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Glycolytic requirements of human cytokine-induced memory-like natural killer cells

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INTRODUCTION

Natural killer (NK) cells are a type of innate lymphoid cells (ILCs) that play a pivotal role fighting against cancer and viral infections. NK cells derive from the $CD34^+$ hematopoietic progenitor cells (HPCs) and since they have cytotoxic activity, it is suggested that NK and cytotoxic T cells might have evolved from a common ancestral cytolytic effector cell (Freud & Caligiuri, 2006; Caligiuri, 2008). Phenotypically, human NK cells are commonly defined by the absence of T cell receptor/CD3 complex or TCR/CD3 (difference from T cells) and the expression of CD56, an isoform of the neural cell adhesion molecule (NCAM). The expression density of the latter marker defines two functionally different NK cell subsets: $CD56^{bright}$ and $CD56^{dim}$. It has been shown that NK cells follow 6 different stages of maturation. $CD56^{dim}CD16^+CD57^+$ NK cells represent the final stage of that maturation, while $CD56^{bright}CD16^-CD57^-$ cells represent the 4^{th} stage of maturation; therefore, $CD56^{dim}$ NK cells are more differentiated (Freud & Caligiuri, 2006; Freud et al., 2017). $CD56^{bright}$ NK cells are abundant in lymph nodes and tonsils, that is, in secondary lymphoid tissues (SLT), and other tissues such as the gravid uterus; but they can also be found in peripheral blood in a very low percentage (Freud et al., 2017). $CD56^{bright}$ NK cells are able to produce abundant amounts of cytokines and chemokines, and they have little capacity to spontaneously kill tumor cell targets (Caligiuri, 2008). $CD56^{dim}$ NK cells, however, are abundant in bone marrow, peripheral blood and spleen, and are able to greatly kill tumor cells, although they have lower ability to produce cytokines and chemokines (Freud et al., 2017; Wagner et al., 2017). Moreover, NK cell subsets are also characterized by their expression of FcγRIII, also known as CD16 ($CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{bright}$ respectively), which gets bound to the Fc region of IgG antibodies and induces antibody-dependent cellular cytotoxicity (ADCC) (Caligiuri, 2008; Freud et al., 2017). Summarizing, we can define NK cell subsets as $CD56^{dim}$ NK cells to be more cytotoxic and the $CD56^{bright}$ to be potent interferon (IFN)-γ producers. However, the total population of NK cells still remains phenotypically heterogeneous (Caligiuri, 2008; Freud & Caligiuri, 2006; Freud et al., 2017; Keating et al., 2016). Indeed, it has been calculated that there could be over 6,000-30,000 NK cell subpopulations in every individual (Horowitz et al., 2013).

NK cells are able to recognize and bind to polymorphic determinants of major histocompatibility complex (MHC) class I molecules (HLA class I molecules in humans), and depending on whether the recognition and binding is carried out by activating (i.e. KIR2DS, CD94/NKG2C) or inhibitory (i.e. KIR2DL, KIR3DL, CD94/NKG2A, ILT2) receptors, NK cells will (or not) secrete cytokine and release cytolytic granules (Borrego et al., 2002). HLA class I molecules are expressed in all healthy nucleated cells, and the inhibitory signals are predominant over the activating signals. Therefore, the consequence is that NK cells do not kill healthy cells (Borrego et al., 2002; Caligiuri, 2008; Freud et al., 2017; Valiante et al., 1997). However, when cells are infected or transformed, the levels of HLA class I molecules decrease and stress induced ligands for NK cell activating receptors are expressed. In this situation, NK cells get activated and become able to recognize and kill those infected and malignant cells (Cerwenka et al., 2016). In addition to the above mentioned KIR2DS and CD94/NKG2C, some very relevant activating cell surface receptors are the natural cytotoxicity receptors (NCR), for example NKp46, NKp44 and NKp30 and others such as NKG2D, 2B4,

DNAM-1 and CD16 (Caligiuri, 2008; Freud et al., 2017; Terrén et al., 2020). NK cells can also get activated by cytokines, as they express receptors for several activating cytokines such as type I interferon (IFN), interleukin (IL)-2, IL-7, IL-12, IL-15, IL-18 and IL-21 (Vivier et al., 2011). At the end, NK cell activation is the result of the balance between inhibitory and activating signals, working along a dynamic equilibrium (Long et al.,2013).

Once activated, NK cells can mediate their functions by three different ways: 1) Via activating receptors that lead to perforin/granzyme B secretion (degranulation) and production of cytokines and chemokines, such as IFNγ, tumor necrosis factor (TNF), macrophage inflammatory protein (MIP)-1β, etc. 2) Via CD16 which recognizes the Fc fragment of antibodies and leads to antibody-dependent cell-mediated cytotoxicity or ADCC, which also induces perforin/granzyme and cytokine secretion (Caligiuri, 2008; Freud et al., 2017; Yawata et al., 2006). Perforin and granzyme are molecules present in cytolytic granules in the cytoplasm of NK cells and induce apoptosis of target cells after their release. CD107a has been described as a marker of NK cell degranulation (Aktas et al., 2009; Alter et al., 2004; Cohnen et al., 2013). Following with NK cell produced cytokines, IFNγ is considered the model NK cell cytokine. $CD56^{bright}$ NK cells produce this cytokine when stimulated through CD16 or in response to different interleukins released by antigen presenting cells (APCs). 3) Last, NK cells also express death receptor ligands such as the first apoptosis signal (FAS)-ligand (FasL) and the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Both receptors induce the process of apoptosis by activation of caspases leading to cell death (Finnberg et al., 2008; Screpanti et al., 2005).

Traditionally, NK cells have been classified as cells of the innate arm of the immune response. However, it has been demonstrated that NK cells can develop immunological memory to some haptens and virus, and perform antigen-specific memory responses (Berrien-Elliott et al., 2015; Cerwenka & Lanier, 2016; Nikzad et al., 2019; Romee et al., 2016). Moreover, it has been demonstrated that a brief exposure (16-18 hours) of NK cells to a mixture of IL-12, IL-15 and IL-18 results in the development of memory-like properties (Cooper et al., 2009). This memory-like behavior could be considered as a pre-activation state, and those pre-activated cells are named cytokine-induced memory-like (CIML) NK cells (Cerwenka & Lanier, 2016; Cooper et al., 2009). CIML NK cells show a major capacity to respond following reactivation with cytokines or via engagement of activating receptors after a resting period (Cooper et al., 2009). Also, these CIML NK cells show an enhanced functionality, exhibiting a significantly increased IFNγ and TNF production, and an increased expression of granzyme B and perforin (Romee et al., 2016). However, this increase in IFNγ is not homogeneous in both NK cell subsets, being more enhanced in $CD56^{bright}$ NK cells. Although both subsets showed similar IFNγ production when restimulated with a mixture of IL-12 and IL-15 (Romee et al., 2012). Moreover, compared to control non-preactivated NK cells, CIML NK cells have shown a higher cytotoxicity after restimulation with targets cells like K562 leukemia cells and primary acute myeloid leukemia (AML) blasts (Romee et al., 2016). All these enhanced functions are correlated with lower expression of inhibitory KIR receptors (Berrien-Elliott et al., 2015; Ewen, Pahl, Miller, Watzl, & Cerwenka, 2018). CIML NK cells have become a potential tool for cancer immunotherapy due to their increased antileukemia effect and the

reduced risk of developing graft-versus-host disease (Wagner et al., 2017). Furthermore, in studies of adoptive cell therapies with CIML NK cells, complete remissions have been observed in patients with refractory AML (Romee et al., 2016). Currently, there are six ongoing clinical trials (NCT04024761, NCT04290546, NCT03068819, NCT04354025, NCT01898793 and NCT02782546, from https://clinicaltrials.gov/ct2/results?cond=&term=CIML&cntry=&state=&city=&dist=) in which CIML NK cells are being used to treat different malignancies.

Until now, CIML NK cells infusion has found to be safe, but moderate efficacy has been achieved (Romee et al., 2016). Therefore, it is necessary to find new ways to improve treatment outcomes. In the last years, immunometabolism has emerged as an interesting target. Metabolism is a key feature for the homeostasis of an organism, and the effector functions of immune cells are highly linked to their metabolism (Gardiner, 2019; O'Brien & Finlay, 2019). However, our understanding of NK cell metabolism is still very limited. NK cells use both oxidative phosphorylation (OXPHOS) and glycolysis to fuel their proliferation processes (O'Brien & Finlay, 2019; O'Neill, Kishton, & Rathmell, 2016). The latter is key for numerous immune responses, thus an enhanced glycolysis enables the immune cells to produce enough ATP and other biosynthetic intermediates in order to carrying out their effector functions (Gardiner, 2019; O'Brien & Finlay, 2019). Hence, an increased glycolysis might be the key for the enhanced effector functions of CIML NK cells. Glycolysis supports cytokine production, cytotoxicity and proliferative capacity (Keppel et al., 2015). On the other hand, inhibition of OXPHOS with oligomycin induces an inhibition of both cytotoxicity and IFNγ production (Chang et al., 2013; Gardiner, 2019; Mah & Cooper, 2016; O'Brien & Finlay, 2019). The effects of the inhibition of glycolysis with 2-deoxy-D-glucose (2-DG) on NK cells are not well known, although it is accepted that a dysfunctional metabolism leads to an inhibition of NK cells effector functions (Gardiner, 2019; Keppel et al., 2015; O'Neill et al., 2016; Terrén et al., 2019). Last, these processes become upregulated when NK cells are activated with IL-2, IL-12, IL-15 and IL-18 (Gardiner, 2019; Pahl et al., 2018), although the metabolic changes induced by cytokine stimulation have been found to be different among distinct NK cell subsets. For example, it has been shown that $CD56^{bright}$ NK cells are more responsive to metabolic changes and they do upregulate several metabolic markers in response to cytokines, while $CD56^{dim}$ NK cells have a low responsiveness (Keating et al., 2016).

Hence, the objective of this work is first to determine the glycolytic requirements of CIML NK cells by studying their glucose uptake rate. Moreover, it will be studied how the inhibition of the glycolytic pathway with 2-DG affects the effector functions of CIML NK cells. Therefore, this work will help to understand the metabolic changes that happen in CIML NK cells and their relationship with effector functions.

MATERIALS AND METHODS

Samples and cell culture

Buffy coats from healthy adult donors were collected through the Basque Biobank for Research. Peripheral blood mononuclear cells (PBMCs) were obtained by a Ficoll density centrifugation and NK cells where

purified from PBMCs using the Human NK Cell Isolation Kit (Miltenyi Biotec). Purified NK cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with GlutaMAX (ThermoFisher Scientific), 10% fetal bovine serum (FBS) (GE Healthcare Life Sciences), 1% non-essential amino acids (ThermoFisher Scientific), 1% sodium pyruvate (ThermoFisher Scientific) and 1% penicillin/streptomycin (ThermoFisher Scientific), from now on, NK medium. Cells were cultured for 16-18 hours, in the presence and absence of recombinant human IL-12 (Milteny Biotec), IL-15 (Miltenyi Biotec) and IL-18 (MBL International) (10, 100 and 50 ng/ml, respectively). Next, cells were washed 3 times with phosphate buffered saline (PBS) (Lonza), resuspended in NK medium and cultured for 7 days at 2 x 10^6 cells/ml in the presence of IL-15 (1 ng/ml) or IL-2 (20 IU/ml) (Figure 1).

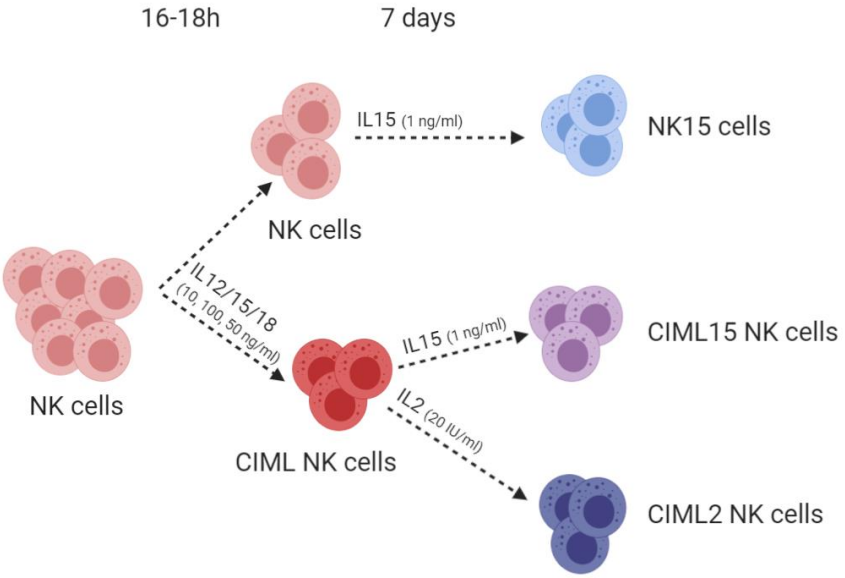

Figure 1. Diagram showing experimental design of NK and CIML NK cells (*made with BioRender*). NK cells were first cultured in the presence (CIML NK cells) or absence (NK cells) of recombinant IL-12, IL-15 and IL-18 (10, 100 and 50 ng/ml, respectively) for 16-18h. Next, cells were washed with PBS, resuspended in NK medium and cultured in presence of IL-15 (CIML15 NK and NK15 cells) or IL-2 (CIML2 NK cells) for 7 days.

Glucose uptake assay

For glucose uptake assay, cells were plated at 1 x 10^6 cells/ml and incubated for 90 minutes with 50 µM of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxy-D-glucose (2-NBDG) (Invitrogen), which is a fluorescent glucose analog. After incubation time, cells were washed and stained with LIVE/DEAD fixable Near-IR cell stain (ThermoFisher Scientific) for 30 minutes on ice. Cells were then washed with PBS+BSA (2.5%) and stained with the following fluorochrome-conjugated mouse anti-human monoclonal antibodies: PE-Vio770 anti-CD56 (REA196, Miltenyi Biotec), BV510 anti-CD14 (MΦP9, BD biosciences) and BV510 anti-CD3 (UCHT1, BD biosciences). Cells were then washed with PBS and acquired in a MACSQuant® flow cytometer. Data analysis was performed with FlowLogic software.

Functional assay

For functional assay, NK cells were plated at 2 x 10^5 cells/well in a U-bottom 96-well plate. Cells were then stimulated for 7 hours at 37ºC either with K562 target cells (1:1 effector:target ratio) or with IL-12, IL-15 and IL-18 (10, 100 and 50 ng/ml, respectively). NK cells were stimulated in the presence and absence of 50 mM 2-deoxy-D-glucose (2-DG) (Sigma-Aldrich), a glycolytic inhibitor. PE anti-CD107a (REA792, Miltenyi Biotec) was added to the wells at the beginning of the stimulation period, and after 1 hour two protein transport inhibitors were also added, one containing Monensin (Golgi Stop, BD biosciences) and the other containing Brefeldin A (Golgi Plug, BD biosciences). After 7 hours of stimulation cells were collected and stained with LIVE/DEAD fixable Near-IR cell stain and with the previously mentioned mAbs. Then, cells were fixed and permeabilized, and stained with the following mouse anti-human monoclonal antibodies: FITC anti-MIP-1β (D21-1351, BD biosciences), BV421 anti-IFNγ (B27, BD biosciences) and APC anti-TNF (Mab11, Biolegend). Finally, cells were washed with PBS and acquired in a MACSQuant ® flow cytometer. Data analysis was performed with FlowLogic software.

Statistical analysis

Statistical analysis and figure representation were made with GraphPad Prism (v8.1) and Funky Cells (v0.1.2). Non-parametric Wilcoxon matched-pairs signed rank tests were performed to check for significant differences between different conditions. Data was represented displaying means ± standard error of the mean (SEM). *$p < 0.05$ and **$p < 0.01$.

RESULTS

CIML NK cells exhibit increased glycolytic activity

Glycolytic pathway in NK cells is fundamental, since it has been reported that glycolytic activity is increased when cells are stimulated (Gardiner, 2019; Keppel et al., 2015; O'Neill et al., 2016; Terrén et al., 2019). Therefore, the first goal was to evaluate the metabolic activity of NK cells after stimulation with IL-12, IL-15 and IL-18 during 16-18 hours. That evaluation was assessed by performing the glucose uptake assay using the fluorescent glucose analogue 2-NBDG, which gives an estimate of the glycolytic activity of the cells. Results show that CIML NK cells highly increased their metabolic activity, showing a value of 8.38 for the median fluorescence intensity (MFI) of 2-NBDG, while the median value for NK cells was 5.31 (Figure 2A, left). Even if the proportion in blood is much higher for $CD56^{dim}$ cells, $CD56^{bright}$ cells were the ones who showed a higher glucose uptake rate in both NK and CIML NK cells, although not statistically significant (Figure 2A, right).

On the other hand, we also analyzed whether those metabolic differences remained in time or not. In different clinical trials, IL-2 and IL-15 are used to promote proliferation and survival of NK cells (from https://clinicaltrials.gov/ct2/results?cond=&term=CIML&cntry=&state=&city=&dist=)so we decided to test *in vitro* the effect of these interleukins on NK cell metabolism. To assess that, non-preactivated and CIML NK cells were incubated with low doses of either IL-2 or IL-15 for 7 days (Figure 1).

Results show similar glucose uptake values for both CIML15 and CIML2 NK cells (Figure 2B). Identification of $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets was possible at day 0 but not at day 7 (Figure 2C).
In short, we have determined that NK cells cultured for 16-18 hours with IL-12, IL-15 an IL-18 induced them to acquire an enhanced metabolic activity. Furthermore, this increased metabolism was a permanent condition for the next 7 days in which the cells were cultured with either IL-2 or IL-15 at low doses.

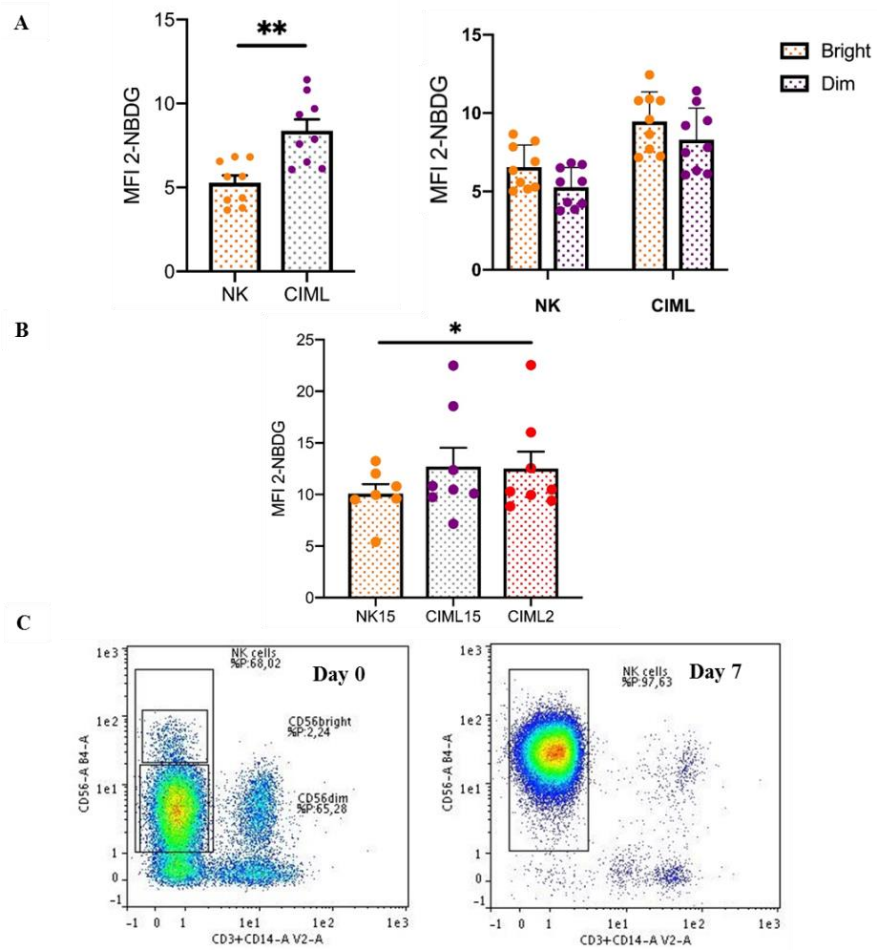

Figure 2. Glucose uptake of non-preactivated natural killer (NK) and cytokine-induced memory-like (CIML) NK cells. **(A)** Bar graph showing glucose uptake, measured as the median fluorescence intensity (MFI) of 2-NBDG, of total control non pre-activated and CIML NK cells at day 0 (left) or $CD56^{bright}$ and $CD56^{dim}$ cells subsets (rigth). **(B)** Bar graph showing glucose uptake, measured as the MFI of 2-NBDG, of control non-preactivated (NK15) and CIML (CIML15 and CIML2) NK cells at day 7. **(C)** Representative pseudocolor plots showing NK cells at Day 0 (left) and at Day 7 (right). Each dot represents an independent experiment. *$p < 0.05$ and **$p < 0.01$.

2-DG inhibits NK cells polyfunctionality

Taking into account that glycolysis supports different important cellular processes and that CIML NK cells exhibit a greater glycolytic activity, the following aim was to evaluate the importance of that metabolic pathway for NK cells effector functions. To assess that, we co-cultured both control non-preactivated NK cells and CIML NK cells with K562 target cells in the presence and absence of 2-deoxy-D-glucose (2DG), a glycolytic inhibitor. After a co-culture period of 7 hours, we measured the production of IFNγ, TNF and

MIP-1β, and the expression of the degranulation marker CD107a, in control non-preactivated and CIML NK cells. IFNγ is considered the model NK cell cytokine and its production is known to activate antigen-presenting cells (APCs) (Capuano et al., 2019). TNF is a major proinflammatory cytokine that is key fighting against virus and tumors, since it activates macrophage killing (Wang, Jaw, Stutzman, Zou, & Sun, 2012). Moreover, MIP-1β develops chemotactic and proinflammatory effects contributing to the recruitment of dendritic cells in the tumor microenvironment (Maurer & von Stebut, 2004; Vilgelm & Richmond, 2019). Last, CD107a is a highly glycosylated lysosomal-associated membrane protein (LAMP-1) that has been described as a marker of NK cell degranulation (Aktas et al., 2009; Alter et al., 2004; Cohnen et al., 2013). Therefore, these four molecules are good markers to determine NK cells functionality.

First, we observed how due to IL-12/15/18 pre-stimulation, CIML NK cells show a higher production of IFNγ, TNFα and MIP1β, and expression of CD107a compared to control NK cells (Figure 3A). When stimulated with K562 target cells, both control NK and CIML NK cells exhibit increased functions (Figure 3B), compared to those NK and CIML NK cells co-cultured with both K562 and 2-DG.. These result regarding upregulation of the effector functions are in accordance with what has been already reported (Cooper et al., 2009; Romee et al., 2016) about CIML NK cells restimulation and their enhanced functionality. Last, we evaluated the polyfunctionality, which is defined as the capacity of cells to produce multiple cytokines and/or degranulate at the same time, of non-preactivated NK and CIML NK cells, in the presence and absence of 2-DG, which is a glycolytic inhibitor that inhibits the glycolytic enzyme hexokinase II (Pajak et al., 2019). We determined the polyfunctionality index using Funky Cell software (Larsen et al., 2012). As expected, incubation with 2-DG not only diminishes the effect in the individual cytokine production and degranulation, but also reduces NK cells polyfunctionality (Table 1 and Figure 3), since it inhibits the glycolytic pathway and, therefore, ATP production. Focusing on CIML NK cells co-cultured with K562 target cells, we observed that triple positive cells (i.e. cells that are positive for three of the four measured functions) are the majority of the cells, followed by the quadruple and double positive cells. When incubated with the glycolytic inhibitor, we see how those percentages diminshe, and the majority of the cells become those that exhibit one effector function (Table 1).

	p_index	*++++*	*+++-*	*++--*	*+---*	*----*
NK	1,58	0,0267%	0,1357%	0,5884%	4,8501%	94,3982%
NK + 2DG	0,46	0,0000%	0,0133%	0,1099%	1,6969%	98,1798%
K562 NK	24,47	3,0697%	5,9911%	16,3250%	21,0209%	47,5933%
K562 NK + 2DG	7,91	0,2400%	0,1445%	5,1500%	17,2060%	75,9548%
CIML	28,14	1,3396%	5,8145%	20,2708%	59,1085%	13,4665%
CIML + 2DG	25,45	0,1300%	1,2266%	18,9479%	60,5660%	19,1295%
K562 CIML	60,48	22,5100%	28,3600%	24,8484%	19,0800%	5,2016%
K562 CIML + 2DG	38,19	2,3669%	11,2711%	32,6839%	43,3720%	12,3061%

Table 1. Polyfunctionality indexes of control and CIML NK cells. First column shows the calculated polyfunctionality index for each cell subset. Adjacent columns show the percentage of cells with 4 (++++), 3 (+++-), 2 (++--), 1(+---) or 0 (----) functions, defining a function as the production of IFNγ, TNF or MIP-1β, or degranulation (expression of CD107a).

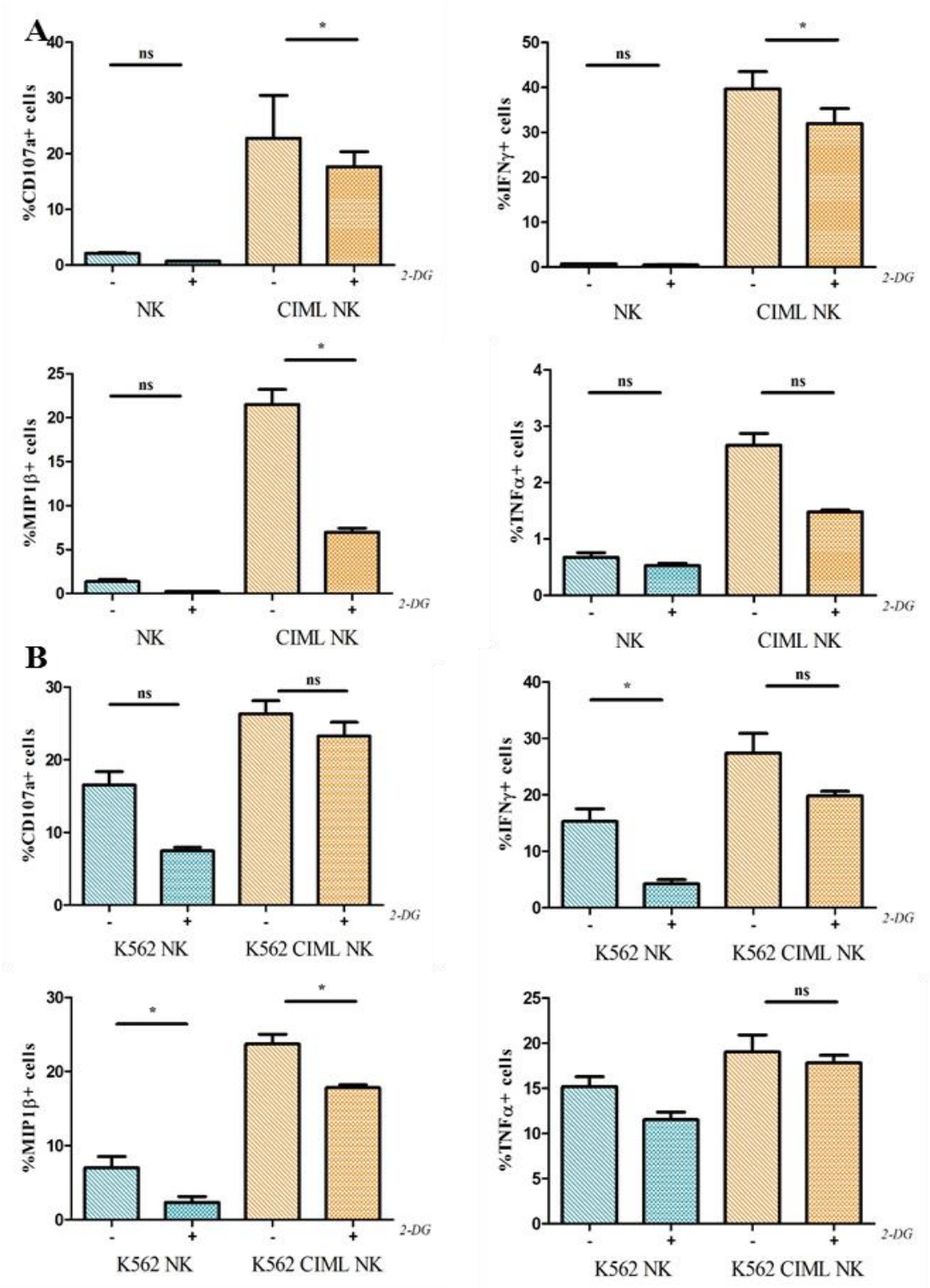

Figure 3. Effect of glycolysis inhibition on the effector functions of control non-preactivated and CIML NK cells. Bar graphs show the percentage of control and CIML NK cells that produce IFNγ, TNF or MIP-1β, or degranulate (measured as CD107a expression), in the presence and absence of 2-DG. Results were obtained (**A**) after 16-18 hours incubation and (**B**) after 16-18 hours incubation plus additional 7 hours co-cultivation with K562 target cells. *$p < 0.05$ and **$p < 0.01$.

DISCUSSION

NK cells, and especially CIML NK cells, have become a promising tool in cancer immunotherapy (Capuano et al., 2019; Ewen et al., 2018; Kim et al., 2019; Ni et al., 2016; Ni et al., 2012; Terrén et al., 2020; Wagner

et al., 2017), in particular against acute myeloid leukemia (AML) (Mao et al., 2016; Uppendahl et al., 2019), where complete remissions have been observed after adoptive cell therapies with CIML NK cells (Romee et al., 2016). NK cells can get activated by different ways, being one via interleukin-mediated signals as it has been described with a mixture of IL-12, IL-15 and IL-18 (Cooper et al., 2009), where NK cells acquired a memory-like state in the absence of antigen stimulation. After activation, NK cells secrete cytokines and cytolytic granules containing perforin/granzyme B and others. These effector functions depend on the metabolic status of the cells (Assmann et al., 2017; Chang et al., 2013; Terrén et al., 2019).

Following NK cell activation with interleukins, it has been demonstrated an increase of both OXPHOS and glycolysis, increasing the expression of glycolytic enzymes and other important nutrient transporters (Gardiner, 2019). In our study, we have observed how NK cells incubated with IL-12, IL-15 and IL-18 acquire a preactivated state (CIML NK cells) and have higher glucose uptake rate, and indirect measure of glycolytic rate (Figure 2A). After incubation of CIML NK cells with low doses IL-15 and IL-2 separately for seven days, we have seen how the high glycolytic rates are maintained (Figure 2B), indicating that the glycolytic pathway is relevant for these cells and that metabolic reprogramming induced by IL-12, IL-15 and IL-18 stimulation is not transient. Previous studies suggest that $CD56^{bright}$ cells have a greater cytokine secretion capacity than $CD56^{dim}$ NK cells (Wagner et al., 2017). Regarding our assays, we see that the $CD56^{bright}$ subset exhibits a major glycolytic rate, also in agreement whit what Keating et al. reported in 2016. Therefore, even if this cell population appears in a very low percentage in peripheral blood, this enhanced cytokine production might be strongly related to that upgraded metabolism and the fact that $CD56^{bright}$ NK cells express high levels of interleukin receptors. Then, in order to define if the glycolytic pathway actually is the primary metabolic pathway that follow CIML NK cells, we studied whether incubation with a glycolytic inhibitor such as 2-DG could affect or not CIML NK cells effector functions. To assess that, we incubated non-preactivated NK and CIML NK cells with 2-DG and stimulated them with K562 targets cells, a myelogenous leukemia cell line (Klein et al., 1976), and last we measured the expression of three critical cytokines (IFNγ, MIP1β and TNF) and the degranulation marker CD107a.

In our experiments we could assessed that CIML NK cells showed major polyfunctional capacity after restimulation with K562 target cells, leading to the appearance of different triple positive functional states (especially $CD107a^{+}$ $MIP1\beta^{+}$ $IFN\gamma^{+}$ $TNF\alpha^{-}$) or even quadruple positive functional state (Table 1). Compared to control non-preactivated NK cells, differences in the increase of effector functions are very significant in CIML NK cells after restimulation with target cells like K562. When incubated with the glycolytic inhibitor 2-DG, polyfunctionality diminished. Nevertheless, the production of IFNγ and expression of CD107a remained somehow high in CIML NK cells, probably due to their pre-activated status (Figure 3). Expression of CD107a confirms that CIML NK cells continue degranulating despite inhibiting glycolysis. Moreover, regarding IFN-gamma expression, it has been suggested that the enzyme Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) appears to bind IFNγ mRNA, thus repressing its translation, as it has been demonstrated in T cells (Chang et al., 2013; Gardiner, 2019; Terrén et al., 2019). When glycolysis is

triggered, this GAPDH acts as a glycolytic enzyme and does not impair the translation of IFNγ mRNA (Gardiner, 2019). This correlates with the importance that has been described for the glycolytic pathway regarding NK cells, since inhibition of that path may cause GAPDH to strongly bind mRNA from IFNγ, inhibiting cells from its production. Therefore, it is again reflected that pre-activation of NK cells with interleukins, among other signals and pathways, is a key tool (Terrén et al., 2018; Uppendahl et al., 2019) since cytokines can affect metabolism, in this case leading to the release of the IFNγ mRNA from GAPDH and enhancing its production and function.

However, not only glycolysis and OXPHOS are key pathways involved in CIML NK cells effector functions. It has been reported that after interaction of non-preactivated NK and CIML NK cells with IL-15, three primary pathways get activated: JAK1-3/STAT3-5, PI3K/Akt/mTOR and Ras/Raf/MEK/ERK (Donnelly et al., 2014; Mao et al., 2016; Marçais et al., 2014). When PI3K and MEK are inhibited, it is observed a decrease on the cytotoxic proteins expression in $CD56^{bright}$ NK cells following IL-15 priming and killing of K562 leukemia target cells is significantly reduced. These two pathways had a lower effect on $CD56^{dim}$ NK cells, but killing was significantly reduced also (Donnelly et al., 2014; Keating et al., 2016; Wagner et al., 2017). Last, the sterol regulatory element-binding protein (SREBP) which controls the citrate-malate shuttle, also appears to be increased in NK cells incubated with IL-2 and IL-12 meaning it is key for controlling NK cells metabolic configuration (O'Brien & Finlay, 2019; Terrén et al., 2019). Moreover, it has been demonstrated that after inhibition of SREBP, CIML NK cells lose their anti-tumor activity (Assmann et al., 2017). However, the role of SREBPs in the metabolic reprogramming of human CIML NK cells is still poorly understood, so it might be an interesting field to study.

CONCLUSIONS

First, this work shows how metabolism, specifically the glycolytic pathway, is a fundamental process for the effector functions by both non-preactivated NK and CIML NK cells. Moreover, we confirmed that the pre-activated state of CIML NK cells leads to higher glycolytic rates, suggesting that these cells are metabolically more active and, therefore, exhibit improved functions. Last, we confirmed that CIML NK cells itself exhibit potent effector functions that are even increased when restimulated with K562 target cells, leading to a very high polyfunctional index. Regarding metabolism, we observed that inhibition of the glycolytic pathway diminished polyfunctionality, although production of IFNγ and expression of CD107a is less affected than TNFα and MIP1β production. All these results suggest that the generation of CIML NK cells is a promising tool for future oncological immunotherapies, and that studies related to their metabolic requirements and reprogramming are needed in order to design more effective adoptive NK cell-based therapies.

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BIBLIOGRAPHY

Aktas, E., Kucuksezer, U. C., Bilgic, S., Erten, G., & Deniz, G. (2009). Relationship between CD107a expression and cytotoxic activity. *Cellular Immunology*, *254*(2), 149–154. https://doi.org/10.1016/j.cellimm.2008.08.007

Alter, G., Malenfant, J. M., & Altfeld, M. (2004). CD107a as a functional marker for the identification of natural killer cell activity. *Journal of Immunological Methods*, *294*(1–2), 15–22. https://doi.org/10.1016/j.jim.2004.08.008

Assmann, N., O'Brien, K. L., Donnelly, R. P., Dyck, L., Zaiatz-Bittencourt, V., Loftus, R. M., … Finlay, D. K. (2017). Srebp-controlled glucose metabolism is essential for NK cell functional responses. *Nature Immunology*, *18*(11), 1197–1206. https://doi.org/10.1038/ni.3838

Berrien-Elliott, M. M., Wagner, J. A., & Fehniger, T. A. (2015). Human Cytokine-Induced Memory-Like Natural Killer Cells. *Journal of Innate Immunity*, *7*(6), 563–571. https://doi.org/10.1159/000382019

Borrego, F., Kabat, J., Kim, D.-K., Lieto, L., Maasho, K., Peña, J., … Coligan, J. E. (2002). Structure and function of major histocompatibility complex (MHC) class I specific receptors expressed on human natural killer (NK) cells. *Molecular Immunology*, *38*(9), 637–660. https://doi.org/10.1016/S0161-5890(01)00107-9

Caligiuri, M. A. (2008). Human natural killer cells. *Blood*, *112*(3), 461–469. https://doi.org/10.1182/blood-2007-09-077438

Capuano, C., Pighi, C., Battella, S., Santoni, A., Palmieri, G., & Galandrini, R. (2019). Memory NK Cell Features Exploitable in Anticancer Immunotherapy. *Journal of Immunology Research*, *2019*, 1–8. https://doi.org/10.1155/2019/8795673

Cerwenka, A., & Lanier, L. L. (2016). Natural killer cell memory in infection, inflammation and cancer. *Nature Reviews Immunology*, *16*(2), 112–123. https://doi.org/10.1038/nri.2015.9

Chang, C.-H., Curtis, J. D., Maggi, L. B., Faubert, B., Villarino, A. V., O'Sullivan, D., … Pearce, E. L. (2013). Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis. *Cell*, *153*(6), 1239–1251. https://doi.org/10.1016/j.cell.2013.05.016

Cohnen, A., Chiang, S. C., Stojanovic, A., Schmidt, H., Claus, M., Saftig, P., … Watzl, C. (2013). Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. *Blood*, *122*(8),

1411–1418. https://doi.org/10.1182/blood-2012-07-441832

Cooper, M. A., Elliott, J. M., Keyel, P. A., Yang, L., Carrero, J. A., & Yokoyama, W. M. (2009). Cytokine-induced memory-like natural killer cells. *Proceedings of the National Academy of Sciences*, *106*(6), 1915–1919. https://doi.org/10.1073/pnas.0813192106

Donnelly, R. P., Loftus, R. M., Keating, S. E., Liou, K. T., Biron, C. A., Gardiner, C. M., & Finlay, D. K. (2014). mTORC1-Dependent Metabolic Reprogramming Is a Prerequisite for NK Cell Effector Function. *The Journal of Immunology*, *193*(9), 4477–4484. https://doi.org/10.4049/jimmunol.1401558

Ewen, E. M., Pahl, J. H. W., Miller, M., Watzl, C., & Cerwenka, A. (2018). KIR downregulation by IL-12/15/18 unleashes human NK cells from KIR/HLA-I inhibition and enhances killing of tumor cells. *European Journal of Immunology*, *48*(2), 355–365. https://doi.org/10.1002/eji.201747128

Finnberg, N., Klein-Szanto, A. J. P., & El-Deiry, W. S. (2008). TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. *Journal of Clinical Investigation*, *118*(1), 111–123. https://doi.org/10.1172/JCI29900

Freud, A. G., & Caligiuri, M. A. (2006). Human natural killer cell development. *Immunological Reviews*, *214*(1), 56–72. https://doi.org/10.1111/j.1600-065X.2006.00451.x

Freud, A. G., Mundy-Bosse, B. L., Yu, J., & Caligiuri, M. A. (2017). The Broad Spectrum of Human Natural Killer Cell Diversity. *Immunity*, *47*(5), 820–833. https://doi.org/10.1016/j.immuni.2017.10.008

Gardiner, C. M. (2019). NK cell metabolism. *Journal of Leukocyte Biology*, *105*(6), 1235–1242. https://doi.org/10.1002/JLB.MR0718-260R

Horowitz, A., Strauss-Albee, D. M., Leipold, M., Kubo, J., Nemat-Gorgani, N., Dogan, O. C., … Blish, C. A. (2013). Genetic and Environmental Determinants of Human NK Cell Diversity Revealed by Mass Cytometry. *Science Translational Medicine*, *5*(208), 208ra145-208ra145. https://doi.org/10.1126/scitranslmed.3006702

Keating, S. E., Zaiatz-Bittencourt, V., Loftus, R. M., Keane, C., Brennan, K., Finlay, D. K., & Gardiner, C. M. (2016). Metabolic Reprogramming Supports IFN-γ Production by CD56 bright NK Cells. *The Journal of Immunology*, *196*(6), 2552–2560. https://doi.org/10.4049/jimmunol.1501783

Keppel, M. P., Saucier, N., Mah, A. Y., Vogel, T. P., & Cooper, M. A. (2015). Activation-Specific Metabolic Requirements for NK Cell IFN-γ Production. *The Journal of Immunology*, *194*(4), 1954–1962. https://doi.org/10.4049/jimmunol.1402099

Kim, N., Lee, H. H., Lee, H.-J., Choi, W. S., Lee, J., & Kim, H. S. (2019). Natural killer cells as a promising therapeutic target for cancer immunotherapy. *Archives of Pharmacal Research*, *42*(7), 591–606. https://doi.org/10.1007/s12272-019-01143-y

Klein, E., Ben-Bassat, H., Neumann, H., Ralph, P., Zeuthen, J., Polliack, A., & Vánky, F. (1976). Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. *International Journal of Cancer*, *18*(4), 421–431. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/789258

Larsen, M., Sauce, D., Arnaud, L., Fastenackels, S., Appay, V., & Gorochov, G. (2012). Evaluating Cellular Polyfunctionality with a Novel Polyfunctionality Index. *PLoS ONE*, *7*(7), e42403. https://doi.org/10.1371/journal.pone.0042403

Long, E. O., Sik Kim, H., Liu, D., Peterson, M. E., & Rajagopalan, S. (2013). Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. *Annual Review of Immunology*, *31*(1), 227–258. https://doi.org/10.1146/annurev-immunol-020711-075005

Mah, A. Y., & Cooper, M. A. (2016). Metabolic Regulation of Natural Killer Cell IFN-γ Production. *Critical Reviews in Immunology*, *36*(2), 131–147. https://doi.org/10.1615/CritRevImmunol.2016017387

Mao, Y., van Hoef, V., Zhang, X., Wennerberg, E., Lorent, J., Witt, K., … Lundqvist, A. (2016). IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells. *Blood*, *128*(11), 1475–1489. https://doi.org/10.1182/blood-2016-02-698027

Marçais, A., Cherfils-Vicini, J., Viant, C., Degouve, S., Viel, S., Fenis, A., … Walzer, T. (2014). The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. *Nature Immunology*, *15*(8), 749–757. https://doi.org/10.1038/ni.2936

Maurer, M., & von Stebut, E. (2004). Macrophage inflammatory protein-1. *The International Journal of Biochemistry & Cell Biology*, *36*(10), 1882–1886. https://doi.org/10.1016/j.biocel.2003.10.019

Ni, J., Hölsken, O., Miller, M., Hammer, Q., Luetke-Eversloh, M., Romagnani, C., & Cerwenka, A. (2016). Adoptively transferred natural killer cells maintain long-term antitumor activity by epigenetic imprinting and CD4 + T cell help. *OncoImmunology*, *5*(9), e1219009. https://doi.org/10.1080/2162402X.2016.1219009

Ni, J., Miller, M., Stojanovic, A., Garbi, N., & Cerwenka, A. (2012). Sustained effector function of IL-12/15/18–preactivated NK cells against established tumors. *The Journal of Experimental Medicine*, *209*(13), 2351–2365. https://doi.org/10.1084/jem.20120944

Nikzad, R., Angelo, L. S., Aviles-Padilla, K., Le, D. T., Singh, V. K., Bimler, L., … Paust, S. (2019). Human natural killer cells mediate adaptive immunity to viral antigens. *Science Immunology*, *4*(35), eaat8116. https://doi.org/10.1126/sciimmunol.aat8116

O'Brien, K. L., & Finlay, D. K. (2019). Immunometabolism and natural killer cell responses. *Nature Reviews Immunology*, *19*(5), 282–290. https://doi.org/10.1038/s41577-019-0139-2

O'Neill, L. A. J., Kishton, R. J., & Rathmell, J. (2016). A guide to immunometabolism for immunologists. *Nature Reviews Immunology*, *16*(9), 553–565. https://doi.org/10.1038/nri.2016.70

Pahl, J. H. W., Cerwenka, A., & Ni, J. (2018). Memory-Like NK Cells: Remembering a Previous Activation by Cytokines and NK Cell Receptors. *Frontiers in Immunology*, *9*(NOV), 1–9. https://doi.org/10.3389/fimmu.2018.02796

Pajak, B., Siwiak, E., Sołtyka, M., Priebe, A., Zieliński, R., Fokt, I., … Priebe, W. (2019). 2-Deoxy-d-Glucose and Its Analogs: From Diagnostic to Therapeutic Agents. *International Journal of Molecular Sciences*, *21*(1), 234. https://doi.org/10.3390/ijms21010234

Romee, R., Rosario, M., Berrien-Elliott, M. M., Wagner, J. A., Jewell, B. A., Schappe, T., … Fehniger, T. A. (2016). Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Science Translational Medicine*, *8*(357), 357ra123-357ra123. https://doi.org/10.1126/scitranslmed.aaf2341

Romee, R., Schneider, S. E., Leong, J. W., Chase, J. M., Keppel, C. R., Sullivan, R. P., … Fehniger, T. A.

(2012). Cytokine activation induces human memory-like NK cells. *Blood*, *120*(24), 4751–4760. https://doi.org/10.1182/blood-2012-04-419283

Screpanti, V., Wallin, R. P. A., Grandien, A., & Ljunggren, H.-G. (2005). Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells. *Molecular Immunology*, *42*(4), 495–499. https://doi.org/10.1016/j.molimm.2004.07.033

Terrén, I., Mikelez, I., Odriozola, I., Gredilla, A., González, J., Orrantia, A., … Borrego, F. (2018). Implication of Interleukin-12/15/18 and Ruxolitinib in the Phenotype, Proliferation, and Polyfunctionality of Human Cytokine-Preactivated Natural Killer Cells. *Frontiers in Immunology*, *9*(APR). https://doi.org/10.3389/fimmu.2018.00737

Terrén, I., Orrantia, A., Mikelez-Alonso, I., Vitallé, J., Zenarruzabeitia, O., & Borrego, F. (2020). NK Cell-Based Immunotherapy in Renal Cell Carcinoma. *Cancers*, *12*(2), 316. https://doi.org/10.3390/cancers12020316

Terrén, I., Orrantia, A., Vitallé, J., Zenarruzabeitia, O., & Borrego, F. (2019). NK Cell Metabolism and Tumor Microenvironment. *Frontiers in Immunology*, *10*(SEP), 1–9. https://doi.org/10.3389/fimmu.2019.02278

Uppendahl, L. D., Felices, M., Bendzick, L., Ryan, C., Kodal, B., Hinderlie, P., … Geller, M. A. (2019). Cytokine-induced memory-like natural killer cells have enhanced function, proliferation, and in vivo expansion against ovarian cancer cells. *Gynecologic Oncology*, *153*(1), 149–157. https://doi.org/10.1016/j.ygyno.2019.01.006

Valiante, N. M., Uhrberg, M., Shilling, H. G., Lienert-Weidenbach, K., Arnett, K. L., D'Andrea, A., … Parham, P. (1997). Functionally and Structurally Distinct NK Cell Receptor Repertoires in the Peripheral Blood of Two Human Donors. *Immunity*, *7*(6), 739–751. https://doi.org/10.1016/S1074-7613(00)80393-3

Vilgelm, A. E., & Richmond, A. (2019). Chemokines Modulate Immune Surveillance in Tumorigenesis, Metastasis, and Response to Immunotherapy. *Frontiers in Immunology*, *10*(FEB), 6–8. https://doi.org/10.3389/fimmu.2019.00333

Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., … Ugolini, S. (2011). Innate or Adaptive Immunity? The Example of Natural Killer Cells. *Science*, *331*(6013), 44–49. https://doi.org/10.1126/science.1198687

Wagner, J. A., Rosario, M., Romee, R., Berrien-Elliott, M. M., Schneider, S. E., Leong, J. W., … Fehniger, T. A. (2017). CD56bright NK cells exhibit potent antitumor responses following IL-15 priming. *Journal of Clinical Investigation*, *127*(11), 4042–4058. https://doi.org/10.1172/JCI90387

Wang, R., Jaw, J. J., Stutzman, N. C., Zou, Z., & Sun, P. D. (2012). Natural killer cell-produced IFN-γ and TNF-α induce target cell cytolysis through up-regulation of ICAM-1. *Journal of Leukocyte Biology*, *91*(2), 299–309. https://doi.org/10.1189/jlb.0611308

Yawata, M., Yawata, N., Draghi, M., Little, A.-M., Partheniou, F., & Parham, P. (2006). Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *The Journal of Experimental Medicine*, *203*(3), 633–645. https://doi.org/10.1084/jem.20051884