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Dissertação no âmbito do Mestrado em Investigação Biomédica orientada pelo Professor Doutor Frederico Guilherme de Sousa da Costa Pereira e pelo Licenciado Paulo Jorge Ferreira Rodrigues dos Santos, apresentada à Faculdade de Medicina da Universidade de Coimbra

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Resumo

As células *Natural Killer* (NK) são células linfócitárias inatas citotóxicas, que desempenham um importante papel na defesa do organismo contra células tumorais ou células infetadas por vírus. Estas células possuem, à sua superfície, uma vasta diversidade de recetores ativadores e inibidores, que permitem a deteção de estímulos e, consequentemente, controlam a sua atividade. Este trabalho foca-se em três membros de um grupo de recetores da superfamília das imunoglobulinas, já descritos como importantes reguladores das funções das NK: o CD96, o CD226 e o TIGIT. O TIGIT é inibidor da ação citotóxica das células NK, enquanto o CD226 desempenha o papel de ativador. O papel do CD96 é ambíguo, uma vez que já foi descrito tanto como inibidor e ativador.

O principal objetivo deste trabalho foi averiguar o efeito de agentes dopaminérgicos nos recetores CD96, CD226 e TIGIT e, consequentemente, nas células NK. Utilizando citometria de fluxo para analisar a expressão destes três recetores nas células NK de dadores saudáveis, concluímos que a percentagem de células a expressar estes recetores não é alterada na presença de agentes dopaminérgicos ou interleucina-2 recombinante (rIL-2). Além disto, há uma maior percentagem de células NK $CD56^{bright}$ a expressar CD96, mas menor percentagem a expressar TIGIT quando comparadas com as NK $CD56^{dim}$. Isto não se aplica ao CD226. A população de recetores $CD96^{-}TIGIT^{-}CD226^{+}$ é a predominantemente expressa nas células NK e NK $CD56^{dim}$.

Adicionalmente, os estudos *in vitro* também revelaram que o repertório CD96, CD226 e TIGIT influencia a atividade das células NK. Mais especificamente, estas células apresentam uma maior percentagem de desgranulação e produção de IFN-γ na ausência de CD96 e TIGIT, quando presente o recetor ativador CD226. A desgranulação também aumenta após 48h de incubação com rIL-2, e este efeito não é alterado pelos agentes dopaminérgicos.

Palavras-chave: *Natural Killer*, Dopamina, CD96, CD226, TIGIT, Desgranulação, IFN-γ.

Abstract

Natural Killer (NK) cells are innate cytotoxic lymphocytes that play an important role in the organism's defense against tumor or virus-infected cells. These cells express, at its surface, a wide variety of activating and inhibitory receptors, that allow the detection of stimuli, and, consequently, control NK cell's activity. This work focusses on three members of a group of receptors of immunoglobulin superfamily, already described as important regulators on NK cell's functions: CD96, CD226 and TIGIT. TIGIT inhibits the cytotoxicity of NK cells, while CD226 activates it. The role of CD96 its ambiguous since it is described both as activator and inhibitor.

The main purpose of this study was to assess the effect of dopaminergic agents on CD96, CD226 and TIGIT receptors and, consequently, on NK cells. Using flow cytometry to analyze the expression of these three receptors on NK cells on healthy donors, we concluded that the percentage of cells expressing these receptors does not change in the presence of dopaminergic agents or recombinant interleukin-2 (rIL-2). Additionally, there is a higher percentage of $CD56^{bright}$ NK cells expressing CD96 but a lower percentage expressing TIGIT receptors when compared to $CD56^{dim}$ NK cells. This does not apply to CD226. Importantly, $CD96^{-}TIGIT^{-}CD226^{+}$ is the predominant population of receptors expressed on both NK cells and $CD56^{dim}$ NK cells.

Additionally, the *in vitro* studies also revealed that CD96, CD226 and TIGIT repertoire influences NK cell's function. More specifically, these cells have a higher percentage of degranulation and production of IFN-γ in the absence of CD96 and TIGIT, in the presence of activating receptor CD226. The percentage of degranulation also increases after a 48h incubation with rIL-2 and this effect is not altered by dopaminergic agents.

Keywords: Natural Killer, Dopamine, CD96, CD226, TIGIT, Degranulation, IFN-γ.

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Abbreviations

α-syn	α-synuclein
cAMP	Cyclic Adenosine Monophosphate
CCL	CC-Chemokine Ligand
CD	Cluster of Differentiation
CFS	Cerebrospinal Fluid
CNS	Central Nervous System
CTLA4	Cytotoxic T-lymphocyte Associated Antigen 4
DA	Dopamine
DR	Dopamine Receptor
DC	Dendritic Cell
E: T	Effector: Target
ERK	Extracellular Signal-Regulated Kinases
FBS	Fetal Bovine Serum
GRB	Growth Factor Receptor-Bound Protein
HD	Healthy Donors
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
ITT	Immunoreceptor Tyrosine tail

LAMP	Lysosomal-Associated Membrane Protein
LFA	Lymphocyte Function-Associated Antigen
mAB	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
NK	Natural Killer
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PI3K	Phosphoinositide 3-Kinase
PK	Protein Kinase
PVR	Poliovirus Receptor
TIGIT	T cell Immunoglobulin and ITIM Domain
TIM-3	T-cell Immunoglobulin and Mucin Domain-Containing Protein 3
Treg	Regulatory T cell
TGF	Transforming Growth Factor
Th	T helper cell
TNF	Tumor Necrosis Factor

1. Introduction

1.1. Immune System

The immune system is a highly complex organ that provides an efficient immune response to protect us from pathogenic agents. There are two distinct types of immune responses: the innate and the adaptive responses.

The innate response is the first line of defense and the most rapidly acting type of immunity since cell-surface receptors and germline-encoded pattern-recognition receptors quickly detect the presence of antigens and create inflammatory responses. This response includes soluble factors, such as proteins, cytokines, and several effector cells, including phagocytic cells that release inflammatory mediators and Natural Killer (NK) cells. The innate immune receptors present at the cell surface of innate cells must be capable of discriminating between self and non-self, allowing an effective innate response against pathogens, or altered cells.

The immune system co-evolves with the nervous systems during aging and, simultaneously, exert a broad influence over all organs. This interaction occurs through cellular communication, which is bidirectional and mediated by direct cellular contacts (e.g., synapse formation between neurons or between immune cells) and by soluble mediators (neurotransmitters or cytokines). Catecholamines, such as dopamine (DA), are neurotransmitters of the sympathetic nervous system, and are released in many peripheral organs. Active concentrations of DA have been reported in the colon, heart, lungs, blood, among others[1]. It has been attributed an immunomodulator role for DA and peripheral blood mononuclear cells (PBMCs) also synthesize DA and express DA receptors and transporters on their cell membrane[1].

1.2. NK cells

NK cells are innate lymphoid cells that play a major role in the organism defense against tumors and virus-infected cells, having earned their name based of their ability to lyse target cells without prior exposure to antigen.

In peripheral blood, these cells constitute about 10% of all lymphocytes, being conventionally defined as $CD3^{-}CD56^{+}$. In humans, the stages of NK cell maturation are demarcated by the expression of specific phenotypic markers. Less mature human peripheral blood NK cells are defined as $CD56^{bright}CD16^{-}$, and, as they mature, NK cells down-regulate

CD56 expression and up-regulate CD16, becoming $CD56^{dim}CD16^{+}$ [2]. $CD56^{bright}$ are more potent cytokine producers, but can become highly cytotoxic when stimulated with different cytokines [3]. On the other hand, $CD56^{dim}$ are known for their cytotoxic capacity, even though they also produce cytokines in response to stimuli [4]. $CD56^{bright}$ NK cells respond better to soluble factors, while the $CD56^{dim}$ subset responds better to receptors binding ligands anchored on other cells [5]. In fact, exposure to exogenous interleukin (IL)-2 induces tenfold greater proliferation of $CD56^{bright}$ cells compared to $CD56^{dim}$ lymphocytes[6]. Furthermore, $CD56^{dim}$ NK cells can up-regulate CD56 expression upon cytokine activation and become $CD56^{bright}$ [7].

1.2.1. Target cell recognition

The activity and responsiveness of Human NK cells is regulated by an array of both co-stimulatory and co-inhibitory cell surface receptors that recognize their respective ligand(s) on target cells or antigen-presenting cells [5]. The differences in the function of the inhibitory and activating NK cell receptors are reflected by the different ligands they recognize, allowing them to distinguish self from non-self. Activating NK cell receptors recognize and interact with pathogen-derived, stress-induced, tumor specific cytokines produced during the early phases of infection, such as IL-12 or IL-18. This phenomenon results in the activation of NK cell cytotoxicity and the secretion of proinflammatory cytokines such as IFN-γ and TNF-α [8,9], which initiates and shapes the adaptive immune response. On the contrary, inhibitory receptors recognize the absence of self ligands, such as major histocompatibility complex (MHC) class I, on potential target cells. This recognition strategy is known as the detection of 'missing self', since pathogenic cells do not express or have a low expression of this molecule [10–13].

Adhesion receptors, such as integrins, are also essential to this contact. In fact, upon NK cell activation, integrins stabilize the synapse between NK and the target cell. Moreover, intracellular vesicles containing cytotoxic molecules, such as perforin or granzyme, are polarized toward the contact site and its content (granules) is released into the synaptic cleft, resulting in the death of the attached target cell [14,15].

1.2.2. NK cells activity

NK cells respond to stimulus with two main functions: cytotoxic activity and cytokine production.

The cytotoxic activity englobes the release of cytolytic granules that contain perforin and granzymes. Perforin is a membrane-disrupting protein that facilitates the entry of granzymes in target cells, while granzymes are able to induce apoptosis of the target cell through the activation of distinct cell death pathways [16–18]. The surface of the cytotoxic granules presents the lysosomal-associated membrane protein (LAMP)-1 (also known as CD107a). As degranulation occurs, CD107a is transported to the surface of NK cells, becoming accessible for antibody binding and making possible to identify NK cells which have been activated for degranulation [19,20].

The recognition of target cells also induces the production and secretion of a wide range of chemokines and pro-inflammatory cytokines. Cytokines are small proteins that regulate the growth, differentiation, and activation of immune cells. These proteins interfere in the interaction and communication between cells in immune responses, as well as stimulate the movement of cells towards the disease site [21,22]. These mainly include tumor necrosis factor (TNF)-α, IL-6, CC-chemokine ligand (CCL) 3, CCL4, CCL5 and interferon (IFN)-γ. The latter has immunostimulatory and immunomodulatory effects, being capable of promoting the activation of macrophages for phagocytosis through tumor immune surveillance and potentiating the differentiation of naïve $CD4^+$T cells into T helper (Th) effector cells, which in turn mediate cellular immunity against non-self, as well as upregulation of the antigen presenting pathways [23,24].

NK cells maturation and effector functions are influenced by their surrounding microenvironment. Some interleukins, such as IL-2, IL-12, IL-18, and IFN-γ can increase cytotoxicity, change chemokines and cytokines properties, enhance pro-inflammatory and innate immune responses and change signaling pathways in NK cells. These changes in signaling and surface/adhesion profile may enable the activated NK cells to migrate and infiltrate tissues where inflammation occur and recruit other effector cells of the immune system[25]. On the contrary, immunosuppressive cytokines, such as transforming growth factor (TGF)-β or adenosine, that can be present in the tumor microenvironment, are capable of inhibiting NK cells maturation and cytotoxic potential[26,27]. Cytokines can enhance degranulation of $CD56^{dim}$ and $CD56^{bright}$ NK cells [3].

1.3. CD96, CD226 and TIGIT

Nectin and nectin-like molecules play important regulatory roles in NK cell functions, such as cell movement, adhesion, proliferation and survival[28]. CD96, CD226 and TIGIT receptors competitively interact with nectin CD112, CD113, CD111, PVRL4 and the nectin-like protein CD155[29]. Therefore, the interactions between these receptors and ligands determine the profile of the immune response.

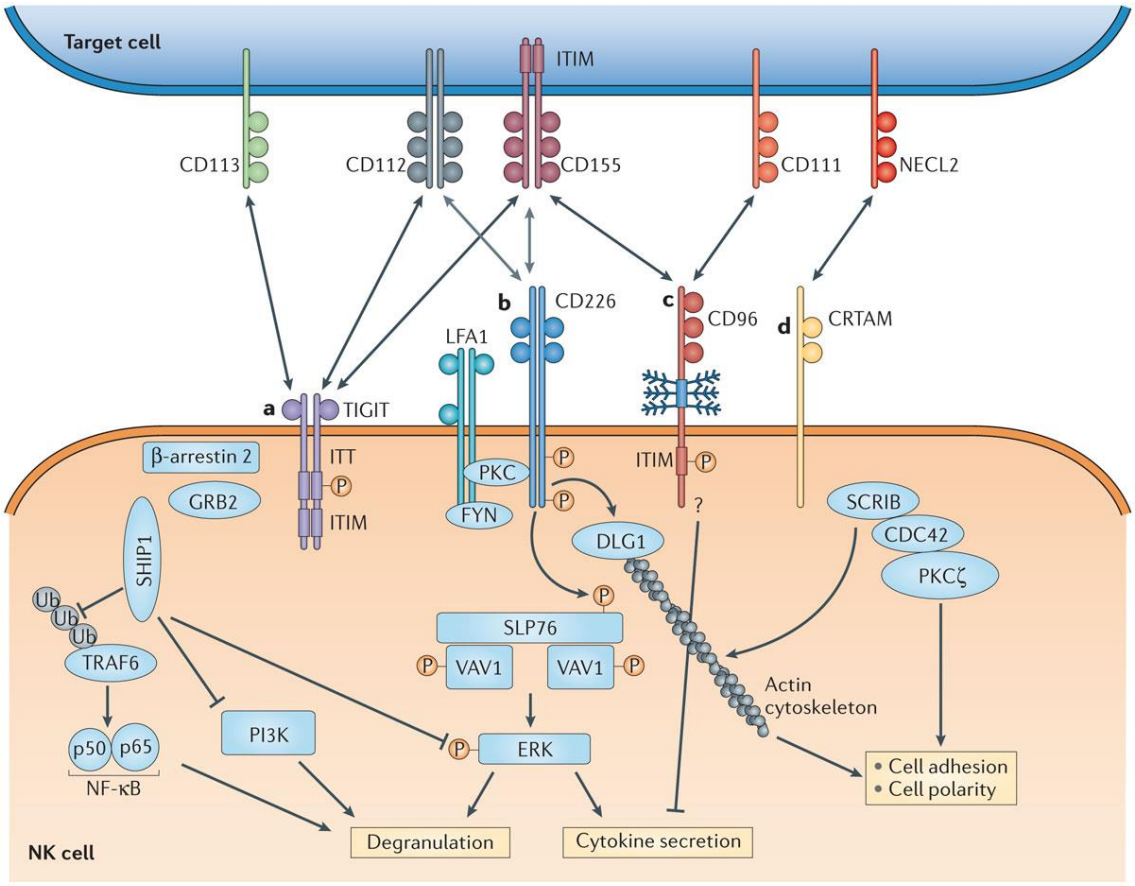

Figure 1 | Interaction between nectins and the receptors CD96, TIGIT and CD226 and its' signaling pathways. **a.** Upon interaction with CD113, CD112 or CD155, the immunoglobulin tail tyrosine (ITT)-like motif of TIGIT is phosphorylated on Tyr225 and binds the cytosolic adaptor GRB2, which in turn can recruit SHIP1 to inhibit PI3K and MAPK signaling. Furthermore, phosphorylated TIGIT recruits SHIP1 through β-arrestin 2 and impairs nuclear factor-χB activation by blocking TNF receptor-associated factor 6 (TRAF6) autoubiquitylation. **b.** The intracellular domain of CD226 is phosphorylated on Ser329 and Tyr322 upon binding to CD112 and CD155. Ser 329 phosphorylation facilitates activation of protein kinase C (PKC) and the association of CD226 with lymphocyte function-associated antigen 1 (LFA1). LFA1 is then required for FYN-mediated phosphorylation of Tyr 322 and CD226 mediated downstream signaling. Additionally, CD226 associates with DLG1 and works together with LFA1 during the initial process of actin cytoskeleton reorganization. **c**. CD96 binds to CD111 and to CD155. The intracellular domain of CD96

contains an immunoreceptor tyrosine-based inhibitory (TITIM)-like motif, however the downstream signaling pathway has not yet been described[30]. Figure from *Martinet et al.* (2015). Used with permission.

1.3.1. CD96

1.3.1.1. Structure and ligands

CD96, also known as Tactile (as in T cell-activated increased late expression), is a transmembrane glycoprotein that contains three extracellular immunoglobulin-like domains and belongs to the Ig superfamily of receptors.

CD96 is expressed on various T cell subsets and in all resting human and mouse NK cells[31–33], but not myeloid or B cells[34,35].

The main ligand for CD96 is CD155. Human CD96 is expressed as two splice variants that express differences in their second immunoglobulin-like domain and their binding affinity for CD155[32]. *Chan et al.* (*2014)* has demonstrated that CD96 actively competes with CD226 for CD155 binding on *naïve* mouse NK cells [33]. However, CD96 have a higher affinity for CD155, suggesting that CD96 is more accessible to CD155 at the surface of *naïve* NK cells than CD226[33].

The downstream signaling pathway of CD96 has not been described in detail yet.

1.3.1.2. Mechanisms of action

CD96 was originally described as an adhesion molecule, able to promote NK and T cells adhesion to CD155-expressing cells and to enhance the cytolytic capacity of NK cells [36–40]. It was also shown to be upregulated late in activation [31] and to enhance the cytotoxicity of NK cell line NK92 in tumor lysis, although less efficiently than other receptors, such as CD226[37].

However, since it was later demonstrated that the cytoplasmic domain of this receptor contains a single putative immunoreceptor tyrosine-based inhibitory motif (ITIM)[37], it can also function as a direct inhibitor receptor under certain conditions[32,33]. Signaling through CD96 has been reported to inhibit the cytotoxicity of NK cells in mouse tumor models, revealing an inhibitory role of CD96. Using $CD96^{-/-}$ mice or anti-CD96 monoclonal Antibody (mAb), it was demonstrated that although the absence or blockade of CD96 did not affect NK cell cytotoxicity against various tumor targets, IFN-γ production was controlled by CD96 upon CD155 binding [33]. On the other hand, the frequency of IFN-γ-producing NK cells was reduced in $CD226^{-/-}$ mice

compared with wild-type and experiments using mice deficient in both CD226 and CD96 ($CD226^{-/-}CD96^{-/-}$ mice) revealed that CD96 also functions independently of CD226 in limiting NK cell IFN-γ production[11]. However, whether human CD96 inhibits or activates human NK cells still needs to be clarified.

1.3.2. CD226

1.3.2.1. Structure and ligands

CD226 or DNAX-Associated Molecule 1 (DNAM-1) is also a transmembrane glycoprotein consisting of an extracellular region with two V-like domains of immunoglobulin, a transmembrane region and a cytoplasmic region containing tyrosine and serine phosphorylated sites[42]. This receptor is expressed on the majority of monocytes/macrophages, T cells, B cells, dendritic cells (DCs) and hematopoietic precursor cells[43–45], as well as in most human NK cells and a large amount of mouse NK cells[33,42,46]. It is upregulated in NK cells activated by, for example, IL-2[25].

Both human and mouse CD226 bind to CD155 and CD112[46–48]. CD226-ligand interactions trigger a canonical activating pathway that enables the recognition of potential targets by NK cells[49]. For example, a strong correlation was found between the expression of CD112 and CD155 and the lysis susceptibility of myeloid leukemia cells by NK cells[50].

1.3.2.2. Mechanisms of action

CD226 functions as an activating receptor in NK cells [51]. Unlike most classical NK cell-activating receptors, CD226 does not associate with any immunoreceptor tyrosine-based activation motif (ITAM)-presenting molecules, but it contains an intracellular domain that has at least two phosphorylation sites — tyrosine residue 322 and serine residue 329 [42]. These residues get phosphorylated upon binding to CD112 and CD155. Ser329 phosphorylation facilitates activation of protein kinase (PK) C and the association of CD226 with lymphocyte function-associated antigen (LFA) 1 at the surface of NK cells[72]. This association with LFA1 facilitates the phosphorylation of Tyr322 by the SRC kinase FYN. The subsequent CD226-induced signaling pathway involves the phosphorylation of SH2 domain-containing leukocyte protein of

76 kilodalton and VAV1 (**Figure 1**). This will lead to the activation of phospholipase Cy2, Ca^{2+} mobilization and, finally, NK cell degranulation [52,53].

A more recent study demonstrated that CD226 engagement in mouse NK cells triggers the phosphorylation and consequent inactivation of Forkhead Box O1 (FOXO1), a key transcription factor involved in the negative regulation of NK cells [54].

Both blocking and knockdown of CD226 expression resulted in deceased cytotoxicity of NK cells against alloantigen activated T cells, while over-expression of CD226 resulted in enhanced cytotoxicity [55]. Also, the combination of CD226 with CD155 and/or CD112, in cooperation with NKp30, can trigger NK cells to eliminate immature DC and promote the proliferation of mature DC [56], which in turn enhance an adaptive immune response [57].

Additionally, CD226 may be one of the markers of mature NK cells [30].

1.3.3. TIGIT

1.3.3.1. Structure and ligands

TIGIT was first identified by *Yu et al. (2009)* as a surface protein containing a single extracellular V-like domain of immunoglobulin, a type I transmembrane region and a short intracellular domain which includes one ITIM motif and one immunoglobulin tyrosine tail (ITT)-like motif [58]. This receptor belongs to the family of poliovirus receptor (PVR)-like proteins [58].

This receptor was named TIGIT for "T cell immunoglobulin and ITIM domain", although it was later demonstrated to have an important role in NK cell activity as well [40]. In fact, TIGIT is expressed on NK and T cells, including $CD4^+$ T cells, $CD8^+$ T cells and Treg, being shown to be up-regulated in T and NK cells upon activation [40,58,59].

TIGIT has been shown to interact with CD155, CD112 and CD113 [47,58], although its interaction with CD112 and CD113 is weaker than the TIGIT-CD155 interaction [60]. TIGIT and CD226 also share the ligand CD112 with CD112R (PVRIG), a more recently discovered immune checkpoint receptor mainly expressed on T cells and NK cells [61].

Based on crystal structure analysis, both TIGIT and CD155 form homodimers and, following ligand-receptor interaction, heterotetramers composed of two molecules of TIGIT and two of CD155, with a TIGIT cis-homodimer at its core [62]. A "lock and key" interaction was

proven to be specific since structural alterations were capable of disrupting TIGIT/CD155 binding [62].

1.3.3.2. Mechanisms of action

TIGIT may be immunosuppressive by competing with CD226 for CD155[57], or by directly delivering inhibitory signals to the effector cell [40].

The ITIM motif is essential for human TIGIT signaling, whereas mouse TIGIT inhibition can be mediated by either the ITIM motif or the ITT motif alone [40,63]. While human TIGIT fails to inhibit NK cell cytotoxic activity with a mutated or blocked ITIM motif [40], mouse TIGIT function is lost when tyrosine residues in both the ITIM and ITT-like motifs are mutated [63].

Moreover, different signaling pathways interfering with NK cell cytotoxicity or IFN-γ production have been highlighted. Following ligand binding, the ITT-like motif becomes phosphorylated and binds with the cytosolic adapter growth factor receptor-bound protein (GRB) 2, which then recruits SH2-containing inositol phosphatase-1 (SHIP-1), leading to the inhibition of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling cascade and the consequently down-regulation of NK cell killing activity[64] (**Figure 1**).

For example, the CD226/TIGIT axis was demonstrated to be involved in the regulation of cytotoxicity of NK cells on alloantigen activated T cells through PI3K-Akt-ERK cascade phosphorylation [55]. Knockdown or blocking of TIGIT expression both resulted in increased cytotoxicity of NK cells against activated T cells, while over-expression of TIGIT resulted in decreased cytotoxicity. Simultaneously, over-expression of CD226 or knockdown of TIGIT expression resulted in an increase of the phosphorylation levels of Akt and Erk1/2 in NK cells after exposure to activated T cells [55]. In addition, allelic variants in the TIGIT/CD226 pathway have been identified as risk factors for the development of human autoimmune diseases [65].

Furthermore, NK cells from $TIGIT^{-/-}$ mice had enhanced IFN-γ production when co-cultured with the YAC-1 target T cell line [59]. This receptor has also been shown to increase its expression after lipopolysaccharides stimulation [33].

In addition, TIGIT, together with MHC class I-specific inhibitory receptors, protects primary fibroblasts from NK mediated killing. Thus, TIGIT-CD155 interacting may provide an "alternative self" mechanism for MHC class I inhibition [40].

1.3.4. Roles of CD96, CD226 and TIGIT in cancer

Malignant cells have developed different mechanisms to escape immune cell recognition and/or killing. For example, tumor cells may hide themselves by down-regulating their antigen presentation machinery or by inhibiting immune cell trafficking to the tumor side [66]. Tumor cells can also create an immune suppressive microenvironment by secreting or promoting the secretion of immunosuppressive cytokines such as IL-10 and TGF-β, recruiting regulatory cells including regulatory T cells (Tregs), myeloid-derived suppressive cells and type 2 macrophages or by affecting immune cell metabolism [67]. However, another mechanism used by tumor cells to evade immune surveillance is the activation of immune checkpoint pathways [68].

Under normal conditions, CD155 is expressed at low levels on many normal cells, helping them to establish adherens junctions[69]. Despite its low level of expression, CD155 could probably be recognized by the inhibitory TIGIT (due to their high binding affinity) and thus the killing of self cells is prevented[70]. However, the expression of CD155 might be up regulated in the presence of developing tumors, to enable its detachment, migration, and proliferation of the developing[70]. The up-regulation of CD155 together with the appearance of tumor-specific activating ligands, such as the stress-induced ligands for NKG2D[71] or the B7-H6 ligand for NKp30[72], can co-stimulate CD226 and CD96 and together activate NK cytotoxicity. However, CD155 preferentially binds TIGIT, for which it has more affinity, and therefore tends to induce an immunosuppressive profile of TIGIT-expressing cells[73]. The progression of cancer is significantly associated with tumor immune escape, which may be associated with NK cell dysfunction and, in turn, can be caused by the dysregulation or inactivation of its receptors, such as CD226, CD96 and TIGIT. In pancreatic cancer, for example, CD226 and CD96 have been suggested to have a tumor surveillance contribution, since the percentage of $CD226^+$ and $CD96^+$ NK cells has been demonstrated to be significantly lower in these patients than in the healthy controls[74]. Also CD226-deficient mice demonstrated enhanced tumor development after transplantation of tumor cells[39,75].

Regarding the activity of NK cells on tumors, TIGIT blockade prevents the exhaustion of these cells and slows tumor growth, since $TIGIT^+$ NK cells present reduced effector function and anti-tumor potential [76], having emerged as an attractive target for cancer therapy.

TIGIT is highly expressed in human tumors and is also correlated with $CD8^+$T cell infiltration. In fact, TIGIT is able to suppress $CD8^+$T cells responses by disrupting CD226 homodimerization and function, since the latter is highly expressed by peripheral and tumor-infiltrating $CD8^+$ T cells[77]. Also, TIGIT was found to be highly expressed on glioblastoma-infiltrating T cells and significantly upregulated in circulating lymphocytes of glioblastoma

patients, in comparison with healthy controls [78]. Expression of CD226 was also increased in glioblastoma patients, but this costimulatory receptor was expressed alongside TIGIT in the majority of tumor-infiltrating T cells, suggesting a functional counteraction [78].

CD226 plays an essential role for in vivo immune surveillance [39,79]. It also plays a role in the control of proliferative disorders, since it was found to be involved in cytotoxicity-triggering in a number of cancer types [80–83]. For example, NK cells from advanced ovarian cancer patients showed an inhibitory phenotype, having lower expression of activating receptor CD226 compared to healthy donors (HD) peripheral blood NK cells, while inhibitory receptor TIGIT and CD96 expression was equal or higher, respectively. TIGIT blockade enhanced degranulation and IFN-γ production of HDs' $CD56^{dim}$ NK cells in response to ovarian cancer tumor cells, as well as in $CD56^{dim}$ NK cells from ovarian cancer patients, especially when CD226/CD155 interactions were in place [84]. Another study reported that CD226 was significantly lower on tumor-associated NK cells and that NK cells in the tumor environment display altered proportions of $CD56^{bright}$ and $CD56^{dim}$ subsets, compared with NK cells isolated from peripheral blood of patients and HD [85].

Furthermore, analysis of peripheral blood from late stage melanoma patients revealed the important role of $CD56^{bright}$ NK cells in melanoma patient survival, since the abundance of these cells inversely correlate with overall patient survival, together with distant metastases and IFN-γ production [86].

Multiple myeloma presents another interesting target. Upon treatment with therapeutic agents, cancer cells increased the expression of CD226 and NKG2D ligands, making them more susceptible to NK-mediated killing[87]. One mouse model showed that together with Ly49H, CD226 was crucial in the formation of NK cells memory[88].

Regarding CD96, the modulation of its pathway enhanced anti-tumor responses in mice[41]. NK cells from acute myeloid leukemia patients presented a reduced expression of CD226 compared with healthy volunteers, while no differences were observed in the expression of CD96. There was an increase in NK and T cells lacking CD226 and co-expressing TIGIT and CD96. Low percentages of $CD226^{-}TIGIT^{+}CD96^{+}$ NK cells were associated with a better survival of these patients [89].

1.3.5. Roles of CD96, CD226 and TIGIT in central nervous system diseases

Normal aging is accompanied by increasing systemic inflammation. In fact, the increased basal inflammatory levels in the periphery that develop with advanced aging are known as 'inflammaging', which suggests that the immune system participates in the aging process of all

organ systems [90,91]. Aging drives cognitive and regenerative impairments in the adult brain that cause increased susceptibility to neurological diseases, such as Parkinson's Disease (PD) [92,93].

NK cells have been suggested to be key players in immune surveillance of the aged brain. For example, NK cells accumulation was discovered to contribute to the decline of neurogenesis and cognition during normal brain aging, a phenomena that was reversed by NK cell depletion [94]. Naturally, receptors such as CD96, TIGIT and CD226, expressed at the surface of these cells, have an essential role in controlling its activity. There are several studies confirming this hypothesis.

In multiple sclerosis models, NK cells have been shown to kill myelin-specific T cells via CD226[95], while TIGIT was found to be nearly absent in these patients[78]. However, TIGIT seemed to be significantly upregulated on glioblastoma-infiltrating T cells, so was CD226[78]. Furthermore, in a murine glioblastoma model, treatment using anti-TIGIT and anti-PD-1 dual therapy was showed to significantly improve survival when compared to control and monotherapy groups, suggesting TIGIT pathway as a valuable therapeutic target. This effect was correlated with both increased effector T cell function and downregulation of suppressive Tregs and tumor-infiltrating dendritic cells[96]. On another study, the dual combination PD-L1/TIGIT or triple PD-L1/TIGIT/TIM-3 blockade with monoclonal antibodies resulted in increases in intracellular cytokine expression in $CD8^+$ T cells after virus stimulation [97].It was even demonstrated that anti-TIGIT and anti-PD-1 combination therapy confers immunologic memory in long-term survivors[96]. TIGIT was also studied as a potential biomarker to predict multiple sclerosis outcome. However, even though patients had significantly lower TIGIT levels, it was the expression levels of LAG-3 and TIM-3 in the peripheral blood at diagnosis that could predict the disease outcome[98].

TIGIT is even expressed on Tim-1^+B cells, being positively regulated by Tim-1 (a phosphatidylserine receptor expressed on B cells) through IL-10 production. *Xiao et al. (2020)* demonstrated that mice with B cell specific Tim-1 deletion develop spontaneous paralysis with inflammation in the Central Nervous System (CNS), which also occurs in mice with B cell specific TIGIT deletion. Therefore, Tim-1 signaling-dependent TIGIT expression on B cells is essential for maintain CNS-specific tolerance. In fact, B cells from $TIGIT^{-/-}$ mice produced less IL-10, indicating that B cells might require TIGIT for optimal IL-10 production[99].

Also, in human T-cell lymphotropic virus type 1 (HTLV-1) infected animals, the expression of TIGIT is altered when comparing to control groups. It was higher in the peripheral organs and the brain of the infected groups comparing to control, while the expression in the spinal cord was elevated almost 2.5 fold in the infected animals compared to control.[100]

Among the genetic risk factors, CD226 Gly307Ser (rs763361) polymorphism has been identified to predispose to type 1 diabetes[101], rheumatoid arthritis[102] and multiple sclerosis[103,104], among other autoimmune diseases[105]. The exon-7 variant (rs763361) in the C-terminal domain can affect the signaling function in T cells, which may affect the CD226 molecule expression. This can lead to activation of T and NK cells that subsequently leads to different phenotypes in inflammatory autoimmune diseases[105].

In fact, CD226 promotes self-reactive $CD4^+$T cell activation in the autoimmune response[106,107]. For example, previous studies have already demonstrated that mice treated with anti-CD226 antibody *in vivo* significantly decreased the susceptibility to autoimmune encephalomyelitis (EAE)[108], caused by the breakdown of self-tolerance and leading to myelin reactive $CD4^+$T cell infiltration of the CNS to mediate neuronal inflammation[109,110]. *Wang et al. (2019)* demonstrated the importance of CD226 in attenuating Treg suppressive capacity associated with the downregulation of CTLA-4 and TIGIT expression levels. Actually, $CD226^{-/-}$ mice were less susceptible to EAE and there were higher levels of Tregs infiltration in the $CD226^{-/-}$ EAE mouse CNS compared with $CD226^{+/+}$ [111]. $CD226^{-/-}$ Tregs are highly proliferative and suppressive *in vivo* and *in vitro*[112] and the loss of CD226 can interfere with the Akt and Erk pathway[112], that had already been associated with the regulation of Tregs. Although most of the previous studies primarily focus on the role of CD226 on the immune system, it has also become appreciated that CD226 might be linked to neuronal functions. For example, CD226 interestingly seems to modulate cognition and anxiety in mice. Indeed, *Fang et al. (2017)* concluded that this $CD226^{-/-}$ mice displayed increased spatial learning and memory than wild-type controls[113]. Additionally, the genetic deletion of CD226 resulted in decreased anxiety-like behaviors, a condition that is often associated with reduced learning and memory[113]. CD226 may also play an important role in the synaptogenesis since it has been demonstrated to be different expression patterns of this receptor in the brain during development and adulthood. According to *Zhang et.al (2009)*, during postnatal development, CD226 could not be detected at its adult locations until postnatal day 12; however, it was temporally expressed in the somata of neighboring or distant nuclei associated with its adult location[114].

1.3.5.1. NK cell's role in Parkinson's Disease

The neuropathological hallmarks of PD include dopaminergic cell loss in the substantia nigra, which causes striatal dopaminergic deficiency, and α-synuclein (α-syn) accumulation in intraneuronal inclusions[115]. Multiple lines of evidence indicate that immune system dysfunction

has a role in PD [116]. Indeed, the number of NK cells is increased in the blood of PD patients and their activity is associated with disease severity[117]. Furthermore, NK cells are present in the human post-mortem PD SN and PD mouse brain[118]. *Earls et al. (2020)* also showed that human NK cells can internalize and degrade α-syn aggregates via the endosomal/ lysosomal pathway. These authors further demonstrated that extracellular α-syn aggregates attenuate NK cells cytotoxicity in a dose-dependent manner and decrease IFN-γ production, although monomeric α-syn alone had no effect on NK cell cytotoxicity. *In vivo* systemic depletion of NK cells in a preclinical mouse PD model resulted in exacerbated motor deficits and increased phosphorylated α-syn deposits, suggesting a protective role of NK cells in Lewis Bodies-related neurodegenerative diseases[118]. Furthermore, *Niwa et al. (2012)* indicated that the percentages of $CD16^+$ and $CD56^+$ cells were significantly higher in patients with PD, concluding that the percentage of NK cells significantly increases[119]. Intriguingly, there is null information regarding the importance of CD96, CD226 and TIGIT receptors in PD.

1.3.6. Dopamine and dopamine receptors in NK cells

DA (3-hydroxytyramine) is a catecholamine neurotransmitter which is well distributed throughout the human body, being present in the human brain (primarily concentrated in the basal ganglia of both humans and rodents)[120], as well as in peripheral tissues and body fluids [121,122].

DA can be quantified in blood and cerebral spinal fluid, although it is difficult to determine their origin because they are produced by CNS and certain peripheral organs, such as kidney and gut [123–125].

DA can influence numerous brain functions, including movement control, emotion, cognition, and neuroendocrine interactions. Peripheral DA functions include metabolic and cardiovascular among other functions[126]. Importantly, the immunomodulatory activities of DA were first proposed in the 1980's and 1990's, when several studies suggested immune cells contained components of the dopaminergic system, including synthesis machinery and DA transporters [127–130]. Recent studies have confirmed the immunoregulatory functions of DA and its importance in neuroimmune communication, exemplified by the DA-induced changes in the functions of lymphocytes, macrophages, neutrophils and monocytes, namely regulating the secretion of inflammatory cytokines and chemokines [131,132].

DA is, therefore, able to modulate adaptive and immune responses through five different DA receptors (DARs) belonging to the G protein-coupled receptor family, which are grouped in

two families: D1-like receptors - D1R and D5R - and the D2-like receptors - D2R, D3R and D4R. Each receptor has a different affinity for DA [133].

The D1-like receptors couple to Gαs/olf, activate adenylate cyclase and stimulate cyclic adenosine monophosphate (cAMP) production, while D2-like DAR couple to Gαi/o and inhibits adenylate cyclase which, in turn, inhibits cAMP production [134]. Modulation of cAMP synthesis by DARs results in the regulation of PKA. One possible PKA-independent but cAMP-dependent mechanism by the D1 receptor is activation of the Rap GTPase by the cyclic AMP-activated guanine nucleotide-exchange factor Epac[135] . In NK cells, cAMP levels regulate target cell adherence and cytotoxic function, since both repression and induction of cAMP inhibit perforin-mediated and target cell lysis [136]. In fact, prolonged increase of the intracellular cAMP concentration leads to inhibition of NK cell-mediated cytotoxicity [137]. In addition to regulating adenylyl cyclase activity, DA receptors may also couple to $G\alpha_q$ protein and modulate phospholipase C[123]**.** It was demonstrated that exposure of human NK cells to lysis sensitive tumor cells activated PKC[138].

These DA receptors also elicit Gβγ responses and crosstalk to other pathways such as MAPK-MEK–ERK[123]**.**

DA has been shown to interact with NK cells mainly through D1-like receptors [139], being the D5 the most expressed receptor. In fact, D5 was found to be the only D1-like receptor subtype expressed on human leukocytes [140]. For example, activation of D1-like receptors with the selective D1 agonist SFK-38393 enhanced NK cell cytotoxicity. However, D2R may also play a role in NK cells. In fact, activation of D2-like receptors with the selective D2 agonist quinpirole attenuated NK cells cytotoxic functions[133].

Some in vivo stress models also suggested an effect of DA signaling on NK cell function, since restrained stress in mice resulted in impairment of NK cell cytotoxicity[141]. Nonetheless, the repertoire of DA actions in NK cells is scarcely known.

2. Objectives

Objectives

The main objective of the present study was to understand the impact of dopaminergic agents on TIGIT, CD96 and CD226 expression in NK cells under a proinflammatory stimulus. We will also characterize the impact of DA on NK degranulation and IFN-γ production.

3. Materials & Methods

3.1. Study population and samples

For this study, a total of 5 peripheral blood samples from HD were collected in leukocyte depletion bags provided by Instituto Português do Sangue e da Transplantação (IPST Coimbra). Of the 5 individuals, 1 was a female and 4 were males. The ages varied between 26 and 49 years old.

This study was previously approved by the Ethics Committee of Faculdade de Medicina da Universidade de Coimbra (CE_Proc. CE-132/2020).

3.2. Enrichment of peripheral blood mononuclear cells

PBMCs were extracted from the samples of HD by density gradient centrifugation that allows the different cell types that are present in peripheral blood to separate in layers, according to their density.

In this method we used Lymphoprep™ solution (StemCell®, Vancouver, Canada). The ratio used was 1:4 (One part of Lymphoprep and 4 parts of diluted blood). The peripheral blood was diluted (1:1) with phosphate buffered saline (PBS) 1x (Corning®, New York, USA), and then added to a 15 mL Falcon® that contained 3 mL of Lymphoprep™. The 15 mL Falcon® was then centrifuged at 400 g for 40 minutes at room temperature (RT). The PBMCs layer was collected and washed with PBS/fetal bovine serum (FBS) 2%. After this procedure, the pellet was added to a volume of 10 mL of RBC Lysis Buffer, after which the solution was immediately centrifuged at 100 g for 10 minutes at room temperature (RT). Finally, cells were washed again with PBS/FBS 2% and were then resuspended in 10 mL of RPMI 1640/FBS 10%.

3.3. Cell counting

PBMCs were counted using a DxH500 (Beckman Coulter, Pasadena, California, USA). This analyzer uses 12 µL of each sample to count and distinguish cell populations, by detecting and measuring changes in the electrical resistance when the cells pass through a small channel.

3.4. Cell culture

Human erythroleukemia K562 cell line, derived from a 53-year-old female with chronic myeloid leukemia in blast crisis and established in vitro, was cultured in RPMI 1640 Medium (Gibco, Waltham, Massachusetts, USA) supplemented with 10% FBS (Gibco, Waltham, Massachusetts, USA) and 1% Antibiotic-Antimycotic (Gibco, Waltham, Massachusetts, USA) and placed in suspension cultures at 37ºC, in a controlled atmosphere with 5% carbon dioxide and about 95% humidity. This cell line was used in cytotoxic assays.

3.5. Cell viability

Trypan Blue was used to assess cell viability. This method is based on the principle that viable (live) cells have its plasmatic membrane intact and, therefore, do not take up trypan blue, whereas non-viable (dead) cells become stained [142].

In this method, 10 µL of the cell suspension were added to 10 µL of Trypan Blue (Sigma-Aldrich®, St. Louis, Missouri, USA). 10 µL of this solution was then transferred to a Neubauer chamber and counted in a microscope.

PBMCs viability was assessed by flow cytometry using NK test, a reagent kit that allows the quantitative determination of the cytotoxic activity of human NK cells. It contains cryopreserved pre-stained K562 target cells, complete medium and a DNA-staining reagent. K562 target cells are labelled with a lipophilic green, fluorescent membrane dye to discriminate effector and target cells. After the incubation period in the cytotoxic assay, killed target cells are identified by a DNA-stain, which penetrates the dead cells and specifically stains their nuclei, allowing the determination of the percentage of target cells killed by effector NK cells.

The volume of DNA-staining reagent (10µL) of NK test was chosen through a dose-response curve (10, 25 and 50µL), from which we chose the 10µL volume for our experiment.

3.6. *In vitro* manipulation

3.6.1. Cell activation: incubation with rIL-2

PBMCs were activated by adding 200 IU/mL of human recombinant IL-2 (rIL-2)[139] during 48 hours-incubation, in order to mimic the pro-inflammatory conditions when in a disease state.

3.6.2. Pharmacological manipulation with dopaminergic agents

PBMCs were manipulated with DA (Sigma-Aldrich®, St. Louis, Missouri, USA), fenoldopam (D1R/D5R agonist; Cayman Chemical Company®, Ann Harbor, Michigan, USA) and with SCH-23390 hydrochloride (D1R/D5R antagonist; Tocris Bioscience™, Avonmouth, Bristol, UK). All dopaminergic agents were used at a concentration of 1µM.

The impact of DA on NK viability was assessed using a concentration-response curve (10^{-3}, 10^{-6}, 10^{-9} and 10^{-12}M) , and the concentration of DAR agonist and antagonist were defined according to the literature[133,139] and previous knowledge from our research group.

DA (1µM; nontoxic concentration) and fenoldopam (1µM) were added 18 hours before the end of the incubation, while SCH-23390 (1µM) was added 30 minutes before DA and fenoldopam.

3.7. Degranulation and IFN-γ release assays

PBMCs and K562 cells were counted and resuspended in RPMI 1640 Medium, GlutaMAX™ Supplement (Gibco™) supplemented with 10% FBS (Gibco™) and 1% Antibiotic-Antimycotic (Gibco™) and then transferred to a culture plate.

Then, isolated PBMCs, at a concentration of $1x10^6$cells/mL, were co-cultured with the target cell line K562 (Effector: Target (E:T) ratio of 25:1) for 4 hours on a 48-well plate. The degranulation and IFN-γ release were analyzed by flow cytometry.

A volume of PE-Cy7-conjugated CD107a antibody (clone H4A3; BD Pharmingen, San Jose, California, USA) and Brefeldin A (Sigma-Aldrich®, St. Louis, Missouri, USA), at a final concentration of 5 ug/mL, were also added to each well 4 hours before the end of the incubation. At the end of this timepoint, cells were harvested and stained for flow cytometry.

3.8. Flow cytometry

3.8.1. Extracellular and intracellular staining

After the 48h incubation, PBMCs were homogenized and transferred to cytometry tubes. The tubes were centrifuged, and the pellet was resuspended in 100 µL of PBS1x.

For NK identification, CD96, CD226 and TIGI expression the following antibodies for extracellular markers were added to the cells PerCpCy5.5-conjugated anti-CD56 (Clone HCD56), V500-conjugated anti-CD3 (Clone UCHT1) and FITC-conjugated anti-CD226 (Clone TX25) were purchased from BD Bioscience® (San Jose, California, USA); APC-conjugated anti-TIGIT (Clone 741182) was purchased from R&D® (Minneapolis, Minnesota, USA), while PE-conjugated anti-CD96 (Clone NK92.39) is from Bio Legend® (San Diego, California, USA). After the addition of these antibodies, the PBMCs were incubated for 15 minutes, at RT in the dark. 100 µL of Fix and Perm A® (BD Biosciences, San Jose, CA, USA) solution was then added, followed by a 10-minute incubation. Thereafter, 2mL of PBS1x were added, after which the cells were centrifuged at 1500 rpm for 5 minutes, the supernatant discarded and 100µL of Fix and Perm B® solution was added.

The intracellular antibody V450 conjugated anti-IFN-γ (Clone B27), purchased from BD Bioscience® (San Jose, California, USA), was then added, followed by a 20-minute incubation in the dark, at RT. The cells were washed with 2mL of PBS1x and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 200 µL of PBS and analyzed by flow cytometry.

3.8.2. Flow cytometry analysis

All samples were acquired in a FACSCanto II™ (BD Biosciences, San Jose, California, USA) with FACSDiva™ software version. Data from the FACSCanto II was analyzed using FlowJo™ 10.7.1.

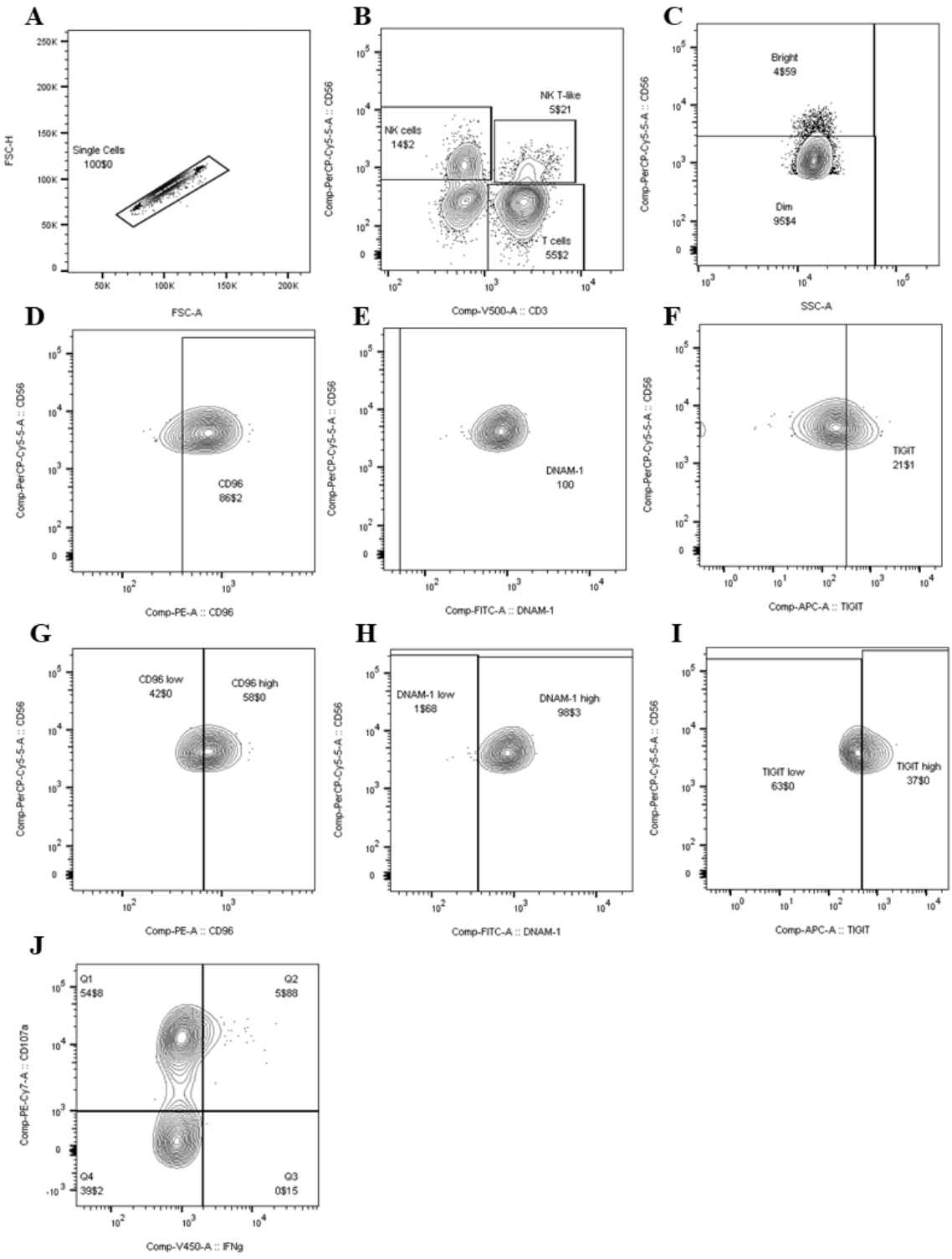

Figure 2 | Gating strategy. PBMCs from HD were isolated and analyzed by flow cytometry. Firstly, PBMCs were selected using Forward Scatter-A (FSC-A) vs. Side Scatter-A (SSC-A) plot, followed by FSC-A vs. FSC-H to isolate single cells. NK cells were identified according to their surface receptors: $CD56^{+}CD3^{-}$. NK cells were further divided into $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells, that were sorted by a CD56 vs. SSC-A plot and identified due to the differences in CD56 expression between the two cell types. Within these cells, CD96 surface expression was acquired through CD56 vs. CD96 plot. The same was performed for CD226 and TIGIT. Data were analyzed using FlowJo software, and population frequencies expressed as percent of cell populations.

Regarding flow cytometry analysis, PBMCs were firstly plotted according to their size and complexity on a Forward Scatter-A (FSC-A) vs. Side Scatter-A (SSC-A) plot, followed by FSC-A vs FSC-H plot to isolate single cells **(Figure 2A)**. NK cells were identified using a gating strategy in the presence of CD56 and the absence of CD3 (detected by distinct fluorochromes conjugated with a respective monoclonal antibody) **(Figure 2B)**. Then, NK cells were divided into $CD56^{bright}$ and $CD56^{dim}$, that were sorted with a CD56 vs. SSC-A plot and identified according to the differences in CD56 expression between the two cell types **(Figure 2C)**. Within these cell types, CD96 surface expression was acquired through a CD56 vs. CD96 plot, and the same was performed for CD226 and TIGIT surface expression **(Figure 2D-F)**. The expression of CD96, CD226 and TIGIT was studied in NK cells, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells **(Figure 2G-I)**, so was degranulation and IFN-γ levels **(Figure 2J)**. The results were presented as percentage of expression. Our results suggest that $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells represent approximately 5% and 95% of NK cells, respectively **(Figure 2C).**

Figure 3 illustrates the experimental workflow.

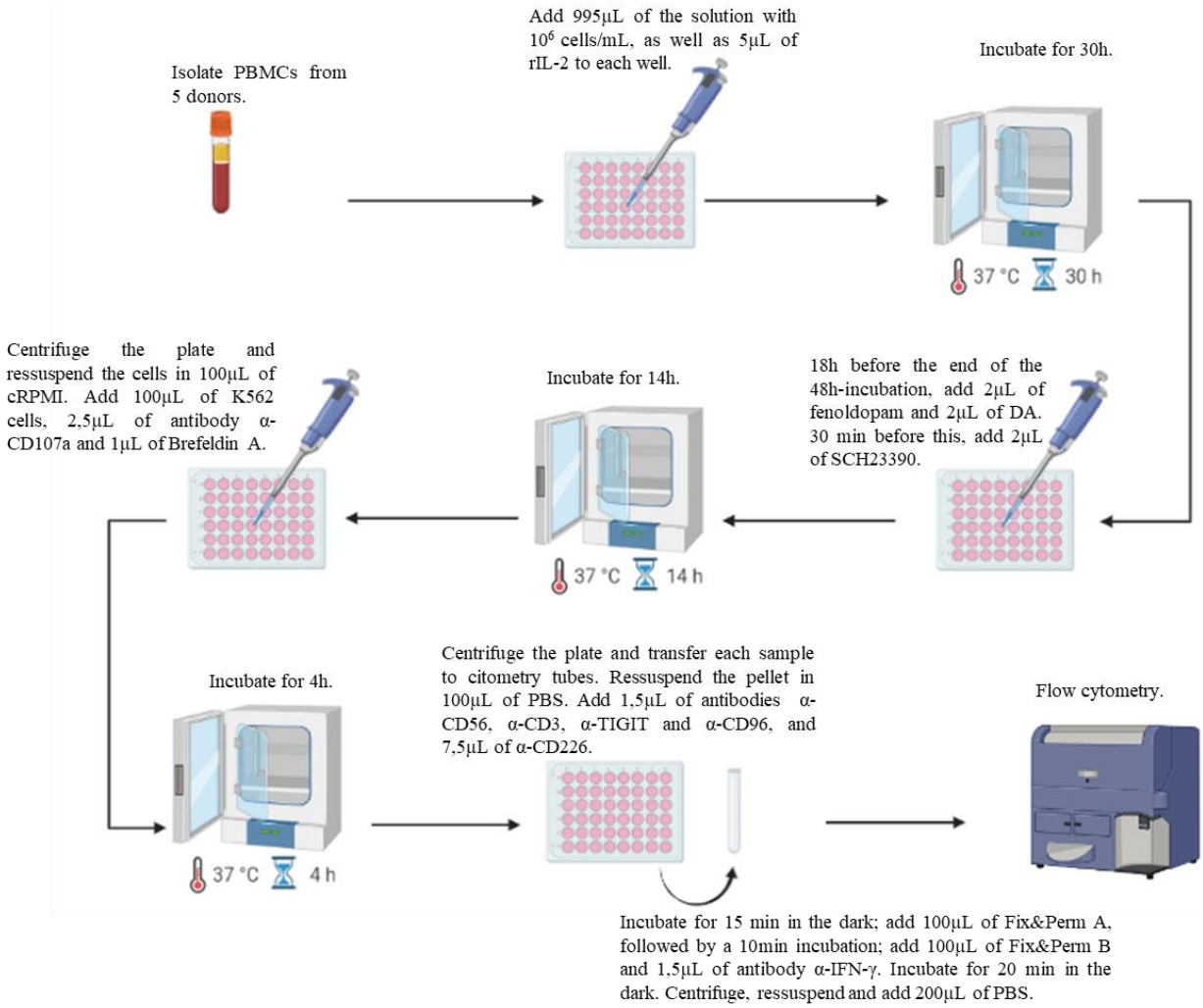

Figure 3 | Schematic representation of the experiment.

3.9. Statistical Analysis

Results are expressed as Mean ± Standard Deviation (SD). Statistical analyses were performed using GraphPad Prism™, version 8 (GraphPad Software, San Diego, California, USA). The significance of the differences between the means was calculated using a Mann-Whitney non-parametric t test or Kruskal–Wallis test when comparing 2 or more groups with not normal distribution. In the presence of normal distribution, we used t-test or one-way analysis of variance (ANOVA), followed by a Turkey's multiple comparisons test or when comparing 2 or

more groups. Differences were considered statistically significant at a p value of <0.05 and are annotated as follows: *, p<0.05; **, p<0,01; ***, p<0.001; ****, p<0.0001.

Values representing specific degranulation and specific production of IFN-γ were obtained by subtracting spontaneous degranulation and IFN-γ to the corresponding values when in the presence of K562 target cells.

4. Results

4.1. Dopaminergic agents do not change rIL-2 induced-degranulation

The first task of our research was to investigate the influence of dopaminergic agents on NK cells, CD56^{bright} NK cells and CD56^{dim} NK cells incubated with rIL-2 regarding degranulation and IFN-γ release.

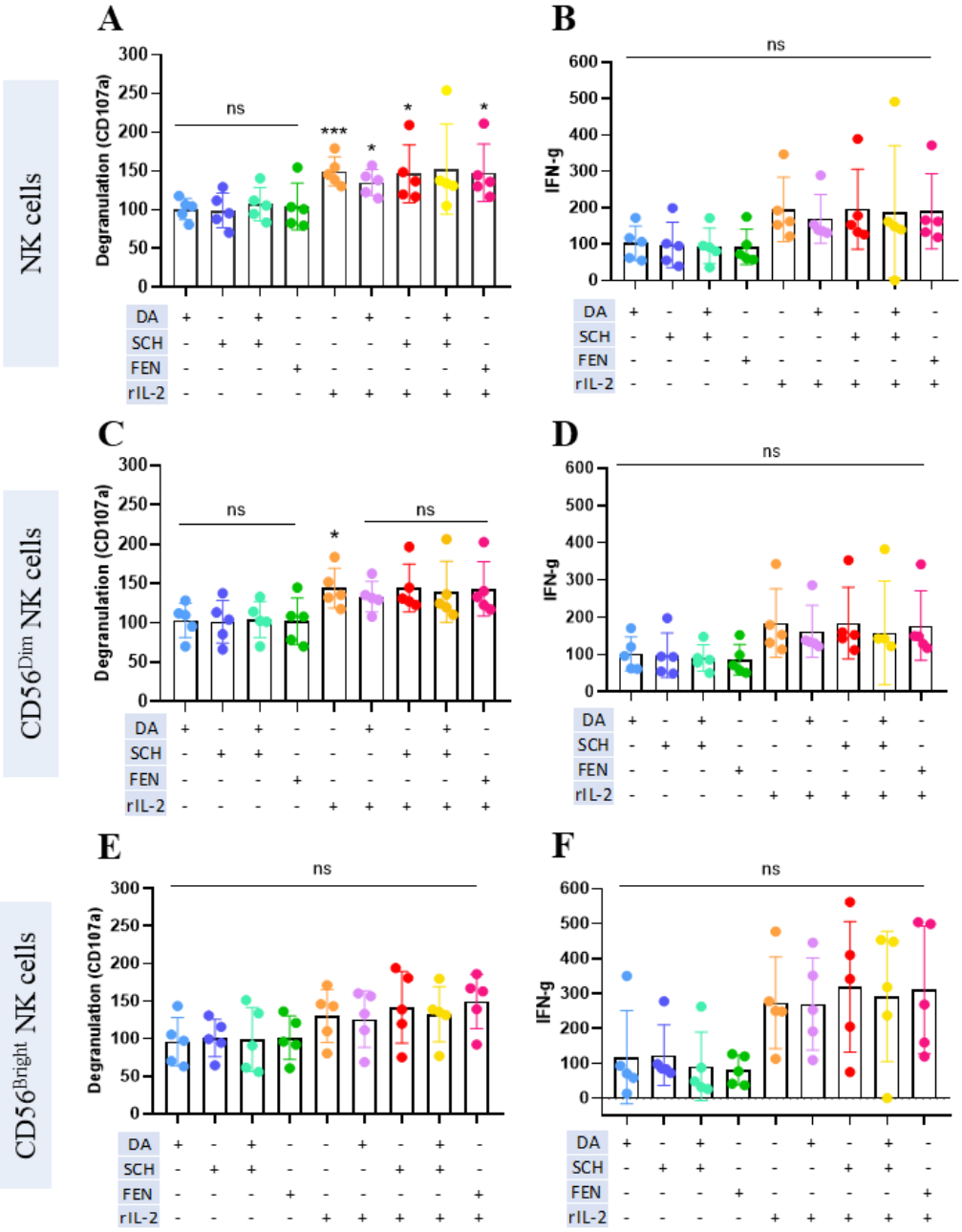

Figure 4 | Specific degranulation and production of IFN-γ regarding NK cells (A, B, respectively), CD56^{dim} NK cells (C, D, respectively) and CD56^{bright} NK cells (E, F, respectively). PBMCs from HD were isolated, submitted to different treatments and analyzed by flow cytometry. Primarily, PBMCs were selected using Forward Scatter-A (FSC-A) vs. Side Scatter-A (SSC-A) plot, followed by FSC-A vs. FSC-H to isolate single cells. NK cells were identified using a CD56^{+}CD3^{-} plot. CD96 surface expression was acquired through CD56 vs. CD96 plot, and the same was performed for CD226 and TIGIT. Data is presented as percentage of the untreated condition, in the absence of K562 cell line. **NK cells**) Regarding degranulation, Kruskal-Wallis test was used for SCH+DA+rIL-2 condition, while One-Way ANOVA was used for the remaining conditions. Regarding production of IFN-γ, Kruskal-Wallis test was used for DA, SCH and FEN conditions. One-way ANOVA was used for SCH+DA. **CD56^{dim} NK cells)** For degranulation, One-Way ANOVA was used for all conditions. Regarding production of IFN-γ, Kruskal-Wallis test was performed for DA, SCH and FEN conditions, while One-Way ANOVA was performed for SCH+DA condition. **CD56^{bright} NK cells)** For degranulation, One-way ANOVA was used for all conditions. Regarding production of IFN-γ, Kruskal-Wallis test was performed for SCH+DA, SCH and DA conditions, while One-Way ANOVA was used for FEN condition. *, p<0,05 vs. untreated; ***, p<0,001 vs. untreated. NS, not significant (p>0,05). All values are Mean with SD (Standard Deviation) and are % of unstimulated cells; n=5.

To study the impact of dopaminergic agents in activated NK cells, PBMCs were cultured with rIL-2 for 48h and then incubated with DA (1 μM) and Fen (1 μM) for the last 18h of the incubation and SCH-23390 (1 μM), 30 min before dopamine.

Our results demonstrated that, after a 48h incubation with rIL-2, NK and CD56^{dim} NK cells significantly increased their degranulation levels (**Figure 4A, C**). However, the apparent increase in degranulation seen in CD56^{bright} NK cells did not reach significance (**Figure 4E**). Moreover, the three type of cells apparently increased production of IFN-γ which did not reach statistical significance (**Figure 4B, D, F**).

None of the three dopaminergic agents seemed to change degranulation and IFN-γ levels from NK (**Figure 4A, B**), CD56^{dim} NK cells (**Figure 4C, D**) and CD56^{bright} NK (**Figure 4E, F**) stimulated with rIL-2. Furthermore, these dopaminergic agents did not seem to change basal degranulation and IFN-γ levels from NK cells (**Figure 4B, D, F**).

4.2. CD96, CD226 and TIGIT expression on NK cells is not changed by rIL-2 and by dopaminergic agents

CD96, CD226 and TIGIT are essential regulators of immune responses by modulating immune cells functions. To better define the role of dopaminergic agents on these receptors on

activated NK cells, we evaluated the percentage of rIL-2 stimulated NK cells, $CD56^{bright}$ and $CD56^{dim}$ NK cells expressing CD96, CD226 and TIGIT.

Even considering a certain degree of variability among all donors tested, our results show that neither rIL-2 nor dopaminergic agents significantly influence the percentage of NK cells expressing CD96, CD226 and TIGIT receptors **(Figure 5A)**. However, in all conditions with rIL-2, there is a tendency to increase the percentage of NK cells expressing TIGIT, when compared to untreated cells (~35% vs 28%). Additionally, there is a higher percentage of $CD56^{bright}$ NK cells expressing CD96 (~70%) than $CD56^{dim}$ NK cells (~20%) **(Figure 5B, C)**. On the other hand, the percentage of $CD56^{bright}$ NK cells expressing TIGI is smaller than $CD56^{dim}$ NK cells (~12% vs. 30%; **Figure 5B, C**).

Regarding CD226, almost every cell expressed this receptor and there were no significant differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells (**Figure 5A-C**).

The percentage of cells expressing these receptors was assessed in the absence of K562 cell line.

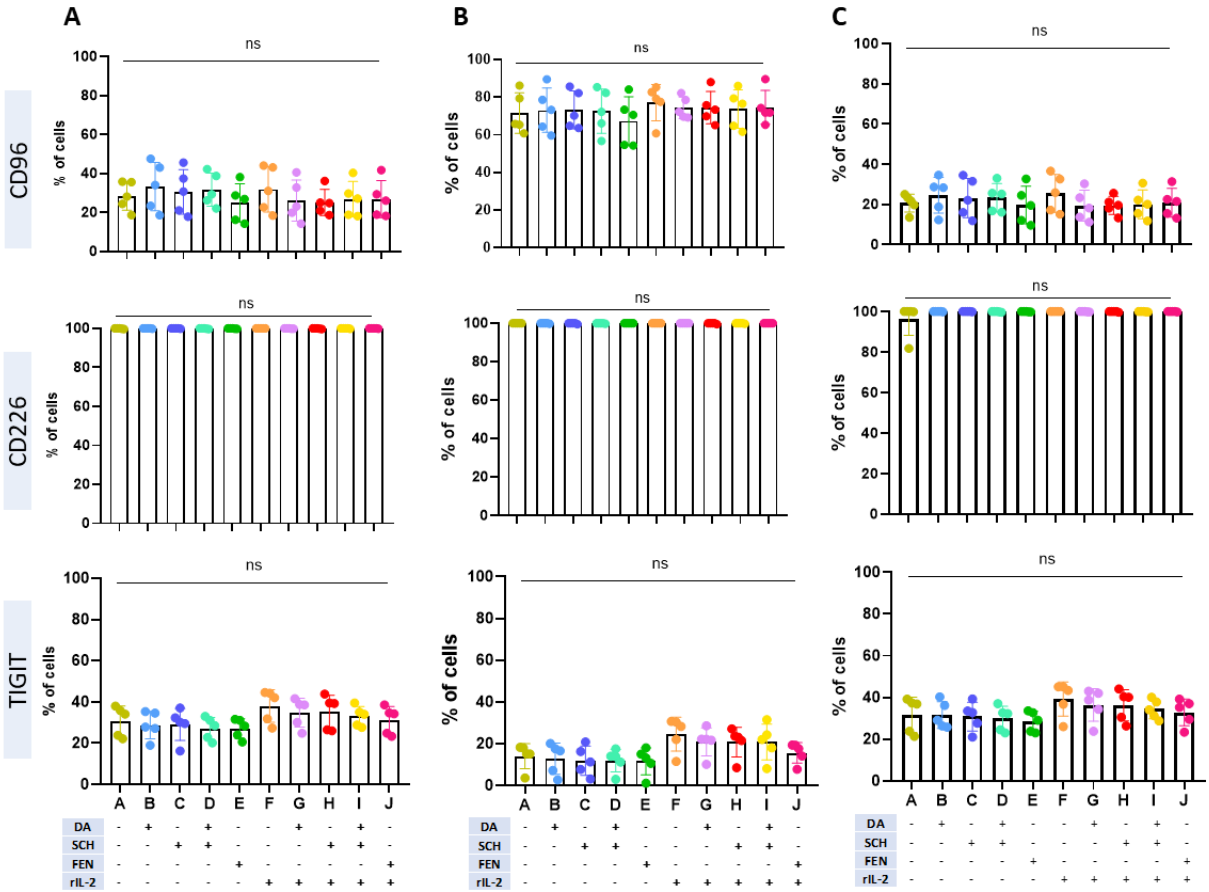

Figure 5 | Percentage of NK cells (A), CD56^{bright} (B) and CD56^{dim} NK cells (C) expressing CD96, CD226 and TIGIT. PBMCs from HD were isolated, submitted to different treatments and analyzed by flow cytometry. Primarily, PBMCs were selected using Forward Scatter-A (FSC-A) vs. Side Scatter-A (SSC-A) plot, followed by FSC-A vs. FSC-H to isolate single cells. NK cells were identified using a CD3^{-}CD56^{+} plot. CD96 surface expression was acquired through CD56 vs. CD96 plot, and the same was performed for CD226 and TIGIT. The results are expressed as percentage of expression in NK cells **(A)**, CD56^{bright} NK cells **(B)** and CD56^{dim} NK cells **(C)**. These parameters were assessed in the absence of K562 cell line. On NK cells and CD56dim NK cells, One-Way ANOVA was used for CD96 and TIGIT, while Kruskal-Wallis test was used for CD226. Regarding CD5bright NK cells, One-Way ANOVA was used for CD96, while Kruskal-Wallis test was used for CD226 and TIGIT. NS, not significant (p>0,05). All values are Mean with SD (Standard Deviation); n=5.

Regarding the percentage of cells expressing each population of receptors, it is possible to assess that CD96^{-}TIGIT^{-}CD226^{+} is the predominant population of receptors expressed on both NK cells and CD56^{dim} NK cells. Moreover, the presence of K562 cell line significantly increased

NK cells expression of this combination of receptors (**Figure 6**) (p value differs for each condition: DA ($p<0,0001$); DA+SCH ($p<0,05$); SCH ($p<0,0005$); FEN ($p<0,005$); rIL-2 ($p<0,0001$); DA+rIL-2 ($p<0,0005$); SCH+rIL-2 ($p<0,0001$); FEN+rIL-2 ($p<0,001$)). Interestingly, the percentage of NK cells expressing the population $CD96^{+}TIGIT^{+}CD226^{+}$ significantly decreased in the presence of K562 cells (in the presence of rIL-2, $p<0,0001$; however, p values varied between conditions). There were no significant differences considering cells expressing the other two populations of receptors. $CD56^{dim}$ NK cells have similar results, as expected. Also, $CD56^{bright}$ NK cells express the same pattern regarding the population $CD96^{-}TIGIT^{-}CD226^{+}$ ($p<0,0001$ for almost every condition). However, there were significantly less cells of this subset expressing $CD96^{+}TIGIT^{-}CD226^{+}$ in the presence of the target cells ($p<0,0001$ for almost every condition).

Comparing the different populations in each condition using a Kruskal-Wallis test, we found that, in the absence of K562 cell line, the only significant differences registered were between $CD56^{bright}$ NK cells expressing $CD96^{-}TIGIT^{+}CD226^{+}$ and the ones expressing $CD96^{+}TIGIT^{-}CD226^{+}$ ($p<0,005$) and it occurred in every condition. However, in the presence of K562 cell line, the significant differences are now between $CD56^{bright}$ NK cells expressing $CD96^{-}TIGIT^{-}CD226^{+}$ vs $CD96^{+}TIGIT^{+}CD226^{+}$ and $CD96^{-}TIGIT^{-}CD226^{+}$ vs $CD96^{-}TIGIT^{+}CD226^{+}$ ($p<0,01$). These were visible in every condition. Regarding NK cells and $CD56^{dim}$ NK cells, the ones that express $CD96^{-}TIGIT^{-}CD226^{+}$ are significantly different than the cells expressing the other three populations, and this applies for both in the presence ($p<0,0001$ for all conditions) and absence (NK cells: $p<0,005$ for most conditions; $CD56^{dim}$NK cells: $p<0,0001$) of K562 cell line. For NK cells and $CD56^{dim}$ NK cells, a one-way ANOVA test was performed.

On the other hand, $CD56^{bright}$ NK cells predominantly express $CD96^{+}TIGIT^{-}CD226^{+}$ population. However, the presence of K562 cell line seems to decrease the percentage of cells expressing this combination and increased the $CD96^{-}TIGIT^{-}CD226^{+}$ population in $CD56^{bright}$ NK cells. The combinations including TIGIT seem to be less represented in NK cells, in $CD56^{dim}$ and in $CD56^{bright}$ NK cells. Finally, no pharmacological stimulus seemed to change this pattern.

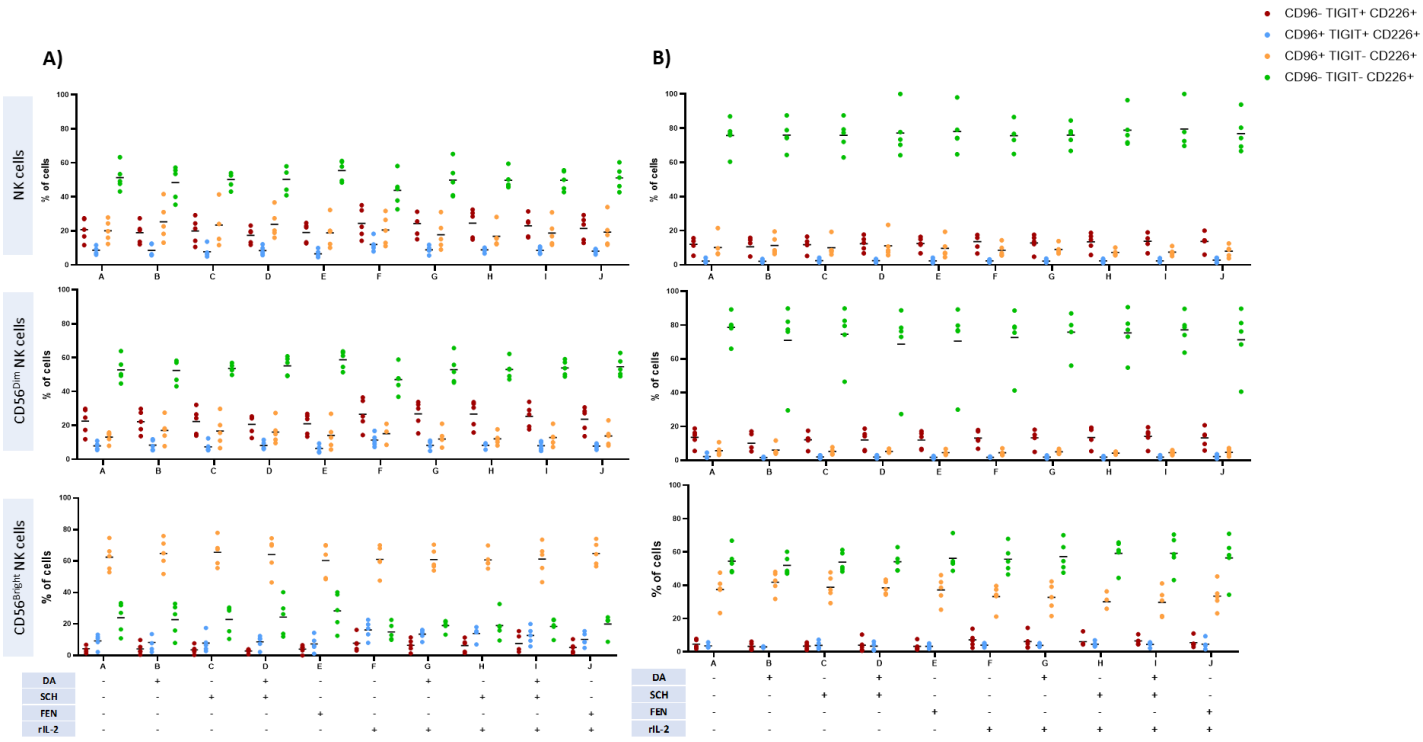

Figure 6 | Percentage of NK cells, CD56^{dim} and CD56^{bright} NK cells expressing each population of receptors. PBMCs from HD were isolated, subjected to different treatments and analyzed by flow cytometry. Primarily, PBMCs were selected using Forward Scatter-A (FSC-A) vs. Side Scatter-A (SSC-A) plot, followed by FSC-A vs. FSC-H to isolate single cells. NK cells were identified using a CD56^{+}CD3^{-} plot. CD96 surface expression was acquired through CD56 vs. CD96 plot, and the same was performed for CD226 and TIGIT. The results are expressed as percentage of NK cells, CD56^{dim} NK cells and CD56^{bright} NK cells expressing each population of receptors, in the absence **(A)** and presence **(B)** of K562 cell line; The statistical analyses performed are described in the text; n=5.

4.3. CD96, CD226 and TIGIT repertoire is determinant on NK cells degranulation and IFN-γ release

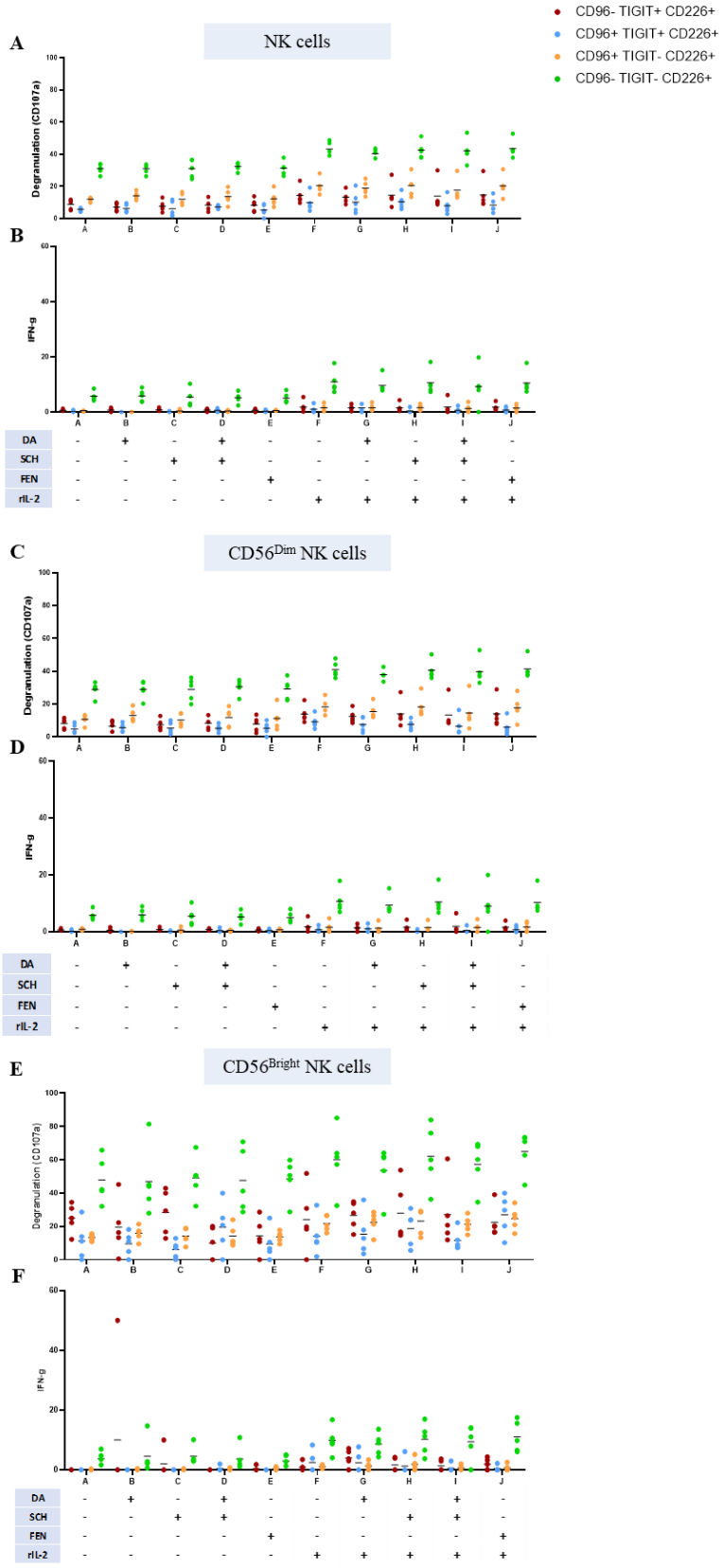
CD96- TIGIT+ CD226+
CD96+ TIGIT+ CD226+
CD96+ TIGIT- CD226+
CD96- TIGIT- CD226+
A
NK cells
Degranulation (CD107a)
B
IFN-g
DA
SCH
FEN
rIL-2
C
CD56Dim NK cells
D
E
CD56Bright NK cells
F

Figure 7 | Specific degranulation and specific production of IFN-γ by NK cells (A, B, respectively), CD56^{dim} NK cells (C, D respectively) and CD56^{bright} NK cells (E, F, respectively) regarding different populations of receptors. PBMCs from HD were isolated, submitted to different treatments and analyzed by flow cytometry. Primarily, PBMCs were selected using Forward Scatter-A (FSC-A) vs. Side Scatter-A (SSC-A) plot, followed by FSC-A vs. FSC-H to isolate single cells. NK cells were identified using a CD56^{+}CD3^{-} plot. CD96 surface expression was acquired through CD56 vs. CD96 plot, and the same was performed for CD226 and TIGIT. The results are expressed as percentage of degranulation and production of IFN-γ from NK cells, CD56^{dim} NK cells and CD56^{bright} NK cells expressing each population of receptors; The statistical analysis performed are described in the text; n=5

After studying the overall expression of these receptors, further analyses were done on whether the expression of these receptors influenced the activity of NK cells, namely its degranulation and production of IFN-γ.

According to **Figure 7**, both degranulation and production of IFN-γ values are higher when cells only express the activator receptor CD226 (CD96^{-}TIGIT^{-}CD226^{+}). This is the case for all studied cells.

On NK cells expressing the population CD96^{-}TIGIT^{-}CD226^{+}, there is a significant increase ($p<0,01$) in the degranulation values when rIL-2 is added, which is also verified in NK cells expressing CD96^{+}TIGIT^{-}CD226^{+} in the presence of rIL-2 + DA and rIL-2 + SCH ($p<0,05$). However, there are no significant differences regarding the production of IFN-γ by NK cells. When comparing the populations to each other through a One-way ANOVA test, we conclude that NK cells expressing the population CD96^{-}TIGIT^{-}CD226^{+} have very significantly higher ($p<0,0001$) degranulation levels than cells expressing the other three combinations of receptors, in every condition of our study. In the presence of DA, cells expressing CD96^{+}TIGIT^{-}CD226^{+} also have significant higher ($p<0,005$) degranulation levels than the cells expressing the populations CD96^{-}TIGIT^{+}CD226^{+} and CD96^{+}TIGIT^{+}CD226^{+}. Regarding the production of IFN-γ, the differences between populations were more subtle. A Kruskal-Wallis test was performed, and there is a significant difference between cells expressing CD96^{+}TIGIT^{+}CD226^{+} and CD96^{-}TIGIT^{-}CD226^{+} ($p<0,05$) in every condition except for rIL-2+SCH+DA and SCH+DA. This difference ($p<0,05$) is also seen between cells expressing CD96^{-}TIGIT^{-}CD226^{+} and CD96^{+}TIGIT^{-}CD226^{+}, in the conditions with DA, SCH, SCH+DA and rIL-2+ DA.

As expected, CD56^{dim} NK cells follow the same patterns as NK cells. Only the ones expressing the population CD96^{-}TIGIT^{-}CD226^{+} have a significant increase ($p<0,05$) in degranulation when comparing the several conditions of our study. This increase is present in

every condition that contains rIL-2. There were no significant differences regarding the release of IFN-γ, and these results were assessed through a Kruskal-Wallis test. The results are also similar to NK cells when comparing the populations to each other. $CD56^{dim}$ NK cells expressing the population $CD96^{-}TIGIT^{-}CD226^{+}$ also have very significantly higher ($p<0,0001$) percentage of degranulation, in every condition of our study. This was assessed through a One-way ANOVA test. Comparing the different populations in each condition using a Kruskal-Wallis test, we also found that $CD56^{dim}$NK cells expressing $CD96^{-}TIGIT^{-}CD226^{+}$ have a significant higher release of IFN-γ than $CD96^{+}TIGIT^{+}CD226^{+}$, in every condition.

Considering $CD56^{bright}$ NK cells, the only significant differences in degranulation levels were regarding cells expressing the population $CD96^{+}TIGIT^{-}CD226^{+}$ in the presence of rIL-2 ($p<0,05$), rIL-2 + DA ($p<0,05$) and rIL-2 + FE ($p<0,01$), when comparing cells expressing the same population in different conditions. There were no significant differences in the release of IFN-γ. This data was assessed through a Kruskal-Wallis test. Then, using a one-way ANOVA test to compare the populations to each other, we confirmed that $CD56^{bright}$ NK cells expressing the population $CD96^{-}TIGIT^{-}CD226^{+}$ also have a significant higher ($p<0,05$ at least) level of degranulation than cells expressing the other three combinations of receptors, in every condition of our study. Regarding the release of IFN-γ the pattern is similar, since cells expressing $CD96^{-}TIGIT^{-}CD226^{+}$ have significant differences ($p<0,05$) in comparison with the other cells expressing the other populations of receptors, in the majority of the conditions.

In conclusion, CD56^{bright} NK cells showed higher values of degranulation in comparison with NK cells and CD56^{dim} NK cells, which presented similar values (**Figure 7**). When considering the release of IFN-γ, all three types of cells presented similar values. This pattern does not seem to be altered by pharmacological manipulations with the four dopaminergic agents.

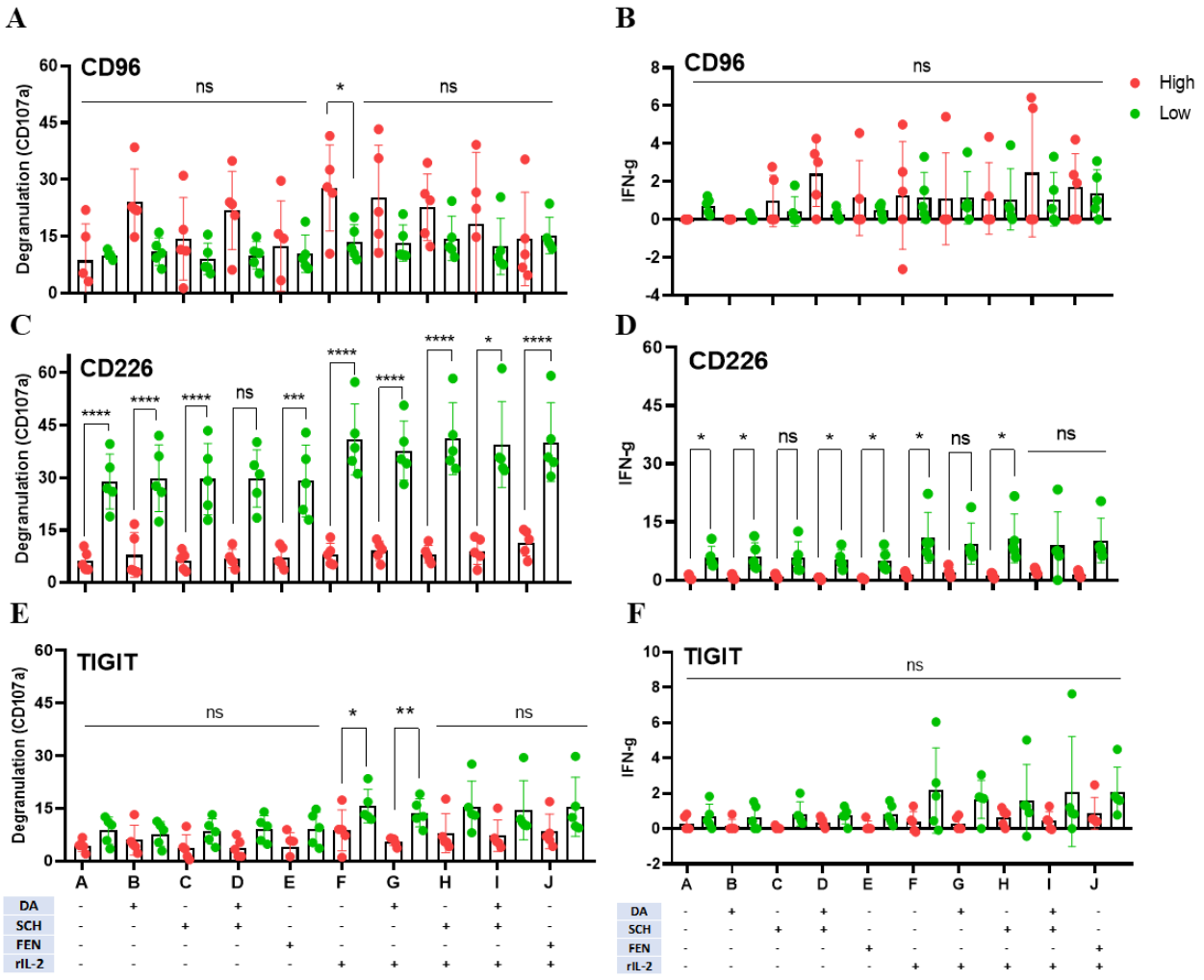

Figure 8 | Specific degranulation and production of IFN-γ of CD56^{dim} NK cells regarding high and low density of CD96, CD226 and TIGIT receptors. A, C, E) Percentage of specific degranulation, in percentage of untreated. CD96 and TIGIT: According to Shapiro Wilk test, not normal distribution. Kruskal-Wallis test, Dunn's multiple comparisons test. CD226: According to Shapiro Wilk test, normal distribution. One-way ANOVA, Sidak's multiple comparison test. **B, D, F)** Percentage of specific production of IFN-γ. CD96, CD226 and TIGIT: According to Shapiro Wilk test, not normal distribution. Kruskal-Wallis test, Dunn's multiple comparison test. *, p<0,05; **, p<0,01; ****, p<0,0001. All values are Mean with SD (Standard Deviation); n=5.

Moreover, CD226 has a measurable impact on degranulation and IFN-γ production of $CD56^{dim}$ NK cells which is significant for most experimental conditions, when compared with CD96 and TIGIT. In fact, we observed that cells with a low CD226 density presented a significantly higher percentage of degranulation and IFN-γ production, in comparison with cells with high CD226 density (**Figure 8C, D**). Additionally, these results were similar for both NK cells and $CD56^{bright}$ NK cells (data not shown).

5. Discussion

The potential targeting of CD96, CD226 and TIGIT receptors for modifying NK cells activity has been suggested by different studies, although many functions of these receptors, mainly CD96, are still being unveiled. The present study focuses on analyzing the effect of rIL-2 and DA on three main parameters: expression of CD96, CD226 and TIGIT on NK cells, NK cell degranulation and IFN-γ production. These receptors were already highly studied in tumors, autoimmune and inflammatory diseases and there are already pharmacological tools, namely antibodies, developed to block these receptors. Therefore, if DA can have an impact in the expression of these receptors, this could help to design disease-modifying therapeutic strategies in PD and other synucleinopathies, which do not exist now.

CD96, CD226 and TIGIT expression on NK cells

The first goal of this study was to make a phenotypic characterization of the expression of CD96, CD226 and TIGIT on NK cells after the 48h incubation with rIL-2 and/or DA. Although the expression of these receptors has already been characterized in NK cells, there is no literature regarding its expression in the presence of DA.

The two major subsets of NK cells are $CD56^{bright}$ and $CD56^{dim}$ NK cells, and $CD56^{dim}$ represent at least 90% of all peripheral blood NK cells[143]. Herein we also confirmed this observation (please see **Figure 4**). The percentage of each receptor varies between NK cells subsets: several studies have demonstrated that CD96 receptor is expressed by most human NK cells [31–33].Our results demonstrated that the percentage of untreated $CD56^{bright}$ NK cells expressing CD96(~70%) is higher than $CD56^{dim}$ NK cells (~20%) (**Figure 5)**. There is no literature available regarding de effect of rIL-2 on NK cells expression of CD96.

A previous study suggested that CD96 is correlated with cytokine production [33]. Thus, the increased percentage of $CD56^{bright}$ NK cells, major cytokine producers[3], expressing CD96 may be a way to regulate its cytokine production.

TIGIT and CD226 are also present at the cell surface of most human NK cells [40,42,58,59]. Almost every cell expressed CD226 and there were no significant differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells. We further show that rIL-2 seems to increase the percentage of cells expressing TIGIT (but not the other receptors), although it does not reach statistical significance. This is important since this receptor mainly influences the cytotoxicity of NK cells [40,63]. Moreover, the percentage of cells expressing CD96, CD226 and TIGIT is also not significantly altered by

dopaminergic agents (**Figure 5**) Importantly, *Hromadnikova et al. (2013)* showed that rIL-2 incubation for 4-5 days increased in a significant manner CD226 expression in NK cells but did not increase the fraction of cells expressing this receptor[144]. Further studies need to be performed including longer time-points (up to 5 days) to establish the impact of rIL-2 on this set of three receptors.

Finally, NK cells seem to follow the pattern of $CD56^{dim}$ NK cells regarding the expression of these 3 receptors. This further confirms that $CD56^{dim}$ NK cells represent most of NK cells.

However, in CNS, the majority of NK cells in cerebrospinal fluid (CSF) are $CD56^{bright}$ NK cells[145]. This may be relevant for neurodegenerative disorders including PD.

We are also demonstrating for the first time that the majority of $CD56^{dim}$ NK cells express the repertoire $CD96^{-}TIGIT^{-}CD226^{+}$ at its surface, and that most $CD56^{bright}$ NK cells express $CD96^{+}TIGIT^{-}CD226^{+}$ population (**Figure 6**). Again, considering that $CD56^{dim}$ represent the majority of all peripheral blood NK cells[143], it is not surprising that their expression pattern of this combination of receptors is similar to total NK cells . Additionally, in the presence of K562 cell line, the percentage of cells with the receptor repertoire $CD96^{-}TIGIT^{-}CD226^{+}$ further increases (**Figure 6**). On the contrary, the percentage of cells with the repertoires $CD96^{+}TIGIT^{-}CD226^{+}$, $CD96^{-}TIGIT^{+}CD226^{+}$ and $CD96^{+}TIGIT^{+}CD226^{+}$, that express at least one of the inhibitor receptors, significantly decreases in the presence of K562 cell line. When exposed to target cells, NK cells, $CD56^{dim}$ NK cells and $CD56^{bright}$ NK cells tend to mainly express the activator receptor CD226. These results strongly suggest that K562 cell primes NK cells to become more reactive and cytotoxic. In fact, previous studies had already demonstrated that the recognition of K562 target cells by activating receptors, such as CD226, stimulates NK cell–mediated cytotoxicity and the secretion of proinflammatory mediators such as IFN-γ [58].

CD96, CD226 and TIGIT repertoire in NK cells' degranulation and IFN-γ production

The relevance of NK cells education through inhibitory and activating receptors has just begun to be elucidated[146,147]. Our results demonstrated that cells that expressed CD226, but not CD96 and TIGIT ($CD226^{+}CD96^{-}TIGIT^{-}$), were the ones with the higher percentage of degranulation and production of IFN-γ (**Figure 7**).

The results regarding the percentage of degranulation and IFN-γ production being higher on cells expressing low CD226 were not according to our expectations and were surprising (**Figure 8**). Being CD226 an activating receptor, we expected to see the opposite effect: cells with a high expression of this activator receptor having a higher percentage of degranulation and IFN-γ production. These results need further confirmation. However, this event might be explained through the downregulation of CD226 after cells having reached the maximum degranulation capacity.

Another possibility is the occurrence of trogocytosis. Cells with a high expression of this activating receptor could have undergone trogocytosis with K562 cell line, decreasing NK cells levels of CD226 after the activation of these cells. When an NK cell stablishes an immune synapse with a target cell, this event is strong enough to allow the transfer of small membrane patches from one cell to another, a process termed trogocytosis [148–152]. Trogocytosis in T and NK cells have been shown to be a metabolically active phenomenon, since it requires signaling in the acceptor cell as well as modulation of the actin cytoskeleton and intracellular Ca^{2+} [153]. This is a very fast process that can occur within 1h or less of co-culturing [154]. Previous experiments have been made to modify the balance between activating and inhibitory signals of NK cells and, consequently, enhance its cytotoxicity against a certain target [155] For example, the chemokine receptor CCR7 has been shown to be transferred from donor cells onto the surface of NK cells via trogocytosis, which stimulated NK cell migration [156,157]. Furthermore, human NK cells can acquire MHC Class I proteins [158] as well as viral receptors [159] from their targets. This transfer is bidirectional, since KIR receptors expressed on NK cells can move onto target cells after cell-to-cell contact [154]. There is no published information regarding trogocytosis between NK cells and K562 cell line. However, this phenomenon could explain the fact that NK cells with low density of CD226 have a higher percentage of degranulation and IFN-γ production.

On the other hand, this can be correlated with other receptors, including CD112 and CD155. CD112 has been identified as a ligand for the activating receptor CD226, mediating cell to cell adhesion by either homophilic interaction with CD112 on a neighboring cell or through heterophilic interaction with nectin 3[38]. This receptor is closely related with tumorigenesis, being overexpressed in different types of cancers such as acute myeloid leukemia, multiple myeloma and epithelial cancers[160–162]. Additionally, *Kearney et al. (2016)* demonstrated that CD112/CD155 expression is required for CD226-dependent killing of acute myeloid leukemia cells, and that the low, or absent, expression of CD112/CD155 on these cells resulted in failure to stimulate optimal NK cell function[163]. Since each nectin contains three Ig-like domains (one V-set and two C-set domains) in their extracellular regions[69], *Liu et al. (2012)* generated a soluble V-set domain of CD112 and demonstrated that it binds to both CD226–expressing cells and the soluble

ectodomain of CD226. This way, they propose that there are two possible models for CD226 binding to CD112: CD226 competes against CD112 dimer formation in a 1:1 (CD226:CD112)-binding mode and CD226 binds nectin-2 dimer in a 1:2 (CD226:CD112)-binding mode[164].

The inhibitory receptor TIGIT also recognizes and interacts with CD112 leading to inhibition of NK cell-mediated cytotoxicity[40,165]. CD155 is recognized by CD226, CD96 and TIGIT receptors [37,46]. It contains an ITIM motif in its cytoplasmic tail, which may lead to the inhibition of various processes. Indeed, it was shown that the CD155 inhibitory signal could facilitate cell detachment from extracellular matrix, leading to cell migration [166], or increase cell proliferation, triggered by growth factors[167]. Unlike mouse cells, human cells express both membranous CD155 and soluble CD155 (sCD155) encoded by splicing isoforms of CD155[168]. The affinity of soluble CD155 (sCD155) for CD226 is greater than that for TIGIT and CD96 on NK cells of both mice and humans[169]. *Okumura et al. (2020)* demonstrated that sCD155 suppressed NK cell activation through the inhibition of CD226-mediated degranulation and immunity activity against lung colonization of B16/BL6 melanoma[169]. Both CD155 and CD112 are widely expressed in a variety of tissue cells, such as nerve cells, epithelial cells, antigen presenting cells, fibroblasts, pathogen infected cells and a variety of tumor cells, as well as on monocytes, DCs, B cells and activated T cells. [38,39,46,47,170,171].

CD112 and CD155 are believed to form homodimers or heterodimers in their functions for cell adhesion, as well as to interact with CD226 to regulate NK cells functions. Interaction with CD226 requires either the homodimerization or engagement of the homodimeric interface of CD112[164]. In fact, it has been shown that CD226 degree of contribution to NK cell-mediated lysis of DCs is correlated with the surface densities of CD112 and CD155[171]. However, to ascertain if these results regarding CD226 are correlated with CD112 and/or CD155, we would have to repeat this study, adding antibodies against these surface receptors as well. Moreover, further analysis is required to analyze if CD112 and CD155 expression on target cells K562 is altered by rIL-2 and DA and if, therefore, their expression has any influence on CD96, CD226 and TIGIT expression and NK cells activity.

Effect of dopaminergic agents on degranulation, production of IFN-γ and expression of TIGIT, CD226 and CD96 of NK cells post-48-hour incubation with rIL-2

Our results demonstrated that, after a 48h incubation with rIL-2, NK and $CD56^{dim}$ NK cells significantly increased their degranulation levels (**Figure 4**). This is consistent with author

Aktas et al. (2009)[20] and *da Silva et al. (2017)*[172] which found that, after 5h stimulation with IL-2 (50 U/ml) and overnight stimulation with IL-2 (1000 U/ml), respectively, the expression of CD107a significantly increased in NK cells. However, the apparent increase in degranulation seen in $CD56^{bright}$ NK cells did not reach significance (**Figure 4**). As seen in Figure 6, there are less $CD56^{bright}$ NK cells expressing the population $CD96^{-}TIGIT^{-}CD226^{+}$, in comparison with $CD56^{dim}$ NK cells and NK cells. In fact, there is a high percentage of $CD56^{bright}$ expressing $CD96^{+}TIGIT^{-}CD226^{+}$, which doesn't verify with NK and $CD56^{dim}$ NK cells. In this case and as was demonstrated by *Chan et al. (2014)*[33], CD96 might compete with CD226 for CD155 binding and inhibit $CD56^{bright}$ NK cell function. The values of degranulation corresponding to $CD56^{bright}$ NK cells differ greatly between donors, probably because of the high percentage of $CD56^{bright}$ expressing $CD96^{+}TIGIT^{-}CD226^{+}$ population (**Figure 6**). Moreover, NK cells apparently increased production of IFN-γ did not reach statistical significance (**Figure 7**). The fact that cytokine secretion and cytotoxicity can be uncoupled when NK cells contact with their target cells[173]may explain our results. Indeed, several signaling pathways can lead to cytotoxicity independently of IFN-γ secretion in NK cells. *Reefman et al. (2010)* demonstrated that cytokines, such as IFN-γ, are secreted via a different pathway than perforin (and consequently CD107a) and its trafficking is nondirectional[174]. Recycling endosomes (RE), the site for regulation and sorting of cytokines, were proven to be functionally required for cytokine release because inactivation of REs or mutation of RE-associated proteins Rab11 and vesicle-associated membrane protein-3 blocked cytokine surface delivery and release. In contrast, REs are not needed for the release of perforin from preformed granules but may be involved at earlier stages of granule maturation[174]. Cytotoxic granules in NK cells are pre-formed before cell activation, and so their maturation and release initially have to be carefully constrained and then enabled upon target cell contact[175].

Rajasekaran et al. (2013) identified a signaling pathway consisting of the tyrosine kinase Fyn, the adaptor ADAP and the CBM signalosome (which consists of the adaptors Carma1, Bcl-10 and MALT1) that exclusively couples the activation of NK cells to cytokine and chemokine production but not cytotoxicity[173]. Additionally, *Jiang et al. (2000)* demonstrated that the signaling routes to induce cytotoxicity are dependent on activation of both the tyrosine kinase Lck and the MAPKs Erk1 and Erk2 (Erk1/2). MEK1-MEK2 and Erk1-Erk2 can be triggered either via PI(3)K-Rac1-PAK downstream of the engagement of NKG2D coupled to the transmembrane adaptor DAP10 or via the GTPase Ras and its effector molecule Raf downstream of the engagement of ITAM-coupled receptors. In humans, NKG2D is coupled only to DAP10, whereas in the mouse, NKG2D can be coupled to DAP10 and/or DAP12 depending on the status of NK cell activation[176]. The coupling of NKG2D and CD137 to ADAP can also trigger cytokine secretion by NK cells but not its cytotoxicity[177]. Analyzing the expression of these signaling pathways could also be an important step to better understand our results.

Importantly, we showed, for the first time, that DA, fenoldopam and SCH-23390 (a D1/D5R agonist and antagonist, respectively) do not change the expression of TIGIT, CD226 and CD96 at both unstimulated and stimulated conditions. Moreover, these dopaminergic agents did not seem to change basal and induced-degranulation and IFN-γ levels from NK.

Dopaminergic pathways are able to modulate the homeostasis or induce the effector functions of several immune cell populations constitutively expressing DARs[178,179]. In fact, DA seems to have an inhibitory function on freshly purified human NK cells[139], although the literature on this effect is very limited at the time. *Mikulak et al. (2014)* argued that DA represented a checkpoint exerted by the nervous system to control the reactivity of NK cells in response to activation stimuli. *Mikulak et al. (2014)* also reported that, in resting human NK cells, DA didn't have any impact on NK cells proliferation, IFN-γ production and degranulation. These authors argued that their results were in contrast to those reported for resting murine NK cells in which the engagement of DARs was associated with modulation of cell homeostasis and functional outcomes [133,178].

Mikulak et al. (2014) further found that NK cells had a significantly increase (>15 fold) in the levels of D5 receptor after stimulation with rIL-2 (200 IU), in comparison with resting NK cells. These results were only verified for the D5 receptor (but not for other DA receptors) and were obtained after 5 days of stimulation, since 1 day did not result in a significant change in DAR expression[139]. Furthermore, *Mikulak et al. (2014)* showed that NK cells do not express D1R.

On one hand, *Mikulak et al. (2014)* demonstrated that an 18-h incubation with DA (10^{-9}M) did not induce significant changes in the percentage of degranulation of rIL-2-activated NK cells. On the other hand, they showed that DA was able to suppress the production of IFN- γ via the engagement of the D5-signaling pathway on activated NK cells, and in the presence of K562 target cells, through the NF-kB pathway. Additionally, other reports indicate that DARs are able to modulate cytotoxicity of NK cells in murine models, after a 4h incubation with dopaminergic agents[133]. Since these data seem to be in contrast with our findings, it is mandatory to assess the expression of dopaminergic receptors, to confirm if at a 48hr incubation with rIL-2 is sufficient to increase D5R receptor. This would be essential to comprehend if the results we obtained were due to the absence of these receptors or if it's in fact DA that does not influence the expression of CD96, CD226 and TIGIT receptors.

6. Conclusion & Future Perspectives

We firstly offered a phenotypic characterization of the expression of CD96, TIGIT and CD226 in NK cells of HD. This paved the way to study the impact of rIL-2 and dopaminergic agents in this receptor repertoire. Analysis of NK cell population lead to the conclusion that the expression of CD96, CD226 and TIGIT is not altered in the presence of rIL-2 and/or dopaminergic agents. There is a big variability between donors, probably because of the different conditions regarding genetics and signaling between the 5 individuals. It is mandatory to increase the number of HD, which would allow the deepening of the evaluation of the role of these receptors on NK cells. However, the increase of the number of samples may allow one to identify clusters of individuals producing different amounts of IFN-γ and CD107a.

Then we focused on the effect of DA on rIL-2 induced NK cytotoxicity and IFN-g release. This evaluation needs to be further characterized using different rIl-2 incubation times (for example 5 days-incubation).

There are many other questions that need to be further studied. For example, the biology and dynamics of the three receptors must be completely understood. Being CD96 the less studied member, further studies focusing on this receptor are required to better understand its function and it's signaling pathways.

Blocking each receptor individually, as well as blocking each receptor-interaction and the different existing combinations would also be necessary to better comprehend the receptor dynamics.

The ligands for these receptors may also be interesting targets for future studies. The receptor-ligand interactions need to be considered since the expression of CD226 and CD96 was reported to be dynamically modulated by ligand exposure. The expression of CD155 in target cells decreased the surface expression of CD226 and CD96 in effector cells [33].

Finally, a future perspective for this study would be to repeat this same analysis using PBMCs from PD patients.

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Appendix I

Table 1 | Synthesis of statistical analysis regarding NK, CD56^{bright} and CD56^{dim} NK cells. *, p<0,05; **, p<0,005; ***, p<0,001, vs Unreated. n=1

		Untreated	DA	SCH	DA+SCH	FEN	rIL-2	rIL-2 + DA	rIL-2 + SCH	rIL-2 + SCH+ DA	rIL-2 + FEN
NK cells	NK cells						***	*	*		*
	CD96						*	*			
	DNAM-1						*				
	TIGIT										
	CD96+ TIGIT+ DNAM-1+										
	CD96- TIGIT- DNAM-1+						***	**	*	*	**
	CD96- TIGIT+ DNAM-1+										
	CD96+ TIGIT- DNAM-1+						*	*	*		
NK Bright	Bright										
	CD96						*	*			**
	DNAM-1										
	TIGIT										
	CD96+ TIGIT+ DNAM-1+										
	CD96- TIGIT- DNAM-1+										
	CD96- TIGIT+ DNAM-1+										
	CD96+ TIGIT- DNAM-1+						*	*			**
NK Dim	Dim						*				
	CD96						*				
	DNAM-1						*		*		
	TIGIT										
	CD96+ TIGIT+ DNAM-1+										
	CD96- TIGIT- DNAM-1+						**	*	*	*	*
	CD96- TIGIT+ DNAM-1+										
	CD96+ TIGIT- DNAM-1+						*				