purified NK cells from day 0 and day 20 of the protocol using quantitative reverse transcription PCR (RT-qPCR). Of the 384 miRNAs profiled, we observed changes in the expression of 64 miRNAs, with especially significant changes in 7 of them. The up-regulated miRNAs of note were miRs-146a, -124, -34a, and -10a, which are key in the regulation of cell survival through the modulation of pro-apoptotic genes such as *PUMA*. The down-regulation of miRs-199a, -223, and -340 was also detected and is associated with the promotion of NK cell cytotoxicity. We validated our analysis using immunoblot and flow cytometry studies on specific downstream targets of both up- and down-regulated miRNAs such as PUMA and Granzyme B. These results corroborate the functional importance of the described miRNA expression patterns and show the wide variety of changes that occur in eNK cells at day 20.

Keywords: NK cells; miRNA; cytotoxicity; immunotherapy

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1. Introduction

Properly capitalizing on allogeneic natural killer (NK) cell-based therapies necessitates a better understanding of the transcriptomic changes that occur during the expansion and activation process. NK cells are critical components of the innate immune system that provide an early defense against viral, bacterial, and aberrant tumor cells. The presence of stress ligands on the surface of tumor cells and, on many occasions, their reduction or loss of HLA-I molecules makes them good targets for the action of properly activated NK cells [1,2]. It is worth noting that, due to the inhospitable tumor microenvironment and other immunosuppressive factors, patient NK cells are unable to carry out their cytotoxic function in many instances. In order to re-capture this function, allogeneic NK cells

produced from healthy human donors have become increasingly of interest as a cancer treatment, and several clinical trials are underway [3,4].

Pioneering work by the group of Dario Campana developed optimal protocols for activation and expansion of allogeneic or autologous NK cells, initially based on the use of K562 feeder cells transfected with membrane-bound Il-15 and the 41BB ligand [5]. Other groups developed successful expansion protocols based rather in stimulation with a cytokine cocktail containing IL-12, IL-15, and IL-18, generating cytokine-induced killers (CIK), with features of memory-like NK cells [6]. Many other protocols have been developed, using not only cells from adult healthy donors or patient NK cells, but also cells from umbilical cord blood or derived from pluripotent stem cells [7], some of them already in clinical trials [4,8]. We developed a NK cell activation and expansion protocol in which we established an optimal combination of cytokines and feeder cells to produce highly cytotoxic expanded and activated NK (eNK) cells from healthy human donor peripheral blood or from umbilical cord blood [9–11]. These expansion protocols would guarantee the use of NK cells from one allogeneic donor to treat one patient in future clinical applications. Over the 20-day expansion period, eNK cells increase expression of CD56 and NKp44 while retaining a constant expression of CD16. In addition, the cytotoxic potential of eNK cells is very much increased compared to freshly isolated NK cells from the same donors, and they exert marked cytotoxicity against cells from a variety of malignancies, especially when combined with therapeutic antibodies directed against tumor antigens such as CD20, EGFR, HER-2 [11], or CD38 [10]. Samples from patients with B-cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM) were treated ex vivo with the eNK cells, alone and in combination with monoclonal antibodies such as daratumumab (anti-CD38) and/or pembrolizumab (anti-PD1) [9,10]. In our MM study, eNK cells were produced from two different sources: umbilical cord blood and healthy adult donor peripheral blood. Each of the eNK cell types exerted a cytotoxic effect against the MM cells in several scenarios. eNK cells from peripheral blood benefited in potency from the addition of daratumumab, thereby suggesting a compelling combination therapy option [10]. Discovering the miRNA changes that produce our eNK cells will present us with previously unknown targets to exploit when creating a more potent cell-based therapy.

MicroRNAs (miRNAs) are small, noncoding RNA molecules that act as regulators of cell development, proliferation, and differentiation [12]. They post-transcriptionally regulate the expression of their target genes, whereby a single miRNA can regulate several genes. miRNAs negatively regulate gene expression by binding to the 3′- untranslated region (3′URTR) of messenger RNAs (mRNAs), which degrades the mRNAs or causes transcript destabilization [13]. Altered miRNA expression, such as overexpression, can promote oncogenes or suppress tumor-suppressing genes [14]. Understanding the impact of miRNA expression changes in healthy cells is equally relevant.

In previous work, we examined the changes in mRNA and miRNA expression in NK cells activated in 5-day protocols with IL2 alone, with IL2+K562 cells, or with EBV^+ R69 cells. These protocols did not result in prominent NK cell expansion, although they increased the cytotoxic potential of the NK cells [15]. miRNA expression analysis resulted in the identification of miR-23a as a key regulator of active granzyme B expression in activated NK cells. In this study, we went beyond the previous 5-day protocol and expanded and activated NK cells for a full 20 days. Using RT-qPCR, we examined the miRNA profiles of day 0 and day 20 NK cells. We then validated our findings using Western blot and flow cytometry studies. We sought to reveal the transcriptomic changes that occurred after the 20-day protocol was completed that allowed for eNK cells to have a greater cytolytic advantage over resting NK cells in NK-cell-based therapies.

2. Results

2.1. Expression of miRNAs in Healthy Donor NKs at Day 0 and Day 20 of Expansion

To establish the miRNA signatures of both resting and expanded NK cells, we purified NK cells from the peripheral blood of healthy human donors. Samples were taken at day 0 and day 20 once the expansion protocol was successfully completed. We tested four matched sets of eNK cells totaling eight samples. The increase in NK cell numbers over the course of these expansions is shown in Figure 1. The mean expansion of NK cells in these four donors was 162-fold, with a maximum expansion of 200-fold in expansion #4, as expected using our protocol (see refs. [9,11]). Each expansion would be sufficient to treat a single patient.

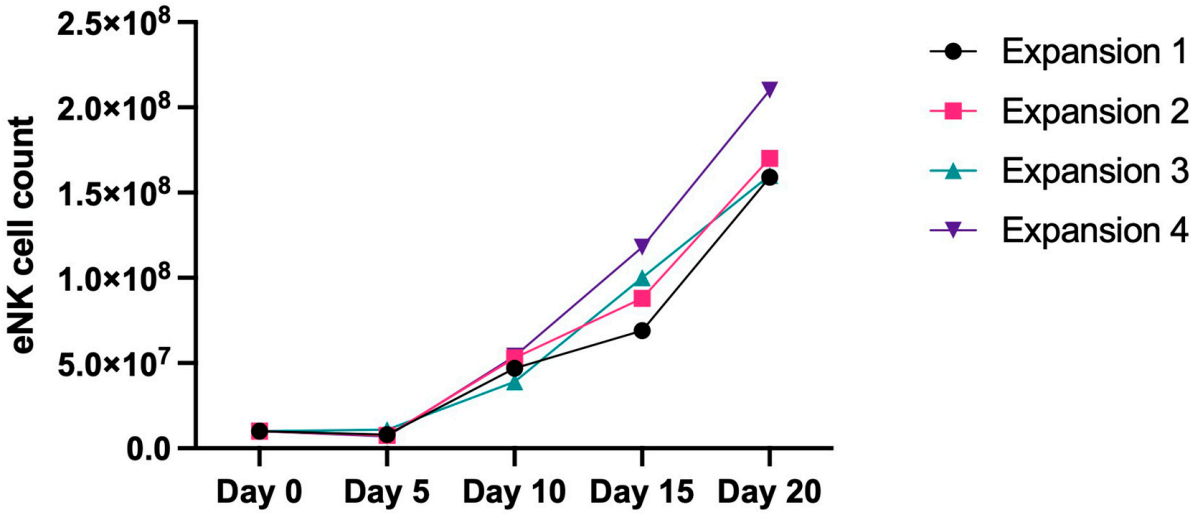

Figure 1. Total number of $CD56^+$ NK cells during the expansion protocols. The graph indicates the total number of NK cells at each stage of the expansion and activation protocol. The four expansions used for transcriptomic analysis are shown. Total number of NK cells is based on the number of $CD56^+CD3^-$ cells in each culture as verified via flow cytometry staining. Greater than 90% eNK cell purity was reached by day 20 on all expansions.

Then, using the TaqMan microRNA assay, we determined the changes in miRNA expression levels prompted by our expansion protocol in purified NK cells at day 0 and at day 20 of the expansions. We chose this assay because it contained all the relevant miRNAs that we wanted to explore. The day 0 samples and day 20 samples were analyzed individually, paired, then compared as two groups using ThermoFisher analysis software version 4.3 (v4.3). The ΔΔCTs were calculated using the day 0 values as a reference. All CT values above 35 were not considered valid as too many PCR cycles were required for detection, indicating low expression levels (Supplementary Figure S1). Of the 384 miRNAs quantified (see Supplementary Figure S2), 64 were considered differentially expressed: 39 were down-regulated and 25 were up-regulated, represented as a volcano plot in Figure 2A. From these data, we calculated the p values with the Applied Biosystems qPCR analysis application based on comparing the ΔΔCt values using multivariate analysis, considering statistically significant p values ≤ 0.05. After this analysis, the statistically significant down-regulated miRNAs were: miR-199a, miR-223, and miR-340 (Table 1). The four significantly up-regulated miRNAs were: miR-124, miR-10a, miR-34a, and miR-146a (Table 1). Figure 2B,C show the expression pattern in each of the samples using heat-maps corresponding to the up- and down-regulated miRNAs, respectively.

Figure 2. miRNA expression profile of eNK cells. (**A**) Volcano plot presenting the sum of eNK miRNAs differentially expressed between day 0 and day 20 of expansion. miRNA microarray

ex-pression profiling from 4 paired NK cell expansions was performed. The threshold to identify up and down regulated genes was a fold change ≥ 2 and $-$log10 (corrected p-value) < 0.1. Red dots in-dicate differentially up-regulated miRNAs and green dots indicate differentially down-regulated miRNAs. The black dot signifies a miRNA with null change while the grey dots indicates miRNAs with insignificant differential expression. (**B**,**C**) Heat maps showing hierarchical clustering of differentially expressed miRNAs from day 20 compared to day 0. Each column represents one NK sample (either from day 20 or day 0) and each row represents one miRNA. Relative expression is indicated using a color scale. Red tones are indicative of up-regulation (-1.1) and green tones are indicative of down-regulation (1). Full hierarchical clustering can be found as Figure S1 in Supplemental Figures.

Table 1. Differentially expressed miRNAs at day 20. The first 3 columns contain the most differentially down-regulated miRNAs at day 20. The last three columns contain the most differentially up-regulated miRNAs at day 20. Listed alongside each target miRNA are their fold change value and p-value when compared to day 0 values. miRNAs that were significantly differentially expressed at day 20 are marked with ** and * indicating p-values < 0.01 and 0.05, respectively.

Differentially Expressed miRNAs					
Down-Regulated miRNAs			**Up-Regulated miRNAs**		
Target	**Fold Change**	***p*-Value, C**	**Target**	**Fold Change**	***p*-Value, C**
hsa-miR-199a-3p	0.004	** 0.01	hsa-miR-124-3p	50,135.445	** 0.01
hsa-miR-223-3p	0.009	* 0.04	hsa-miR-34a-5p	159.077	** 0.01
hsa-miR-126-3p	0.012	0.19	hsa-miR-10a-5p	38.856	** 0.01
hsa-miR-495-3p	0.025	0.19	hsa-miR-589-5p	38.322	0.33
hsa-miR-193a-3p	0.026	0.12	hsa-miR-146a-5p	33.218	** 0.01
hsa-miR-145-5p	0.033	0.19	hsa-miR-615-3p	20.92	0.39
hsa-miR-193a-5p	0.036	0.17	hsa-miR-210-3p	15.214	0.12
hsa-miR-101-3p	0.04	0.12	hsa-miR-135b-5p	14.119	0.22
hsa-miR-130a-3p	0.047	0.19	hsa-miR-135a-5p	10.394	0.46
hsa-miR-338-3p	0.051	0.23	hsa-miR-330-3p	6.473	0.1
hsa-miR-340-5p	0.058	** 0.01	hsa-miR-342-5p	5.791	0.38
hsa-miR-365a-3p	0.092	0.39	hsa-miR-342-3p	4.618	0.2
hsa-miR-190a-5p	0.095	0.31	hsa-miR-363-3p	4.526	0.39
hsa-miR-450b-5p	0.105	0.31	hsa-miR-155-5p	4.361	0.27
hsa-miR-125b-5p	0.111	0.23	hsa-miR-130b-3p	3.951	0.27
hsa-miR-499a-5p	0.186	0.12	hsa-miR-193b-3p	3.038	0.3
hsa-miR-532-5p	0.19	0.12	hsa-miR-181b-5p	3.003	0.37
hsa-miR-26a-5p	0.196	0.31	hsa-miR-222-3p	2.749	0.43
hsa-miR-339-5p	0.226	0.36	hsa-miR-132-3p	2.701	0.44
hsa-miR-27a-3p	0.227	0.39	hsa-miR-195-5p	2.653	0.35
hsa-miR-30b-5p	0.242	0.27	hsa-miR-625-5p	2.631	0.19
hsa-miR-188-3p	0.247	0.27	hsa-miR-214-3p	2.488	0.27
hsa-miR-328-3p	0.255	0.23	hsa-miR-181a-5p	2.346	0.33
hsa-miR-29c-3p	0.258	0.27	hsa-miR-598-3p	2.274	0.27
hsa-miR-326	0.258	0.27	hsa-miR-152-3p	2.248	0.35
hsa-let-7b-5p	0.277	0.23			
hsa-miR-32-5p	0.293	0.31			
hsa-miR-140-3p	0.304	0.46			
hsa-miR-660-5p	0.319	0.34			
hsa-miR-194-5p	0.34	0.39			
hsa-miR-502-5p	0.36	0.19			
hsa-miR-30e-5p	0.376	0.39			
hsa-miR-421	0.381	0.48			
hsa-miR-361-5p	0.393	0.36			
hsa-miR-197-3p	0.4	0.34			

Table 1. *Cont.*

Differentially Expressed miRNAs					
Down-Regulated miRNAs			Up-Regulated miRNAs		
Target	Fold Change	*p*-Value, C	Target	Fold Change	*p*-Value, C
hsa-miR-339-3p	0.4	0.31			
hsa-miR-141-3p	0.406	0.24			
hsa-miR-192-5p	0.41	0.19			
hsa-miR-20a-5p	0.467	0.37			

2.2. Down-Regulated Differentially Expressed miRNAs

The miRNA with greatest down-regulation at day 20 when compared to expression levels at day 0 was mir-199a. By day 20, mir-199a expression was down-regulated with a fold change (FC) of 0.004 ($p < 0.01$), a statistically significant reduction. This miRNA has been studied in relation to different neoplastic and neurodegenerative diseases, particularly hepatocellular carcinoma [16–21]. The expression of miR-199a is down-regulated in hepatocellular carcinoma (HCC) compared with healthy donor samples, and this has been associated with the regulation of the pro-apoptotic gene *PUMA* [22]. Low levels of mir-199a in HCC cells correspond to low levels of PUMA, resulting in an anti-apoptotic effect, prolonging the survival of cancer cells. As such, mir-199a has become a therapeutic and diagnostic target in HCC and many other malignant tumors. In our study, mir-199a was also down-regulated at day 20, indicating that eNK cells can undergo changes in apoptotic pathways in order to prevent cell death.

The progression of PUMA expression was analyzed in eNK samples taken at D0, D10, and D20 of expansion and activation (Figure 3A). The PUMA levels at day 0 were extremely low and used as the point of comparison. Day 10 presented the highest levels of PUMA in the samples analyzed, with a mean expression of 23.8. However, by day 20, we observed a marked reduction in PUMA expression, averaging 5.05; a 79% decrease from day 10. Graphs detailing each expansion's PUMA progression can be found in Figure 3B,C. In the case of miR-222, there was a greater than two-fold increase at day 20, although not statistically significant. While mir-222 has not been directly studied in NK cells, it has been studied in a variety of cells, particularly in various cancers [23–25]. In these studies, miR-222 has been established as a powerful negative regulator of PUMA. The expression of PUMA is suppressed by miR-222, preventing the induction of cellular apoptosis [21,26]. The down-regulation of miR-199a and the up-regulation of miR-222 contribute to reduce PUMA expression levels at day 20, having an anti-apoptotic and pro-survival effect on eNK cells.

Although not statistically significant, miR-326, miR-140, and let-7b were also down-regulated, presenting FC values of 3.88, 3.29, and 3.61, respectively. These miRNAs have been shown to directly regulate Bcl-x_L, an anti-apoptotic protein from the Bcl-2 family, in a variety of cell types. miR-326 and let-7b have been shown to inhibit Bcl-x_L expression in stored platelets [27,28]. In vascular smooth muscle cells, the increased expression of miR-140 induced a down-regulation of Bcl-x_L [29]. In hepatocellular carcinoma, let-7b also negatively regulates Bcl-x_L expression, potentiating the effect of sorafenib-induced apoptosis by curtailing the anti-apoptotic function of Bcl-x_L [30]. In our eNK cells, we examined Bcl-x_L expression alongside PUMA using immunoblot analysis (Figure 3A). The day 0 expression levels were very low and were used as the point of comparison. Both of the eNK cell expansions analyzed had increased levels of Bcl-x_L at day 10 when compared to day 0; expansion 1 had its peak expression at day 10 with a slight decrease at day 20 (Figure 3D, while expansion 2 had its peak Bcl-x_L expression at day 20 (Figure 3E). Altogether, the eNK cell expansions maintained elevated expression of Bcl-x_L throughout the expansion when compared to day 0.

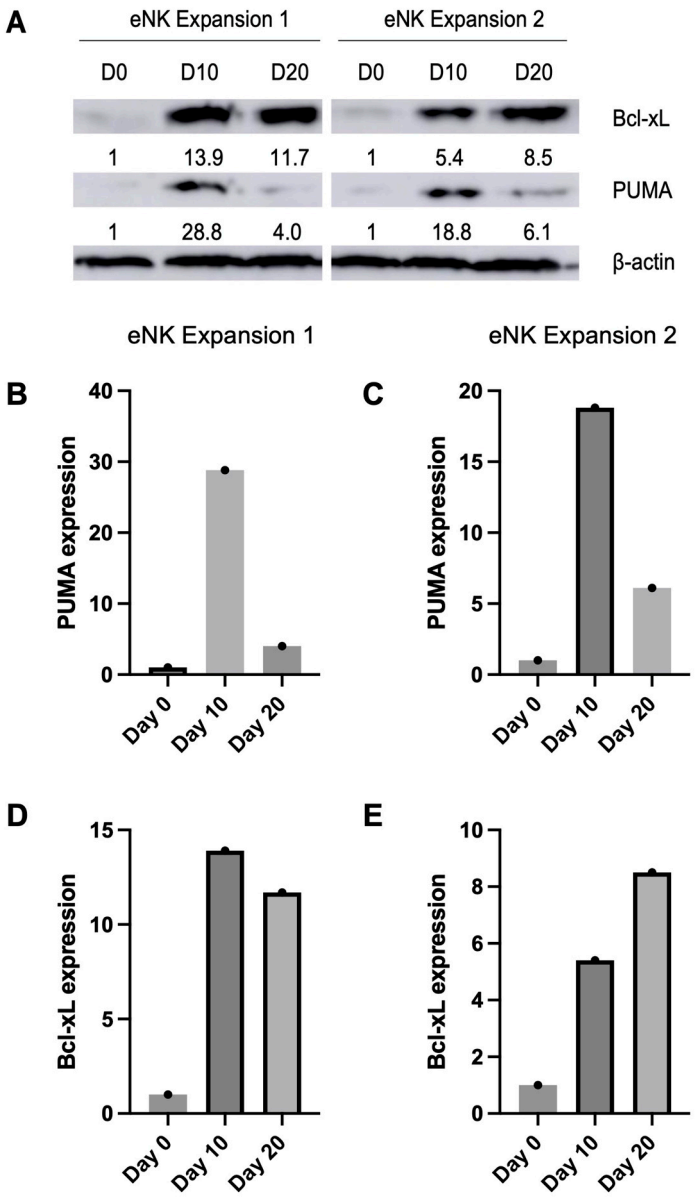

Figure 3. Validation of PUMA and Bcl-x_L expression levels using Western blot based on miR-199a findings. (**A**) Expression levels of Bcl-x_L and PUMA were determined via Western blotting in expansions 1 and 2. Samples were taken at day 0, 10, and 20 of the expansion. (**B**,**C**) Bar graphs show the quantified expression of PUMA at each stage of the expansion for eNK expansion 1 (**B**) and 2 (**C**). (**D**,**E**) Bar graphs showing the quantified expression of Bcl-x_L at each stage of the expansion for eNK expansion 1 (**D**) and 2 (**E**). Day 0 is used as point of reference and protein control was β-actin. Intensities of the bands were determined densitometrically. Protein samples obtained from two independent samples in each expansion were subjected to Western blot analysis, and results are shown as mean ± SD.

Our second most down-regulated miRNA was miR-223-3p (fc = 0.009; $p < 0.05$ *). This miRNA has been identified as being down-regulated in NK cells in a time-dependent manner upon exposure to IL-15 [31]. Granzyme B has also been identified as its direct target in NK cells. At rest, baseline miR-223 expression prevents granzyme B production. However, upon activation through the use of IL-15, miR-223 is down-regulated and granzyme B expression is increased. We observe this same pattern of expression in eNK cells with a steady increase in granzyme B being produced over the course of 20 days, with the highest levels recorded on day 20 (Figure 4A). Granzyme B is also regulated by

miR-27a [15]. As part of the miR-23a~27a~24-2 locus, miR-27a negatively regulates both granzyme B and perforin expression in NK cells [32]. Our analysis showed a greater than 80% down-regulation of miR-27a, which, while not statistically significant, did correlate to the increase of both granzyme B and perforin in eNK cells over the 20-day expansion (Figure 4A,B).

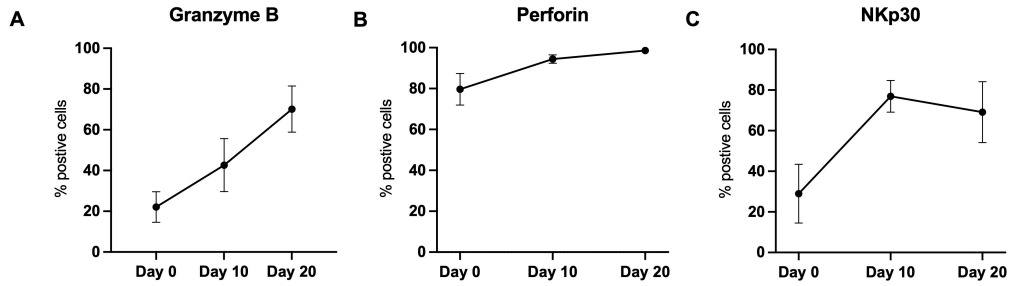

Figure 4. Flow cytometry analysis of granzyme B, perforin, and NKp30 expression in NK cells throughout the 20-day activation and expansion protocol. Levels of each were studied at 10-day intervals. (**A**) Granzyme B expression was analyzed intracellularly with a significant increase at day 20 as compared to day 0. (**B**) Perforin levels were studied intracellularly and remained steady throughout the 20 days. (**C**) NKp30 was studied extracellularly and had significant peak expression as day 10 as compared to day 0. * $p \leq 0.05$.

The third miRNA that was significantly down-regulated was miR-340-5p (fc = 0.058, $p < 0.01$). No studies are available on the role of miR-340-5p in NK cells. The down-regulation of miR-340 promotes the migration, cell proliferation, drug resistance, and invasion of several types of cancer cells, including colorectal and squamous cell carcinoma [33,34]. The possible downstream targets include ANXA3, PERK, and PNO1, all of which are negatively regulated [35,36]. Regarding NK cells, these properties can be translated to increased migration to target tissues, cell proliferation, and resistance to apoptosis.

2.3. Up-Regulated Differentially Expressed miRNAs

Of the 384 miRNAs analyzed, 4 were significantly up-regulated: miR-124, miR-34a, miR-10a, and miR-146a.

The role of miR-124 has not been directly studied in NK cells. However, miR-124 directly regulates Signal Transducer and Activator of Transcription 3 (STAT3) in several cell types [37]. STAT3 is constitutively activated in many cancers and its inhibition is being explored in clinical trials [38–40]. On the other hand, the loss of STAT3 in murine NK cells resulted in increased expression of granzyme B, perforin, and DNAM-1 [41]. Hence, the increase in miR-124 could be associated with this effect, correlating also with the effect of down-regulation of miR-233 and miR-27a. We described their effects in the previous section and could also correlate with the observed increases in cytotoxicity and in perforin and granzyme B expression (Figure 4A,B).

NKG2D and NKp30 expression has also been linked to STAT3, with conflicting reports [42,43]. We previously compared the expression of the activating receptors NKG2D and NKp30 at the beginning and end of our activation protocol [9]. We also explored the changes in NKp30 expression in our present study. NKG2D was highly expressed at day 0 and maintained expression throughout the expansion process [9]. NKp30 expression increased significantly with peak expression at day 10, with an average of 78% eNK cells expressing the receptor, a level that was maintained at day 20 (Figure 4C). Thus, the up-regulation of miR-124 can be taken as a negative regulator of STAT3, inhibiting the impact of STAT3 on the expression of NKp30.

Known as a tumor suppressor and regulator of apoptosis, miR-34a is a transcriptional target of p53 [44]. While miR-34a has not been directly linked to NK cell function, it was significantly up-regulated in eNK cells at day 20 (fc = 159.077; $p < 0.01$). This miRNA is down-regulated in several types of tumor cells when compared to healthy cells [45–47]. In

cervical cancer, among others, the overexpression of miR-34a results in an increase in cell death and decrease in proliferation due to cell cycle arrest [48]. As evidenced by previously published expansion data and shown in Figure 1, our eNK cells proliferated over 200-fold by day 20; thus, this negative effect on cell growth was not observed in non-tumoral eNK cells.

miR-10a is significantly up-regulated at day 20 (fc = 38.856; $p < 0.01$). The up-regulation of miR-10a has been described in several cancer types [49,50]. In acute myeloid leukemia, miR-10a directly binds to several key p53-dependent genes, suppressing p53 central role in cell cycle arrest and apoptosis [51]. The high levels of miR-10a expressed in eNK cells may have a role in eNK cell proliferation and further strengthen the antiapoptotic changes described in the previous section. As far as we know, this is the first reported analysis of miR-34a and miR-10a expression in activated NK cells.

Our eNK cells have a unique phenotype and miRNA profile compared to those of standard, non-activated NK cells. This is clear when analyzing the pattern of miR-146a expression, significantly up-regulated at day 20 (fc = 33.218; $p < 0.0.1$). miR-146a has been described as a negative regulator of IFN-γ production in NK cells and is one of the miRNAs most relevant to NK cell function [31]. In agreement with that, our eNK cells show high cytotoxicity but low cytokine production [9].

Previous studies focused on NK cells from diseased patients or resting $CD56^{dim}CD16^{+}$ cells [52,53]. When miR-146a expression was examined in various NK cell subsets, it was found that $CD56^{bright}CD16^{-}$ NK cells expressed high levels of miR-146a with corresponding high levels of IFN-γ after stimulation with IL-12 and IL-18 [54]. However, our eNK cells are $CD56^{bright}CD16^{+}$ effector cells [9], with a high capability to exert antibody-dependent cellular cytotoxicity (ADCC) [10,11]. As also shown in Figure 5, there is a progression of CD56 expression from dim to bright during the generation of eNK cells. However, the $CD56^{bright}$ cells that are attained at day 20 are not the same as the regulatory $CD56^{bright}$ cells found in the resting state in lymph nodes. In fact, eNK cells maintain high levels of CD16 throughout the expansion process, as opposed to $CD56^{bright}CD16^{-}$ NK cells [10,11].

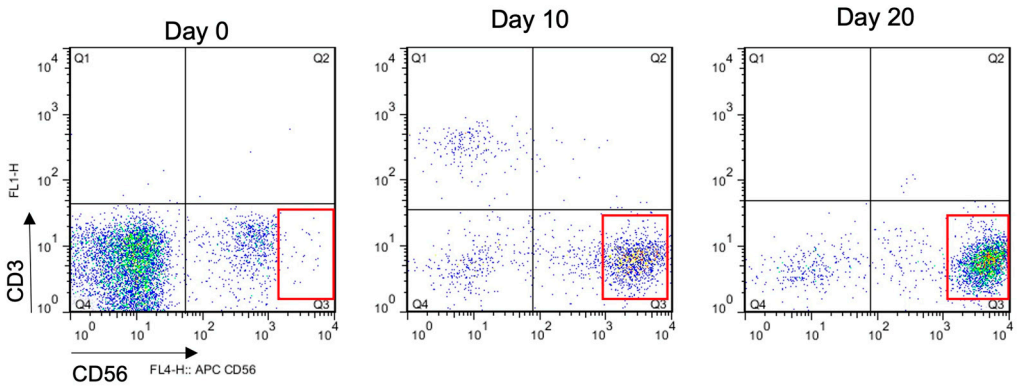

Figure 5. Phenotype progression of NK cells during expansion and activation. Representative dot plots from flow cytometry with X-axis showing CD56 expression and Y-axis showing CD3 expression. Red squares indicate the $CD56^{bright}$ NK cell population that expands and proliferates preferentially during the expansion protocol.

To examine the downstream effects of miR-146a up-regulation, we quantified IFN-γ intracellularly in our eNK cells throughout the 20-day expansion. The percentage of NK cells positive for IFN-γ expression remained very low throughout with peak expression at day 10 that never surpassed 20% of the NK cell population (Figure 6A). In addition, we explored the effect of CD16 ligation by the 3G8 mAb on IFN-γ expression. We confirmed that the basal level of IFN-γ expression in primary NK cells or in day 20 eNK cells is null (Figure 6B,C). When stimulated with 3G8, IFN-γ production levels increased significantly and averaged 40.9% in primary NK cells; meanwhile, although increasing, these levels reached a lower level in eNK cells (only 30.9%). All these results confirm that miR-146a

negatively regulates IFN-γ expression in eNK cells. These results confirm the unique changes that occur at both the transcriptomic and protein levels in order to produce effective eNK cells.

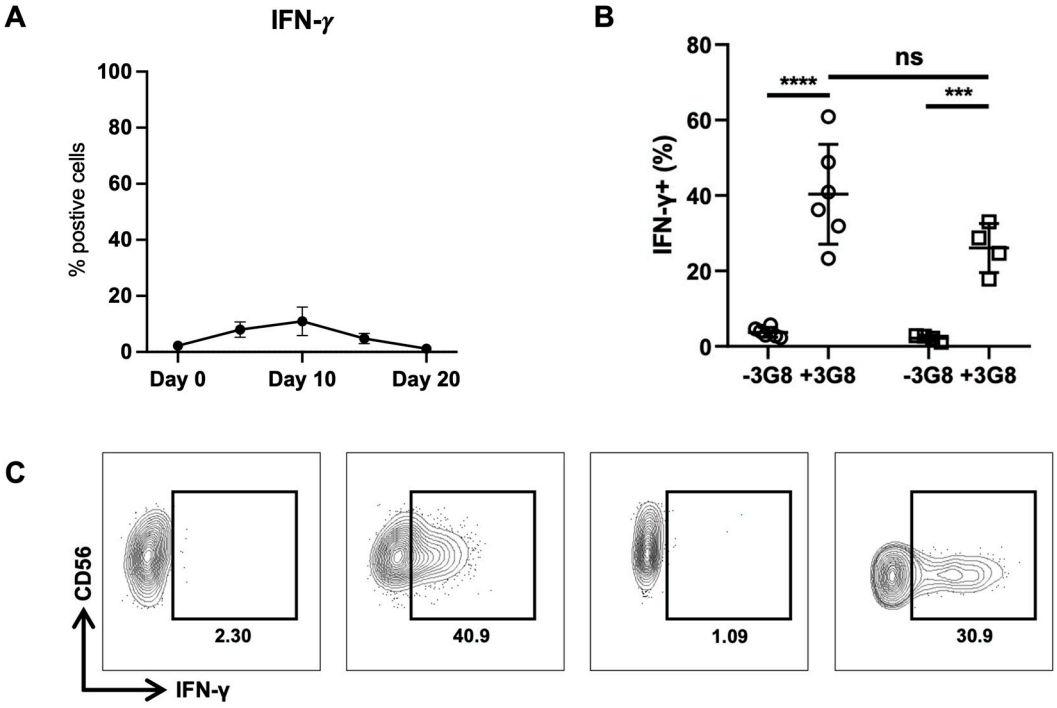

Figure 6. IFN-γ expression in eNK cells. (**A**) Flow cytometry analysis of intracellular IFN-γ during the 20-day expansion protocol. Expression was measured in NK cells every 10 days. (**B**) The percent of IFN-γ production in primary NK cells (left) and eNK cells (right) treated or not with the anti-CD16 agonist mAb 3G8. (**C**) Representative flow cytometry contour plot of IFN−γ secretion in primary (left panels) and eNK cells (right panels) treated (panels 2 &,4) or not (panels 1 & 3) with 3G8. **** $p \leq 0.0001$, *** $p \leq 0.001$.

3. Discussion

In this study, we report changes in the NK cell miRNA profile after expansion. Our expansion protocol is optimized for producing NK cells with high natural cytotoxicity and ADCC against multiple tumor targets in vitro and in vivo [9–11,55,56]. Of the 378 miRNAs in the array, we identified 3 miRs significantly down-regulated and 4 significantly up-regulated at day 20, while a total of 64 miRNAs showed some fold variation. Remarkably, the miRNAs significantly affected by the expansion protocol played roles in cell proliferation, apoptosis, and NK cell function. Moreover, we found strong consistency in the miRNAs that increased and decreased, their biological role, and the NK cell physiology. In fact, it is tempting to hypothesize that all these miRNAs are regulated in a "synchronous" manner after NK cells encounter target cells, leading to proper proliferation/expansion and activation.

A distinct pattern emerged amongst the up- and down-regulated miRNAs. The down-modulated miRNAs are mainly regulated by cytokines, particularly IL-2 and IL-15. Specifically, IL-15 is associated with the down-regulation of mir-223-3p and of the locus miR-23a~27a~24-2, resulting in the increased expression of perforin and granzyme B [15,31,32]. This type of miRNA-dependent gene regulation correlates with the effective increase in cytotoxicity observed in eNK cells, the most important functional feature of these cells for their use as an anti-tumoral treatment [9–11]. In relation to the miR-27a locus, our previous studies in 5-day activated NK cells have pointed to the importance of mir-23a in the generation of active granzyme B by the regulation of cathepsin C expression [15]. However, we did not detect the down-regulation of mir-23a in day 20 eNK cells. This could

indicate that by day 20, the relevance of mir-23a on granzyme B production waned and there was a change in emphasis towards regulation by miR-27a and miR-223-3p during the prolonged expansion.

This brings us to a large caveat in comparing our findings to previous studies in which the durations of activation are different [15]. In most of the studies performed, NK cells were activated for 1.5 to 5 days [15,57,58]. Therefore, it is difficult to compare relatively short activation protocols with the generation of cells that have largely proliferated and show high cytotoxic activity. As seen in our immunoblot and flow cytometry data, day 10 is a point of inflection, where the expression of activating receptors and cytokine production take off, and day 20 marks peak viability. Further studies of the transcriptomic changes that occur between day 0 and day 20 are necessary to fully understand the molecular mechanism(s) activated during expansion. However, it is clear that dramatic changes occur at day 10, allowing for the production of robust eNK cells at day 20.

Most studies related to gene regulation by down- or up-regulated miRNAs were performed in cancer cells [33,36,37,50]. We found that eNK cells use similar mechanisms as cancer cells to proliferate and evade apoptosis. The regulation of the pro-apoptotic member of the Bcl-2 family *PUMA* is especially interesting. The expression of this gene is inhibited by mir-199a down-regulation and by miR-222 up-regulation, both being events observed in day 20 eNK cells as compared with day 0 freshly isolated NK cells. While previous reports have shown that NK cells have higher cytotoxic ability beginning at day 5 of stimulation, the exponential expansion of eNK cells does not occur until day 10 [9–11,59,60]. This is also the peak of PUMA expression. It seems that, from day 10 on, there is a miRNA-regulated inhibition of PUMA expression, allowing for subsequent exponential growth until day 20. As previously discussed, the concerted action of several miRNAs is required to achieve full NK cell expansion. Additionally, the expression of Bcl-x_L remained high at day 20 in comparison to day 0 levels, indicating that eNK cells, once activated, express high levels of Bcl-x_L and maintain elevated levels throughout their expansion. Multiple miRNAs contributed to this sustained anti-apoptotic protein expression, allowing for the prolonged life of the eNK cells. The increase in PUMA expression at day 10 was not enough to overcome the anti-apoptotic expression of Bcl-x_L, as seen by the continued eNK cell expansion after day 10. A simplified schematic showing the effects of these miRNA changes is shown in Figure 7.

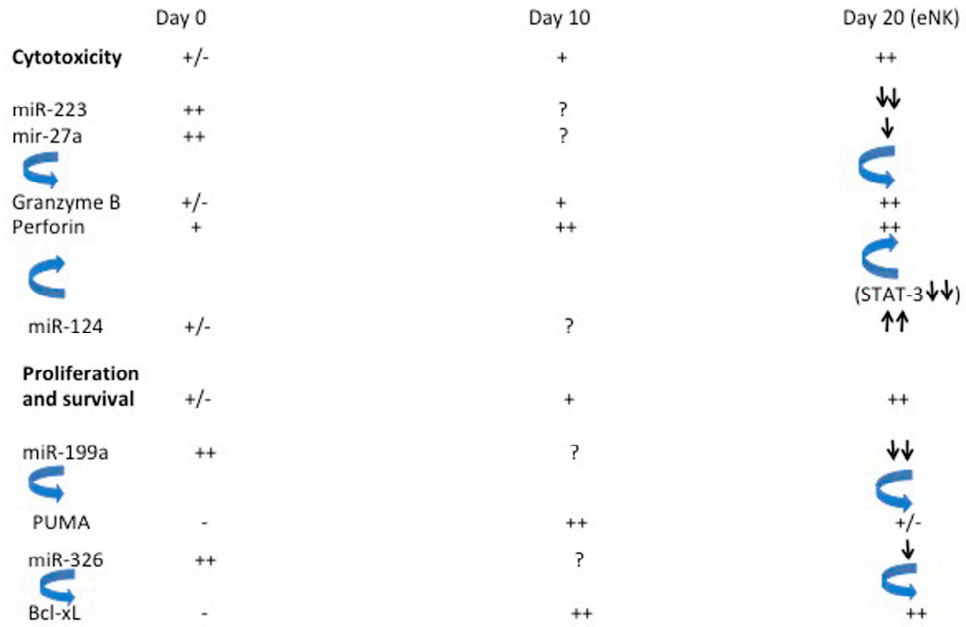

Figure 7. Simplified scheme of the changes in relevant genes and their regulation by specific changes in miRNA expression.

4. Materials and Methods

4.1. NK Cell Activation and Expansion Protocol

As previously described [9,10] healthy human donor leukopacks were obtained from the Blood and Tissue Bank of Aragon, Spain. Peripheral Blood Mononuclear Cells (PBMCs) were isolated using density gradient centrifugation with Histopaque-1077, density 1.077 g/mL (Sigma Life Sciences, Madrid, Spain). The $CD3^+$ cell fraction of PBMCs was partially depleted using the EasySep Human CD3 Positive Selection Kit II (Stemcell Technologies, Grenoble, France). The remaining cells, including the $CD56^+CD3^-$ NK cell fraction, were cultured alongside 25 IU/mL of IL-15 and 100 IU/mL of IL-2. Then, 721.221 cells, an EBV+ lymphoblastoid B cell line, were inactivated and used as feeder cells at a ratio of 1:5 with the total number of cells in culture. The combination of these concentrations of IL-15 and IL-2 were used based on our previous studies in which the addition of IFN-α was also tested, but did not offer clear advantages over the combination of IL-15 and IL-2 [9]. IL-15 is a cytokine needed to maintain the viability of activated NK cells [61] and IL-2 is needed to guarantee NK cell proliferation, together with the presence of feeder cells [62]. As 721.221 cells are HLA-null, this abrogates inhibitory KIR signaling, lowering the threshold for NK cell activation. The cytokines and feeder cells were refreshed every 5 days of the 20-day protocol. Samples of NK cells were taken at day 0, 10, and 20 of expansion for further study. The NK cell expansion and purity were monitored through flow cytometry every 5 days. The four expansions reached greater than 90% NK cell purity at day 20 as judged by $CD56^+CD3^-$ staining, similarly to results previously published using this protocol [10]. In any case, NK cells were purified before the miRNA determinations via positive selection using anti-CD56 mAb-coated beads from Milteny and magnetic separation.

4.2. Total RNA Extraction

The total RNA, including the miRNA fraction, was isolated from 8 NK samples (4 paired samples at day 0 and day 20) using the mirVANATM miRNA isolation kit (Thermo Fisher Scientific, Madrid, Spain) and performed according to manufacturer's protocol. We harvested 10^6 cells from day 0 and day 20 of each expansion and preserved them using RNA later (Thermo Fisher Scientific, Madrid, Spain) to preserve RNA integrity. The small RNA fraction was not enriched in order to better quantify the amounts of miRNAs in the total RNA fraction. The extracted RNA was quantified using the Qubit Fluorometer (Thermo Fisher Scientific, Madrid, Spain).

4.3. RT-qPCR and miRNA Quantitative Analysis

The TaqMan™ Advanced miRNA Assay (Thermo Fisher Scientific, Madrid, Spain) was the kit used for RT-PCR. We began by preparing cDNA templates for each sample using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Madrid, Spain). We used 2uL of total RNA for each sample. Once the mature miRNAs were reverse transcribed to cDNA, the Universal miR-Amp Primers were added to amplify the amount of cDNA for each target. Once amplified, RT-PCR was performed. The amplified cDNA template was mixed with TaqMan® Fast Advanced Master Mix. A final volume of 10 uL was loaded into the ports of each 384-well Taq-Man® Advanced miRNA Human A card (Thermo Fisher Scientific, Madrid, Spain). The control wells present on each card contained hsa-miR-16-5p, cel-miR-39-3p, and ath-miR159a. RT-qPCR was performed on a ViiATM 7 Real-Time PCR System (Thermo Fisher Scientific, Madrid, Spain). The polymerase activation was performed at 95 °C for 20 s. This was followed by 40 cycles of PCR consisting of denaturing at 95 °C for 1 s then annealing/extending at 60 °C for 20 s. The data were analyzed and the relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method from the Applied Biosystems™ qPCR analysis application (Thermo Fisher Scientific, Madrid, Spain). A Ct (cycle threshold) of 35 was chosen as a cutoff for analyzing samples, as 35 was the cycle before the background signal from control wells began to be amplified (Ct 37+). Wells containing samples with Ct higher than 35 were

considered as not expressing the analyzed miRNA and thus not used in our study. The p-values shown for RT-qPCR results were derived from the Applied Biosystems qPCR analysis application based on comparing the ΔΔCt values using multivariate analysis. p values ≤ 0.05 were considered to be statistically significant. All materials and software used for RT-PCR were sourced from Thermo Fisher Scientific.

4.4. Immunoblot

The NK cells were lysed in lysis buffer (1% Triton-X-100; 150 mM NaCl 50mM Tris/HCl ph7.6; 10% v/v glycerol; 1 mM EDTA; 1mM sodium orthovanadate; 10 mM sodium pyrophosphate; 10 µg/mL leupeptin; 10 mM sodium fluoride; 1 mM methyl sulfide, Sigma) on ice for 30 min and ultra-centrifugated for 20 min at 12,000 rpm at 4 °C. The protein concentrations were calculated using a BCA assay (Thermo Fisher Scientific). For each sample, 10 µg of protein was loaded and mixed with 3x lysis buffer (SDS 3% v/v; 150 mM Tris/HCl; 0.3 mM sodium molybdate; 30% v/v glycerol; 30 mM sodium pyrophosphate; 30 mM sodium fluoride; 0.06% p/v bromophenol blue; 30% v/v 2-mercaptoethanol, purchased from Sigma). For SDS-PAGE protein separation, a 12% polyacrylamide gel was used. Transfer was performed onto nitrocellulose membranes using a semi-dry electro-transfer method (GE Healthcare, Madrid, Spain). The membranes were subsequently blocked using TBS-T buffer (10 mM Tris/HCl, pH 8.0; 120 mM NaCl; 0.1% Tween-20, 0.1 g/L thimerosal, Sigma, Madrid, Spain) with 5% skimmed milk. Anti-PUMA (SR42-09, Novus Biologicals, Móstoles, Spain) and anti-Bcl-x_L (54H6, Cell Signaling, Barcelona, Spain) mAbs were used for Western blot and were incubated overnight in agitation at 4 °C. Appropriate anti-rabbit or anti-mouse peroxidase labeled secondary antibodies (Sigma) were incubated for 1 h at RT. The antibody dilutions used were specified by the manufacturer. The blots were analyzed with Pierce EL Western Blotting Substrate (Thermo Fisher Scientific, Madrid, Spain) using Amersham Imager 680 (GE Healthcare Life Sciences, Madrid, Spain). β-actin expression was used as a reference to normalize the data (Cell Signaling, Barcelona, Spain; Ref. 3700). ImageJ software (NIH, Bethesda, MD, USA) was used to quantify protein expression through densitometry.

4.5. Flow Cytometry

The intracellular and extracellular staining of NK cells was performed throughout the expansion. We washed 2.5 ×105 cells with PBS and stained them with the appropriate combination of fluorochrome-conjugated monoclonal antibodies for 30 min in the dark. The following antibodies were used in this study: CD56–APC, CD3–FITC (Miltenyi Biotec, Pozuelo de Alarcón, Spain); NKp30 (CD337)–PE (BD Biosciences, Madrid, Spain). Human NK cells are defined as being positive for CD56 staining and negative for CD3 expression, while NKp30 is a characteristic NK-cell-activating receptor. For intracellular staining, 5×10^5 cells were harvested and washed in ice-cold PBS, then fixed in 4% formaldehyde for 20 min at RT. The cells were then permeabilized using 0.1% saponin for 20 min at RT. After fixation/permeabilization, the cells were stained for perforin – REAfinity FITC, IFN-γ–REAfinity FITC (Miltenyi Biotec), and granzyme B–FITC (BD Biosciences). Appropriate isotype-matched controls were included. All stained cells were measured using a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using FlowJoTM v7.0 Software (BD Life Sciences, Ashland, OR, USA). To assess NK IFN-γ production using immobilized anti-CD16 Ab 3G8, Nunc™ Immuno Maxisorp 96-Well Microplates were used to coat anti-CD16 agonist 3G8 and the unstimulated group was replaced with 1 × PBS. After overnight incubation at 4 °C, 0.2 million PBMCs or eNK cells were added to each well, along with Brefeldin A, Monensin, and anti-IFN-γ fluorescent conjugated antibodies. The cells were cultured for 6 h in an incubator at 37 °C and 5% CO_2 before being harvested for flow cytometry. For these stainings, the gating strategy was first the selection of living cells by FSC/SSC, and then the selection of double positive cells for CD56 and for the specific marker in each case.

4.6. Statistical Analysis

Statistical analysis using Student's *t*-test was performed using GraphPad Prism v9 (GraphPad Software Inc., Boston, MA, USA) unless otherwise noted. p values ≤ 0.05 were considered significant.

5. Conclusions

We recently reviewed the current status of NK cell-based immunotherapies in clinical trials as they are of great interest as an off-the-shelf, non-GVHD inducing cellular therapy; several autologous and allogenic NK cell products are currently in clinical trials [4]. As there are several approaches to producing NK cells for clinical use, it is important to identify the transcriptomic changes associated with optimal anti-tumor activity. Identifying key miRNAs and their downstream targets will help in the production of more efficient anti-tumoral eNK cells. Establishing an eNK miRNA-based identity will also help differentiate the treatment of eNK cells from existing patient NK cells. In the present study, we demonstrated that the down-regulation of mir-223-3p and the locus miR-23a~27a~24-2 resulted in the increased expression of perforin and granzyme B, correlating with the effective increase in cytotoxicity observed in eNK cells. On the other hand, the expression of the pro-apoptotic protein PUMA is inhibited by mir-199a down-regulation and by miR-222 up-regulation in eNK cells. Together with the increase in Bcl-x_L expression, these changes favor the survival of the highly cytotoxic eNK cells generated.

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Paper IV

Harnessing the Potential of NK Cell-Based Immunotherapies against Multiple Myeloma.

Chantal Reina-Ortiz, David Giraldos, Gemma Azaceta, Luis Palomera, Isabel Marzo, Javier Naval, Martín Villalba, and Alberto Anel

cells

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Review

Harnessing the Potential of NK Cell-Based Immunotherapies against Multiple Myeloma

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Abstract: Natural killer (NK) cell-based therapies have emerged as promising anticancer treatments due to their potency as cytolytic effectors and synergy with concurrent treatments. Multiple myeloma (MM) is an aggressive B-cell malignancy that, despite development of novel therapeutic agents, remains incurable with a high rate of relapse. In MM, the inhospitable tumor microenvironment prevents host NK cells from exerting their cytolytic function. The development of NK cell immunotherapy works to overcome this altered immune landscape and can be classified in two major groups based on the origin of the cell: autologous or allogeneic. In this review, we compare the treatments in each group, such as autologous chimeric antigen receptor (CAR) NKs and allogeneic off-the-shelf NK cell infusions, and their combinatorial effect with existing MM therapies including monoclonal antibodies and proteasome inhibitors. We also discuss their placement in clinical treatment regimens based on the immune profile of each patient. Through this examination, we would like to discover precisely when each NK cell-based treatment will produce the maximum benefit to the MM patient.

Keywords: NK cells; multiple myeloma; daratumumab; isatuximab; autologous; allogeneic

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1. Introduction

From Dr. William B. Coley's first foray with bacterial injections into tumors in the 1890s to the 2018 Nobel Prize in Physiology or Medicine being awarded to Jim Allison and Tasuku Honjo for immune checkpoint inhibitors, immunotherapy has always looked towards being a cancer treatment. Immunotherapeutic approaches have focused on harnessing the adaptive immune response. Here we focus on Natural Killer (NK) cells, powerful members of the innate lymphoid cell family [1] that possess features of "adaptive" or rather, trained immunity [2]. They respond rapidly, without antigen specificity, during cellular transformation or viral infection. As innate lymphoid cells, NK cells target tumor cells through direct target killing and by the release of inflammatory cytokines.

1.1. NK Cell Function

With their discovery in the early 1970s, NK cells were thought to be specialized cells geared towards eliminating cancer cells. Specifically, they were described as small granular lymphoid cells that exerted a cytotoxic function against leukemia cells [3,4]. Once it was identified that NK cells were not of the monocyte or T cell lineage, their function and biology became easier to clarify [5]. As opposed to other cells also originating from the

common lymphoid progenitor, NK cells do not require prior sensitization to attain cytolytic activity. While they develop in non-nodal sites, such as the bone marrow and liver, they comprise 10–15% of total lymphocytes found in peripheral blood. NK cells constitute an ideal adoptive transfer treatment because of their multifaceted cytolytic biology and diverse mechanisms for activation. Their main function is antiviral, especially against viruses that induce loss of MHC-I expression, such as the herpes virus family. Accordingly, patients with impaired NK cell function are prone to viral infection [6]. Their role in immune surveillance against tumors is also well established [1,7,8].

1.2. Major Subsets of NK Cells

As NK cells express the NCAM-1 molecule, which clusters as CD56, they are identified as $CD56^{+}CD3^{-}$ lymphocytes. The difference in CD56 expression intensity divides NK cells into two major subsets: $CD56^{bright}$ and $CD56^{dim}$ cells, both types with unique functions and capabilities [9]. $CD56^{bright}$ cells produce pro-inflammatory cytokines, have a low expression of killer immunoglobulin-like receptors (KIR) and show a low level of cytotoxic activity. $CD56^{dim}$ NK cells constitute the majority of NK cells in peripheral blood and express greater amounts of CD16 on their cell surface than their bright counterpart. $CD56^{dim}$ NK cells are cytotoxic and are also able to induce potent antibody-dependent cellular cytotoxicity (ADCC) through CD16 binding to the Fc fraction of IgG [10]. In lymph nodes, $CD56^{bright}$ cells are the most common population and NK cell development probably follows the path: $CD56^{brigth}CD16^{-}$ to $CD56^{brigth}CD16^{dim}$ to $CD56^{dim}CD16^{dim}$ to $CD56^{dim}CD16^{+}$ [11].

1.3. NK Cell Activation

In order to carry out any cytotoxic effect, NK cells must first discriminate between target and healthy cells. NK cells have transmembrane receptors known as KIRs that recognize HLA-I haplotypes [12], and that are able to inhibit or activate NK cell function. The KIRs containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) recruit tyrosine phosphatases and inhibit cell function. KIR haplotypes are classified into groups A and B. Haplotypes in Group A encode only inhibitory receptors with a fixed number of genes, KIR2DS4 being the only exception. Haplotypes in Group B have variable types of KIR, also including genes encoding for activating receptors [13,14]. As per Ljunggren and Karre's "missing-self" hypothesis, NK cells select and target cells that do not express MHC-I [15]. This would allow NK cells to target only those cells "stressed" by viral infection or by neoplastic transformation and not healthy self-tissue. Another important inhibitory NK cell receptor is the C-type lectin-like heterodimeric receptor NKG2A and its isoform NKG2B, which recognize the non-classical MHC class I molecule HLA-E [16,17].

On the other hand, NK cells express numerous and variable activating receptors, which recognize viral determinants and/or stress-induced cellular ligands. Between them, natural cytotoxicity receptors (NCR) were one of the first families of activating receptors to be described. This family includes NKp46, NKp30 and NKp44, with NKp46 and NKp30 being constitutively expressed while NKp44 is only expressed after NK cell activation. NCR ligands include influenza virus hemagglutinin for NKp46, B7-H6 for NKp30 [1] and a tumor-associated ligand for NKp44 [18]. Another activating receptor is NKG2D, a C-type lectin-like receptor, which is constitutively expressed as a homodimer on the surface of NK cells. NKG2D have several types of ligands: the MHC class I chain-related molecules A and B (MICA and MICB) and the UL16-binding proteins (ULBP; [19]). Other NK cell activating receptors are the heterodimeric receptor CD94/NKG2C, which interacts with HLA-E [20], and DNAM-1, which compete with TIGIT and CD96 to bind to its ligands, CD155 and CD112 [21].

NK cell activation depends on signals mediated by activating and inhibitory receptors, the final functional outcome being the result of the balance between those activating and inhibitory signals [22]. It is remarkable that in a hematopoietic transplant setting, unlike T cells, NK cells do not elicit graft versus host disease (GvHD; [23]). However, the missing-self theory predicts that donor NK cells should react against recipient cells that do not express

the HLA molecules on which donor NK cells have been educated. The experimental result means that NK cells do not attack healthy, non-stressed cells which do not express ligands for their activating receptors, although NK cells were not receiving the KIR-mediated inhibitory signals. On the contrary, as indicated below, donor NK cells are able to attack recipient leukemic cells, since in this case, in addition to the absence of inhibitory signals, tumor cells provide ligands for donor NK cell activating receptors.

Between the activating NK cell receptors, CD16 is particularly important as it mediates potent ADCC. This Fc receptor is crucial for the anti-tumor activity of therapeutic monoclonal antibodies normally directed against molecules over-expressed by tumor cells [24].

2. Killing of Tumor Cells by NK Cells

Once a target cell has been identified and the correct NK cell activating receptors have been engaged, granule exocytosis is activated inside the NK cell. Granules containing perforin and granzymes are released with perforin creating a pore in the membrane of the target cell [25]. Both perforin [26] and at least granzyme A and B [27] are needed for tumor cell killing by NK cells. Granzymes enter the cytosol of the tumor target cell through the perforin pore and are able to induce several types of cell death, including apoptosis, necroptosis or pyroptosis, depending on the tumor target [28]. The presence of Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) on the surface of NK cells also provide secondary pathways through which to exercise their lytic actions [25]. NK cells have been shown to exert cytotoxicity through FasL expression [29], but it seems that TRAIL is more relevant for NK cell cytotoxicity [30]. NK cells also carry out anti-tumor activities through the production of IFN-gamma. This pleiotropic cytokine is able to induce apoptosis in some circumstances, but also to inhibit angiogenesis and contribute to the activation of both innate and adaptive immune anti-tumor responses [31].

Frequently, tumor cells, and especially metastatic tumor cells, reduce their MHC-I expression, allowing them to escape from recognition by cytotoxic T cells (CTL) and from immune surveillance [32]. In fact, mutations in β2-microglobulin that result in the impairment of MHC-I expression, are associated with resistance to anti-tumor CTL and the generation of evading lesions [33] and also with resistance to immune checkpoint inhibitor immunotherapy [34]. In this case, those tumor cells should be more sensitive to NK cell-mediated elimination, making NK-cell adoptive cell transfer a therapy of choice.

In addition, and in connection with the absence of GvHD mediation by NK cells in hematopoietic transplants, NK alloreactivity can also be exploited for the treatment of hematological cancers. This has been clearly demonstrated in pioneer studies that exploited NK cell alloreactivity for the treatment of blood-borne cancers [35–37]. Remarkably, it has been shown, at least on ex vivo cells from B-CLL patients, that KIR mismatch is not relevant when properly expanded and activated NK cells are used as effectors [38].

Phase I/II clinical studies have been performed using NK cells expanded through different approaches on multiple myeloma and acute myeloid leukemia (AML) including pediatric patients [39–42].

We will look at the dysfunctional aspects of the MM microenvironment that cause NK cell dysfunction and the clinical trials using autologous or allogeneic NK cell therapies to overcome these immunogenic hurdles.

3. NK Cells in Multiple Myeloma

3.1. Current Therapies for Multiple Myeloma

Multiple myeloma (MM) is the second most common hematological malignancy, characterized by the clonal expansion of plasma cells in the bone marrow [43,44]. Once a disorder without effective treatment, over the past two decades, new treatments such as autologous stem cell transplant (ASCT), immunomodulatory drugs (IMiDs), proteasome inhibitors and monoclonal antibodies have improved the survival rates of myeloma patients [45]. Current median survival is 6 years, and relapse, even after complete remission, is very common [45–47]. Regarding IMiDs, preclinical data showed that lenalidomide

enhances anti-myeloma cellular immunity mediated by $CD8^+$ T cells and by NK cells [48]. Later on, it was described that lenalidomide was able to reduce the expression of PD-1 in $CD8^+$ T lymphocytes and in NK cells, and of PD-L1 in MM cells and bone marrow accessory cells [49]. The combination of expanded NK cells with proteasome inhibitors has also been demonstrated to increase their cytotoxic potential [50]. Currently, through the identification of tumor cell surface markers, e.g., CD38 [51], the development of targeted antibodies and adoptive cell therapies have been developed. While many of the available therapies induce remission in new onset cases, relapse, often with acquired resistance, occurs [52]. There is thus a persistent need for novel and combinatorial therapies. As it will be indicated below in the description of the NK cell-based clinical assays, the combination with proteasome inhibitors and especially with IMiDs is given the best clinical results.

3.2. Antibody-Based Therapy of Multiple Myeloma

Antibody-based therapies rely on unique or over-expressed proteins on the surface of aberrant cells. In MM, the cell surface single-chain transmembrane glycoprotein CD38 is highly expressed and used as part of the definitive phenotype for MM cells. As MM cells have a high surface density of CD38, it has become the target for antibody therapy [53]. Daratumumab, a fully humanized IgG1 κ mAb, was the first to target CD38 and gain approval for MM treatment. Daratumumab has been approved as both monotherapy and in combination with several regimens of proteasome inhibitors and chemotherapeutic agents [54,55]. It causes the death of myeloma cells primarily through ADCC. This process occurs through the crosslinking of CD38-bound antibody on MM cells by the CD16 receptors on NK cells. Other pathways of action include antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC, [56]). Of note, NK cells also express CD38 on their surface, albeit at a lower level than myeloma cells. It has been reported that daratumumab causes fratricide between NK cells [57,58]. However, CD38 expression on myeloma or NK cells can be balanced to favor myeloma killing by daratumumab [59,60]. In addition, it has been demonstrated using two different expansion protocols that the deleterious effect of daratumumab is reduced on properly activated and expanded NK cells, demonstrating that the potential of the combination therapy is stronger than the possible fratricidal side effects [57,61]. Daratumumab augments, at least in an ex vivo model, the dysfunctional NK cell activity caused by the hypoxic and acidified MM microenvironment [62].

Isatuximab is a novel chimeric mouse/human IgG1κ mAb that also targets CD38. It is currently approved in combination with pomalidomide/dexamethasone in relapse/refractory patients with at least two prior therapies [63]. Although directed at the same target as daratumumab, it is principally dependent on the ADCC pathway and less on CDC [64]. There are several ongoing clinical trials examining its effectiveness alone and in combination with classic chemotherapies and proteasome inhibitors [65].

Elotuzumab is a humanized immunoglobulin G1 immunostimulatory mAb that targets the signaling lymphocytic activation molecule family member 7 (SLAMF7; [66]). This mAb is currently approved for relapsed and refractory MM [67]. SLAMF7 is expressed on both NK and myeloma cells and exerts its effect by activating NK cells directly and also by mediating CD16-dependent ADCC [68].

The use of mAbs has greatly improved treatment outcomes in MM, however, patients continue to relapse. Part of this is due to the heavy reliance of mAbs on functional NK cells to mediate ADCC. The suppressive MM microenvironment actively inhibits the function of immune cells, including NK cells. To overcome drug resistance and improve long-term treatment outcomes, the MM microenvironment must be explored, and its deleterious effects limited.

3.3. MM Microenvironment NK Cell Dysfunction

Alterations within the bone marrow microenvironment (BMM) guide the progress and ongoing persistence of MM. MM patients show variable infiltration of immune cells,

even in the early disease stage [69]. Remarkable changes in immune cell populations begin during precursor stages of MM, particularly MGUS [45]. The BMM contains NK cells, T and B-lymphocytes, a balance of osteoclasts and osteoblasts, fibroblasts, bone marrow stromal cells, endothelial cells, the extracellular matrix and blood vessels. Important growth factors, chemokines and cytokines are secreted by the stroma. Progressive immune deregulation impairs T, B, APCs and NK cell function in the MM niche [70]. Deficits in the humoral immune response are common in MM due to a reduction of bone marrow B-cell progenitors [71]. An immunosuppressive microenvironment is generated through the increased infiltration of regulatory T cells (Tregs), generating also aberrant CD4/CD8 ratios [72,73]. Additionally, an expansion of the myeloid-derived suppressor cells (MDSCs) population correlates with disease progression and with negative patient outcome [74]. On the other hand, BM stromal cells (BMSCs) favor myeloma cell proliferation and survival by cell-to-cell contacts and by the secretion of soluble factors [75]. Finally, several studies have shown impaired dendritic cell function in the MM microenvironment [76,77]. Genetic abnormalities in myeloma cells deregulate signaling and transport pathways and increase the expression of anti-apoptotic molecules [78], favoring their escape from immune surveillance as disease progresses [79]. Expression of immune checkpoint inhibitors is believed to be a mechanism of tumor escape in multiple types of cancers, including MM. Increased expression of PD-L1 on MM cells, combined with PD-1 expression on T cells are indicators of poor prognosis [80,81].

Consequently, the anti-myeloma effect of NK cells is be tempered by the immunosuppressive MM tumor microenvironment that leads to dysfunctional cytotoxicity. Despite this, MM patients have NK cells that are actively interacting and killing MM cells [82,83]. In fact, while Garcia-Sanz et al. reported an increase in NK cells found in the peripheral blood and bone marrow of MM patients, other studies detected a decrease in overall NK cell numbers [84,85]. On the other hand, of all lymphocyte subsets, only NK cell numbers increased after autologous stem cell transplantation in patients with long-term disease [86]. Total numbers aside, NK cells present in the tumor microenvironment are impaired and their activation and function decline as disease-stage progresses [87]. Indeed, the changes undergone by NK cells in the BMM have been implicated in the progression of MGUS to MM [88].

As previously discussed, NK cells require activating signals such as those provided by NCRs, NKG2D or DNAM-1 ligation in order to lyse target cells. The ligand for NKG2D most abundantly expressed by MM cells is MICA. As disease progresses, MICA is shed from the surface of MM cells and NKG2D is internalized, avoiding NK cell activation by this pathway [89,90]. DNAM-1 expression is also reduced as MM progresses while its ligand PVR is upregulated. Likewise, SLAMF7 and the FcyRIII CD16 have lower expression levels than in healthy controls [91].

In conjunction with the increase in PD-L1 expression on MM cells, NK cells also present up-regulation in the expression of PD-1 [92]. While the PD-1/PD-L1 axis is a promising avenue of treatment in most cancers, clinical assays in MM combining those checkpoint inhibitors and IMiDs have been underwhelming and have been discontinued (see the NTC02579863, NTC02576977, NCT02726581 and NCT02431208 clinical assays [93,94]). However, a recent study combining pembrolizumab with lenalidomide, dexamethasone and autologous transplant gave good results [95]. Presence of PD-1 on NK cells, even when the axis is blocked through the use of immune checkpoint inhibitors, do not result in increased cytolytic NK cell activity. Indeed, expression of PD-1 on NK cells appears to be a marker of exhaustion [61,96].

The most abundant cytokines in the MM microenvironment are IL-6 and IL-10, secreted primarily by perivascular cells and Tregs, respectively [97,98]. While IL-6 has both pro- and anti-inflammatory roles, in MM it exerts an immunosuppressive effect on NK cells and CTLs. In NK cells, IL-6 initiates a signal transduction pathway that implicates NF-κB and STAT3, resulting in reduced perforin expression and other down-regulations of NK cell cytotoxicity [99,100]. IL-6 also stimulates tumor cell proliferation, metastasis and survival [101]. Both MM cells and Tregs infiltrated in the MM microenvironment secrete the immunosuppressive cytokine TGF-β [102].

The most important effects of the MM tumor microenvironment on the inhibition of NK cell function are shown in Figure 1.

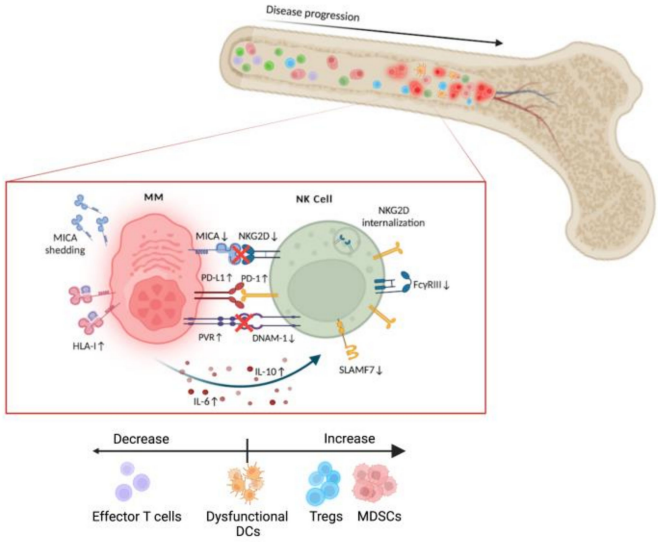

Figure 1. Schematic representation of the immunosuppressive MM microenvironment and the deleterious effect on NK cell anti-myeloma function. Created with BioRender.com.

Due to this extremely unfavorable microenvironment, patient NK cells frequently lack the robustness necessary to have an impact on MM development. In consequence, adoptive treatment with functionally activated NK cells will be key to augmenting the abilities of existing MM therapies. To this effect, there are several ongoing clinical trials that explore this immune niche, which are going to be described in the next section.

4. NK Cell-Based Treatment for MM

In order to overcome the above-described NK-cell dysregulation, several clinical studies have and are being carried out with NK cells as adoptive cell transfer therapies in MM patients (see also the recent review by Liu et al. [103]). There are 22 clinical trials registered in the clinicaltrials.gov database that use NK cells for the treatment of MM, alone or in combination with other therapies. The studies can be stratified based on the source of the NK cells used, whether they are autologous or allogeneic as described in Tables 1 and 2, respectively. In order to reach their full potential, NK cells should be activated and expanded ex vivo or supported through the addition of cytokines alongside NK cell infusions. A variety of "feeder" cells engineered to express ligands that activate the NK cells are used along with cytokines such as IL-2 and IL-15 for the maintenance and expansion of healthy, cytotoxic NK cells. The utilization of NK cells in adoptive cell transfer therapy was first trialed in 1985 on patients with metastatic cancers. The results showed no long-term clinical benefit, but NK cells were detected in the patients' fluids weeks after the infusions [104]. More recent NK cell-based trials have tried to capitalize on NK cell persistence while improving clinical results.

Table 1. Clinical trials based on autologous NK cell infusions. Bor—bortezomib, Elo—elotuzumab, Mel—melphalan, Len—lenalidomide, Isa—isatuximab, NR—not recruiting, * MM indicates that the trial was done in a variety of tumor types and that the number of MM patients was not specified.

Trial ID.	Specific NK Cell Source	Additional Treatment	Cytokine Support	Phase	Status (# Patients)	Trial Title
NCT01884688	K562-mb15-41BBL	-	IL-2	II	Completed (1)	A Phase II Study of Autologous Expanded Natural Killer Cell Therapy for Asymptomatic Multiple Myeloma
NCT01313897	K562-mb15-41BBL	Bor	IL-2	II	Completed (10)	UARK 2010-35, A Phase II Study of Expanded Natural Killer Cell Therapy for Multiple Myeloma
NCT03003728	K562-mb15-41BBL	Elo, Mel	IL-15 (ALT-803)	I	Withdrawn (10)	2015-10: A Phase II Pilot Study of Expanded Natural Killer Cells and Elotuzumab to Eradicate High-Risk Myeloma Post Autologous Stem Cell Transplant
NCT04558853	Autologous	-	-	I/II	Active, NR (6)	A Safety Study of CellProtect, an Autologous ex Vivo Expanded and Activated Natural Killer (NK) Cell Product, in Patients with Multiple Myeloma
NCT00720785	Autologous	Bor	-	I	Completed (35, * MM)	Safety and the Anti- Tumor Effects of Escalating Doses of Adoptively Infused Ex Vivo Expanded Autologous Natural Killer (NK) Cells Against Metastatic Cancers or Hematological Malignancies Sensitized to NK-TRAIL Cytotoxicity with Bortezomib
NCT02481934	K562-mb15-41BBL	Len, Bor	-	I	Completed (5)	Phase 1 Clinical Trial to Evaluate Security and Dose of Expanded and Activated Autologous NK Cells Infusions in Consolidation of Multiple Myeloma Patients Treatment on Second or Later Relapse
NCT04558931	Autologous	Isa	-	II	Recruiting	An Open, Randomized, Controlled, Phase II Trial of CellProtect in Combination with Isatuximab Antibody Versus Isatuximab Antibody Alone as Maintenance Treatment in Patients with Multiple Myeloma Undergoing High Dose Treatment

Table 2. Clinical trials based on allogenic NK cell infusions. Dex—dexamethasone, Cyc—cylophosphamide, Mel—melphalan, Flu—fludarabine, Bor—bortezomib, Elo—elotuzumab, Len—lenalidomide, Myc—mycophenolate mofetil, Rit—rituximab, ATG—anti-thymocyte globulin, Bus—busulfan, Clo—clofarabine, NR—not recruiting, NRP—no results posted, * MM indicates that the trial was done in a variety of tumor types and that the number of MM patients was not specified.

Trial ID	Specific NK Cell Source	Additional Treatment	Cytokine Support	Phase	Status (# Patients)	Trial Title
NCT0008945	KIR-L mismatch haploidentical	Dex, Cyc, Mel, Flu, Bor	IL-2	I/II	Completed, NRP (10)	UARK 2003-18, A Phase II Study of KIR-Ligand Mismatched Haplo-Identical Natural Killer Cells Transfused Before Autologous Stem Cell Transplant in Relapsed Multiple Myeloma
NCT00569283	Allogenic	-	-	I	Completed, NRP (18, * MM)	Donor Natural Killer Cell Infusion for the Prevention of Relapse or Graft Failure After HLA-Haploidentical Familial Donor Bone Marrow Transplantation-A Phase I Study
NCT00660166	HLA Class I Haplotype Mismatched	Ben	-	I	Completed, NRP (13, * MM)	HLA Class I Haplotype Mismatched Natural Killer Cell Infusions After Autologous Stem Cell Transplant for Hematological Malignancies
NCT00789776	Allogenic	-	-	I/II	Completed (41, * MM)	A Phase I/II Study Evaluating the Safety and Efficacy of Adding a Single Prophylactic Donor Lymphocyte Infusion (DLI) of Natural Killer Cells Early After Nonmyeloablative, HLA-Haploidentical Hematopoietic Cell Transplantation—A Multi-Center Trial
NCT00823524	Allogenic	-	-	I/II	Completed (47, * MM)	Donor NK Cell Infusion for Progression/Recurrence of Underlying Malignant Disorders After HLA-haploidentical HCT—a Phase 1-2 Study
NCT00990717	NK-92 cells	-	-	I	Completed (11)	A Dose Escalation Study of NK-92 Cell Infusions in Patients with Hematological Malignancies in Relapse After Autologous Stem Cell Transplantation
NCT02955550	PNK-007	Mel	rhIL-2	I	Completed (15)	A Phase 1, Multicenter, Open-label, Safety Study of Human Cord Blood Derived, Culture-expanded, Natural Killer Cell (PNK-007) Infusion Following Autologous Stem Cell Transplant for Multiple Myeloma
NCT01040026	Haploidentical	Mel	-	I/II	Active, NR (10)	A Phase I/II Single Center Study to Assess Tolerability and Feasibility of Infusions of Allogeneic Expanded Haploidentical Natural Killer (NK) Cells in Patients Treated with High Dose Melphalan Chemotherapy and Autologous Stem Cell Transplantation for a Multiple Myeloma
NCT04309084	CYNK-001	-	-	I	Active, NR (29, * MM)	A Phase I Study of Human Placental Hematopoietic Stem Cell Derived Natural Killer Cells (CYNK 001) in Multiple Myeloma Patients Following Autologous Stem Cell Transplant in the Front-line Setting

Table 2. *Cont.*

Trial ID	Specific NK Cell Source	Additional Treatment	Cytokine Support	Phase	Status (# Patients)	Trial Title
NCT01619761	UCB	Mel, Len, Flu, Myc, Cyc, Rit	-	I	Active, NR (12)	Natural Killer Cells in Allogeneic Cord Blood Transplantation
NCT01729091	UCB	Elo, Len, Mel	-	II	Active, NR (72)	Phase II Study of Umbilical Cord Blood-Derived Natural Killer Cells in Conjunction with Elotuzumab, Lenalidomide and High Dose Melphalan Followed by Autologous Stem Cell Transplant for Patients With Multiple Myeloma
NCT02890758	Non-HLA matched donor	-	ALT803	I	Active, NR (14, * MM)	Phase I Trial of Universal Donor NK Cell Therapy in Combination With ALT-803
NCT02727803	NK-92	ATG, Flu, Cyc, Clo, Bus	-	II	Recruiting	Personalized NK Cell Therapy in Cord Blood Transplantation
NCT03019666	NAM-NK	Cyc, Flu	-	I	Recruiting	A Phase I Trial Testing NAM Expanded Haploidentical or Mismatched Related Donor Natural Killer (NK) Cells Followed by a Short Course of IL-2 for the Treatment of Relapsed/Refractory Multiple Myeloma and Relapsed/Refractory CD20+ Non-Hodgkin Lymphoma
NCT04754100	agent-797	-	-	I	Recruiting	A Phase I Open-Label Study of the Safety, Tolerability and Preliminary Clinical Activity of Allogeneic Invariant Natural Killer (iNKT) Non-transduced Cells (agenT-797) in Patients with Relapsed/Refractory Multiple Myeloma

4.1. Autologous NK Cells

Autologous SCT after induction therapy for MM remains the standard of care for patients who are transplant eligible. While effective in temporarily holding the disease at bay, it is not curative. The addition of autologous cell transfer therapies can help prolong the effect of ASCT [105].

Of the clinical trials presented in Table 1, the team of Dr. Frits van Rhee at the University of Arkansas has led three: NCT01884688, NCT01313897 and NCT03003728. The two first assays showed that NK cells from heavily pre-treated MM patients could be expanded using K562-mbIL15- 41BBL feeder cells and that the combination with bortezomib, a proteasome inhibitor, before the NK cell infusion improved the results. Of the 10 patients enrolled in NCT01313897, 5 used autologous NK cells and 3 used haploidentical NK cells. Two patients were able to reach 6 months without further therapy (1 PR, 1 decrease in disease progression). The other 5 patients did not see a change in disease progression [42]. NCT03003728, which used eNK cells combined with elotuzumab, was withdrawn without definitive results.

The FDA recently gave orphan drug status to CellProtect, an autologous NK cell product that is frozen and delivered to clinics (NCT04558853). This product was trialed at Karolinska University hospital in newly diagnosed MM patients who had undergone ASCT. No severe adverse events were reported and of the 6 participants, 4 continued to have measurable disease after ASCT but then responded to NK cell treatment. The clinical trial NCT04558931 using this same CellProtect product with the addition of isatuximab in newly diagnosed MM patients is under the recruitment phase at this moment.

The combination of autologous NK cells and bortezomib was also studied in two further trials. NCT00720785 consisted of 35 participants with a variety of cancers including multiple myeloma. Each patient was treated with 3 escalating doses of ex vivo expanded NK cells. Those with hematological malignancies were sensitized to NK cell cytotoxicity through the addition of bortezomib. In NCT02481934, patients were stratified into two arms, 3 patients received lenalidomide while 2 received bortezomib. After infusions, there was an increase in NK cells and in the expression of its activating receptors NKp30 and NKG2D [40]. One patient was able to maintain partial response for 13 months after infusion. Of the others who completed the NK treatment, each achieved disease stabilization, which persisted for at least 4 months before disease progression. The only patient that was unable to finish the NK infusion due to unrelated toxicity experienced disease progression after 2 months, indicating the need to receive the full dose of NK cells.

The completed studies clearly show that autologous activated NK cells have anti-myeloma activity and that infusions from patient NK cells are possible.

4.2. Allogeneic NK Cells

Allogeneic NK cells are a convenient option that does not rely on the viability of patient NK cells. As each patient presents with a different treatment history and unique set of markers, expansion of autologous cells can be difficult and may fail. Induction of remission in patients with advanced acute leukemia was shown in pioneer clinical trials using allogeneic NK cells [106]. All studies are necessarily based on patients that have undergone some form of stem cell transplant. The success of lymphocyte reconstitution, particularly that of NK cells, is associated with better progression-free survival [107]. This applies to patients having undergone allogeneic hematopoietic stem cell transplantation due to the possible NK cell-mediated effects of graft-versus-tumor (GVT) [108].

To provide a more universal, off-the-shelf product, there have been several clinical trials in MM based on different forms of allogeneic NK cell therapy, summarized in Table 2. These studies are based on patients that have received allogeneic HSCT. The addition of allogeneic NK cells to augment the lymphocyte population should help in the prolongation of PFS. Several studies showed that treatment with allogeneic NK cells was well tolerated with no evidence of GvH disease (GvHD). However, relapse, progression-free survival

and overall survival were not significantly different from patients only having undergone ASCT (clinical trial NCT01040026; [109]).

Not all studies are based on haploidentical-donor-derived NK cells. In NCT04309084, allogeneic CYNK-001 NK cells are used. They originate from human placental $CD34^+$ cells and are enriched for the $CD56^+CD3^-$ subset after generation. These cells are infused in patients post-ASCT as a front-line treatment, in 3 dosing cohorts.

While many studies use adult-donor-derived NK cells, there is also an arm of clinical trials that use umbilical-cord-blood-derived NK cells (see Figure 2). In a multi-disease study out of MD Anderson, 13 patients with hematological malignancies, which included MM, were treated with ex vivo expanded umbilical cord blood NK cells (NCT01619761). The treatment plan consisted of 2 arms. In both plans, patients received a high dose of lenalidomide and fludarabine phosphate. Melphalan was added to arm 1 and cyclophosphamide plus total body irradiation (TBI) was part of arm 2. All patients received cord blood NK (CB-NK) cells prior to undergoing allogeneic umbilical cord blood transplants. The primary outcomes of this study were to generate a robust NK cell presence and to establish the treatment-related mortality. Focusing on MM, this team continued to study the safety and tolerance of CB-NKs after a high dose of melphalan and a low dose of lenalidomide (clinical trial NCT01729091; [110]). No toxicities resulted from CB-NK infusions. Of the 12 participants, 10 achieved a partial response or better. At the 21-month follow-up, only 4 patients had progressed, 2 having expired. Phase II continued with CB-NKs being used at doses of 1×10^8 CB-NK/kg, employing the previous treatment scheme. Three additional studies using this therapeutic approach are in the recruiting phase: NCT02727803, NCT03019666 and NCT04754100.

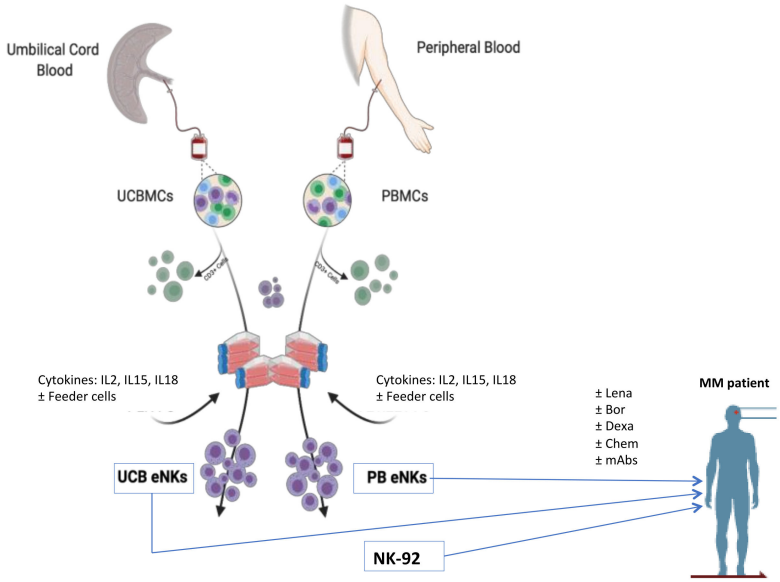

Figure 2. Schematic representation of the main therapeutic protocols described in Section 4.2. Abbreviations are the same as used in Tables 1 and 2.

Another category of allogeneic NK cells is that based on cell lines. In the phase I trial NCT00990717, NK cells from the NK-92 cell line are irradiated and given to patients in 3 escalating doses in 6 cycles [111]. Treatment resulted in minimal toxicity and was deemed safe. While 8 patients received only a fraction of the cycle due to disease progression, 3 patients did receive the full 6 cycles. Eventually patients did relapse, but the tolerability of NK-92 cells was established. Further modifications to this product are ongoing. A schematic representation of the different therapeutic protocols described is shown in Figure 2.

Overall, the clinical trials described here establish the safety and dose limitations of adoptive cell therapies with both autologous and allogeneic NK cells. Due to the variety

of allogeneic products available, their ease of manipulation and off-the-shelf formatting, trials are focusing on the development of this product. Further studies need to be done to ameliorate the long-term therapeutic outcomes of these treatments.

5. Future Prospects

The future of cancer immunotherapy, particularly that of harnessing the cytolytic power of NK cells is wide open. As summarized above, there are several avenues being explored for effective future treatments in MM. In order to fully realize the potential of NK cell therapies, we must have strategies for addressing challenges. Beginning with NK cell functionality, there is a large range of NK cell types being tested.

For example, in [61] results showed how expanded NK cells from umbilical cord blood were highly cytotoxic against MM patient bone marrow aspirates without the addition of monoclonal antibodies. However, expanded NK cells derived from healthy human peripheral blood did have a synergistic effect when combined with daratumumab. Each type of expanded NK cells would serve well in a situation based on possible treatment combinations and patient history.

While personalized treatments such as autologous CAR NKs or CAR T cells have their place in immunotherapy, the goal should be to make viable NK cell therapies universally available in all markets. For this, allogeneic NK cells would help MM cell therapy, as they do not elicit GvHD. This therapy would be scalable causing a decrease in overall cost and would be ready off-the-shelf. Having the treatment ready for the patient immediately would greatly reduce the waiting times caused by the production of autologous cell therapies. Collaborations between the private and public sector will also make this a competitive, highly available option. Combining NK cellular therapy with existing approved immunotherapies such as mAbs is also a promising avenue. In this sense, it is important that grafted activated NK cells maintain CD16 expression to mediate ADCC [24]. As we add to the number of successful existing therapies under the pillar of cancer immunotherapy, the possible combinations available to patients in every progressing situation also increases. Personalized medicine will venture beyond only the use of autologous cells and respond to fluctuations in the patients' genetic, phenotypic profile, and provide cellular treatments geared towards those changes. While there is still no definitive cure for multiple myeloma, the treatment landscape continues to evolve, and NK-cell-based therapy is on the horizon as an exciting, viable option that will create better outcomes for future patients.

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Chapter IV

Discussion

General Discussion

NK cells are powerful mediators of cellular cytotoxicity that can swiftly kill virally infected and transformed cells. However, in cancer, the tumor microenvironment's immunosuppressive mechanisms impacts the ability of NK cells and other immune cells to properly perform their function. As new immunotherapy treatments increasingly rely on functional NK and immune cells, the development of NK cell-based immunotherapies has gained central importance.

NK cell-based treatments are not currently approved for use in neither B-cell chronic lymphocytic leukemia (CLL) nor multiple myeloma (MM). Although the past decade has seen improvements in the rates of progression free survival and overall survival in these hematological cancers, they remain incurable in the majority of patients, especially those with MM. Harnessing the power of NK cells through the use of cellular immunotherapy presents an avenue of hope for these fatal cancers.

In this doctoral thesis, we developed highly cytotoxic expanded and activated NK (eNK) cells. A 5-day protocol was previously established that focused on activating NK cells sourced from the peripheral blood of healthy human donors[340]. In order to produce a sufficient number of NK cells to be used in the treatment of a patient, we developed a 20-day protocol that focused not only on activating NK cells, but also on expanding NK. Thus, we have titled them eNK cells, to signify their expansion. Over the course of 20 days, we optimized the cytokine cocktail used, relying on culturing with IL-2 and IL-15 alongside the feeder cells 721.221. These cells are an immortalized EBV+ HLA-I negative B

lymphoblastoid cell line. Lack of HLA-I allows for proper activation of NK cells without the inhibitory signals present on HLA-I positive cells used in other eNK cell-based therapies.

The function of NK cells is based on the signal balance between inhibitory and activating receptors on their cell surface. We established the changes undergone by eNK cells by comparing their phenotypic profile throughout the expansion protocol. eNK cells had substantially elevated levels of CD56 while maintaining steady levels of CD16. The maintenance of CD16 is key to eNK cells being able to work synergistically with mAbs that rely on CD16 throughout which they bind and induce cell death in target cells. Of note was NKp44, an activating receptor on NK cells, that had elevated expression at day 20.

Once the protocol for producing eNK cells was optimized, we began by performing cytotoxicity assays with our eNK cells against ex vivo CLL patient samples. Profiling both eNK cells for their KIR repertoire and the HLA-I on CLL cells was important in estimating a possible relationship between cytotoxicity and KIR-HLA match or mismatch. We had previously established the correlation between KIR ligand mismatch and sensitivity to NK cell mediated lysis in 5-day activated NK cells[340]. In our study, we initially had five eNK/CLL patient pairs with KIR ligand match. However, four of the five matches were highly susceptible to eNK cell induced death. The remaining case was one of two patients that presented resistance to eNK cells. As the previous study relied on NK cells that were activated for only 5 days without the presence of cytokines, the lack of response in KIR ligand matched cells could be caused by deficits in NK cell cytotoxicity. Our cells were sufficiently activated and expanded to overcome the limitation of KIR ligand match in 4 out of 5 cases presented. We conclude that properly activated and expanded NK cells can overcome KIR-HLA match.

Due to patient privacy and anonymity rights, we were only able to receive follow-up samples throughout the course of the disease from a limited number of patients. Two of the patients presented with *de novo* resistance to lysis by eNK cells, when they had previously been highly sensitive to eNK cell lysis. The patients presented vastly different clinical cases including disease state, treatment history, and KIR status. However, both patients had acquired substantially increased expression of PD-L1 when compared to their initial samples. PD-L1, the ligand of PD-1, an immune checkpoint of great clinical interest, inhibits NK cell cytotoxicity in vitro[345]. Blocking of either PD-1 on immune cells or PD-L1 on CLL cells using immune checkpoint inhibitors such as pembrolizumab has promising clinical benefit[346]. The increase of PD-L1 on CLL cells from resistant patients presents an interesting therapeutic target for immune checkpoint inhibitors and shows cases where eNK cells may not be effective therapy.

We were fortunate to obtain a third sample from one of the patients that developed resistance to eNK cells. During the intervening months between samples, the patient relapsed and had been treated with idelalisib, a PI3Kδ inhibitor. Upon examination of these cells, we found the patient had lost expression of PD-L1. When cytotoxicity assays were performed, the CLL cells had regained sensitivity to eNK cell mediated cell death. Since the effect of idelalisib on PD-L1 expression was only observed in one patient, we examined this effect on a PD-L1 expressing CLL cell line. Idelalisib treatment was able to partially reduce PD-L1 expression in these cells. These same cells were then utilized in cytotoxicity assays where their increased sensitivity to eNK cell mediated lysis was confirmed. It is important to note this occurred in a minority of cases where eNK cells expressed a substantial amount of PD-1, established as expression in more than 30% of the population. Studies in anaplastic large cell

lymphoma recently established the dependent relationship between the PI3K/Akt signaling pathway and PD-L1 expression[347]. Taken together, we conclude PD-L1 expression on CLL cells can possibly be reversed through the disruption of the PI3K/Akt pathway using idelalisib.

The success of eNK cells in CLL patient samples led us to test eNK cell cytotoxicity in MM *ex vivo* patient samples. Armed with the optimized eNK cell production protocol, we performed cytotoxicity assays using samples from patients with either MM or monoclonal gammopathy of undetermined significance (MGUS). We also produced eNK cells sourced from umbilical cord blood (UCB eNKs) using a similar expansion and activation protocol. Phenotypically, both peripheral blood eNKs (PB eNKs) and UCB eNKs had increased expression of CD56 while also exhibiting CD16 expression of more than 80% of their population. Expression of CD16 was of particular importance due to our addition of monoclonal antibodies (mAbs) to the cytotoxicity assay. CD16 expression on NK cells is necessary for mAbs to perform antibody dependent cellular cytotoxicity to lyse target cells[341]. The mAbs forming part of the assays were daratumumab, an anti-CD38 mAb approved for use in MM, and pembrolizumab, an anti- PD-1 mAb immune checkpoint inhibitor.

UCB eNKs when used as single therapy showed high average cytotoxicity when compared to PB eNK cells. The combination therapy of PB eNK plus daratumumab equaled the cytotoxicity level observed in UCB eNKs. Neither daratumumab nor pembrolizumab had an appreciable cytotoxic effect on patient samples when used as single therapy. A few of the eNK cell expansions presented with elevated PD-1 expression and produced low levels of cell death on the samples tested. Addition of pembrolizumab was not sufficient to restore the

cytotoxicity of PD-1^{+} eNK cells, regardless of the PD-L1 expression on MM cells. We concluded eNK cells, particularly UCB eNK cells, with PD-1 expression are nonfunctional and this is independent of the PD-1 signaling pathways as blocking this pathway has no appreciable effect on regaining cytotoxic function. The results of this study demonstrated the increased cytotoxic capacity of eNK cells and their efficacy in causing cell death in MM cells obtained from patients, alone or in combination with mAbs.

To understand the mechanisms behind the increased cytotoxic capability of eNK cells, we performed RT-qPCR on the miRNA of eNK cells at day 0 and day 20 of expansion. This elucidated the post-transcriptional modifications taking place throughout the protocol. We examined 384 miRNAs, in which 64 miRNAs presented fold changes. Of those 64, the changes in 7 miRNAs were especially significant at day 20 when compared to day 0. The four up-regulated miRNAs were miRs-146a, -124, -34a, and -10a and the three down-regulated miRNAs were miRs-199a, -223, and -340. Although the differentially expressed miRNAs were few in number, they are all key in cell proliferation, apoptosis, and NK cell function.

As most miRNA studies were performed in cancer cells, we were able to compare the function of certain miRNAs in cancer to their possible function in eNK cells. Many of the miRNAs that are up/down regulated to help cancer cells survive and thrive are also present in eNK cells. PUMA, a pro-apoptotic gene member of the Bcl-2 family, is of particular importance in both cancer cells and eNK cells. At day 10 of the protocol, the day in which expansion of eNK cells begins, we observed down-regulation of miR-199a and up-regulation of miR-222, both of which inhibit PUMA expression. Inhibition of PUMA expression could be responsible for the exponential growth of eNK cells after day 10. The down-regulation of

miR-223 is associated with IL-15 treatment, an important cytokine used in eNK cell expansion, and the subsequent expression of granzyme B and perforin, mediators of apoptosis in target cells. This correlates with the increased cytotoxic capacity observed in eNK cells and the steady increase of granzyme B in eNK cells throughout the 20-day expansion. Further studies of miRNAs at day 10 alongside mRNA studies would give us deeper insight into the mechanisms behind the success of eNK cells as effectors of cell death. Taken together, we conclude that the transcriptomic changes observed in the miRNA of eNK cells clearly correlate with the observed changes in protein and in practice.

Our eNK cell-based therapy presents a promising immunotherapeutic option that is both highly cytotoxic against transformed cells but can also work in combination with other effective immunotherapies. Our last article was a review on the current state of NK cell-based immunotherapies in clinical trials for approval as MM treatments. We divided the studies as using either autologous or allogenic NK cells. Although there are no currently approved NK cell-based treatments in MM, all these studies present an exciting future in the feel of cellular therapy. Currently, chimeric antigen receptors T cell (CAR T cell) therapy garners high levels of attention in the realm of cellular therapies. However, due to the steep cost and list of side effects, allogenic NK cell-based therapies present a viable alternative. Due to their biology, NK cells have a reduced risk of graft-versus-host disease (GvHD) and cytokine release syndrome(CRS), both of which are adverse effects encountered in CAR T cells. NK cell therapies have the potential to be scalable and an off-the-shelf product. This would reduce the cost to the patient and ensure a reliable, steady source of treatment. Of note is the ability of NK cells to combine and augment other therapies. At present, we present our potent eNK cell-based immunotherapy as an exciting, feasible option for treatment of hematological cancers, especially in combination with approved therapeutic mAbs.

Chapter V

Conclusions

Conclusions

1. Highly cytotoxic eNK cells were produced from either healthy human donor peripheral blood NK cells or NK cells sourced from human umbilical cord blood.
2. The 20-day protocol relies on an optimized cocktail of IL-2, IL-15 and 721.221 feeder cells.
3. The majority of eNK cells produced using our protocol have little to no expression of PD-1. eNK cells with greater than 30% PD-1^{+} cells should not be considered for use in a therapeutic setting as PD-1 was found to be a marker of functional exhaustion. eNK cells with high levels of PD-1 expression are unable to effectively lyse target cells.
4. The phenotypic profile of eNK cells consists of increased expression levels of CD56 and NKp44 while CD16 remains consistent or increases throughout the 20-day expansion.
5. KIR ligand mismatch is not necessary for eNK cells to lyse leukemic cells as eNK cells are sufficiently activated to overcome KIR ligand match limitations.
6. More than 90% of CLL cells are sensitive to eNK cell mediated cell death.
7. Sporadic cases of resistance to eNK cells are associated with high PD-L1 expression. Although this could not formally be proven as most eNK cells batches produced did not express PD-1.
8. Idelalisib, a PI3Kδ inhibitor, can reduce the expression of PD-L1 on hematological cancer cells through the disruption of the PI3K/Akt pathway.
9. MM cells are susceptible to eNK cell mediated cell death. UCB eNKs should be used as solo therapy against MM while PB eNKs benefit greatly from the addition of daratumumab to garner higher cytolytic activity against MM.

10. Treatment with anti-PD-1 mAbs does not restore the cytotoxic function in PD-1^{+} eNK cells, especially those eNK cells produced from UCB samples.
11. The transcriptomic changes that occur between day 0 and day 20 correlate with the expansion of eNK cells and their increased cytotoxic ability through inhibition of pro-apoptotic genes and proteins and increase in granzyme-B expression levels.
12. eNK cells produced using our optimized protocol are excellent effectors of cell death and have a future as an immunotherapeutic option for hematological cancers.

Conclusiones

1. Células eNKs con alta capacidad citotóxica fueron producidas de NKs derivadas de sangre periférica de donante sano o de sangre de cordón umbilical.
2. El protocolo de expansión y activación de 20 días depende de un coctel de citoquinas optimizado que contiene IL-2, IL-15, y células 721.221 llamadas 'feeder cells'.
3. La mayoría de las células eNKs producidas usando nuestro protocolo tienen poca expresión de PD-1. Las células eNKs con positividad para PD-1 en más de 30% de la población no se deben consideradas aptas para uso como tratamiento porque consideramos que la expresión de PD-1 es un marcador de agotamiento funcional. Células eNKs con alta expresión de PD-1 no tienen la capacidad adecuada para causar muerte celular en las células dianas.
4. El fenotipo de células eNK consiste en el incremento de expresión de CD56 y NKp44, mientras CD16 se mantiene o incrementa durante la expansión de 20 días.
5. La falta de coincidencia de los ligandos KIR (KIR ligand mismatch) no es necesario para que las células eNKs realizan lisis celular en células de leucemia como las eNKs están suficientemente activadas para superar las limitaciones proporcionadas por la coincidencia de ligandos de KIR (KIR ligand match).
6. Mas de 90% de las células de CLL son sensibles a la muerte celular inducida por las células eNKs.
7. Casos esporádicos en CLL de resistencia a células eNKs está asociado con altos niveles de expresión de PD-L1. Esta correlación no fue formalmente concluida como la mayoría de nuestras células eNKs producidas no expresaban PD-1.
8. Idelalisib, un inhibidor de PI3Kδ, puede reducir la expresión de PD-L1 in células de canceres hematológicos a través de la vía PI3K/Akt.

9. Células de MM son susceptibles a la muerte celular mediada por las células eNKs. Las células UCB eNKs deben ser usadas como terapia sola contra MM y las células PB eNKs se benefician de la adición de daratumumab para producir altos niveles de citotoxicidad en MM.
10. Tratamiento con anticuerpos monoclonales anti-PD-1 no restaura la función citotóxica en células eNKs PD-1^{+}, especialmente las células eNKs procedente de muestras de UCB.
11. Los cambios transcriptómicas que ocurren entre día 0 y día 20 se correlacionan con la expansión de eNKs y el incremento en su habilidad citotóxica a través de la inhibición de genes y proteínas pro-apoptóticas y el incremento en el nivel de expresión de granzima B.
12. Las células eNKs producidas usando nuestro protocolo optimizado son excelente efectoras de muerte celular y tiene un futuro como una opción como inmunoterapia para canceres hematológicos.

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Appendices

Appendix A

Articles in JCR

Journal Rankings in JCR of ISI Web of Knowledge

The tables indicate the ranking of each journal in their subject categories based on Impact Factor (IF).

Paper I

Expanded and activated allogeneic NK cells are cytotoxic against B-chronic lymphocytic leukemia (B-CLL) cells with sporadic cases of resistance. Calvo T*, Reina-Ortiz C*, Giraldos D, Gascón M, Woods D, Asenjo J, Marco-Brualla J, Azaceta G, Izquierdo I, Palomera L, Sánchez-Martínez D, Marzo I, Naval J, Vilches C, Villalba M, Anel A.

For **2020**, the journal **SCIENTIFIC REPORTS** has an IF of **4.38**.

Category Name	*Journal Rank in Category*	*Total Journals in Category*	*Quartile in Category*	*Journal IF Percentile*
Multidisciplinary Sciences	17	72	**Q1**	77.08

Paper II

Expanded NK cells from umbilical cord blood and adult peripheral blood combined with daratumumab are effective against tumor cells from multiple myeloma patients. Reina-Ortiz C, Constantinides M, Fayd-Herbe-de-Maudave A, Présumey J, Hernandez J, Cartron G, Giraldos D, Díez R, Izquierdo I, Azaceta G, Palomera L, Marzo I, Naval J, Anel A, Villalba M.

For **2020**, the journal **ONCOIMMUNOLOGY** has an IF of **8.11**.

Category Name	*Journal Rank in Category*	*Total Journals in Category*	*Quartile in Category*	*Journal IF Percentile*
Immunology	23	162	**Q1**	86.11
Oncology	37	242	**Q1**	84.92

Paper III

Dynamic Changes in miRNA Expression during the Generation of Expanded and Activated NK Cells. Reina-Ortiz C, Mozas MP, Ovelleiro D, Gao F, Villalba M, Anel A.

For **2022***, the journal **INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES** has an IF of **5.60**.
*Although the paper was published in 2023, the last available data is for 2022.

Category Name	*Journal Rank in Category*	*Total Journals in Category*	*Quartile in Category*	*Journal IF Percentile*
Cell Biology	60	191	**Q2**	68.8

Paper IV:

Harnessing the Potential of NK Cell-Based Immunotherapies against Multiple Myeloma. Reina-Ortiz C, Giraldos D, Azaceta G, Palomera L, Marzo I, Naval J, Villalba M, Anel A.

For **2022**, the journal **CELLS** has an IF of **6.00**.

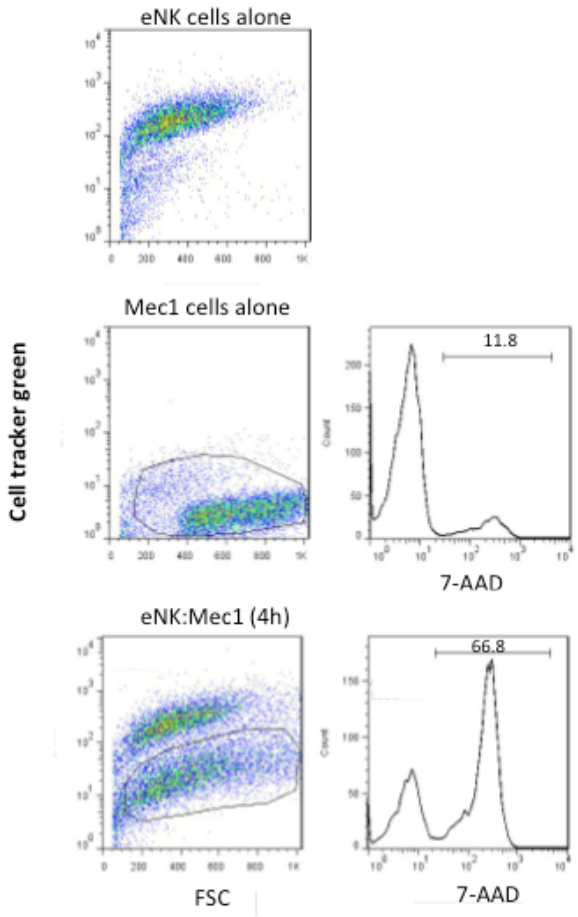
Category Name	*Journal Rank in Category*	*Total Journals in Category*	*Quartile in Category*	*Journal IF Percentile*
Biochemistry & Molecular Bio	66	285	**Q1**	77.0
Chemistry, Multidisciplinary	52	178	**Q2**	71.1

Appendix B

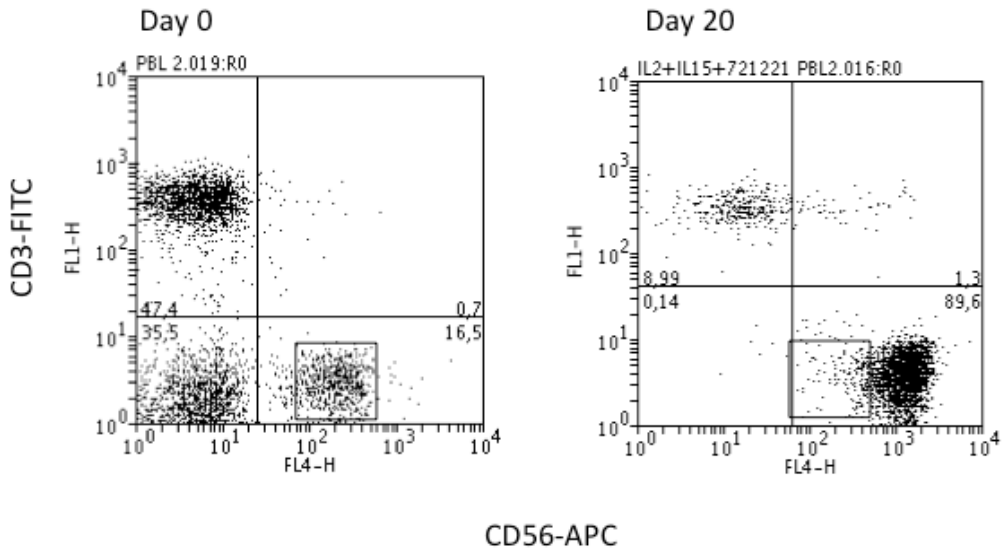
Supplementary Materials

Paper I

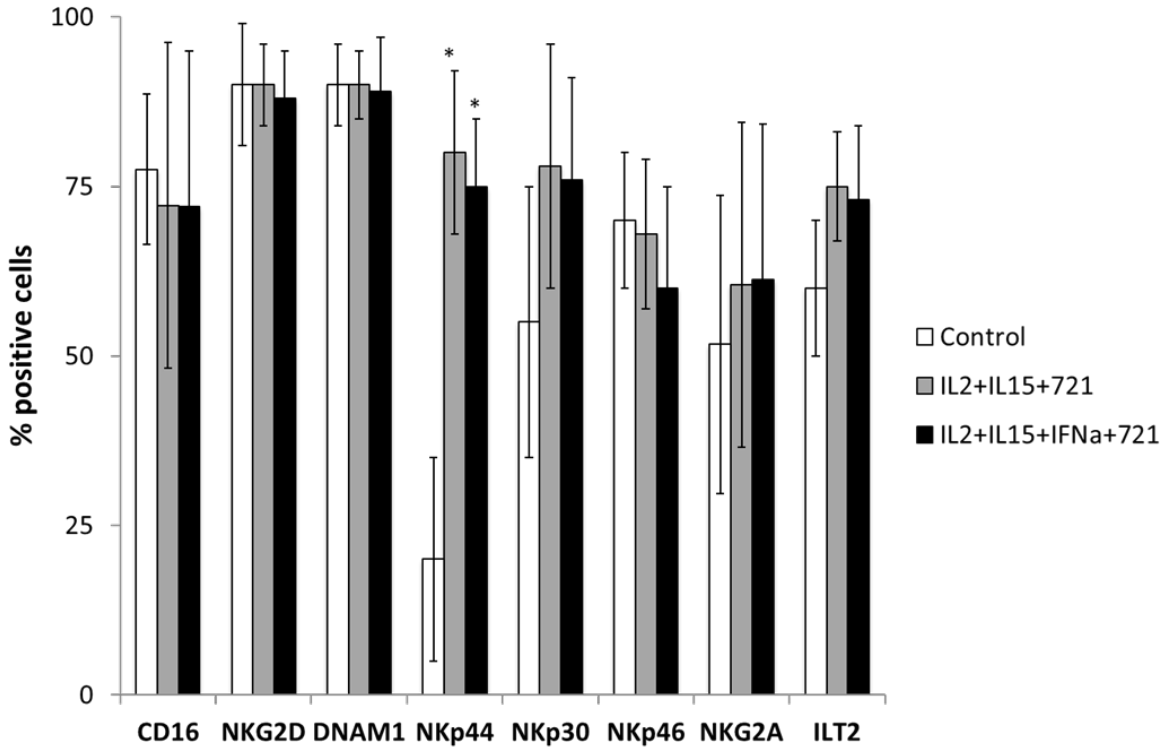
Supplementary Materials



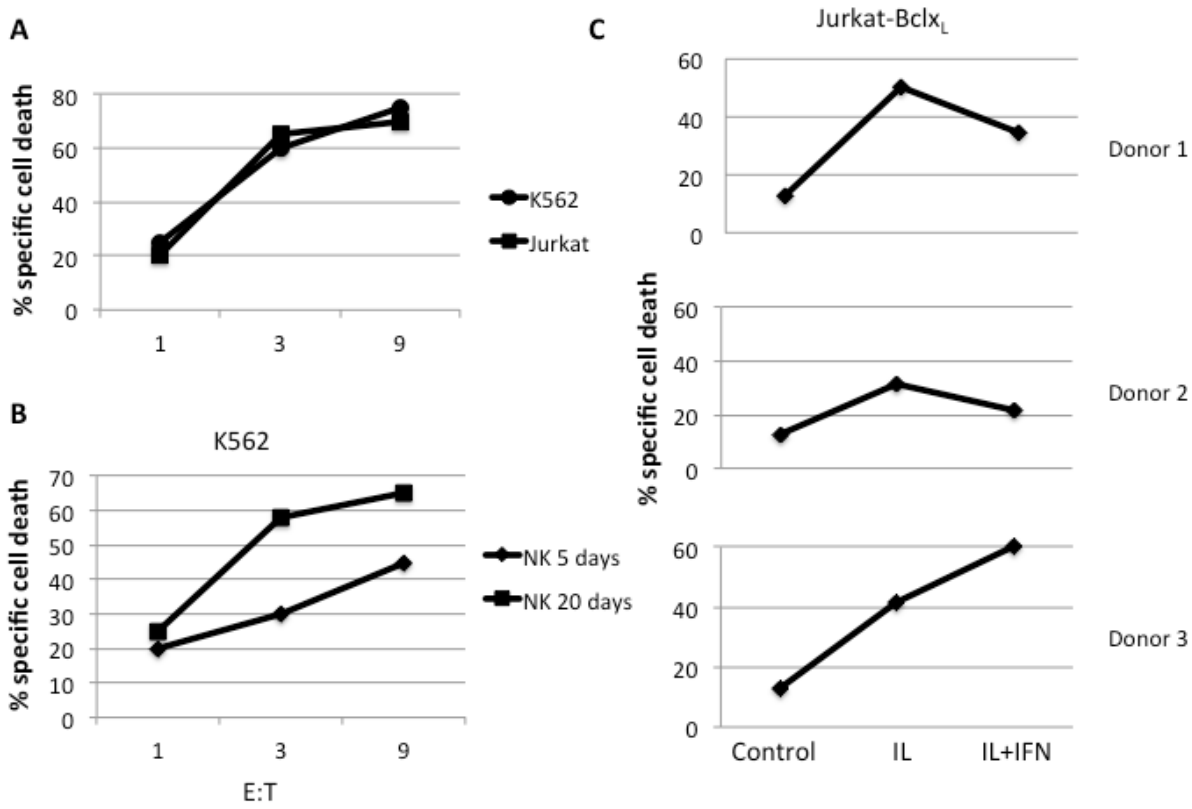
Supplemental Figure 1. Example of a representative cytotoxicity assay using eNK cells against leukemic Mec1 cells. eNK cells were labeled with Cell Tracker Green (CTG), and this labeling alone is shown in the upper dot plot vs. FSC. Mec1 cells were not labeled with CTG and analyzed at time 0 vs. FSC (middle dot plot). This was used for gating cells and to analyze basal Mec1 cell death on the gated population by 7-AAD staining (middle histogram). eNK cells were mixed at a 1:1 E:T ratio and the cytotoxicity assay developed during 4h. After this time, CTG labeling was analyzed on the mixed population vs. FSC, allowing gating of the Mec1 population (lower left dot plot). This gating was used to analyze cell death induced by eNK cells on Mec1 cells by 7-ADD staining (lower histogram).



Supplemental Figure 2. Example of the variation in the CD56 phenotype in eNK cells (NK3; day 20) with respect to NK cells at day 0; at day 0, most NK cells are $CD56^{dim}$ (squared population), while at day 20, most NK cells are $CD56^{bright}$ and are outside the square.



Suppl. Figure 3. Phenotype of expanded NK (eNK) cells. Percentage of NK cells positive for the expression of the indicated surface receptors at day 0 (white bars), and after 20-day expansion following the protocol indicated in Material and Methods in the presence (IL2+IL15+IFN□+721, black bars) or in the absence of IFN□α□(IL2+IL15+721, grey bars). Data are the mean ± SD of data obtained in cells from the same 10 donors used in the expansion experiments (Supplemental Table I) and in the cytotoxicity assays shown in Fig 2A, except NK5 and NK6. Significance was determined by Student's t-test; *, $P<0.05$.



Supplemental Figure 4. eNK cells were labeled with cell tracker green (CTG) and tested against different leukemic target cells for 4h at the E:T ratios indicated. Then, target cells were gated as shown in the previous Figure, and cell death was tested by nuclear 7-AAD incorporation. Results are shown as percentage of specific cell death induction, subtracting basal cell death, which was never higher than 15%. A, expanded NK cells were tested at the indicated E:T ratios against HLA-I negative K562 or against HLA-I positive Jurkat cells; B, NK cells activated for 5 or for 20 days, as indicated, with IL-2+IL-15+721.221 feeder cells, were tested on K562 target cells at the indicated E:T ratios; C, non-activated NK cells (control), or eNK cells generated in the presence of IL-2 plus IL-15 (IL) or in the presence of IL-2, IL-15 and IFN-α (IL+IFN) from three different donors were tested at a 5:1 E:T ratio against Jurkat cells over-expressing the anti-apoptotic molecule Bcl-x_L (Jurkat-Bcl-x_L).

CODE	AGE	HEMOGRAME (HGB,LK(Ly),PL)	STAGE AT DIAG.	TREATMENT (TT)	TIME UNTIL TT	RESPONSE	DURATION OF RESPONSE	GENETICS	ZAP70	CD38	STAGE AT 21/02/2017
CLL 001	52	15,9/10,100(6,400)/263,000	IB	/	/	/	/	DELETION 11q22-q23 (ATM gene)	/	54	B. PROGRESSION
CLL 01	72	13,1/23,49(18)/178	IIA	Fluda, Chlor, R-Chlor	4y	Complete	5y	N.D.	26	/	EXITUS
CLL 1	77	11,2/232.000/112	0	2012 rituximab + chlorambucil.	10y	Partial	18m	N.D.	/	5	IVC.PROGRESSION.
CLL 2	89	5/140.000(133.000)/134	IIIC	Chlorambucil + Prednisone	5m	Partial	In treatment	48XX +12 +21	/	/	EXITUS
CLL 3	66	11,1/1,9(1)/100	IIIC	Prednisone, R-Benda x1, COP x2	6y	No	In treatment	46xy T(2;14)	32	7	IIIC. PROGRESSION
CLL 4	74	12,4/40,8(36)/109	0	/	/	/	/	N.D.	/	20	0. W/O TT.
CLL 5	75	12,7/114/122	0	R-COP x6, R-Benda x1	3y	Partial	3y	FISH: del(13q) heterozygosis(84%)	10	70	IVC.PROGRESSION.IN TT.
CLL 06	58	12,2/155,000/65	IA	FC x4, R-FC x6, R-Benda x6	6m	Complete	3y	del 11(q22.3)	67	73	IA. 13,9/127(121,7)/125
CLL 6	74	15,2/49,7(42,3)/179	0	/	/	/	/	IgVH Mut	2	neg	IA.14,9/52,2(43,6)/224
CLL 7	75	8,7/279(250)/113	IA	R-FC x6, R-Benda x1	13	Complete	3y	N.D.	6	18	IVC.PROGRESSION.IN TT.
CLL 8	89	11,2/40,2(35,3)/174	0	/	/	/	/	N.D.	/	/	0. W/O TT.11,2/40,2(35,5]/174
CLL 9	81	12,6/4(2,3)/76	IA	Chlorambucil, Ritu-Prednisone	8y	Partial	In treatment	N.D.	/	neg	IIA. PROGRESSION
CLL 10	85	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.	/	/	IA. 12,6/126,8(119,1]/113
CLL 11	80	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.	/	/	0. W/O TT. 12,5/30,6(25,5]/171
CLL 012	63	12,1/2,2(0,1)/165	0	Ig-Prednisone RFC	7y	Complete	In treatment	del 13q (33%) ATM + (21%)	4	/	IV. PROGRESSION.
CLL 12	83	9,3/19,7(18,6)/27	IIIA	Chlorambucil, R-chlorambucil	1y	Partial	2y.In TT	N.D.	/	/	IV.PROGRESION.9,3/19,7(18,6]/27
CLL 13	44	15,6/125,1(118,5)/154	0	/	/	/	/	del 13q	/	neg	0. W/O TT. 15,5/125,1(118,5]/154
CLL 14	77	11,3/6,3(3,8)/43	0	R-chlorambucil	14y	/	In treatment	del 13q	/	neg	IVC. IN TT. 11,3/6,3(3,8]/43
CLL 15	66	11,6/17(12,6)/36	0	Rituximab, RFC	1y	Partial	In treatment	del (13q14.3)	/	49	IN TREATMENT
CLL 16	77	15/20,4(16,3)/109	0	/	/	/	/	N.D.	/	neg	0.W/O TT.15/20,4(16,3]/109
CLL 17	71	14,8/13,9(10,4)/168	0	/	/	/	/	N.D.	/	/	0.W/O TT.14,8/13,9(10,4]/168
CLL 0000018	71	N.A.	0	/	/	/	/	/	/	neg	0. W/O TT. 15.1/17(13,6]/184
CLL 000018	61	14/9,8(4,9)/224	0	/	/	/	/	/	/	neg	0. W/O TT. 14/9,8(4,9]/224
CLL 00018	82	12,4/49,5(45)/142	0	/	/	/	/	N.D.	/	/	0. W/O TT. 12,4/49,5(45]/142
CLL 0018	79	13,4/23,8(20,4)/123	0	/	/	/	/	46XX.Del 13(q)	1,6	neg	0.W/O TT.13,4/23,8(20,4]/123
CLL 6 (2016)	BIS	BIS	BIS	R-Bendamustine	BIS	BIS	BIS	BIS	BIS	BIS	C. PROGRESSION.
CLL 5 (2016)	BIS	BIS	BIS	BIS	BIS	BIS	BIS	BIS	BIS	BIS	IN TT.12,2/219,2(205]/121
CLL 18	76	9,2/310(292)/59	0	R-Benda	11y	Complete	2y	46 XY. Del (13q) heterozygosis	0,05	1,5	IVC. PROGRESSION
CLL 19	89	13,4/32(13,6)/190	0	/	/	/	/	N.D.	/	/	0. W/O TT. 13,4/32(13,6]/190
CLL 20	87	12,2/6,9(1,4)/22	0	Leukeran	3y	In TT	/	ATM mut. IgVH mut.	/	/	IIB W/O TT. 14,6/143(130]/133
CLL 21	76	15,5/14,9(8,8)/166	0	/	/	/	/	N.D.	/	/	0.W/O TT. 14,1/21,3(15,8]/194
CLL 8 (2016)	BIS	BIS	BIS	BIS	BIS	BIS	BIS	BIS	BIS	BIS	0.W/O TT.11,5/18,2(14,5]/166
CLL 22	74	10/39,2(34,3)/198	0	/	/	/	/	N.D.	/	/	0.W/O TT.10/39,2(34,3]/198

Supplemental Table I (previous page). Clinical data of the 30 patients enrolled in the study. Hemograme expressed as hemoglobin value in g/dL/number of leukocytes x $10^3/\mu$L (of which number of lymphocytes x $10^3/\mu$L)/number of platelets x $10^3/\mu$L; Fluda,fludarabine; Chlor, chlorambucil; R-Chlor, rituximab plus chlorambucil; R-COP, rituximab plus cyclophosphamide, vincristine and prednisone; R-Benda; rituximab plus bendamustine; FC, fludarabine plus cyclophosphamide; R-FC, rituximab plus fludarabine and cyclophosphamide. Genetics performed: karyotype, deletion of chromosome 13, deletion of chromosome 11q (ATM gene), trisomy of chromosome 12, deletion of chromosome 17 (17p) and IgVH mutation analysis.

Protocol	Fold expansion	Mean
IL2+IL15+721.221	Donor 1 (NK1) – 34 Donor 2 (NK2) – 32 Donor 3 (NK3) – 33 Donor 4 (NK4) – 42 Donor 5 (NK5) – 30 Donor 6 (NK6) – - Donor 7 (NK7) – 195 Donor 8 (NK8) - 32 Donor 9 (NK9) - 30 Donor 10 (NK10) - 91	58
IL2+IL15+IFNα+721.221	Donor 1 (NK1) – 21 Donor 2 (NK2) – 16 Donor 3 (NK3) – 61 Donor 4 (NK4) – 67 Donor 5 (NK5) – 55 Donor 6 (NK6) – 50 Donor 7 (NK7) – 121 Donor 8 (NK8)- 55 Donor 9 (NK9) - 16 Donor 10 (NK10) - 144	61

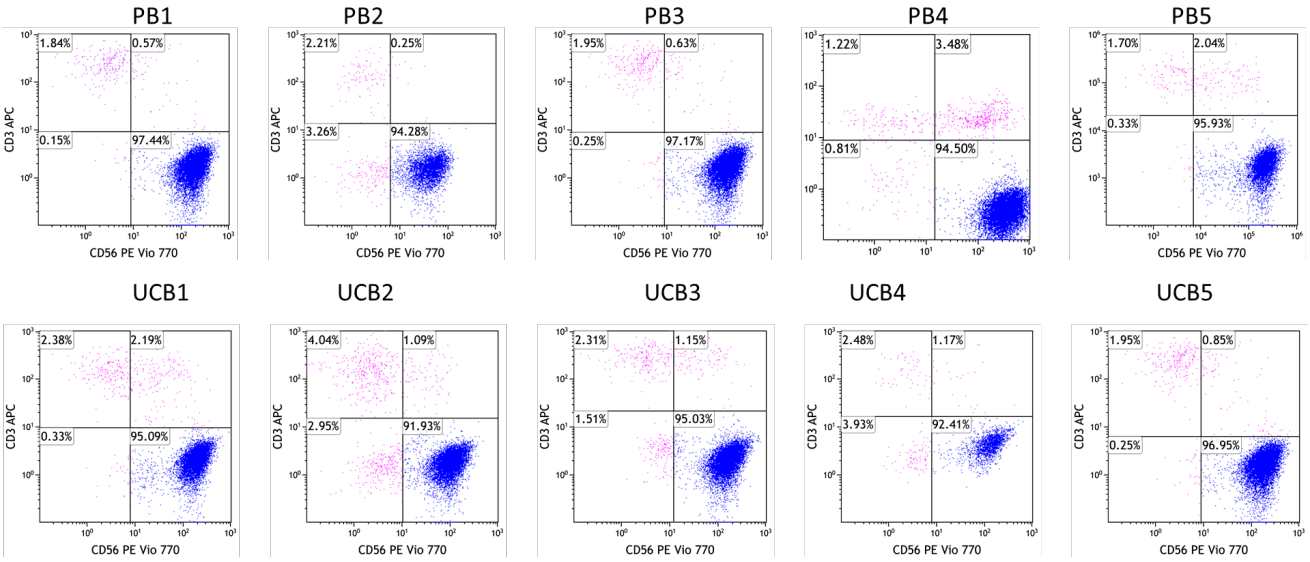
Supplemental Table II. Expansion of NK cells following the two protocols tested, expressed as fold expansion with respect to time 0.

	Age	Stage	Treatment	Response	%CD38	%ZAP70
CLL 5	75	IA (2015)	R-COP x6 R-Benda x1	Partial	70	10
		IV-C (2016)	R-Benda	In treatment	Bis	Bis
CLL 8	**89**	0 (2015)	No	·	0	·
		0 (2016)	No	·	Bis	Bis

Supplemental Table III. Clinical data of patients 5 and 8 at the time of the first test (2015) and at the time of the second test (2016) with eNK cells

Paper II

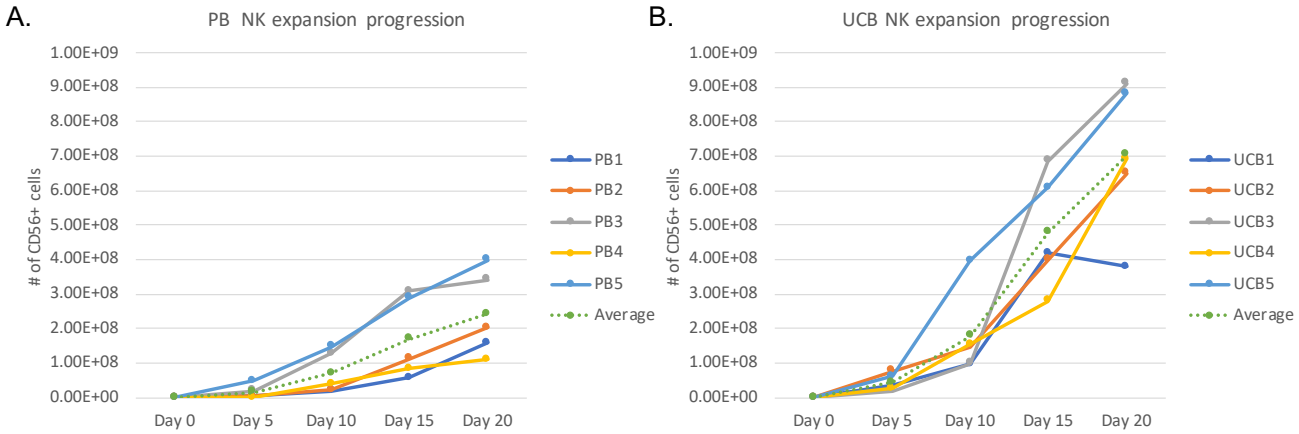
Supplementary Materials



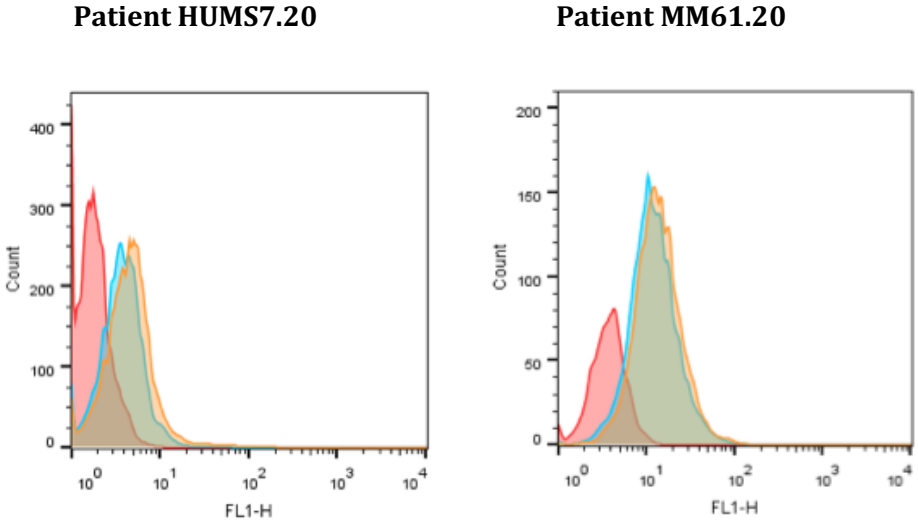
Supplemental Figure 1. CD3 and CD56 expression at day 20 in the 10 NK cell expansions used in this study.

	NK	**T**	**NKT**
UCB1	95.09	2.38	2.19
UCB2	91.93	4.04	1.09
UCB3	95.03	2.31	1.15
UCB4	92.41	2.48	1.17
UCB5	96.95	1.95	0.85
Average	**94.28 ± 2.08**	2.63 ± 0.81	1.29 ± 0.52
PB1	97.44	1.84	0.57
PB2	94.28	2.21	0.25
PB3	97.17	1.95	0.63
PB4	94.5	1.22	3.48
PB5	95.93	1.70	2.04
Average	**95.86 ± 1.46**	1.78 ± 0.37	1.39 ± 1.35

Supplemental Table I. Purity of NK cells after the 20 days of expansion.



Supplemental Figure 2. Expansion of the eNK cells used in the assays. The total number of $CD3^{-}CD56^{+}$NK cells present in the cultures is represented at the different time points analyzed.



Supplemental Figure 3. Expression of NKp44 ligands on the surface of MM cells from two patients. The expression of ligands for NKp44 on the surface of MM cells from patients was analyzed by flow cytometry using a chimeric NKp44-Fc molecule, kindly provided by Dr. Ofer Mandelboim, plus a secondary FITC-labelled anti-human IgG1 antibody. The MM population in the medullar aspirate was previously gated using an anti-CD38 mAb conjugated with APC. The red histograms show the labeling of the cells alone, the blue histograms those using the secondary antibody alone and the orange histograms those corresponding to the specific labeling.

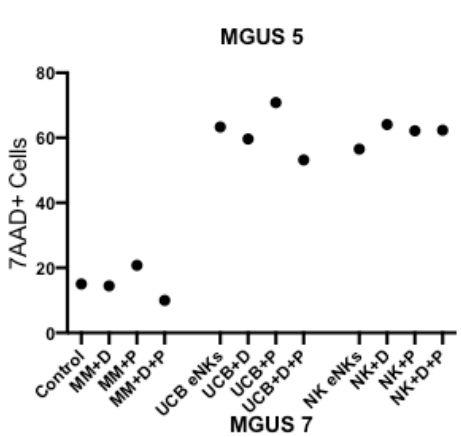
CD16 expression in NK cells from UCB

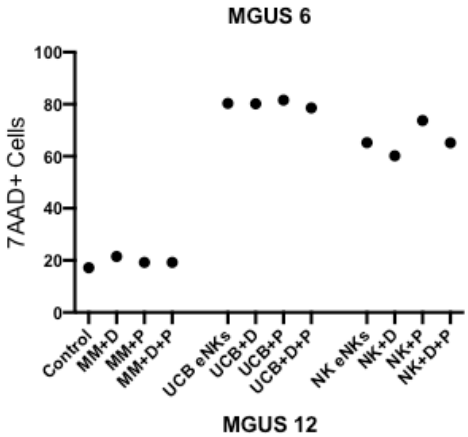
CD16 expression	Day 0	Day 5	Day 10	Day 15	Day 20
Percentage	87.3 ± 4.5	76.3 ± 10	86 ± 6.5	89 ± 6.1	84 ± 1.7
MFI	37635 ± 4621	11163 ± 3032	12647 ± 2701	22813 ± 2205	18617 ± 1992

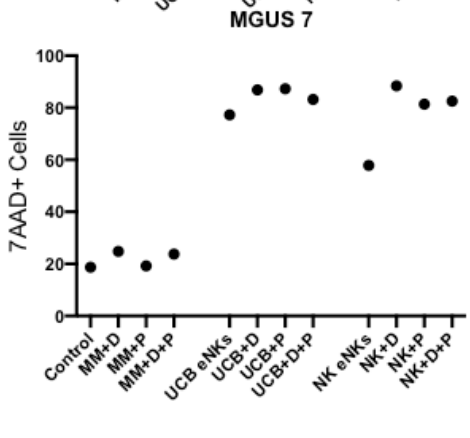
CD16 expression in NK cells from PB

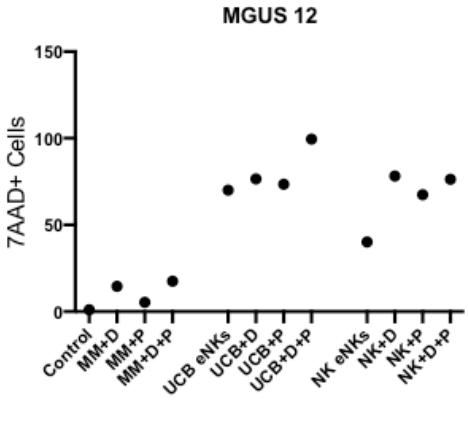
CD16 expression	Day 0	Day 5	Day 10	Day 15	Day 20
Percentage	82.4 ± 3.5	70.3 ± 9.6	89 ± 3.5	90 ± 7.5	87 ± 2.5
MFI	19921 ± 3220	8102 ± 1030	11304 ± 1605	12558 ± 1220	10551 ± 1090

Supplemental Table II. Expression of CD16 in NK cells during the expansion protocol. Results are expressed as percentage of positive cells and as MFI ± SD.


MGUS 5
7AAD+ Cells
0
20
40
60
80
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MGUS 6
7AAD+ Cells
0
20
40
60
80
100
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MGUS 7
7AAD+ Cells
0
20
40
60
80
100
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MGUS 12
7AAD+ Cells
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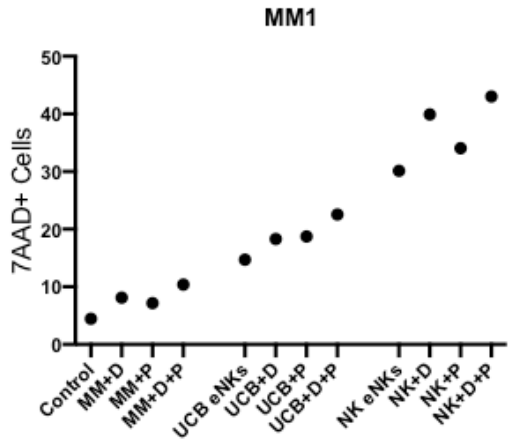
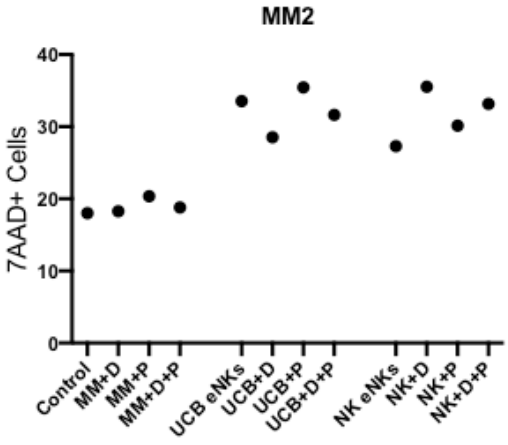
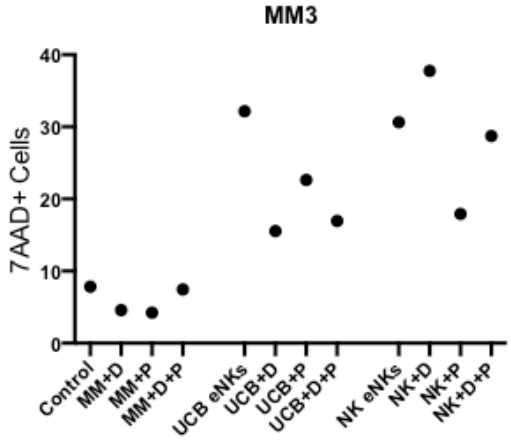
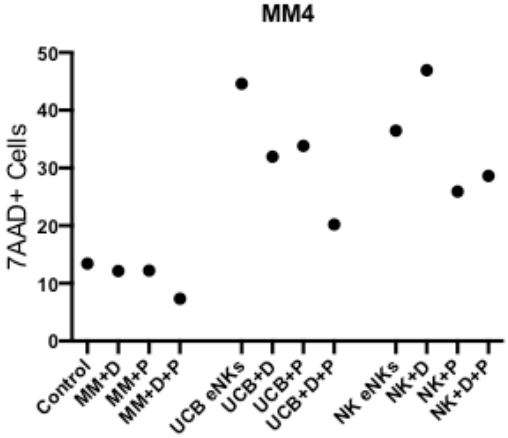
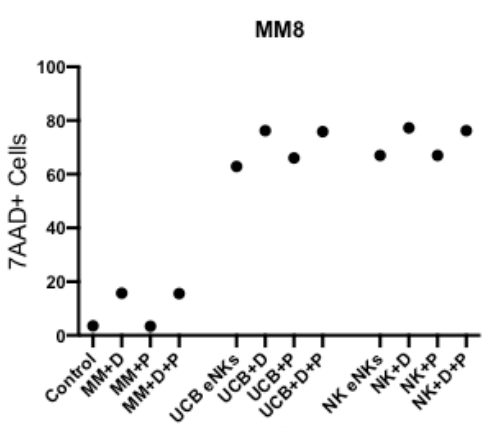
50
100
150
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P

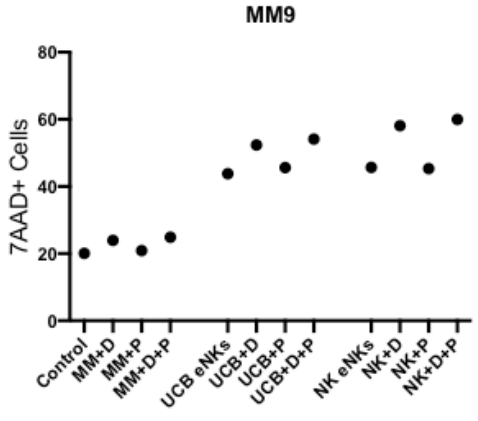

MM1
7AAD+ Cells
0
10
20
30
40
50
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P

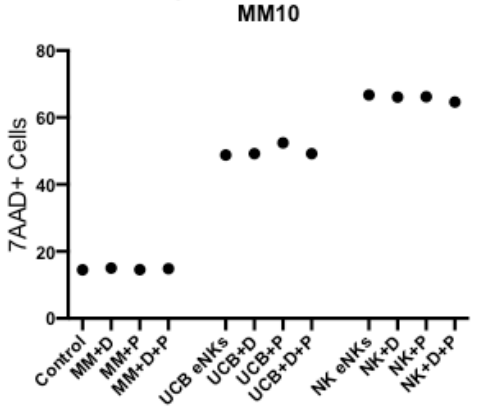

MM2
7AAD+ Cells
0
10
20
30
40
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P

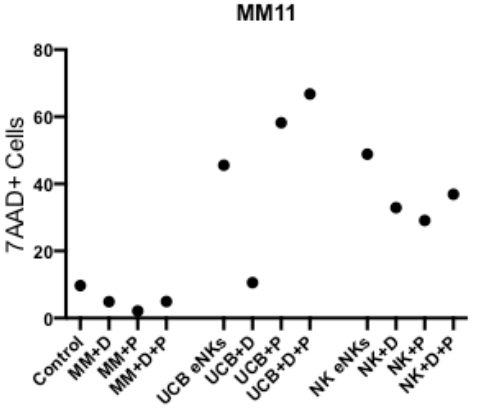

MM3
7AAD+ Cells
0
10
20
30
40
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P

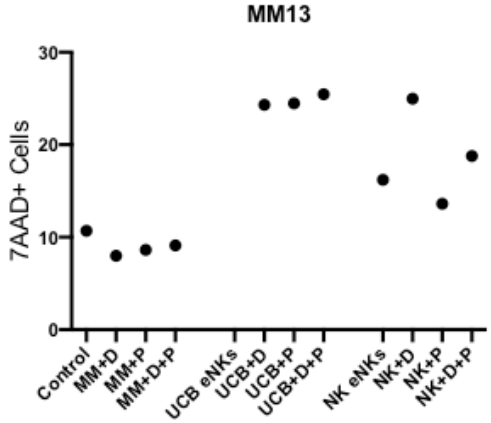

MM4
7AAD+ Cells
0
10
20
30
40
50
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MM8
7AAD+ Cells
100
80
60
40
20
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MM9
7AAD+ Cells
80
60
40
20
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MM10
7AAD+ Cells
80
60
40
20
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MM11
7AAD+ Cells
80
60
40
20
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MM13
7AAD+ Cells
30
20
10
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
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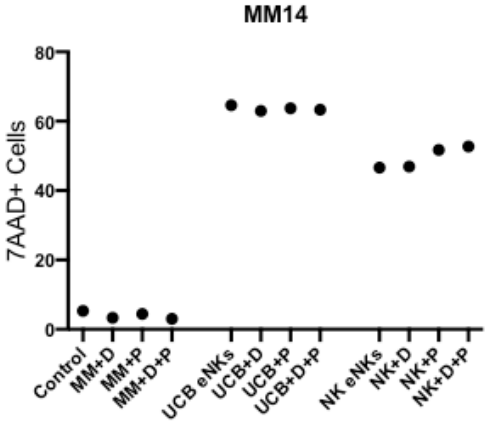
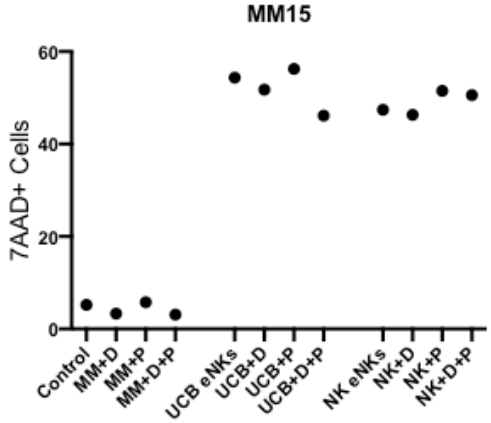
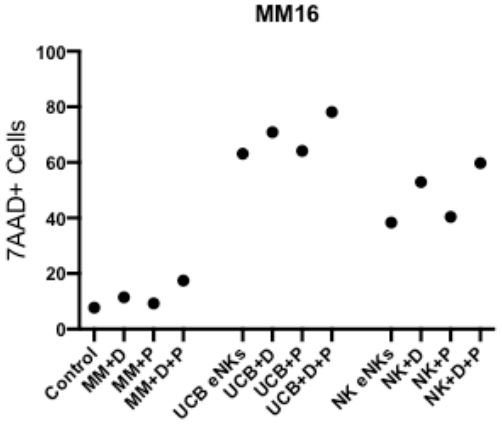
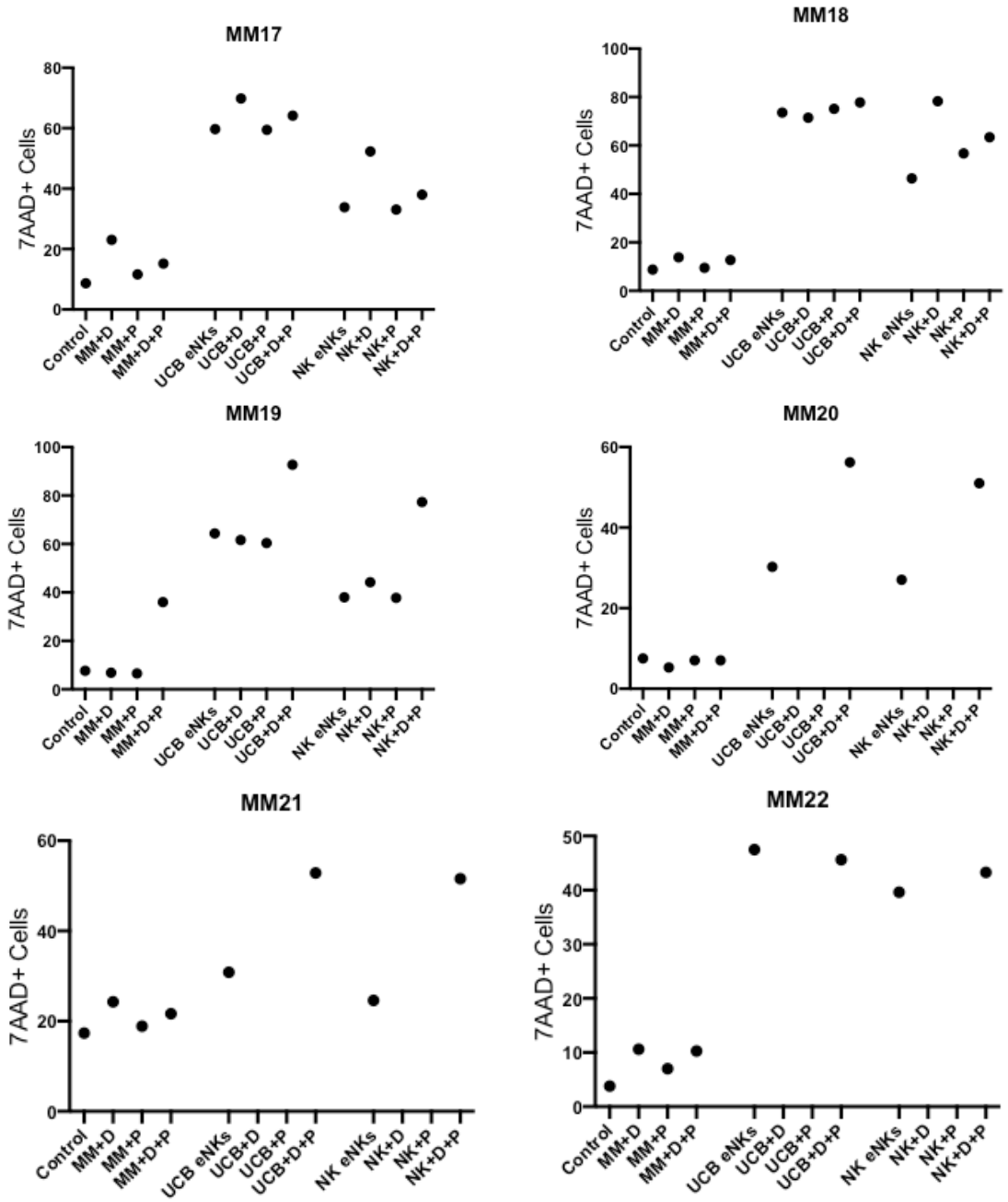
NK eNKs
NK+D
NK+P
NK+D+P

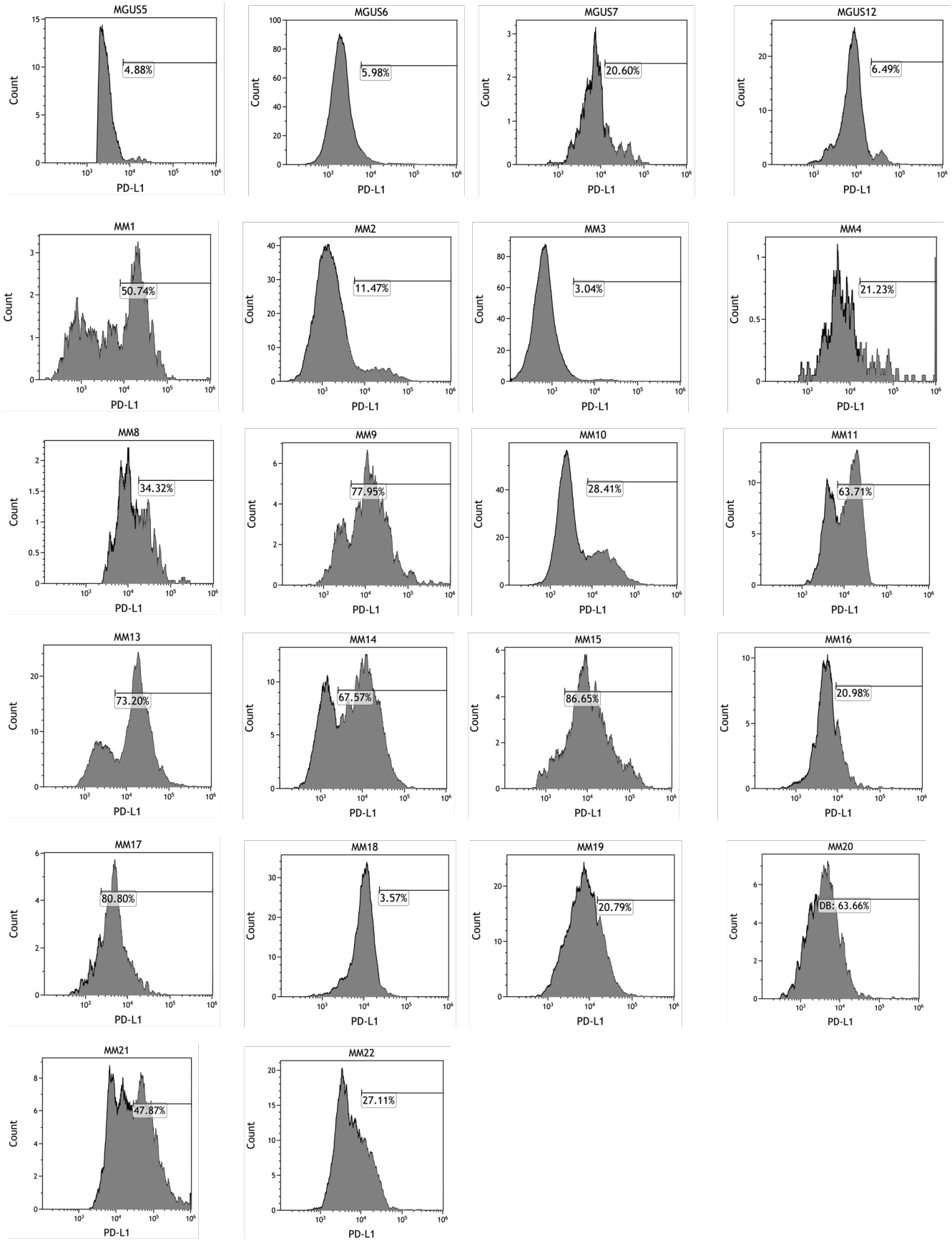

MM14
7AAD+ Cells
80
60
40
20
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P


MM15
7AAD+ Cells
60
40
20
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P

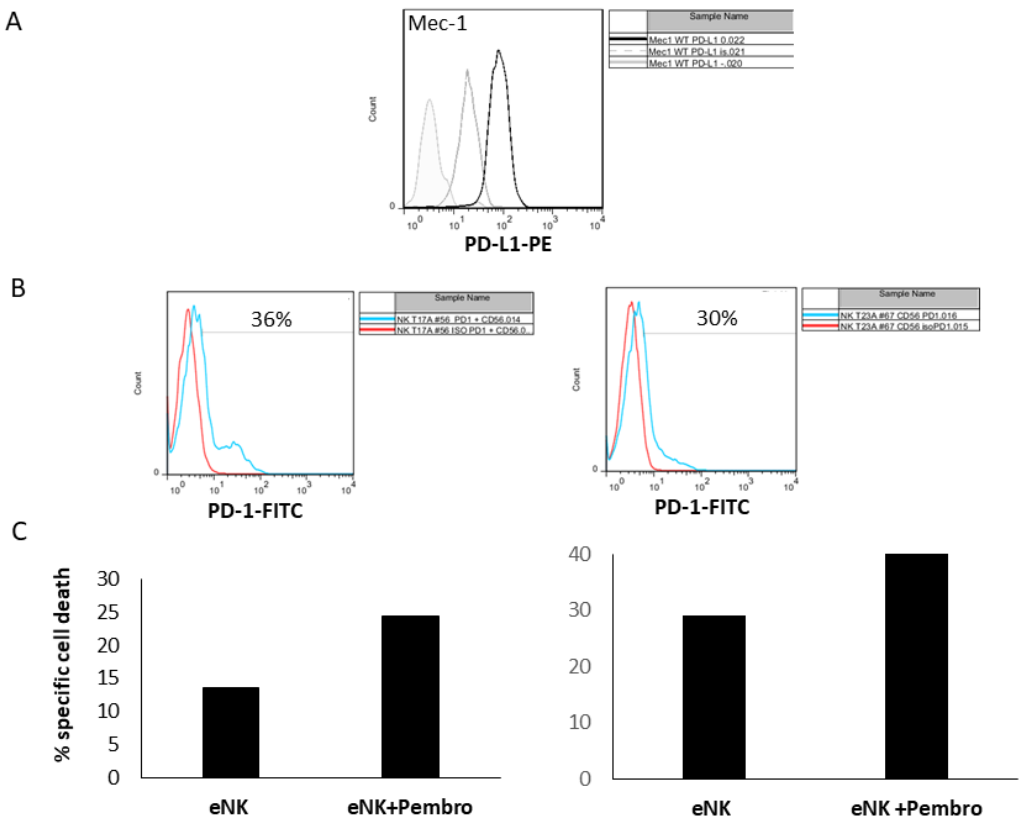

MM16
7AAD+ Cells
100
80
60
40
20
0
Control
MM+D
MM+P
MM+D+P
UCB eNKs
UCB+D
UCB+P
UCB+D+P
NK eNKs
NK+D
NK+P
NK+D+P



Supplemental Fig 4. Individual cytotoxicity tests on samples from the 22 MM or MGUS patients used in the present study. Results are expressed as explained in the Figure legend of Figures 4A and 5A of the manuscript. The eNK cells from UCB or from PB used in each case is delineated in Tables IB and IC of the manuscript.



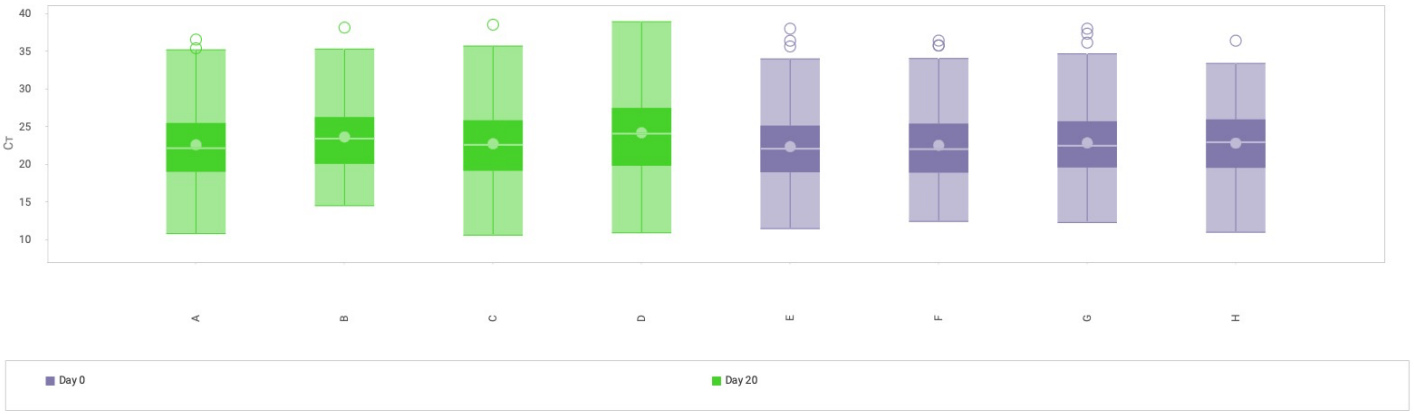
Supplemental Fig 5. Pattern of PD-L1 expression on samples from the MGUS (upper 4 panels) or from the MM patients (lower 18 panels) used in this study.



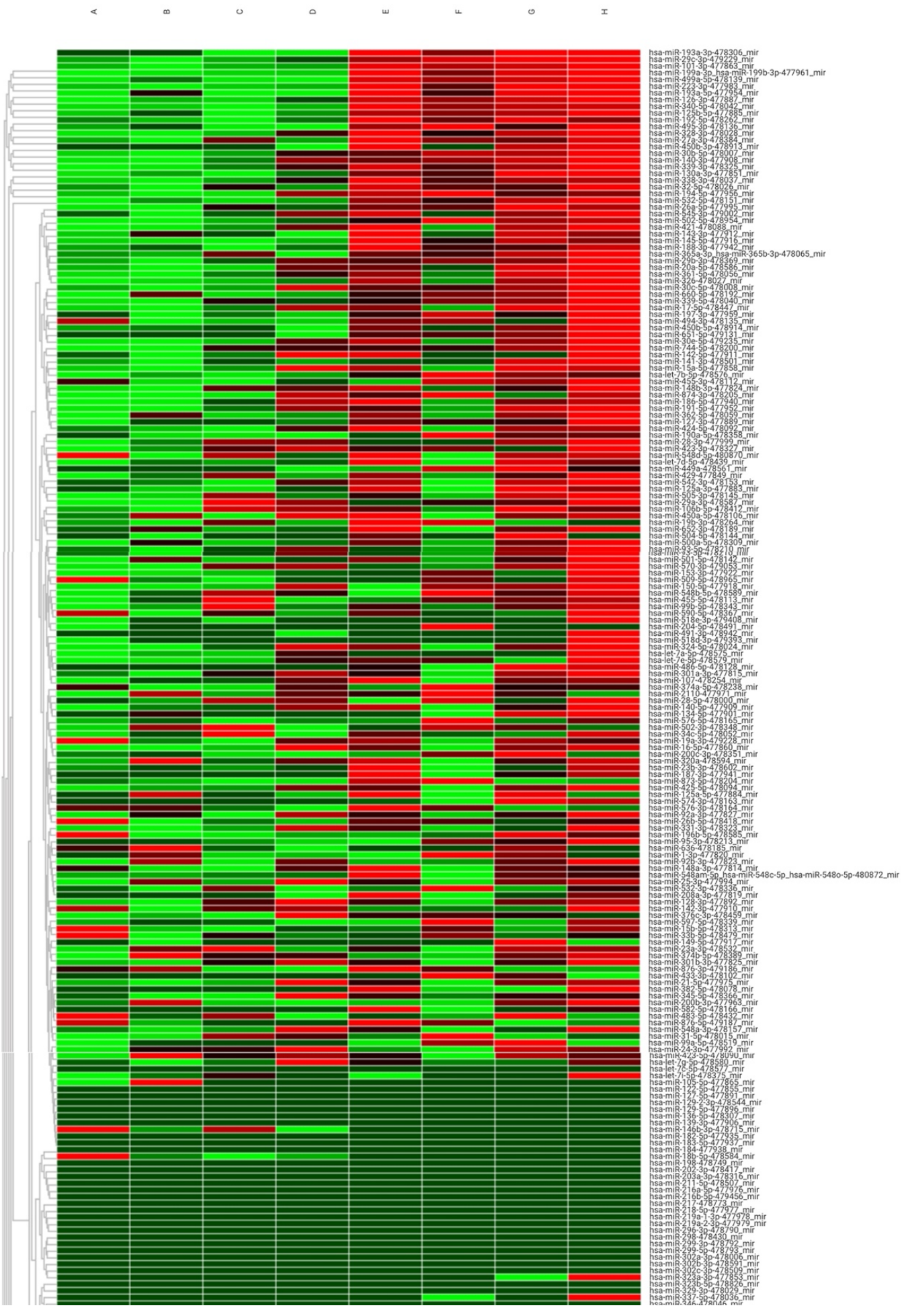
Supplemental Figure 6. Effect of pembrolizumab on cytotoxicity of eNKs on the PDL-1⁺ B-CLL cell line Mec-1. A) The expression of PDL-1 on the surface of the B-CLL cell line Mec-1 was analyzed by flow cytometry using a specific anti-PD-L1 mAb labelled with PE (black histogram). The grey histogram shows the labeling using an irrelevant mouse antibody of the same isotype and labeled with the same fluorophore. B) The expression of PD-1 in two PB eNKs that were used in cytotoxicity assays was determined by flow cytometry using an anti-PD-1 mAb labelled with FITC (blue histograms). The red histograms show the labeling with an irrelevant mAb of the same isotype. C) The eNK cells analyzed in B for PD-1 expression were tested overnight at a 1:1 E:T ratio against Mec-1 cells in the absence (eNK) or in the presence of 10 □g/ml of the anti-PD1 blocking mAb pembrolizumab (eNK+pembro), Mec-1 cell death was tested by annexin-V-APC labelling.

Paper III

Supplementary Materials



Supplementary Figure S1. CT values for each sample during RT-PCR. All CT values above 35 were not used when calculating ΔΔCTs as they represent miRNAs with expressions too low to be detected through RT-qPCR. Green bars indicate the 4 different day 20 samples and purple bars indicate the corresponding 4 day 0 samples. Pairs are as follow: A-E, B-F, C-G, D-H (day 20 - day 0).


A
B
C
D
E
F
G
H
hsa-miR-193a-3p-478306_mir
hsa-miR-29c-3p-479229_mir
hsa-miR-101-3p-477863_mir
hsa-miR-199a-5p_hsa-miR-199b-3p-477961_mir
hsa-miR-499a-5p-478139_mir
hsa-miR-223-3p-477983_mir
hsa-miR-193a-5p-477854_mir
hsa-miR-126-3p-477887_mir
hsa-miR-340-5p-478042_mir
hsa-miR-125b-5p-477885_mir
hsa-miR-192-5p-478262_mir
hsa-miR-495-3p-478136_mir
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hsa-miR-27a-3p-478384_mir
hsa-miR-450b-3p-478913_mir
hsa-miR-30b-5p-478007_mir
hsa-miR-140-3p-477908_mir
hsa-miR-339-3p-478325_mir
hsa-miR-130a-3p-477851_mir
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hsa-miR-145-5p-477916_mir
hsa-miR-188-3p-477942_mir
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hsa-miR-20a-5p-478586_mir
hsa-miR-361-5p-478056_mir
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hsa-miR-19a-3p-479228_mir
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hsa-miR-320a-478594_mir
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hsa-miR-92b-3p-477823_mir
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hsa-miR-376c-3p-478459_mir
hsa-miR-597-5p-478339_mir
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hsa-miR-33b-5p-478479_mir
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hsa-miR-374b-5p-478389_mir
hsa-miR-301b-3p-477825_mir
hsa-miR-876-3p-479186_mir
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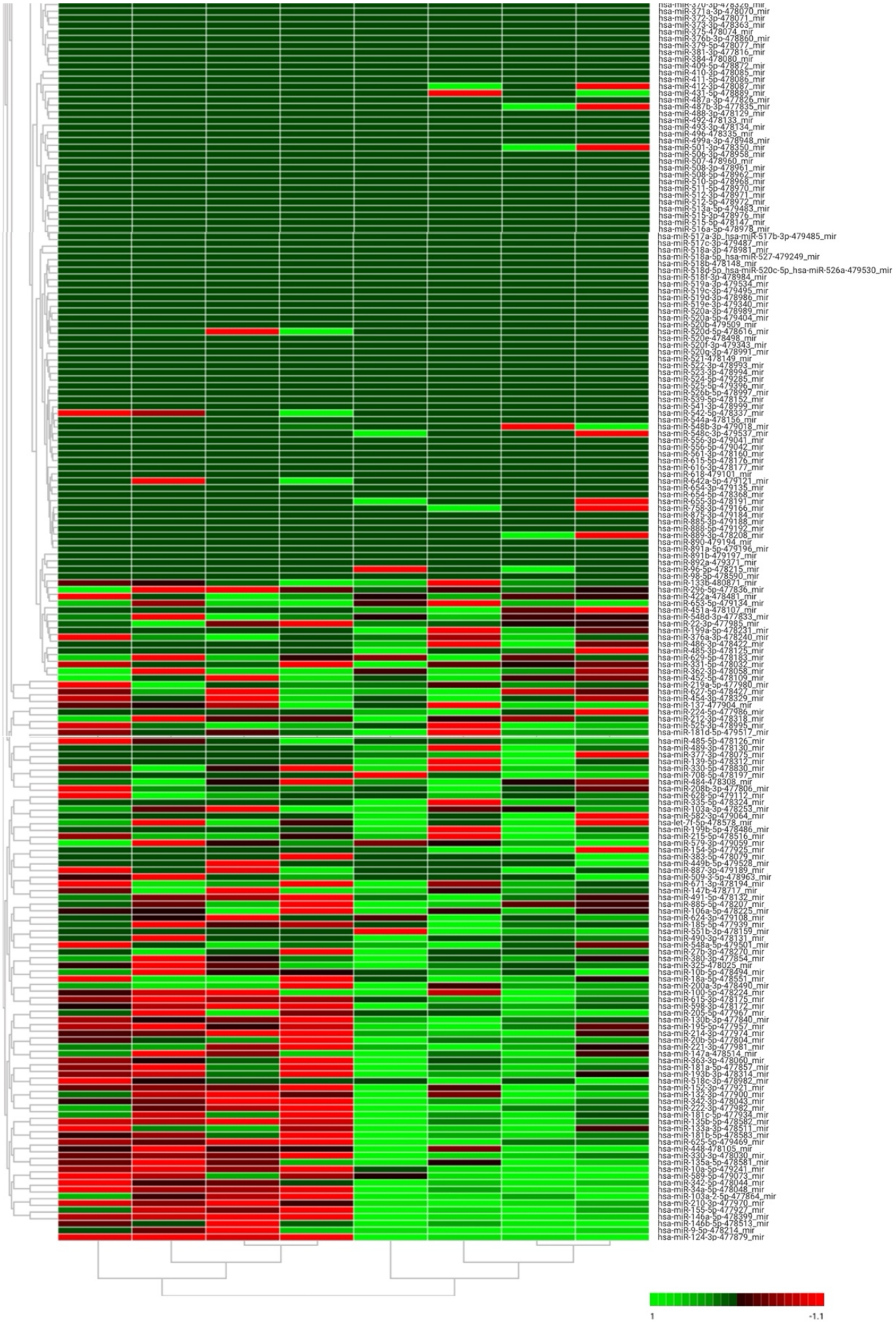
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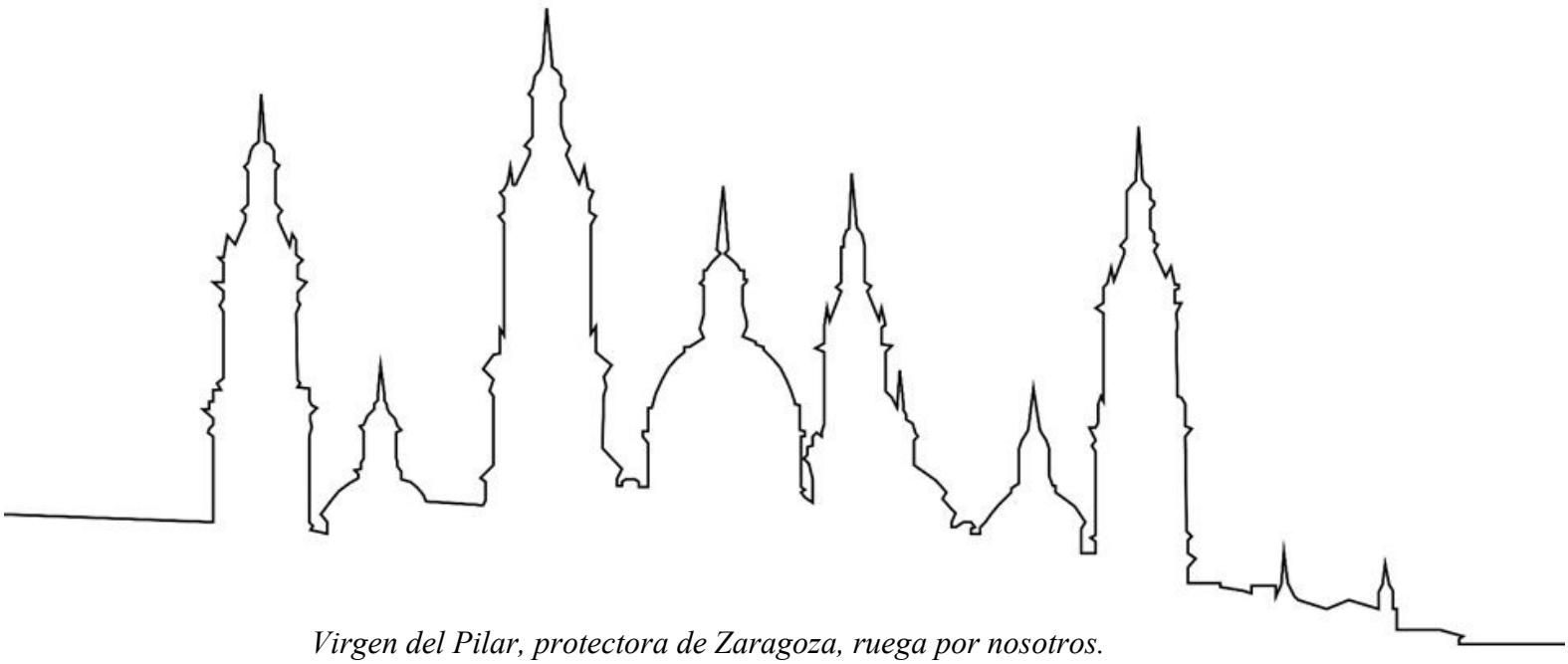
Supplementary Figure S2. Heatmaps of the 378 miRNA targets analyzed. Columns represent each sample. A- D are day 20 samples. E-H are day 0 samples. Each row represents a single miRNA target. Red represents an increase in expression and green represents diminishing expression.



Virgen del Pilar, protectora de Zaragoza, ruega por nosotros.