DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Characterization of Natural Killer Cells in Chronic Myeloid Leukemia Patients

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Paulo Rodrigues Santos (Faculdadede Medicina da Universidade de Coimbra) e da Professora Doutora Ana Luísa Carvalho (Faculdade de Ciências e Tecnologias da Universidade de Coimbra)

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<< What doesn't kill us make us stronger>>

Friedrich Nietzsche

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Natural Killer (NK) cells are lymphocytes of the innate immune system killing skills against infected or transformed cells, without prior signaling. NK cells represent 5-20% of peripheral blood lymphocytes and based on their functions NK cells are subdivided into two subsets $CD56^{bright}CD16^{-}$ (cytokine production) and $CD56^{dim}CD16+$ (cytotoxicity). Chronic myeloid leukemia (CML) is a genetic myeloproliferative disease and previous studies indicate that NK cells are deficient in CML patients, although the mechanisms behind the dysfunction are not completely understood. The main goal of this work was to contribute to the cellular understanding of the insufficient control of NK cells over malignant CML cells. For this propose, we analyzed 62 blood samples from CML patients and 4 from healthy donors. All samples are labelled with extra or intracellular antibodies and through flow cytometry, we analyzed the expression of some surface markers, receptors and cytokine production NK cells from CML patients. In this study we demonstrate that NK cells from CML patients are reduced in number and the expression of cell surface markers and receptors, and cytokine production are altered. Besides that we reported that different biologic and TKI therapies affect in different ways the NK cells as well as imatinib dosage. We conclude that CML patients actually have dysfunctional NK cells, which reveal a dysfunction in the immune response against the disease. Thus, understand how these cells are affected can bring new developments in the treatment of these patients, particularly through modulation of NK cells, strategies to boost its activity.

As células Natural Killer (NK) são linfócitos granulares com capacidade de matar células infectadas ou transformadas sem sinalização prévia, para além de produzirem citocinas que permitem modular a resposta imune inata e adaptativa. Representam cerca de 5-20% dos linfócitos no sangue periférico e, com base na sua função, são divididas em duas subpopulações $CD56^{bright}CD16^{-}$ (produção de citocinas) e $CD56^{dim}CD16+$ (citotoxicidade). A Leucemia Mielóide Crónica (CML) é uma doença genética mieloproliferativa e estudos anteriores indicam que células NK de doentes com CML são disfuncionais, mas os mecanismos que estão por base destas disfunções não estão ainda bem definidos. Desta forma, o principal objectivo deste trabalho é contribuir para o entendimento do controlo insuficiente das células NK sobre as células leucémicas. 62 Amostras de sangue de doentes com CML e de 4 dadores saudáveis foram analisadas. Todas as amostras foram marcadas com anticorpos e, por citometria de fluxo, analisamos a expressão de alguns marcadores, receptores e a produção de citocinas nas células NK. Neste estudo demonstramos que a frequência das células NK de doentes com CML está diminuída e a expressão de marcadores de superfície, receptores e a produção de citocinas está alterada. Para além disso, reportamos também que diferentes tipos de terapia ou a dosagem do fármaco têm diferentes efeitos nestas células. Concluímos então que os doentes com CML têm disfuncionalidades ao nível das células NK, o que indica uma disfuncionalidade na resposta imune do organismo contra a doença. Assim, entender como estas células estão a ser afectadas pode trazer novos desenvolvimentos no tratamento destes doentes, nomeadamente por modulação das células NK, estratégias que permitam potenciar a sua actividade.

ABL	Abelson tyrosine kinase
ADCC	Antibody-dependent cell cytotoxicity
AP	Accelerated phase
APC	Antigen presenting cell
Ara-C	Cytosine arabinoside
AML	Acute Myeloid Leukemia
ATP	adenosine triphosphate
BC	Blast crisis
BCR	B-cell receptor
BCR	Breakpoint cluster region
CML	Chronic myeloid leukemia
CMR	Complete molecular response
CTL	Cytotoxic T cell
DAMP	Danger-associated molecular pattern molecules
DC	Dendritic cell
EMEA	European Medicines Evaluation Agency
Fas-L	Fas-ligand
FDA	Food and Drug Administration
FSC	Side-scattered light
GM-CSF	Granulocyte monocyte colony stimulating factor
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activating motif
ITGAM	Integrin alpha M
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer immunoglobulin-like receptors
KLFR	killer cell lectin-like receptor subfamily F
MFI	Mean of fluorescence intensity
MHC-1	Major histocompatibility complex class 1
NCR	Natural cytotoxicity receptor
NCAM	Neural Cell Adhesion Molecule
NK	Natural killer
NKp	Natural killer protein
PAMP	Pathogen-associated microbial patterns
PBMC	Peripheral blood mononuclear cells
Ph	Philadelphia chromosome

PRR	Pattern recognition receptor
PVR	Poliovirus receptor
SSC	Forward-scattered light
TCR	T-cell receptor
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitors
TNF	Tumor necrosis factor
TNFSF	Necrosis factor superfamily members
TNFRST	Tumor necrosis factor receptor super family

Introduction

Results

Introduction

The immune system is complex machinery, involving an intricate molecular and cellular organization with specific and specialized roles against pathogens, substances that can cause illness or death (2).

Classically, there are two different types of immune frontiers, external and internal defenses (2). Nowadays, the organization of immune system consists into three phases based on the timing of response (3). Mechanical defenses such as the skin and mucous membranes that line digestive and respiratory tracks act instantly to prevent body tissues, innate immune defenses and adaptive/acquired immune defenses, both comprise a variety of specialized cellular and humoral components (3). Innate and adaptive systems combined represent the internal defenses seen as cellular antigen- specific processes (2).

Hematopoietic stem cells are produced lifelong and same cells produce all the classes of leukocytes(3). Based on function these cells are divided into three major groups, phagocytes, lymphocytes and cells containing cytoplasmatic granules (3).

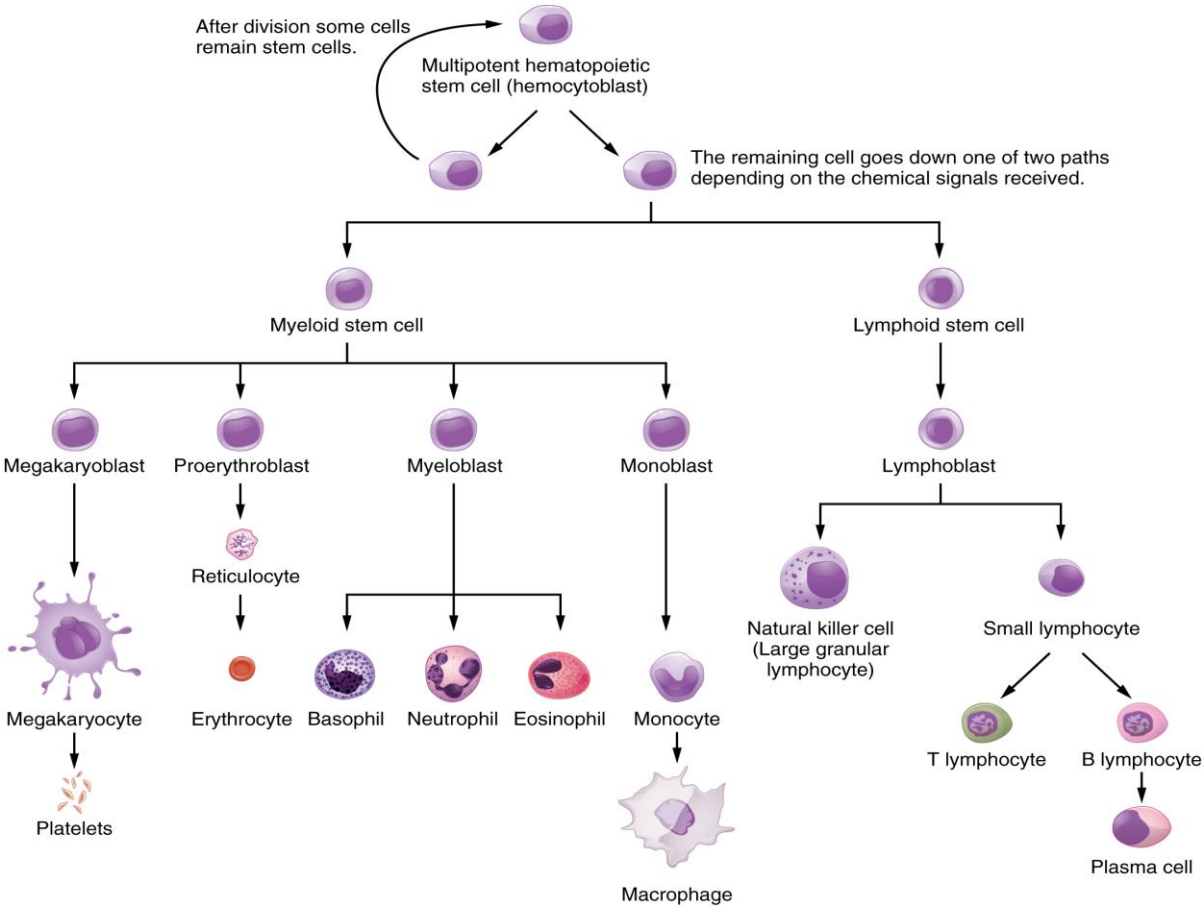


Figure 1: Hematopoietic stem cells differentiation. Cells of the immune response as well as of the blood arise by differentiation from hematopoietic stem cells. Platelets are cell fragments involved in the clotting of blood (3).

Innate & Adaptive Defenses

Fundamentally, the immune system is been allocated into two branches, innate and adaptive immunity. Both immune responses results from an unbalanced integration of positive and negative signals that have impact on both innate and adaptive cells (4). When positive signals prevail, cell activation and pro-inflammatory responses succeed with resultant elimination of microorganisms and viruses, when inhibitory signals dominate or in absence of productive stimulation cell activation is blocked (4). The duel between stimulation/inhibition occurs through cell-cell contact, by cytokines and signaling pathways downstream (4).

Innate immune system is fast and less specific and can be divided in cellular and humoral immunity. Cellular components are NK cells, APCs and other phagocytes, representing the organism first internal defense (2).

APCs, dendritic cells and macrophages, have the ability to recognize internal dangers through antigen recognition, and thus exert its effector action or present them to other immune cells from both innate and adaptive system, acting like a dynamic bridge between them (2). Humoral compounds include complement proteins and cytokines (2).

The adaptive system is highly specific and can be divided in humoral compounds related to antibodies release by the B lymphocytes, and cellular components associated with T lymphocytes and memory response, allowing an improvement on repeated exposure to a given infection (2). Adaptive immunity in mammals is characterized by two types of lymphocytes, T and B lymphocytes are the main self-defensive weapons of the adaptive immune responses, triggered by antigen exposure (5).T and B cells clonally expressing a large repertoire of antigen receptors produces by

site-specific somatic recombination, TCRs and BCRs (6). Functionally, naïve B and T cells target antigens in lymphoid organs and promote cell division and maturation before exerting their effector functions (6). The innate immunity distinctive features are commonly refer to fast "time of reaction" and broadly variety of myeloid and lymphoid cells that only need a few minutes of exposure to exert their effector functions, put forward a protective inflammatory response and if necessary lead off an adaptive response (5,6).

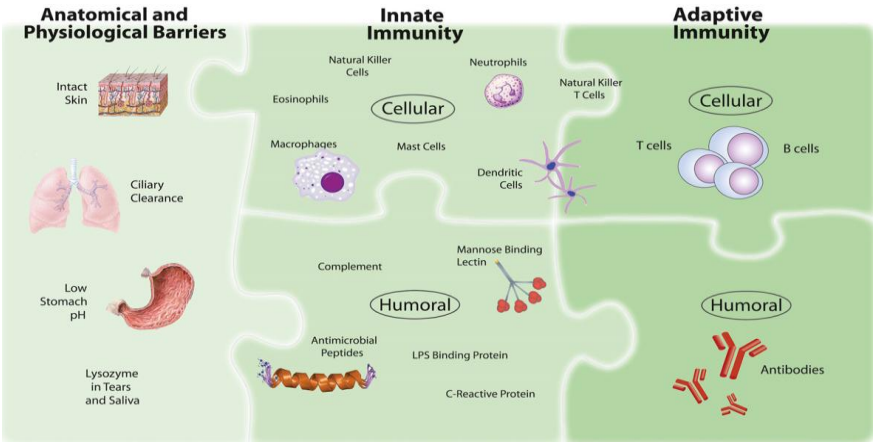


Figure 2: Integrated human immune system. The human microbial defense system can be simplistically viewed as consisting of 3 levels: anatomical and physiological barriers; innate immunity; adaptive immunity (5).

Innate and adaptive responses work by themselves and together with the same purpose, organism protection from danger (7). The combination of both enables the immune system to recognize and eliminate invading pathogens with maximal efficacy and minimal damage to self, also providing protection from re-infection (7).

From a phylogenetic standpoint, the innate immunity evolved long before the adaptive immune system, and homologous components can be found in invertebrates and plants (8). Although it might appear primitive, the innate immune system has recently been recognized to be more complex and sophisticated than previously thought and it is now increasingly clear that several aspects of the adaptive immune response are controlled by the innate response (9).

The focus on innate immunity dates to the 1908 Nobel Prize winning efforts of Ilya Mechnikov, but until the last decade its study has been covered by severe discoveries in adaptive immunity field (5). Currently, the recent molecular definition of how the innate immune system senses infection to enhance protective immune responses has precipitated a renaissance in this campus. The title "nonspecific immunity" is no more connected with innate immune system, now being regarded as a powerful partner of adaptive system (5).

Innate immune responses are not specific to a particular pathogen in the way that the adaptive immune responses are (2). The innate immune cells distinguish infectious agents and/or abnormal "self" from normal "self" molecules, using germline encoded receptors. The cellular compartment is represented by granulocytes, mast cells, monocytes, macrophages, DC and NK cells (9). Although each cell type has specific functions, they cooperate to orchestrate effective immune responses. Besides that they

provide direct elimination of infectious agents and induce the adaptive immunity developing, manipulating the quality and strength of the immune response (5,9).

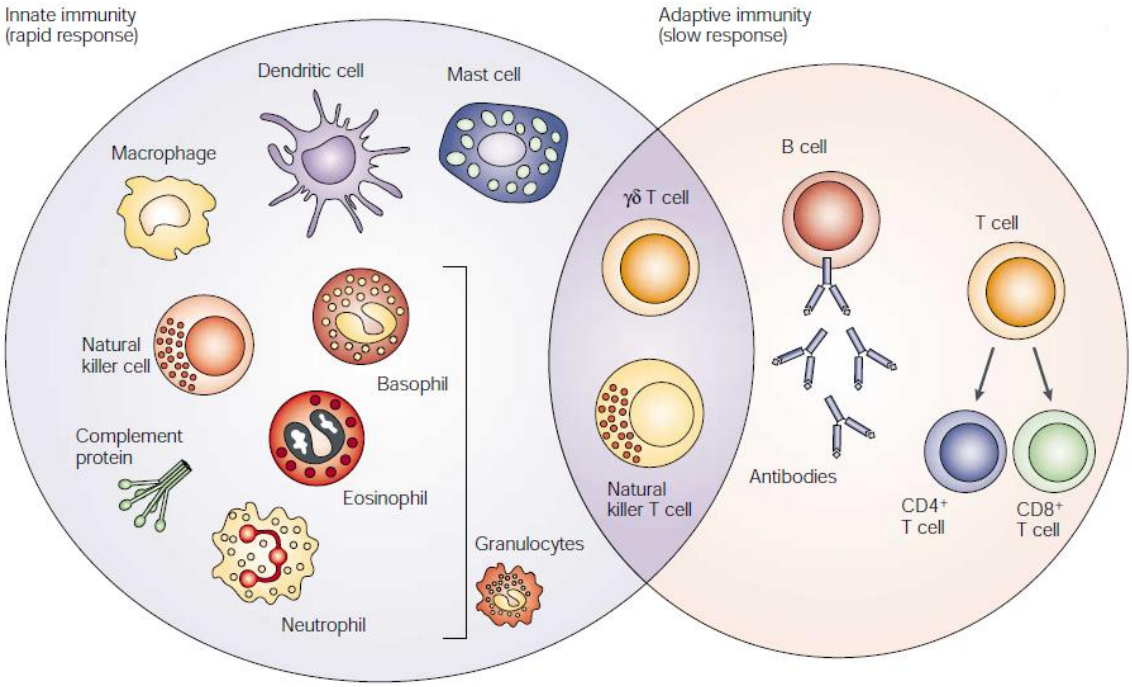


Figure 3: The innate and adaptive immune cells and soluble components. Innate immunity consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. Adaptive immunity consists of antibodies, B cells, and CD4+ and CD8+ T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity (10).

Innate Immune Recognition

The innate immune system uses at least three strategies in order to recognize invading microorganisms (5). Cells from innate defenses recognize "microbial nonself" conserved molecular structures that are expressed by a variety of microorganisms. Indeed, Charles Janeway invented the terms PRRs to collectively describe such receptors and PAMP to nominate the microbial structures recognized by the PRRs (5). TLRs are PRRs that are expressed on both lymphoid and non-lymphoid tissues, especially APC such as DC and macrophages (11). TLR-PAMP targeting initiates intracellular signal transduction cascades that lead NF-kB activation and the upregulation of adhesion and costimulatory molecules, and cytokines that are essential to aware the immune system for the presence of an intruder (5) (figure 4). The innate system has the capacity to detect immunologic danger in form of DAMPs related molecules that are commonly released and upregulated during the cell lysis and tissue damage, molecules often found in the tumor microenvironments (5).

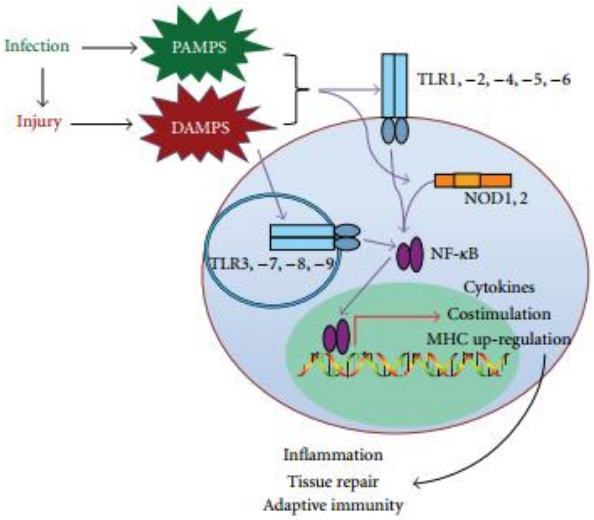


Figure 4: Inflammatory response via PAMPS/DAMP-TLR. Infection and cell injury result in the production of PAMPs and DAMPs that promote the inflammatory response via TLRs located on the cell membrane (11).

Another strategy of recognition was described, based on the "missing self" hypothesis, innate immune receptors, namely Natural Killer cells (NK cells) receptors, detect molecules expressed by normal healthy cells but not expressed by infected cells or microbes. Recognition and tolerance of "self" induces an inhibitory signal, preventing the action of the immune system against host healthy cells (5).

NK cells were originally described in 1975 as large granular lymphocytes with capacity to kill in vitro tumor target cells without prior sensitization (12,13). At present, NK cells have gained recognition for their ability to secrete proinflammatory and antiviral cytokines, to recognize and eliminate tumors, viral and parasitic infections as well as normal allogeneic cells and to have regulatory functions in the context of the adaptive immune response (14,15).

NK cells are usually considered to be constituents of innate immune defense, being actually one of its pillars, because they lack antigen-specific cell surface receptors (14). NK cells have been shown in humans and mice to participate in the early control against virus infection, especially herpesvirus infection (16), and in tumor immunosurveillance (15,17).

Immunobiology of NK Cells

Natural killer (NK) cells are innate immune lymphocytes capable of killing target cells and producing immunoregulatory cytokines.

NK Cells Subsets, Distribution and Characterization

NK cells are large granular cells that have a common progenitor with B and T cells. All of them are originated in the bone marrow from CD34+ hematopoietic progenitor cells and are widespread throughout lymphoid and non-lymphoid tissues. They constitute 5-25% of PBMCs and up to 5% of the whole lymphocyte population in lymph nodes (14). Furthermore, NK cells can be found throughout most tissues including liver, spleen and lungs (18). Several studies have also described the existence of NK cells in the uterine mucosa, where they promote vascularization of implanting embryos during pregnancy (19).

NK cells are conventionally defined as CD56+CD3-, in peripheral blood NK cells comprise approximately 10% of all lymphocytes based on surface density of CD56 (NCAM) and CD16 (FccRIII). Thus, NK cells are usually divided into two subpopulations $CD56^{bright}CD16^-$ and $CD56^{dim}CD16+$ NK cells (20). $CD56^{bright}CD16^-$ has a more immunoregulatory character and has a higher rate of cytokine production, whereas $CD56^{dim}CD16+$ has more cytotoxic power and produce less cytokines (21). $CD56^{bright}$ NK cells account for around 10% in peripheral blood and are the predominant subset found in secondary lymphoid tissues. $CD56^{dim}$ NK cells represent the vast majority in peripheral blood accounting for up to 90% of all NK cells, but are rare in lymph nodes. They express high levels of FcγRIII (CD16) and are cytotoxic. Both subsets also differ in the distribution of NK cells receptors and in their proliferative response (22).

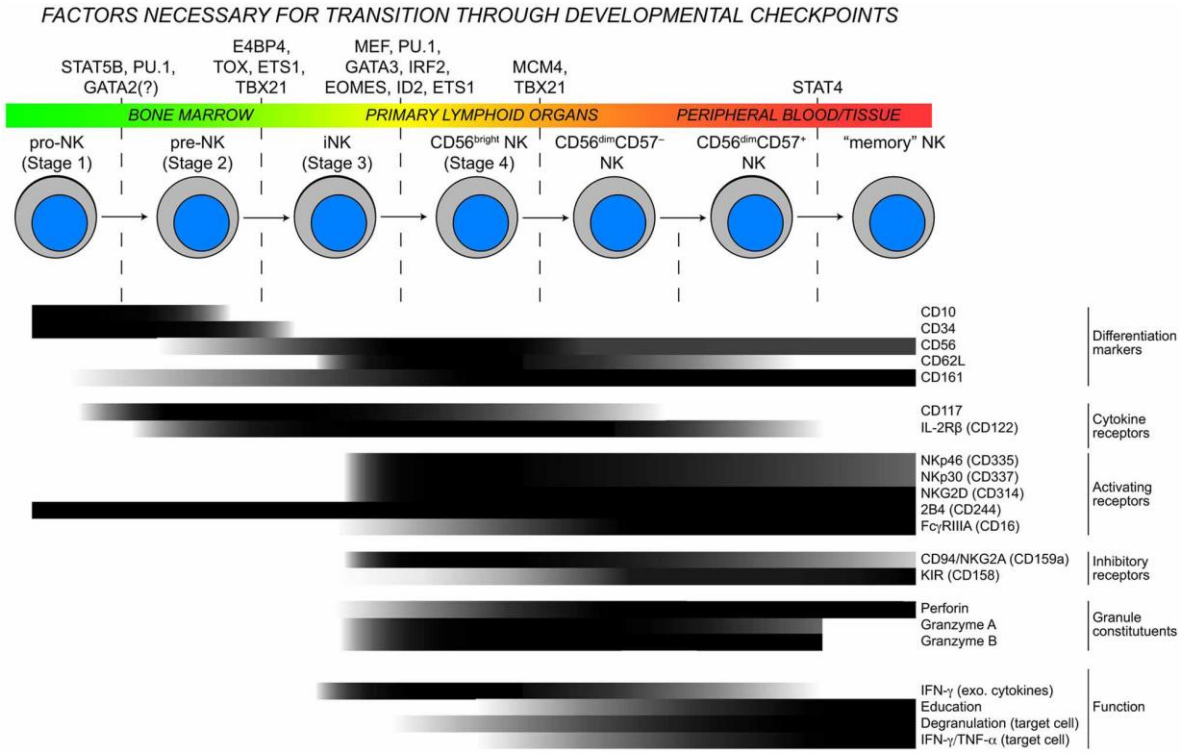


Figure 5: Model of NK cell developmental checkpoints. Checkpoints in NK cell development, expression of developmental markers, cytokine receptors, natural cytotoxicity receptors, and functional competencies (23).

NK Cells Effector Functions

In literature, two major functions are associated to NK cells, cytokines and chemokines production and cytotoxic activity. Thus, NK cells could be classified as immunoregulatory and cytotoxic cells (15). NK cells are also involved in shaping adaptive immune responses through their production of cytokines (12). Recently, mouse NK cells demonstrate immune memory, similar to T lymphocytes, after viral infection (12). These kinds of developments in the NK cells area have been particularly intriguing, given the interest in developing strategies to apply NK cells as therapeutic agents against a broad range of malignancies as augmenting the activity of NK cells during chronic infections (12). NK cells exert two major effector mechanisms: 1 – Recognition and killing of target cells through perforin and granzyme release, as well as expression of death receptor ligands. 2 – Activation and recruitment of other immune

cells by secretion of cytokines and chemokines (24)(figure 6). Transformed or infected cells upon direct contact and recognition by NK cells leads to the activation of NK cell mediated cytotoxicity, as well as via ADCC through engagement of FcγRIII receptor (CD16) (25).

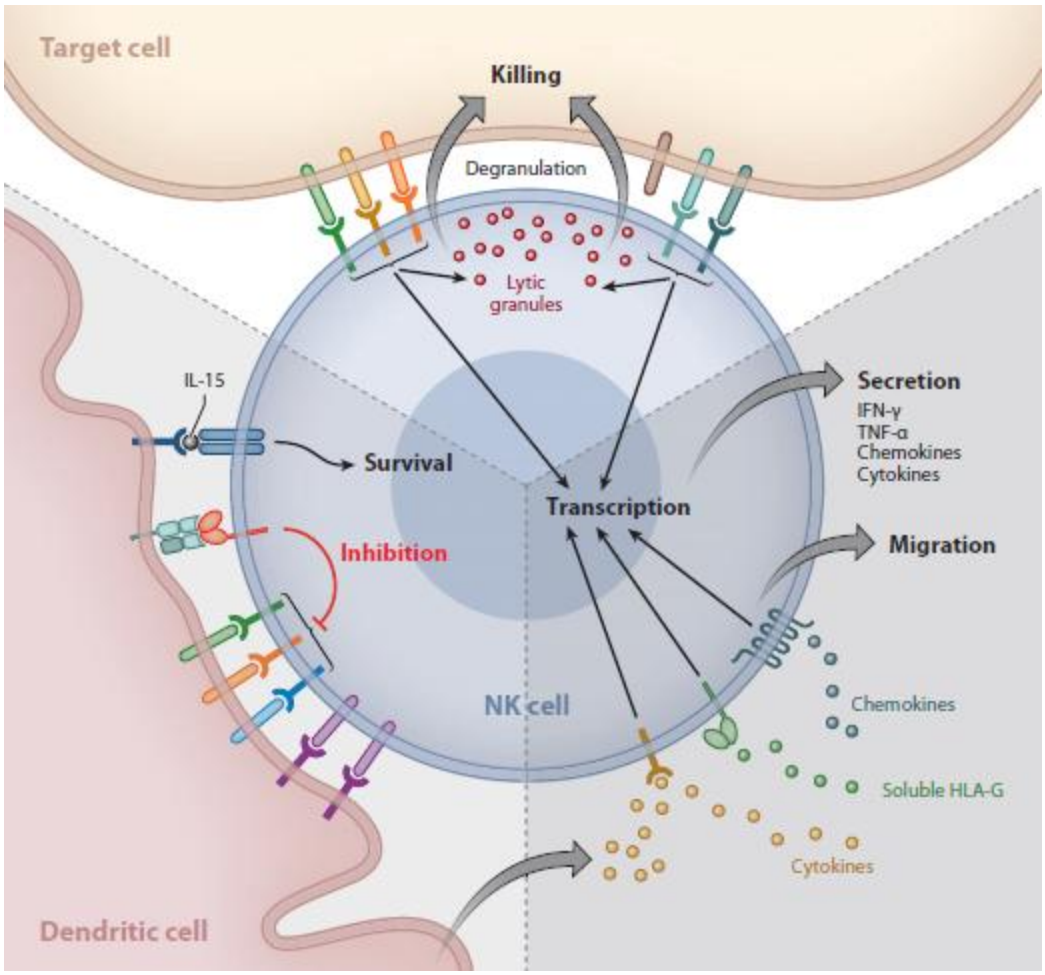


Figure 6: Multiple signals control NK cell responses. An array of NK cell activation receptors bind to ligands on other cells. Target cell killing requires signals for lytic granule polarization and degranulation. Combinations of coactivation receptors synergize to induce killing and a secretory response. Some of the secretory response is independent of transcription. Inhibitory receptors for MHC-I exert dominant inhibition of cytotoxicity and secretion, as seen in the NK cell–dendritic cell interaction. Dendritic cells also receive signals from ligands on NK cells. In the absence of inhibition through MHC-I, target cells that express ligands for activation receptors are killed. NK cells respond to various soluble activators, such as cytokines and chemokines, that deliver signals for functions such as migration and survival (26).

Perforin/granzyme via represent the main cytotoxic pathway, perforin and granzymes are stored in granules inside NK cells and get released by exocytosis upon

target cell recognition. Perforin molecules form a pore in the target cell membrane which enables granzymes to enter into the cytoplasm and initiate the apoptotic process (27). Other mechanisms include induction of apoptosis via TRAIL or engagement of the Fas receptor on the target cell surface via Fas-L (28).

Have been described NK cells regulatory capacities, which are mediated by various cytokines, such as IFN-γ, TNF-α, IL-10, IL-13 and/or GM-CSF, released upon engagement of triggering NK cell receptors or signaling by other cytokines (29).

Finally, the intensity and the quality of the NK cell response are regulated and depend on the NK cell microenvironment (e.g. presence of cytokines), as well as on the interactions with other cells of the immune system that cooperate to promote NK cell "priming" (13). IFN-γ and some interleukins (e.g. IL-1, IL-12 and IL-18) are potent activators of NK cell effector functions, whereas other interleukins (e.g. IL-15 and IL-2) are important for NK cell maturation, proliferation, and survival, cytotoxicity and cytokine secretion. Instead, NK cell function can be negatively regulated by TGF-β), and regulatory T cells through a TGF-β-dependent mechanism (13).

Target Cell recognition

The mechanism that allows NK cells to discriminate target cells remained a mystery for a long time. In 1981, Klas Kärre made a key contribution postulating the "missing self hypothesis". This concept was based on the observation that NK cells kill target cells lacking self MHC class I molecules, whereas normal MHC class I surface expression levels protects healthy autologous cells from lysis (30).

NK cells acquire tolerance to "self" during their development and maturation through a process called "licensing or "education". Tolerance to "self" requires recognition by NK cell inhibitory receptors of self MHC-1 molecules that are expressed in the steady state condition (31). Yet, in order to activate NK cells, other signals triggered by the engagement of specific activating receptors are required (32). According to the current "dynamic equilibrium paradigm", the balance between inhibitory and stimulatory signals received by a NK cell determines the outcome of the interaction with the target cell (32).

NK cells can recognize a variety of stressed cells in the absence or in the presence of antibodies, NK cells can directly trigger stressed cells and kill them – "Natural Killing" or can act by the antibody dependent cellular cytotoxicity – ADCC, leading to target cell death by apoptosis (28). NK cells preform three mechanisms of recognizing and became or not activating in line with ligands express by the target cells (33). These mechanisms are based essentially in the expression of MHC-1 molecules NK cells inhibitory receptors recognize MHC-I molecules and then block the activating signaling cascade which confer tolerance by the NK cells (33). So, if the NK cells recognize a target cell and don't became active means that these cells are "self" material, if the target cell lacks the presence of MHC-I molecules activating signals will

prevail and NK cells act in order to destroy them, these constitutes the hypothesis of "missing-self" recognition (figure 7). Furthermore, NK cells can act against cells that express MHC-I molecules too, since the activating signals overcomes the inhibitory signals, induced-self recognition (33).

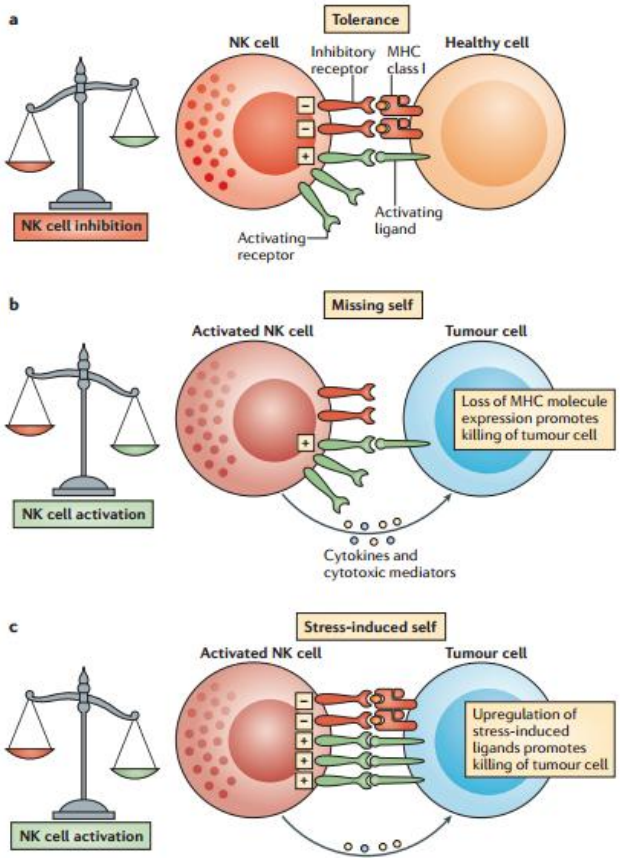


Figure 7: Recognition of tumor cells by NK cells. a) Natural killer (NK) cells are tolerant to healthy host cells; b) NK cells become activated when tumor cells lose expression of MHC class I molecules; c) 'Stress-induced self' triggering, NK cells are selectively activated by 'stressed' cells, which upregulate activating ligands for NK cells and thereby overcome the inhibitory signaling delivered by MHC-I molecules (13).

Recently, NK cells and DC have been demonstrated some cross-talk with each other in many different ways, including the NK cell killing of immature DC and the promotion of DC maturation, through cytokines released by NK Cells. Besides that DC can also promote the activation of NK Cells, and all together have crucial effects on adaptive responses (28).

Inhibitory and Activating NK Cells Receptors

To perform its functions NK cells require the expression of a highly organized diversified repertoire of activating and inhibitory receptors, the balance between positive and negative signals, respectively, controls the tolerance of NK cells to the other cells, and allows the immunosurveillance of the organism (15). NK cells have a lot of receptors, of different families, but the most crucial difference between all of these receptors is the negative or positive sort of signaling. The complex interactions, which regulate both the quality and magnitude of the ultimate response, depend on two short conserved motifs found in intracellular domain of various signaling proteins (15,34).

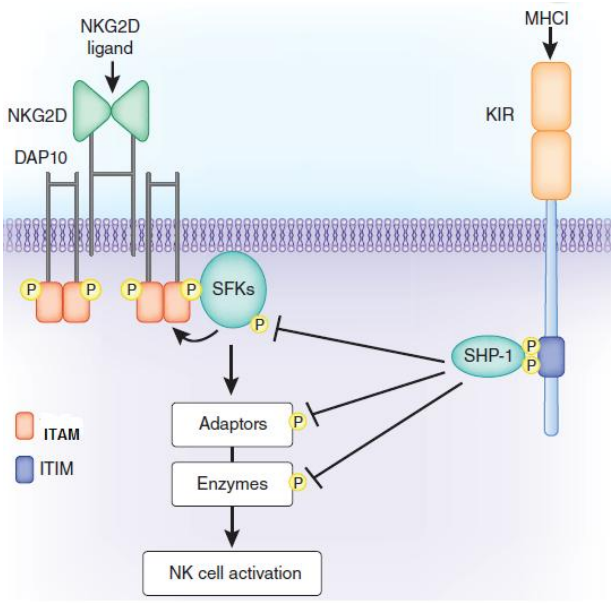


Figure 8: Control of NK cell activation by SHP-1. In NK cells, engagement of NKG2D by its ligands on target cells triggers tyrosine phosphorylation of DAP10 by SFKs (at the tyrosine-isoleucine-asparagine-methionine motif). This phosphorylation triggers the recruitment and tyrosine phosphorylation of downstream effectors. When a KIR is engaged by its ligand, class I major histocompatibility complex molecules (MHCI), also present on target cells, SFKs phosphorylate the ITIM of the KIR, thus triggering the recruitment and activation of SHP-1. SHP-1 mediates the inhibition of NK cells by dephosphorylating the activating tyrosine residues of SFKs, as well as various intracellular tyrosine-phosphorylation substrates (34).

ITAMs and ITIMS are the terms for immunoreceptor tyrosine-based activation (or inhibitory) motifs, provide the basis for two opposed signaling modules that compete for NK cell activation control within the immune system (35).

Upon ligand binding, activator receptors like TREM2, SIRP1, FcRI or FcγRIIIA associate with ITAM containing adaptor proteins such as DAP12. Subsequently, members of Src kinase family (SKF) phosphorylate tyrosine residues of ITAMs. Phosphotyrosine residues are docking sites for Syk protein kinases that upon activation mediate cellular activation via a number of downstream cascades. Upon ligand binding, inhibitory receptors recruit SHP-1 and SHP-2 which can in turn terminate intracellular signals emanating from ITAM receptors (34,35). Hence the balance between these two classes of receptors is the major target of NK cell modulation (35) (figure 8).

NK Cells – Fighting Cancer

The relationship between the immune response and tumors was addressed by the concept of cancer immunosurveillance that was presented in 1950s by Lewis Thomas Paul and then developed by Sir Macfarlane Burnet (36). This hypothesis supports the proposal of Ehrlich and other researchers that the immune system protects self-cells and destroys non-self cells. The hypothesis advocates that the human body develops tumor cells all the time, but these cells are routinely recognized and eliminated by the immune system. Therefore, tumor progression is a result of the immune system's failure to clear tumor cells (24,37).

Different mechanisms are known to be involved in the destruction of tumor cells by NK cells (10) (figure 9). Three types of mechanisms have been described until now, perforin/granzyme-mediated cytotoxicity, death receptor mediated apoptosis and Interferon-γ effector functions (38). The perforin/granzyme-mediated cytotoxicity is the release of cytotoxic granules composed of perforin and granzymes is the fastest and also the most powerful way to lyse tumor cells (38). By creating a synapse with the target cell, NK cells will drop, at this junction, perforin and granzyme molecules inducing the lysis of the target cell (10,38). Death receptor mediated apoptosis refers to the death of the target cell, induced by apoptosis via TNF family ligands, Fas ligand and TRAIL, an alternative way to release granules (38). IFN-γ effector functions occurs after NK cells activation, these cells secrete various cytokines like IFN-γ, TNF-α, GM-CSF, IL-10, or IL-13 and their antitumor activities can be mediated by IFN-γ (38). Indeed, IFN-γ produced by NK cells contributes to eliminate tumor metastases, inhibition of proliferation of tumor cells, and has been also described to enhance NK cell cytotoxicity

by overexpressing adhesion molecules or by increasing the sensitivity of tumor cells to cytotoxicity mediated by granule release or death receptor engagement (10,38).

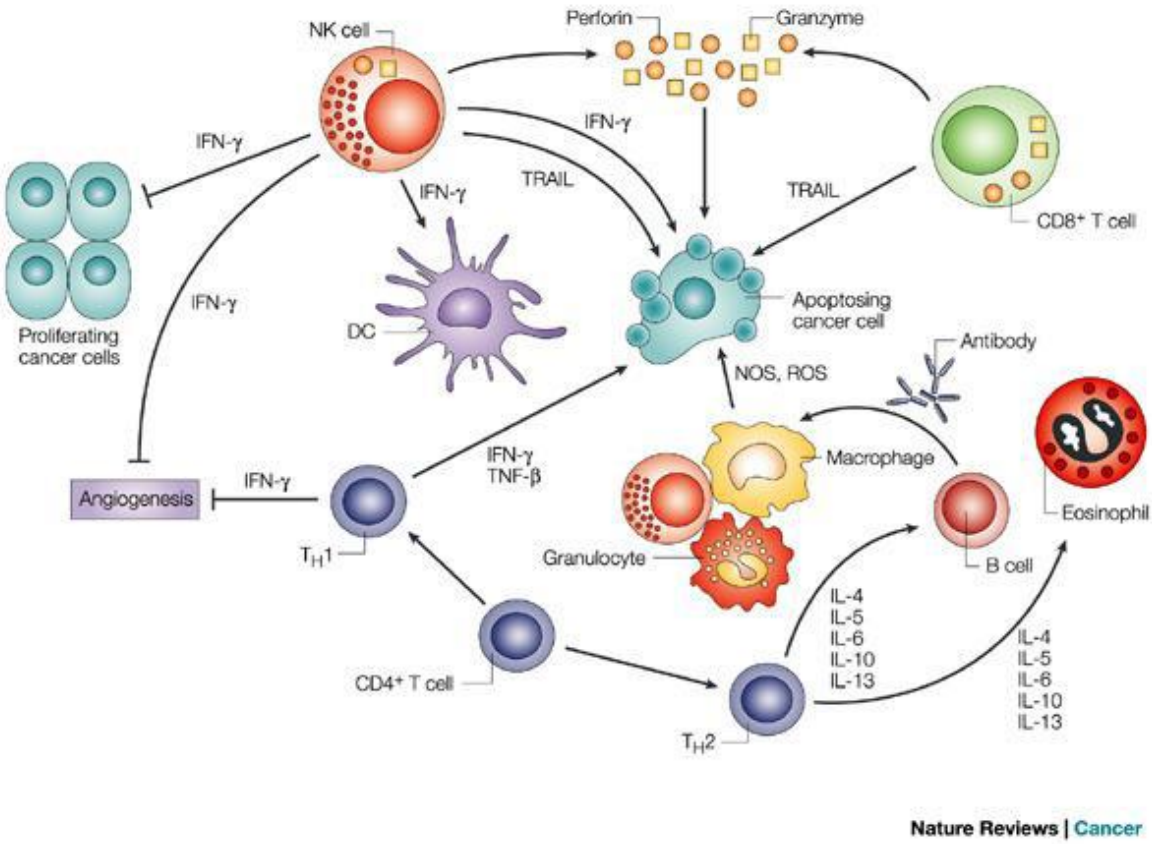


Figure 9: A coordinated cellular and humoral reaction by NK cells mediates tumor destruction. Following stimulation, NK cells can lyse tumors through the perforin/granzyme pathway or apoptosis-inducing ligands such as TRAIL. NK cells secrete IFN-γ), which inhibits tumor-cell proliferation, enhances tumor-cell apoptosis, improves tumor antigen presentation and inhibits angiogenesis. NKT cells also execute cytotoxicity and cytokine production. Cytotoxic CD8+ T cells lyse tumors through death ligands, such as TRAIL, and the perforin/granzyme pathway. CD4+ T cells can differentiate into T helper 1 (TH1) cells that secrete IFN-γ and TNF-β or T helper 2 (TH2) cells that secrete IL-4, IL-5, IL-6, IL-10 and IL-13. The latter cytokines enhance eosinophil function and increase antibody production by B cells. Antibodies to cancer cell-surface molecules can inhibit oncogenic signaling and/or stimulate tumor destruction through engaging Fc receptors on macrophages, granulocytes and NK cells (not shown). Antibodies can further promote tumor antigen presentation by DCs through immune-complex formation. Macrophages can lyse tumors through the production of nitric oxide and reactive oxygen species. Tumor blood vessels can also be attacked by lymphocytes and granulocytes (10).

Sawyers's in a review article defines CML as "a malignant clonal disorder of hematopoietic stem cells that results in increases in not only myeloid cells but also erythroid cells and platelets in peripheral blood and marked myeloid hyperplasia in the bone marrow" (39). Chronic myeloid leukemia (CML) is a clonal disorder in which cells of the myeloid lineage undergo inappropriate clonal expansion, caused by a BCR-ABL fusion gene resulting from a balanced translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11), also known as the Ph (40). The resulting fusion protein BCR-ABL is a cytoplasmic oncoprotein of 210 kDa with constitutively activated tyrosine kinase responsible for the clinical features of CML. The disease typically progresses through three distinct phases, chronic phase, accelerated phase, and blast crisis, during which the leukemic clone progressively loses its ability to differentiate (41).

Molecular Biology

The t(9;22)(q34;q11) translocation juxtaposes the 30 segment of the c-ABL oncogene (normally encoding the Abelson tyrosine kinase) from the long arm of chromosome 9 to the 50 part of the BCR gene on the long arm of chromosome 22, resulting in one shortened chromosome 22 (22q) (the Ph chromosome) and one elongated chromosome 9 (9q) (41) (figure 10). At diagnosis, the Ph is present in approximately 95% of CML cases (41). The molecular consequence of t(9;22)(q34;q11) translocation origins a gene that is expressed as a BCR-ABL mRNA transcript, translated into a 210-kDa protein known as p^{210} BCR-ABL (41,42).

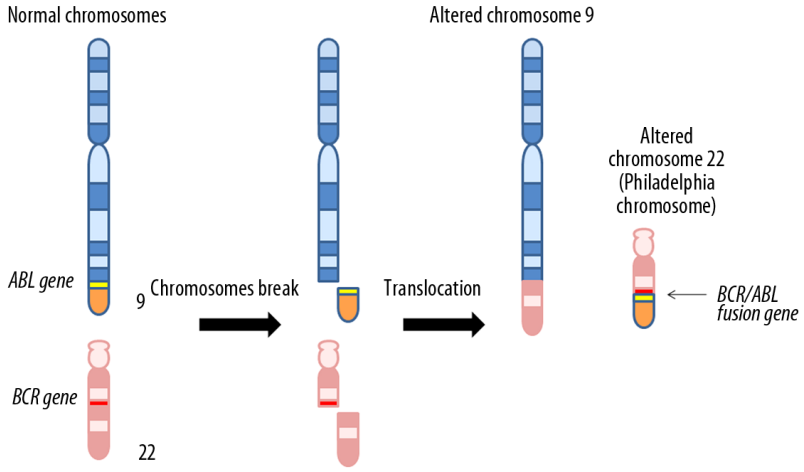


Figure 10: Philadelphia chromosome. Balanced translocation t(9,22)(q34: q11) translocation. This mutation is present in most cases of CML (>95%) (42).

BCR-ABL oncoprotein acts as a constitutively active tyrosine kinase that can phosphorylate a large range of cytoplasmic substrates, giving advantage to proliferation, differentiation, adhesion, and survival (43). The effects of BCR-ABL oncogene leads to an uncontrolled signal transduction, effectively by increasing proliferation, motility and myogenic outcomes, and decreasing cell adhesion, differentiation and apoptosis (42,43). The leukemic clone in CML has a tendency to acquire additional oncogenic mutations over time, usually associated with progression to accelerated phases of disease or resistance to tyrosine kinase inhibitors (TKIs) (43,44).

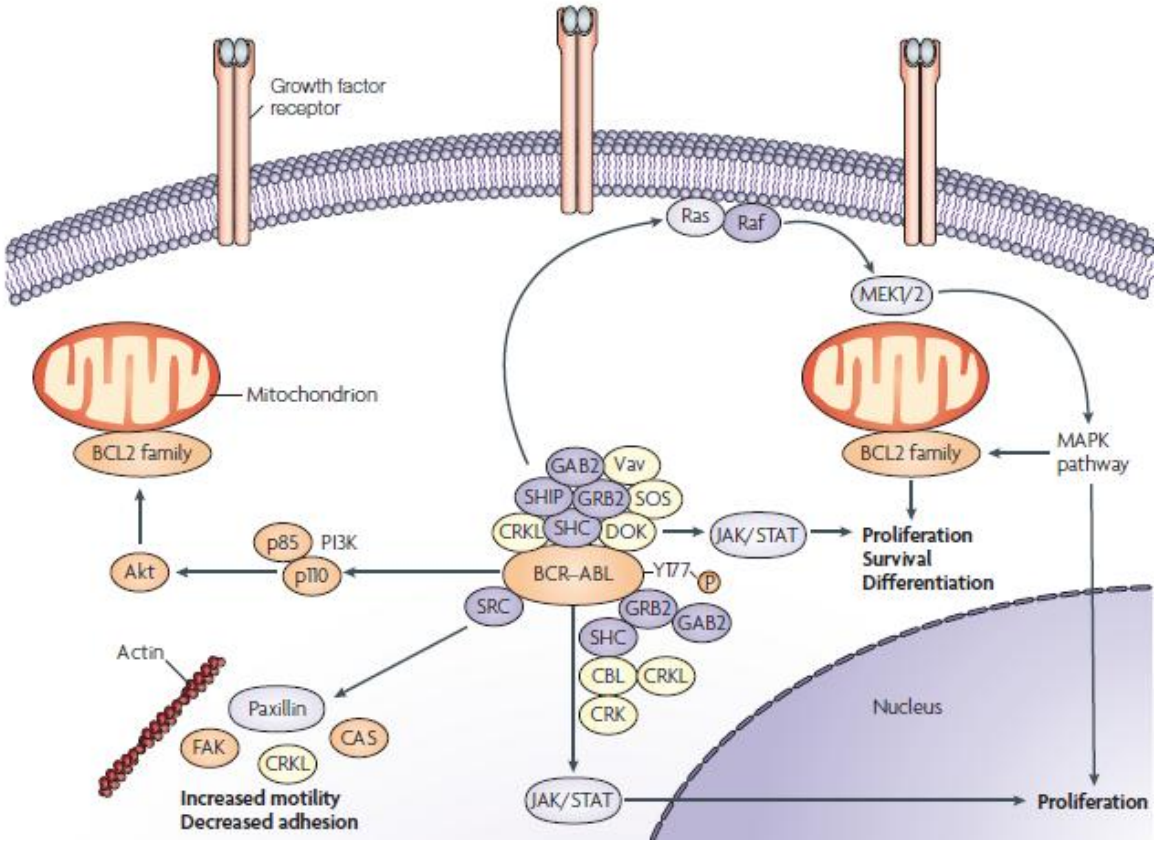


Figure 11: BCR-ABL signalling in chronic myeloid leukemia. With the aid of several mediator proteins, BCR-ABL associates with Ras and stimulates its activation. The adaptor protein, growth factor receptor-bound protein 2 (GRB2), interacts with BCR-ABL through the proximal SRC homology 2 (SH2)-binding site that develops when the tyrosine 177 (Y177) residue of BCR-ABL is autophosphorylated. GRB2, when bound to BCR-ABL, interacts with the son of sevenless (SOS) protein. The resulting BCR–ABL–GRB2–SOS protein complex activates Ras. The adaptor proteins CRKL (CRK-like) and SHC (SH2-containing protein) can also mediate the BCR-ABL activation of Ras. Ras and the mitogen activated protein kinase (MAPK) pathway are coupled by Raf (a serine/threonine kinase). Raf catalyses the phosphorylation of the mitogenactivated and extracellular-signal regulated kinase kinases 1 and

2 (MEK1 and MEK2); this results in their activation. Through the stimulation of the Ras–Raf pathway, BCR-ABL increases growth factor-independent cell growth. BCR-ABL also associates with and activates the phosphatidylinositol-3 kinase (PI3K) pathway, suppressing programmed cell death and increasing cell survival. BCR-ABL is associated with components of the focal adhesion (that is, actin, paxillin and focal adhesion kinase, or FAK); the activation of CRKL–FAK–PYK2 leads to a decrease in cell adhesion. BCR-ABL also associates with the Janus kinase and signal transducer and activator of transcription (JAK–STAT) pathway. Finally, BCR-ABL activates pathways that lead to atypical responses to chemotactic factors, which leads to an increase in cell migration. BCR-ABL also associates with survival proteins that interact with the mitochondrial-based BCL2 family. CAS, p130 CRK-associated substrate; GAB2, GRB2-associated binding protein 2; SHIP, SH2-containing inositol-5-phosphatase (44).

Once well established the molecular biology of CML cells, the researchers had the "open doors" to develop targeting drugs for BCR-ABL metabolic pathways (45). The BCR-ABL protein is unique to leukemic cells, being expressed in high levels and its tyrosine kinase activity is essential for its ability to induce CML. Thus, BCR-ABL protein is an ideal and attractive target for CML therapies (46).

Clinical Features

With an annual incidence of approximately $1.5/10^5$ CML is a rare genetic disease that affect most males than females (ratio 1.7:1) and the incidence greatly increases in older population (average 45-50years), but doesn't show significant ethnic or geographical predisposition. Symptoms comprise fever, weight loss, and abdominal fullness, but the majority of patients are asymptomatic at diagnosis (47). The clinical course of CML is three-phased with gradually severe over each phase, chronic and accelerated phase, and blast crisis. Most patients are diagnosed in the chronic/stable phase – expansion of the myeloid cell compartment, cellular differentiation and function is maintained – and after a variable length of time the disease progresses to blast crisis, which look like an acute myeloid (70%) or lymphoid (30)% leukemia (39). Before the drug therapy approval for CML the patients were treated with interferon-α-based regimens, the median duration of the chronic phase was approximately five years (48).

Timeline Treatment

In 1960, investigators in Philadelphia identify a chromosomal abnormality linked to a specific form of leukemia called chronic myelogenous leukemia (CML). A decade later, further research by Janet Rowley leads to the discovery that this abnormality occurs when two chromosome fragments – chromosomes 9 and 22 – switch places in a phenomenon called balanced translocation (40).

Since 1970s, allogenic stem cell transplant (allo-SCT) has been used as the only therapy that effectively could eradicate leukemic clones. However, a higher risk of mortality has been associated with this therapy and in addition some constraints like age, disease status, time from diagnose and recipient-donor gender combinations must be taken in account (49). In the early 1980's, Interferon alpha (IFN-α) entered clinical trials and was the first effective therapy for CML, being the treatment of choice for CML patients, before the TKIs era (46). IFN-α is a glycoprotein of biological origin and displays antiviral and antiproliferative properties. This drug induces cytogenetic response in 35% to 55% of patients and longer survival achieved in combination with chemotherapy, however CML clones were rarely completely eliminated (49).

In 2001, FDA approves imatinib (Gleevec®) after just three months of review – the fastest approval in FDA history – based on data showing the drug halted the growth of CML clones in the most patients (40). Imatinib is the first drug proven to shut down a molecular "imperfection" related to a specific kind of cancer – mutation on the so-called "philadelphia chromosome" (40). Since, has become the care standard for CML, and its effectiveness and easily-administered pill formulation allows a relatively normal life to patients despite the chronic disease they carry (40). In 2006, researchers identify a

second targeted treatment, dasatinib (Sprycel®), for patients with chronic myelogenous leukemia who cannot tolerate or develop resistance to imatinib. The drug is approved based on reports screening that more than 90 percent of these poor prognosis patients had no evidence of their cancer following Dasatinib treatment (40). Dasatinib targets the same mutated protein as imatinib, but through a different mechanism (40) . In 2007, research shows that Dasatinib – initially approved for use only when treatment with Imatinib fails (40) – should also be an option for initial treatment of early-stage chronic myelogenous leukemia. The FDA approves the drug for this broader indication in 2010, giving CML patients more solutions (40). In 2010, two studies show that Dasatinib and nilotinib (Tasigna®) may be more effective than the current standard drug, Imatinib, for the initial treatment of chronic myeloid leukemia. These drugs target the same active genetic pathway on the "Philadelphia chromosome," studies suggest that newer drugs possibly prompt faster and stronger responses, and less side effects than Imatinib (40).

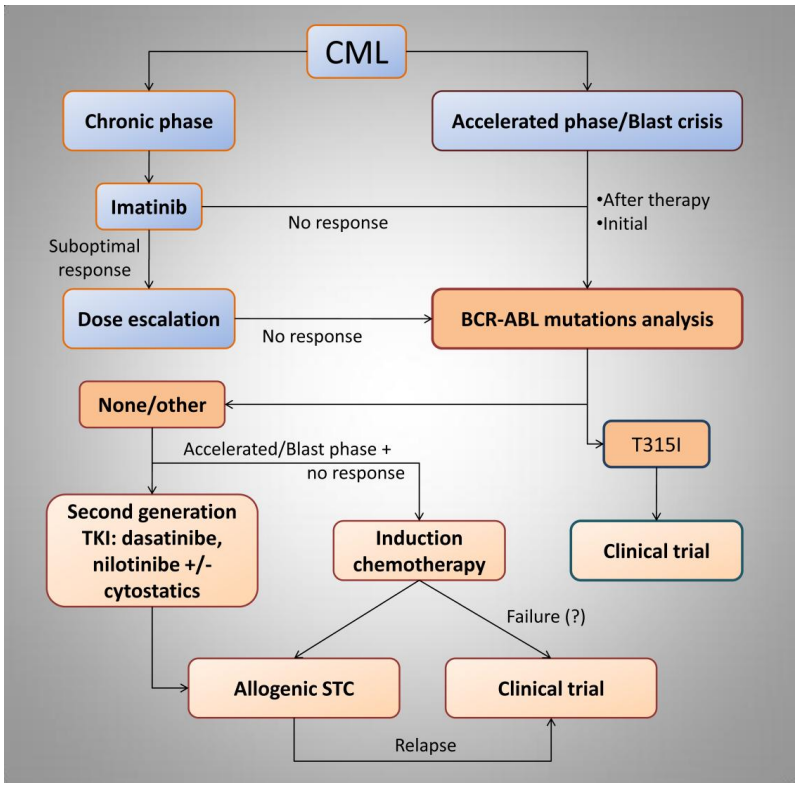


Figure 12: Clinical algorithm for CML therapy: chronic, accelerated and blast phase (50).

TKI based therapy is a type of targeted therapy used to treat CML. Targeted therapy uses drugs that target a specific or unique feature of cancer cells not generally present in normal cells. Because these drugs specifically target cancer cells, they may be less likely to harm normal cells throughout your body (45). TKIs target the abnormal BCR-ABL protein that causes the overgrowth of abnormal white blood cells (CML cells). The BCR-ABL protein belongs to tyrosine kinase proteins family located on or near the cell surface and control the growth and division of other cells (45). TKIs work in slightly different ways, but the final propose of each is inhibition of BCR-ABL protein activity (49). They are made in the form of a pill that is swallowed and the drug dosage is measured in mg. The FDA approved the first TKI for CML treatment in 2001 (40). Since then, several new TKIs have been developed to treat CML. These newer drugs are mentioned as "second and third-generation" TKIs (45).

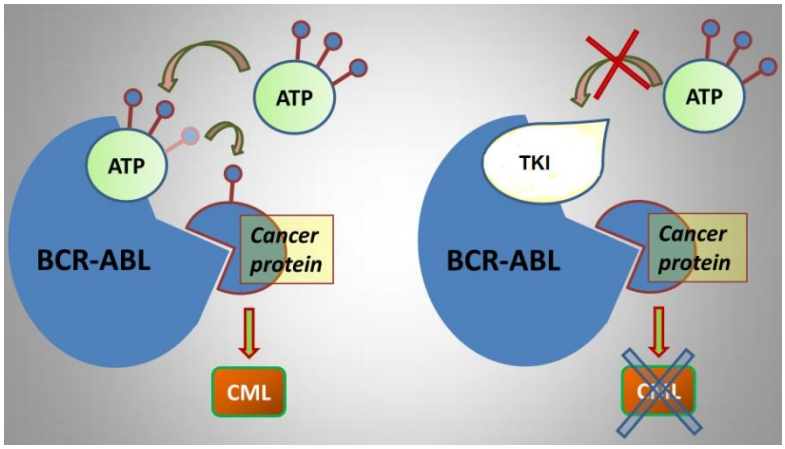


Figure 13: Action mechanism of Imatinib. Imatinib blocks the ATP binding center of BCR-ABL thus inhibiting its phosphorylation activity (51).

Imatinib

Imatinib mesylate (STI 571, Glivec®) is the most successful synthetic ATP inhibitor designed, first being approved by Food and Drug Administration (FDA) in the United States, later licensed for use in the United Kingdom by the European Medicines Evaluation Agency (EMEA) (40,49). Arise of Imatinib has dramatically changed the life style and survival of CML patients. Currently, Imatinib is considered as the "gold standard" in treating CML, approved for the first line treatment of adult patients at all disease stages (1).

Imatinib belongs to the "first generation" TKIs and was the first drug order to CML patients (49). As a mimic molecule, the drug will block the interaction between the tyrosine kinase and ATP, avoiding the phosphorylation of potential subtracts, thus Imatinib competes with ATP for the ATP-binding site on BCR-ABL protein (1,40,45). This mechanism decline the constitutive activity of the BCR-ABL protein, blocking some of the uncontrolled pathways characteristic of the $BCR\text{-}ABL^{+}$ leukemic cells, furthermore decrease the metabolic features of CML (1).

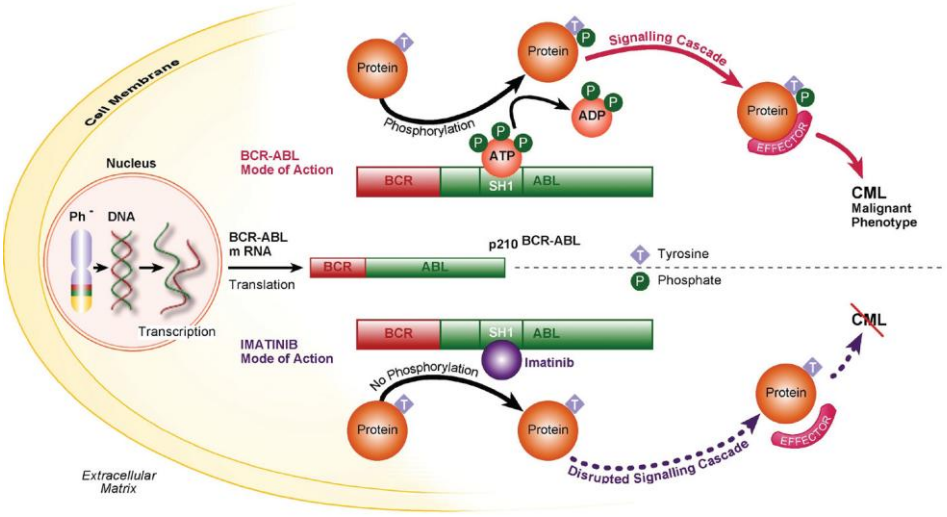


Figure 14: Comparing the mode of action of BCR-ABL and Imatinib in CML pathogenesis. Imatinib functions as a mimic of ATP, in the ATP binding pocket in the BCR-ABL SH1 domain. A further characteristic of Imatinib is its striking degree of specificity for the ATP binding pocket, as its effect on other cellular tyrosine kinases is negligible (46).

Imatinib Resistance

Although the remarkable efficacy of Imatinib in treating CML, a minority of patients do not respond to the therapy at all or in some cases the patients develop resistance to the drug. This involves the occurrence of resistant leukemic clones after continued drug therapy. Commonly, acquired resistance is caused most by mutations in the BCR-ABL kinase domain, preventing successfully Imatinib binding (49,51). Other resistance mechanisms could occur, outside the domain, like over expression of BCR-ABL gene, acquired additional mutations, clonal evolution (chromosomal aberrations), and pharmacological mechanisms. These kind of structural or biochemical modifications affect, direct or indirectly, the linkage between the drug and the kinase domain, reducing the Imatinib effect in the mutated cells (49).

2nd and 3RD Generation Tyrosine-Kinase Inhibitors

With the new highlights in target therapies the Imatinib became the support for CML treatment (52). Despite the really good results obtained some patients discontinue therapy in long-term due to failure and or intolerance (52). In order to overcome these limitations new drugs emerged, second and third-generation TKIs.

Second-generation TKIs are like an upgrade of the first generation TKIs, they are similar but the new ones have expanded inhibition against a broad spectrum of mutations resistance to Imatinib. Nilotinib **(**AMN107, Tasigna®), Dasatinib **(**BMS-354825, Sprycel®) and Bosutinib (SKI-606, Bosulif®) have demonstrated in vitro and in vivo activity against different types of mutations and various forms of resistance (52). However, some patients carry the T3151 mutation and don't obtain better results with these drugs, but a third generation inhibitor ponatinib (AP24534, Iclusig®) was tested and hopeful results (52).

Chronic myeloid leukemia is a slowly progressing blood and bone marrow disease that usually occurs during or after middle age, and rarely occurs in children. CML is a genetic disease that affects most directly the myeloid lineage, an abrupt increase in granulocytes is a hall mark for this specific leukemia (51). The NK cells belong to the lymphoid blood lineage and although the higher percentage of autologous NK cells in chronic phase CML are not derived from the malignant clone, some studies report that NK cells are reduced in numbers and have limited cytolytic capacity. NK cell function in untreated patients with chronic phase CML has not been systematically addressed (53).

Furthermore, treatment with TKIs may have additional effects on cellular immune function. Tyrosine kinase receptors are involved in multiple cellular processes and are critical in tumor development and progression (52). Recent observations indicated that TKIs have inhibitory and/or stimulatory effects on immune cells, via modification of markers in surface of tumor cells (1). Some reports focused on NK Cells show that Imatinib have no impact on NK cytotoxicity or cytokine production, whereas nilotinib negatively influenced cytokine production and Dasatinib additionally abrogated cytotoxicity *in vitro* (52,53).

Table 1: Overview on the impact of TKI agents on NK cells (43,52,54,55).

TKIs

Imatinib	• DC stimulation leading to increased NK cell activity in human and mice. • Increased NK cell numbers post therapy.
Nilotinib	• No impact on cytotoxicity and cytokine production *in vitro.*
Dasatinib	• Inhibition of cytotoxicity in mice. • Inhibition of cytotoxicity toward K562 and cytokine production.

Understanding how the immune system affects cancer development and progression has been one of the most challenging questions in immunology. Now appreciate that the immune system plays a dual role in cancer: It can not only suppress tumor growth by destroying cancer cells or inhibiting their outgrowth but also promote tumor progression either by selecting for tumor cells that are more fit to survive in an immunocompetent host or by establishing conditions within the tumor microenvironment that facilitate tumor outgrowth (24,56). In the last decades researchers have reported that cancer development and progression is highly affected by the host immune system through a process termed cancer immunoediting (56). Cancer immunoediting is a three-step strategy that consists in elimination (host immune cells destroy immune cells), equilibrium (residual tumors persist but their growth is under control of host immunity) and escape (outgrowth of tumor cells with reduced immunogenicity and/or increased capacity to attenuate or subvert host immunity) (24,56). Based on this the immune system starts to be a powerful target for tumor elimination.

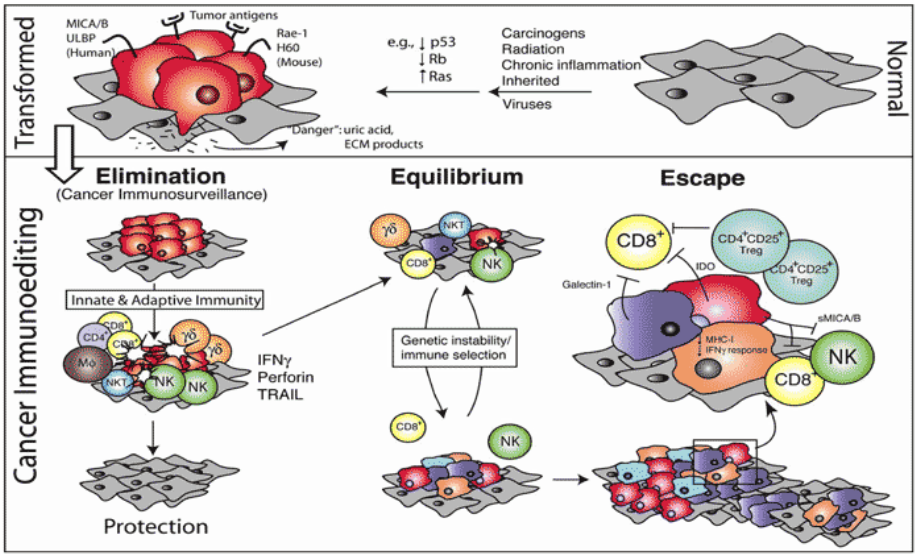


Figure 15: Cancer immunoediting: host protective versus tumor sculpting actions of immunity. Following cellular transformation and the failure of intrinsic tumor suppressor mechanisms, a developing tumor is detected by the immune system and its ultimate fate is determined by whether or not it is eliminated by the host protective actions of immunity (Elimination phase), maintained in a dormant or equilibrium state (Equilibrium phase) or escapes the extrinsic tumor suppressor actions of immunity by either becoming non-immunogenic or through the elaboration of immunosuppressive molecules and cells (Escape phase) (24).

The immunotherapy principle lies on stimulate and/or restore our immune defenses, in general these science fall into major fields, antigen-targeted immunotherapy and immunomodulatory immunotherapy (57). In antigen-targeted immunotherapy, tumor-associated antigens are introduced into the patient as a vaccine to elicit an immune response that targets the tumor. In immunomodulatory immunotherapy, the immune system, presumably primed by endogenous tumor-associated antigens, is potentiated either by blocking inhibitory immune effectors or by triggering immune activators. Both strategies qualify immune cells to become activated and target the tumor (57).

Focus on NK Cells

As long as the immune system emerged as a source of many hopes for cancer treatment, the researchers had focused their attention in immune cells self-defense mechanisms, especially and more recently in NK Cells (56). NK cell immunotherapy is rapidly developing since simple methodologies like manipulation of NK cell activity using antibodies or interleukins, or more complex tasks like engineering gene-modified NK Cells (37).

Modulation of NK cell activity

Antibodies – Use of antibodies to block inhibitory receptors can mimic the missing self-environment, in order to augment NK cell cytotoxicity. Recently, the development of human monoclonal antibodies that prevent signaling via KIR2DL1, KIR2DL2 and KIR2DL3 was developed, monoclonal antibody 1-7F9 cross-reacts with KIR2DL1, KIR2DL2 and KIR2DL3 to increase NK cell-mediated lysis of tumors expressing HLA-C (37).

Interleukins – Il-2 activation of NK cells can promote cytotoxic activity against targets that were previously NK cell resistant. NK cell co-cultured with fresh tumor cells have also proved that IL-2 activation in vitro enhances the killing potential of NK cells. Il-15 is another promising subject for immunotherapy, as it provides NK cells, CD8+ T cells and NKT cells activation (37).

NK cell Adoptive Therapy – NK cells can be isolated, expanded or produced in vitro to be used either in autologous or an allogeneic setting. It is clear that short-term

activation is not sufficient for increase NK cell count or NK cell cytotoxicity, but a long-term infusion of ex vivo activated cells may present a benefit (37).

Gene modification of NK cells – Genetically modified NK cells hold great potential for cancer immunotherapy. Certain tumors, such as carcinomas lacking MHC class I, are resistant to NK cell killing. Hence, different methodologies have been developed to modify genetically these immune cells (37).

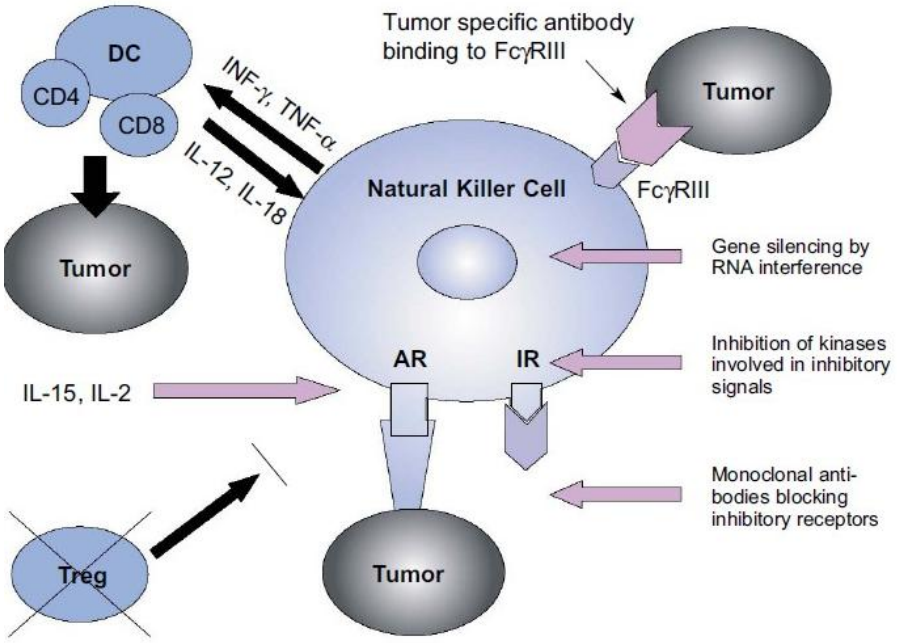


Figure 16: Strategies to modulate the NK cell activity and augment their anti-tumor effect. AR, activating receptors; IR, inhibitory receptors; DC, dendritic cell; IL, interleukin; Treg, T regulatory cell (58).

Aims

NK killer cells are innate lymphocytes capable of mediating multiple effector functions. NK cells express a number of receptors through which they can recognize microbial products, can sense transformed cells or even mediate regulatory functions by cytokine release. Specialized NK subsets display distinct functions according to their typical cell surface phenotype. Namely, the $CD56^{bright}CD16^{-}$ NK cell subset that present a more regulatory character and the $CD56^{dim}CD16+$ NK cell subset that displays cytotoxicity and also cytokine production in response to stimuli. In the last decade several studies have been done in order to understand the immunobiology of these cells and several studies indicate that NK cells have an important role in immune response (59). In chronic diseases like CML the immune response is impaired and NK cell dysfunction could also affect the immune system. In addition, a variety of reports have shown that chronic infections and some types of cancer negatively affect the NK cell function (26,59).

With this entire brand new knowledge, the importance of understand NK cells have been drastically increased. In the context of our project some researchers reported that NK cells are reduced in CML patients and other ones demonstrated that the dosage and the type of TKI could directly or indirectly affect NK cells (1,52,53). Thus, characterize the NK cells of CML patients is the main goal of our study, in order to understand how these cells behave and how we can modulate its activity in order to increase the effectiveness of therapies that are in vogue today for CML. To achieve this complex goal we focused our report on three objectives: study the immunoprofile of CML patients NK cells; study the effects of different

TKIs at different doses; study the role of $BCR^{-}ABL^{+}$ leukemic cells in NK cells populations.

Material & Methods

Object of study

All samples used in this research were collected in heparin tubes from Chronic Myeloid Leukemia patients (n=62) at Centro Hospitalar e Universitário de Coimbra for further blood samples analysis at the Laboratório de Imunologia e Oncologia, Centro de Neurociências e Biologia Celular, Universidade de Coimbra. The patients concerned are being treated with different drugs (Imatinib n=40, Dasatinib n=6, Ponatinib n=1) and within who are taking Imatinib dosing also varies (400mg/day n=17, 600mg/day n=10 and 800mg/day n=13), besides that some of them are taking combination therapy (IFN-α + Imatinib 400/600mg/day n=5) or only IFN-α (n=4) or Cytarabine (Ara C, cytosine arabinoside; n=1). We also have the chance to include in this study two naive patients, some patient's therapy is unknown.

This study was previously approved by Ethnics Committee of the Faculty of Medicine of the University of Coimbra.

Sample Handling

The samples were all treated equally, 100uL of whole blood in was taken in cytometry tube and extracellular antibodies were added, then 15 minutes incubation in the dark at room temperature. After incubation 2mL of BD Lysis buffer was added, the tubes were vortex and incubated for more 10 minutes in the same conditions. Following the tubes were centrifuged at 314g for 5 minutes, supernatant was discarded and 2mL of PBS 1x was added and next tubes were centrifuged in the same way. The final steps before the tubes are taken to the flow cytometry was to discard the supernatant and add 300uL of PBS 1x. In case of intracellular labelling the protocol is similar, but the intracellular antibodies were only added after fix and permeabilize the cellular membranes with two unlike solutions. Then tubes were incubated in the dark for 20 minutes. After incubation 2mL of PBS 1x was added and the following steps are identical to the extracellular labeling protocol. The lymphogram were performed in the same way for extracellular labelling, except the volume of blood 50uL instead of 100uL and the

Lymphogram™ kit from Cytognos S.L was used., a 3-color mixture of monoclonal antibodies to determine the major lymphocyte subpopulations, including total T-cells (CD3+), B-cells (CD19+) and NK-cells (CD3- CD56+) as well as helper/inducer (CD3+ CD4+) and suppressor/cytotoxic (CD3+ CD8+) T-cell subsets.

Flow Cytometry Analysis

All the samples were performed according to type of labelling, after that all marked tubes were read and analyzed on flow cytometer – FACS CANTO II from BD Biosciences with FACS Diva software version 6.1.3. A panel of fluorochrome-labeled antibodies was used in order to identify and characterize specific NK cell subsets. This panel includes CD3-PB (clone UCHT1), CD3-PerCP/Cy5.5 (clone HIT3a), CD14-PerCP/Cy5.5 (clone M5E2), CD19 PerCP/Cy5.5 (clone HIB19), CD56-PE/Cy7 (clone HCD56) and CD16-APC/Cy7 (clone 3G8) which allowed specific NK cell subset identification. A combination of monoclonal antibodies was selected for characterization as follows: CD4-V500 (clone RPA-T4), CD8-APC (clone HIT8a), CD8-APC/Cy7 (clone SK1), CD7-PE (clone CD7-6B7), CD57-PB (clone HCD57), CD27-FITC (clone O323) , CD11b-PB (clone ICRF44), CD62L-FITC (clone DREG-56), CD69-FITC (clone FN50), CD137-APC (clone 4B4-1), CD137L-PE (clone C65-485),CD94-FITC (clone DX22), NKp46-PE (clone 9E2), NKp44-PE (clone P44-8), NKp30-PE (P30-15), NKp80-PE (clone 5D12), NKG2A-PE (clone 16A11), NKG2C-PE (clone 134591), NKG2C-APC (clone 134591), CD158a-PE (clone HP-MA4), CD158b-PE (clone DX27), IFN-γ-FITC (clone 4S.B3), TGF-β-FITC (clone TW4-6H10) and IL-4-FITC (clone 8D4-8), IL-10-FITC (clone JES3-9D7), TNF-α-PE (clone MAb11), 1F12-FITC (clone 1F12H7A2), 8C11-FITC (clone 8C11F11D4), CD279-APC (clone MIH4), CD274-FITC (clone MIH18), CD273-PE (clone MIH18), CD226-FITC (clone TX24), CD96-PE (clone NK92,39), CD155-PE (clone TX24) and TIGIT-APC (clone IgG2B). All antibodies used were purchased from Biolegend (San Jose, CA, USA).

Data Analysis

FlowJo® (Tree Star Inc, Ashland, USA) is powerful software that allows analyzing flow cytometry data on a computer. In the present study the FlowJo version 10 was used, with this software all the samples added to the "Worksheet" frame were hierarchical organized and analyzed according to their lineage and surface or intracellular markers.

Statistical Analysis

GraphPad Prism® is commercial scientific 2D graphing and statistics software published by GraphPad Software, Inc (CA, USA). This informatics program combines scientific graphing, comprehensive curve fitting (nonlinear regression) understandable statistics, and data organization. All the statistical analysis was performed by GraphPad Prism version 6 using one-way or two-way ANOVA with Dum or Bonferroni posttests, respectively. Statistically significant *p*-values are addressed as follows: * $P<0.05$, ** $P<0, 01$, *** $P<0.001$ and **** $P<0.0001$.

Results

Lymphocytes and their subpopulations in CML

To elucidate about the relative frequencies of lymphocytes major populations in CML patients, blood samples were analyzed by flow cytometry. For cellular labelling a Lymphogram kit was used in order to identify and calculate the percentage of B cells, T cells and NK cells as well as CD4+ and CD8+ cells. The lymphogram kit is a combination of 5 antibodies in 3 different fluorescences, CD3/CD56 –FITC, CD19/CD8 – PE and CD4 – PerCPCy.5. First, total lymphocytes were selected according to SSC and FSC know characteristics (figure 17.A). After total lymphocytes selection, B cells, T cells and NK cells were distinguished based on, respectively, CD19, CD3 and CD56. These three markers are specific for its population (figure 17.B). Then, within T and NK cells we selected the CD4 and CD8 positive cells (figure 17.C/D).

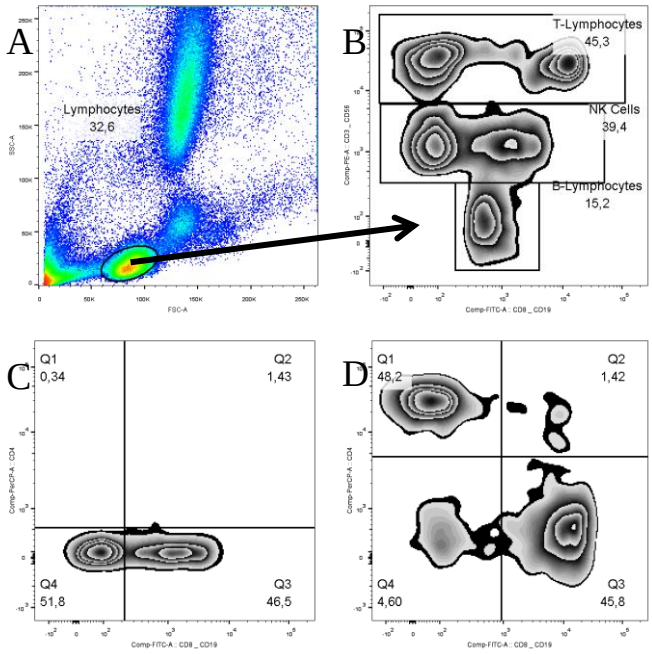


Figure 17: Representative gating strategy. A – Representative dot plots of total lymphocytes; B – Representative dot plots of B cells, T cells and NK cells; C – Representative zebra plot of CD4 and CD8 positive cells on NK cells; D – Representative zebra plot of CD4 and CD8 positive cells on T cells.

Fifty-five blood samples (four from healthy donors, two from naïve CML and forty-nine from treated CML) were analyzed by flow cytometry and then the relative frequencies of each cell type was calculated by FlowJo software. Representative graphs were made by GraphPad software.

Relative frequency of total lymphocytes is decreased in CML patients.

Patients on therapy have higher percentage of lymphocytes than at diagnosis, however didn't achieve the values of lymphocytes from healthy donors (figure 18.A). However the amount of lymphocytes/µL collected from CML patients on treatment is decreased in comparison with those collected at diagnosis (figure 18.B).

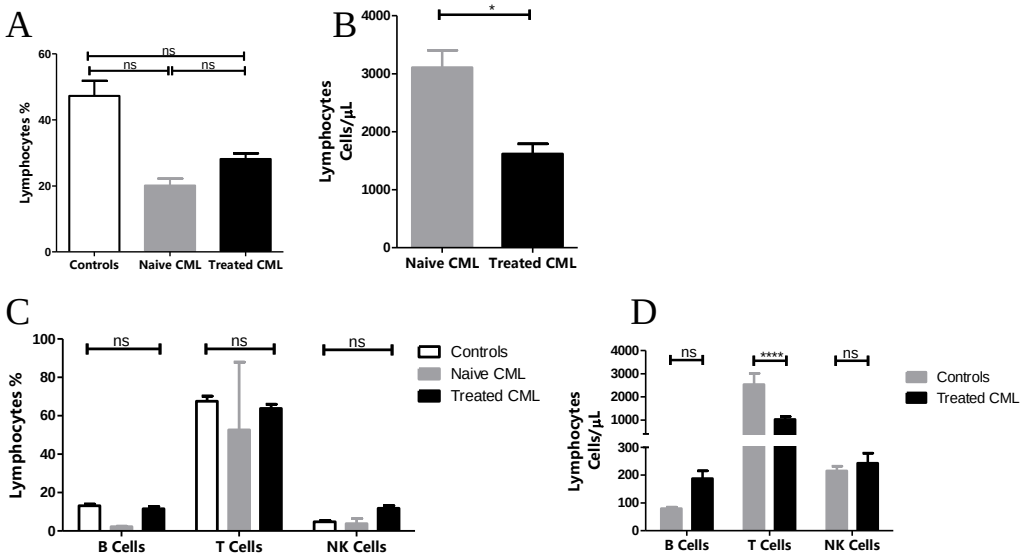


Figure 18: Relative frequencies of lymphocytes in CML patients. A – Percentage of lymphocytes from PBMCs in healthy donors (n=4), naïve (n=2) and CML patients (n=49). B – Absolute numbers of lymphocytes in peripheral blood of naïve (n=2) and CML patients (n=20). C – Analysis of lymphocytes major populations, B cells, T cells and NK cells from PBMCs of healthy donors (n=4), naive (n=2) and CML patients (n=38). D – B cells, T cells and NK cells absolute numbers in healthy donors (n=4) and CML patients (n=20).

Relative frequency of T, B and NK cells at diagnosis is lower than in healthy individuals.

After gating the lymphocytes population with the combination of antibodies used allows separate the three big families of lymphocytes. B cells ($CD19^+CD3^-CD56^-$), T cells ($CD3^+CD56^-CD19^-$) and NK cells ($CD56^+CD3^-CD19^-$) are selected based on density of their specific markers. The 3 cell types are reduced in blood samples collected at diagnosis and with therapy these values recover to normal standards, according to controls (figure: 18.C). Individuals who are receiving treatment appear to have a higher percentage of NK cells in relation to the amount of total lymphocytes; however the absolute numbers of NK cells from CML patients are lower than NK cells from healthy donors on average (figure 18.D). The absolute numbers of CML B cells

are lightly higher than from controls and the CML T cells are drastically reduced (figure 18.D).

CD4 and CD8 expression on T and NK cells surface is altered in naïve and CML patients.

With the populations of interest rightly identified another parameters were analyzed. The combination of Abs in the lymphogram kit has CD4 and CD8 besides the specific markers, allowing the study of CD4 and CD8 positive cells contribution. In naïve T^-Lymphocytes the CD4+ subpopulation is decreased in terms of lymphocyte percentage (figure 19.A). Although the CD8 subset of individual without treatment is increased relatively to controls and in individuals undergoing treatment this percentage decrease to normal values (figure 20.A), the absolute numbers of CD4+ and CD8+ T cells from CML patients are lower than those from naïve (figure 19.B). Moreover CML patients have a huge decrease in CD8+ T cells with statistically significance.

In NK cells from CML patients the percentage of CD4 positive cells is decreased but its absolute numbers are enlarged at diagnosis, after treatment these values tend to decrease to minimal levels (figure 19.C/D). The percentage of CD8+ NK cells is reduced at diagnosis and it is augmented in treated patients, yet CD8+ NK cells absolute numbers are ^{dim}inished in treated CML cells (figure 19.C/D).

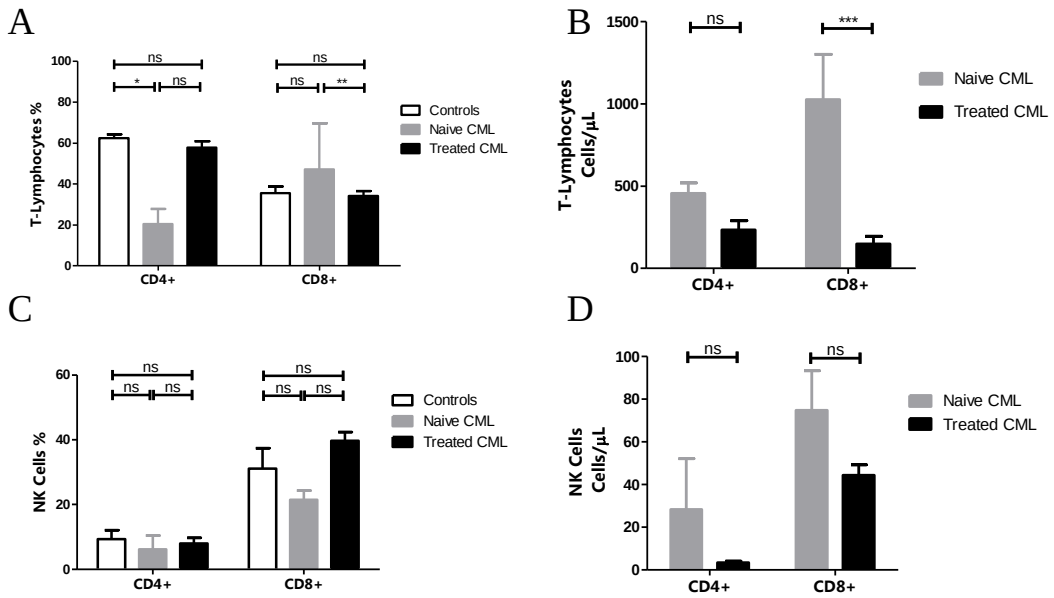


Figure 19: Relative frequencies of CD4+ and CD8+ in lymphocytes and NK cells. A – Frequencies of CD4+ and CD8+ T^-Lymphocytes. B – Absolute numbers of CD4+ and CD8+ T^- Lymphocytes in peripheral blood. C – Frequencies of CD4+ and CD8+ NK cells. D – Absolute numbers of CD4+ and CD8+ T^- NK cells in peripheral blood. Controls (n=4); Naïve CML (n=2); Treated CML (n=34).

Immunoprofiling of NK cells from CML patients

$CD56^{bright}CD16+$ and $CD56^{dim}CD16+$ NK cells are until now the two more distinct subsets of NK cells. Based on this and in the previous knowledge about their intrinsic functionalities whole this study is developed around $CD56^{bright}$ and $CD56^{dim}$ cells rather than looking at the overall NK cells.

All the experiments are performed at the same way as explained in the chapter before. After load the samples in cytometer data results are analyzed by informatics software (FlowJo), at the early beginning the gating strategy is the same (figure 20). First of all lymphocytes are selected based on forward/scattered properties, and then the gated population is displayed in a CD56/ (CD3CD14CD19) plot which avoid NK cell identification (CD56+ $(CD3CD14CD19)^-$). Identified NK cells are divided in CD56brigh and $CD16^{dim}$ NK cells according to their expression of CD56 and CD16.

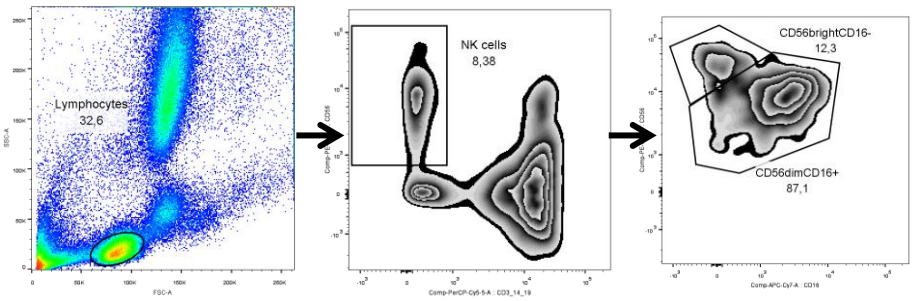


Figure 20: Gating strategy for CD56brighCD16- and CD56dimCD16+ NK cells from CML patients. From left to right, representative dot plot of lymphocytes, next representative zebraplot of NK cells followed by representative zebraplot of $CD56^{bright}CD16^-$ and $CD56^{dim}CD16+$ NK cells.

NK cells are increased in CML patients undergoing treatment.

NK cells from healthy donors are 6.1% of lymphocytes on average and NK cells from naïve patients (which mean that the samples are collected at diagnosis) represent a similar rate. Treated CML patients have a relative increase on NK cells percentage,

although without statistical significance (figure 21.A). Besides that NK cell absolute count increase from healthy donors to naïve patients and then with the higher rate the treated patients (figure 21.B).

The proportion of CD56^{bright}CD16^{-} and CD56^{dim}CD16+ NK cells is independent of the disease.

In healthy donors, naïve and in CML patients the relative frequency of the CD56brigh and CD56^{dim} cells doesn't significantly change. The CD^{bright}CD16^{-} subset represents around 7% of total NK cells whiling the CD56^{dim}CD16+ subset represents around 90% in all three groups (figure 21.C).

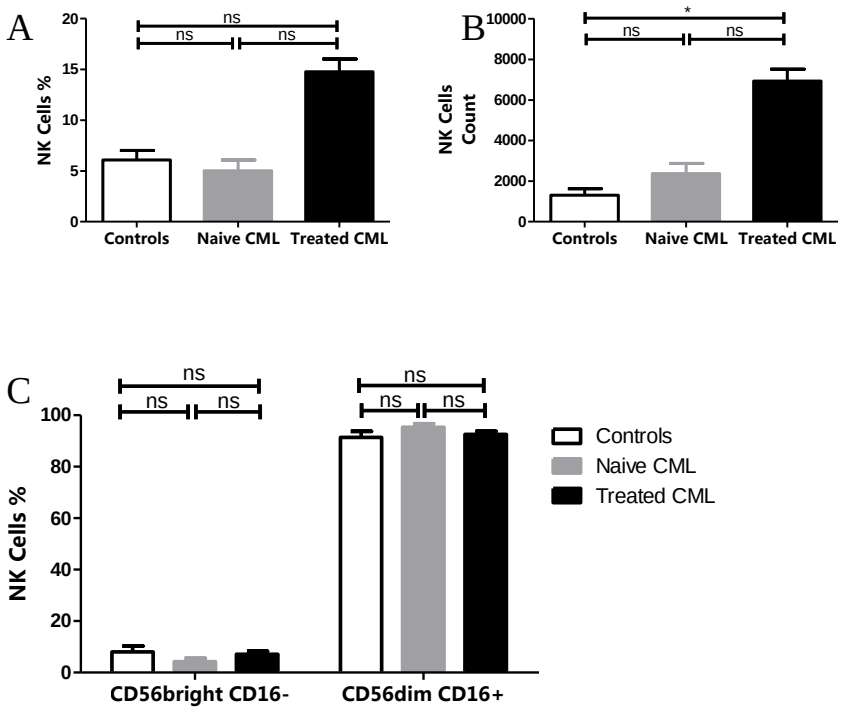


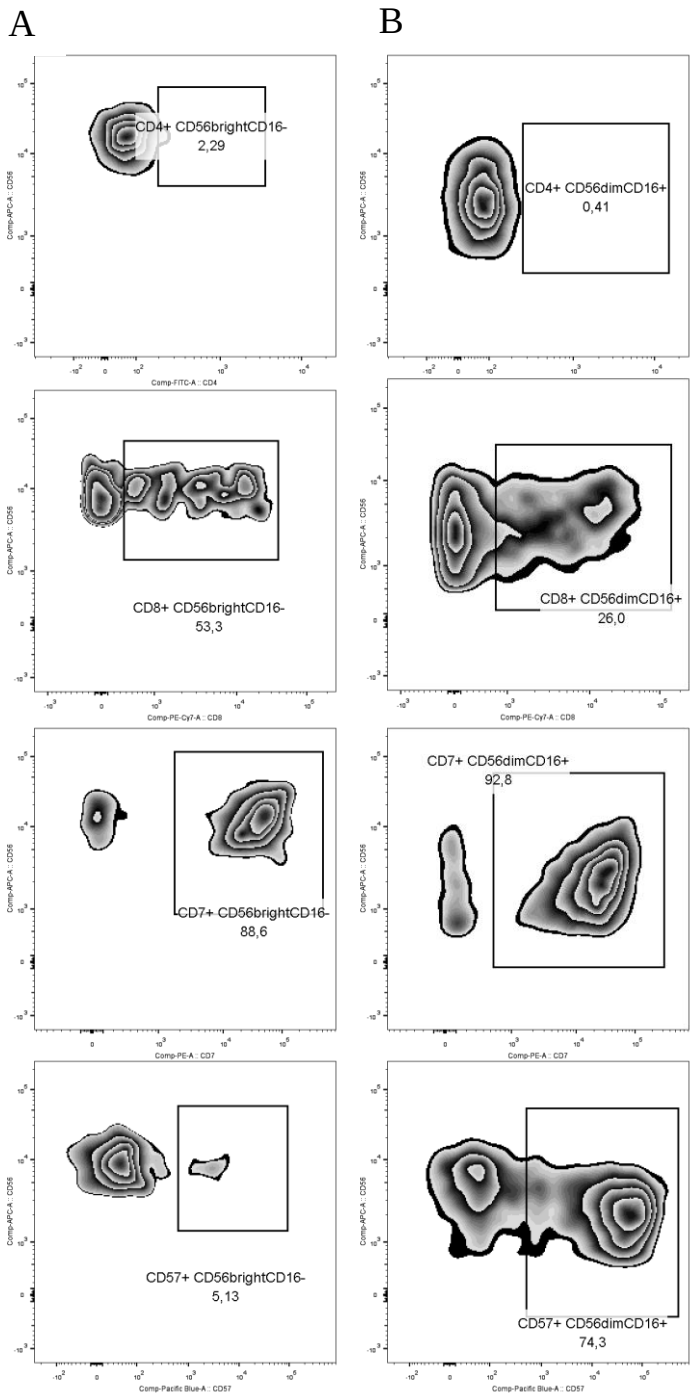
Figure 21: Relative proportions of NK cells and their subpopulations. A – NK cells percentage from all lymphocytes in healthy donors (controls), naïve and CML patients based on positive selection for CD56 marker and negative selection for CD3 marker. B – NK cells

absolute count to each group. C^- $CD56^{bright}CD16^-$ and $CD56^{dim}CD16+$ NK cells percentage also for each group. Controls (n=4); naïve (n=2); treated CML (n=49).

Surface markers, receptors and cytokine production are crucial factors for effectiveness of NK cells. In CML patients some reports show that NK cells are affected by leukemic cells in this line we selected a few markers of interest in order to address some alterations in CML patients that could be involved with the low activity of NK cells against leukemic cells in some patients.

Expression of cell surface markers by CD56brightCD16- and CD56dimCD16+ NK cells in CML patients

After $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells identification we analyzed other parameters. With extracellular labelling for multiparametric flow cytometry a variety of markers can be evaluated simultaneously, so information about cell surface markers is provided. The frequency of positive labelling for CD4, CD8, CD7, CD57, CD27, CD11b CD62L and CD69 were studied and analyzed by informatics software described on chapter II (figure 22). The samples were divided into the same 3 groups as previous, healthy donors (controls), naïve (naïve CML) and CML patients (treated CML).


A
B
CD4+ CD56brightCD16-
2,29
CD4+ CD56dimCD16+
0,41
CD8+ CD56brightCD16-
53,3
CD8+ CD56dimCD16+
26,0
CD7+ CD56brightCD16-
88,6
CD7+ CD56dimCD16+
92,8
CD57+ CD56brightCD16-
5,13
CD57+ CD56dimCD16+
74,3

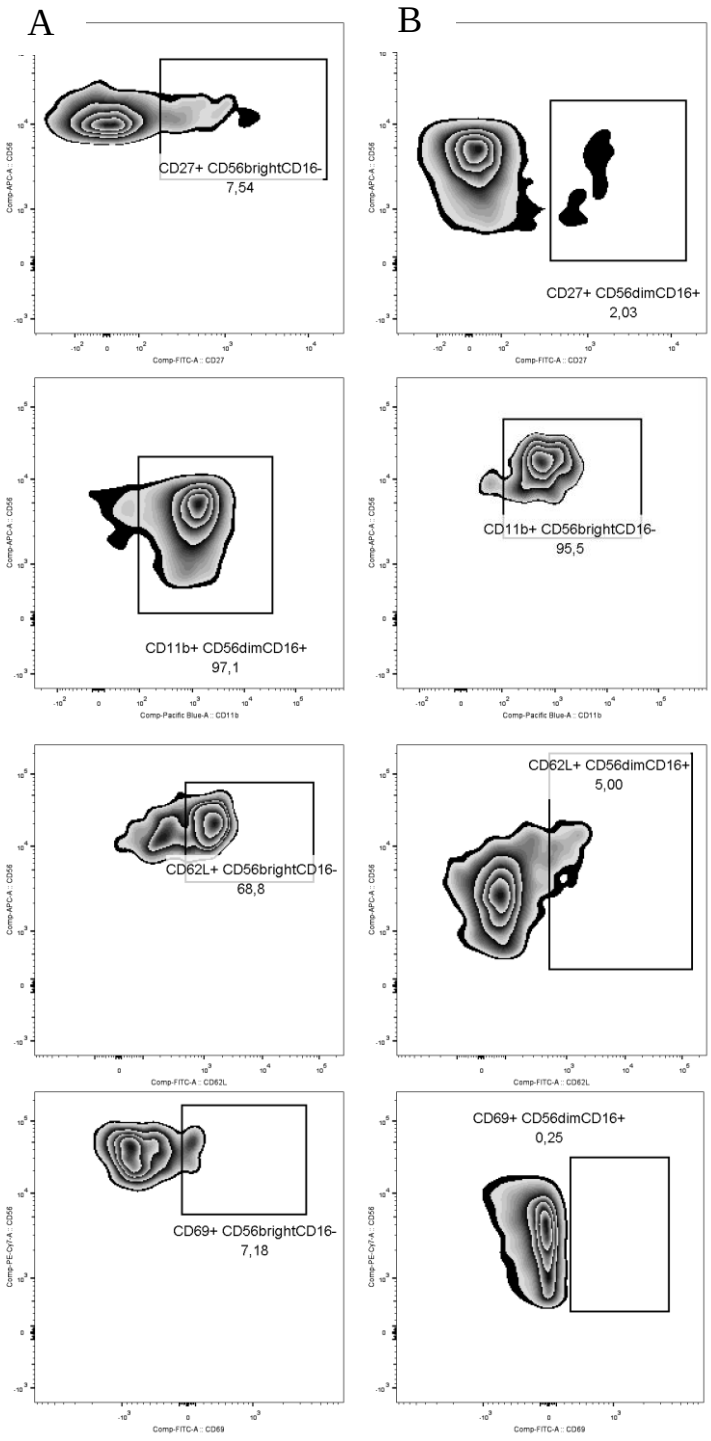


Figure 22: Expression of cell surface markers on CD56brighCD16^{-} and CD56dimCD16+ NK cells, from CML patients. A – Representative zebraplots of positive labelling of CD4, CD8, CD7 and CD57, CD27, CD11b, CD62L and CD69 in CD56^{bright}CD16^{-} NK cells (up to down). B^{-} Representative zebraplots of positive labelling of same markers in CD56^{dim}CD16+ NK cells.

After $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16+$ NK cells identification we analyzed other parameters. With extracellular labelling for multiparametric flow cytometry a variety of markers can be evaluated simultaneously, so information about cell surface markers is provided. The frequency of positive labelling for CD4, CD8, CD7, CD57, CD27, CD11b CD62L and CD69 were studied and analyzed by informatics software described on chapter II (figure 22). The samples were divided into the same 3 groups as previous, healthy donors (controls), naïve (naïve CML) and CML patients (treated CML).

Relative frequency of $CD4^{+}CD56^{bright}CD16^{-}$ NK cells is reduced in CML patients and the percentage of $CD8^{+}$ $CD56^{bright}CD16^{-}$ NK cells is higher in CML patients.

CD4+ $CD56^{bright}CD16^{-}$ cells are null in naïve patients and in those who undergoing treatment is a little higher but even so don't achieve healthy donors percentage (figure 23A). The MFI results displayed higher fluorescence in cells from naïve and CML patients than for controls both in $CD56^{bright}$ and $CD56^{dim}$ NK cells (figure 23.B). CD8+ $CD56^{bright}CD16^{-}$ NK cells percentage is augmented in naïve and CML patients, being the higher percentage in patients doing therapy. The $CD8+CD56^{dim}CD16+$ NK cells percentage is diminished at diagnosis, but in patients under therapy this values reach near controls values (figure 23.C). The MFI for both CD8+ NK cells subsets is increased in CML patients and there not much difference between the values for the controls and naïve (figure 23.D).

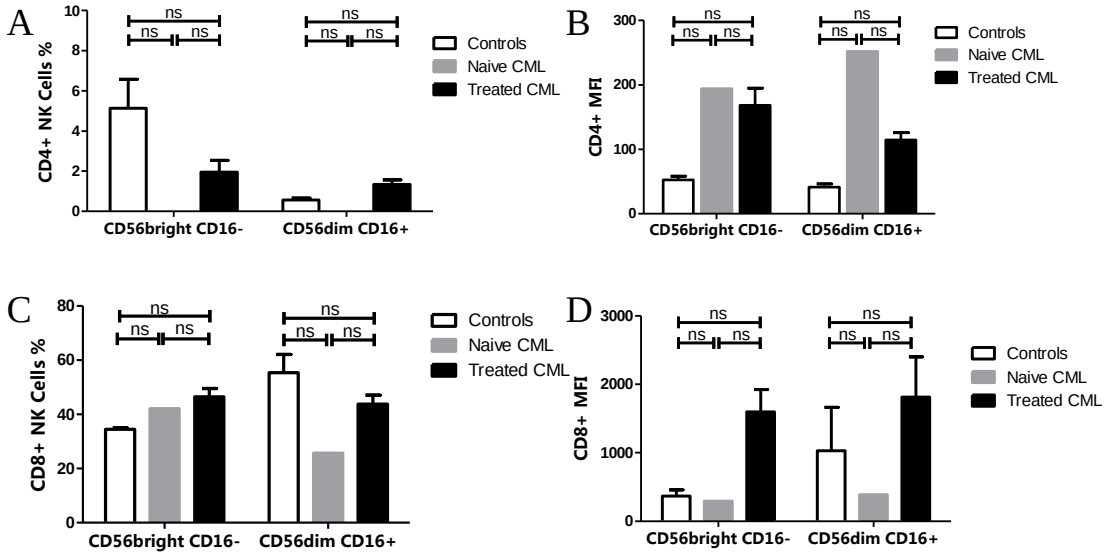


Figure 23: Percentage of CD4+ and CD8+ CD56brightCD16^{-} NK cells and of CD4+ and CD8+ CD56dimCD16+ NK cells from healthy donors (n=4), naïve (n=2) and CML patients (n=41). A – Percentage of CD4+ CD56^{bright}CD16^{-} NK cells and CD4+ CD56^{dim}CD16+ NK cells. B – Mean of fluorescence intensity of CD4 in CD56^{bright} and CD56^{dim} populations. C – Percentage of CD8+ CD56^{bright}CD16^{-} NK cells and CD8+ CD56^{dim}CD16+ NK cells. D – Mean of fluorescence intensity of CD4 in CD56^{bright} and CD56^{dim} populations.

CD56^{bright}CD16^{-} NK cells from CML patients have a higher expression of CD7 per cell and CD56^{dim}CD16+ NK cells from naïve patients have a higher expression of CD57 per cell, in comparison with healthy controls.

With the same strategy as before lymphocyte population is gated, NK cells are identified and then the CD7 and CD57 positive cells are calculated. The percentage of CD7+ cells within CD56^{bright} and CD56^{dim} population approximated for healthy controls, naïve and CML patients (figure 24.A). However, the MFI for CD7 in CD56^{bright}CD16^{-} NK cells is quite elevated, with statistically significance (figure 24.B). The MFI provides information about the fluorescence intensity, as the name says, of certain parameters (markers added to the samples) giving us an idea about the density of each marker per cell, so in this case the surface expression of CD7 by CD56^{bright}CD16^{-} NK cells from CML patients is highly increased. Healthy donors have a very low

percentage of CD57+ CD56^{bright}CD16^{-} NK cells (~2%), the same population in naïve and CML patients is relatively higher (~5%) (figure 24.C). The MFI for CD57 in CD56^{bright} subset is null for the three groups and the MFI of CD57 in CD56^{dim} subset is drastically increased in naïve group, CML patients have much lower MFI values although greater then healthy donors (figure 24.D).

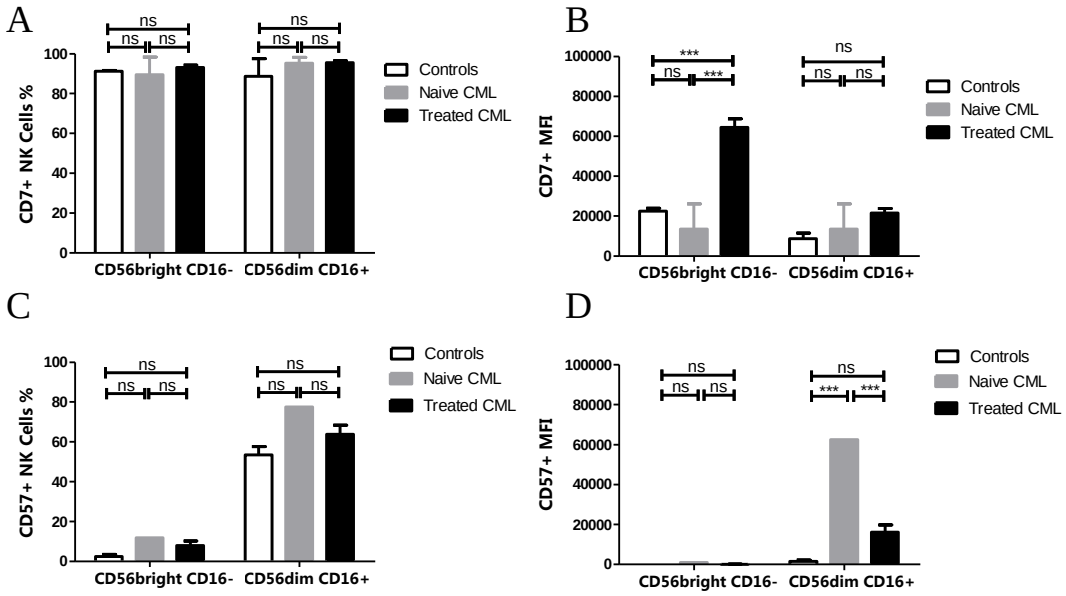


Figure 24: Percentage of CD7+ and CD57+ CD56brightCD16^{-} NK cells and of CD7+ and CD57+ CD56dimCD16+ NK cells from healthy donors (n=4), naïve (n=2) and CML patients (n=20). A – Percentage of CD7+ CD56^{bright}CD16^{-} NK cells and CD7+ CD56^{dim}CD16+ NK cells. B – Mean of fluorescence intensity of CD7 in CD56^{bright} and CD56^{dim} populations. C – Percentage of CD57+ CD56^{bright}CD16^{-} NK cells and CD57+ CD56^{dim}CD16+ NK cells. D – Mean of fluorescence intensity of CD57 in CD56^{bright} and CD56^{dim} populations.

CML patients NK cells have higher expression of CD27 in CD56^{bright}cd16^{-} subset. The percentage of CD11b+ NK cells is augmented in CD56^{bright} subset of CML patients and the surface density of CD11b is higher in both CD56brigh and CD56^{dim} NK cells.

For these two markers only two groups of individuals are taking in account, healthy donors (controls) and CML patients (treated CML), samples of naïve patients were not available. The percentage of CD27 positive cells in CD56^{bright}CD16+ population don't vary from group to group so as in CD56^{dim}CD16^{-} population (figure 25.A). The CD27 MFI is much more superior for CD56^{bright}CD16^{-} NK cells from CML patients (with statistical significance); regarding CD56^{dim}CD16+ NK cells the values are similar (figure 25.B). The percentage of CD11b+ cells is higher in the CD56^{bright} subset from CML patients (with statistical significance), with respect to CD56^{dim} subset there is no relevant difference (figure 25.C). Relative to CD11b MFIs for both NK subsets there is an increased in CML patients comparing with controls (figure 25.D).

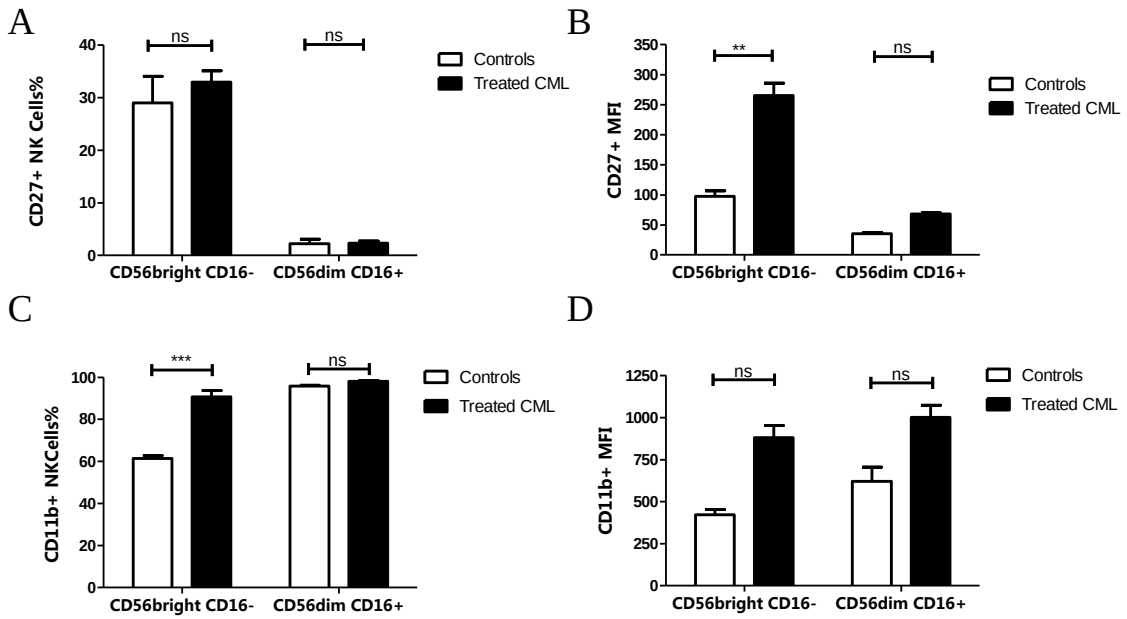


Figure 25: Percentage of CD27+ and CD11b+ CD56brightCD16$^-$ NK cells and of CD27+ and CD11b+ CD56dimCD16+ NK cells from healthy donors (n=4) and CML patients (n=33). A – Percentage of CD27+ CD56^{bright}CD16$^-$ NK cells and CD27+ CD56^{dim}CD16+ NK cells. B – Mean of fluorescence intensity of CD27 in CD56^{bright} and CD56^{dim} populations. C – Percentage of CD11b+ CD56^{bright}CD16$^-$ NK cells and CD11b+ CD56^{dim}CD16+ NK cells. D – Mean of fluorescence intensity of CD11b in CD56^{bright} and CD56^{dim} populations.

In CD56^{bright}CD16$^-$ NK cells, the percentage of CD62L+ and CD69$^+$ cells and their surface density is augmented in CML patients.

As we can see in figure below (figure 26.A) the percentage of CD62L positive cells is augmented in both CD56^{bright} and CD56^{dim} NK cells, notably in CD56^{bright} subset this percentage increase drastically, with statistically significance. The CD62L MFI is also much higher for CD56^{bright} subset of CML patients (with statistically significance), for CD56^{dim} subset the advantage for CML patients is tenuous (figure 26.B). For CD69 positive cells, the two subsets behave differently. CD69$^+$CD56brigh cells reveal lower percentages of CD69$^+$ cells in healthy donors, at diagnosis an increase is notice and

CML patients achieve the highest values (figure 26.C), with statistically significance. In terms of CD69 MFI measurements, CML cells have higher intensities in both subpopulations (figure 26.D).

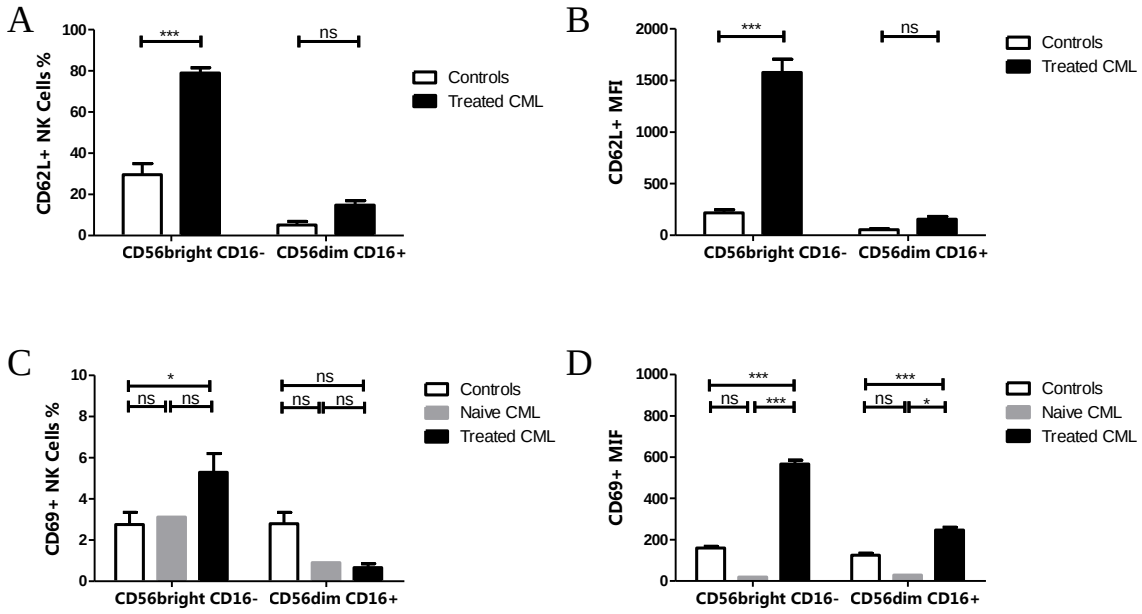


Figure 26: Percentage of CD62L+ and CD69+ CD56brightCD16- NK cells and of CD62L+ and CD69+ CD56dimCD16+ NK cells from healthy donors, naïve and CML patients. A – Percentage of $CD7^{+}$ $CD56^{bright}CD16^{-}$ NK cells and $CD7^{+}$ $CD56^{dim}CD16^{+}$ NK cells. B – Mean of fluorescence intensity of CD7 in $CD56^{bright}$ and $CD56^{dim}$ populations. C – Percentage of $CD57^{+}$ $CD56^{bright}CD16^{-}$ NK cells and $CD57^{+}$ $CD56^{dim}CD16^{+}$ NK cells. D – Mean of fluorescence intensity of CD57 in $CD56^{bright}$ and $CD56^{dim}$ populations. For CD62L samples are collected from healthy donors (n=4) and CML patients (n=43). For CD69 samples are collected from healthy donors (n=12), naïve (n=1) and CML patients (n=30).

The surface density of CD137L and CD137 and the CD137^{+} cell percentage is augmented in both CD56^{bright} and CD56^{dim} NK cells from CML patients.

The CD137L^{+} cells percentage within the two NK subpopulations is similar from healthy to CML individuals, but the naïve samples have lower percentages of CD137L^{+}CD56^{bright}CD16^{-} NK cells (figure 27.A). CD137L MFI increases in naïve and in CML individuals, those who are undergoing treatment have the highest number (figure 27.B). The percentage number of CD137^{+} CD56brigh and CD56^{dim} NK cells is augmented in CML patients, more in ^{bright} then in ^{dim} subset (figure 27.C). The MFI value for CD137 in the same populations is higher for CML patients (figure 27.D).

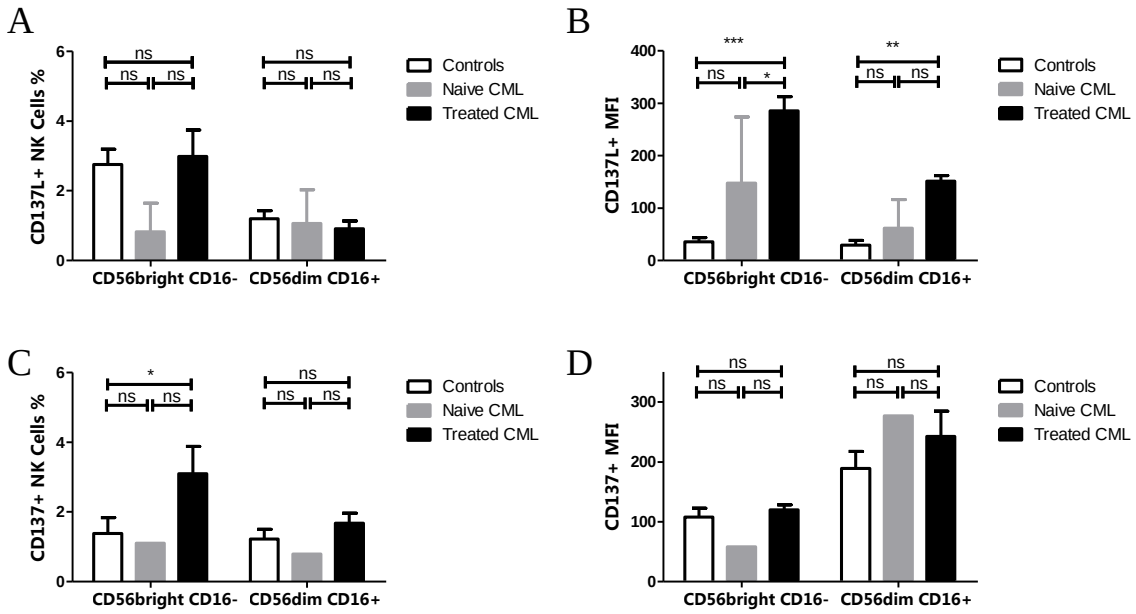


Figure 27: Percentage of CD137L+ and CD137+ CD56brightCD16- NK cells and of CD137L+ and CD137+ CD56dimCD16+ NK cells. From healthy donors (n=4), naïve (n=2) and CML patients (n=20). A – Percentage of CD137L+ CD56brightCD16- NK cells and CD137L+ CD56dimCD16+ NK cells. B – Mean of fluorescence intensity of CD137L in CD56^{bright} and CD56^{dim} populations. C – Percentage of CD137^{+} CD56^{bright}CD16^{-} NK cells and CD137^{+} CD56^{dim}CD16^{+} NK cells. D – Mean of fluorescence intensity of CD137 in CD56^{bright} and CD56^{dim} populations.

The ratio CD137/CD137L in $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells is augmented in CML patients.

By statistical analysis performed by GraphPad Prism we calculated the ratio $CD137^{+}CD56^{bright}CD16^{-}$/$CD137L^{+}CD56^{bright}CD16^{-}$ NK cells and the ratio $CD137^{+}CD56^{dim}CD16^{+}$/CD137L$CD56^{dim}CD16^{+}$ NK cells from both healthy donors and CML patients. The results show that CML patients have an increase in both ratios (figure 28).

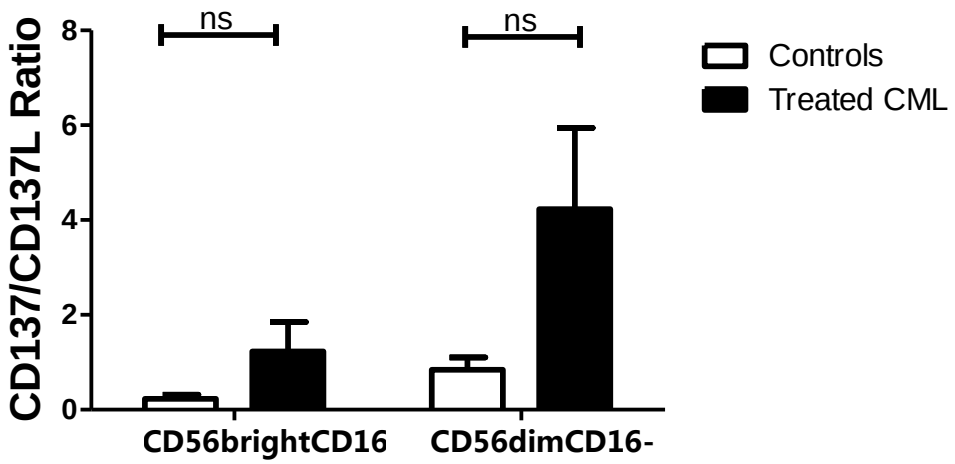


Figure 28: CD137/CD137L ratio in CD56brightCD16- NK cells and in CD56dimCD16+ NK cells, from healthy donors (n=4) and CML patients (n=20).

Receptor repertoire of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells from healthy donors and CML patients

To access information about the receptors expressed by NK cells the same procedure was performed, changing only the surface markers. This study allows us to identify differences in the receptor repertoire of NK cells between healthy controls and CML patients, so in this context we measure by flow cytometry the expression of some KIRs (KIR2DL1, KIR2DL2 and KIR2DS1), C-type lectin-like receptors (NKG2 family and NKp80), and NCRs (NKp30, NKp44, and NKp46).

KIR2DL1, KIR2DL2 and KIR2DS1 expression is altered in CML patients.

The percentage of KIR2DL1, KIR2DL2 and KIR2DS1 is measured by the combination of four markers/Abs CD158a, CD158b, 8C11 and 1F12 as described in (60) and the results that allows us calculate de percentage number of KIRs are in the appendix (figure 29).

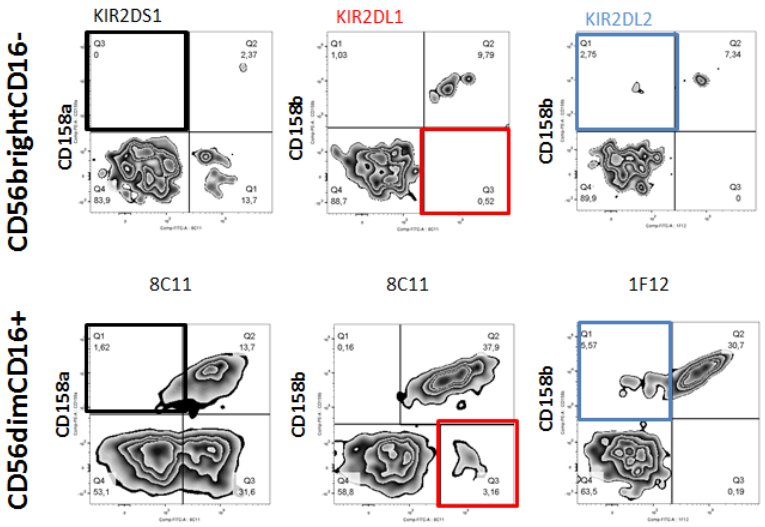


Figure 29: Representative zebraplots of KIR2DS1, KIR2DL1 and KIR2DL2 expression on CD56brightCD16- and CD56dimCD16+ NK cells from CML patients. Multiparametric analysis of CD158a, CD158b, 8C11 and 1F12 markers.

The expression of KIR2DL1 by $CD56^{bright}CD16^{-}$ NK cells is drastically decreased (with statistical difference) in CML patients, whereas in $CD56^{dim}CD16^{+}$ NK cells the KIR2DL1 expression doesn't vary significantly (figure 30.A). KIR2DS1 expression by $CD56^{bright}$ cells is decrease and by $CD56^{dim}$ cells is increased in CML patients comparing with controls (figure 30.B). KIR2DL2 expression is higher in CML patients than in healthy individuals for both NK cells subsets (figure 30.C).

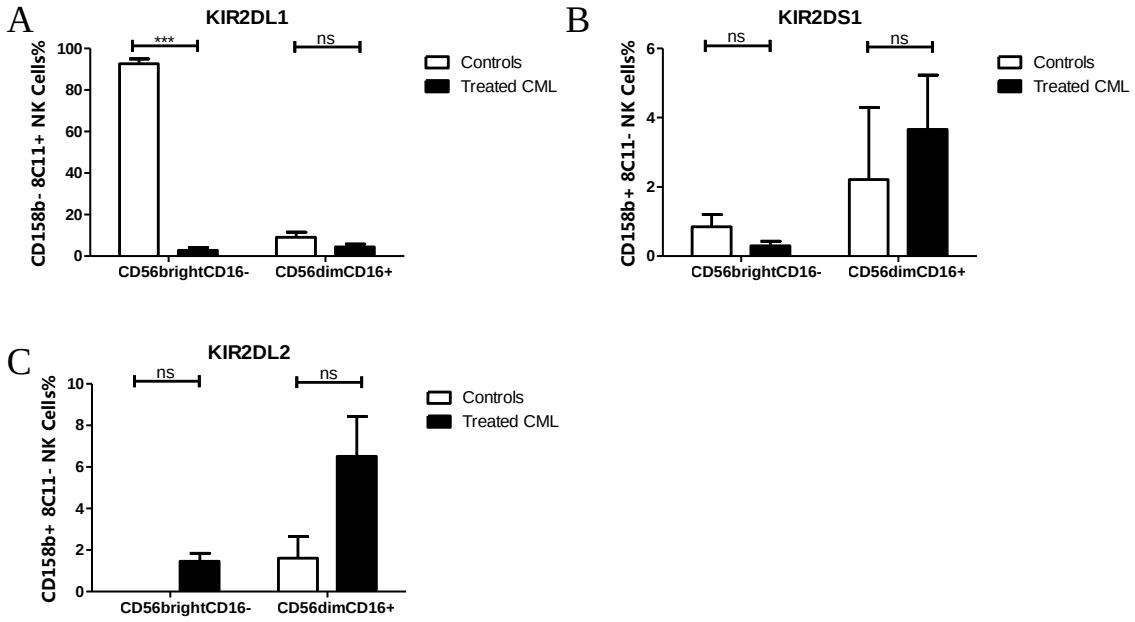


Figure 30: KIR2DL1/L2/S1 expression by CD56brightCD16- and, from healthy donors (n=4) and CML patients (n=10). A – KIR2DL1 expression by $CD56^{dim}CD16^{+}$ NK cells; B – KIR2DS1 expression by $CD56^{dim}CD16^{+}$ NK cells; C – KIR2DL2 expression by $CD56^{dim}CD16^{+}$ NK cells.

The NKG2A expression and surface density on $CD56^{bright}CD16^{-}$ NK cells and the surface density of NKG2C on $CD56^{dim}CD16^{+}$ NK cells is decreased in CML patients.

NK cells express other receptors like NKG2A and NKG2C, the NKG2A is an inhibitory receptor and the NKG2C is an activating receptor. In this study we access to their expression in CML patients (figure 31) and in healthy controls.

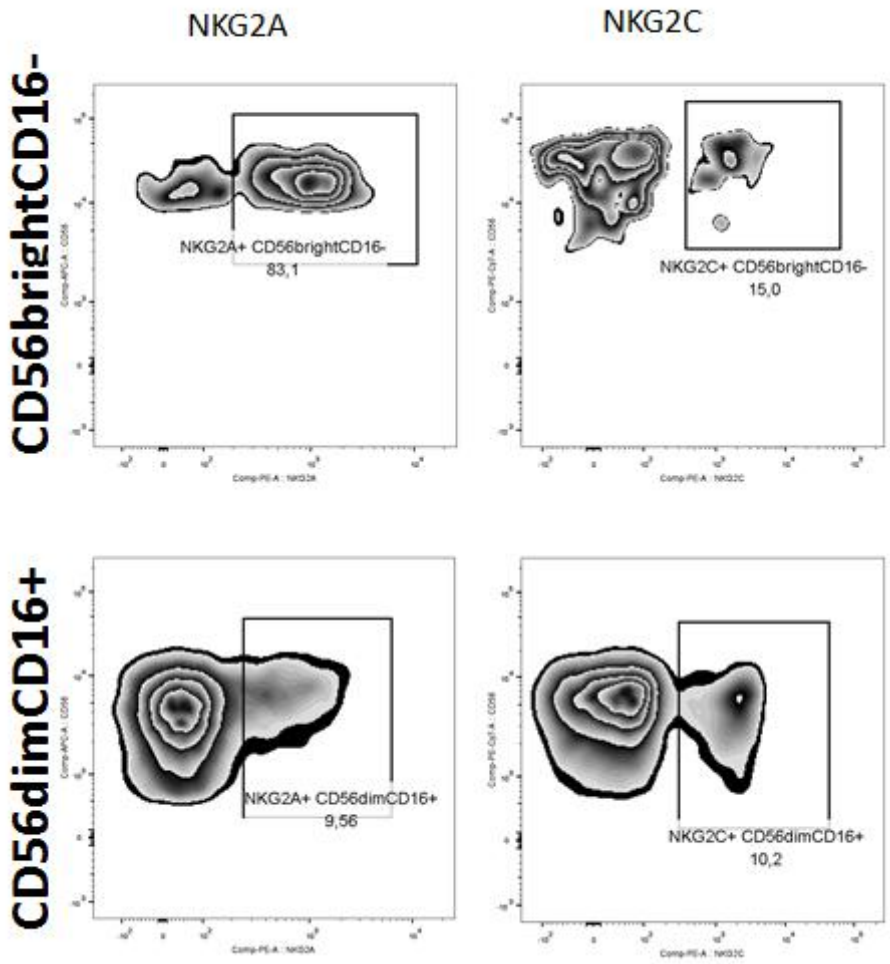


Figure 31: Representative zebraplots of NKG2A and NKG2C expression on $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells from CML patients.

The expression of NKG2A is decreased essentially in the $CD56^{bright}CD16^{-}$ NK cells (with statistical significance) comparing with healthy controls, in the $CD56^{dim}$ subpopulation the percentage of NKG2A positive cells is almost the same in both groups (figure 32.A). The surface density of NKG2A is drastically decreased in $CD56^{bright}CD16^{-}$ NK cells (with statistically significance), in the $CD56^{dim}CD16^{-}$ subpopulation the surface expression don't differ significantly (figure 32.B). The percentage of NKG2C positive cells in NK cells don't change significantly, but in the $CD56^{bright}CD16^{-}$ subpopulation a relative decrease is notice and in the $CD56^{dim}CD16^{+}$

subpopulation the percentage of NKG2C positive cells is augmented (figure 32.C). The surface density of this receptor in both groups is similar in $CD56^{bright}CD16^{-}$ subpopulation, however in the $CD56^{dim}CD16^{+}$ subpopulation the NKG2C surface density, in CML patients, is severely decreased (with statistical significance) (figure 32.D).

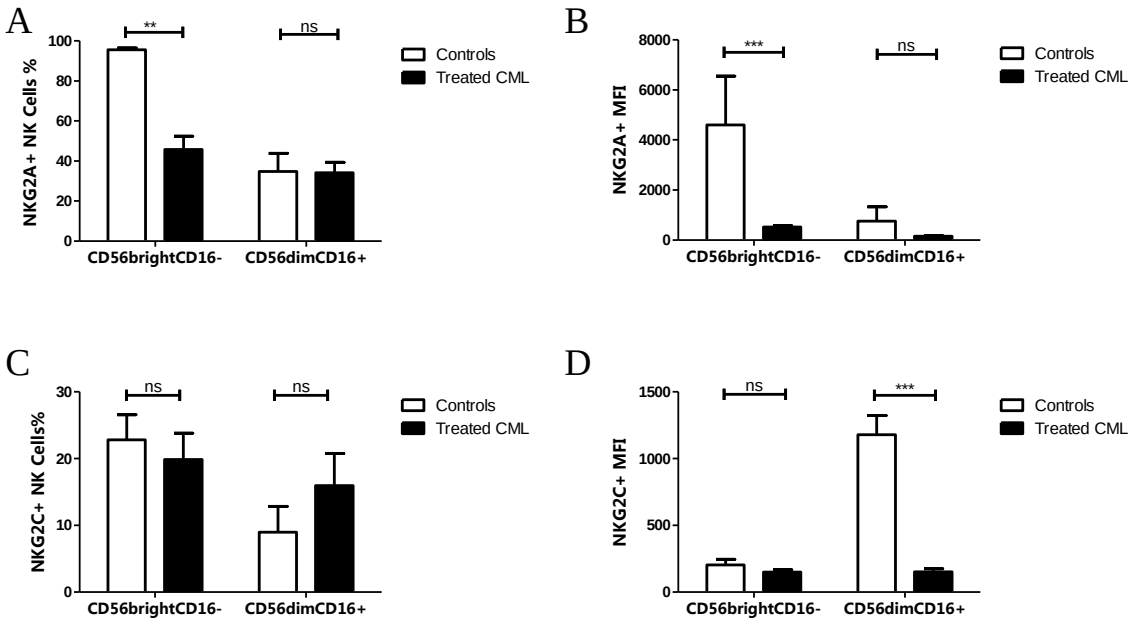


Figure 32: Percentage of NKG2A+ and NKG2C+ CD56brightCD16- NK cells and of NKG2A+ and NKG2C+ CD56dimCD16+ NK cells. From healthy donors (n=4) and CML patients (n=10). A – Percentage of NKG2A+ CD56brightCD16- NK cells and NKG2A+ CD56dimCD16+ NK cells. B – Mean of fluorescence intensity of NKG2A in $CD56^{bright}$ and $CD56^{dim}$ populations. C – Percentage of $NKG2C^{+}$ $CD56^{bright}CD16^{-}$ NK cells and $NKG2C^{+}$ $CD56^{dim}CD16^{+}$ NK cells. D – Mean of fluorescence intensity of NKG2C in $CD56^{bright}$ and $CD56^{dim}$ populations.

The ratio NKG2C/NKG2A is increased in CML patients, both in $CD56^{bright}CD16^{-}$ NK cells and $CD56^{dim}CD16^{+}$ NK cells.

In order to identify significant differences in the relationship between inhibitor and activator receptors we study the ratio NKG2C/NKG2A, what give us an idea about the contribution activators/inhibitors in NK cells from CML patients and healthy controls. In present research we saw that in both subsets of NK cells the ratio NKG2C/NKG2A is increased in CML patients comparing with controls (figure 33).

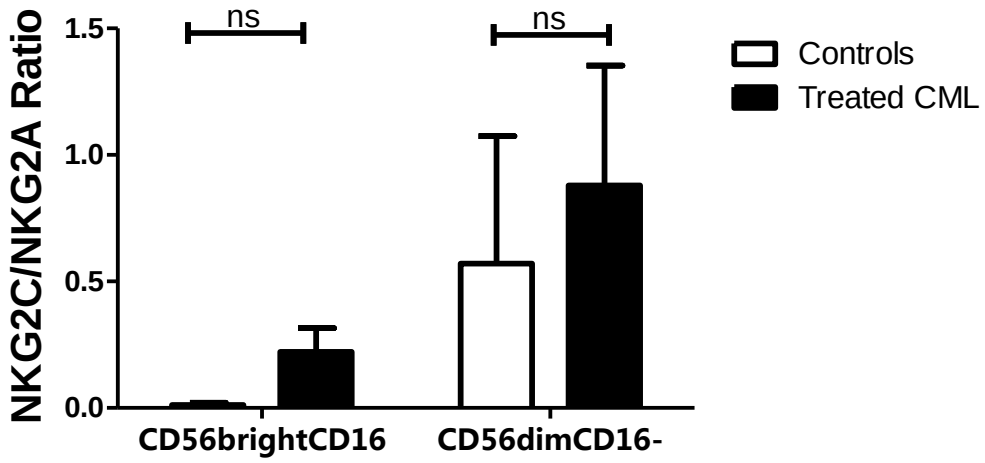


Figure 33: NKG2C/NKG2A ratio in $CD56^{bright}CD16^{-}$ NK cells and in $CD56^{dim}CD16^{+}$ NK cells, from healthy donors (n=4) and CML patients (n=10).

NKp30, NKp44, NKp46 and NKp80 expression by NK cells in CML patients is different from healthy controls.

The natural cytotoxic receptors are activating receptors. We analyzed the percentage of NKp30, NKp44, NKp46 and NKp80 in CML patients and we noticed that, with exception for NKp44, the other receptors are more expressed by the cytotoxic NK cells, $CD56^{bright}CD16^{-}$ subpopulation (figure 34).

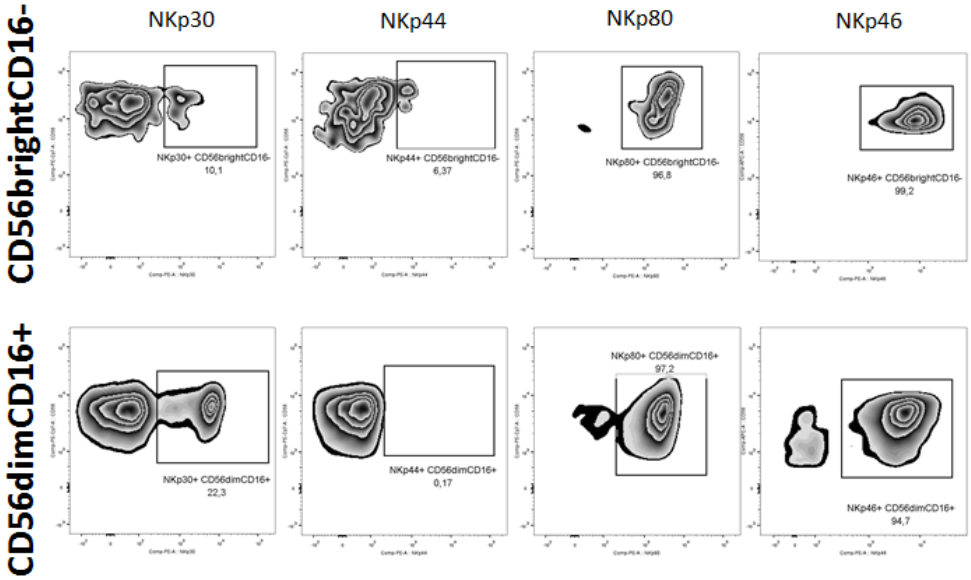


Figure 34: Representative zebraplots of NKp30, NKp44, NKp80 and NKp46 expression on $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells from CML patients.

When we compared the expression of the concerned receptors in CML patients and in healthy controls differences were observed. In CML patients the expression of NKp30 is slightly augmented in the $CD56^{bright}CD16^{-}$ NK cells and in the $CD56^{dim}CD16^{+}$ NK cells the expression of NKp30 is ^{dim}inished with statistical significance (figure 35.A). In terms of MFI, the intensities in both NK subsets are comparable (figure 35.B). The expression of NKp44 in CML patients is reduced in $CD56^{bright}$ NK cells and augmented in $CD56^{dim}$ NK cells (figure 35.C). The MFI shows

us that the surface density of this receptor in the $CD56^{bright}$ population is higher for patients then for healthy donors (with statistical significance), however for the $CD56^{dim}$ population the opposite happens (figure 35.D). In CML patients the expression of NKp46 is a little high for both NK subpopulations, comparing with healthy donors (figure 35.E). The surface density of this receptor is higher in CML patients, mainly in $CD56^{bright}CD16^{-}$ NK cells (with statistical significance) (figure 35F). Finally, for NKp80 both expression and surface density is equal in CML patients and healthy donors even though the MFI for CML patients is a bit high (figure 35.G/H).

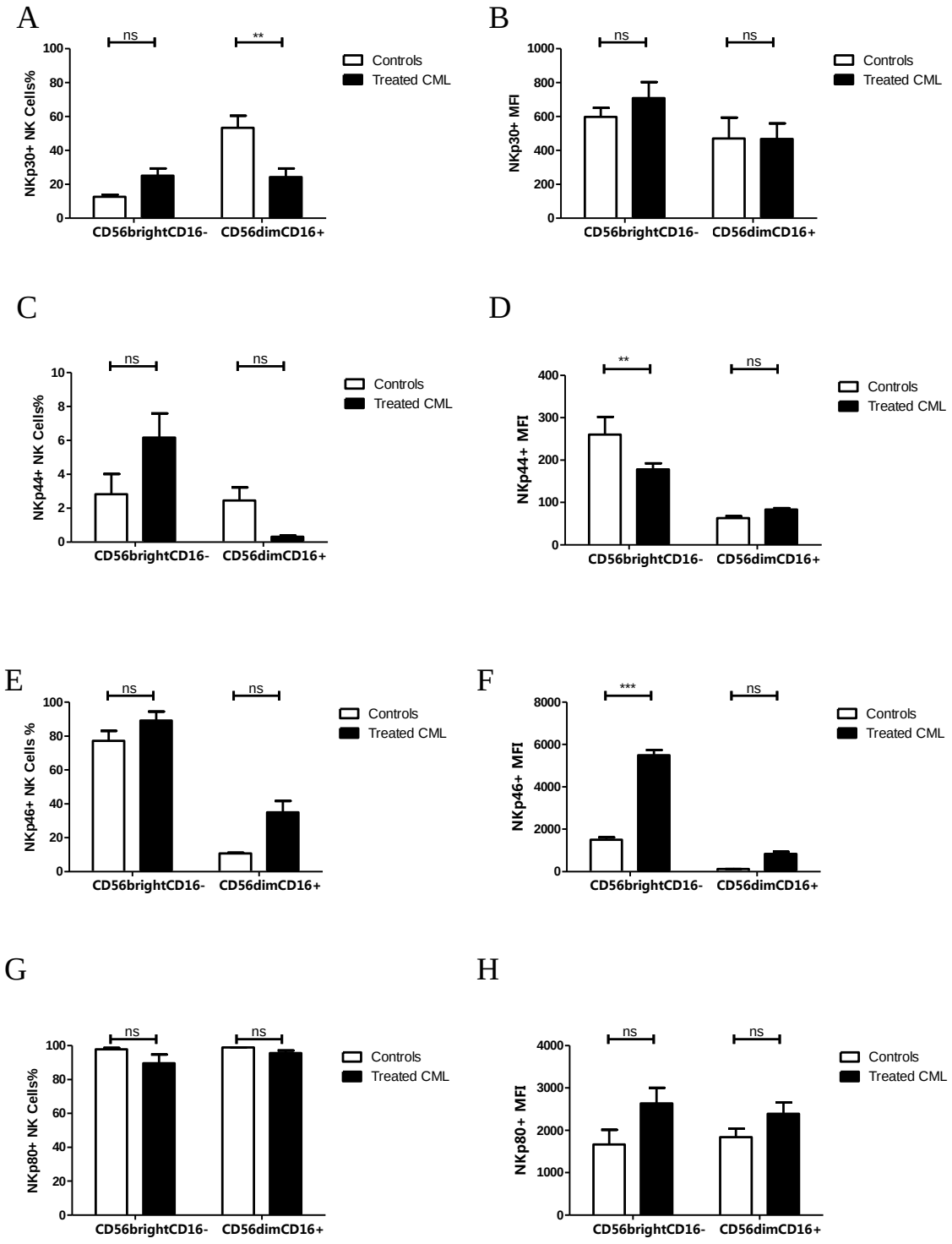


Figure 35: Percentage of NCRs expression on CD56brightCD16- NK cells and of NCRs expression on CD56dimCD16+ NK cells. From healthy donors (n=4) and CML patients (n=10). A – Percentage of NKp30^{+} CD56^{bright}CD16^{-} NK cells and NKp30^{+} CD56^{dim}CD16^{+} NK cells. B – Mean of fluorescence intensity of NKp30 in CD56^{bright} and CD56^{dim} populations. C – Percentage of NKp44^{+} CD56^{bright}CD16^{-} NK cells and NKp44^{+} CD56^{dim}CD16^{+} NK cells. D – Mean of fluorescence intensity of NKp44 in CD56^{bright} and CD56^{dim} populations. E – Percentage of NKp46^{+} CD56^{bright}CD16^{-} NK cells and NKp46^{+} CD56^{dim}CD16^{+} NK cells. F – Mean of

fluorescence intensity of NKp46 in $CD56^{bright}$ and $CD56^{dim}$ populations. G – Percentage of $NKp80^{+}$ $CD56^{bright}CD16^{-}$ NK cells and $NKp80^{+}$ $CD56^{dim}CD16^{+}$ NK cells. H – Mean of fluorescence intensity of NKp80 in $CD56^{bright}$ and $CD56^{dim}$ populations.

Cytokine production by $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells from healthy donors, naïve and CML patients

The production and release of cytokines allows the signaling transduction between NK cells and other immune cells. In the current project we measure by flow cytometry the percentage of IFN-γ, TNF-α, TGF-β, IL-4 and IL-10 (figure 36).

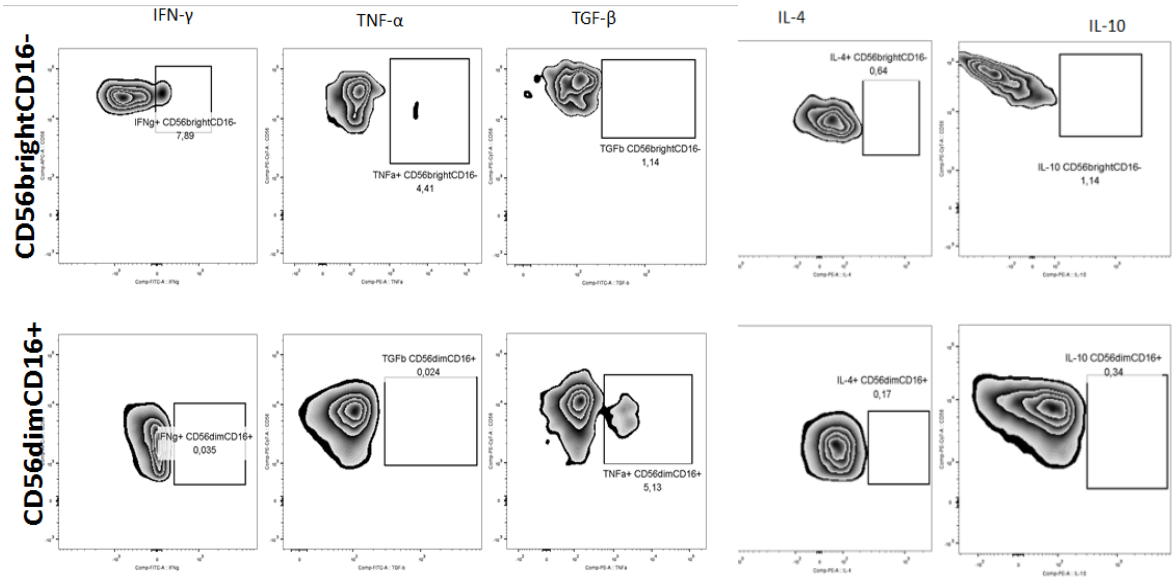


Figure 36: Representative zebraplots of IFN-γ, TNF-α, TGF-β, IL-4 and IL-10 positive cells on $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells from CML patients.

The IL-4 and IL-10 production by NK cells is augmented in naïve individuals and reduced in CML patients, comparing with healthy donors.

For interleukins, we measure the percentage of IL-4 and IL-10 positive NK cells of healthy donors (n=4), naïve patients (n=2) and CML patients (n=40). In the naïve group we can see that the percentage of both IL-4 and IL-10 is increased comparing with controls, and in the CML patients this percentage decrease achieving lower percentages then in controls, with statistical significances (figure 37.A/C). In the other hand the MFIs for IL-4 and IL-10 are diminished in naïve group and augmented in CML patients, comparing with healthy donors (figure 37.B/D).

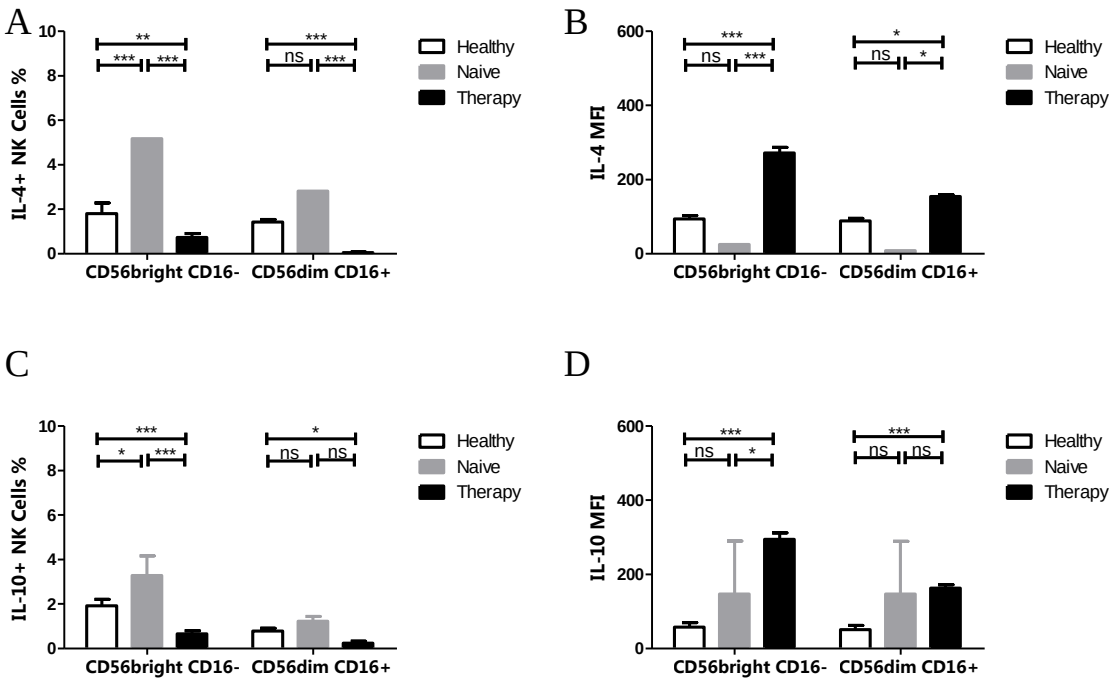


Figure 37: Percentage of IL-4 and IL-10 CD56brightCD16- positive NK cells and of IL-4 and IL-10 CD56dimCD16+ positive NK cells. From healthy donors (n=4), naïve (n=2) and CML patients (n=40). A – Percentage of IL-4+ $CD56^{bright}CD16^{-}$ NK cells and IL-4^{+} $CD56^{dim}CD16^{+}$ NK cells. B – Mean of fluorescence intensity of IL-4 in $CD56^{bright}$ and $CD56^{dim}$ populations. C – Percentage of IL-10^{+} $CD56^{bright}CD16^{-}$ NK cells and IL-10^{+} $CD56^{dim}CD16^{+}$ NK cells. D – Mean of fluorescence intensity of IL-10 in $CD56^{bright}$ and $CD56^{dim}$ populations.

NK cells production of TGF-β, TNF-α and IFN-γ is affected by the disease.

Using the same procedure we measure the percentage of other cytokines present in NK cells. For TGF-β and TNF-α we don't have chance to study naïve individuals, so for these two markers we just have two groups, healthy donors and CML patients. For IFN-γ we study two naïve patients beyond healthy donors and CML patients. The percentage of positive TGF-β NK cells is drastically reduced in CML patients, with statistical significance (figure 38.A), in both $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ cells. However, the MFIs for this cytokine are increased in CML patients, with statistical significance (figure 38.B). In CML patients the percentage of positive cells for TNF-α is relatively increased in both NK subsets (figure 38.C) and the same thing happens for the MFIs (figure 38.D). In case of IFN-γ we had the opportunity to study naïve patients too. Our results show that CML patients have the higher percentage of IFN-γ^{+} $CD56^{bright}CD16^{-}$ NK cells (with statistical significance), followed by the naïve group and the lowest value is correlated with healthy donors (figure 38. E), in the $CD56^{dim}CD16^{+}$ population the percentages for IFN-γ is similar. The MFI for IFN-γ is augmented for CML patients mainly in the $CD56^{bright}$ subset, the IFN-γ MFIs for controls and naïve groups are equal (figure 38.F).

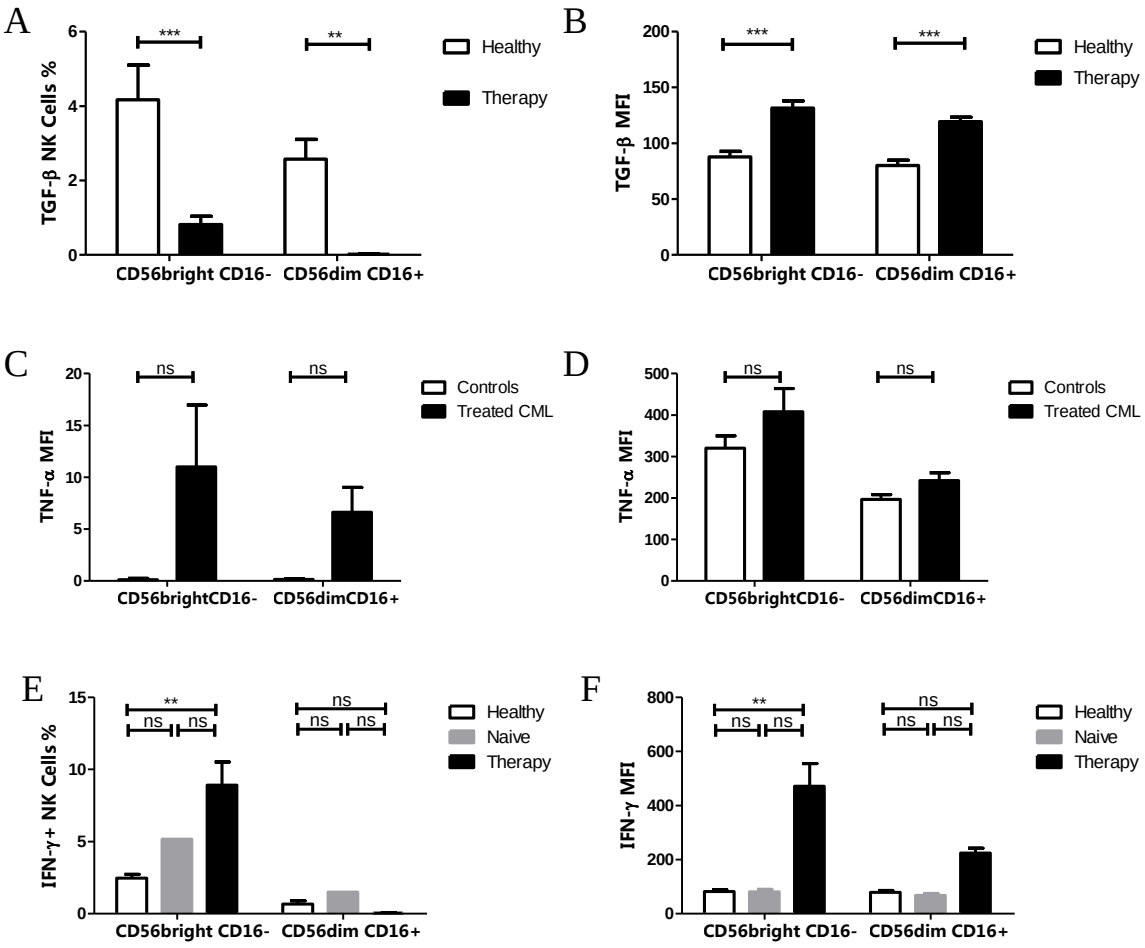


Figure 38: Percentage of TGF-β, TNF-α and IFN-γ CD56brightCD16- positive NK cells and of TGF-β, TNF-α and IFN-γ CD56dimCD16+ positive NK cells. From healthy donors (n=4), naïve (n=2) and CML patients (n=10 for TGF-β, n=6 for TNF-α and n=40 for IFN-γ). A – Percentage of TGF-β+ CD56brightCD16- NK cells and TGF-β+ CD56dimCD16+ NK cells. B – Mean of fluorescence intensity of TGF-β in CD56bright and CD56dim populations. C – Percentage of TNF-α+ CD56brightCD16- NK cells and TNF-α+ CD56dimCD16+ NK cells. D – Mean of fluorescence intensity of TNF-α in CD56bright and CD56dim populations. E – Percentage of IFN-γ+ CD56brightCD16- NK cells and IFN-γ+ CD56dimCD16^{+} NK cells. F – Mean of fluorescence intensity of IFN-γ in CD56^{bright} and CD56^{dim} populations.

Briefly research of some special markers in NK cells from CML patients

Additionally, we did a quick research about other important markers that are emerging as powerful targets for modulating NK cell activity. So we did a briefly research about the expression of DNAM-1, CD155, CD96, TIGIT, PD-1, PD-L1 and PD-L2 in NK cells.

The expression and surface density of CD96, DNAM-1, CD155 and TIGIT of CML patients don't differ, significantly, from healthy donors.

We study the expression of CD96, DNAM-1, CD155 and TIGIT on $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells. For NK cells the percentage and MFI for the present markers in CML patients don't change significantly comparing with controls (figure 39).

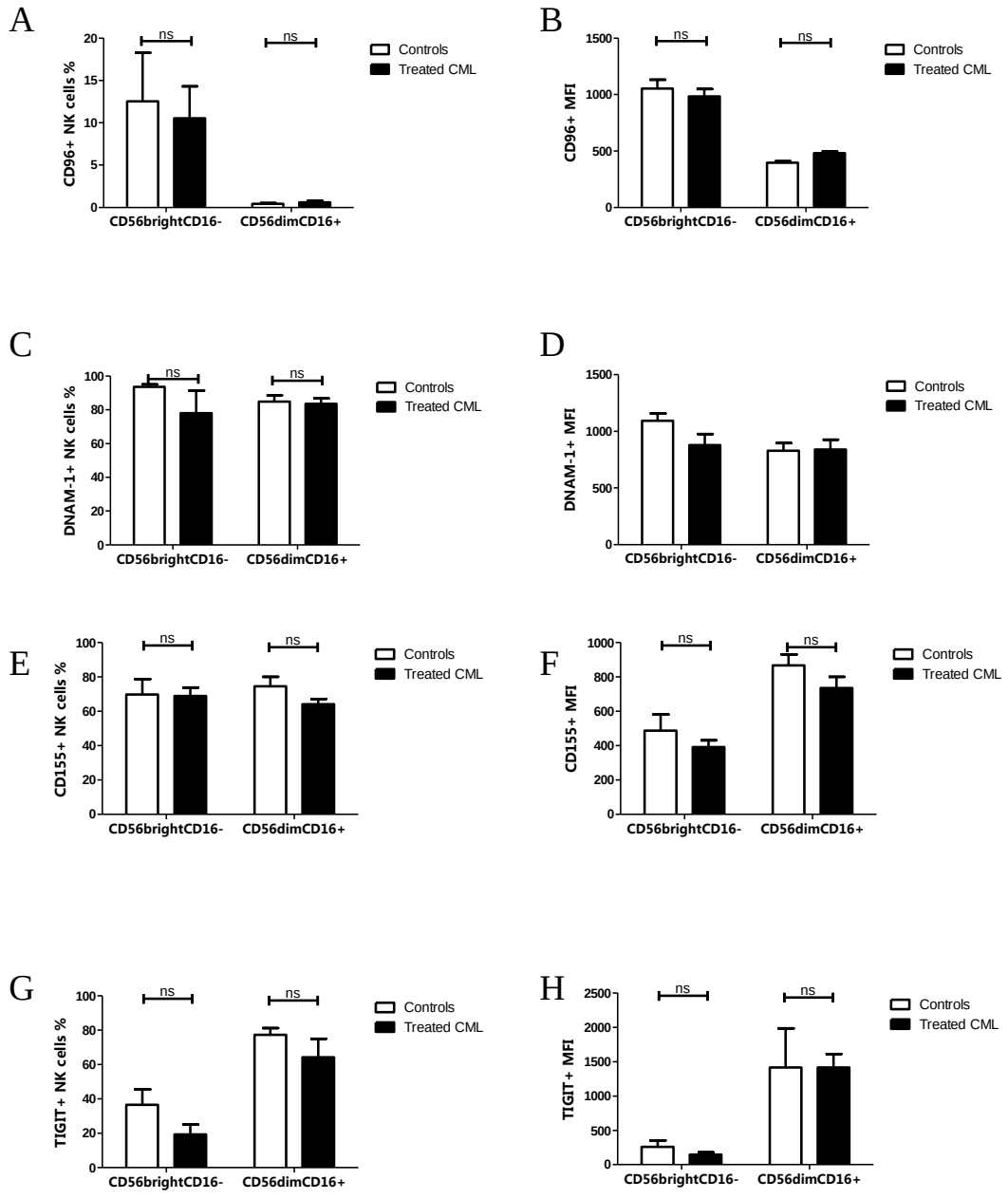


Figure 39: Percentage of CD96, DNAM-1, CD155 and TIGIT on CD56brightCD16$^-$ and CD56dimCD16 NK cells from healthy donors (n=4) and CML patients (n=7). A – Percentage of CD96 positive CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells. B – Mean of fluorescence intensity of CD96 in CD56^{bright} and CD56^{dim} NK cells. C – Percentage of DNAM-1 positive CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells. D – Mean of fluorescence intensity of DNAM-1 in CD56^{bright} and CD56^{dim} NK cells. E – Percentage of CD155 positive CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells. F – Mean of fluorescence intensity of CD155 in CD56^{bright} and CD56^{dim} NK cells. G – Percentage of TIGIT positive CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells. H – Mean of fluorescence intensity of TIGIT in CD56^{bright} and CD56^{dim} NK cells.

The expression and surface density of PD-1, PD-L1 and PD-L2 of CML patients don't differ, significantly, from healthy donors.

In our results we can't see any differences in the expression or surface density of PD-1, PD-L1 and PD-L2 in NK cells from CML patients relatively to healthy controls (figure 40), with the exception for PD-1 MFI in NKT cells, with statistically significance (figure 40.B).

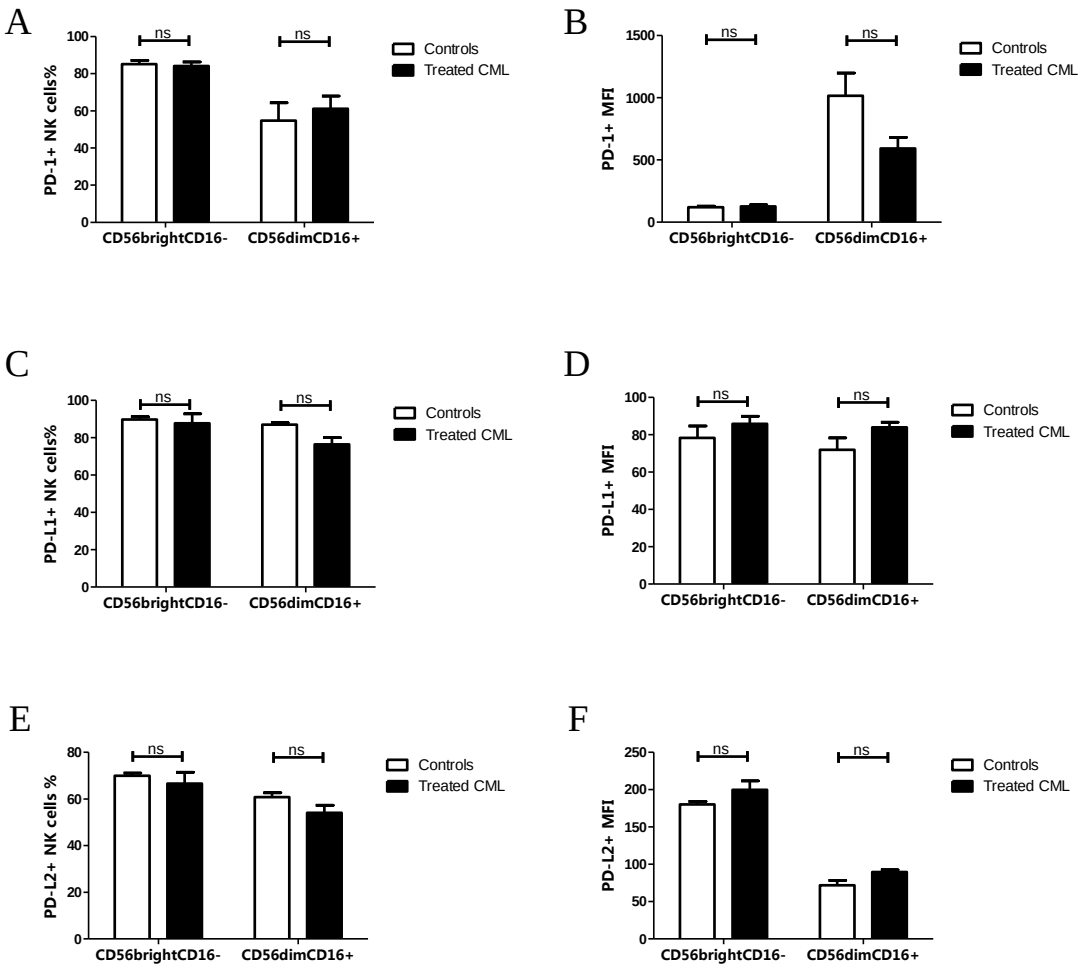


Figure 40: Percentage of PD-1, PD-L1 and PD-L2 on CD56brightCD16- and CD56dimCD16+ NK cells from healthy donors (n=4) and CML patients (n=7). A – Percentage of PD-1 positive CD56brightCD16^{-} and CD56dimCD16^{+} NK cells. B – Mean of fluorescence intensity of PD-1 in CD56bright and CD56dim NK cells. C – Percentage of PD-L1 positive CD56brightCD16^{-} and CD56dimCD16^{+} NK cells. D – Mean of fluorescence intensity of PD-L1 in CD56bright and CD56dim NK cells. E – Percentage of PD-L2 positive CD56brightCD16^{-} and CD56dimCD16^{+} NK cells. F – Mean of fluorescence intensity of PD-L2 in CD56bright and CD56dim NK cells.

Effect of CML therapies, biologic or TKI therapy, and dosage in NK cells

The major goal of this study is try to understand how NK cells are affected in CML patients, further that we also try to understand if those alterations in NK CML cells are more evidenced in patients with certain type of therapy or certain dosage. After all the data exported from cytometer analyzed we divided our results according to the therapy and the dosage. So, in general, we have seven different groups. Eighteen CML patients are doing Imatinib 400, eight are doing Imatinib 600, eight are doing Imatinib 800, five are doing Dasatinib 100, one are doing ponatinib, three are doing IFN-α and seven are doing other therapies like Ara^{-}C or combination therapy with IFN-α plus Imatinib 100. These data are based on the previous results for the immunoprofiling of NK cells in CML patients. The statistical analysis is based on comparing all the groups with the Imatinib 400 group, because is the standard therapy for CML patients.

The percentage of lymphocytes is independent of therapy and dosage, but the percentage of NK cells differs.

The percentage of total lymphocytes in CML patients is more or less the same in all groups, being independent of therapy and dosage (figure 41.A). Relatively to NK cells we saw that the Dasatinib group has the highest percentage and count, and the lowest percentage and count correspond to those patients that are doing ponatinib or other therapies, with statistical significance (figure 41.B/C). We observed to that the percentage and count of NK cells don't differ significantly with the Imatinib dosage (figure 41.B/C). Within the NK cell population we measure the percentage of CD56^{bright} and CD56^{dim} subsets. For the CD56^{bright}CD16^{-} NK cells percentage we verified that the ponatinib and others group ensured the highest percentage (with statistical significance) and for the CD56^{dim}CD16^{-} NK cells the percentage for all the groups is around 80%

(figure 41.D). Further that we revealed that the percentage of CD56^{bright} NK cells decreased in accordance with the increase in dose of Imatinib (figure 41.D).

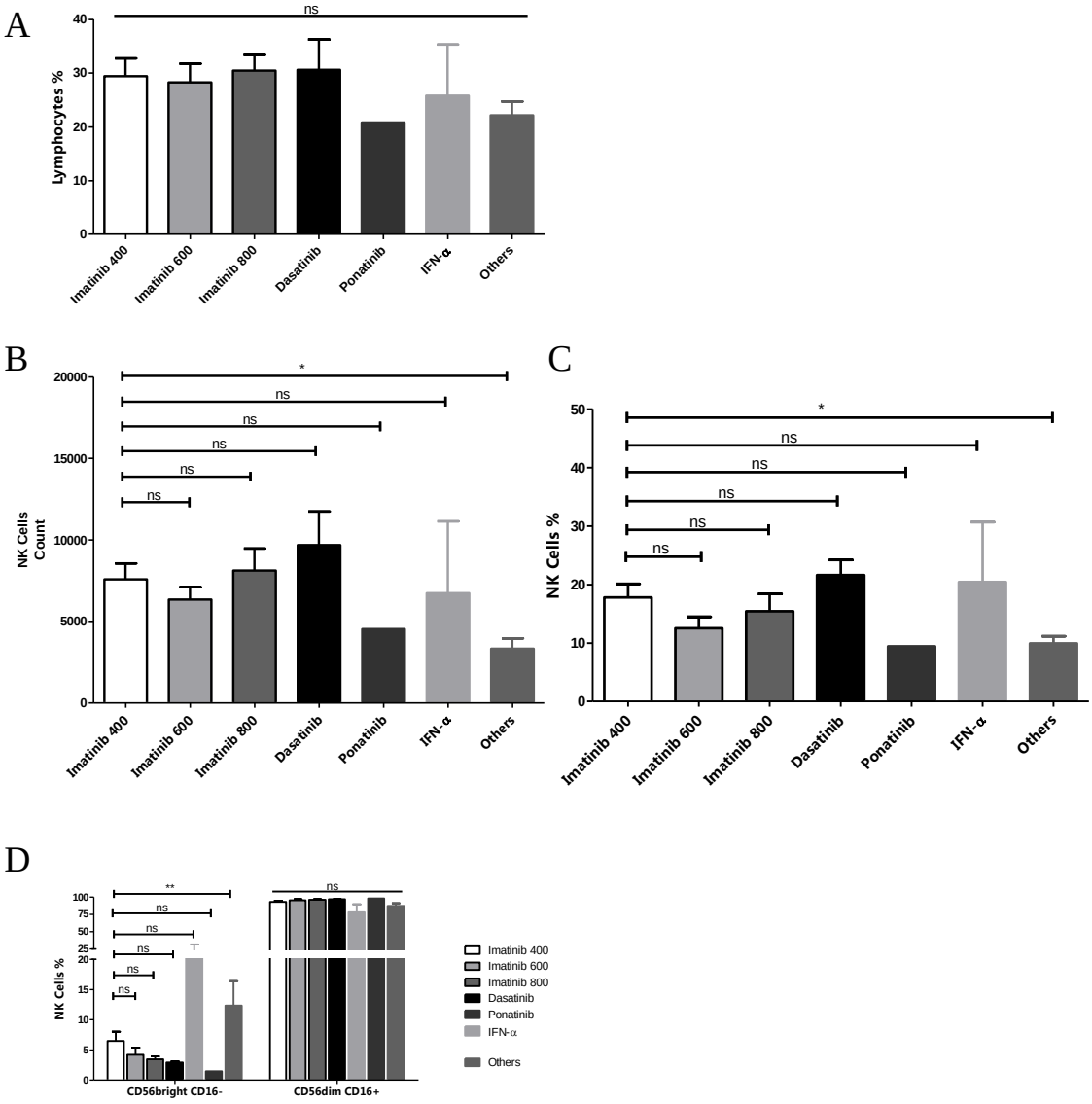


Figure 41: Percentage of total lymphocytes, NK cells and NK cells subpopulations from CML patients according to therapy and dosage. A – Percentage of lymphocytes. B – Count of NK cells. C – Percentage of NK cells. D – Percentage of CD56brightCD16- and CD56dimCD16+ NK cells. Imatinib 400 (n=18); Imatinib 600 (n=8); Imatinib 800 (n=8); Dasatinib (n=5); Ponatinib (n=1); IFN-α (n=3); Others (n=7).

The expression of cell surface markers by CD56brightcd16- and CD56dimcd16+ NK cells from CML patients varies with therapy and dosage.

In this study we look to the expression of surface markers in CML patients that are doing different therapies. So we did this additional analysis for the expression of CD4, CD8, CD7, CD57, CD62L, CD69, CD27, CD11b, CD137 and CD137L in $CD56^{bright}$ and $CD56^{dim}$ NK cells.

For CD4 positive NK cells we demonstrate that the percentage of $CD4^{+}$ $CD56^{bright}$ NK cells decrease proportionally with the increase of Imatinib dose, NK cells from ponatinib patient don't seem to express $CD4^{+}$ NK cells but we just had one patient so this result is poor and in the $CD56^{dim}CD16^{+}$ population the percentage of $CD4^{+}$ cells is decreased in Dasatinib and other groups (figure 42.A). The percentage of CD8 positive NK cells is equal for all groups in $CD56^{bright}$ NK cells, in $CD56^{dim}$ NK cells the Imatinib 400 group is the one with highest percentage of CD8 positive cells and the others group had the lowest percentage, with statistical significance (figure 42.B). The $CD7^{+}$ NK cells are highly expressed by the two subpopulations with no significant differences among patient groups (figure 42.C). CD57 positive NK cells don't differ much between the groups in both $CD56^{bright}$ and ^{dim} NK cells, with the exception for the others group that show a decrease in $CD8^{+}$ $CD56^{dim}CD16^{+}$ NK cells (figure 42.D). The expression of CD62L is high in the $CD56^{bright}$ population from all groups with no significant differences between them, unless for the Dasatinib group where the CD62L positive cells tend to be low (figure 42.E). In the $CD56^{dim}$ population the percentage of CD62L positive cells increase a bit in the others and ponatinib groups (figure 42.E). $CD56^{bright}CD16^{-}$ NK cells from the Imatinib 400 and others group have the highest percentage of $CD69^{+}$ cells in the $CD56^{dim}CD16^{+}$ NK cells the percentage of $CD69^{+}$ cells is low in all groups (figure 42.F). For the expression of CD27 only in the

$CD56^{bright}CD16^{-}$ NK cells a decrease is noticed, for the remaining groups no differences are shown, independent of the NK cell subpopulation (figure 42.G). The percentage of CD11b positive cells no differences were observed between the groups in study in both $CD56^{bright}$ and $CD56^{dim}$ NK cells (figure 42.H). Finally, we study the differences in the same groups for CD137 and CD137L positive cells. The percentage of CD137 positive NK cells is highest in the Dasatinib and ponatinib groups and the lowest percentages are related with the IFN-α and others groups comparing with the individuals doing Imatinib 400 (figure 42.I). The percentage of $CD137L^{+}$ $CD56^{bright}CD16^{-}$ NK cells is highest for the Imatinib 400, decreasing with the dosage of Imatinib, as we can see in the Imatinib 600 group. Further that a more accentuated decreased is noticed for the Dasatinib group and the lowest frequencies belong to the $IFN^{-}\alpha$ and others groups. In the $CD56^{dim}CD16^{+}$ subpopulation the percentage of $CD137L^{+}$ cells don't differ much, although in Imatinib 600 and Dasatinib groups this percentage is relatively reduced comparing with others groups (figure 42.J).

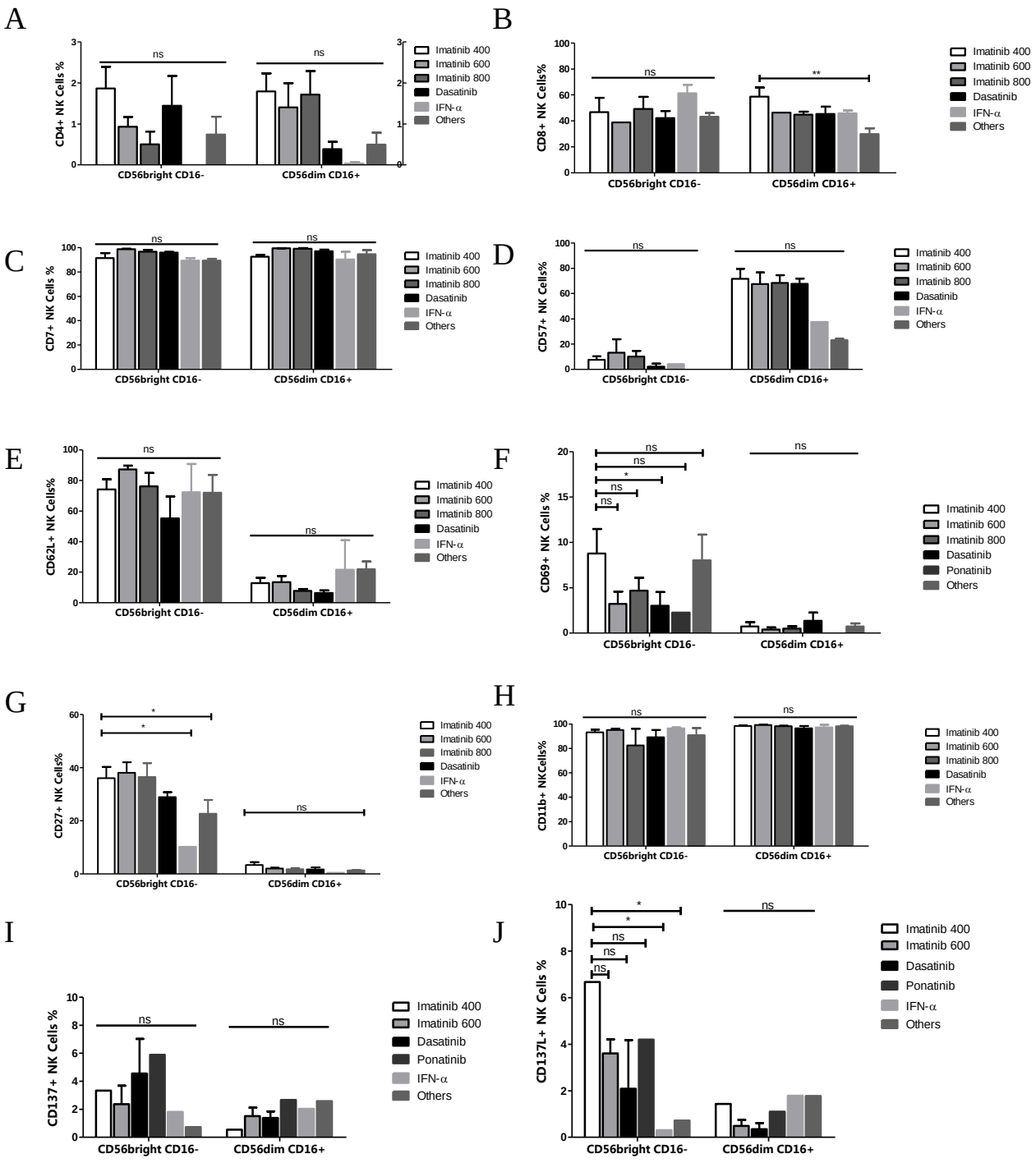


Figure 42: The expression of cell surface markers in CD56brightCD16- and CD56dimCD16+ NK cells, according with the defined groups. Imatinib 400 (n=18); Imatinib 600 (n=8); Imatinib 800 (n=8); Dasatinib (n=5); Ponatinib (n=1); IFN-α (n=3); Others (n=7). A – The expression of CD4 in CD56bright and CD56dim NK cells from the different groups of individuals. B – The expression of CD8 in CD56bright and CD56dim NK cells from the different groups of individuals. C – The expression of CD7 in CD56bright and CD56dim NK cells from the different groups of individuals. D – The expression of CD57 in CD56bright and

CD56dim NK cells from the different groups of individuals. E – The expression of CD62L in CD56bright and CD56dim NK cells from the different groups of individuals. F – The expression of CD69 in CD56bright and CD56dim NK cells from the different groups of individuals. G – The expression of CD27 in CD56bright and CD56dim NK cells from the different groups of individuals. H – The expression of CD11b in CD56bright and CD56dim NK cells from the different groups of individuals. I – The expression of CD137 in CD56bright and CD56dim NK cells from the different groups of individuals. J – The expression of CD137L in CD56bright and CD56dim NK cells from the different groups of individuals.

NK cell receptors expression in CD56brightCD16- and CD56dimCD16+ populations varies with different therapies.

As we saw before NK cells have a vast repertoire of receptors. In order to understand how different therapies affect these cells in terms of receptors. All samples are performed with extracellular labelling and then acquired on cytometer as described in chapter II. For the receptor expression by NK cells we study five groups of individuals, Imatinib 400 (n=18), Imatinib 600 (n=8), Imatinib 800 (n=8), Dasatinib (n=5) and others (n=7).

For the KIRs we study KIR2DL1, KIR2DS1 and KIR2DL2. The receptor KIR2DL1 is highly expressed by $CD56^{bright}CD16^{-}$ NK cells from Dasatinib group and those who are doing Imatinib 800 also express more KIR2DL1 comparing with Imatinib 400 group, with statistical significance (figure 43.A). In the $CD56^{dim}CD16^{+}$ population the Imatinib 400 group is the one that express more KIR2DL1, whereas the other groups have reduced expression of this receptor (figure 43.A). KIR2DS1 expression is poor by $CD56^{bright}CD16^{-}$ NK cells independently of therapy. About the $CD56^{dim}CD16^{+}$ population we observed a higher expression in the Dasatinib group comparing with the Imatinib 400 group (with statistical significance) and the other groups express less this receptor (figure 43.B). CD56brighCD16^{-} NK cells almost not express KIR2DL2 with no significant differences between the groups in study (figure 43.C). In the $CD56^{dim}CD16^{-}$ population the expression of KIR2DL2 is higher for the Imatinib 600 group in

comparison with Imatinib 400 group and the Dasatinib group seems to express less this receptor (figure 43.C). The expression of these KIRs is independent of dosage.

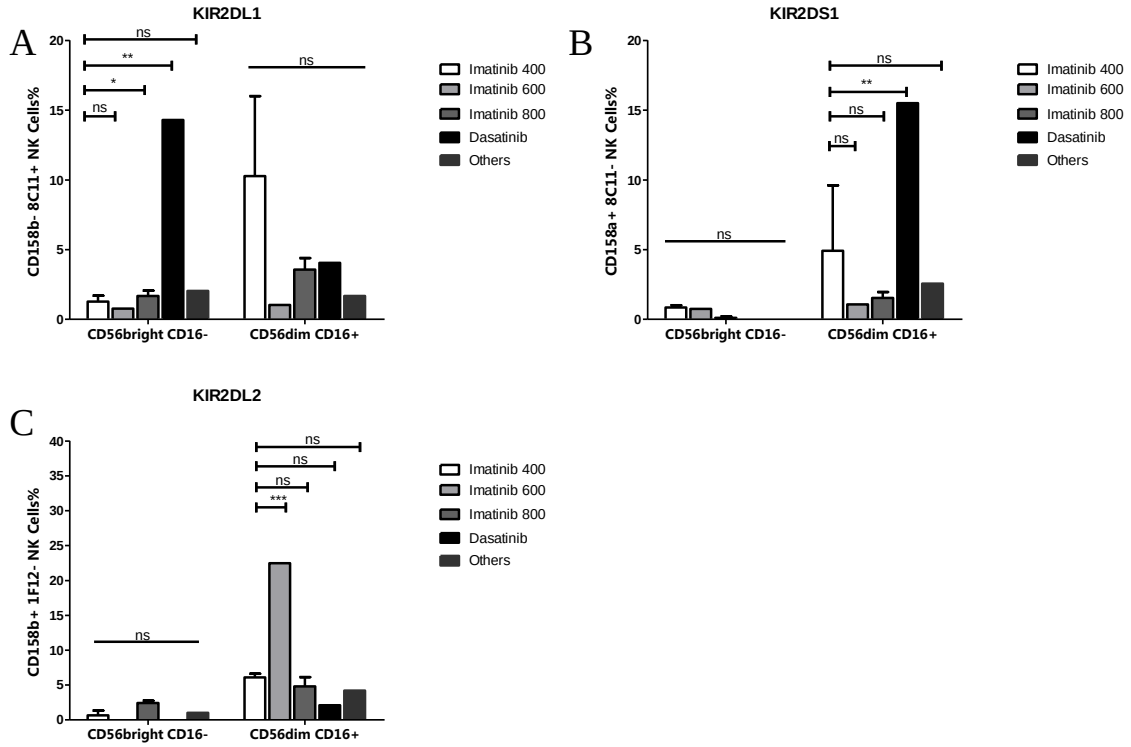


Figure 43: The expression of KIRs in CD56brightCD16- and CD56dimCD16+ NK cells, according with the defined groups. Imatinib 400 (n=18); Imatinib 600 (n=8); Imatinib 800 (n=8); Dasatinib (n=5); Others (n=7). A – The expression of KIR2DL1 in CD56bright and CD56dim NK cells from the different groups of individuals. B – The expression of KIR2DS1 in CD56bright and CD56dim NK cells from the different groups of individuals. C – The expression of KIR2DL2 in CD56bright and CD56dim NK cells from the different groups of individuals.

Beyond KIRs we also analyzed the effect of therapy in the expression of NCRs by NK cells, namely NKp30, NKp44, NKp46 and C-type lectin-like receptor NKp80. Comparing the expression of NKp30, by $CD56^{bright}CD16^{-}$ NK cells, between the Imatinib 400 group and the other groups, we demonstrated that only the others group express less this NCR (figure 44.A). Doing the same comparison for the $CD56^{dim}CD16^{+}$ cells we saw that the Imatinib 600 group express more and the Dasatinib group practically not express NKp30 (figure 44.A). The NKp44 expression is augmented in the Imatinib 600, 800 and Dasatinib groups for $CD56^{bright}$ NK cells and for $CD56^{dim}$ NK cells hardly noticed expression in all groups (figure 44.B). For NKp46 expression the $CD56^{bright}$ cells have a higher rate than $CD56^{dim}$ cells, but between groups no significant differences were found. Although in the $CD56^{dim}CD16^{+}$ NK cells apparently occurs an effect of Imatinib dosage, because the NKp46 expression increases with the higher dose of this drug (figure 44.C). NKp80 is greatly expressed by both subpopulations of NK cells, being independent of therapy or dosage, with exception for the $CD56^{bright}$ NK cells in Dasatinib group, where NKp80 expression is reduced (figure 44.C).

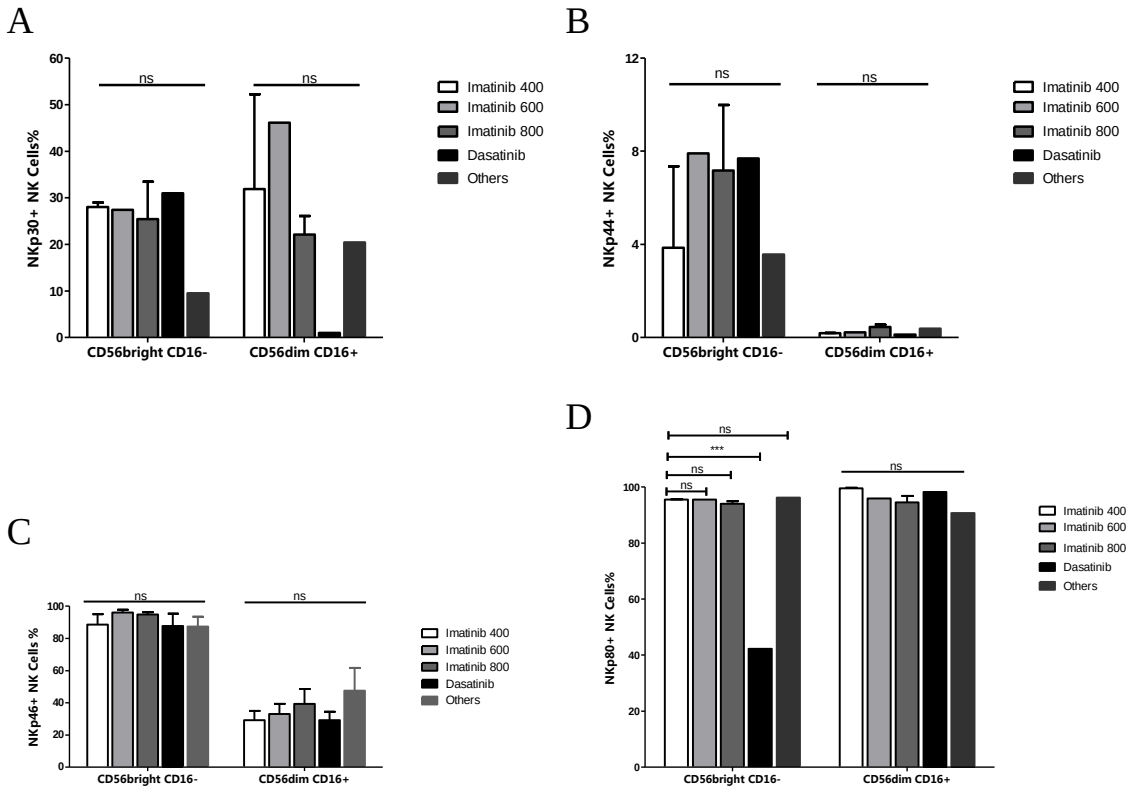


Figure 44: The expression of NCRs in CD56brightCD16- and CD56dimCD16+ NK cells, according with the defined groups. Imatinib 400 (n=18); Imatinib 600 (n=8); Imatinib 800 (n=8); Dasatinib (n=5); Others (n=7). A – The expression of NKp30 in CD56bright and CD56dim NK cells from the different groups of individuals. B – The expression of NKp44 in CD56bright and CD56dim NK cells from the different groups of individuals. C – The expression of NKp46 in CD56bright and CD56dim NK cells from the different groups of individuals. D – The expression of NKp80 in CD56bright and CD56dim NK cells from the different groups of individuals.

Cytokine production by NK cells is affected by CML therapy.

Finally, we made a research about the effect of CML in the cytokine production by NK cells. By intracellular labelling we study the NK cell production of IL-4, IL-10, TGF-β and IFN-γ in seven different groups of CML patients. However, for some groups not all cytokine information can be assessed. Looking to the $CD56^{dim}CD16^{+}$ NK population we saw that these cells are very deprived of cytokine production, at least for the cytokines studied (figure 45). So we concentrated our attention in the $CD56^{bright}CD16^{-}$ population. Comparing with the Imatinib 400 group, the $CD56^{bright}$ NK cells from CML patients doing IFN-α produce more IL-4, whereas Imatinib 600 and Dasatinib patients produce less (figure 45.A). IL-10 production is augmented in the Dasatinib, ponatinib and others group, with statistical significance (figure 45.B). For TGF-β the ponatinib and others group shows a higher production in comparison with Imatinib 400 group and the Imatinib 600 group express less, with statistical difference (figure 45.C). About the IFN-γ production all groups express less than the Imatinib 400 group, namely the Imatinib 600, 800 and ponatinib group (figure 45.D).

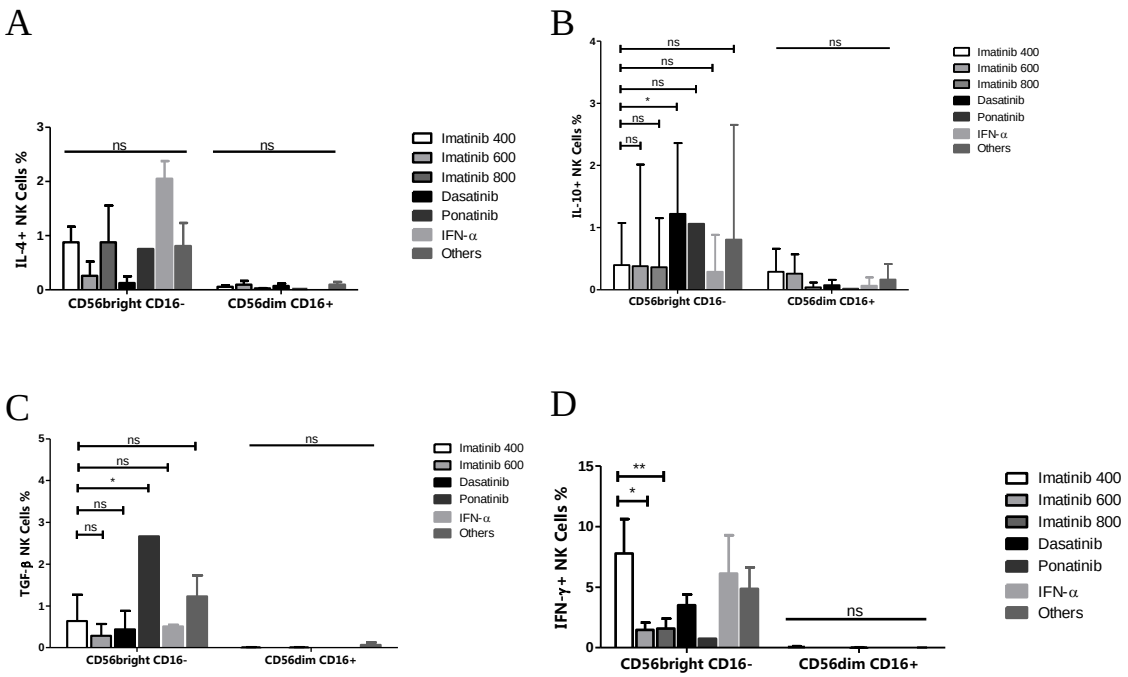


Figure 45: Cytokine production by CD56brightCD16- and CD56dimCD16+ NK cells from patients undergoing different therapies. Imatinib 400 (n=18); Imatinib 600 (n=8); Imatinib 800 (n=8); Dasatinib (n=5); Ponatinib (n=1); IFN-α (n=3); Others (n=7). A – The percentage of IL-4+ CD56brightCD16- and IL-4+ CD56dimCD16+ NK cells. B – The percentage of IL-10+ CD56brightCD16- and IL-10+ CD56dimCD16+ NK cells. C – The percentage of TGF-β+ CD56brightCD16- and TGF-β+ CD56dimCD16+ NK cells. D – The percentage of IFN-γ+ CD56brightCD16- and IFN-γ+ CD56dimCD16+ NK cells.

Effect of BCR-ABL clones in NK cells from CML patients

Another objective of this study is tried to understand if the amount of BCR-ABL clones affect in some way NK cells. In our lab, the amount of BCR-ABL is quantified in order to monitorize the CML patients. With access to this information we divided CML patients in five groups, one negative for BCR- ABL – until 0, 01% BCR/ABL (n=34) and four positives for BCR- ABL – until 0, 1% (n=2), 1% (n=4), 10% (n=4) and 100% (n=5) of BCR/ABL. The negative group served as control.

The percentage of total lymphocytes, NK cells and NK cells subpopulations don't differ with the ratio BCR/ABL.

The percentage of lymphocytes is higher for the negative group, in the other groups the percentage of lymphocytes varies but not much (figure 46.A). For the proportion and count of NK cells the 0, 1% BCR/ABL group of individuals has the upper percentage and in the other groups this percentage doesn't differ significantly from the negative group (figure 46.B.C). Within the NK cell population no differences are reported in the groups of study (figure 46.D).

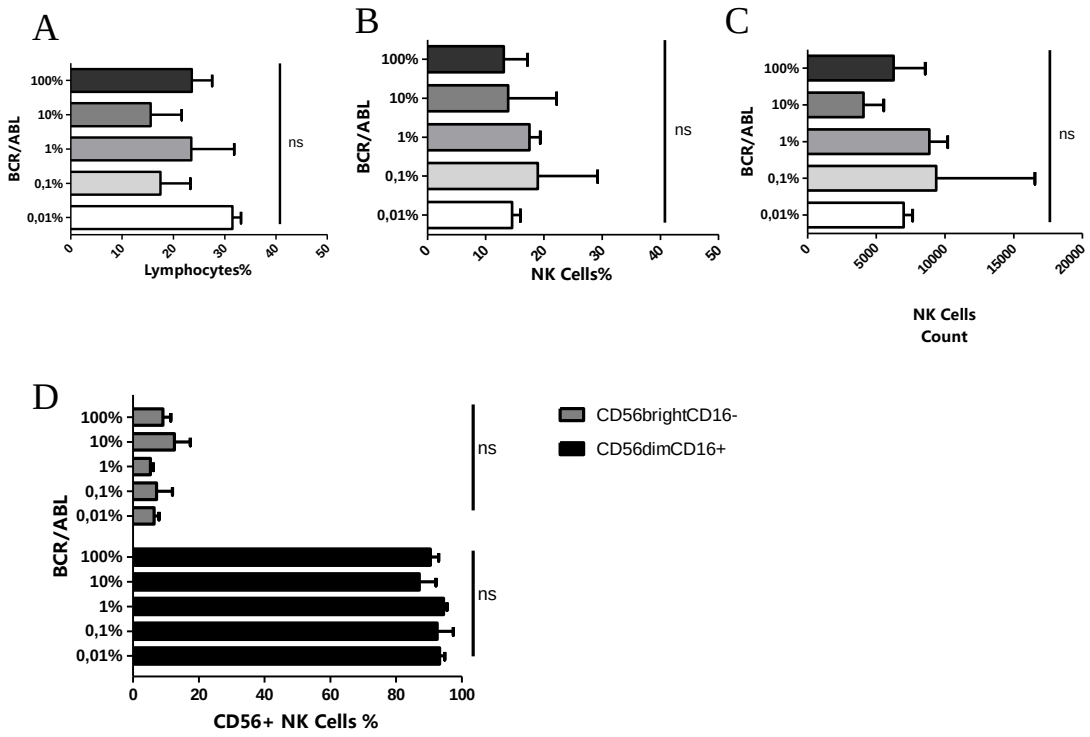


Figure 46: Percentage of total lymphocytes, NK cells and NK cells subpopulations from CML patients according to BCR/ABL ratio. A – Percentage of lymphocytes. B – Percentage of NK cells C – Count of NK cells. D – Percentage of CD56brightCD16- and CD56dimCD16+ NK cells. 0, 01% BCR/ABL (n=34) 0, 1% BCR/ABL (n=2), 1% BCR/ABL (n=4), 10% BCR/ABL (n=4) and 100% BCR/ABL (n=5).

Discussion

NK cells are potent and aggressive killers of tumor cells, so tumor cells need to change and modify their microenvironment to escape recognition and attack by NK cells (61). CML is a myeloproliferative disease subdivided in three characteristic phases; chronic phase (CP) of CML is characterized by excess numbers of myeloid cells that proliferate extensively. Between 90% and 95% of patients will be diagnosed in this phase of the disease. If untreated, within an average of 4 to 6 years, the disease transforms through an accelerated phase (AP) to an invariably fatal acute leukemia, also known as blast crisis (BC). Disease progression is likely due to the accumulation of molecular abnormalities that lead to a progressive loss of the capacity for terminal differentiation of the leukemic clone. The current treatment tries to maintain remission and prevent progression of the disease to AP and BC while minimizing any therapy-related toxicity (40,41). Early studies were shown that NK cells selectively lyse CML progenitor cells *in vitro.* In accelerated CML and blast crisis, NK cell proliferation, lytic function and frequency seems to decline, but until now remains unclear whether this decline is a cause rather than an effect of disease progression. Additionally, donor vs recipient NK cell alloreactivity could eliminate leukemia in human transplants (62). As follows, understanding the mechanisms of the NK cell dysfunction in CML could be useful in elucidating how this disease develops and in identifying therapeutic strategies.

In this study we analyzed the NK cell immunoprofile from CML patients and healthy donors, including surface molecules (e. g. CD57, CD7), receptors (KIRs, C-type Lectin-like receptors and NCRs), some adhesion molecules (e.g. DNAM-1) and some cytokines (e.g. IFN-γ, TGF-β). After characterization of CML NK cells we investigated whether therapy and drug dosage incomes or not effect on NK cells.

The percentage of total lymphocytes and their subpopulation is decreased in CML patients.

Lymphocytes are immune cells originated from lymphoid hematopoietic stem cells, after differentiation in thymus they become T cells, B cells or NK cells. As potent tolls of the immune system is important to study how these immune cells are affected by malignant cells, many studies reported that tumor microenvironment could suppress the immune system but the mechanisms behind such evidences stands unclear (8). In leukemia patients the T cell dysfunction is associated with disease progression, which may be due to a disorder in the thymic output function, the abnormal expression of TCR and, possibly, abnormal TCR signal transduction through altered CD3 gene expression (54). The percentage of lymphocytes is reduced in CML patients as described for other diseases. The T cells and B cells are diminished in naïve patients however in CML patients these percentages return to normal levels, revealing a positive effect of therapy. About NK cells we will discuss later. Additionally, in de novo CML have been reported decreased levels of $CD4^+$ and $CD8^+$ T cells (63), what is coherent with our results where the absolute numbers of $CD4^+$ and $CD8^+$ cells are decreased compared with healthy controls, specially the $CD8^+$ T subpopulation is drastically reduced. Other investigators reported a decrease in $CD8^+$ T cells in other diseases and they hypothesized that such decrease may underlie the persistent immunodeficiency found in patients (64).

The proportion of $CD56^{bright}$ and $CD56^{dim}$ NK cells in healthy donors and CML patients is comparable, but CML patients have higher percentage of total NK cells.

Preceding studies suggest that NK cells play an important role in controlling growth of CML cells and sustaining complete molecular response (CMR) and , recently, CML patients who sustained a CMR after Imatinib discontinuation were shown to exhibit higher levels of functional NK cells than either normal subjects (63). In scientific literature some studies evaluate the NK cells in patients with viral infections, but in peripheral blood no quantitative alterations were described. Except in three kids

with herpetic encephalitis, that present an important reduction in percentage and in absolute number of NK cells (65). Our results confirm that CML patients under treatment have an increase on NK percentage and cell count, and this increased wasn't observed in naïve patients, what suggest that therapy potentiate the immune response by increasing the NK cells frequency, at least in peripheral blood. About the relative proportions of $CD56^{dim}$ and $CD56^{bright}$ NK cells in disease less has been known, and in our reports no differences between healthy donors and CML patients are conveyed.

Expression of cell surface markers by $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells from CML patients

The expression of cell surface markers displays some characteristics of NK cells, like activated NK cells (e.g. CD69, CD4), maturation stages (e.g. CD11b, CD27 and CD57), and structural properties involved in NK cell mechanisms (e.g. DNAM-1) (49,53). Analyzing some of these markers we conclude that NK cells from CML patients have different expression profiles which imply differences in NK cell recognition and effector functions.

The percentage of CD4 positive $CD56^{bright}CD16^{-}$ NK cells is reduced in CML patients and the percentage of CD8 positive $CD56^{bright}CD16^{-}$ NK cells is higher in CML patients.

CD4 is normally expressed on helper T cells, where it plays an important role in the recognition of MHC molecules on the surface of adjacent cells (66). Recently, studies demonstrated that activated NK cells express CD4 molecule on their surface (67). Our data confirms that NK cells express CD4 on their surface and interestingly NK cells from CML patients express less this marker. But there is no clues so far that explain the role of CD4 on NK cells functions. CD8 is another antigen usually expressed on CTLs, which interacts with HLA-I molecules. Previous studies demonstrated that CD8 engagement delivers an activating signal which increases the synthesis and secretion of $Fas^{-}L$ and ultimately it leads to NK cell apoptosis through the interaction of soluble Fas-L with Fas (68). The expression of CD8 by NK cells from CML patients is slightly increased compared with healthy donors, which in accordance with prior studies reflects an increase in NK cell apoptosis against tumor cells, in this case treatment seems to induce a higher expression of CD8.

$CD56^{bright}CD16^{-}$ NK cells from CML patients have a higher expression of CD7 per cell and $CD56^{dim}CD16^{+}$ NK cells from naïve patients have a higher expression of CD57 per cell, in comparison with healthy controls.

The function of CD7 on NK cells is barely unstudied. CD7 is a member of the immunoglobulin superfamily(67). $CD56^{bright}$ NK cells from CML patients have a higher expression of CD7 per cell, but further studies about the contribution of CD7 on NK cells are needed to understand what this CD7 increase actually means. The expression of CD57, a cell surface glycoprotein, on NK cells is well known, until now the expression of CD57 is associated with NK cell and T cells terminal maturation and differentiation. Thus CD57 allows the differentiation between $CD56^{bright}$ and $CD56^{dim}$, which express less and more CD57 respectively (49). Herein we demonstrate that $CD56^{dim}$ NK cells are most of all $CD57^{+}$, in naïve patients the expression of CD57 per cell is highly augmented compared with healthy controls but in CML patients this not occurs. Maybe NK cells from naïve patients are "so old", elucidated by the increase in CD57 surface density, that loss their capacities.

CML patients NK cells have higher expression of CD27 in $CD56^{bright}CD16^{-}$ subset. The percentage of $CD11b^{+}$ NK cells is augmented in $CD56^{bright}$ subset of cml patients and the surface density of cd11b is higher in both CD56brigh and $CD56^{dim}$ NK cells.

Recent studies have reported that CD27 belongs to TNF receptor family and allows the differentiation between NK^{-}cell subsets in murine and human. The surface density of CD27 and CD11b in NK cells may reflect different stages of differentiation too (69). Our results confirm that $CD56^{bright}$ cells has higher expression of CD27 and less expression of CD11b than $CD56^{dim}$NK cells, in agreement with the definition of $CD56^{dim}$ NK cells as the more mature NK cells. The CD27 expression by NK cells from healthy or CML patients is equivalent. However, the percentage of CD11b in $CD56^{bright}$ NK cells is increased in CML patients, which indicates a greater degree of maturation in $CD56^{bright}$ population. Such premature development could affect the function of $CD56^{bright}$ NK cells, by losing their immunomodulatory character.

In $CD56^{bright}CD16^{-}$ NK cells, the percentage of $CD62L^{+}$ and $CD69^{+}$ cells and their surface density is augmented in CML patients.

CD62L is an adhesion molecule that mediates the primary interaction of immune cells with the vascular endothelium and the homing of $CD56^{bright}$ NK cell subset to secondary lymphoid tissues (29). The lack of CD62L expression has been linked to NK cell degree of cytotoxicity and the majority of $CD62L^{-}$ NK cells were found to be $CD56^{dim}$ NK cells (29), so higher expression of CD62L means a minor degree of cytotoxicity by NK cells. Our results show an increase in CD62L expression in CML patients in both NK cell subsets. Some studies in T lymphocytes reported that CD62L down-regulation accompanies T lymphocyte activation and is thought to redirect cells away from lymph nodes to sites of infection, parallel with NK cells maybe in CML patients the upregulation of CD62L prevents that NK cells turn activated (70). By contrast, CD69

molecule is rapidly upregulated on NK cells and other lymphocytes when they are activated (71). Human peripheral blood NK cells express CD69 after their stimulation by IL-2 or IFN-α. This activation antigen represents a triggering surface molecule in NK cells as its stimulation triggers the cytolytic machinery of these cells. However, so far the, mechanisms regulating the expression of CD69 in are unknown (71,72). In our studies CML patients have a higher percentage of $CD69^{+}$ $CD56^{bright}$ NK cells whereas have a lower percentage of $CD69^{+}$ $CD56^{dim}$ NK cells. However, for both NK subsets the surface density of CD69 is bigger in CML patients. Thus we conclude that NK cells from CML patients express more CD69 in order to increase their activity.

The surface density of CD137L and CD137 and the $CD137^{+}$ cell percentage is augmented in both $CD56^{bright}$ and $CD56^{dim}$ NK cells from CML patients; the ratio CD137/CD137L in $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells is augmented in CML patients.

There are numerous studies in mice supporting the notion that NK cells are involved in the elimination of tumor cells. CD137 belongs to the TNFRSF and has been reported to enhanced the proliferation and survival of activated $CD4^{+}$ and $CD8^{+}$ T cells in humans (73). Furthermore, its expression has been reported on NK cells (74). In AML it was demonstrated that CD137L was expressed on leukemic cells and interacts with CD137 on activated NK cells. The interaction CD137/CD137L induces cytokine release (IL-10 and TNF-α) by AML cells, which defect granule mobilization, cytotoxicity and IFN-γ production. In our studies we detected a higher expression of CD137 by $CD56^{bright}$ and $CD16^{dim}$ NK cells from CML patients as demonstrated for AML. And the ratio CD137/CD137L is also augmented in NK cells from CML patients (73–75). Additionally, as we saw the expression of TNF-α is increased in CML patients evidencing the correlation between CD137/CD137L and the production of TNF-α in NK cell, as demonstrated in AML patients.

The expression and surface density of CD96, DNAM-1, CD155 and TIGIT of CML patients don't differ, significantly, from healthy donors.

DNAM-1, CD96 and TIGIT belong to an new family of receptors that interact with nectin and nectin-like proteins. DNAM-1 induces NK cell-mediated cytotoxicity, whereas TIGIT counterbalances the effect of DNAM-1.By contrast, despite CD96 shares the ligand CD155 with DNAM-1 and TIGIT their role has remained unclear (76). Previous studies shown that DNAM-1 plays a crucial role in tumor immune surveillance mediated by NK cells and CTLs and, in human cancers, NK cells may express low levels of DNAM-1 as a result of tumor-mediated activity. For example blocking the interaction of DNAM-1 with its ligands results in inhibition of NK cell cytotoxicity against carcinomas and hematopoietic tumors (76,77). In our study the particular frequency of these four markers is equal for healthy donors and CML patients, but further studies are needed to deduce if expression of DNAM-1, TIGIT and CD96 on NK cells from CML patients could effectively be involved in the disease development.

The expression and surface density of PD-1, PD-L1 and PD-L2 of CML patients don't differ, significantly, from healthy donors.

PD-1 is a programmed death receptor T cell expression of PD-1 downregulatas the immune response against malignancy by engagement of them cognate ligands (PD-1 or PD-2) expressed on tumor cells (54,78). However, little is known concerning PD-1 and NK cells. Recent studies in chronic HIV-1 infection described that raised levels of PD-1 were associated with limited NK cell proliferation, which may have consequences for their maintenance during disease (79). We just did a quick research about PD-1, PD-L1 and PD-L2 in T cells, NKT cells and NK cells from CML patients, and comparing with healthy donors there is no evidence for PD-1 role in CML.

Receptor repertoire of $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells from healthy donors and CML patients

NK cells are cells with the mission to defend the body immediately against pathogens or in the early stages of tumor development. As a consequence, the recognition receptors of NK cells are displayed on the cell surface without the need of assembly, i.e., rearrangements from multiple DNA segments after antigenic encounter. After recognition NK cells are regulated by a balance of signals delivered from activating and inhibitory receptors as we saw before.

KIR2DL1, KIR2DL2 and KIR2DS1 expression is altered in CML patients.

The highly polymorphic KIR receptor family encodes the main MHC monitoring molecules on primate NK cells and includes both inhibitory and activating members. KIRs are transmembrane glycoproteins containing two (D1 and D2) or three (D0, D1 and D2) extracellular C2 type Ig like domains. KIRs with two Ig like domains are designated KIR2D; KIRs with three Ig like domains are designated KIR3D. Whereas KIR2D receptors bind HLA C alleles, KIR3D receptors bind HLA A and HLA B alleles, In addition, they have either short (S) or long (L) intracytoplasmatic tails which transduce activating or inhibitory signals, respectively(80). However our results shows that KIR2DL1 in heathy donors is highly expressed in $CD56^{bright}$ NK cells and poorly expressed by $CD56^{dim}$ NK cells, in literature KIRs are expressed preferably on $CD56^{dim}$ NK cells than in $CD56^{bright}$NK cells (80). KIR2DL1 is an activating receptor of NK cells and its expression is drastically decreased on $CD56^{bright}$ NK cells from CML patients, in healthy control almost $CD56^{bright}$ NK cells express KIR2DL1 whereas in CML patients $CD56^{bright}$ NK cells practically not express this receptor. So KIR2DL1 is downregulated by the disease or treatment condition leading to an impaired NK cell activity. In contrast, KIR2DL2, also an activating receptor, is relatively upregulated on

$CD56^{dim}$ NK cells what it means that NK cells are expressing more activating KIR2DL2 improving their cytotoxic activity. In the other hand, KIR2DS1 expression seems to be a little high in CD56brigh NK cells. Different KIR repertoires were identified in CML patients and healthy donors, but the mechanisms behind such modifications remain unclear.

The NKG2A expression and surface density on $CD56^{bright}CD16^{-}$ NK cells and the surface density of NKG2C on $CD56^{dim}CD16^{+}$ NK cells is decreased in CML patients, and the ratio NKG2C/NKG2A are increased in both NK subsets.

Human NKG2 molecules belong to the C-type lectin family (81). CD94 forms $hetero^{dim}$ers with NKG2A or with NKG2C that bind with its ligand HLA-E. Binding HLA-E with CD94:NKG2A conveys an inhibitory signal, by contrast with CD94:NKG2C an activating signal is transduced to NK cells (82). The CD94/NKG2A complex is expressed on most normal NK cells, namely on $CD56^{bright}$ NK cells, whereas CD94/NKG2C complex is poorly express on resting blood NK cells although the expression can be regulated by the cytokine environment and might be affected by ongoing infections (83). Our studies confirm that NKG2A is expressed essentially on $CD56^{bright}$ NK cells, further that in CML patients the NKG2A expression is drastically reduced which could represent a positive effect of CML treatment on NK activity, once NK cell inhibiting control is less effective. In the other hand, NKG2C expression is augmented in $CD56^{dim}$ NK cells, cytotoxic NK cells. This receptor is an activating counterpart for the inhibitory CD94/NKG2A $hetero^{dim}$er (83) and the ratio between NKG2C/NKG2A is increased on CML patients. Furthermore the expression of CD94 is unimpaired in CML patients (data not shown). We hypothesized that NKG2A and NKG2C expression on NK cells is inversely correlated, and in CML patients something is upregulating NKG2C expression inhibiting the expression of NKG2A, consequently

the NK cells overcomes the rest state and be active against leukemic cells. However, further studies are needed to understand if these facts are consequence of disease rather than cure.

NKp30, NKp44, NKp46 and NKp80 expression by NK cells between CML patients and healthy controls is different.

Another NK cell receptor family consists exclusively of activating receptors known as NCRs, including NKp30, NKp44, and NKp46. The expression of such markers is restricted to NK cells as well as NKp80. NKp80 is a C-type lectin-like receptor that function as a co-receptor with NCRs to induce activation of NK cell-mediated cytolysis (41,84) and in our results there is no difference for NKp80 expression in NK cells from CML patients . NCRs trigger lysis of tumors and virus infected cells (85). Earlier studies shows that NKp30 and NKp46 are constitutively and selectively expressed on all peripheral NK cells whereas NKp44 is expressed only on activated NK cells (84,85). NKp46, NKp30 and NKp44 bind molecules expressed by tumor cells(86), and in some diseases as acute myeloid leukemia (AML) the down-regulation of activating receptors NKp30 and NKp46 correlates with defective NK cell cytotoxicity and poor leukemia prognosis(87). In our studies the expression of NKp30 is downregulated mainly on $CD56^{dim}$ NK cells, what suggests a weaker NK cell cytotoxic activity, since activating receptor its less expressed NK cell recognition of tumor cells through NKp30 is deficient. NKp46 expression is increased in NK cells from CML patients, such increased should be correlated with the recognition of molecules expressed by leukemic cells, increasing NKp46 upregulation on NK cells and activating them. NKp44 is a characteristic receptor of activated NK cells, and in our studies we identify a decrease in NKp44 expression on NK cells from CML patients, possibly tumor microenvironment upregulate the expression of ligands recognized by inhibitory

receptors on NK cells inhibiting their activation, these could explain the low frequency of NKp44^{+} NK cells in CML patients.

Cytokine production by CD56^{bright}CD16^{-} and CD56^{dim}CD16^{+} NK cells from healthy donors, naïve and CML patients

With a multiplicity of receptors evolved to sense cellular homeostasis and distress, NK cells are well equipped to act as primary initiators of immune responses upon recognition of infected or neoplastic cells. Such responses are not confined to cytotoxic effector mechanisms, but also involve the secretion of cytokines and chemokines. In this line we observed the frequency of some cytokines like interleukins (IL-4 and IL-10), TNF-α, TGF-β and IFN-α in NK cells from CML patients.

The cytokine production by NK cells is impaired in naïve and CML patients, comparing with healthy donors.

Our results shows that NK cells from naïve patients produced more interleukins (IL-4 and IL-10) than healthy controls, although NK cells from treated CML have a reduced production of ILs, less than controls. Interleukins are proteins that mediate cellular communication. The biological effect of IL-10 is anti-inflammatory and immune suppressive(88). So an elevated production of IL-10 by NK cells could suppress immune response against leukemic cells in untreated CML patients, in order to escape to NK cell activity. The normal levels are restored after treatment, since NK cells from CML patients have lower production of IL-10. Other studies confirm that NK cell expression of IL-10 downregulate the immune response (29). About IL-4 production by NK cells not much is known but in our report IL-4 expression is similar to IL-10 expression by NK cells in healthy donors, naïve and CML patients. Like IL-10, TGF-β is also a cytokine with immune suppressive effects. TGF-β antagonizes IL-15, a cytokine that induces NK cell proliferation and activation (87). Our results show that the

percentage of TGF-β^+ NK cells from CML patients is reduced; however the expression of TGF-β per cell is augmented in both NK subsets from CML patients. Previous studies shown that TGF-β mediates inhibition of surface expression of NKp30 (89) and in our study low expression of NKp30 in $CD56^{dim}$ NK cells is confirmed. Suggesting that TGF-β suppresses NKp30 expression, thus inhibiting NK cells cytotoxicity. Besides that some reports defend that release of TGF-β upregulate MHC-I expression on tumor cells and downregulate expression of NK cell activating receptors such as NKG2D and their ligands (89). Among all NK cells produced cytokines, TNF-α and IFN-γ are critical in early host defense pathways, to help to control the spread of intracellular pathogens (29). Following activation, NK cells are well known to produce and express several types of cytokines, for instance IFN-γ that influence other types of immune cells such as T cells and DCs. IFN-γ, as our results confirmed, is predominantly produced by $CD56^{bright}$ NK cells (90). In CML patients rate of IFN-γ produced by NK cells its greatly increased, which means that the production of IFN-γ is positively affected in NK cells from CML patients, signifying higher NK cell cytotoxicity. TNF-α, another proinflammatory cytokine produced by NK cells, member of the tumor necrosis factor receptor super family (TNFRST) and their ligands in the tumor necrosis factor superfamily members (TNFSF) have been suggested to be involved in the activation and maturation of NK cells (91). In patients with CML the NK cells CD56brigh and $CD56^{dim}$ express $TNF^-\alpha$ in higher amounts then NK cells from healthy donors, so NK cells from CML patients are more cytotoxic than those from healthy donors.

Effect of CML therapies, biologic or TKI therapy, and dosage in NK cells

As we saw before, TKI therapy revolutionized the CML treatment. Imatinib was the first drug used in CML treatment and stands as standard drug for CML patients. Despite the success of Imatinib, some patients do not respond or develop resistance against Imatinib. Concerning these patients novel TKIs are introduced in market, second and third generation TKIs that overcomes some limitations of Imatinib (1,49,52). Recent observations indicated that TKIs have inhibitory and/or stimulatory effects on immune cells, via modification of markers in surface of tumor cells. Some reports focused on NK Cells show that Imatinib have no impact on NK cytotoxicity or cytokine production, whereas nilotinib negatively influenced cytokine production (1). Several studies try to report the effect of Dasatinib on NK cells and although immunosuppressive effects were initially observed in preclinical studies, recent evidence suggests Dasatinib may activate and mobilize anti-leukemic immune responses, improving efficacy (92). About ponatinib effect on NK cells little information is available until now. Herein, we report the latter results but dividing CML patients according to therapy as described is results.

The percentage of lymphocytes is independent of therapy and dosage, but the percentage of NK cells differs, evidenced on CD56^{bright} NK cells percentage.

Although total lymphocytes are reduced in CML patients, no differences exist between the therapy groups which means that the current treatment for CML don't affect the total lymphocyte frequency in peripheral blood cells (49). NK cells are decreased in number in CML patients; however the Dasatinib group has increased NK cell number, which indicates that Dasatinib induce NK cell restore by the immune system (92). Another interesting finding is that those patients who are doing IFN-α have a higher percentage of CD56^{bright} NK cells compared with other therapies, in accordance

with this report, increased counts of NK cells have also been reported for IFNα-treated CML patients who were able to discontinue treatment without relapse (63). Recent studies shows that IFN-α combined with TKIs is more effective than TKI by himself (93), and the increase in NK cell frequencies could be another positive insight for IFN-α in current treatment of CML. Plus, $CD56^{bright}$ NK cells seems be lower in patients with higher doses of Imatinib (42,53) but in our studies this difference is minimal.

NK cells from patients undergoing different therapies have different expression of surface markers and receptors as well as different cytokine production.

Bringing together our results in order to compare the different kinds of therapy that the patients in study are doing we conclude that different therapy, biologic or TKI, different TKI drug and its dosage are crucial factors in disease development. As we can see in table 1 many direct or indirect effects have been reports about TKIs on NK cells (1,42). In this specific study we demonstrate that Imatinib 600 and Imatinib 800 patients have less production of IFN-γ and TNF-α and less expression of NKG2C. Furthermore KIR2DL1 and KIR2DL2 expression is augmented in Imatinib 800 and Imatinib 600 patients, respectively. Both cytokines and NKG2C are players in active immune response. Thus, weak expression by NK cells from Imatinib 600 and 800 patients in addition with the high expression of inhibitory KIRs results in a defective NK cell function, confirming the critical role of Imatinib dose on NK cells. Other researchers reported that Dasatinib induce alterations on NK cells, but the critical effects in this cellular population remain undiscovered (92). NK cells from Dasatinib patients have reduced expression of CD69 and higher production of IL-10, CD69 data indicates that NK cells are less activated and the production of IL-10 seems to suppress NK cell activity, both occurrences point to NK cell dysfunctional activity probably caused by Dasatinib drug. Plus, KIR2DL1 expression is augmented in NK cells such as the

expression of NKp80 and NKp30 is decreased, one more time the inhibitor receptors are overexpressed whereas activating receptors are under expressed. The decrease in NKp30 expression was before related with other leukemias like AML (87). Ponatinib is the newest TKI used in CML treatment, so few studies exist about this drug until now. In our research we analyzed one ponatinib patient so the results for Ponatinib group are poorly founded. However an increased in TGF-β production is reported. For IFN-α and others subject was evidenced that the expression of CD27 is decreased in $CD56^{bright}$ NK cells, showing that IFN-α didn't affect so strongly NK cells like TKIs. At present, IFN-α has been combined with Imatinib for CML treatment and investigators have reported a higher effectiveness and less parallel effects on immune cells from those patients (54).

Effect of BCR-ABL clones in NK cells from CML patients

Several studies reported that NK cells from blood of CML patients are progressively decreased in number as the disease progresses from chronic phase to blast crisis (94). So is hypothesized that $BCR^{-}ABL^{+}$ clones per patient may be directly responsible by interfering with NK cell differentiation and function(93,94). As we saw in the introduction the $BCR^{-}ABL$ oncogene encodes a constitutively activated tyrosine kinase that affects proliferation, mobilization and differentiation of $BCR^{-}ABL^{+}$ cells (41).

The percentage of total lymphocytes, NK cells and NK cells subpopulations don't differ with the ratio BCR/ABL.

The percentage of BCR-ABL clones is directly correlated with disease severity. Disease remission is achieved when the ration BCR/ABL is below than 0.01%, when the BCR-ABL ratio exceeds this value means that therapy is not being effective. Thus the BCR-ABL ratio allows monitorize the treated CML patients (95). In our study we conclude that the higher ratio of BCR-ABL clones in blood samples from CML patients not significantly affect neither the percentage of lymphocytes or percentage and NK cells count.

Conclusions and Future Perspectives

The concluding remarks of the present research are that the frequency of total lymphocytes and NK cells is decreased at diagnosis and in treated CML patients. The expression of surface markers such as CD7, CD57, CD27, CD11b, CD62L, CD69, CD137 and CD137L are altered in CML patients. The receptor NK cell repertoire from CML patients is different from healthy donors, namely KIR2DL1, NKG2A, NKG2C, NKp30, NKp44 and NKp46. NK cells from CML patients produce more IFN-γ and less IL-4, IL-10 and TGF-β. Besides that the current therapies used in CML treatment have different effects on NK cells subpopulations, specially the patients undergoing Dasatinib or IFN-α, the effects of dosage is not so evident. A briefly research was made for patients with low to high percentage of BCR/ABL and no significant differences are found but further studies are needed in this field. NK cells as we demonstrate in this study are altered in CML patients and these alterations disturb the NK cell homeostasis preventing NK cell effector mechanisms against leukemic cells. In literature have been reported NK cell changes in other diseases, viral infections and cancer, and in the last decade many researches focus their attention into understand NK cell biology and mechanisms in order to potentiate cytokine production and cytotoxicity against tumor cells. Therefore NK cells are emerging as a powerful tool in immunotherapy.

Next, we hope to be able to analyze others interesting markers that could be key points of the disease and preform genetic studies of NK cells from CML patients to confirm the present results and bring new discoveries. Concluding, I think that NK cells may be the future of immunotherapy not only in CML as well as other diseases.

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Appendix

Table 2: Antibodies used in present research.

1F12	Monoclonal antibody
8C11	Monoclonal antibody
CD11b (ITGAM)	Cluster of Differentiation 11b
CD137 (4-1BB)	Cluster of Differentiation 137
CD137L	Cluster of Differentiation 137 Ligand
CD14	Cluster of Differentiation 14
CD155 (PVR)	Cluster of Differentiation 155
CD158a	Cluster of Differentiation 158a
CD158b	Cluster of Differentiation 158b
CD16	Cluster of Differentiation 16
CD19	Cluster of Differentiation 19
CD27	Cluster of Differentiation 27
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD56 (NCAM1)	Cluster of Differentiation 56
CD57	Cluster of Differentiation 57
CD62L (L-selectin, LAM-1)	Cluster of Differentiation 62L
CD69	Cluster of Differentiation 69
CD7	Cluster of Differentiation 7
CD8	Cluster of Differentiation 8
CD96	Cluster of Differentiation 96
DNAM-1 (CD226)	DNAX Accessory Molecule-1
NKG2A	Natural killer group 2A
NKG2C	Natural killer group 2C
NKp30 (NCR3)	Natural Killer protein 30
NKp44 (NCR2)	Natural Killer protein 44
NKp46 (NCR1)	Natural Killer protein 46
NKp80 (KLRF1)	Natural Killer protein 80
PD-1	Programmed death receptor 1
PD-L1	Programmed death receptor ligand 2
PD-L2	Programmed death receptor ligand 1
TIGIT	T cell immunoreceptor with Ig and ITIM domains

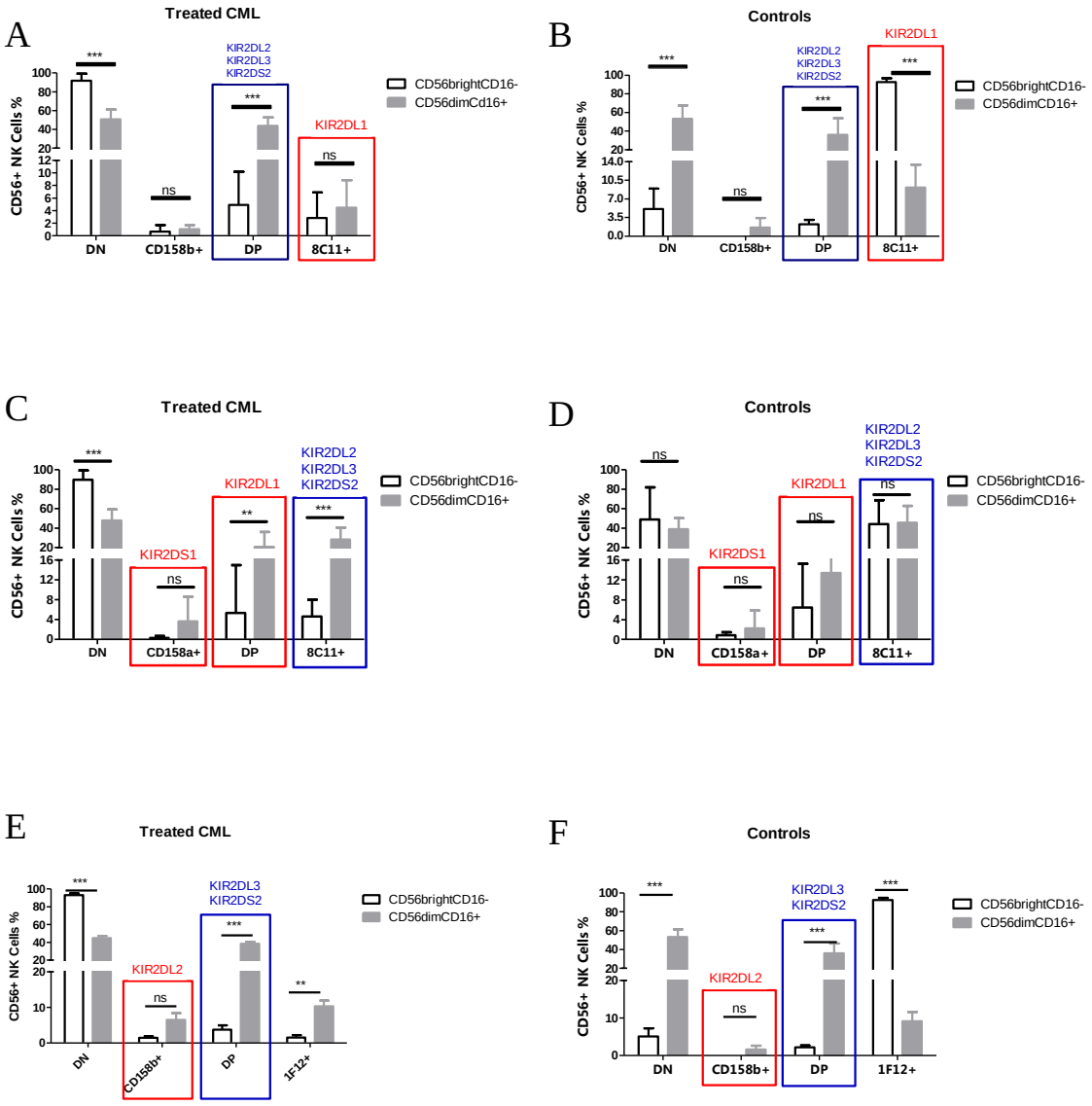

Figure 47: Analysis of the distribution of KIR2DL1, KIR2DL2 and KIR2DS1 on natural killer (NK) cells by combining the antibodies 1F12, 8C11, CD158a and CD158b. A/B – 8C11 and CC158b combined to KIR2DL1 measurement; C/D – 8C11 and CD158a combined to KIR2DS1 measurement; E/F – 1F12 and CD158b combined to KIR2DL2 measurement. The graphs on the left are related to CML patients (n=10) and the graphs on the right are related to healthy controls (n=4).

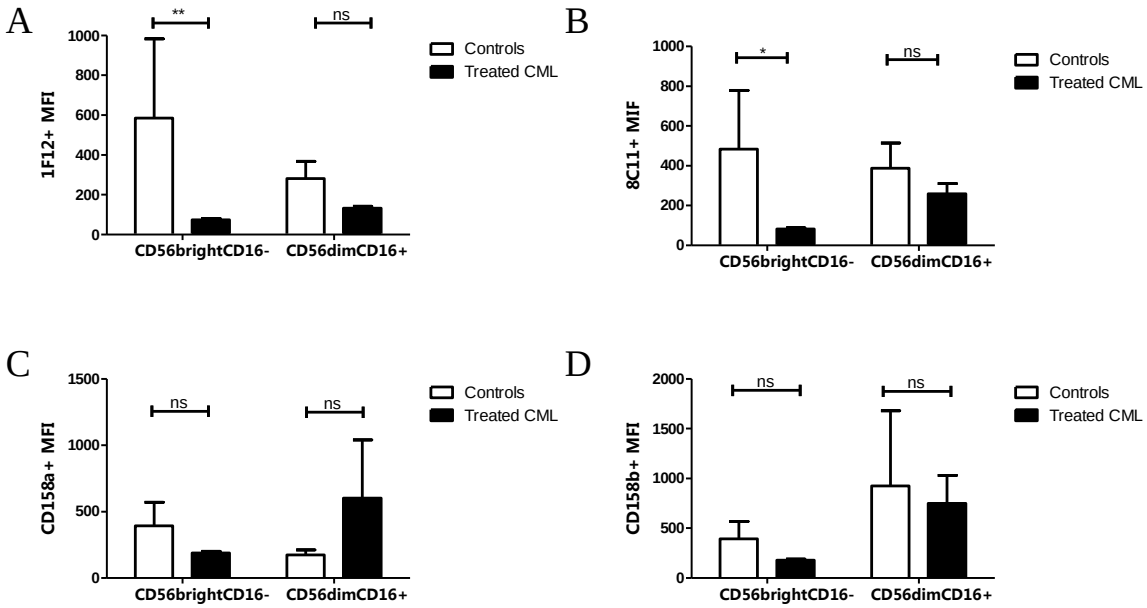

Figure 48: Mean of fluorescence intensity of 1F12, 8C11, CD158a and CD158b in CD56bright and CD56dim populations. A – Mean of fluorescence intensity of 1F12 in $CD56^{bright}$ and $CD56^{dim}$ NK cells. B – Mean of fluorescence intensity of 8C11 in $CD56^{bright}$ and $CD56^{dim}$ NK cells. C – Mean of fluorescence intensity of CD158a in $CD56^{bright}$ and $CD56^{dim}$ NK cells. D – Mean of fluorescence intensity of CD158b in $CD56^{bright}$ and $CD56^{dim}$ NK cells. The blood samples are collected from CML patients (n=10) and healthy donors (n=4).