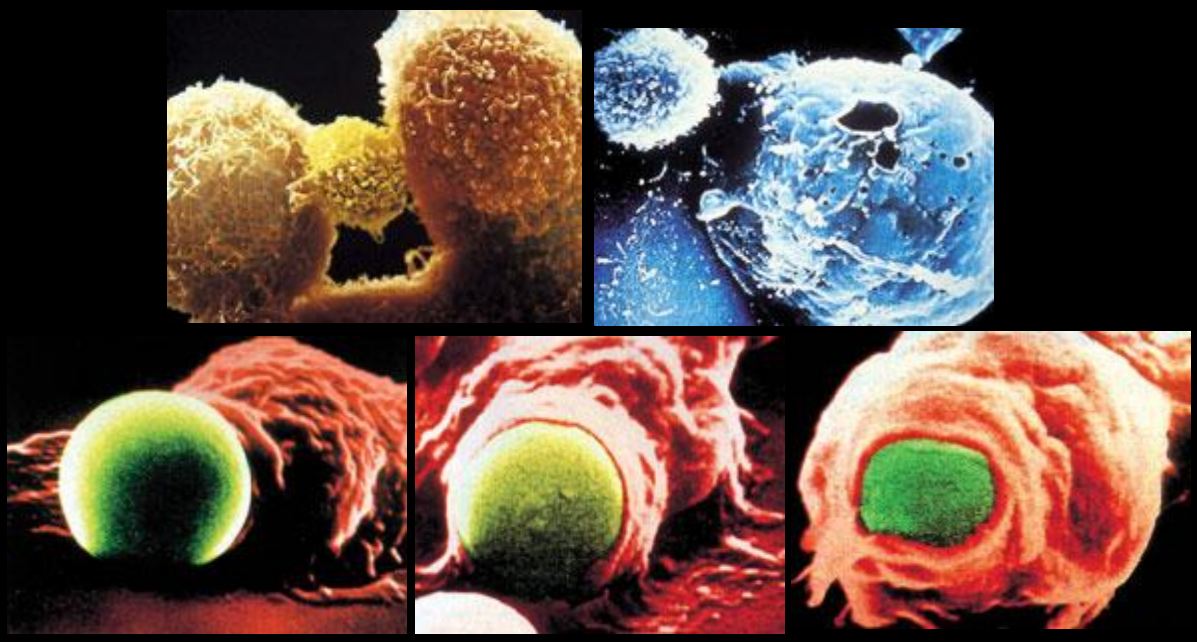

Diogo Gomes da Silva

ALTERAÇÃO DAS CÉLULAS NATURAL KILLER EM FUNÇÃO DA PRODUÇÃO DE ESPÉCIES REACTIVAS DE OXIGÉNIO

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Alteração das células Natural Killer em função da produção de espécies reactivas de oxigénio

Diogo Gomes da Silva

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica, realizada sob a orientação científica do Doutor Paulo Rodrigues Santos e da Professora Doutora Helena Sá.

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ADCC	Antibody-dependent cell cytotocicity
AML	Acute myeloid leukemia
APC	Antigen presenting cells
CCR	C-C chemokine receptor
CD	Clusters of differentiation
CGD	Chronic granulomatous disease
CML	Chronic myeloid leukemia
CTL	Cytotoxic T cells
DC	Dendritic cells
EGF	Epidermal growth factor
FSC	Forward scatter cell
GM-CSF	Granulocyte monocyte Colony Stimulating Factor
HC	Healthy controls
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
iDC	Immature dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer immunoglobulin-like receptors
LILR/ LIR	Leukocyte Ig-like receptor
mAbs	*Monoclonal antibodies*
MFI	Mean of fluorescence intensity
MHC	Major histocompatibility complex.
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural Killer
NCAM	Neuronal cell adhesion molecule
NCR	Natural cytotoxicity receptor
PAMP	Pathogen-associated microbial patterns
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptors
RBC	Red Blood Cell

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SSC	Side scatter cell
TAM	Tumor-associated macrophages
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
T_H	T helper
TNF	Tumor Necrosis Factor
VEGF	Vascular endothelial growth factor

ABSTRACT

Natural Killer (NK) cells are key components of the innate immune system due to cytotoxicity and cytokine release, without previous stimulation, in the early response against infected or malign cells. Their action is totally dependent on the balance between a varied repertoire of inhibitory and activator receptors. Although previous stimulation is not mandatory, the communication with cells of innate immune system especially antigen presenting cells can exacerbate their function. The production of intracellular reactive oxygen species (ROS) through NADPH oxidase during phagocytosis by antigen presenting cells is a simple mechanism to help in the destruction of the pathogen. However the NADPH oxidase enzymatic system also produces extracellular ROS with negative impact over NK cells. In many pathologies associated with chronic inflammation, ROS are increased and NK cells fail to deliver cytotoxic action. In *Mycobacterium tuberculosis* infection NK cells can have a important role, producing IFN-γ and lysing infected macrophages. However *M. tuberculosis* survives in macrophages avoiding ROS produced, without immune system responding appropriately. In chronic myeloid leukemia the uncontrolled expansion of myeloid cells are correlated with the decrease of antitumor lymphocytes. The main goal of our study is to investigate alterations of NK cells in tuberculosis and chronic myeloid leukemia due to production of reactive oxygen species.

In this work we analyzed 88 samples of peripheral blood from 50 chronic myeloid leukemia patients, 13 samples from tuberculosis patients and 20 samples from healthy individuals. The production of ROS and NK cell surface expression of some important receptors and markers (CD56/CD16, CD57, CD11b and NKp46) were evaluated through flow cytometry to all samples. NK cells and monocytes from healthy controls were sorted through fluorescent activating cell sorting and cultured in different conditions to evaluate NK cell capacity to resist to ROS production.

In tuberculosis and chronic myeloid leukemia patients, NK cells, monocytes and granulocytes from peripheral blood showed an increased of ROS production comparing with healthy controls. It was not detected any difference between subsets of NK cells. The stimulation of NK cells with IL-2, IL12 and IL-15 revealed to have a protective role to decrease ROS liberation.

In conclusion, in tuberculosis and chronic myeloid leukemia patients, NK cells have high levels of ROS being one of the possible immunosuppressive mechanisms that are associated with immunescape. The stimulation with a combination of interleukins could have a possible protective role.

As células Natural Killer (NK) são componentes chave do sistema inato devido à citotoxicidade e libertação de citocinas, sem estimulação prévia, na resposta inicial contra células infetadas e malignas. A sua ação é totalmente dependente do balanço entre um variado repertório de recetores ativadores e inibidores. Embora uma prévia estimulação não seja obrigatória, a comunicação com células do sistema inato especialmente com células apresentadoras de antigénio pode exacerbar a sua função. A produção de espécies reativas de oxigénio (ROS) intracelulares pela NADPH oxidase durante a fagocitose pelas células apresentadoras de antigénio é um mecanismo simples que contribui para ajudar na destruição do agente patogénico. No entanto o sistema enzimático NAPDH oxidase também produz ROS com impacto negativo sobre as células NK. Em muitas patologias associadas com inflamação crónica, ROS estão aumentadas e as células NK são incapazes de realizar ação citotóxica. Na infeção por *Mycobacterium tuberculosis,* as células NK podem ter um papel importante produzindo IFN-γ e lisando macrófagos infectados. No entanto o *M. Tuberculosis* pode sobreviver nos macrófagos evitando as ROS produzidas, sem que o sistema imune responda apropriadamente. Na leucemia mielóide crónica a descontrolada expansão das células mielóides está correlacionada com a diminuição linfócitos antitumorais. O principal objetivo deste trabalho é investigar as alterações das células NK em indivíduos com tuberculose e leucemia mielóide crónica devido à produção de ROS.

Estudámos 88 amostras de sangue periférico de 50 doentes com leucemia mielóide crónica, 13 amostras de doentes com tuberculose e 20 amostras de indivíduos saudáveis. A produção de ROS e a expressão de alguns recetores e marcadores da superfície de células NK (CD56/CD16, CD57, CD11b, NKp46) foram avaliadas por citometria de fluxo para todas as amostras. As células NK e os monócitos de indivíduos saudáveis foram separadas através da separação de células ativas por fluorescência e colocadas em cultura em diferentes condições para avaliar a capacidade das células NK para resistir à produção de ROS.

Nos doentes com tuberculose tal como nos doentes com leucemia mielóide cronica, as células NK, os monócitos e os granulócitos do sangue periférico apresentam um aumento de ROS comparando com controlos saudáveis. Não foi detetada nenhuma diferença entre as subpopulações de células NK. A estimulação de células NK com IL-2, IL-12 e IL-15 revelou ter um papel protetor protector ao diminuir os níveis de ROS

Em conclusão em doentes com tuberculose e leucemia mielóide cronica, as células NK têm os níveis de ROS sendo esse um dos possíveis mecanismos immunosupressores. A estimulação com uma combinação de interleucinas pode ter um papel protector

THEORICAL BACKGROUD

Immune system

The immune system is a versatile defense mechanism evolved in animals and humans against pathogenic microorganisms and cancer. With an enormous variety of cells and molecules, it provides the means to rapidly and specifically recognize and eliminate a myriad of potential variety of foreign invaders. The dynamic complexity of the immune system, using cellular and molecular mechanism all working together, can solve a sort of immunological challenges being severe or sustained infections quite rare.

Any immune system, since from more primitive organisms until the most recent, must be able to: recognize the pathogens, kill them and not affect the host tissue in this process (Beutler, 2004). These are the fundamental characteristics that allow the health of a multicellular organism. To do so, the immune system has a broad of mechanisms generally divided into two different components based on their own characteristics and functionality - the innate immunity and adaptive ("specific" or "acquired") immunity. Although different, each system is able to solve these fundamental problems (Kimbrell and Beutler, 2001) (Figure 1).

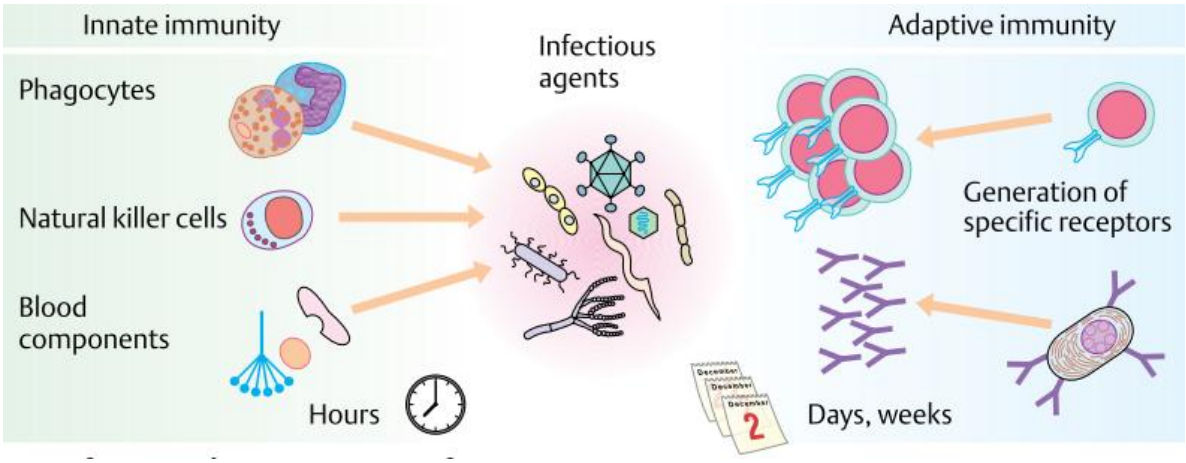

Figure 1- Different cellular and humoral components of the immune system *divided in innate and adaptive immunity (adapted from Burmester et al., 2003).*

Innate immune system

The innate immunity is the most universal and instantaneous way of fight infections and many multicellular organisms survive only with this type of immunity, being vertebrates the only that have also the contribution of the adaptive immune system (Kimbrell and Beutler, 2001, Beutler, 2004). Considered sometimes as primitive, this type of immunity has been shaped longer than adaptive immunity. Furthermore many of the infectious agents that an organism encounters in his life are controlled only with innate immunity (Beutler, 2004).

In most organisms, the first tier of defense is a physical barrier like the skin (Chaplin, 2010). If this barrier has been breached, various specialized cells and molecules released from cells or normally present in fluid bodies are responsible for recognize and fight the infectious agent (Kimbrell and Beutler, 2001)

The innate immunity is largely depending on myeloid cells. These cells, with a common progenitor, can be divided in mononuclear and polymorphonuclear phagocytes. In the mononuclear phagocytes there are monocytes, macrophages and dendritic cells (DCs), being all of them highly efficient in phagocytosis and in antigen presentation to cells of the adaptive immune system. Belonging to polymorphonuclear phagocytes are neutrophils, basophils and eosinophils (Chaplin, 2010).

The process of recognition of the invaders is the nucleus of innate immunity. This recognition is based on existent receptors in plasmatic membrane of the innate immune cells. (Kimbrell and Beutler, 2001).The diversity of mechanisms and receptors available by the immune cells can be divided in three different strategies: recognition of microbial nonself (also called pattern recognition which allows recognition of microrganisms); recognition of missing self (can detect small differences in cells allowing the recognition as not constituent of the body) and recognition of induced or altered self (can detect cells from the own body that can be dangers to the organism) (Medzhitov, 2010).

Although innate immunity cells have a limited type of receptors, they recognize molecules of indispensable components that exist on microbes and cannot suffer mutations. These host receptors are able to recognize characteristic components of microbes and are dseginated as “pathogen-associated microbial patterns” (PAMPs) and the receptors that recognize them are pattern recognition receptors (PRRs) (Beutler, 2004).

Another important cellular element of innate immunity is the Natural Killer (NK) cell. These cells are the only member of lymphocytes classified as innate immune cells and can be defined as large granular lymphocytes. They are developed in the bone marrow under the influence of IL-2, IL-15 and bone marrow stromal cells. Although they are classified as

lymphocytes they do not have antigen-specific receptors. NK cells have cytotoxic action in the absence of self-MHC molecules in the cells and can release cytokines with important roles in adaptive immune response (Chaplin, 2010).

Adaptive immune system

The adaptive immune system is, in terms of evolution, more recent and was build based on the older innate immune system, whereby is controlled and assisted. Without innate immunity the adaptive immune system offers a weak protection. (Kimbrell and Beutler , 2001)

The key feature of this type of immunity is the capacity to, after appropriate stimulation, express effector functions against a specific antigen and not the type of cell, and at the same time initiate mechanisms that allow a more effective response in the next infection, even if it occurs after decades (Chaplin, 2010). The cells that belong to adaptive immunity are B and T lymphocytes which express an almost unlimited and randomly array of recombinant receptors – immunoglobulin (Ig) and T-cell receptors (TCR), respectively - that are able to recognize any antigen (Kimbrell 2001), and once activated maintain a specific long-term memory. The genes responsible for TCR and Ig receptors are assembled by somatic rearrangements (Cooper and Adler, 2006).

The mains characteristics of T and B cells and therefore, of the adaptive immunity are specificity and memory. Specificity allows the immune system to recognize subtle differences among antigens like thus created by single mutations. The diversity of recognition molecules is so vast that the adaptive system is able to recognize billions of antigens. After the recognition of a specific antigen, memory T and B cells, formed through clonal expansion, when in contact with the same exactly antigen a second encounter, react faster than the first time, a characteristic unique in these cells (Kindt *et al.,* 2006). In the first contact with the antigen, effector and memory cells only become ready after a few days situation that is faster in posterior encounters. Meanwhile, while T and B lymphocytes suffer clonal expansion, the innate immunity is responsible for at least mitigate the infection. After adaptive immunity becomes ready the innate immunity still helps to amplify the immune response (Chaplin, 2010)

As previous described, innate immunity is the only way of fight infections in invertebrates. But in mammals there is a necessity of both indicating that the evolution shape innate and adaptive immunity in a way to unify and fortify the immune response. Although the innate response is capable of fight many infections and can control when and how adaptive response is activated, the opposite also occur. The adaptive immune system gave to vertebrates the ability to minimize immunopathology by specifically orientate host defenses

to pathogens, and due to memory, prevent repeated infections. At some point in evolution these two components of immune system evolve to cooperate but nowadays they are codependents of each other. (Palm and Medzhitov, 2007)

Natural Killer cells

Natural Killer cells are a sub-group of lymphocytes because they share a common lymphoid progenitor cell in bone marrow, have lymphoid markers and also due to typical lymphoid morphology. However they do not share the major characteristics that make T and B lymphocytes part of adaptive immunity and therefore are considered components of the innate immunity (Vivier *et al.,* 2011). They are a transitional cell type that helps the interaction between innate and adaptive immune systems (Lanier, 2005). NK cells represent 10-15% of all lymphocytes circulating in blood and phenotypically they express CD56 and in opposite to T lymphocytes do not express CD3 (Robertson and Ritz, 1990). It is possible to find high amounts of NK cells in the blood, spleen and other tissues.

During earlier steps of inflammation, NK cells are recruited to tissues in response to chemokines and can interact with other immune cell types having these cells a critical role in initiation, amplification and polarization of adaptive responses (Moretta *et al.,* 2005). NK cells have many roles in the immunologic response and are regularly associated with the early control against virus infection (Lee *et al.*, 2007) and tumor immune surveillance (Smith *et al.,* 2002), but unlike cytotoxic T cells, they do not need specific immunization inducing directly the death of tumor and virus infected cells. NK cells need to sense if cells are transformed, infected or “stressed” to discriminate between abnormal and healthy cells (Lanier, 2005).

There are few reports of complete NK cell deficiencies in humans, normally resulting in fatal infection during childhood, leading to the idea that NK cells must serve a very important role in host defense. In patients with some NK cells deficiency normally a persistent acute viral infection occurs especially with *Herpes simplex* virus (Orange, 2002). Although in humans there is limited information, the importance of NK cells probably extends beyond viral infections. In mouse models with defects in NK cells, is clearly demonstrated an increased susceptibility to neoplasic diseases as animals become older. (Alderson e Sondel, 2011)

The full capacity of NK cells is only achieved with stimulation of cytokines responsible for different functions like IL-2 IL-12 and IL-15 that are produced by others cells of immune system. The IL-15 is required for the maturation and survival of NK cells (Caligiuri, 2008) and IL-12 induce strong cytolytic activity against tumor cells (Marcenaro *et al.,* 2005). Like T and B cells, NK cells participate in the immune response from different ways depending on which

cytokines are present in their microenvironment and the state of functional maturation that these cells have in that moment (Vivier *et al.,* 2011).

Beyond the cytotoxic action, NK cells are the major producers of interferon-y and produce also others cytokines as tumor necrosis factor-α (TNF-α), interleukin (IL)-10 among others (Vivier *et al.,* 2011). These cells can mediate perforin and granzyme dependent lysis and proliferate. Despite their natural killing mechanism, NK cells can be activated and become more cytotoxic with IFN-α/β. Both the cytotoxicity and IFN-y production occur in few hours after infection contrary to the other lymphocytes (Biron *et al.,* 1999).

Subsets of Natural Killer cells

Two different populations of NK cells are identified based on the expression of CD56 (isoform of neuronal cell adhesion molecule- NCAM) in the cell surface. About 90% of human NK cells are classified as $CD56^{dim}$ because express medium levels of CD56 whereas the rest of NK cells are $CD56^{bright}$ (high levels of CD56). The $CD56^{dim}$ subset also expresses high levels of Fcy receptor III (FcyRIII or CD16) and in $CD56^{bright}$ subset it is possible to find $CD56^{bright}CD16^{+}$ and $CD56^{bright}CD16^{-}$ cells. The level of CD56 that is expressed in the membrane is associated different functions. The $CD56^{dim}$ cells are more cytotoxic and the $CD56^{bright}$ cells are responsible for the interleukin production (Figure 2) (Cooper *et al.,* 2001). In peripheral human blood only 5-15% of NK cells are $CD56^{bright}$ whereas in lymph nodes most NK cells are $CD56^{bright}$ (Fehniger *et al.,* 2003; Ferlazzo and Munz, 2004).

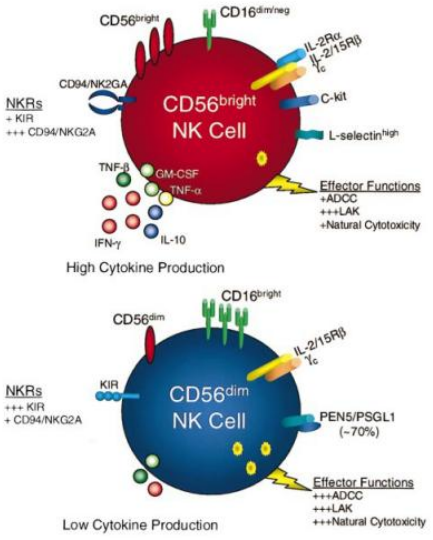

Figure 2-Differences between two subsets $CD56^{dim}$ and $CD56^{bright}$. Both subsets differ in receptor expression on the membrane cytotoxicity and cytokine release (adapted from Cooper *et al.,* 2001).

The proportion between $CD56^{dim}$ and $CD56^{bright}$ found in healthy individuals is not static and there are several changes in the relative levels of these populations associated with certain diseases. For example, was already discovered an expansion of $CD56^{bright}$ in different pathologies like transporter associated with antigen processing (TAP) deficiency, (Zimmer *et al.,* 2007), multiple sclerosis (Saraste *et al.,* 2007), hepatitis C virus (HCV) (Poli *et al.,* 2009), systemic lupus erythematosus (Shepis *et al.,* 2009), among others. The lack or decreased levels of $CD56^{bright}$ was already observed in coronary heart disease (Hak *et al.,* 2007) and in juvenile rheumatoid arthritis (Villanueva *et al.,* 2005)

Another difference between the subsets is the transcriptome. NK cells populations differ in 473 transcripts, 176 are exclusively expressed in $CD56^{dim}$ and 130 exclusively in $CD56^{bright}$ (Went *et al.,* 2006)

NK cell receptors

NK cells recognition is a very complex mechanism, more complex than B and T cells, because there is no antigen receptor in NK cells that can be considered the main responsible for differentiation, activation and effector functions as in cells of the adaptive immunity. In NK cell the recognition is dependent of different receptors that can transmit activator or inhibitory signals to the cytoplasm. This complex process requires initial binding to the potential target cell, interaction between ligands and activating and inhibitory receptors which mediate internal signals that will determinate whether NK cells detaches and moves on or stays and respond. In this mechanism, NK cell reorients the relevant receptors creating a synapse between target and NK cell (Lanier*,* 2005). In the immune synapse that occurs between NK and the target cell, the type and number of receptor/ligand interactions determinates the survival or death of the target cell. Because in an autologous synapse, cells from the own body express high amounts of self-HLA class I molecules that bind to inhibitory receptors and lack or express low ligands for activating receptors these cells are spared of the cytotoxicity delivered by NK cells, a process called missing self-hypotheses. However, in tumor or infected cells, a decrease of self-HLA and/or up-regulation of ligands for activating NK cells receptors activate NK cells (Bellora *et al.,* 2010).

In inhibitory receptors it is possible to catalog two groups: members of the immunoglobulin (Ig) receptors superfamily where are included the killer immunoglobulin-like receptors (KIRs) that bind to classical MHC class Ia ligands (HLA-A, B C) and leukocyte Ig-like receptor (LILR/LIR) and in the other group are C-lectin type receptors mainly composed of CD94-NKG2A therodimeric receptores that bind the nonclassical MHC class Ib (HLA-E) and ly49 homodimer (Chen *et al.,* 2009). The NK cell must always have an inhibitory receptor

that recognizes at least one of the MHC class I. Although already was found more than a dozen individual KIR molecules, each individual NK cell expresses only a fraction of the available KIRs (Zorn *et al.,* 2006). All the inhibitory receptors share the same signaling mechanism that is dependent of the immunoreceptor tyrosine-based inhibition motif (ITIM) (Binstadt *et al.,* 1996).

Regarding to activating receptors, they bind to some ligands expressed by stressed cells and can also be specific for classic MHC Class I, nonclassic MHC class I or MHC class I-related (Bakker *et al.,* 2000). Natural cytotoxicity receptors (NCRs) include NKp46, NKp30 and NKp44 (Moretta, A. *et al.,* 2000). They are expressed in NK cells selectively and are associated with immunoreceptor tyrosine-based activating motif (ITAMs). NCRs have a major role in NK-mediated lyses of various tumors including myeloid leukemia's (Pende *et al.,* 2005). One of the most studied receptor in NK cells is CD16 that is responsible for detection of antibody-coated cells and is necessary to exert antibody-dependent cell cytotocicity (ADCC) (Vivier *et al.,* 2011).

Recent studies have shown that there is a very complex recognition process by NK cell receptors by far more complex than the initial "missing self-hypotheses" (Caligiuri, 2008). NK cell recognition is dependent of the balance between activating and inhibitory signals that are simultaneously delivered to NK cells and the alteration of this balance determines the action of NK cells (Lanier, 2001, Vivier *et al.,* 2004). Although the missing self-hypotheses is still correct new data are showing that NK cells can kill transformed cells that express ligands for activating NK cell receptors despite normal expression of self–MHC class I molecules (Cerwenka *et al*., 2001). The opposite can also occur because NK cells do not kill some cells with low (neural cells) or none MHC molecules (erythrocytes). This can be explained by one of two theories: or these cells do not have ligands to activating receptors or others inhibitory receptors to non-MHC ligands are expressed and protect these cells (Lanier, 2005)

Natural Killer cells in immune response against tumor cells

What makes NK cells so prominent and unique in immune surveillance in cancer context is because they do not require specific priming by APCs and MHC expression in tumor cells. They can respond directly to cells with decreased/lost MHC molecules and upregulated stress signals that occur after DNA damage. Such characteristic is necessary in T cell immune response (Chan *et al*., 2008). The capacity of NK cells to respond to human tumors depends largely on NKG2D and also on the NCRs NKp46, NKp44 and NKp30 (Fauriat *et al*., 2007).

NK cells can infiltrate solid tumors and their presence is considered a good prognostic indicator (Coca *et al.,* 1997) but normally they do not interact with tumor cells, because they are clustered around the stroma. The role of NK cells in the immune response context against tumors is the elimination of tumors at the beginning of proliferation or in the residual state of the disease that occurs after post-surgery or other interventions. However the control of "dormant" tumor state depends mainly on the adaptive immunity (Chan *et al.,* 2008). This early action of NK cells can be responsible for immune response not only in solid tumors but also in hematological malignancies (Chan *et al.,* 2008) like chronic myeloid leukemia (CML).

NK cells can participate in immune response against CML cells. However is normally observed a low number and defective function during CML progression (Pierson and Miller 1997). CML cells induce apoptosis in $CD56^{dim}$ NK cells and in less extend in $CD3^{+}$ T cells (Mellqvist *et al*., 2000). The importance of NK cells in the immune surveillance can be proven by the fact that when stimulated NK cells from patients with CML show lytic activity *in vitro,* there is lower risk of relapse after allogeneic bone marrow transplantation (Hauch *et al.,* 1990). The lower number of NK cells in the progression of this disease is well established as the less cytotoxic capacity of NK cells from blood of CML patients (Fujimiya *et al*., 1986) and other chronic leukemia's (Sørskaar *et al.*, 1988).

Chronic Myeloid Leukemia is a type of cancer where cells of the myeloid lineage undergo a massive clonal expansion genetically characterized by a translocation between chromosomes 9 and 22 t(9:22)(q32:q11) giving rise to a defective chromosome applied Philadelphia chromosome (Deininger *et al.,* 2000). From this translocation, results a chimeric protein - Bcr-Abl –which constitutively expressed as tyrosine kinase in about 90% of patients with CML. The new protein created have a central role in pathogenesis of this type of leukemia (Sawyer, 1999; Deininger *et al.,* 2000) The formation of BCR-ABL is implicated in alteration of adhesion to stroma cells and extracellular matrix (Gordon *et al.,* 1987) constitutively activation of mitogenic signals and reduced apoptosis (Bedi *et al.,* 1994).

The disease has three different phases –choric phase, accelerated phase and blast crisis (Drunker *et al*., 2001). Although was already proved that the tumor phenotype is dependent on the BCR-ABL fusion, this new protein can lead to new mutations (Melo and Barnes*,* 2007; Rassool *et al*.*,* 2007). Today, new therapies are being tested, being the inhibition of gene expression at translational level by antisense strategies and the stimulation of the immune system to recognize and destroy leukemic cells some of the most prominent (Ciarcia *et al.,* 2010).

Natural Killer cells in infection

The NK cells are normally associated with immune responses against malignant and virus infected cells. However, NK cells also participate in responses to bacteria, fungi and protozoa (Steveson and Riley, 2004; Chiche *et al.*, 2011). NK cells have long been demonstrated to be activated in vitro by virus-infected cells (Lee *et al.*, 2007). Other types of intracellular pathogens have also been shown to activate NK cells for IFN-γ production or increase cytotoxicity (Lee *et al*., 2007; Korbel *et al.*, 2004). NK cell secretion of the cytokines TNF-α and IFN-γ is known to play a crucial role in granuloma formation following challenge with intracellular bacteria, including *Mycobacterium avium* and *Francisella tularensis* (Smith *et al.*, 1997; Bokhari *et al.,* 2008). NK cell activation occurs in infections by intracellular bacteria, such as *Listeria monocytogenes* (Luca *et al.*, 2007), or protozoa, such as *Leishmania* (Schleicher *et al*., 2007) or *Plasmodium* (Newman *et al.*, 2006).

In tuberculosis infection, NK cells are the immune cells to arrive secondly to the site of bacilli presence, after neutrophils. The capacity of NK cells to produce IFN-y has an important role in killing infected cells with mycobacterium (Molloy *et al.,* 1993). Beyond IFN-γ release, NK cells can directly lyse M. *tuberculosis*-infected monocytes and macrophages *in vitro* a process dependent of NKp46 and NKG2D (Vankayalapati *et al.*, 2002, Vankayalapati *et al.*, 2005). However a reduced activity of NK cells has found in active pulmonary TB patients (Nirmala *et al.*, 2001).

NK cells are recruited to the lung within the first days of influenza infection in humans and in murine models. The depletion of lung NK cells leads to increased morbidity and mortality, within days of infection. NK cells reciprocally regulate the adaptive response in influenza because they are required for activation of the cytotoxic T lymphocyte (CTL) response and T-cell IL-2 production augments NK cell IFN-γ production in recall responses. The NKp46 is a key activating receptor which is critical for protecting mice against lethal influenza infection, and is one of the few known examples of direct binding of viral glycoprotein to an NK cell-activating receptor. In addition to possible direct activation of NK cells via TLR by pathogen-derived molecular structures (PAMP, e.g., LPS, RNA, DNA), accumulating evidences over recent years have linked NCRs on NK cells with direct or indirect recognition of pathogen-associated structures. Given their role for sensing intracellular pathogen-infected cells, under particular conditions, these observations may bear relevant importance in the outcome of an immune response. NCRs have been found to interact with infected cells through recognition of virus-encoded molecules (Culley, 2009).

HIV-1 infection is associated with significant changes in NK cell subset distributions and functions in the peripheral circulation which were detected already at the beginning of

the infection. In addition to changes in NK subpopulations associated with HIV infection, there are also marked changes in NK surface receptor expression that are related with loss of function. With HIV viremia, there is an overall decrease in surface receptor density of NKp46 and NKp30 (Marras *et al.,* 2011). The role of NK cells are being now revealed in many more infections, with more and more data showing their importance in the immune response.

Regulator role of Natural Killer cells

Through cytokines production, NK cells are able to change future adaptive immune responses due to IFN-γ, TNF, IL-10 growth factor such GM-CSF (granulocyte macrophage colony stimulating factor), and IL-3 production. NK cells can also secrete many chemokines, including CCL2 (MCP-1) CCL3 (MP1-α), CCL4 (MIP1-β), CCL5 (RANTES), XCL1 (lymphotactin) and CXCL8 (IL-8) (Walzer *et al.,* 2005). Expose of NK cells to IL-12 induce strong cytoytic activity against tumor cells and iDCs (Marcenaro *et al.,* 2005)

The magnitude of NK cells to produce IFN-γ, can be seen by the fact that the mRNA that codifies the protein is constitutively expressed and they can immediately produce IFN-γ (Stetson *et al.,,* 2003) The production of IFN-γ is known to shape the T_H1 adaptive immune response, activate APCs to up-regulate MHC class I expression, activate macrophages to kill intracellular pathogens and have antiproliferative effects on viral and malignant-transformed cells (Caligiuri 2008). The $CD56^{bright}$ cells need two signals to produce IFN-γ where IL-12 is almost one of them and the second could be IL-1, IL-2, IL-15 or IL-18 or even engagement of NK activating receptors such CD16 or NKG2D (Cooper *et al.*, 2001, Koka *et al.,* 2003). These cytokines can be released from monocytes, macrophages and/or DCs suggesting an important role of this cross-talk during immune activation (Figure 3).

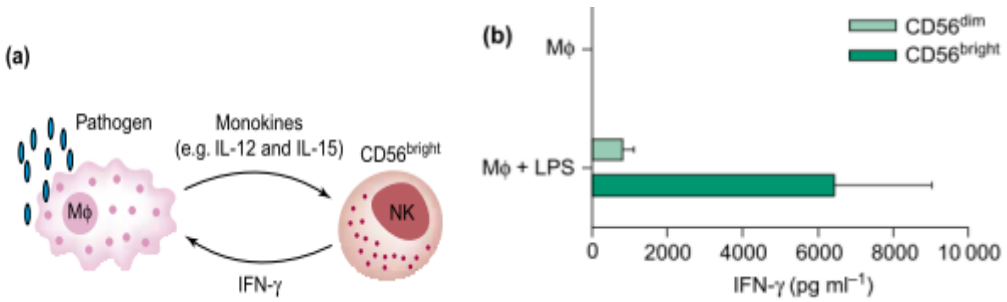

Figure 3- Influence of macrophages over CD56^{bright}. *(**A**) After a macrophage encounter a pathogen it produce IL-12, IL-15 and other cytokines production, leading to IFN-γ by NK cells with several consequences to immune response.**(B)** IFN-γ is released mainly by CD56^{bright} after contact with activated macrophages (adapted from Cooper et al., 2001)*

The immediately and innate cytotoxic action of NK cells is only one part of the role of an NK cell in the immune response. The capacity to activate or increase adaptive immune response is another great importance of the NK cells in immune surveillance. They can do that by at least three mechanisms. They can influence dendritic cells leading to T_H1 response by creating a favorable cytokine milieu, can kill some subset of APCs that are responsible for inhibition of immune response or can initiate immune activation after stimulation by somatic cells (Chijioke and Münz C, 2011). However, NK cells can also negatively influence T and B cell immunity (Andrews *et al.,* 2010). So although the cytotoxic effects normally referenced is related with capacity to kill non-healthy cells, NK cells can also regulate DCs, macrophages and neutrophils (Moretta *et al.,* 2005) and affect specific T and B cell responses (Vivier *et al.,* 2011)

Although NK cells and cells derived from myeloid lineage are different in many aspects their capacity to restrain infections occurs not in isolation but they constantly influence and activate each other for a more efficient innate immunity (Münz *et al*, 2005a).The capacity of NK cells to polarize future steps of immune response is intrinsically attached to the ability to kill different subsets of APCs. This process must be very controlled. Resting NK cells can not kill any subset of macrophages or DCs. However activated NK cells have cytolytic activity against M0 and M2 macrophages and against immature DCs. M1 and mature DCs are more resist against NK cell cytotoxicity (Bellora *et al*., 2010).

Although the cross-communication with DCs and macrophages can occur, having in account the tissue distribution of the different types of immune cells, the principal compartment of NK cells is the peripheral blood so, this type of cells are more commonly in presence of monocytes (Kloss *et al.,* 2011).

Monocytes

Monocytes are cells of the innate immunity with irregular shape, a nucleus with oval or kidney form, cytoplasmic vesicles and high cytoplasm-to-nucleus ratio. They are a subset of circulating white blood cells that can differentiate into a range of tissue macrophages and DCs (Shi and Pamer, 2011). Based on the expression of CD14 and CD16, it is possible to define three distinct subsets: $CD14^{++}CD16^{-}$, $CD14^{+}CD16^{+}$ $CD14^{dim}CD16^{++}$ (Passlick *et al*., 1989). The cells that belong to three subsets differ in size, trafficking, and innate immune receptors and also in the ability to differentiate after stimulation (Shi and Pamer, 2011). The $CD14^{++}CD16^{-}$ subset is the major subset present in the blood (80 - 90% of total monocytes), has higher phagocytic and myeloperoxidase activity, and superoxide release but have lower cytokine production than $CD16^{+}$ subsets. The monocytes from these subsets are applied as

classic monocytes. The subsets $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{++}$ are less studied and are able to release high amounts of IL-1 and to mediate antibody-dependent cytotoxicity (Auffray *et al*., 2009 e Ziegler-Heitbrock *et al*., 2010). Among $CD16^{+}$ cells the two subsets that can be identified, $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{++}$ are referred as intermediate and non-classical monocytes, respectively (ZiegleHeitbrock *et al*., 2010). Between both subsets there are little differences. The $CD14^{+}CD16^{+}$ cells have more phagocytic activity and are the only that produce TNF-α and IL-1 in response to LPS. Monocytes with low levels of CD14 but express high levels of CD16 ($CD14^{dim}$ $CD16^{++}$) are poorly phagocytic and do not produce TNF-α or IL-1 and their function is not entirely established (Grage-Griebenow *et al*., 2001).

The different subsets have also different places in the body and different time span. In murines and possibly in humans, $CD14^{++}CD16^{-}$ monocytes are present in inflamed tissues, have short life and can trigger immune responses and $CD16^{+}$ subsets are present in non-inflamed tissues and live longer (Geissmann *et al*., 2003).

One important function of monocytes is that act as myeloid precursors for renewal of some macrophages and dendritic cells. Monocytes can be recruited to tumor sites and inhibit some immune defenses against transformed cells (Peranzoni *et al*., 2010). During inflammatory process, monocytes are recruited into tissues and only there suffer a differentiation to M0 macrophages and then can be polarized to the M1 or M2 functional phenotype (Martinez *et al*., 2006, Mosser and Edwards, 2008). The M1 macrophages are immunostimulatory cells with T_H1-oriented properties, can kill intracellular pathogens and have anti-tumoral activity. The M2 macrophages have poor Ag-presenting capacity, promote angiogenesis, tissue remodeling and repair, and in opposition to M1 suppress T_H1 adaptive immunity and supports T_H2 responses against parasites (Bellora *et al*., 2010)

Monocytes mediate host antimicrobial defense (Serbina *et al.*, 2008) and when stimulated can produce large concentration of ROS, complement factors, prostaglandins, cytokines such as TNF-α, IL-1β, CXCL8, IL-6, and IL-10, vascular endothelial growth factor (VEGF) and proteolytic enzymes which are associated with defense against pathogens (Auffray *et al*., 2009).

NADPH oxidase and Reactive oxygen species

One of the mechanisms used by some innate immune cells is the ingestion of extracellular material by phagocytosis. The phagocytes are a group of cells that can internalize and then digest bacteria and other cells, and in this process, produce ROS and reactive nitrogen species (RNS), scavenge toxic compounds, produced by metabolism, and

produce inflammatory mediators that can also kill bacteria, virus and parasites leading eventually to activation of other immune cell types (Auffray *et al*., 2009)

In professional phagocytes, the production of ROS occurs in NADPH oxidase, a small transmembrane transport system. When this enzymatic system is activated, one molecule of NADPH suffers oxidation in the cytoplasmic surface and occurs the generation of superoxide on the other surface of the membrane (Oliveira-Junior *et al*., 2011). The NOX family of NADPH oxidase, the first to be identified, (Nauseef, 2008) is highly expressed in granulocytes, monocytes and macrophages and is necessary to kill microbes. This enzymatic mechanism is responsible for the respiratory burst (Rossi and Zatti, 1964). The enzymatic system is composed by gp91^{phox}, p22^{phox} p47^{phox} p67^{phox} p40^{phox} proteins (Oliveira-Junior *et al*., 2011) which assemble to produce an active form of NADPH oxidase (Figure 4).

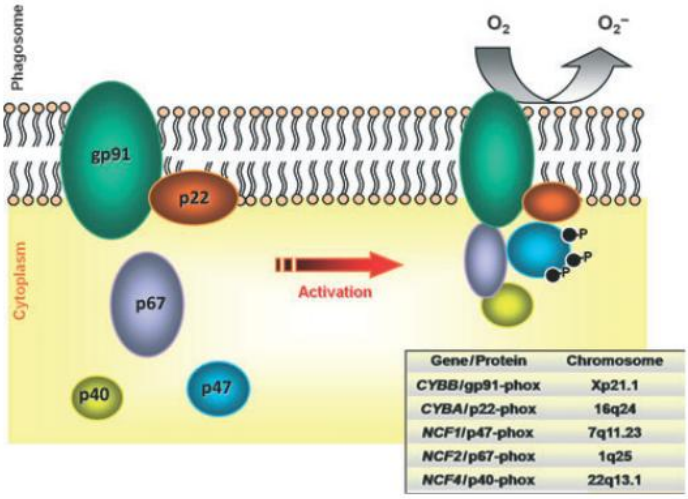

Figure 4- Components of enzymatic system NAPDH oxidase*. The five proteins must be linked to create a functional NADPH oxidase (adapted from Oliveira-Junior et al., 2011).*

Two subunits, the p22^{phox} and the gp91^{phox} are permanently bounded to membrane and are called cytochrome b. In dormant state the cytosolic components, p40^{phox}, p47^{phox} and p67^{phox}, are not associated with cytochrome b (Bylund *et al*., 2010).The activation of NADPH oxidase is a simple mechanism that starts with phosphorylation of the p47^{phox} subunit that leads to conformational changes allowing the interaction with p22^{phox}. The translocation of p47^{phox} to cytochrome b is the final step to assemble all the other subunits- p67^{phox}, p40^{phox}, and others. After this activation are finished, occurs the fusion of vesicles with the plasma membrane or the phagosomal membrane (Oliveira-Junior *et al*., 2011). Although p22^{phox} is expressed in almost cells, the gp91^{phox} is highly expressed in phagocytes and B cell lineages (Parkos *et al.,* 1988; Condino-Neto and Newburger, 1988; Dinauer *et al.*, 2000).

The NADPH oxidase is able to transport electrons from cytoplasmic NADPH to extracellular or phagosomal oxygen and generate superoxide, a reactive oxygen species that can be converted to other ROS like hydrogen peroxide and singlet oxygen (Ross *et al.,* 2003). The production of ROS by monocytes can occur both spontaneously or in response to certain stimuli (Asea *et al*., 1996).

The gp91 subunit generates superoxide (O_2^-) a free radical of O_2 in the following reaction: $2O_2 + NADPH \rightarrow 2O_2^{\bullet -} + NADP + H^+$

A second reaction occurs catalyzed by superoxide dismutase to produce another reactive oxygen species, the hydrogen peroxide (H_2O_2): $2H^+ + 2O_2^{\bullet -} = H_2O_2 + O_2$

Then H_2O_2 can generate more reactive metabolites such as hydroxyl radical ($OH^{\bullet}$) or hypochlorous acid (HOCL) which requires myeloperoxidase (MPO) an enzyme located in neutrophils (Bylund *et al*., 2010).

Reactive Oxygen Species in cell biology

Because ROS are so reactive, and because their lifetimes generally are very short, their very existence has often been denied. Their importance to cell biology was, during many decades, controversial but nowadays it is clear that they are vital in the functioning of every air-living organism. The discovery of superoxide dismutase (SOD) and then the prostaglandin enzyme system, with reactions that involve ROS, that play vital roles in many biological processes reveal a new world undiscovered until then (Davies and Pryor, 2005).

It is becoming increasingly clear that localized expression of enzymes responsible for ROS production along with a fine balance between production and metabolism of ROS have effects in different cell functions ranging from basic cell division, migration and differentiation to vestibular balance, neuronal signaling, angiogenesis, and thyroid hormone synthesis (Lambeth, 2007). The extracellular ROS (Figure 5) can act as secondary messengers and interfere with the expression of a number of genes and signal transduction pathways (Bylund *et al*., 2010). The ROS homeostasis is critical in cell signaling and in the regulation of cell death (Rakshit *et al.,* 2010).

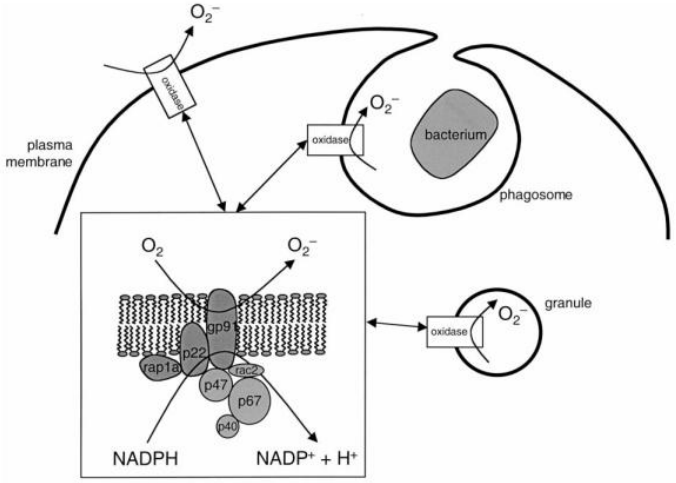

Figure 5- Different places where NADPHoxidase can be assembly*. ROS from NADPH oxidase are produce in the phagossome to destroy the bacterium but also are produced in outer side of plasmatic membrane (adapted from Dahlgren and Karlsson 1999).*

However ROS can damage a lot of molecules especially proteins, lipids, DNA and there are more than 100 disorders somehow associated with ROS formation (Canakci *et al*., 2009) including hyper-tension, diabetes, atherosclerosis, Parkinson's disease, Alzheimer's disease, and cancer. Oxidative stress results from an imbalance between the production of ROS and the antioxidant capabilities of a given system. This imbalance can culminate in a stimulation of degenerative signaling pathways often leading to weak control of tissue growth and neoplasia, inflammation, and dysfunctional of innate immune reactions (Bedard *et al*., 2007).

Oxidative stress in inflammation

The mitochondria is normally classified as the main source of ROS production (Boveris and Chance, 1973). However, in hematopoietic stem cells 50% of the oxygen consumption occurs in NADPH oxidase instead of the mitochondria (Piccoli *et al*., 2005)**.** The ROS produced by NADPH oxidase have well-defined roles in the inflammatory process. In one hand, ROS can contribute to observed dysfunction of lymphocytes in malignant disorders and chronic infections (Hellstrand, 2003; Kiessling *et al.,* 1999; Kono *et al.,* 1996; Schmielau and Finn, 2001 Dobmeyer *et al*., 1997). In the other hand, oxygen radicals can suppress autoimmunity and arthritis development (Hultqvist *et al.*, 2004; Gelderman *et al.*, 2006). ROS not only can be considered harmful to organism but can also have immune regulatory functions especially when produced in lower amounts (Hultqvist *et al*., 2009). Patients with chronic granulomatous disease (CGD) suffer more frequently from autoimmune diseases and hyperinflamation, when compared with healthy individuals (Kraaij *et al*., 2010).

This disease is characterized by a mutation in any of the four subunits of NADPH oxidase complex with consequent inability to generate ROS (Chan *et al*., 2001). This reveals that ROS from NADPH oxidase keep inflammatory reactions under control.

Some neurological diseases with autoimmune background like multiple sclerosis can be linked with altered levels of ROS. Phagocytes from these patients produce less ROS after stimulation than healthy individuals (Mossberg *et al.,* 2009). These give the idea of a protective role of ROS in autoimmunity.

According to Thorén *et al*., (2011), ROS can be a complementary mechanism to signals from inhibitory receptors acting as emergency brake for potentially dangerous immune responses from NK cells. In many pathologies associated with ROS several studies have shown that while $CD56^{bright}$ survive, $CD56^{dim}$ cells succumb. This is the case for head and neck cancer, breast cancer, hepatitis C, tuberculosis and chronic inflammatory diseases (Bauernhofer *et al*., 2003; Dalbeth *et al.,* 2004; Lin *et al.,* 2004; Schierloh *et al.,* 2005). It is expected that $CD56^{bright}$ may affect autoimmune disease by promoting T-cell activation in lymphoid tissues and subsequent B cell response (Martín-Fontecha *et al.,* 2004)

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Oxidative stress in infection

The ROS and also the RNS that are produced by innate immune cells are considered a relative effective mechanism against microbial pathogens (Chan *et al*., 2001). However ROS produced by phagocytes must be produced near or on microorganisms to be effective (Bassøe *et al*., 2003).

Their importance to immune system can be confirmed by the inability of patients with CGD to kill infections with *Staphylococcus aureus*, Aspergillus species, and Nocardia species. Despite of healthy individuals could produce normal ROS levels some pathogens developed mechanisms to control or escape to ROS production as is the case of *Mycobacterium tuberculosis. Mycobacterium tuberculosis*, the pathogen responsible for the tuberculosis infection, is an obligatory aerobic, intracellular pathogen, which normally infects lung tissue (Raja, 2004) more precisely macrophages and DCs (Cooper, 2009). In the first 2 to 6 weeks of infection, the immune response starts with an influx of lymphocytes and activated macrophages formatting a granuloma (Raja, 2004). Innate immune system plays a critical role in antimicrobial host response in the early steps (Sohn *et al*., 2011). The bacilli may remain alive and forever in granuloma, can be re-activated later or may get exonerated into the airways after increases in number, necrosis of bronchi and cavitation (Raja, 2004).

Alveolar macrophages are the key effector cells in immune response against *M. tuberculosis* creating a situation where the bacteria can survive and replicate within phagocytes (Hingley-Wilson *et al.,* 2011). The interaction of *M. tuberculosis* with macrophages is in the key process in the immune response. To limit multiplication of pathogen, macrophages can enter in apoptotic cell death (Molloy *et al.,* 1994). After *M. tuberculosis* was phagocytosed, macrophages generate ROS and RNS to help in the kill of the pathogen. The infection by *M. tuberculosis* induces the accumulation of macrophages and ROS production in the lungs (Selvaraj *et al.,* 2004). However *M. tuberculosis* has the ability to evade immune strategies. It can produce superoxide dismutase and catalase as antagonistic mechanism to escape to ROS effect (Andersen *et al.,* 1991). Macrophages infected with bacteria have diminished ability to present antigens to $CD4^+$ T cells and can lead to persistent infection. The virulent mycobacterium is able of escape from fused phagosome, multiply (Moreira *et al.,* 1997) and inhibit apoptosis of infected macrophages (Sohn *et al.,* 2011). After endocytosis *M. tuberculosis* normally resides in a phagossomal compartment that does not suffer maturation towards an acidified phago-lysosome because is blocked by the bacteria (Koul *et al.,* 2004; Ehrt and Schnappinger, 2009). Tuberculosis remains a major infectious disease killing about 3 million people a year, about five deaths every minute and approximately 8-10 million people are infected every year (Raja, 2004).

It is possible to find also in virus, like hepatitis C virus (HCV), examples where ROS production is important to chronic infection. The liver contains around 80% of mononuclear phagocytic system (Saba, 1970) and was already documented an increase of ROS production by a factor of 100,000 in HCV-infected liver tissue (Valgimigli *et al*., 2003). This ROS production and decreased NK cell cytotoxicity observed (Corado *et al.,* 1997) leads to the proposed idea that excessive formation of ROS can be in the basis of HCV immunescape. In addition, there was reported a depletion of reduced glutathione in lymphocytes and the consequent decrease in cytotoxicity in HCV infected patients (Barbaro *et al*., 199). It was demonstrate that NS3 a protein from HCV leads to ROS production of macrophages and resultant decrease in NK cells cytotoxicity (Thorén *et al*., 2004). Similar results can be found using a derived peptide from the bacteria *H. pylori* that activates monocytes with suppressive properties over NK cells and T cells and triggers apoptosis. (Betten *et al*., 2001)

Oxidative stress in cancer

The immunological system as others non-immunological mechanisms are responsible for detection and elimination of transformed cells. The immune system acts as the last barrier

in our natural mechanisms of protection against cancer. Our own cells of the immune system evolved to detect and act against tumors and while functional are able to prevent the development of neoplastic disease. The tumors detected in patients are assumed as the exceptions when immune system was unable to detect and eliminate cancer cells (Jakobisiak *et al*., 2003).

A developing tumor influences and is influenced by its stroma, and interacts with both the adaptive and innate immune systems (Levy *et al*., 2011). The $CD8^+$ cytotoxic T cells (CTL) and NK cells are the most important effector cells against tumors (Zitvogel *et al*., 2006) The production of IFN-γ by NK cells is important to stimulate $CD4^+$ T_H1 cells for fighting tumors (Flavell *et al*., 2010). However their immunesuppression and tumor escape from immune recognition is seeing as the major responsible for cancer although the mechanisms by which it occurs are so far not completely understood.

Inflammation and cancer are linked together (Mantovani and Sica, 2010). In fact a new paradigm emerged in recent years with more and more data revealing a link between chronic inflammation and cancer (Vakkila and Lotze 2004). This cross-communication may lead to some pressures that shape the microenvironment that surrounds the tumors, favoring the growth, expansion and invasion of malignant cells. Clearly solid tumors are not composed of just malignant cells (Hanahan and Weinberg 2000) and among the complexity of cells in this pathology the presence of leucocytes with unrevealed functions are changing the knowledge of the immune system in cancer. In the microenvironment of a tumor it is possible to find leucocytes, cytokines and chemokines (Allavena *et al*., 2008). In this case inflammation associated with cancer can promote tumor growth (Balkwill *et al*., 2005; Balkwill and Mantovani, 2001) The complexity of tumor environment is in part based in the attraction and education suffered from tumor-infiltrating leucocytes (especially macrophages, neutrophils and mast cells) to act not as anti-tumor but instead as pro-tumor functional cells (Figure 6) (Coussens and Werb, 2001; Murdoch *et al.,* 2004; Pollard, 2004).

Macrophages present in tumors use growth factors, proteases, angiogenic mediators and ROS to promote tumor growth, angiogenesis, metastasis and genomic instability (Balkwill and Mantovani, 2001; Lin and Pollard, 2004; Wyckoff *et al.,* 2004; Chen *et al.*, 2005). The ROS have also another defective role, by their capacity to be toxic to antitumor lymphocytes such as NK cells and T cells (Hansson *et al.,* 1996; Kono *et al.,* 1996; Malmberg, 2004). The ROS can be produced not only by activated granulocytes and macrophages during inflammation but also by tumor cells (Szatrowski and Nathan, 1991).

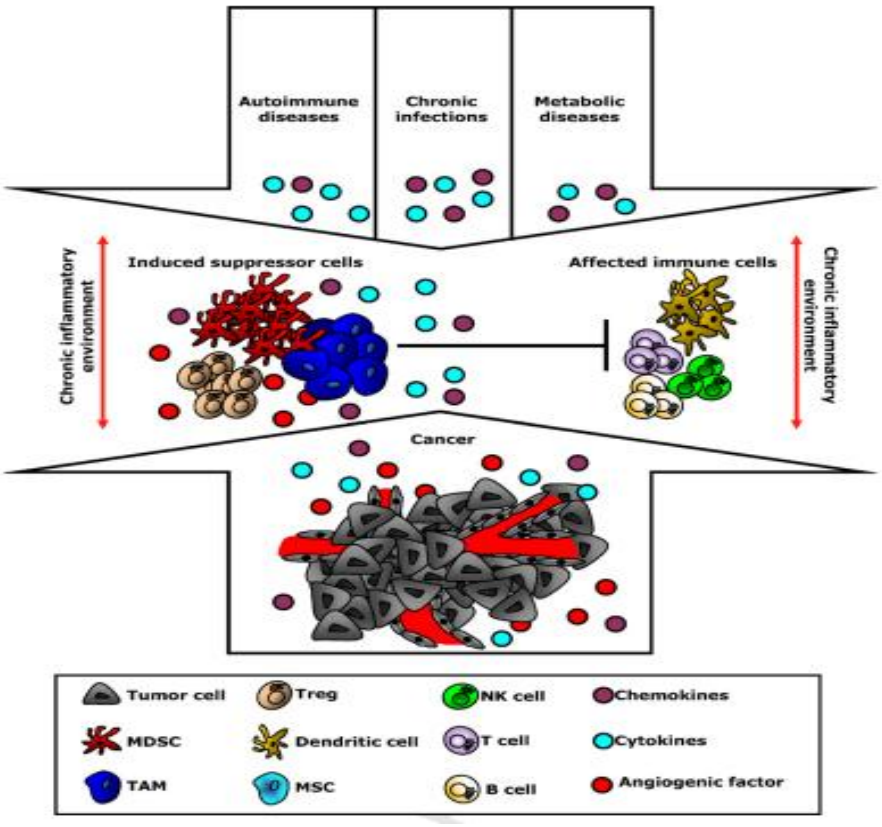

Figure 6- Role of immune cells in different diseases associated with chronic inflamation. *In many chronic diseases some cells of the immune system as TAMs Treg, and soluble factors have immunosuppressive role over NK, T, and dendritic cells (adapted from Kanterman et al., 2012).*

Due to opposite effects of macrophages in tumor microenvironment the macrophage balance hypothesis, was created (Mantovani, 1992). Macrophages can produce both pro or anti-angiogenic molecules (Figure 7) (Allavena *et al*., 2008).

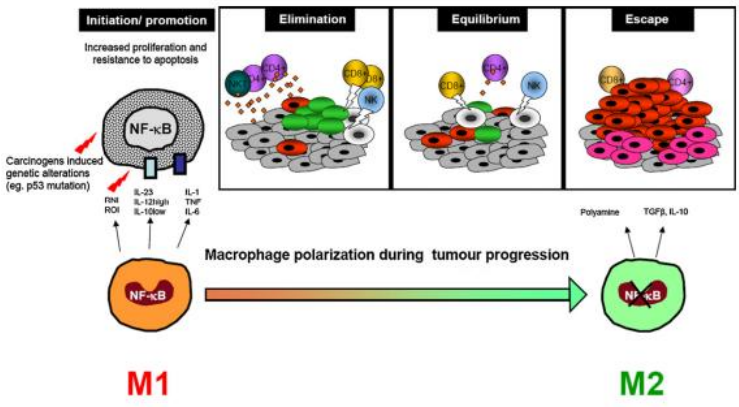

Figure 7- Macrophage polarization over tumor progression*. During all phases of tumor progression macrophages are converted from M1 anti-tumor to M2 pro-tumoral macrophages.*

In leukemia as in other cancers, the genetic alterations that are being discovered as associated to tumor-like phenotype are responsible for aberrant activation of signal transduction pathways. The oncogenes not only are responsible for increasing the cell survival but also lead to ROS production (Sattler *et al*., 2000). Tumor cells have higher levels of ROS compared with non transformated cells (Trachootham *et al*., 2009). The production of ROS in this type of cells has the effect to increase the number of mutations which leads to more resistance capacity of tumor cells (Rassol *et al*., 2007). The BCR-ABL protein found in patients with Chronic Myeloid Leukemia are responsible for the increase in ROS production but how this happens is unknown (Sattler *et al*., 2000, Nowicki *et al*., 2004)

Several studies show a decrease in cytotoxic activity in NK cells obtained from peripheral blood of patients with different types of cancer. Many mechanisms are been discovered as responsible for NK cell inactivation in presence of cancer cells, like over-expression of Fas ligand, loss of mRNA for granzyme B (Mulder *et al*., 1997), decreased CD16 expression (Nakagomi *et al*., 1993), some secreted factors like Interleukin (IL)-6, vascular Endothelial Growth Factor and Granulocyte Monocite Colony Stimulating Factor (GM-CSF), and ROS, all with immunosuppression properties (Jewett and Tseng, 2011)

Many reports demonstrate that in tissue within or adjacent to tumors there is an increased oxidative stress probably from monocyte/macrophages that infiltrate in tumors (Betten *et al*., 2001). Tumor cells have higher levels of ROS when comparing with normal counterparts (Trachootham *et al*., 2009). This ROS production is believed to be one important immunosuppressive mechanism especially against NK cells and also T cells (Mantovani, 1992 Kiessling, R 1996).

Several cancers differ in their prognosis based in a various aspects being one of them the presence and function of NK cells like in acute myeloid leukemia (Aurelius *et al*., 2012). The presence of monocytes and neutrophils circulating in blood in a phase II trial study was considered a bad factor for the success of IL-2 based immunotherapy against metastatic renal cell carcinoma. High amounts of monocytes/neutrophils and the decrease in cytotoxicity and NK cells has opposite correlation (Donskov *et al*., 2006).

Reactive oxygen species from phagocytes inhibit NK cells

NK cells when in contact with ROS (either from phagocytes or added exogenously *in vitro*) have their normal capacities affected. Among the affected characteristics found in NK cells are decrease cytotoxicity and proliferation, alteration of transcription of cytokine genes and incapacity to be activated by IL-2 (Hellstrand *et al*., 1994; Kono *et al*., 1996). This ROS are produced by NADPH oxidase, because the monocytes recovered from patients with CGD are unable to inhibit NK cells and only catalase (a scavenger of H_2O_2) is able to prevent this inhibition (Hellstrand *et al*., 1994; Hansson *et al* 1999). As ultimate effect, NK cells die by apoptosis after cell-to-cell contact with monocytes (Hansson *et al*., 1996).

Although IL-2 can induce the expression of CD69 (marker of NK cell activation) in NK cells, its expression is abolished in the presence of hydrogen peroxide (Betten *et al*., 2001). Beyond IL-2 incapacity to stimulate or at least recover NK cells also IFN-α, a stimulus to NK cells that is being used as therapy in hematology, oncology and infectious diseases, has the same weak ability when phagocytes are co-culture with NK cells (Hansson *et al*., 2004). H_2O_2 has been proposed as an effector molecule able to inhibit NK cell normal function (Figure 8) (Kono *et al*., 1996, Hellstrand *et al*., 1994 Samlowski *et al*. 2003). Besides this molecule, MPO-dependent radicals that can be converted directly from H_2O_2 are also able to NK cell inhibition. A third group of ROS which can inhibit NK cells but are MPO-independent are also present (Betten *et al*., 2004). This gives the idea of a very complex system depending on a huge diversity of ROS that can compensate for the lack of others (Wentworth *et al*., 2002).

The ROS inhibition mechanism is dependent of contact because monocyte-derived supernatants or separation of these two cell types by a semipermeable membrane cannot inhibit NK cells (Hellstrand and Hermodsson, 1991). However formation of a soluble mediator is required which gives the idea that H_2O_2 inhibition requires strait contact between cells. After H_2O_2 formation, several mechanisms can occur and one of them is the conversion by mieloperoxidase (MPO) in more radicals that contribute significantly to NK cell inhibition. Serotonin can inhibit myeloperoxidase (Betten *et al*., 2001). However the pharmacologic inhibition of MPO activity was not able to suppress NK cell inhibition by phagocytes (Betten 2004) probably because other ROS as H_2O_2 itself can have the same deleterious action.

After exposure to H_2O_2 $CD56^{dim}$ cells have defective capacity to perform ADCC. Clearly the decrease number of NK cells and low expression of CD16 in NK cells in cancer microenvironment are responsible for the lack of immunity against tumor cells in many cancers (Izawa *et al*., 2011). Not only CD16, but also other important activating receptors have low expression as NKp46 and NKG2D (Harlin *et al*., 2007). It is possible that the effect

of ROS-derived phagocytes cannot have only the effect of decrease the expression of receptors as was demonstrated by the fact that in $CD56^{bright}$ NK cells the expression of NKp46 increased, after co-cultured with granulocytes (Romero *et al*., 2005).

CML cells inhibit cytotoxicity of NK cells even after stimulation. In this specific type of cancer this inhibition is based on ROS production because histamine (inhibitor of NADPH oxidase) (Figure 8) and catalase almost rescue NK cytolytic activity and prevent apoptosis. The paracrine ROS production may contribute to the inhibition of NK cells in CML (Mellqvist *et al*., 2000). The merely presence of granulocytes in vitro was enough to reduce NK cell activation induced by IL-2 (Mellqvist *et al*., 2000) because ROS production through NADPH oxidase can occur spontaneously without any stimulus (Asea *et al*., 1996). Not only in this type of cancer but probably in many others the balance between ROS production and histamine released by mast cells and basophils is in favor of ROS production leading to NK cells inhibition (Mellqvist 2000). When leukemic sorted-cells from patients with acute myeloid leukemia (AML) are cultured with NK cells, is also detected an increase apoptosis in lymphocytes and the use of catalase and histamine are able to reduce NK cell apoptosis (Aurelius *et al*., 2012) revealing the same mechanism seeing in CML patients.

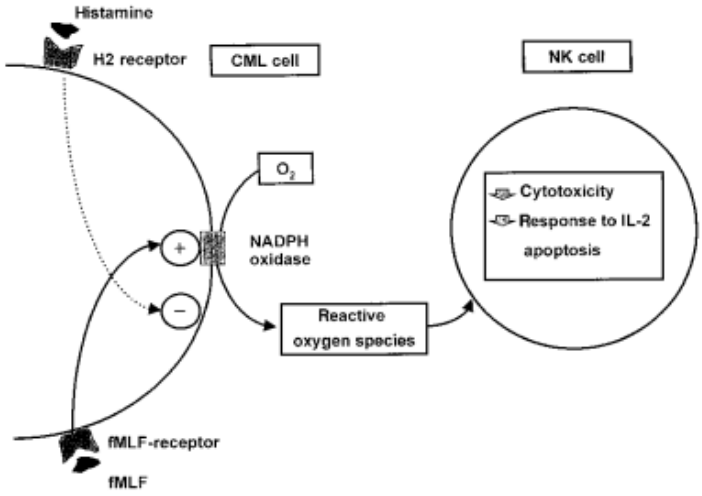

Figure 8- Ilustrated mechanism of ROS production as the effect in NK cells*. The activation of NADPH oxidase leads to external ROS production which decrease cytotoxicity and response to IL-2 by NK cells and increase apoptosis (adapted by Melqvist 2001)*

The possible idea of competition between NK cells and APCs should be excluded by the fact that unexpectedly NK cells, through cytokines production and after stimulation, can prolong the life of granulocytes, and also increase expression of CD11b and CD62L (two markers of granulocyte activation) reduce granulocyte apoptosis and especially increase

phagocytosis action and ROS production (Bhatnagar *et al*., 2010). These data are unexpected having in consideration the deleterious effects that phagocytosis and more precisely ROS production have in NK cells. More interesting is the fact that both $CD56^{bright}$ and $CD56^{dim}$ are able to produce TNF-α, IFN-γ and GM-CSF in quantities enough to induce all the described effects. Taking into account that these two types of immune cells are some of the first to appear in inflammatory sites this cross communication reveal a more complicated communication being ROS a complex immunomodulator.

The infiltrating ratio of NK cells especially $CD56^{dim}$ cells in the tumor gradually decrease according to disease progression and most important $CD56^{dim}$ cells decrease with H_2O_2 production in tumor microenvironment (Figure 9) (Izawa *et al*., 2011).

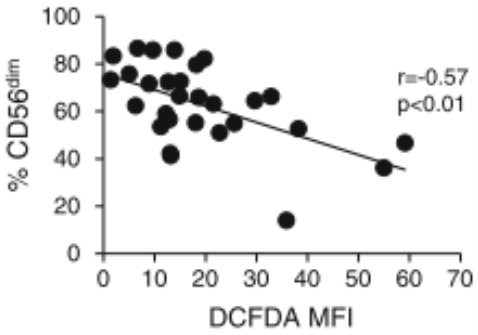

Figure 9- Correlation between CD56^{dim} NK cells and H2O2*. The percentage of CD56^{dim} is inversely correlated with H2O2 production in tumor microenvironment*

NKp46 and NKG2D, two of NCRs more studied, although almost invariably expressed in healthy subjects, are normally absent or expressed at low densities in more than 80% of AML patients (Costello *et al*., 2002). This deleterious decrease in such important receptors to NK cell immune response is due to contact with mono and polymorphonuclear phagocytes and soluble mediators produced by them (Romero *et al*., 2005)

Histamine: friend or foe?

Histamine is a bioamine with multiple physiological activities that is released from stimulated mast cells and basophils. Among very different functions in immune response, histamine can synergistically augment inflammatory stimuli as TNF-α, IL-1 and lipopolysaccharide LPS (Nagai *et al*., 2012). Histamine can also inhibit ROS formation by NADPH oxidase in phagocytes Hellstrand *et al*., 1994). In CML cells the use of histamine can protect NK cells by ROS-dependent mechanism. (Mellqvist 2000) In attempt to promote NK cells cytotoxicity in tumor environment has been using IL-2 as activator and more recently histamine. So far, the administration of both compounds combined in humans are in a phase III trial (Brune *et al*., 2006; Aurelius *et al*., 2012), with good results. Therefore, NK cells

remain viable and can be stimulated by IL-2 (Donskov *et al*., 2006). It was already proven the beneficial effect of histamine in leukemic cells reducing ROS formation and subsequently decreased inactivation and apoptosis of NK cells in AML patients (Aurelius *et al*., 2012). The administration of histamine and IL-2 combined to protect and activate NK cells, respectively is well tolerated in terms of side effects and are showing less probability of relapse with higher leukemia-free survival in AML patients (Brune *et al*., 2006). In melanoma this combination is also showing better results than monotherapy with IL-2 (Asemissen *et al*., 2005). Clearly IL-2 alone is unable to restore ROS-inhibited NK cells and the results from treatments with IL-2 alone are not meeting the expectations. I*n vitro,* the histamine concentration normally used to restore NK cells is much higher than the levels found in blood. However, local concentrations of this compound in inflamed tissues are even higher than *in vitro* conditions (Asea 1996).

However, the negative effects of histamine are now being revealed with a decrease of NKG2D ligands in leukemic cells. Furthermore, although IFN-γ can increase NKG2D ligands in the same cells, the presence of histamine reduce the positive action of IFN-γ. It was already shown a decrease of NK cell cytotoxicity using in a cell line of leukemia, when in contact with histamine. This reveal that tumor susceptibility to NK cells is decreased with histamine because leds to the decrease of NKG2D ligands expression (Nagai *et al*., 2012). This occurs in NK cell purity medium or with PBMCs. Histamine does not potentiate directly NK cells although can induce NK cell chemotaxis (Damaj *et al*., 2007). Interestingly, although as previously described, histamine is now being tested in oncology therapies, its levels are increased in various human tumors, such as melanoma (Darvas *et al*., 2003), breast cancer (Sieja *et al*., 2005), and small cell lung carcinoma (Graff *et al*, 2002). Histamine can act as an autocrine growth factor. If histamine activates H_1R, the proliferation of melanoma cells is decreased but it is enhanced if H_2R is activated (Falus *et al*, 2001). The hypothesis of histamine to be responsible for immune evasion was already tested as demonstrated by the fact that histamine derived from mast cells exhibited grown-inducing activity of human thyroid carcinoma cells (Melillo *et al*., 2010). Histamine are also been tested in other ROS-associated diseases as in HCV as supplement with IFN therapy (Lurie *et al*., 2002)

AIMS

Aims

Although NK cells are known since 1970s, in the last decade, the information regarding NK cell immunobiology are increasing exponentially revealing more and more an important role of NK cells in the immune response. However this also means that in chronic pathologies where an immune response is not correctly assemble, NK cell dysfunction is one of the characteristics with negative impact to a healthy organism. NK cell dysfunction is being found in many types of cancer and chronic infections (Bauernhofer *et al*., 2003; Dalbeth *et al.,* 2004; Lin *et al.,* 2004; Schierloh *et al.,* 2005). Reactive oxygen species are seen as one of the main factors for such NK cell impairment.

With all these information regarding NK cell dysfunction in tuberculosis and chronic myeloid leukemia, the main goal of our study is to show the ROS negative function on tuberculosis patients and demonstrate an increase of ROS in NK cells from chronic myeloid leukemia. So far the studies in this field reveal deleterious action of ROS, more specific H_2O_2 without having in account how exactly they affect NK cells. Without this question revealed, becomes more difficult to design therapies directed to NK cells. It is important to clarify the role of ROS over NK cell in immunopathogenesis and hypothetical contribution as targets for therapy interventions.

MATERIAL AND METHODS

Material and Methods

Study population

The samples used in this study are from three different groups: healthy donors from Instituto Português do Sangue of Coimbra, blood from chronic myeloid leukemia patients from Hospitais da Universidade de Coimbra, and pulmonary Tuberculosis patients from Centro de Diagnóstico Pneumológico from Vila Nova de Gaia. Samples from tuberculosis patients and chronic myeloid leukemia were collected in lithium heparin tube. Samples from healthy donors were collected in leukocyte depletion filters. These filters are used to separate leucocytes from whole blood to future transplantation retaining almost all leucocytes that pass through them. Blood from Tuberculosis patients (TB) were collected within the first two weeks of anti-mycobacterial therapy and samples with concomitant conditions including autoimmune disease, HIV infection, cancer, extrapulmonary TB and other systemic disease were excluded from the present analysis. Were studied 13 TB patients 68% male, age 45±16. Regarding to CML, were collected 88 samples from 50 different patients 56% male, age 55±15 colleting along their treatment and no more than three samples were collected from each patients during treatment. Twenty samples from healthy donors were used which 55% were male, age 44±14. As controls to CML and TB blood samples, a small proportion of blood still in collecting bags from filters were used. To each CML blood sample, the ratio of BCR-ABL over ABL of the mRNA expression was provided.

Leucocytes from healthy donors collected were removed from leukocyte depletion filters before their use. Briefly, Red Blood Cell (RBC) buffer (NH4Cl 0,115M, KHCO3 0,01M, EDTA 0,1mM) were added to one side of the filters and recovered from the other side, centrifuged at 1250 rpm 5 minutes, leave 10 minutes with RBC buffer to properly lysis of erythrocytes and wash once with PBS with 2mM EDTA. At the end were added 5 mL of RPMI 1640 with glutamax and 10% feat bovine serum to leucocytes until being used.

Blood samples were analyzed in maximum 24 hours as cells from leukocyte depletion filters were removed and placed in culture medium in less than one day. The sample number used in each experience is referred in the legend of each graphic.

All samples collection was previously approved by Ethics Committee of the Faculty of Medicine of the University of Coimbra

Cells count

Buffy coats from healthy donors, after their removal from filters, were counted in COULTER A^C •T diff Analyzer (Beckman Coulter) using 12µL of leucocytes in RPMI 1640 with glutamax and 10% feat bovine serum. The number of leucocytes was recorded. The Coulter method accurately counts and sizes by detecting and measuring changes in electrical resistance when a particle in a conductive liquid passes through a small aperture.

Cell viability

Cell viability was determinate after leucocyte isolation from healthy donors trough: trypan blue exclusion method. This test is based on the principle that live cells with intact cell membranes exclude the dye, whereas dead or injured cells with damaged membranes do not. About 5 µL of samples suspension was added to equal volume of trypan blue (0,2%) and 10 µL of the misture were transferred into a Neubar chamber and then observed and counted in a microscope (Leitz). Viable cells appear brilliant with a clear cytoplasm whereas nonviable cells appear blue. Cell viability corresponds to the percentage of viable cells relative to the total number of cells. Cells exhibiting up to 90% viability were used in all experiments.

Cell sorting

The Cell sorting was performed recurring to fluorescent activated cell sorting. About $4x10^7$ cells were incubated with CD16 FITC, CD3 PE, CD14 PerCP-Cy5 and CD56 APC during 30 minutes at 4ºC in the dark, washed once with cold PBS and resuspended in PBS. Cell sorting was performed in FACSAria III cell sorting (BD Biosciences Erembodegem Belgium) with FACS Diva software version 6.1.3. Monocytes and lymphocytes were gated according to side-scatter and forward-scatter characteristics. In the lymphocyte gate, only $CD56^+CD3^-$ were chosen (Robertson 1990). In monocytes population $CD14^-CD16^-$ subset was excluded to sort keeping all known subsets classified as monocytes ($CD14^{++}$ $CD16^-$; $CD14^+CD16^+$ $CD14^{dim}CD16^{++}$) (Ziegler-Heitbrock 2010)

.

Reactive oxygen species detection

Reactive oxygen spcies production in blood samples was analyzed using kit Phagoburst test from Glicotope Biotechnology with some modifications. About 100µL of blood was collected in test tubes to nonstimulated and stimulated conditions. To stimulate ROS production phorbol 12-myristate 13-acetate (PMA) from the kit (1:200) was used during 30 minutes at 37ºC. Then 20µL of dihidrorhodamine 123 as fluorogenic substrate was added to blood during 10 minutes at dark and 37ºC. Dihidrorhodamine 123 is one of the most frequently used cellular probes for the detection of ROS. This nonfluorescent dye gains fluorescence when oxidized by ROS. The oxidized compound that emits fluorescence is rhodmine 123 (Bylund *et al* 2010). The oxidative burst occurred with the production of ROS. Antibodies to cell surface receptors were incubated at room temperature during 15 minutes. Erythrocytes were removed using lysing solution for 20 min at room temperature, centrifuged (5 min, 250 g/min, 4 °C), Samples were washed again (washing solution from kit), centrifuged (5 min, 250 g/min, 4 °C) and the supernatant was decanted. About 200µL of PBS was added and analyzed by flow cytometry in FACSCanto II (BD Biosciences, Erembodegem Belgium).

In ROS detection to cultured cells from healthy donors the protocol from the kit Phagoburst test was adapted. After cell sorting, 100.000 cells/well (if not otherwise stated) were added to 96-well microplate in 200µL RPMI 1640 with glutamax, 10% fetal bovine serum, streptomycin (100U/ml), penicillin (100ng/ml) and amphotericin B (250ng/ml). Two different experiment conditions were performed. In one, sorted NK and monocytes were added in different wells. Monocytes were stimulated with LPS (100ng/mL) during 30 minutes, the culture medium was removed and NK cells were co-cultured with previous stimulated monocytes. For each sample one well with NK cells were incubated previously with IL-2, IL-12 and Il-15 (100ng/mL) during 30 minutes and in other well histamine (100mM) were added simultaneously with LPS to monocytes. NK cells and monocytes were co-cultured during 2 hours.

In other experiment, sorted cells were cultured together since the beginning of cell culture. In one well for each sample IL-2, IL-12, IL-15 all at 100 ng/mL, were added 24 hours before LPS stimulus and catalase (200 U/mL) was added together with LPS.

About 20 µL of dihidrorhodamine 123 was added to microplates during 10 minutes at RT in the dark, centrifuged (5 min, 250 r/min, 4 °C) resuspended in 100µL to a correct concentration for incubation with antibodies at 4ºC in the dark during 30 minutes. Cells were washed with cold PBS, and resuspended in 200 µL of PBS and analyzed in FACSCanto II Flow Cytometry.

Natural killer cells and monocytes phenotyping

In order to enumerate leucocytes, especially monocytes and NK cells, in each experiment, samples were labeled with surface monoclonal antibodies (mAbs) anti-human that are described in table 1. Samples were incubated with 30µL of mABS mix (diluted 1,5:100) in 1xPBS.

***Table 1**- Antibodies used to label NK cells and monocytes in different experiments.*

Fluorochrome	FITC	PE	PerCP-Cy5.5	Pe-Cy7	APC	APC-Cy7	Pacific Blue
mAbs (clone)	**CD16** (3G8)	**CD3** (UCHT1)	**CD3** (HIT3a)	**CD27** (O323)	**CD56** (HCD56)	**CD16** (3G8)	**CD57** (HCD57)
		NKp46 (.9E2.)	**CD14** (M5E2)				**CD11b** (ICRF44)
			CD19 (HIB19)				**CD3** (UCHT1)

Flow cytometry analysis

All sample data was acquired in FACSCanto II from BD Biosciences with FACS Diva software version 6.1.3 except for cell sorting experiments that was used FACSAria III (BD Biosciences) with same software. Data from flow cytometry were analyzed using FlowJo 7.6.5 (Tree Star Inc, Ashland, USA)

Statistical analysis

Statistical tests were performed using Graph Pad Prism Version 5.0 software (CA, USA). The non-parametric Mann-Whitney was used for comparison of all samples. Statistically significant P values are annotated as follows: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Compounds

The following compounds were used: PBS, RPMI 1640 with Glutamax, Heat Inactivated Fetal Bovine Serum, Penicillin, Streptomycin, Fungizone® (amphotericin B) (GIBCO, Invitrogen), Histamine, Catalase and Trypan blue (Sigma-Aldrich). Phagoburst kit

(Glicotope Biotechnology); Lipopolysaccharide (LPS) (InvivoGen); RealTime ready Cell Lysis Kit (Roche, Germany); All antibodies and IL-2, IL-12 and IL-15 used were purchased from Biolegend (San Jose, CA, USA);

RESULTS

Tuberculosis

To elucidate about the negative contribution of ROS in blood from TB patients, 13 samples with pulmonary tuberculosis were analyzed to ROS production in granulocytes, monocytes and within lymphocytes only NK cells and their subsets (Figure 10). Granulocytes, monocytes and lymphocytes where selected according to SSC and FSC known characteristics. In monocytes three subsets were distinguished based on CD14 and CD16 surface markers (classic – $CD14^{++}CD16^{-}$ intermediate- $CD14^{+}CD16^{+}$ and nonclassic- $Cd14^{dim}CD16^{++}$) (Ziegler‑Heitbrock 2010). Within the lymphocytes NK cells were selected as $CD56^{+}CD3^{-}$ (Robertson 1990). The mean of fluorescence intensity and the percentage of positive stained cells for rhodamine 123 (rhodamine^{+}) were used to evaluate ROS production in the mentioned cell types.

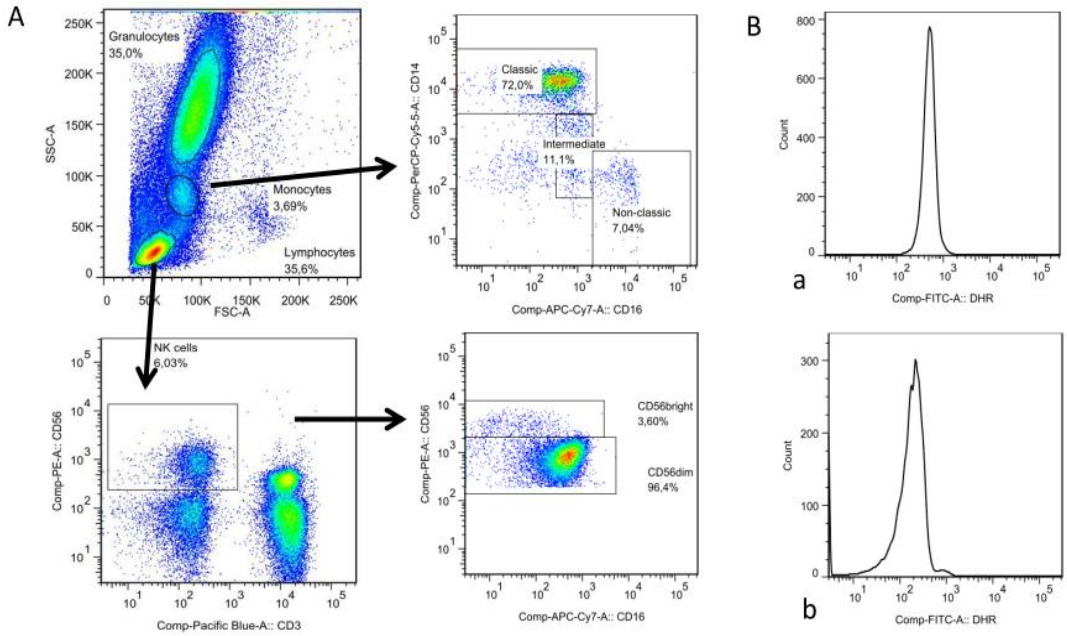

Figure 10- A- *Representative dot plots to NK cells, monocytes and their subsets and granulocytes.* ***B-*** *Representative histogram of rhodamine in TB patients (a) and controls (b)*

Granulocytes from TB patients have higher percentage of cells positive to rhodamine 123

Taking into account the results of granulocytes, TB patients have, in nonstimulate state, cells with high levels of ROS contrary to healthy controls. This difference is statistically significant. After stimulation TB patients still have a superior percentage of cells rhodamine^{+} (Figure 11).

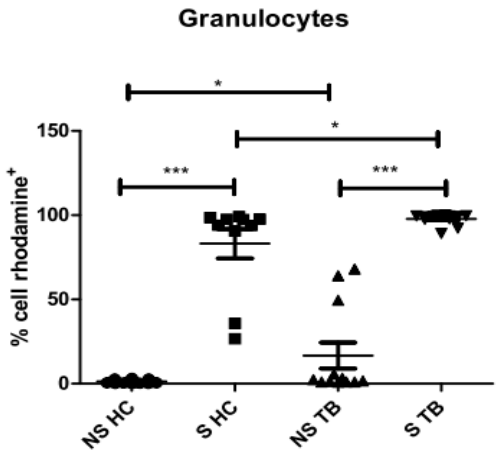

Figure 11- *Analysis of granulocytes with positive fluoresce to rhodamine 123 (HC n=20 and TB n=13).*

Regarding to the mean of fluorescence intensity in total granulocytes, comparing healthy controls with tuberculosis patients the small increase of ROS in TB granulocytes is not statistically significant (Figure 12 A). The same happens after half hour of stimulation with an expected increase in rhodamine 123 conversion to nostimulated cells but small differences between healthy and tuberculosis patients (Figure 12 B).

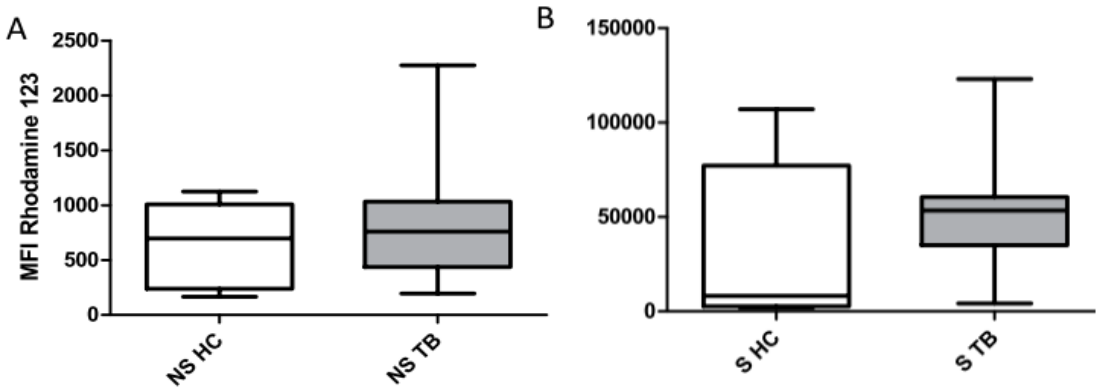

Figure 12- *A- Mean of fluorescence intensity of rhodamine 123 in not stimulated (NS) granulocytes (A) and after stimulation (S) (B) (HC n=20 and TB n=13)*

Taking in account these results TB patients have in their blood more granulocytes activated and but the total amount of ROS produced showed no difference to healthy controls.

Monocytes from TB patients have increased ROS production

The analysis made for granulocytes was also performed for monocytes showing different results. Taking into account the percentage of positive monocytes to rhodamine 123 there was not detected any statically difference between nonstimulated healthy controls and TB patients. However besides the expected increase after stimulation in healthy controls and TB patients, monocytes from TB patients had higher percentage of cells rhodamine^{+} with statistical significant increase higher than found in granulocytes (Figure 13 A). Looking for the mean of fluorescence intensity to rhodamine 123, there is a statistical significant increase of ROS production associated with the disease. After stimulation, monocytes from TB patients showed even higher differences of ROS comparing with controls (Figure 13 B). Considering both results in TB patients there is the same percentage of cells with positive stain for rhodamine however the levels of ROS produced is higher.

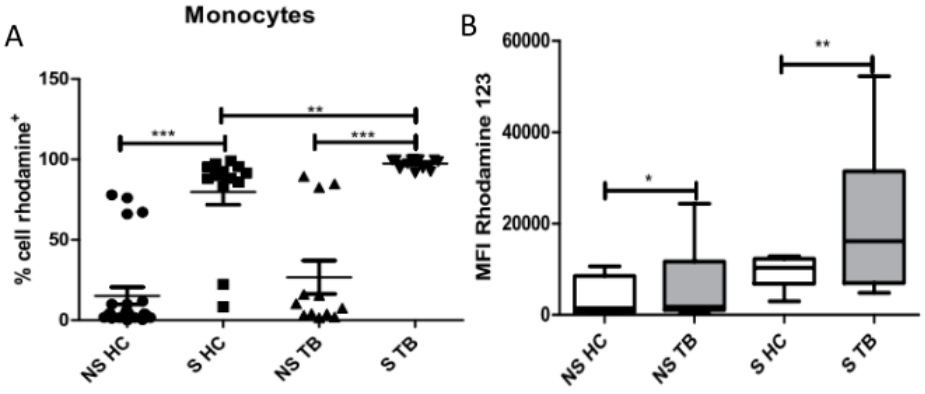

Figure 13- A- *Analysis of granulocytes with positive fluoresce to rhodamine 123 (HC n=20 and TB n=13).* ***B-*** *Mean of fluorescence intensity of rhodamine 123 in monocytes from controls and TB patients before and after stimulation (HC n=20 and TB n=13)*

It should be noted that, comparing ROS production of granulocytes and monocytes, the mononuclear subtype produces less ROS than polymorphonuclear cells after stimulation (Figures 12 and 13).

ROS production is higher in intermediate monocyte subset

Due to increased ROS production of monocytes in tuberculosis patients detected by mean of fluorescence intensity, all three monocyte subsets were analyzed separately to ROS production (Figure 14 A). In not stimulated state the differences between all three subtypes were not statistically significant having the nonclassical ($CD14^{dim}CD16^{++}$) subtype the lower levels of ROS. However after stimulation, the differences appear, with intermediate ($CD14^{+}CD16^{+}$) subtype showing to be the main responsible for increase of ROS. Regarding to classical ($CD14^{++}CD16^{-}$) monocytes there was no statistically significant increase, while in the nonclassical subtype after stimulation there was a statistically significant increase of ROS production (Figure 14 B).

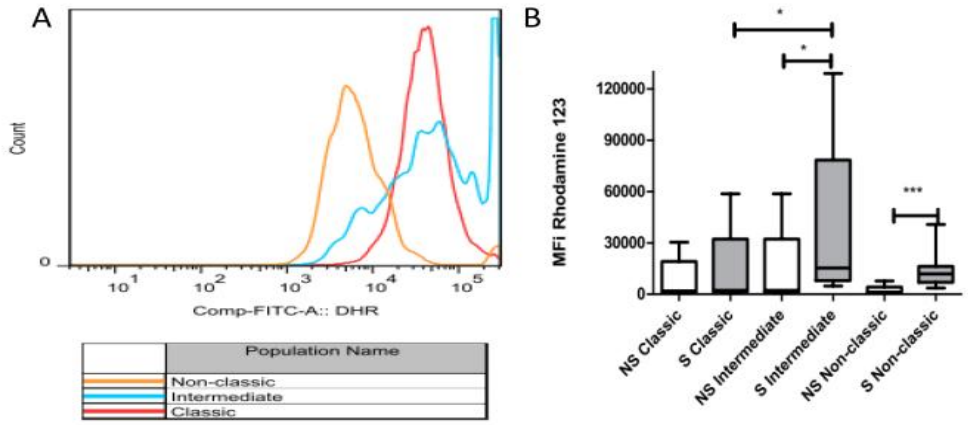

Figure 14- A- *Representative histogram of rhodamine 123 to three different monocyte subsets.* ***B-*** *Mean of fluorescence intensity of rhodamine 123 in three subsets of monocytes from healthy controls and TB patients before and after stimulation (HC n=20 TB n=13)*

NK cells have higher ROS in TB patients

The main focus in this work was to analyze ROS in NK cells. This immune cell was not known as able to modulate ROS production in the immune context. However our results show that NK cell population from tuberculosis patients had increased percentage of positive cells to rhodamine. This increase detected is statistically significant. After stimulation there is no difference between stimulated cells of healthy controls and TB patients.

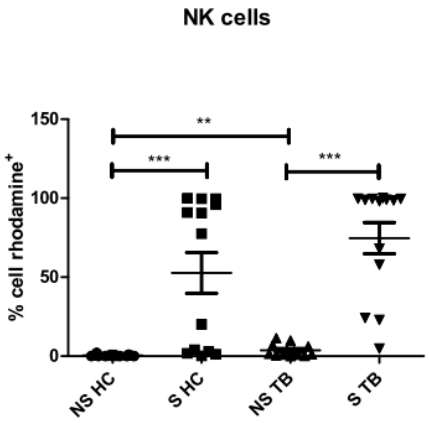

Figure 15- *Analysis of NK cells with positive fluoresce to rhodamine 123 (HC n=20 and TB n=13).*

Regarding to the mean of fluorescence intensity, NK cells from TB patients had higher ROS than controls with statistic significant difference (Figure 16 A). The differences detected in nonstimulated cells are even higher after stimulation (Figure 16 B).

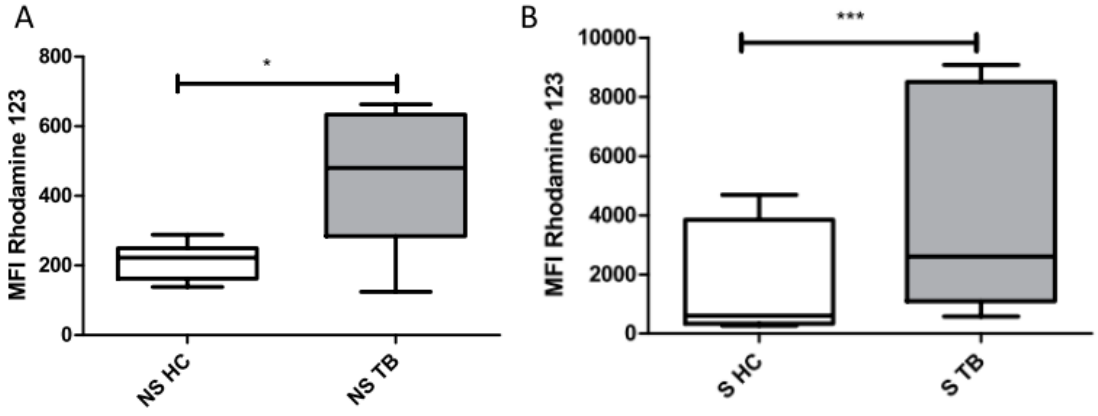

Figure 16- *Mean of fluorescence intensity of rhodamine 123 in NK cells from controls and TB patients before (A) and after stimulation (B). (HC n=20 and TB n=13)*

CD56^{dim} subset has higher levels of ROS

Having in account the literature showing different sensitivities of CD56^{dim} and CD56^{bright} to ROS, both subsets were analyzed separately. The percentage of positive cells to rhodamine 123 was higher with statistically significant results in opposition to CD56^{bright} that did not reveal any difference (Figure 17 A, B). However when the mean of fluorescence intensity was analyzed, the increased ROS in NK cells depend equally from CD56^{dim} and CD56^{bright,} judging by the fact that both subsets had equal differences from healthy controls to TB patient, without any statically significant difference between them (Figure 18 A and B).

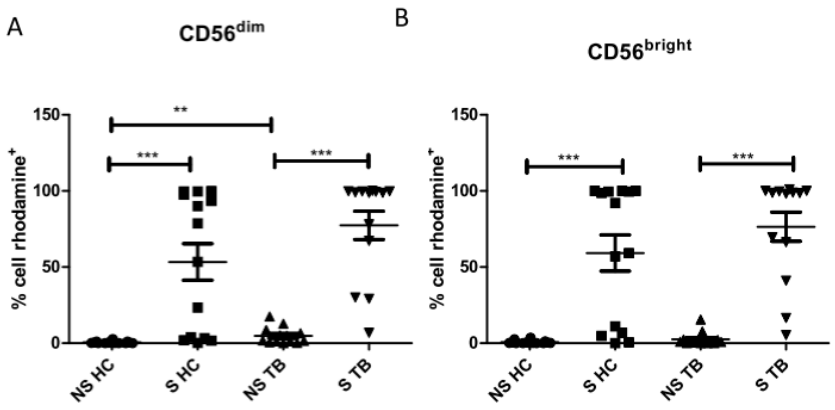

Figure 17- *Analysis of CD56^{dim} (A) and CD56^{bright} (B) NK cells with positive fluoresce to rhodamine 123 (HC n=20 and TB n=13).*

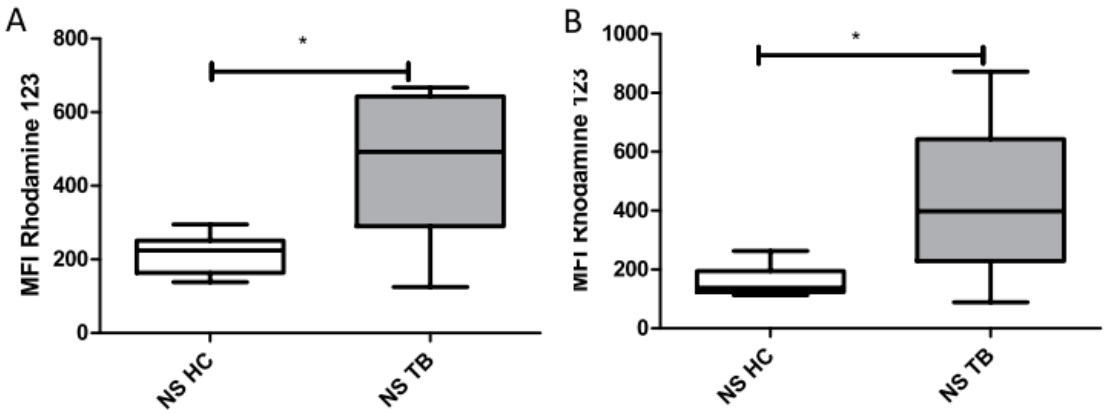

Figure 18- *Mean of fluorescence intensity of rhodamine 123 from controls and TB patients in CD56^{dim} NK cells (A) CD56^{bright} NK cells (B). (HC n=20 and TB n=13)*

These results show that there is an increased percentage of CD56^{dim} NK cells with positive stain to rhodamine in total population comparing with CD56^{bright} but the levels of ROS detected in all cells on both subsets was statistically equal.

After stimulation CD56^{dim} have higher ROS

Although nonstimulated CD56^{dim} and CD56^{bright} did not show differences in the mean of fluorescence intensity, after stimulation CD56^{dim} still showed an statistical significant increase in TB patients (Figure 19 A) unlike CD56^{bright} which, although suffered an increase in rhodamine conversion, the results shown that there is not any statistical significant differences between healthy controls and TB patients (Figure 19 B)

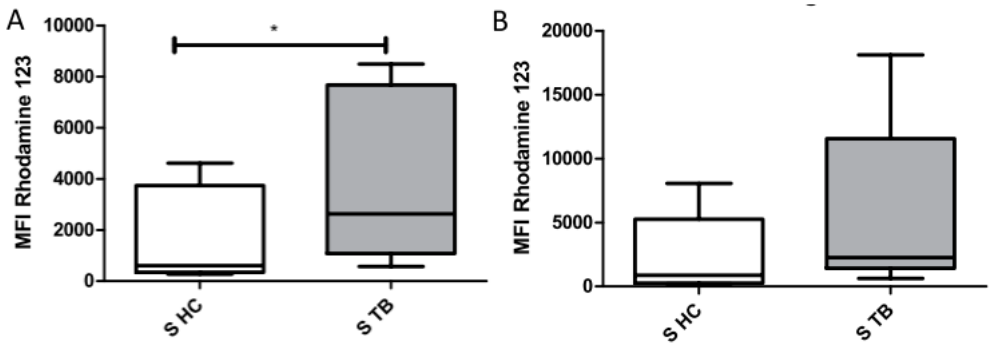

Figure 19- *Mean of fluorescence intensity of rhodamine 123 from controls and TB patients after stimulation in CD56^{dim} NK cells (A) CD56^{bright} NK cells (B). (HC n=20 and TB n=13)*

Stimulated TB patients have lower NK cells

The percentage of NK cells within lymphocytes and its subsets were also analyzed. The TB patients had a small, yet not statistical significant difference to healthy controls in NK cell percentage. After stimulation, TB patients had a statistical significant decrease of NK cells comparing nonstimulated cells (Figure 20). In healthy controls, this decrease did not reveal statistical significant difference. Stimulated NK cells from TB patients had the lowest percentage of NK cells.

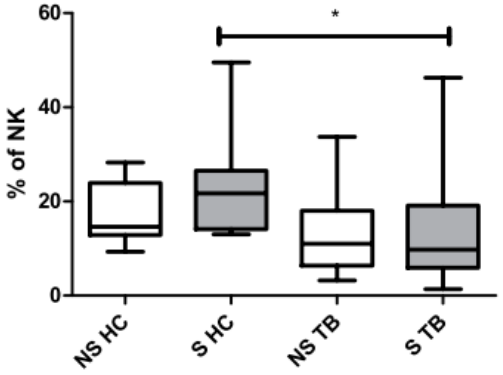

Figure 20- *Analysis of NK cells in peripheral blood of healthy controls and TB patients (HC n=20 and TB n=13)*

Taking into account all the results regarding the NK cells, there was a decrease of NK cells in lymphocyte population but their levels of ROS increased. These observations are even higher after stimulation.

TB patients have lower CD56^{dim} and higher CD56^{bright} subsets

Taking into account NK cell subsets, very small differences were detected with a minor decrease in CD56^{dim} and a minor increase in CD56^{brigh}, not being statistically significant (Figure 21).

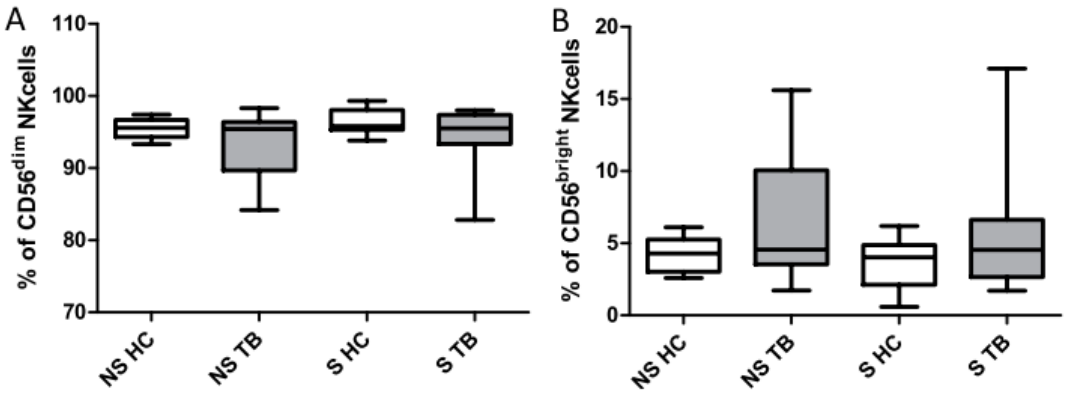

***Figure 21**- Analysis of CD56^{dim} (A) and CD56^{bright} (B) NK cells in peripheral blood of healthy controls and TB patients (HC n=20, TB n=13)*

Chronic myeloid leukemia

In the presented work where analyzed 88 samples from 50 CML patients to evaluate the ROS production in the blood of these patients. It was also provided for each sample the ratio BCR-ABL/ABL used to diagnose and evaluate the disease progression. The samples used in most of analysis were only those whose results were positive for the presence of BCR-ABL mRNA. Granulocytes and monocytes and lymphocytes where selected according to SSC and FSC know characteristics. Within the lymphocytes NK cells were selected as CD56^{+}CD3^{-} (Figure 22). The mean of fluorescence intensity and the percentage of positive stained cells for rhodamine 123 (rhodamine^{+}) were used to evaluate ROS production in mentioned cell types.

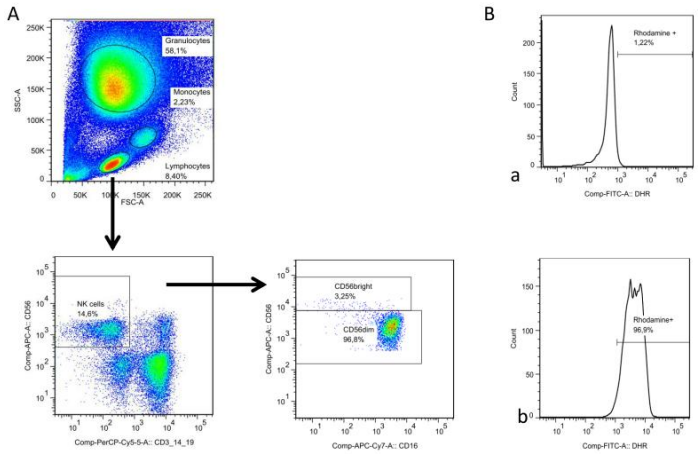

Figure 22 ***A****-Representative dot plots to NK cells and their subsets, monocytes and granulocytes.* ***B****- Representative histogram of positive stain to rhodamine in NK cells from healthy controls (a) and CML patients (b)*

Granulocytes in CML patients have higher ROS production.

The percentage of granulocytes rhodamine^{+} from CML patients had a statistically significant increase compared with healthy controls before and after stimulation (Figure 23). Almost 50% of granulocytes in nonstimulate state from CML patients were active to ROS production.

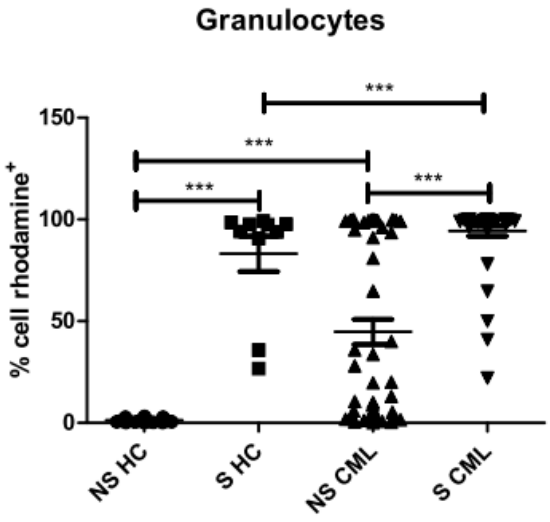

Figure 23*-Analysis of granulocytes with positive fluoresce to rhodamine 123 in CML patients (HC n=20 and CML n=51).*

Looking for the mean of fluorescence intensity, nonstimulated granulocytes in CML patients had an increase of ROS production compared with healthy controls with statistical

significant difference (Figure 24 A). After the stimulation, granulocytes from CML patients and healthy controls produce equal levels. (Figure 24 B)

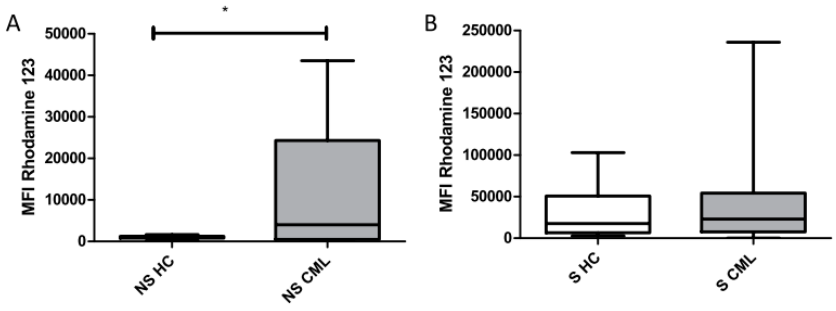

Figure 24*- Mean of fluorescence intensity of rhodamine 123 in granulocytes of healthy controls and CML patients (HC n=20 CML n=51)*

Monocytes have higher levels of ROS production in CML patients

Monocytes positive to rhodamine from CML patients were compared with their counterparts from healthy controls. As in granulocytes, CML patients have higher percentage of activated monocytes producing ROS than healthy controls. This difference is statistically highly significant. However no differences where found between healthy controls and CML patients after stimulation (Figure 25 A)

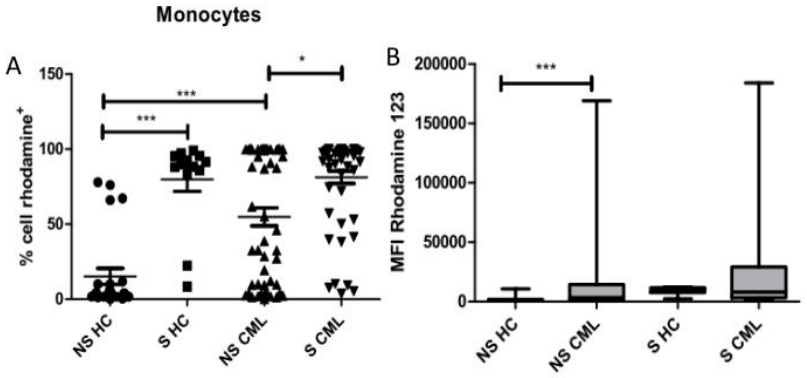

Figure 25- A- *Analysis of monocytes with positive fluorescence to rhodamine 123 in CML patients* ***B-*** *Mean of fluorescence intensity of rhodamine 123 in monocytes of healthy controls and CML patients (HC n=20 CML n=51)*

Regarding to the mean of fluorescence intensity, monocytes from CML patients produced higher levels of ROS being this increase statistically significant. After stimulation the registered differences were not statistically significant comparing to healthy controls (Figure 25 B).

NK cells from CML patients have higher ROS

NADPH oxidase, the enzymatic system responsible for oxidative burst is not active in NK cells because some of the proteins responsible for correct assemble were not express in this type of cell (Bedard 2007). Nevertheless after stimulation the percentage of cells with positive stain to rhodamine increases substantially. This demonstrates that NK cells can have increased ROS levels. The same analysis that was already performed to phagocytes where also performed to NK cells with similar results. In CML patients the percentage of NK cells with positive stain to rhodamine where considerable higher than in healthy controls. This result is statistical highly significant (Figure 26). The percentage of NK cells positive to rhodamine in CML patients was almost the same that was found in stimulated healthy controls.

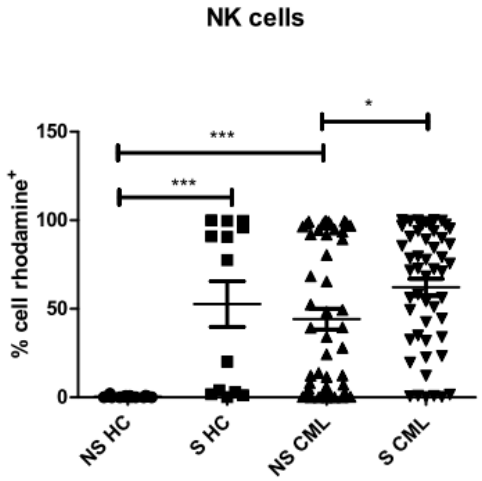

***Figure 26**- Analysis of NK cells with positive fluorescence to rhodamine 123 in CML patients (HC n=20 CML n=51)*

Taking into account the level of ROS by the mean of fluorescence intensity in NK cells, this type of cell in CML patients had higher statistical significant ROS than in healthy controls (Figure 27 A). After stimulation, like in granulocytes and monocytes, the differences were not statistically significant (Figure 27 B).

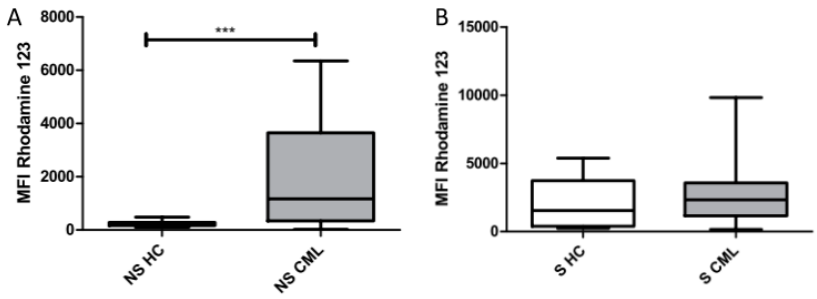

Figure 27- *Mean fluorescence intensity of rhodamine 123 in NK cells from blood of healthy controls and CML patients before (A) and after stimulation (B) (HC n=20 CML n=51)*

CD56^{dim} and CD56^{bright} have the same levels of ROS

Like it was made to TB patients, the CD56^{dim} and CD56^{bright} subsets were analyzed separately to evaluate possible differences in these subsets. The analysis of the percentage of NK cells positive to rhodamine for both subsets reveal that there are not any difference between these subsets and the statistical significant results found in NK cells are the same to CD56^{dim} (Figure 28 A) and CD56^{bright} (Figure 28 B). Regarding to the mean of fluorescence intensity, was not detected any difference between these subsets (Figure 29) revealing the contribution of both CD56^{dim} and CD56^{bright} is the same to the differences showed in total NK cells. In both cases nonstimulated CML patients had a statistical significant increase to healthy controls that was not present after stimulation.

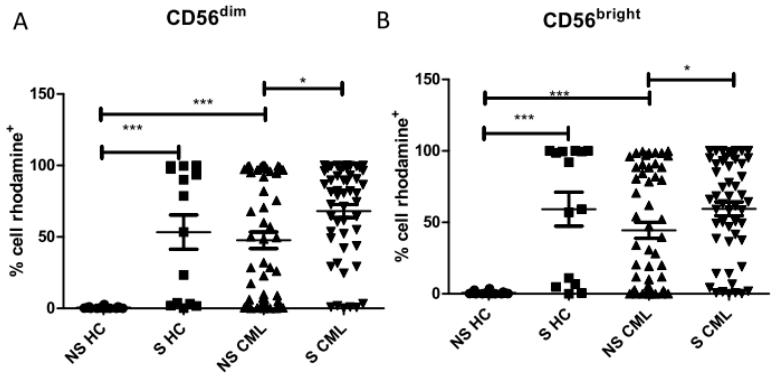

Figure 28- *Analysis of CD56^{dim} (A) CD56^{bright} (B) NK cells with positive fluorescence to rhodamine 123 in CML patients (HC n=20 CML n=51)*

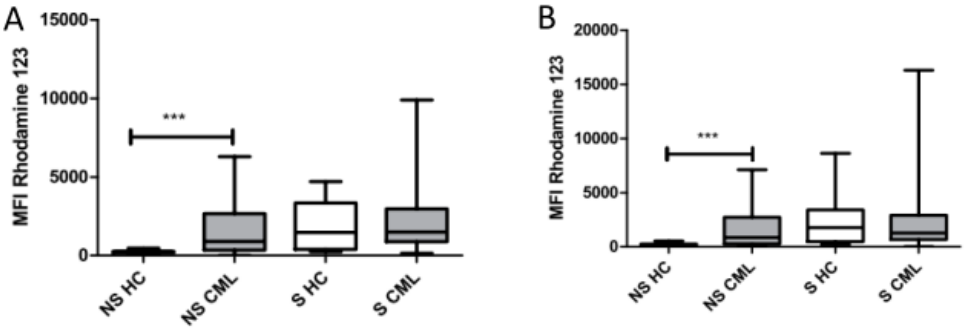

Figure 29- *Mean fluorescence intensity of rhodamine 123 in CD56^{dim} (A) and CD56^{bright} (B) NK cells from blood of healthy controls and CML patients before (NS) and after (S) stimulation (B) (HC n=20 CML n=51)*

One of the theories to explain why CD56^{dim} NK cells were more sensitive to ROS inhibition than CD56^{bright} is based in the fact that CD56^{bright} NK cells had more antioxidants in plasmatic membrane (Thoren 2011). Comparing directly the levels of ROS in these subsets, though the mean of fluorescence intensity, there was not found any difference (Figure 30). Clearly the differences demonstrated between NK CD56^{dim} and CD56^{bright} to take cytotoxic action and cytokine release, respectively, were not due to ROS detected by rhodamine 123.

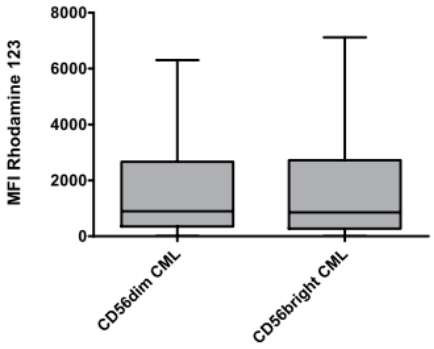

Figure 30- *Mean fluorescence intensity of rhodamine 123 in CD56^{dim} and CD56^{bright} in CML patients (n=51)*

ROS production is dependent of BCR-ABL expression in granulocytes and monocytes

During this work 88 samples from CML patients were collected along there ratio BCR-ABL/ABL as diagnostic to their progression. With the propose to evaluate separately ROS production in different phases of disease, taking into account all the samples collected, was created three different groups- one with negative expression to BCR-ABL, another with higher expression (more than 30) of the mRNA and an intermediate population. Analyzing

the results to granulocytes (Figure 31 A) and monocytes (Figure 31 A), a statistical significant increase was detected from patients without the expression of BCR-ABL to patients with higher expression of the mutated gene in both cell types. The difference was more pronounced in granulocytes.

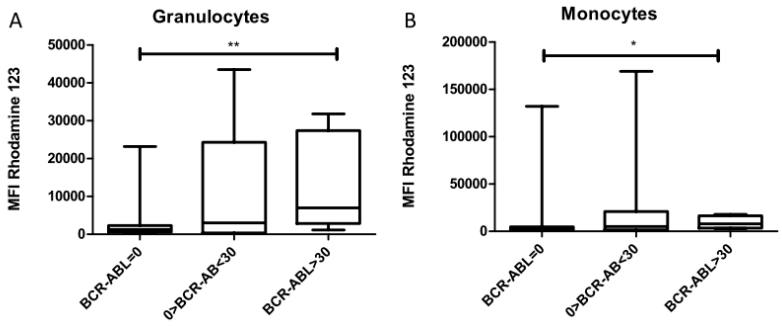

Figure 31- *Mean of fluorescence intensity of rhodamine 123 in granulocytes (A) and monocytes (B) from blood of CML patients with three population separated according to negative, intermediate and higher levels of BCR-ABL mRNA (BCR-ABL=0 n=37; 0>BCR-ABL<30 n= 43 and BCR-ABL>30 n=8)*

ROS levels in NK cells is independent of BCR-ABL expression

Although in granulcoytes and monocytes a statistical significant increase was detected in patients with higher levels of ROS, after the same analysis was performed to NK cells there was no statistically differences registered (Figure 32).

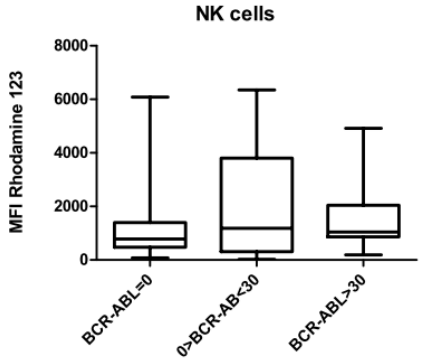

Figure 32- *Mean of fluorescence intensity of rhodamine 123 in NK cells from blood of CML patients with three population separated according to negative intermediate and higher levels of BCR-ABL mRNA (BCR-ABL=0 n=37; 0>BCR-ABL<30 n= 43 and BCR-A and BCR-ABL>30 n=8)*

This reveals that after the diagnostic to the patients was negative, NK cells from CML patients did not suffer a decrease of ROS.

ROS from NK cells are correlated with ROS from granulocytes

To elucidate if ROS detected in phagocytes are related with ROS in NK cells both data was confronted in a linear regression. It can be observed that ROS increase in granulocytes is associated with ROS increase in NK cells this correlation is still weak (Figure 33).

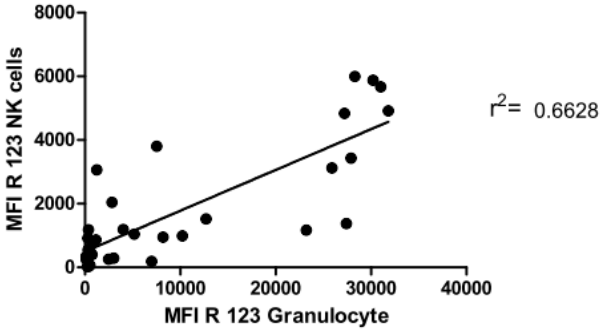

***Figure 33**- Correlation between mean fluorescence intensity of rhodamine 123 in NK cells and in granulocytes in CML patients (n=51).*

Based on these results it is plausible that increase of ROS in NK cells are associated with oxidative burst in granulocytes but others variables are also contributing.

The Percentage NK cells do not change in CML patients even after stimulus

NK cell apoptosis is one of the negative effects observed with ROS increase (Betten 2001). In this work it was not observed a decrease in percentage of NK cells comparing controls and CML patients (Figure 34).

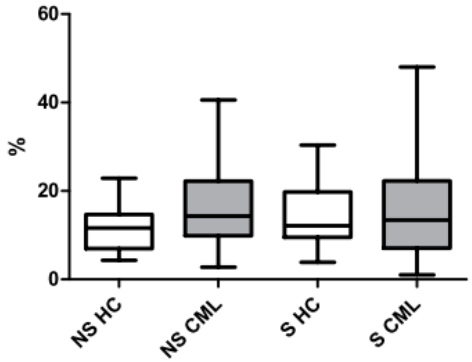

***Figure 34**- Analysis of NK cells in peripheral blood of healthy controls and CML patients before (NS) and after (S) stimulus. (HC n=20, CML n=51)*

This is unexpected but may be explained by the fact that blood from CML patients were collected during treatment and in some cases there is a positive effect of some treatments in NK cells (Therme 2008).

$CD56^{dim}$ is reduced and $CD56^{bright}$ is increased in CML patients.

Considering the NK cell subsets a very small decrease in $CD56^{dim}$ (Figure 35 A) and small increase in $CD56^{bright}$ (Figure 35 B) was observed but, for both cases, without statistically significant results.

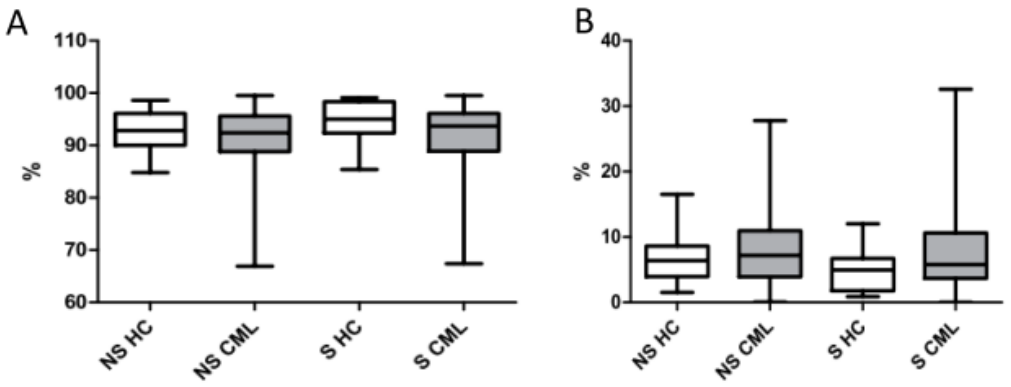

Figure 35- *Analysis of $CD56^{dim}$ (A) and $CD56^{bright}$ (B) in peripheral blood of healthy controls and CML patients before (NS) and after (S) stimulus. (HC n=20, CML n=51)*

The expression of NK cell receptors are reduced in CML patients

Many studies showed that the reason for NK cell decreases their cytotoxic action is due to lower expression of essential receptors in the membrane to detect altered ligands (Romero 2005). From NK cell receptors analyzed, NKp46 and CD16 showed a very significant decrease in their expression without the stimulus and after stimulation (Figure 36 A and B).

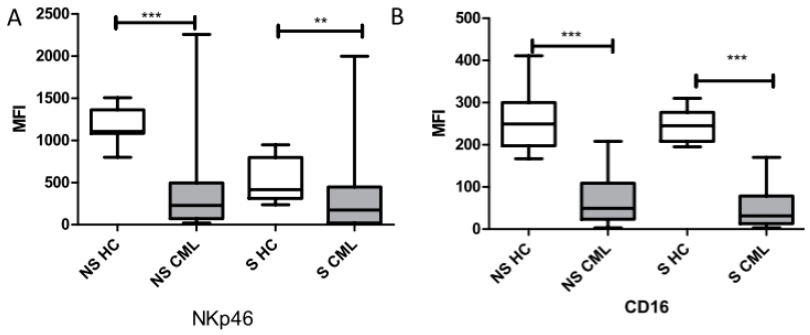

***Figure 36**- Mean of fluorescence intensity of NKp46 (A) and CD16 (B) in NK cells from healthy controls and CML pateints before (NS) and after (S) stimulation (HC n=20 CML n=51).*

Being NKp46 and CD16 two important activator receptors, their down regulation was clearly associated with lower cytotoxic capacity from NK cells. However CD57, CD56 and CD11b did not show any relevant difference between healthy controls and CML patients (Figure 37 A, B and C).

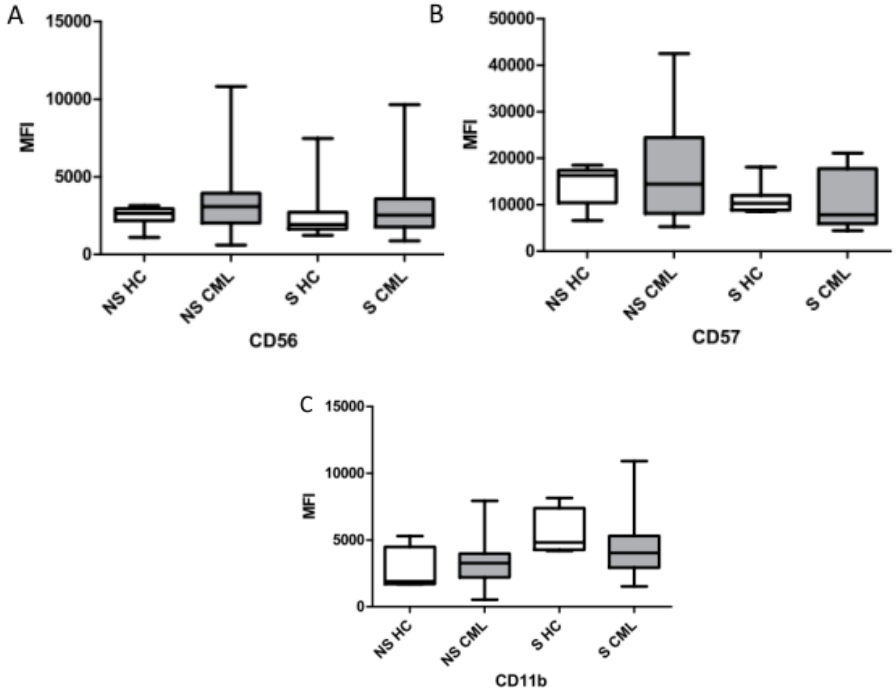

***Figure 37**- Mean of fluorescence intensity of CD56 (A) CD57 (B) and CD11b (C) in NK cells from healthy controls and CML patients before (NS) and after (S) stimulation (HC n=20 CML n=51)*

The only difference registered in the subsets of NK cells was in CD56^{bright} where CD56 expression was increased in nonstimulated blood from CML patients. After stimulation, CD56 expression acquire de same level than healthy controls (Figure 38)

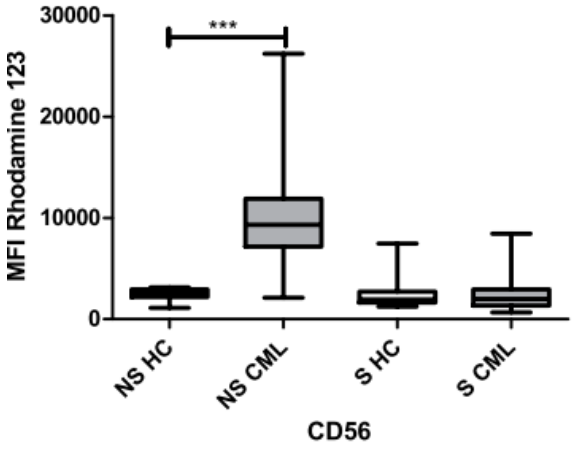

Figure 38- *Mean of fluorescence intensity of CD56 in CD56^{bright} NK cell from healthy controls and CML patients before (NS) and after (S) stimulation (HC n=20 CML n=51)*

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NK and Monocyte ROS communication

To better understand how monocytes can influence NK cells, both type of cells were sorted previous to cell culture and then stimulate. In a first experiment, NK cells and monocytes were cultured separately and LPS was added during 30 minutes to monocytes. After 30 minutes the medium was removed and NK cells were added to monocytes and leave for 2 hours to give the idea how monocytes influence ROS in NK cells without the last one were in contact with stimulus.

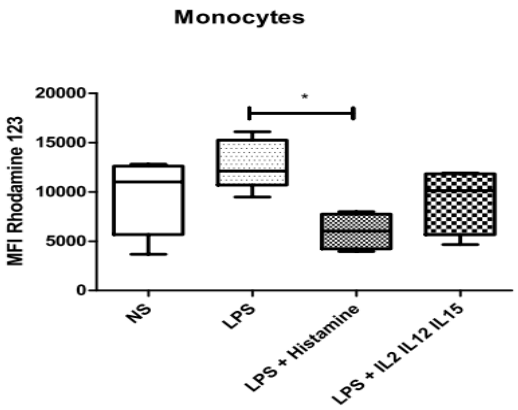

Figure 39- *Mean fluorescence intensity of rhodamine 123 in monocytes in different cell culture conditions*

After 30 minutes of stimulus there was a small increase in ROS production in monoyctes. But when histamine was added to medium with the same stimulus, the quantity of ROS detected decrease significantly (Figure 39). The use of histamine as a coadjuvant in

therapy against some type of tumors is based on its capacity to reduce dramatically ROS production by phagocytes. However is always used with IL-2 to activate antitumor action of NK cells. But when a combination of IL-2, IL12 and IL-15 was added only to NK cells during 30 minutes, the result show the same effect as not stimulation of monocytes. For some reason, stimulate NK cells before they were in contact with monocytes led to the same ROS production in monocytes as no LPS added. The use of IL-2 Il-12 and IL-15 in NK cells abolished the LPS effect. When NK cells were analyzed however no differences were detected in ROS levels (Figure 40)

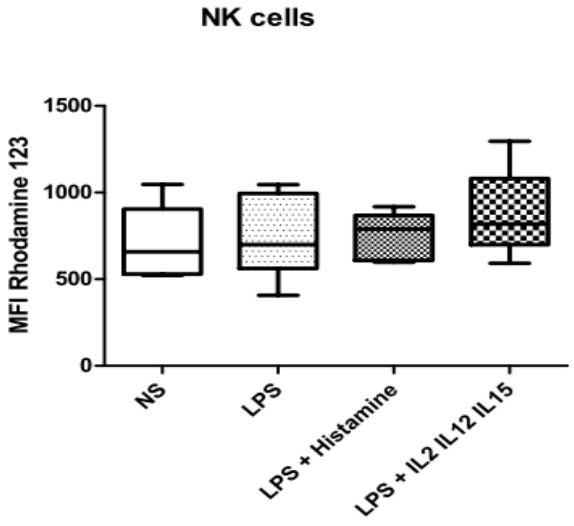

***Figure 40**- Mean fluorescence intensity of rhodamine 123 in NK cells from healthy controls in different cell culture conditions (NS- not stimulate)(n=6)*

ROS production occur in the first minutes after stimulation

The evaluate the velocity of the decrease in ROS production, the cells were stimulated during 10 and 30 minutes. In 10 minutes it is possible to detect a huge amount of ROS but after 30 minutes was already decreasing (Figure 41).

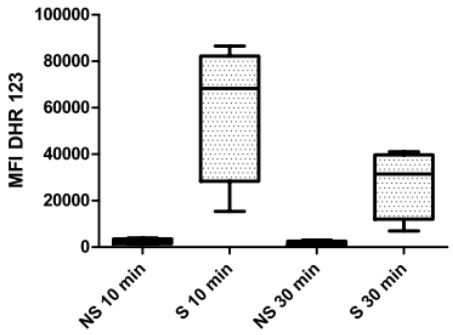

Figure 41- *Mean fluorescence intensity of rhodamine 123 in leucocytes from healthy controls before (NS) and after (S) stimulation during 10 and 30 minutes. (n=6)*

Ratio of NK cells/monocyte and IL-2/12/15 influence the levels of ROS

In a second experiment, NK cells and monocytes were cultured and stimulated together. In this case NK and monocytes where cultured not only in 1:1 ratio but also with just 10% of monocytes. According to Asea *et al.,* 1996, 10% or less of monocytes has a positive effect over NK cells. Analyzing ROS production in monocytes, when just 10% of these cells were in culture the production of ROS had the lowest levels. When catalase was added to medium no especial effect over monocytes were detected. This is because catalase did not prevent ROS production but remove it from the medium. The condition with less ROS production occurs when LPS was added with IL-2 IL-12 and IL-15 (Figure 42). Between all conditions mentioned there was not any statistical significant difference.

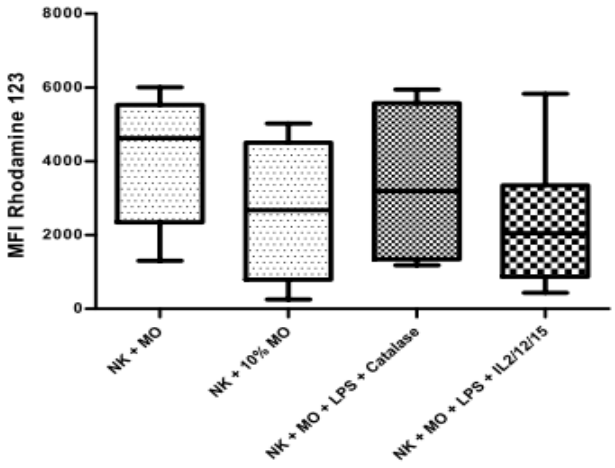

Figure 42- *Mean of fluorescence intensity in monocytes co-cultured with NK cells from healthy controls in different cell culture conditions (n=6)*

The effects in NK cells were dramatically different (Figure 43). It was possible to demonstrate a small increase in ROS detected in NK cells when monocytes were cultured together with NK cells without stimulus comparing to NK cells a alone. The merely presence of monocytes were enough to increase the levels of ROS on NK cells. However such results were not statistically significant. So far, it was never demonstrate that the presence of monocytes influence directly the amount of ROS in NK cells. When only 10% of monocytes were added, the quantity of ROS detected had the smallest amount with significant differences to NK cells cultured alone and especially to NK cells cultured with monocytes. This information is in concordance with the results from Asea *et al.,* 1996 revealing a positive effect of monocytes in lower numbers clearly demonstrates a very complex mechanism of cross communication where not only the secondary messengers have an important role as the ratio of the cells that produce them. Adding IL-2, IL-12 and IL-15 with LPS almost had the same effect to NK cells as the condition with 10% of monocytes and with statistically significant lower ROS levels in NK cells with monocytes without any stimulus. All three interleukins were added 24 hours after LPS stimulation. Clearly with this data, the combination of these three interleukins can have a very interesting protecting role. Adding the interleukins previously to LPS stimulation leads to a significant less ROS in NK cells than before any stimulation. Adding catalase together with LPS led to the same results that the condition without any stimulus. Clearly ROS produced by monocytes was scavenged by catalase so the ROS in NK cells in this situation was equal to nonstimulated cells..

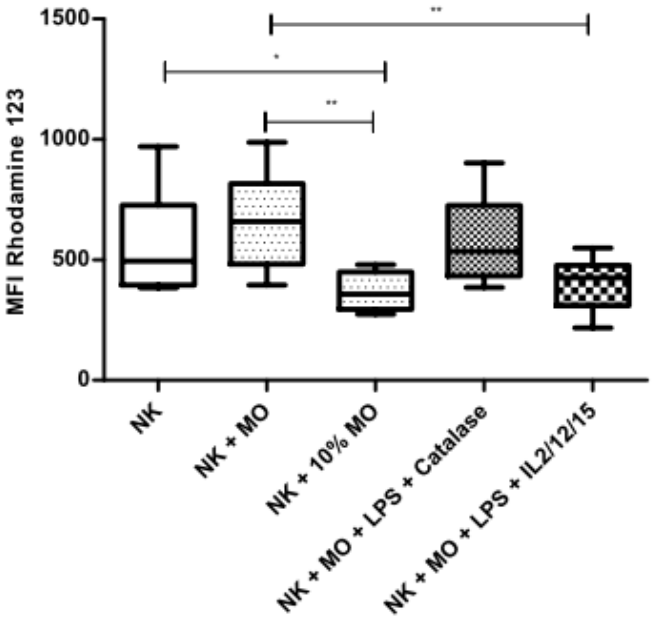

Figure 43- *Mean fluorescence intensity of rhodamine 123 in NK cells co-cultured with monocytes from healthy controls in different cell culture conditions (n=6)*

DISCUSSION

Discussion

In the last years, many efforts were made to understand how tumors and infected cells can escape to immune system and how it is possible to reverse the immunescape characteristics developed in such diseases. Several data revealed an inhibition of NK cells in different pathologies. Part of this inhibition is caused by reactive oxygen species created by phagocytes. Many researches demonstrate the negative role of ROS but in all cases, because ROS derived from NADPH oxidase are characteristic of phagocytes no data demonstrate the redox state in NK cells. In this work it was demonstrated for the first time an increase in ROS within NK cells in tuberculosis and chronic myeloid leukemia patients. Associated with an increase in ROS was also demonstrated lower expression of important activating receptors as was already demonstrated by others investigators (Romero 2005, Peraldi 2009) Furthermore, a different protective of NK cells form phagocytosis-derived ROS can be possible recurring to interleukins. Although histamine, as already was demonstrated, is an effector inhibitor of ROS production in monocytes, the results from this work prove that the combination of IL-2, IL-12 and IL-15 can be almost protective as histamine without some negative impact that histamine can provoke in tumor microenvironment.

Phagocytes from TB patients had higher ROS production

The ROS production through NADPH oxidase is considered part of the immune system as a mechanism that destroys bacteria and other phagocytosed components. Many authors showed their importance in infection with *M. tuberculosis* and how the bacterium can escape to death by ROS (Chan 2001 Simone 2011). But the constant presence of *M. tuberculosis* in lungs without the capacity of macrophages to destroy them and constant ROS production can be a possible inhibitor to lymphocytes, especially NK cells. As in many chronic infections, the prolonged activation of humoral and cellular components of the immune system can have a deleterious effect. Therefore, we studied the ROS production in cellular components of blood from tuberculosis. The phagocytes in peripheral blood showed increased ROS production, however with some differences. The granulocytes in the blood of TB patients have an increased percentage of cells positively stained to rhodamine but the mean of fluorescence intensity in all granulocyte population did not reveal significant differences to healthy controls. The same analysis performed for monocytes reveal the opposite- there is not statistically significant increase of positive monocytes to rhodamine but the mean of fluorescence intensity is higher in TB patients. The reason for this could be to

their different roles in the immune response. Granulocytes are cells with short life span that act in the first ours of the immune response. Monocytes, despite their antimicrobial action, can also suffer differentiation for macrophages. It is however clear that both phagocytic cells have higher levels of ROS in TB patients.

In monocytes, the subset that after stimulation showed higher ROS production was intermediate subset. This is not expected because classic subset is considered the most phagocytic with more myeloperoxidase activity. However, the conversion of dihidrorhodamine 123 is not dependent of all ROS that can be produced by NADPH oxidase and posterior reactions (Bylund 2010). The incapacity for detect all ROS using one single probe can hide the full ROS efficiency for each monocyte subset. In fact the higher activity of myeloperoxidase can led to H_2O_2 conversion to other ROS that are not detected by dihidrorhodamine 123.

After stimulation granulocytes and monocytes continued to have higher levels of ROS than healthy controls. Because it was analyzed cells from the blood and not from the tissue it is expected that the results from this work after stimulation are in more concordance with inflammation in lungs were the bacteria itself act as a stimulus. Therefore it is expected that in the lungs of TB patients the levels of ROS are even higher than those who were found in the blood. It is also important to consider that in lungs the main responsible for ROS production are macrophages that can produce even higher amounts than their phagocytic counterparts from blood (Golderman 2007 de kraaij 2010).

NK cells in TB patients had more ROS

The effect registered in phagocytes from blood of TB patients can only be considered negative to lymphocytes especially NK cells if act directly over them. Through flow cytometry, we demonstrate that the redox unbalance from oxidative burst can also affect NK cells which showed a very significant increase in ROS. The NADPH oxidase is not active in NK cells therefore the increased ROS cannot be attributed to the activity of the enzymatic system. However, ROS can cross the membrane and act directly as second messengers in nonproducing cells (bylund 2010). The theory that explains how NK cells suffer a decrease in the expression of NK cell surface receptors, activate apoptosis and suffer other negative effects is based in the action of ROS as second messengers. In this work for the first time was proven that NK cells in a chronic inflammation as tuberculosis infection had an unbalance in their redox state with higher levels of ROS. After stimulation, the level of ROS in NK cells like in monocytes increased.

$CD56^{dim}$ NK cells had higher levels of ROS

It has been reported an increase in $CD56^{bright}$ in detriment of $CD56^{dim}$ in chronic inflammatory conditions, possibly due to the fact that $CD56^{bright}$ NK cells are more resistant to ROS induced apoptosis than $CD56^{dim}$ NK cells. Besides ROS production is increased in chronic inflammatory conditions (Batoni 2005 Schierloh P, 2005, Harlin 2007). The explanation to $CD56^{dim}$ be more affected by ROS can be due to more antioxidative compounds within the cell like paraoxonase-2 and lysozyme (sixfold upregulated in $CD56^{bright}$ comparing with $CD56^{dim}$) that can protect better $CD56^{bright}$ NK cells (Schierloh *et al.,* 2005, Hanna 2004). Also, $CD56^{bright}$ NK cells express higher levels of cell-surface thiols and are more efficient at neutralizing hydrogen peroxide (Thoren 2011). In these work it was tested if the NK cell subsets had differences in the levels of ROS. The $CD56^{dim}$ subset had a statistical significant increase of ROS levels in TB patients, but $CD56^{bright}$ cells did not reveal such difference. However when the mean of fluorescence intensity was analyzed for both subsets there was not found great differences. However after stimulation, $CD56^{dim}$ had increased ROS levels with statistical significant difference and $CD56^{bright}$ did not. Taking into account all this results it is clear that $CD56^{dim}$ had higher levels of ROS reinforcing the data that reveals different capacities of both subsets to respond to ROS.

Decrease of NK cells in tuberculosis

In the presented work, the percentage of NK cells after stimulation was statistically significant lower than healthy controls. In nonstimulated cases, there was not any statistical difference. Taking into account that stimulated cells can be a more approximate model for the redox state in the lungs, and with these results, the levels of NK cells are decreased in TB patients in a stimulated environment. Regarding to NK cells subsets there was not any statistical significant difference but there is a trend to a decrease of $CD56^{dim}$ NK cells and to a increase of $CD56^{bright}$ in TB patients. Although was already proved that NK cells can kill *M. tuberculosis-infected* macrophages in vitro their role *in vivo* it is still unknown. The decrease of their number and especially the decrease of the cells with cytotoxic action points to an important role where ROS produced during macrophage phagocytosis could be responsible for their lower numbers.

Chronic myeloid leukemia

Much of the work in this field is focus in tumor pathology both hematologic as solid tumors. The use of myeloid leukemia proved to be an excellent model to study this mechanism because the collection of samples is easy, and specially, because tumor cells have NADPH oxidase active. Even a small amount of ROS detected in myeloid cells, along with increase in their number can lead to higher levels of ROS in blood.

Granulocytes and monocytes from CML patients had higher ROS production

In some types of cancer, the presence of neutrophils and monocytes circulating in the blood is associated with a bad prognostic (Donskov *et al*., 2006). This negative impact of myeloid cells is due to their ROS production with direct impact in antitumor lymphocytes. In the presented work was detected the production of ROS in monocytes and granulocytes separately. In both cases the percentage of cells with high levels of ROS and the mean of fluorescence intensity of rhodamine 123 were significantly higher in CML patients comparing with healthy controls. Clearly in the CML patients analyzed the phagocytes in the blood produced more ROS. These results are in concordance with Mellqvist and colleagues however with some differences (Mellqvist *et al.,* 2000). In the cited work, granulocytes from healthy controls were more prone to inhibit NK cells than granulocytes from CML patients. In this work the percentage of granulocytes rhodamine^{+} were higher than healthy controls with statistical significant difference. However the mean of fluorescence intensity reveal no difference between both types of samples. Nevertheless, in our results even from monocytes reveal that healthy controls produce less ROS than in CML patients.

Increased ROS in NK cells

Like it was stated for the results of tuberculosis, NK cells cannot produce ROS by NADPH oxidase. However like in TB patients the levels of ROS in NK cells from the blood of CML patients had a statistical significant increase comparing to healthy controls. Therefore the levels of ROS detected must be from other source. Because ROS can enter in the membrane and is necessary the strait contact between phagocytes and NK cells (Hellstrand

K, Hermodsson S. 1991) it is plausible that the detected ROS came from NADPH oxidase from phagocytes acting as second messengers. Nevertheless, increased ROS is associated with NK cell dysfunction and despite some works revealing that ROS from phagocytes is able to inhibit NK cells, it was the first time that was demonstrated a significant increase of ROS in NK cells from CML patients. The levels of ROS detected in nonstimulated NK cells in CML patients were almost the same as in stimulated healthy controls. Taking into account the different experiments in literature sowing the negative impact of ROS in NK cells against tumor cell lines after stimulation in healthy controls (Betten *et al.*, 2001, Betten *et al.,* 2004 Mellqvist *et al.,* 2000) this could explain why NK cells from CML patients are unable to lyse tumor cells.

ROS levels are the same in $CD56^{dim}$ and $CD56^{bright}$ in CML patients

The cytotoxic action of NK cells is affected in more extension than their IFN-γ release. Like in TB patients, to elucidate if there was any difference in the levels of ROS between $CD56^{dim}$ and $CD56^{bright}$ in CML patients, these two subsets were analyzed separately and compared. The levels of ROS in both subsets were equal without any statistically significant difference. This cannot prove that cytotoxicity is more affected than the capacity to IFN-γ production but reveal that both subsets have the same ROS levels and the different sensitivities to cytotoxicity and IFN-γ already demonstrated (Thorén *et al*., 2011) were not from different levels of ROS in NK cells.

ROS production depends on the number of tumor cells

Among the samples collected in this work some of them were from patients in final stage of treatment with negative results to malignant cells. To analyze the levels of ROS in different cells during different phases of treatment, three groups were created, one with negative presence of mRNA for BCR-ABL, a group with high values and a group with intermediate levels. In granulocytes and monocytes, the level of ROS in the samples with higher level of mRNA (patients in the beginning of the treatment) had a statistical significant increase comparing with samples with negative values. However when the same analysis was performed for NK cells any statistical difference was noted. This shows that NK cells in the final steps of treatment are under the same redox state as patients in the beginning of treatment and could be an explanation for relapses. NK cells are considered important when

lower amounts of tumor cells are present, being cells from adaptive system responsible for fighting more complex tumor situations. After the treatment had destroyed the myeloid tumor cells, the incapacity to reduce ROS in NK cells can explain why, in a possible replace, these cells are not able to detect and lyse tumor cells.

Important NCRs have lower expression in CML patients

Associated with lower cytotoxicty is the decreased expression of some important NCRs. With lower expression of activating NK cell receptors, NK cells are unable to detect ligands associated with tumor cells and cannot activate cytotoxic machinery. The lower expression of NK cell receptors was already demonstrated by others (Romero 2005, Peraldi 2009). In this work it was demonstrated that NK cells from CML patients had lower expression of NKp46 and CD16. The NKp46 is one important receptor to NK cells in the response against human tumors (Fauriat *et al*., 2007). The decreased expression of CD16 prevents NK cells to develop an immune response against antibody-coated cells. Beyond NKp46 and CD16, the expression of CD56, CD57 and CD11b NK cell receptors were analyzed. The CD57 is preferentially expressed in more matured cells. The $CD57^{+}$ cells are in terminal phase of differentiation, have lower proliferative capacity and produce low levels of IFN-γ (35). The CD11b can also be a used as a marker of differentiation because more mature NK cells in peripheral blood express CD11b in opposition to more immature forms. For these three receptors there was not any statistical significant difference in NK cells. However, in $CD56^{bright}$ NK cells the expression of CD56 is highly increased. This result is unexpectedly however Romero and colleagues also showed increased expression of NKp46 after increase ROS by granulocytes. Although the receptor is different and such increase was not detected in NKp46 analysis, this could mean that ROS cannot just provoke downregulation of the receptors in NK cells.

Protective role of interleukins in ROS production from monocytes

To elucidate better some aspects of ROS communication, NK cells and monocytes from healthy controls were separated using FACSAria III. After cultured in appropriate medium different conditions were tested. As expected the use of LPS activate NAPDH oxidase and an increase in ROS levels were detected comparing to not stimulate monocytes. However in this case the increase was not significant as it was in the experiments with blood.

This could be to *in vitro* limitation. *In vitro* conditions are not usually in agreement with *in vivo* oxygen concentration. The 20% oxygen level that cultured cells are in contact is enough to change metabolism, gene expression and function of primary cells. Genes associated with detoxification, inflammation cell death and cell repair is more expressed in higher levels of oxygen and the intracellular environment is more oxidative at atmospheric conditions (atkuri 2007). With a higher oxidative stress the difference between a not stimulate over LPS stimulate cultured cells cannot reveal the same differences that probably occur *in vivo.* It should be also considered that the sorting using antibodies is also a source of stress to the cells.

One possible theory to explain why NK cells have so much ROS in the two studied diseases could be the production from phagocytes that across the plasmatic membrane. This theory was already proposed giving the idea that ROS changes directly metabolic pathways inside NK cells. According to Bylund 2010 in dense cell population, extracellular ROS may accumulate and diffuse across membranes to react with dihidrorhodamine-123 intracellularly. (Bylund 2010). In fact, when stimulus were added to monocytes and the medium was changed before contact with NK cells differences were detected in monocytes and not in NK cells In the light of this experiment it was not possible to detect ROS probably because removing the medium can remove H_2O_2 from it. However when type of cells were stimulated together the rdox state in NK cels change. The LPS is a stimulus to Toll-like receptor 4 which is not expressed in NK cells (54 55) therefore cannot stimulate directly NK cells.

Another possible explanation is that ROS production should be from mitochondria. The ROS production from this organelle is normally considered as none regulated. However stimulation of some TLRs can leads to mitochondrial ROS production (West 2011). It was never tested if this mechanism can happen in NK cells, only in macrophages and if ROS production in mithocondria is enough to increase dramatically its levels. But, nevertheless is undeniable the increase of ROS in NK cells although its mechanism is still not completely understood.

When histamine is added together with stimulus there is a dramatic reduction in ROS production. The capacity of histamine to inhibit NADPH oxidase was already demonstrated in other work (Melqvist 2000). But when IL-2 IL-12 and IL-15 are added to stimulate NK cells, the quantity of ROS produced by monocytes, decrease to levels detected without any stimulus. Apparently NK cells after specific stimulation can reduce ROS production by monocytes. It was already demonstrated that NK cells can influence positively ROS production through cytokine release (Bhatnagar *et al.,* 2010) but was never demonstrated that can also have the opposite impact. Also when just 10% of monocytes were cultured with NK cells their level of ROS reach lowest levels almost the same as interleukine stimulation. It is possible that both mechanisms are connected because the source of IL-12 and IL-15 to

NK cells is from phagocytes. The decreased number of monocytes leads to lower levels of ROS and but with the production of interleukins. For example in the work of Asea *et al* 2005, with a density of less than 10% of monocytes in culture with NK cells, the levels of IFN-γ released reached maximum quantities after 72 hours but with higher densities of monocytes IFN-γ is not detected. Although the addiction of catalase to medium not increased IFN-γ when less than 10% of monocytes are present, when higher densities are in contact with NK cells, the previous inhibition detected was suppressed (Asea *et a.,l* 2005).

All this information reveals a very complex signaling mechanism to control or stimulate ROS from monocytes. If ROS can be a way to reduce NK cell cytotoxic action over target cells from the own body, it is not possible that in all cases its production have negative impact. Is necessary to control how NK cells can be released by its inhibitory action not only by histamine release but also with communication between NK cells and phagocytes. New data are starting to reveal a positive effect from phagocytes as the work of Thorén *et al.,* 2007 which demonstrate increase antioxidant defenses in NK cells after synapse formation with DCs. A more complex and probably bi-directional communication can occur between phagocytes and NK cells as it was proved by the presented work where NK cells can influence monocyte ROS production.

CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions and future perspectives

In the last decades many efforts are being made to understand and remove inhibition suffered by antitumor effector cells of the immune system. Among such cells NK cells has gained more and more attention not only due to their importance in anti-tumor response but also due to immunotherapeutic potential. However oxidative stress that is associated with chronic pathologies reveals to be an important immune inhibitor. Although acute inflammation is an important mechanism to healthy maintenance when it passes to a chronic state as in many diseases the health of the organism is compromised. Malignant and infected cells use sometimes immune mechanism to own vantage. Reactive oxygen species, produced by phagocytes in oxidative burst, beyond have properties of kill foreign organisms, can also have a second role acting as immunosuppressive agents.

In tuberculosis, ROS failed, in most of the cases, to be a determinate agent in immune response against *M. tuberculosis.* Due to dual role of ROS and taking together the presented results, NK cells in peripheral blood show higher levels of ROS, a sign of inhibition. NK cells can have an important role not only as main cell in IFN-γ release but also due to cytotoxic action over permanent infected cells. Not only phagocytes but also NK cells show very significant differences in ROS compared with controls. Since the NK cells analyzed were from peripheral blood and not from infected tissue, is expected that the differences are even higher in the. Taking in account all literature showing negative impact of ROS over NK cells in different pathologies, and based in results from this work, ROS can act in tuberculosis as inhibitor over NK cells. The differences found in monocytes and NK cells, are in concordance with expected due to a known chronic inflammation with increased ROS production by macrophages in lungs.

The effect of ROS over NK cells are well studied in tumors both solid and hematologic. Analyzing the literature over this subject, it was never studied the redox state within NK cells despite some theories relating ROS with their capacity to enter in cells and, as second messengers, act negatively over metabolic pathways. In this work, it was showed that NK cells from chronic myeloid leukemia patients have higher levels of ROS as monocytes. Such as in tuberculosis both these cells have increased ROS. However in all studies performed so far the increase of ROS in co-cultured monocytes and NK cells were attributed to monocytes never deposit any attention in redox balance in NK cells. The increase of ROS production by phagocytes is linked with ROS increase in NK cells. It was

also confirmed in this work that NKp46 and CD16 are highly decreased in NK cells from CML patients with consequent lower cytotoxic action.

Based on *in vitro* and *in vivo* studies, histamine is the only compound used with success to reverse the action of ROS produced from phagocytes. However a negative and opposite effect is also pointed to histamine over some type of tumors. IL-2 is a therapeutic interleukin used with disappointing results because is not able to stimulate NK cells inhibited by ROS. However in this work the use of IL-2 combined with Il-12 and IL-15 reveals to be a protector stimulus almost as the use of histamine. NK cells stimulated with all three interleukins showed lower ROS. These results suggest a possible therapeutic use of combined interleukins in pathologies associated ROS.

With this work, we hope in the near future to analyze mRNA of NK cells from CML patients to understand which metabolic effects ROS can have to better understand the implications of detected ROS in NK cells. It is also important to perform more experiments to evaluate the potential role of IL-2, IL-12 and IL-15 and possibly in combination with histamine and to dissect histamine action over phagocytes and tumors cells using the same samples used in this work in combination with cells with silenced NADPH oxidase.

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