Faculdade de Medicina da Universidade de Coimbra

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Role of Natural Killer cells in the progression of *Mycobacterium tuberculosis* infection to pulmonary disease

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INDEX

FIGURE INDEX

TABLE INDEX

ABBREVIATIONS INDEX

ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen-presenting cell
BCR	B cell receptor
BM	Bone marrow
CD	Cluster of differentiation
DC	Dendritic cell
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HPC	Hematopoietic progenitor cells
IFN-γ	Interferon – gamma
IL	Interleukin
iNK	Immature natural killer
KIR	Immunoglobulin-like receptor
LN	Lymph nodes
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MFI	Mean fluorescence intensity
Mtb	*Mycobacterium tuberculosis*
NCR	Natural cytotoxic receptor
NK	Natural killer
NKR	Natural killer receptor
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated pattern

PRR	Pattern recognition receptor
TB	Tuberculosis
TCR	T cell receptor
TGF-β	Transforming growth factor – beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor – alpha
TST	Tuberculin skin test

ABSTRACT

Introduction: Natural killer (NK) cells are key components of the innate immune system and participate in the early response against infected or transformed cells. They are characterized by the expression of a varied repertoire of receptors, named inhibitors and activators, which balance mediates their function. NK cell contribution in infection remains unclear. *Mycobacterium tuberculosis* infection is still a significant health problem and it will probably become even more significant in coming years because of the high prevalence of human immunodeficiency virus (HIV). The main goal is to clarify the role of NK cells in immunopathogenesis and hypothetical contribution as targets for therapy interventions.

Material and methods: Peripheral blood from 38 TB patients and 15 HC was analysed. Complete Blood Cells count as well as enumeration of lymphocyte subsets was made. NK cell surface expression of some important receptors and markers (CD56/CD16, CD27/CD11b, CD57, CD94/NKG2D, NKp30, NKp44, NKp46, NKp80, KIR2DL1, KIR2DL2, KIR3DL1, KIR2DS1) were evaluated and also the intracellular expression of IFN-γ.

Results and Discussion: A moderate lymphopenia was observed in TB patients, with a decrease of all lymphocyte subsets, except for B cells. NK cells from TB patients present a higher level of maturation and IFN-γ production. The most marked increase in surface markers expression was observed for CD57.

Conclusion: The general NK cells phenotyping here presented can help in the understanding of NK cell role in pulmonary tuberculosis infection and progression to disease, giving some highlights for further research.

Introdução: As células Natural Killer (NK) são componentes chave do sistema imune inato e participam numa primeira fase da resposta contra células infectadas ou danificadas. São caracterizadas pela expressão de um variado repertório de receptores, incluindo inibidores e activadores, cujo balanço irá mediar as funções destas células. A contribuição das células NK na infecção não se encontra definida. A infecção pelo *Mycobacterium tuberculosis* continua a ser um grave problema de saúde pública, podendo vir-se a agravar devido ao aumento da prevalência da infecção com o vírus da imunodeficiência humana (HIV). O principal objectivo deste trabalho foi clarificar o papel das células NK na imunopatogénese desta doença e a sua hipotética contribuição como alvos terapêuticos.

Material e Métodos: Neste estudo foram analisadas amostras de sangue periférico de 38 doentes com tuberculose e 15 contactos saudáveis. Foi feito um hemograma seguido da enumeração dos subtipos dos linfócitos presentes. A expressão na superfície das células NK de alguns receptores e marcadores importantes (CD56/CD16, CD27/CD11b, CD57, CD94/NKG2D, NKp30, NKp44, NKp46, NKp80, KIR2DL1, KIR2DL2, KIR3DL1, KIR2DS1) foi avaliada juntamente com a expressão intracelular de IFN-γ.

Resultados e discussão: Foi observada uma linfopenia moderada nos doentes com tuberculose, com um decréscimo em todos os subtipos de linfócitos, à excepção das células B. As células NK dos doentes com tuberculose apresentaram níveis mais elevados de maturação e de produção de IFN-γ. O aumento mais acentuado registou-se na expressão do CD57.

Conclusão: Os dados aqui apresentados relativos à fenotipagem das células NK poderão ajudar na compreensão do papel destas células na infecção pela *Mycobacterium tuberculosis* e progressão para doença, abrindo perfectivas de utilização das células NK como alvos terapêuticos.

INTRODUCTION

Immune System

The immune system is a specialized network of organs, cells and soluble mediators that all together forms a defence mechanism against invading pathogens, infectious agents and transformed cells. The immune system can be divided into two branches: the innate and the adaptive systems. Both systems interact and complement each other to provide the protection of the body.

Innate immune system

The innate immune system offers a first barrier against penetration and is quickly activated after tissue injure or infection and targets pathogens non-specifically. However, it does not have memory, so the immune response will not increase with next exposure to the same pathogen. Physical barriers (skin and mucosal membranes), which prevent the infection by a pathogen, are combined with a set of cellular mechanisms and soluble factors that are able to destroy a pathogen once the infection occurred.

The most important cells in a response to an infection are phagocytic white blood cells like macrophages and neutrophils, competent to ingest and kill microbes by producing toxic chemicals and degradative enzymes, and Natural Killer (NK) cells, which mediate lysis of target cells. During the early phase of the innate immune response, both cell types produce cytokines, which cause a local inflammation and active the adaptive immune system. NK cells are crucial for early defence against infections and tumour surveillance and also represent a connecting cell type between innate and adaptive immune system.

As phagocytic cells, macrophages and neutrophils are important in the elimination of pathogens. Macrophages migrate to the site of infection after neutrophils but are also involved in other functions, such as initiating healing and stimulating the adaptive immune response. Like macrophages, dendritic cells serve as antigen-

presenting cells (APCs) and stimulate the adaptive response. On the other hand, natural killer cells are quickly activated lymphocytes that attack tumours and cells infected with virus.

In order to be alerted, the innate immune system displays a set of receptors, which recognize many related molecular structures called pathogen-associated patterns (PAMPs). PAMPs are molecular motifs consistently found on pathogens and not in the host. They are recognized by toll-like receptors (TLRs) and other pattern recognition receptors (PRRs), such as dectins and nucleotide-binding oligomerization domain containing (NOD). The binding of PAMP to cells of the innate immune system results in killing of the pathogens and secretion of pro-inflammatory cytokines. The innate immune system acts within minutes to hours after an infection and many of the effector and costimulatory molecules generated during this early phase of the immune response play an important role for the slower developing adaptive response.

Adaptive immune system

Although 90% of infections are eliminated by mechanisms of the innate immune system, some pathogens escape the defences and the adaptive immune system has to be activated. Soluble factors that belong to the complement system and chemokines and cytokines secreted by innate immune system induce recruitment of lymphocytes and the activation of the adaptive immune system. Adaptive immunity is mainly exerted by two types of lymphocytes, namely T cells and B cells. In contrast to innate immune cells, T and B lymphocytes express antigen specific receptors (TCR and BCR, respectively) which undergo genetic recombination in somatic cells. This process provides with a highly diverse repertoire of receptors able to recognize plenty different pathogen-derived antigens. Adaptive immunity not only contributes to pathogen clearance but is also essential for the formation of an immunological memory allowing fast acting responses in case of reinfection. The protection of the extracellular fluids and spaces is mediated by humoral immune response, in which antibodies produced

by B cells bind to extracellular pathogens and toxins. Activation of naive B cells is triggered by binding of the BCR to its specific antigen and usually requires the help of T cells. Following antigen binding to the BCR, B cells become activated and differentiate into antibody secreting plasma cells. The secreted antibodies bind specifically to the antigen on the pathogen surface, subsequently leading to complement activation and phagocytosis of the pathogen. Also NK cells recognize target cells coated with antibodies, leading to lysis of the target cell by so-called antibody dependent cellular cytotoxicity (ADCC).

Different from B cells, T cells are not able to recognize the pathogens directly, needing the help of a professional APC which have been activated by pathogen-derived PAMP degrade the pathogen and present the antigens on major histocompatibility complex (MHC) molecules expressed on their surface leading to T cell activation and clonal expansion. Among T cells, two populations can be distinguished: $CD4^+$ T helper (T_H) and $CD8^+$ cytotoxic T cells. $CD8^+$ T cell activation and release of cytotoxic molecules leads to killing of the infected target cells and $CD4^+$ T cell do not only stimulate innate cells but also play a crucial role in activating other adaptive cells, contributing to the elimination of the pathogen.

Summing up, innate and adaptive immune responses intensively cooperate with each other contributing to pathogen elimination. While the innate immune system has a crucial importance during the early phase of a primary infection but does not provide immunological memory, the adaptive system takes longer to be activated but have immunological memory, which allows an even quicker response in case of re-exposure to the same pathogen. (1, 2)

Natural Killer Cells

Natural killer (NK) cells represent the third largest lymphoid cell population in mammals, are key components of the innate immune system and participate in the early response against infected or transformed cells (3). They constitute a first line of defence and can kill infected and tumor cells. These cells are large, granular, bone-marrow – as well as lymph node-derived lymphocytes. However, NK cells are distinct from T cells or B cells and have distinct morphologic, phenotypic and functional properties. NK cells do not require sensitization for the exertion of their activity differently from T cells or B cells (4).

NK cells are present in blood as circulating cells and to other organs of the body as resident cells. In peripheral blood, they are characteristically described as having the morphology of large granular lymphocytes (5), whereas in tissues, the microenvironment of the organ has influence on phenotype and activity of NK cells. They are characterized by the expression, on their cell surface, of a varied repertoire of receptors, named inhibitors and activators, which balance mediates their function (6). The balance of inhibitory and stimulatory signals received by a NK cell determines the outcome of interactions with target cells. Normal target cells are protected from killing by NK cells when inhibitory signals delivered by self MHC class I molecules compensate the signals delivered by stimulatory ligands (7).

Development and maturation

NK cells are believed to be relatively short-lived lymphocytes. They derived from $CD34^+$ hematopoietic progenitor cells (HPC) and some observations indicate that the bone marrow (BM) and lymph nodes (LN) are important for their development/maturation (8, 9).

Primarily, NK cell development occurs in the BM, and despite the critical factors necessary for development are still unknown, some analyses demonstrated the

presence of an enriched microenvironment with $CD34^+$ HPC, including a fraction of NK cell precursors (pre-NK) in BM(10). IL-2 has been used to study NK cell development from $CD34^+$ HPC *in vitro* (10-12), however, this cytokine is not found within the BM stroma (11, 13, 14), so other factors that bind to the IL-2R are critical for NK cell development. IL-15 is produced by human BM stromal cells and its function have demonstrated to facilitated the differentiation of cytolytic NK cells from $CD34^+$ HPC (15) because this cytokine shares common signalling receptor subunits with IL-2, which form an intermediate-affinity heterodimeric receptor complex, IL-2/IL-15Rβγ (16). It has been demonstrated that only the $CD34^+$ $CD45RA^+$ phenotype is all-inclusive for human IL-2/IL-15-responsive pre-NK cells (17, 18). Other BM stromal cell factors such as c-kit ligand (KL) and flt3 ligand (FL), the ligands for members of the class III receptor tyrosine kinase family (includes c-kit and flt3), have been shown to potentiate significantly the expansion of NK cells from $CD34^+$ HPC in combination with IL-15, however alone these molecules have no effect on cell differentiation into NK cell (19, 20). In development, NK cell it may be divided into an early phase in which FL acts synergistically with IL-15 to generate an exclusive $CD34^+CD122^+CD38^+$ NK cell intermediate subset from $CD34^+$ HPC, and where IL-15 is necessary to increase the mature NK cells characteristics such as CD56 and NKR expression, cytotoxic activity and the ability to produce abundant cytokines and chemokines (11). CD56 is a 140-kDa isoform of neural cell adhesion molecule (NCAM).

LN are also naturally and selectively enriched with $CD34^{dim}CD45RA^+$ HPC and are able to differentiate these cells into $CD56^{bright}$ NK cells in the presence of either IL-2 or IL-15 (17). Fehniger *et al.* (21) showed that endogenous T cell-derived IL-2 may trigger, through the NK high-affinity IL-2 receptor, $CD56^{bright}$ NK cells to produce IFN-γ. This selective enrichment of both $CD34^{dim}CD45RA^+$ HPC and $CD56^{bright}$ NK cells within LN compared with the BM or blood is suggesting of LN as a site for NK cell development.

There are two distinct blood subsets of human NK cells identified by cell surface density of CD56 (22). NK cells in human peripheral blood are majority $CD56^{dim}$, express high levels of CD16 and killer cell immunoglobulin-like receptor (KIR) and a minority are $CD56^{bright}$ $CD16^{dim/neg}$, having low cytotoxic activity and secrete more cytokines in response to stimulation (22). Furthermore, Romagnani *et al.* (23) demonstrated that $CD56^{dim}$ NK cells from peripheral blood exhibit shorter telomeres than peripheral and LN-derived $CD56^{bright}$ NK cells.

The NK cell development stages in human are not yet well established but based on the presence of different expression kinetics of surface markers could define distinct stages of their development. Freud *et al.* (24) based on the principle that: more than 99% of NK cells within LN express at least CD34, CD117, and/or CD94; CD34 and CD94 are independent antigens, indicating that NK cells intermediate stages would first lose CD34 and then express CD94; and NK cell functional maturity (cytotoxic and IFN-γ secretion) as well as acquisition of surface CD56 in humans are acquired at a later stage of development (25, 26), have proposed a marker panel set using the combination of CD34, CD117, CD94 and CD16 to differentiate the functionally distinct stages of human NK cell development (figure 1).

The first stages of NK cells differentiation are dependent of concomitant IL-15 responsiveness and not all $CD34^{+}$ $CD117^{+}$ $CD94^{-}$ cells are compromised with NK cell lineage. Immature NK cells (iNK cells), are different from the first developmental stage of NK cell because these cells are completely incapable to generate T cells and DC, being this way committed with NK cell lineage (8, 24). iNK cells express antigens including CD2, CD7, CD56, CD161 and NKp44, besides lack of CD10, integrin β7 and HLA-DR, characteristics that further will distinguish the iNK cells phenotype from pre-NK cells. In this third developmental stage, iNK cells are exclusively in NK cell branch however they are not capable to produce IFN-γ or mediate perforin-dependent cellular cytotoxicity against MHC-I negative target cells (24). $CD56^{+}$ cells can express different

levels of CD117 and are subdivided in $CD56^{+}CD117^{high}$ and $CD56^{+}$ $CD117^{low/-}$ subsets (27). These populations are considered different since the latter expresses NKp30, NKp46, NKG2D, NKG2A and CD94, while $CD56^{+}CD117^{high}$ cell do not. It were been shown that $CD56^{+}CD117^{high}CD94^{-}$ cells are not cytotoxic and $CD56^{+}CD117^{low/-}CD94^{+}$ effectively kill target cells and express high levels of FasL and IFN-γ, representing differentiation at stage 4. KIR^{+} NK cells are primarily within the $CD56^{dim}CD94^{+/-}CD16^{+}$ fraction of cells in both LN and peripheral blood, whereas the $CD56^{bright}$ $CD94^{+}CD16^{+/-}$ do not express KIR, which is consistent with evidence indicating that KIR acquisition is rather a late event during NK cell maturation (28-30).

Stage 1	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**
(Pro-NK)	(Pre-NK)	(iNK)	($CD56^{bright}$)	($CD56^{dim}$)
$CD34^{+}$	$CD34^{+}$	$CD34^{-}$	$CD34^{-}$	CD34-
$CD117^{-}$	$CD117^{+}$	$CD117^{+}$	$CD117^{+/-}$	$CD117^{-}$
$CD94^{-}$	$CD94^{-}$	$CD94^{-}$	$CD94^{+}$	$CD94^{+/-}$
$CD16^{-}$	$CD16^{-}$	$CD16^{-}$	$CD16^{-}$	$CD16^{+}$

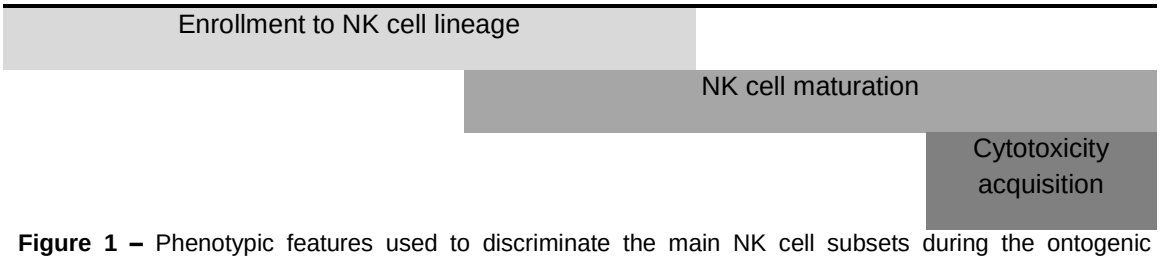

Figure 1 – Phenotypic features used to discriminate the main NK cell subsets during the ontogenic process. This figure brings the more important markers acquired during the five ontogenic stages (31)

The dominant NK cell subset in LN are $CD56^{bright}$ (75% median value), however, in peripheral blood and spleen, the majority of NK cells are a more mature subset: $CD56^{dim}$ (95% and 85%, respectively) (32). Moreover, the $CD56^{dim}$ subsets in the spleen and in the peripheral blood express CD16; $CD56^{bright}$ NK cells in LN are negative for CD16 and express low levels of activation markers (HLA-DR and CD69) (32). These observations suggest that the $CD56^{bright}$ cells are more immature and will give rise to $CD56^{dim}CD16^{+}$ NK cells (the last stage of NK cell life) inside the LN and

then go to peripheral blood. It is important to mention that despite these stages representing a possible developmental NK cell pathway, it is still possible that some cells from each stage may still be terminally differentiated with essential functions for body homeostasis.

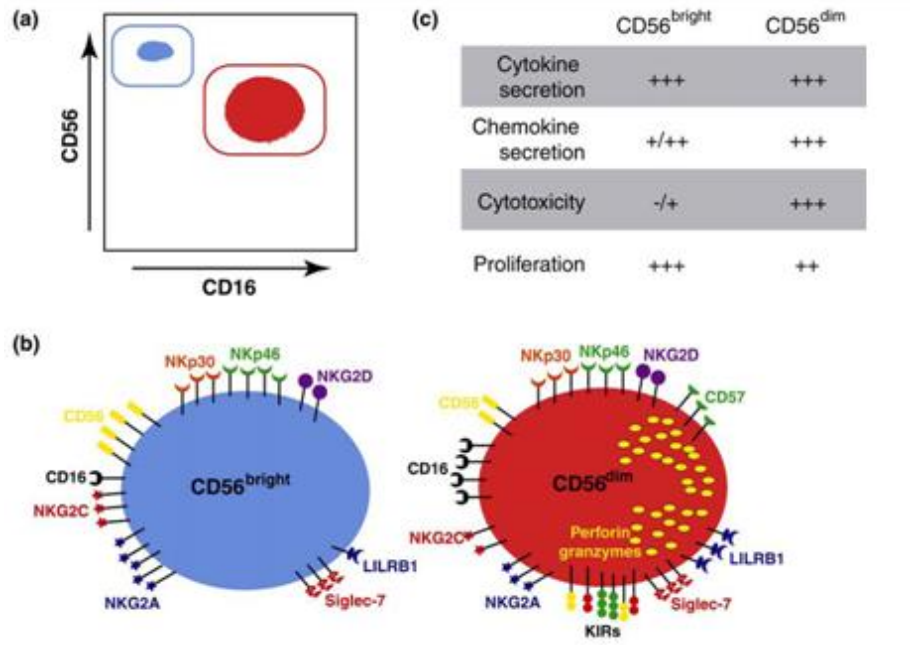

Figure 2 – Phenotypical and functional properties of $CD56^{bright}$ and $CD56^{dim}$ NK cells. (a) Schematic illustration of CD56 and CD16 expression on $CD3^{-}CD4^{-}CD14^{-}CD19^{-}$ lymphocytes with gates on $CD56^{bright}$ (blue) and $CD56^{dim}$ (red) NK cells. (b) Relative expression levels of activation and inhibitory receptors on $CD56^{bright}$ (blue) and $CD56^{dim}$ (red), and NK cells from peripheral blood. (c) Functions of $CD56^{bright}$ and $CD56^{dim}$ NK cells from peripheral blood (adapted from Björkström *et al.* Trends Immunol 2010 (33))

Fu *et al.* (34) recently found that according to the differential CD27/CD11b expression NK cells could be characterized in terms of maturation state. This work refer that during NK cell maturation, they acquire CD27 having a highest cytokine expression, suggesting that NK cells had the ability to produce cytokines, low expression of CD16, suggesting a diminished cytotoxic capacity. After acquisition of CD27, NK cells acquire CD11b and lost CD27. These NK cells had the highest CD16 expression, suggesting that they have the strongest cytotoxic capacity. Summary, NK cells during development/maturation pass for four stages according to CD27/CD11b expression: $CD27^{-}CD11b^{-}$, $CD27^{+}CD11b^{-}$, $CD27^{+}CD11b^{+}$ and finally $CD27^{-}CD11b^{+}$.

Recently, CD57 was described as a marker for NK cell terminal differentiation as happens in CD8+ T cells (35). Authors refer $CD57^+$ NK cells as subset of highly mature cells, having lower frequency of IFN-γ^+ cells comparatively to $CD57^-$ cells, lower proliferative capacity and with a mature phenotype.

Function properties

NK cell functions can be classified in three categories: cytotoxicity, cytokine and chemokine secretion and contact-dependent cell costimulation.

Cytotoxicity

NK cells can kill certain infected cells and tumor target cells apart of their MHC expression (36). NK cells have a relatively large number of cytolytic granules (secretory lysosomes) containing perforin and various granzymes. After the contact between an NK cell and its target, these granules travel to the contact zone with the susceptible target cell (immunological synapse), and the contents are released to effect lysis. Perforin-dependent cytotoxicity is the major mechanism of NK cell lysis, but NK cells have also other ways of killing, namely in a perforin-independent manner utilizing FAS ligand, TNF or TNF-related apoptosis-inducing ligand (TRAIL), although with less efficiency and in a slower time kinetic.

Cytokine and chemokine secretion

NK cells are best noted for their ability to produce IFN-γ but also produce a number of other cytokines and chemokines which contribute to the resistance against infectious agents (37-39). Killing and cytokine secretion are mediated by two different subsets of human NK cells characterized by the intensity of expression of CD56 and CD16 on their surface.

Contact-dependent cell costimulation

NK cells express several costimulatory ligands including CD40L (CD154) and OX40L, allowing them to provide a costimulatory signal to T cells or B cells (40, 41). NK cells may serve as a bridge in an interactive loop between innate and adaptive immunity. Dendritic cells (DC) stimulate NK cells, which then deliver a costimulatory signal to T or B cells allowing for an optimal immune response. NK cells also stimulate cells from the innate immune systems after being stimulated by them.

Cytokines and chemokines

The cytokines play a key role in NK cell activation. They are immunomodulating molecules, once secreted by infected cells, they signal NK cells for the presence of pathogens. NK cells produce cytokines after being stimulated, in part by the monocyte-derived cytokines during the early pro-inflammatory response to infection and also by the a subset of NK cells present at the site of inflammation (42).

NK cells produce a range of cytokines, including haematopoietic factors such as IL-3 and granulocyte–macrophage colony-stimulating factor (GM-CSF), TNF-α and regulatory cytokines such as transforming growth factor (TGF-β) and IFN-γ (42). In both viral and bacterial models of infection, IFN-γ production by NK cells has been shown to be a crucial event in successful resolution of infection (43). These molecules secreted by NK cells will stimulate phagocytosis of bacteria by macrophages and facilitate their elimination via a number of mechanisms including the generation of reactive oxygen and nitrogen species important in immune response (44). In a very early phase of infection, IL-12 is produced and will be responsible for driving NK cells to produce IFN-γ (45).

The $CD56^{bright}$ NK cell subset produces significantly more IFN-γ following IL-18 and IL-12 stimulation compared with $CD56^{dim}$ NK cells (45).

Natural Cytotoxicity Receptors (NCRs) and other activating receptors

Natural cytotoxicity receptors are an important group of activating receptors consisting of NKp30 (CD337), NKp44 (CD336) and NKp46 (CD335) expressed exclusively on NK cell surface (46). NCRs play a major role in the NK-mediated killing of most tumor cells (47). NKp46 and NKp30 are constitutively expressed on resting or activated NK cells, enabling a precise identification of all NK cells (which is not true for other widely used NK cell markers including CD56 and CD16) and NKp44 is selectively expressed only by IL-2 activated NK cells (47, 48). The ligands for the NCRs are not well characterized but seem to exist on tumor cells and virally infected cells.

NK cells also express other triggering receptors that contribute to cell activation and target cell killing, including NKG2D, NKp80, NTB-A (CD352), 2B4 (CD244), DNAM-1 (CD226), and NKG2C. Among these molecules, NKG2D forms homodimers, is not structurally related to the other NKG2 receptors and represents a major triggering receptor that is known to specifically recognize the stress-inducible MHC class I-related chain molecules and plays a role in NK-mediated cytolysis (47, 49). On the contrary, NKp80, NTB-A, 2B4, and NKG2C appear to synergize with NCRs and NKG2D in the NK-mediated cytolysis, working as co-receptors (47).

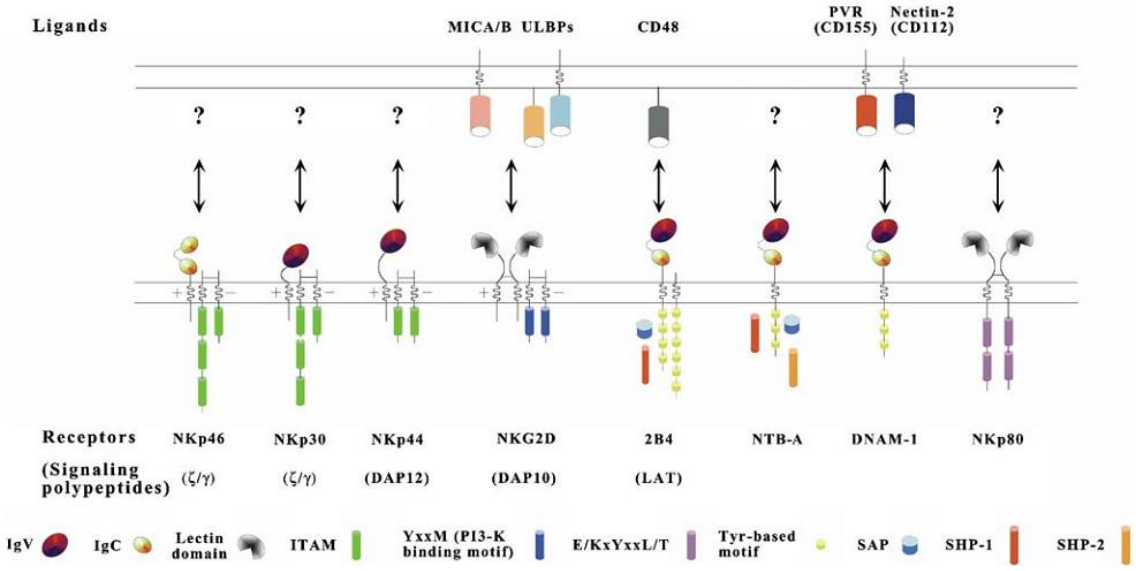

Figure 3 – Activating NK receptors and coreceptors and their cellular ligands. This figure illustrates the molecular structure of the NK receptors NKp46, NKp30, NKp44 and NKG2D as well as of the NK coreceptors 2B4, NTB-A, DNAM-1 and NKp80. Their interaction with signaling polypeptides or with relevant cytoplasmic molecules is also shown. The known cellular ligands are illustrated in a simplified form. (Moretta *et al.* EMBO J 2004 (46))

Cross-talk with other immune cells

NK cells have a regulatory action that influence various other immune cell types, such as macrophages, DCs, T cells and B cells. NK cells can interact with DCs in peripheral tissues, as well as in secondary lymphoid organs, and can have two distinct ways of action (50-52). First, NK cells are capable to kill immature DC, influencing DC homeostasis, but they can also limit DC-based vaccination efficiency (53, 54). However, the lysis of target cells by NK cells can cause cross-presentation of antigens from apoptotic NK cell targets by some DCs' subsets. This mediated cytotoxicity exerted by NK cell on target cells induces robust antigen-specific adaptive immune responses that involve $CD8^+$ T cells, $CD4^+$ T cells and immunoglobulin G (55). Second, by the secretion of IFN-γ and tumor necrosis factor (TNF), NK cells can help the development of DCs, which will also activate NK cells by secretion of IL-12 (50-52). This interaction between NK cells and DCs might thus lead to anti-inflammatory applications.

In addition to the influence exercised in DC function, NK cells also influence adaptive immune responses by direct action on T and B cells. The IFN-γ secretion by NK cells can help the priming of $CD4^+$ T helper type 1 (TH1) (56, 57). NK cells have also the capacity of killing activated T cells, except T cells express sufficient amounts of classical or non-classical MHC I molecules (58). Blockade of CD94-NKG2A inhibitory receptors results in NK cell lysis of activated $CD4^+$ T cells. This knowledge can be useful in $CD4^+$ T cell-dependent autoimmunity therapy by the use of blocking antibodies to NKG2A to prevent it (58).

NK cells not only protect the host against pathological agents, but also control the immune response exerted by other immune cells.

Killer-cell Immunoglobulin-like Receptors (KIRs)

Natural Killer cells and some subsets of T cells express in its surface receptors belonging to the immunoglobulin-like receptors (KIRs). KIR nomenclature is based on its structure: they can have two (KIR2D) or three (KIR3D) extracellular immunoglobulin-like domains (59, 60). The cytoplasmic tail varies in length, a property connected to functional activity: inhibitory KIRs have long (L) cytoplasmic tails with ITIM motifs and activating KIRs have short (S) cytoplasmic tails which can associate with the ITAM-containing DAP12 adaptor protein.

Currently, about 14 KIR genes and 2 pseudogenes have been described in the KIR gene cluster on chromosome 19 in humans. The number of genes varies greatly between individuals and there are only three commonly shared framework genes present in all individuals (KIR2DL4, KIR3DL2 and KIR3DL3). KIR genes are highly homologous so it is likely that the variability is due to gene duplications or non-allelic homologous recombinations during evolution (61).

Based on the gene content two types of haplotypes have been defined, where B haplotypes have more activating KIRs compared to A haplotypes. KIR haplotype B

have one or more of the KIR genes; 2DS1, 2DS2, 2DS3, 2DS5, 3DS1 and 2DL5 and haplotype A lack all of these and can as an alternative possess inhibitory KIRs including 2DL1, 2DL3 and 3DL1 as well as the activating 2DS4 (62). An extensive allelic variation in several genes confers an even higher diversity to KIR genes that will influence the amount of KIR expressed on each NK cell (63-65). In addition, some allelic variants do not produce functional proteins expressed at the cell surface (64). This highly diverse expression is believed to be important for giving a broad NK cell response against different pathogens.

The KIR proteins are also expressed in a diverse way on NK cells (59, 60, 66). However, once an NK cell clone has started to express a certain KIR gene during development, the expression is established and does not appear to be affected by cytokines (64, 67). In recent studies, it was described that KIR gene transcription is controlled by epigenetic mechanisms such as methylation, and by the presence of a bidirectional promoter able to stochastically switch direction of transcription during maturation of the NK cell, determining if the NK cell will express the KIR gene or not (68, 69). Resulting in a diverse KIR expression repertoire in NK cells that can recognize almost every MHC class I molecule (66).

Natural Killer cells in infection

NK cells have long been demonstrated to be activated in vitro by virus-infected cells (70). Other types of intracellular pathogens have also been shown to activate NK cells for IFN-γ production or increase cytotoxicity (70, 71). Evidence for an implication of NK cells in the control of extracellular pathogens is not defined (72).

NK cells are activated by a variety of intracellular pathogens, including many viruses and also bacteria or protozoa having potential to contribute to the immune defence against a variety of infections. However, in certain infections were a high of NK

cell activation is observed, there is no evidence that NK cells play a direct role in the control of the pathogen (73). Thus, the modulation of NK cell functions by an infection is not enough to indicate that NK cells contribute directly to the clearance of the pathogen.

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* (74, 75). Granulomas help protect the host from bacterial dissemination by isolating infectious foci.

NK cell activation resulting of infections by intracellular bacteria, such as *Listeria monocytogenes* (76), or protozoa, such as *Leishmania* (77) or *Plasmodium* (78), involves the production of IL-12 and IL-18 by innate immune cells such as DCs, monocyte or macrophages and also the direct interactions between these cells and NK cells. Activation receptors on primary NK cells can add to these cells the capacity of IFN-γ production and also to the cytotoxic activity (38, 79). Finally, NK cells can respond to a variety of chemokines being essential for their recruitment to the site of inflammation quickly after infection, as demonstrated in the model of murine cytomegalovirus infection in a seminal report from Salazar-Mather and colleagues (80), more recently in *Toxoplasma gondii* infection (81) and reviewed elsewhere (82).

Tuberculosis

The World Health Organization (WHO) defines Tuberculosis as:

"*… an infectious bacterial disease caused by Mycobacterium tuberculosis, which most commonly affects the lungs. It is transmitted from person to person via droplets from the throat and lungs of people with the active respiratory disease. In healthy people, infection with Mycobacterium tuberculosis often causes no symptoms,*

since the person's immune system acts to "wall off" the bacteria. The symptoms of active TB of the lung are coughing, sometimes with sputum or blood, chest pains, weakness, weight loss, fever and night sweats. Tuberculosis is treatable with a six-month course of antibiotics."

Mycobacterium tuberculosis (Mtb) infection remains a major international health problem that is probable to become even more significant in coming years because of the high prevalence of human immunodeficiency virus (HIV). Although it is estimated that one-third of the world population is currently infected by *M. tuberculosis*, the majority never develop the active disease (83), indicating the ability of human immune responses to control the infection. On the other hand, approximately 10% of these individuals develop active pulmonary disease. Immune mechanisms involved in this differential response by each individual are not clearly explained. Genetic mechanisms involved in immune response can also be one of the reasons.

Mtb is an obligatory aerobic, intracellular pathogen, which preferentially infects lung tissue rich in oxygen, but can also spread to other parts of the body. The tubercle bacilli enter the body via the respiratory and are phagocytosed by alveolar macrophages as first event in the host-pathogen relationship that decides the outcome of infection. Then, an influx of lymphocytes is observed and activated macrophages migrate to the site of infection, granuloma is formed. The exponential growth of the bacilli is verified and dead macrophages form a *caseum* containing the *bacilli*. The bacilli can remain forever within the granuloma, get re-activated later or may get released into the airways after enormous increase in number, necrosis of bronchi and cavitation.

"*Fibrosis represents the last-ditch defence mechanism of the host, where it occurs surrounding a central area of necrosis to wall off the infection when all other mechanisms failed*" (84).

There are some other mechanisms that can be described in immune response against TB: the binding of Mtb to monocytes/macrophages by complement, mannose and other surface receptors; the fusion of phagolysossome that will allow the Mtb degradation; the recruitment of accessory immune cells for the local of inflammatory response; the role of reactive oxygen and nitrogen intermediates in the signalling of the infection; the IFN-γ and TNF-α mediated anti-mycobacterial effects; the NK cells action upon pathogens or infected monocytes; the antigen presentation by APCs to T lymphocytes for the development of adaptive response; and finally, the role of B cells or antibody in response to Mtb infection (84).

Natural killer cells in Tuberculosis

As integrant part of innate immune system, NK cells has been implicated in early immune response to a variety of pathogens because they are capable of rapidly producing IFN-γ and other immunoregulatory cytokines, as well as lysing specific target infected cells always in the absence of prior activation. Some researches have demonstrated that NK cells from the peripheral blood contribute for protective immunity though IFN-γ of cytotoxic mechanisms, having a huge bactericidal role against Mtb (85).

The role of NK cell receptors in cytotoxic-mediated killing of mononuclear phagocytes infected with an intracellular bacterium has already been reported (85) in the same work were they propose that the diminished NK activity during tuberculosis infection is probably the 'effect' and not the 'cause' for the disease. Human NK cells are known to directly lyse *M. tuberculosis*-infected monocytes and macrophages *in vitro* (86, 87). In this work, it was found that NKp46 and NKG2D receptors contribute to NK cell-mediated lysis of cells infected with Mtb and that reduced functional capacity of NK cells is associated with severe manifestations in disease. Denis' data (88) suggest an

important involvement of NK cells in host resistance to TB because of their elevated lytic activity against Mtb-infected monocytes. Another study demonstrate the direct binding of NKp44 to the mycobacterial surface (89), suggesting that ligands for other NK cell receptors may play a role in the specific NK-mediated recognition of Mtb.

But the NK cells activity in Mtb-infection is not just lyse Mtb-infected cells, these cells also actively restrict the infectious agent growth in an apoptosis-dependent but Fas/FasL independent manner (90, 91) and this action can be further higher by addition of IL-2, IL-12 and glutathione (90). Consistent with the protective role of NK cells in tuberculosis (TB), reduced activity of NK cells has been found in active pulmonary TB patients (85). Higher levels of pre-NK cells were observed in positive tuberculin skin test (TST+) and in TB patients, and in addiction TST+ individuals presented levels greatly increased of these cells in comparison to TB (92). Barcelos *et al.* (92) also observed a selective increase in putative activated NK cells of TST+ individuals and demonstrated for TST+ and TB patients a distinct correlation profile between NK cells and macrophage-like monocytes, suggesting that high levels of activated NK cells together with macrophage-like monocytes may be involved in protective mechanisms in putative TB-resistant individuals. This finding could be important to explain the immunopathogenic context, since these cells contribute for protective mechanisms because NK cells have a great ability to proliferate and their potential to differentiate into $CD3^{-}CD16^{+}CD56^{+}$ cells with higher cytotoxic activity.

Infection with the intracellular pathogen Mtb also results in local lung NK cell accumulation and activation, however, their importance in clearing the infection is not clear yet (93, 94). Junqueira-Kipnis *et al.* (94) explained that NK cells become activated during the early response to Mtb infection, but their removal does not substantially affect the expression of host resistance. Contrary, Roy *et al.* (95) identified a potential new role for NK cells in maintaining the balance between the regulatory and effector arms of the immune response to Mtb infection with the Treg cells lysis. Other findings

also suggest NK cells role upon other immune cells in response to Mtb infection, promoting expansion of γδ T cells forming immune synapse and by soluble factors TNF-α, GM-CSF, and IL-12, but not IFN-γ concluding the NK cells action might be beneficial to prevention and control this infection (96).

Aim

In the present research on TB patients and healthy contacts it was intended to analyze NK cell subsets, surface receptors and intracellular production of cytokines (IFN-γ), accounting for the effect in *Mycobacterium tuberculosis* infection and to the progression to pulmonary disease. Establishment of regulatory, cytotoxic and cell-dependent contact status of NK cells in TB is aimed for an extended characterization of NK cells in TB. The main objective is to clarify the role of NK cells in immunopathogenesis and hypothetical contribution as targets for therapy interventions.

MATERIAL AND METHODS

Study population

The samples used in this study are from two different groups: healthy contacts (HC) and pulmonary tuberculosis patients (TB) from *Centro de Diagnóstico Pneumológico (CDP) de Vila Nova de Gaia.* Were studied 38 TB patients 68% male, age 45±16 and 15 HC 73% male, age 38±14. Sample number in each experience is referred in results (figure or table). Patients with concomitant conditions including autoimmune disease, HIV infection, cancer, extrapulmonary TB and other systemic disease were excluded from the present analysis. Samples from TB patients were collected within the first two weeks of anti-mycobacterial therapy.

It was obtained informed consent from participants and approval from the Ethics Committee of the Faculty of Medicine of the University of Coimbra and of the Faculty of Medicine of the University of Porto.

Blood sampling

Peripheral blood samples were collected using one K_3EDTA tube (3mL), one Lithium heparin tube (4mL) and one tube for serum separation (5mL). Serum and plasma aliquots were frozen at -80ºC. PBMCs from K_3EDTA and samples from Lithium heparin tubes were used to flow cytometry and cell culture, respectively.

Complete Blood Cell (CBC) count

CBC were released in COULTER A^C •T diff Analyzer (Beckman Coulter) using 12µL of whole blood from K_3EDTA tube. 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.

Enumeration of Lymphocyte subsets

In order to enumerate lymphocytes and subpopulations, leukocytes from 100µL of each peripheral blood sample were labeled with surface monoclonal antibodies (mAbs) anti-human: anti-CD4 FITC (clone: OKT4), anti-CD56 PE (clone: HCD56), anti-CD3 PerCP-Cy5.5 (clone: OKT3), anti-CD8 PE-Cy7 (clone: HIT8a), anti-CD19 APC (clone: HIB19), anti-CD16 APC-Cy7 (clone: 3G8) and anti-CD11b Pacific Blue (clone: ICRF44). All mAbs were purchased from Biolegend (San José, CA, USA). PBMCs were incubated with 30µL of mAbs mix (diluted 1,5:100 in 1x PBS), after red blood cells were lysed with 1x RBC lysis buffer (NH_4Cl 0.15M, $KHCO_3$ 10mM, EDTA 0.1mM), during 30min in the dark at RT. Were added 100µL 4% Formalin and incubated 10min at the previous conditions. Cells were washed with cold 1x PBS, for 10min at 300g. Supernatant were discarded and cells resuspended in 200µL 1x PBS. Samples were analysed in FACSCanto II Flow Cytometer (BD Biosciences, Erembodegem, Belgium).

NK and KIR phenotyping

To phenotype NK and KIR were used flow cytometry. Whole blood from K_3EDTA tube were lysed with 1x RBC for 10min at 4ºC and then washed with cold 1x PBS. Cells were labeled with extracellular mAbs according to Table 1 scheme during 30min in the dark at RT. Samples without intracellular labeling were fixed with 100µL 4% Formalin and incubated 10min at the previous conditions and washed with cold 1x PBS for 10min at 300g. Samples with intracellular labeling were fix with 100µL Fix & Perm® Medium A (Invitrogen, Carlsbad, CA, USA) for 10min in the dark at RT, washed with cold 1x PBS 10min at 300g, were added 100µL Fix & Perm ® Medium B and the intracellular mAbs, incubated 30min at RT in the dark and washed for 10min at 300g. Supernatant from all samples were discarded and cells resuspended in 200µL 1x PBS. Sample data was acquired in FACSCanto II Flow Cytometer.

Table 1 – Labeling plan for NK and KIR phenotyping. mAb (clone).

Fluorochrome	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7	Pacific Blue
mAbs anti-human	CD27 (O323)	CD57 (HCD57)	CD3 (UCHT1)	**IFN-γ (4S.B3)**	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)
	1F12	CD158a/h (HP-MA4)	CD3 (UCHT1)	-	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)
	8C11	CD158a/h (HP-MA4)	CD3 (UCHT1)	-	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)
	1F12	CD158b (DX27)	CD3 (UCHT1)	-	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)
	8C11	CD158b (DX27)	CD3 (UCHT1)	-	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)
	CD27 (O323)	CD158a/h (HP-MA4)	CD3 (UCHT1)	CD56 (HCD56)	CD335 (9E2)	CD16 (3G8)	CD11b (ICRF44)
	CD27 (O323)	CD158e1 (DX9)	CD3 (UCHT1)	CD56 (HCD56)	CD335 (9E2)	CD16 (3G8)	CD11b (ICRF44)
	CD94 (DX22)	CD56 (HCD56)	CD3 (UCHT1)	**IFN-γ (4S.B3)**	CD314 (1D11)	CD16 (3G8)	CD11b (ICRF44)
	CD27 (O323)	CD336 (P44-8)	CD3 (UCHT1)	**IFN-γ (4S.B3)**	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)
	CD27 (O323)	CD337 (P30-15)	CD3 (UCHT1)	**IFN-γ (4S.B3)**	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)
	CD27 (O323)	NKp80 (5D12)	CD3 (UCHT1)	**IFN-γ (4S.B3)**	CD56 (CMSSB)	CD16 (3G8)	CD11b (ICRF44)

Note: intracellular staining referred in bold.

Flow Cytometry acquired data were analyzed using FlowJo 7.6 software (Tree Star Inc, Ashland, USA).

Statistical analysis

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

RESULTS AND DISCUSSION

Complete Blood Cell (CBC) count

A CBC is a record of the findings that give the numbers, proportions and morphological features of cell components present in peripheral blood. It was performed a CBC in TB patients (TB) and in healthy controls (HC) to evaluate the possibility of significant changes not only in numbers of lymphocytes, but in all the blood components since there are interactions between them that can be important in infection and disease. The values of both groups are represented in Table 2.

Table 2 – Absolute and relative frequencies of white blood cells (WBC) from HC and TB.

Parameters	Units	HC	TB	P
Leucocytes	x10^3/µL	5.88 ±1.03	6.30 ±1.72	0.8106
Lymphocytes	%	31.07 ±5.84	25.40 ±7.73*	0.0423
Monocytes	%	4.95 ±0.83	4.53 ±1.40	0.1735
Granulocytes	%	63.98 ±5.23	69.24 ±8.90	0.0512
Lymphocytes	x10^3/µL	1.82 ±0.43	1.57 ±0.52	0.1845
Monocytes	x10^3/µL	0.28 ±0.06	0.30 ±0.14	0.8178
Granulocytes	x10^3/µL	3.76 ±0.78	4.41 ±1.47	0.2964

Values represent mean ±SE (HC n=10; TB n=26). *p<0.05 compared to HC (Mann Whitney test).

All the parameters analysed in the CBC were within the reference values for Portuguese populations (97). Comparing the two groups, there is only statistically significant difference in the percentage of lymphocytes (p<0,05). This decrease of lymphocytes is supported by several studies (98-100), other study found this decrease

in 46% of the untreated TB patients but a lymphocytosis in 6% of TB patients (101) and two other reports, relate the lymphopenia with the severity of the disease (102, 103).

Enumeration of lymphocyte subsets

Lymphocyte subsets reference values are used to monitor infectious diseases including tuberculosis. Using Flow Cytometry, these subsets were evaluated by the differential surface expression of some markers: $CD3^+$ (T cells); $CD3^+CD4^+$ (helper T cells); $CD3^+CD8^+$ (cytotoxic T cells); $CD3^-CD19^+$ (B cells) and $CD3^-CD56^+$ (NK cells). With Flow Cytometry data (percentage of cells) and the absolute values for lymphocytes of the CBC it was possible to calculate the absolute numbers of each lymphocyte subset.

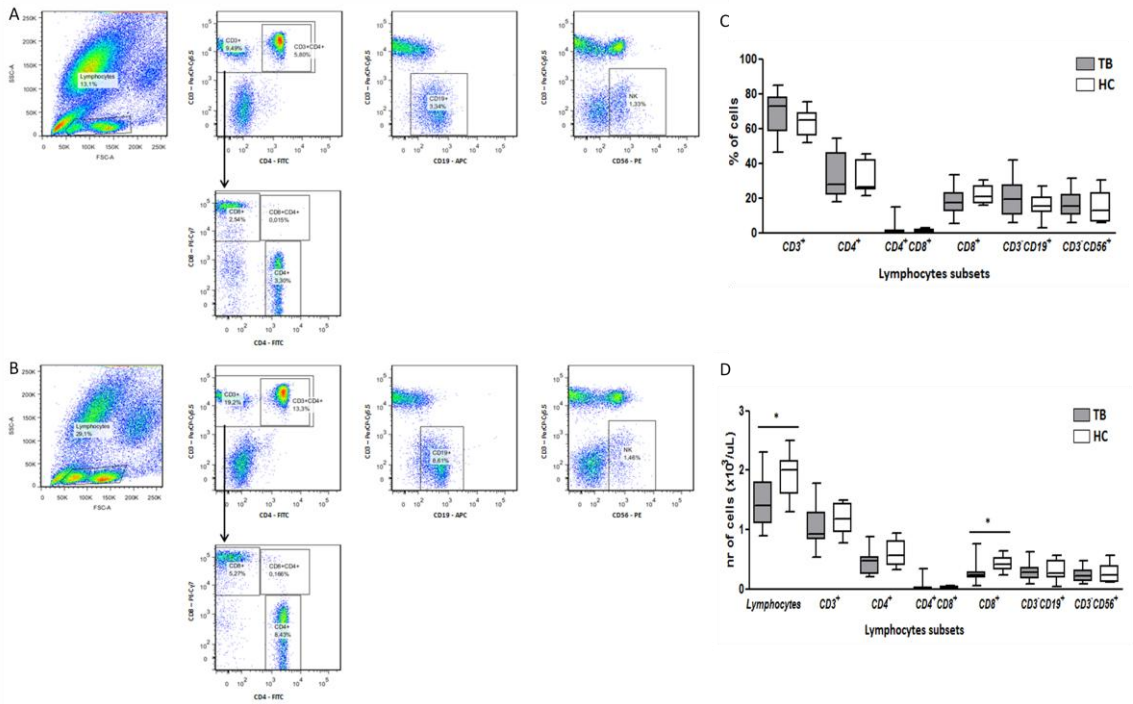

Figure 4 – A, B – Representative dot plots of lymphocyte sub-populations in TB and HC, respectively. **C** – Lymphocyte sub-populations percentage in TB (gray; n=11) and HC (white; n=9). **D** – Absolute number of lymphocyte sub-populations in TB and HC. *p<0.05 compared to HC (Mann Whitney test).

In terms of percentages of cells there are no statistically significant differences and the distribution is similar between both groups. When the comparison is made in

number of cells there are important changes to report. All the lymphocytes subsets are decreased in TB group, particularly cytotoxic T cells (p=0.0184), except B cells subset. Since the number of lymphocytes was lower in TB, as was observed in CBC, was expected that the same happen in its subsets. In the literature, the results for this type of lymphocyte counts are not consensual. Wu *et al.* (104) observed a decrease in $CD3^+$ and $CD3^+CD4^+$, an increase in B cells and $CD3^+CD8^+$ and a similar numbers in NK cells between TB and controls. Ainslie *et al.* (105) also found decrease in $CD3^+CD4^+$ and an increase in $CD3^+CD8^+$. Rodrigues *et al.* (106) and Beck *et al.* (107) describe a decrease in both $CD3^+CD4^+$ and $CD3^+CD8^+$. Uppal *et al.* (108) observed a decrease in $CD3^+CD4^+$ but similar levels for $CD3^+CD8^+$, Hernandez *et al.* (109) found significant lower values for B cells and similar for $CD3^+$. These contrasting results may be explained by the possibility that the modifications of PBMCs change over time since immunological factors seem to change in concentrations over the time.

Total NK cells

NK cells are lymphocytes phenotypically characterized as $CD3^-CD56^+$. Once the work focused the NK cells, it was performed another evaluation of this cells in TB and HC using labelling with mAbs in other fluorochromes.

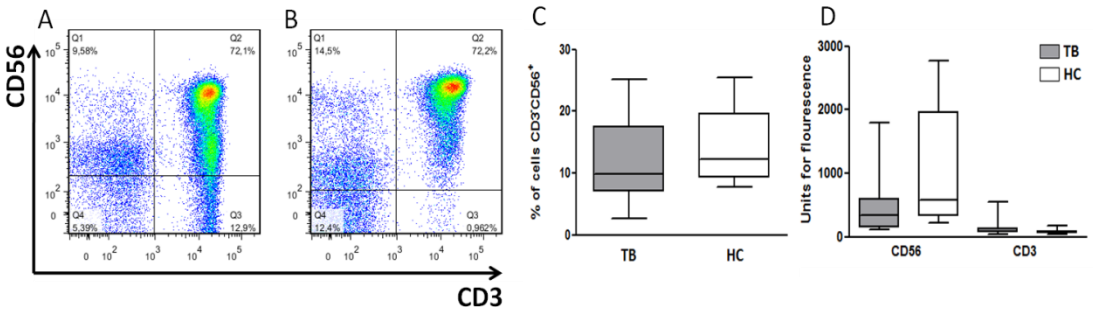

Figure 5 - A, B – Representative dot plots of total NK cells (CD3 *vs.* CD56) in TB and HC, respectively. **C** – Analysis of total NK cells ($CD3^-CD56^+$) in peripheral blood of TB (gray, n=27) and HC (white, n=9). **D** – Mean fluorescence intensity of CD56 and CD3 in NK cells of TB and HC. Gated lymphocytes were used in analysis. Not significant results. (Mann Whitney test).

Once again the results only present a trend to a decrease of NK cells in TB comparatively to HC. As was referred above, other studies also didn't find significant differences between these two groups. It is important to report that the mAbs used in this labelling seems to be unstable since the population $CD3^+CD56^+$ is not according to what was expected. The mean of fluorescence intensity for CD3 and CD56 in $CD3^-$ $CD56^+$ population in both groups was analysed and CD56 was no significantly increase in HC population. These increase that mean a higher expression of this molecule can be due to the instability of the fluorochrome so, in the future, different fluorochromes should be tested.

For $CD3^-CD56^+$ populations in both TB and HC were evaluated theirs expression of CD16 as well as their distribution in terms of CD27/CD11b. The results are represented in Table 3.

Table 3 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $CD3^-CD56^+$ in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	62.83 ±20.70	57.21 ±17.45	0.5837
	MFI CD16	1336.33 ±381.58	1077.04 ±684.55	0.0860
$CD11b^-$ $CD27^+$	%	1.25 ±1.23	1.17 ±1.23	0.9272
	MFI CD27	2177.75 ±1450.53	1240.08 ±997.29*****	0.0140
	MFI CD11b	261.25 ±54.00	243.95 ±73.40	0.7453
$CD11b^+$ $CD27^+$	%	1.92 ±1.40	7.69 ±11.54*****	0.0235
	MFI CD27	1163.33 ±289.48	841.96 ±337.14*****	0.0137
	MFI CD11b	1822.22 ±479.18	1739.70 ±800.74	0.4650
$CD11b^+$ $CD27^-$	%	64.94 ±20.51	65.59 ±15.28	0.8982
	MFI CD27	13.52 ±28.14	65.33 ±84.43	0.0531
	MFI CD11b	2228.78 ±503.08	2144.59 ±807.88	0.6348
$CD11b^-$ $CD27^-$	%	31.89 ±20.63	25.57 ±14.33	0.4216
	MFI CD27	37.77 ±30.89	49.09 ±37.20	0.5211
	MFI CD11b	135.29 ±53.40	122.73 ±51.06	0.6090

Values represent mean ±SE (HC n=27; TB n=9). *p<0.05 compared to HC. (Mann Whitney test)

The distribution of these surface makers will be explained in more detail below, where it is only important to refer that the CD16 expression tends to decrease and it is

observed a higher percentage in $CD11b^{+}CD27^{+}$ subset in TB patients, probably due to a decrease in NK cell maturation. The partial decrease in CD16 expression does not corroborate a previous study that reports a great increase of CD16 expression in TB patients (110).

Proposal of NKp46 for NK cell definition

NKp46, a natural cytotoxicity receptor, is expressed almost exclusively in NK cells. Recently this molecule is being referred as a better markers for NK cells than CD56, so it as analysed its expression of $CD3^{-}$ subset of lymphocytes (111).

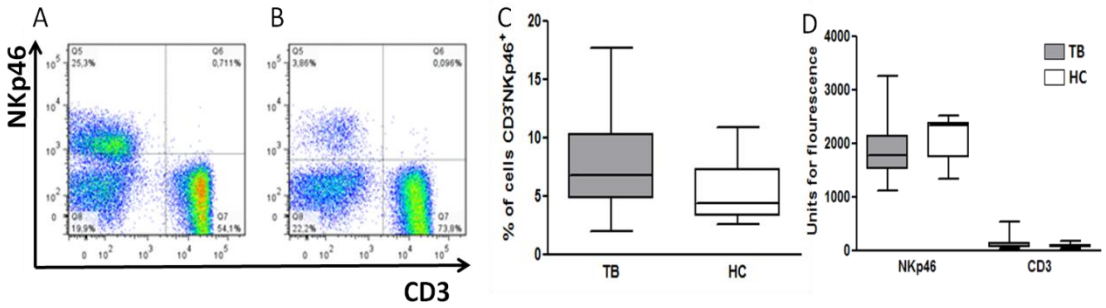

Figure 6 – A, B – Representative dot plots of NK cells (CD3 *vs*. NKp46) in TB and HC, respectively. **C** – Analysis of NK cells ($CD3^{-}NKp46^{+}$) in peripheral blood of TB (gray, n=27) and HC (white, n=9). **D** – Mean fluorescence intensity of NKp46 and CD3 in NK cells of TB and HC. Gated lymphocytes were used in analysis. (Mann Whitney test).

It was observed a higher percentage of $CD3^{-}$ cells expressing NKp46 in TB relatively to HC, but with a decrease level of expression of this receptor. The role of NKp46 in lysis of infected monocytes with Mtb is already defined. One study correlates this capacity of lyse with increased expression of mRNA of NKp46 receptor and also refers that this expression in reduced in TB patients (86). Results presented here are concordant because despite of the higher percentage of $NKp46^{+}$ cells present in TB, its expression tends to decrease.

When the expression of CD16 and subset distribution of CD27/CD11b was evaluated (Table 4), once again it was observed an increase in $CD11b^{+}CD27^{+}$ (not

statistically significant) in TB patients, with a slight decreased expression of CD27 in this subset. This result points towards a more immature form of cells in TB patients.

Table 4 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $CD3^-NKp46^+$ in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	84.51 ±6.09	80.84 ±13.35	0.7285
	MFI CD16	1478.56 ±453.33	1174.26 ±717.53	0.0734
$CD11b^-$ $CD27^+$	%	0.26 ±0.38	0.43 ±0.86	0.7549
	MFI CD27	892.75 ±764.06	622.40 ±224.02	0.5249
	MFI CD11b	127.71 ±79.46	232.96 ±93.04*****	0.0102
$CD11b^+$ $CD27^+$	%	5.29 ±3.71	11.45 ±15.41	0.4762
	MFI CD27	685.25 ±107.39	524.15 ±139.35******	0.0045
	MFI CD11b	2095.00 ±218.10	1752.31 ±611.03*****	0.0403
$CD11b^+$ $CD27^-$	%	91.32 ±3.89	85.03 ±15.04	0.4321
	MFI CD27	17.07 ±38.57	44.83 ±58.09	0.1001
	MFI CD11b	2476.22 ±513.43	2351.07 ±829.02	0.4876
$CD11b^-$ $CD27^-$	%	3.12 ±3.07	3.10 ±3.10	0.9418
	MFI CD27	16.75 ±22.38	61.53 ±62.26	0.0929
	MFI CD11b	113.43 ±31.22	177.76 ±89.13*****	0.0258

Values represent mean ±SE (HC n=27; TB n=9). *p<0.05 **p<0.01 compared to HC (Mann Whitney test).

When the values of $CD3^-CD56^+$ were compared to $CD3^-NKp46^+$, the first observation was that $CD3^-NKp46^+$ population is smaller, meaning that if only $CD3^-NKp46^+$ was considering as NK cells, probably would be lost a lot of information. So, $CD3^-CD56^+$ still remain as the better characterization for NK cells.

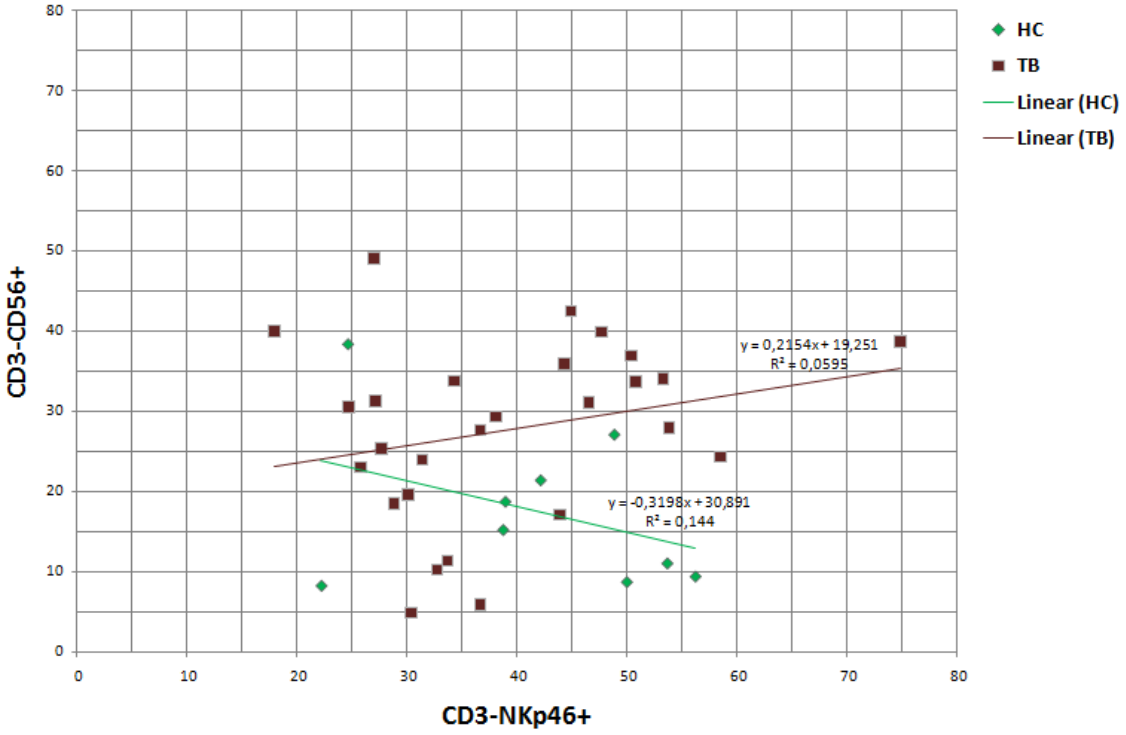

Figure 7– CD56 *vs*. NKp46 as NK cell-speciifc phenotype markers: correlation of CD3-CD56+ and CD3-NKp46+ cells in peripheral blood of pulmonary tuberculosis (TB) patients and healthy contacts (HC). Gated lymphocytes were used for comparisons. No correlations were found in TB (r^2=0.0595) and HC (r^2=0.144).

Then $CD3^-CD56^+NKp46^+$ population was studied and the results are represented in Figure 8 and Table 5.

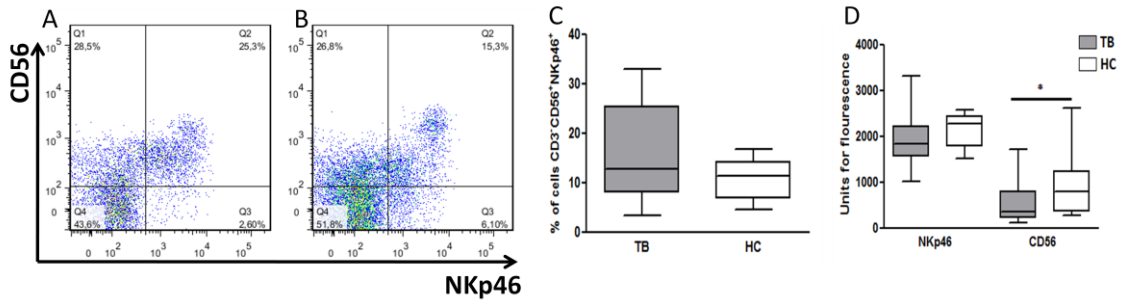

Figure 8 – A, B – Representative dot plots of $CD3^-$ using CD56 and NKp46 as surface markers to distinguish different subsets in TB and HC, respectively. **C** – Analysis of NK cells ($CD56^+NKp46^+$) in peripheral blood of TB (gray, n=27) and HC (white, n=9). **D** – Mean fluorescence intensity of NKp46 and CD56 in NK cells of TB and HC. Gated lymphocytes were used in analysis. *p<0.05 compared to HC. (Mann Whitney test).

Percentage of cell $CD3^-CD56^+NKp46^+$ doesn't have significant variation between two groups. Looking to mean of fluorescence intensity, there is a significant

higher expression of CD56 in HC group. CD16 percentage of cells and expression are similar in both groups. CD27/CD11b subsets are concordant with previous results here presented: increase of $CD11b^{+}CD27^{+}$ in TB patients. In the literature are not studies of these characterizations in $CD3^{-}CD56^{+}NKp46^{+}$.

Table 5 – Percentage of cells $CD16^{+}$ and in each CD27/CD11b subset for $CD3^{-}CD56^{+}NKp46^{+}$ in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^{+}$	%	81.97 ±9.00	78.22 ±13.85	0.5588
	MFI CD16	1372.78 ±411.39	1126.74 ±685.40	0.1002
$CD11b^{-}$ $CD27^{+}$	%	0.14 ±0.19	0.43 ±1.03	0.8810
	MFI CD27	925.71 ±1169.03	618.88 ±229.07	0.3326
	MFI CD11b	104.50 ±68.72	207.88 ±80.04**	0.0083
$CD11b^{+}$ $CD27^{+}$	%	5.68 ±3.98	11.33 ±16.18	0.7701
	MFI CD27	723.00 ±114.56	527.69 ±147.48**	0.0028
	MFI CD11b	2089.75 ±353.92	1692.81 ±685.73	0.1180
$CD11b^{+}$ $CD27^{-}$	%	91.33 ±4.51	85.09 ±15.74	0.5226
	MFI CD27	12.39 ±26.55	44.47 ±57.55	0.1048
	MFI CD11b	2392.56 ±497.24	2257.30 ±806.19	0.5346
$CD11b^{-}$ $CD27^{-}$	%	2.85 ±3.40	3.15 ±3.63	1.0000
	MFI CD27	4.23 ±6.98	48.48 ±51.14*	0.0130
	MFI CD11b	107.13 ±55.34	168.31 ±71.41*	0.0194

Values represent mean ±SE (HC n=27; TB n=9). *p<0.05 **p<0.01 compared to HC (Mann Whitney test).

Total NK cell – Classic subsets (CD56/CD16)

Classically, NK cells are characterized by $CD3^{-}$ and by the intensity of CD56 (dim or bright). Approximately 90% of peripheral NK cells present low density for CD56 and high levels of CD16. The remaining 10% express high levels of CD56 and are

negative for CD16 (112). This last subset is more immature, it is thought that during their development and cytotoxicity acquisition NK cells pass through three stages: $CD56^{dim}CD16^{-}$ (immature), $CD56^{bright}CD16^{-}$ ("regulatory") and $CD56^{dim}CD16^{+}$ (cytotoxic). In this study was considered another stage: $CD56^{bright}CD16^{+}$ as a "pro-inflammatory" stage.

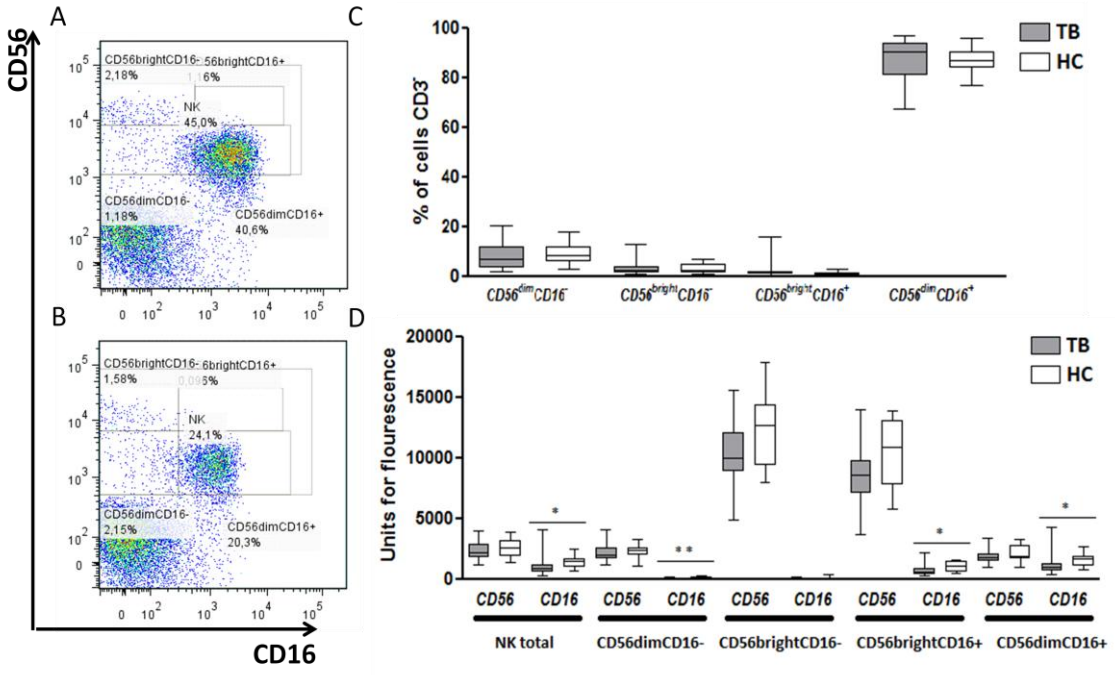

Figure 9 – A, B – Representative dot plots of NK cell subsets according to CD56 and CD16 surface expression in TB and HC, respectively. **C** – Analysis of NK cell sub-sets in peripheral blood of TB (gray, n=29) and HC (white, n=10). **D** – Mean fluorescence intensity of CD56 and CD16 in NK cell subsets of TB and HC. Gated lymphocytes were used in analysis. *p<0.05 **<0.01 compared to HC. (Mann Whitney test)

Data represent NK cells normalized for a total of 100%. As can be observed in first graph (C) there are no statistically significant changes in both of groups for NK cell subsets, but the values for more mature subsets in TB patients tend to be increased. The mean of fluorescence intensity has some variations in CD16 expression with a significant decrease in TB patients. This means that although TB patients present these increased values in more mature subsets, NK cells have less cytotoxic capacity in comparison with HC.

Previous data supports these results referring a decrease in $CD3^{-}CD16^{-/+}CD56^{dim}$ and an increase in $CD3^{-}CD16^{-/+}CD56^{bright}$ in TST+ controls (92). However,

Bozzano *et al.* (113) found decreased proportions of $CD16^{+/-}CD56^{bright}$ and increased values of $CD16^{+}CD56^{dull}$ subsets in TB.

CD27/CD11b expression was evaluated for each subset. Only $CD56^{bright}CD16^{-}$ subset presented differences in these markers as well as in mean of fluorescence intensity (Table 6).

Table 6 – Percentage of cells in each CD27/CD11b subset for $CD3^{-}CD56^{bright}CD16^{-}$ in TB and HC. Means of fluorescence intensity for CD27 and CD11b.

		HC	TB	P
$CD11b^{-}$ $CD27^{+}$	%	0.46 ±0.93	2.98 ±3.46*	0.0355
	MFI CD27	2365.00 ±481.09	1757.06 ±735.53	0.2040
	MFI CD11b	346.33 ±66.51	208.84 ±72.65*	0.0300
$CD11b^{+}$ $CD27^{+}$	%	23.17 ±14.43	18.85 ±13.72	0.4556
	MFI CD27	1242.75 ±171.97	1193.86 ±250.42	0.7144
	MFI CD11b	2163.25 ±570.82	1485.43 ±465.76**	0.0068
$CD11b^{+}$ $CD27^{-}$	%	74.31 ±15.84	71.42 ±16.45	0.5516
	MFI CD27	121.09 ±52.79	80.22 ±55.90	0.0592
	MFI CD11b	2396.90 ±416.10	1785.07 ±609.98**	0.0033
$CD11b^{-}$ $CD27^{-}$	%	2.07 ±2.38	6.75 ±6.84*	0.0295
	MFI CD27	137.97 ±84.49	120.73 ±92.21	0.6321
	MFI CD11b	261.86 ±81.04	304.56 ±76.96	0.2740

Values represent mean ±SE (HC n=29; TB n=10). *p<0.05 **p<0.01 compared to HC (Mann Whitney test).

Significant changes were found in $CD11b^{-}$ subsets, with an increased expression in TB patients. This alteration is also observed in $CD11b^{+}$ subsets with a sharp significantly lower mean of fluorescence intensity. CD11b expression is related with gain of maturity, as will be explained next. The subset of these findings in the second more immature of the NK cells, and this lack of CD16 expression gives an even more immaturity phenotype to TB patients cells in $CD3^{-}CD56^{bright}CD16^{-}$ subset.

CD27/CD11b

Recently, Fu *et al.* (34), made some progress in NK cell phenotyping relatively to CD27/CD11b expression and defined four stages that describe NK cell development: $CD11b^{-}CD27^{-}$, $CD11b^{-}CD27^{+}$, $CD11b^{+}CD27^{+}$ and $CD11b^{+}CD27^{-}$. So, during their maturation cells acquire and lose CD27 and acquire CD11b. In peripheral blood NK cells have their more mature stage: $CD11b^{+}CD27^{-}$.

Data of this kind of analysis is represented in Figure 10. There are no differences in subset distribution of NK cells in TB and HC. In other words, according to this characterization NK cells in terms of CD27/CD11b, TB and HC have the same level of development.

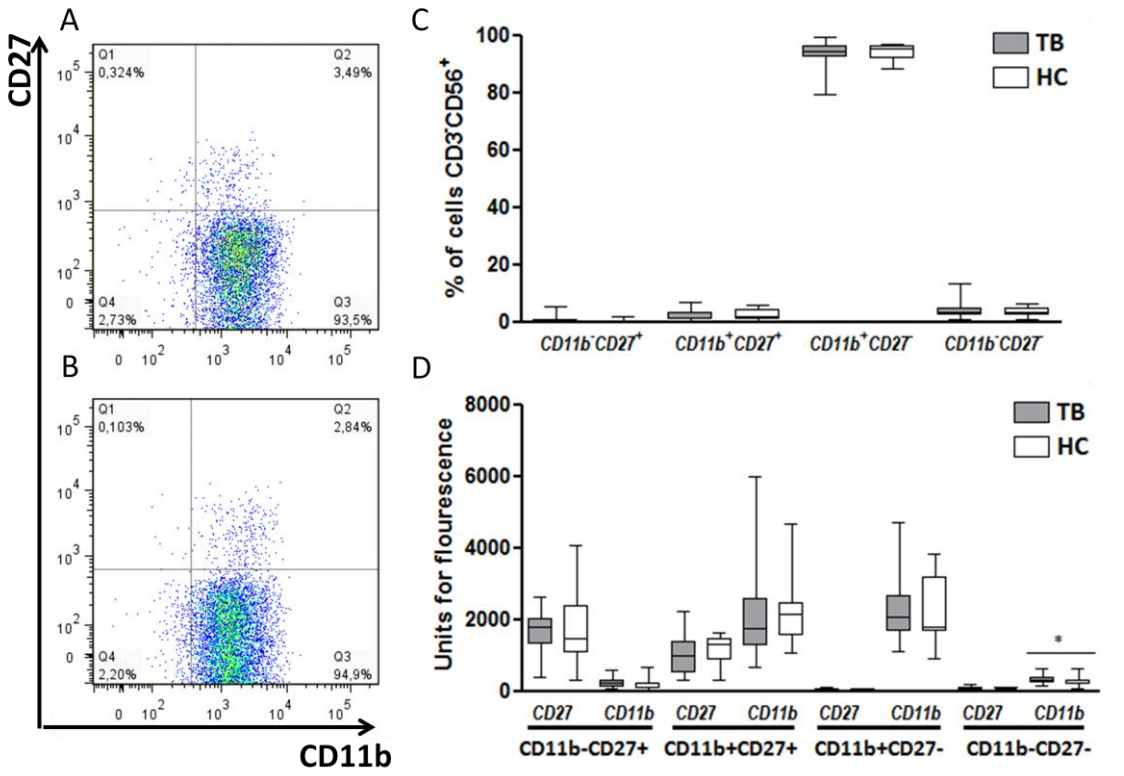

Figure 10 – A, B – Representative dot plots of NK cells and NK cell subsets according to CD27 and CD11b surface expression in TB and HC, respectively. **C** – Analysis of NK cell subsets in peripheral blood of TB (gray, n=29) and HC (white, n=11). **D** – Mean fluorescence intensity for CD27 and CD11b in NK cell subsets of TB and HC. Gated lymphocytes were used in analysis. *p<0.05 compared to HC (Mann Whitney test).

IFNγ production by NK cells

The IFNγ is the key cytokine for a protective immune response against Mtb (114). This cytokine is produced mainly by $CD4^+$, $CD8^+$ T cells and NK cells (114). Here it was evaluated the intracellular IFNγ production by NK cells. As observed in Figure 11, TB patients present a significantly higher percentage of $IFN\gamma^+$ NK cells comparatively to HC. This can be explained because in TB patients, NK cells are producing great levels of this cytokine to recruit macrophages to kill intracellular Mtb.

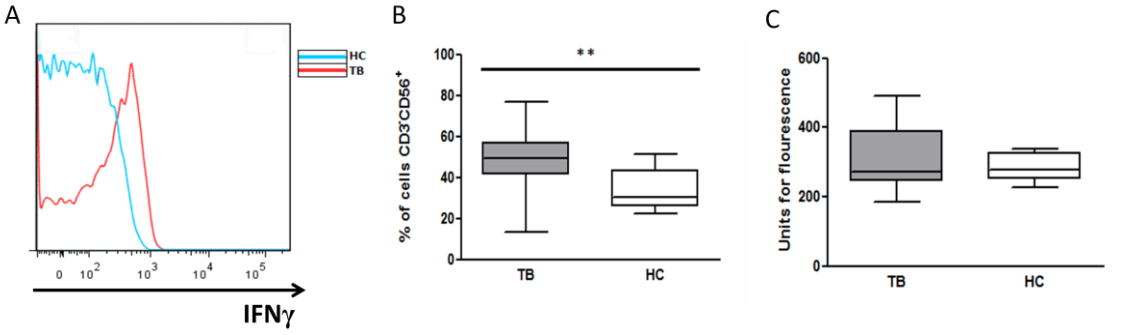

Figure 11 – A – Representative histogram of IFNγ intracellular expression in NK cell ($CD3^-CD56^+$) of TB (red) and HC (blue). **B** – Analysis of IFNγ intracellular expression in NK cell in peripheral blood of TB (gray, n=23) and HC (white, n=10). **C** – Mean fluorescence intensity of IFNγ in NK cells of TB and HC. Gated lymphocytes were used in analysis. **p<0.01 compared to HC (Mann Whitney test).

Bozzano F *et al.* (113) made some observations in NK cell phenotype as well as IFNγ production related with the course of treatment. They report that before treatment cells were producing less IFNγ than controls, but after treatment these levels of production were equivalent. What was observed here was a massive production of IFNγ in TB comparatively with HC, that can be due to treatment.

CD57 as a marker for NK cell terminal differentiation

Lopez-Verges *et al.* (35) suggested recently that NK cells expressing CD57 represent a subset of mature and terminally differentiated cells. Data presented here demonstrate a significantly increase (p<0.001) of CD57 expressing cells in TB patients

with a partial increase in intracellular IFNγ in these cells represented in Figure 12. In terms of CD27/CD11b distribution, results for $CD57^+$ subset are concordant with what was expected: almost of cells are $CD11b^+CD27^-$, confirming their high degree of maturation (Table 7). CD16 expression is not according with this state of differentiation; it was expected higher percentage of cells expressing this marker. This may be due to instability of marker once cells were first extracellular labelled and than permeabilized, so there may be some interference with extracellular labelling of this marker.

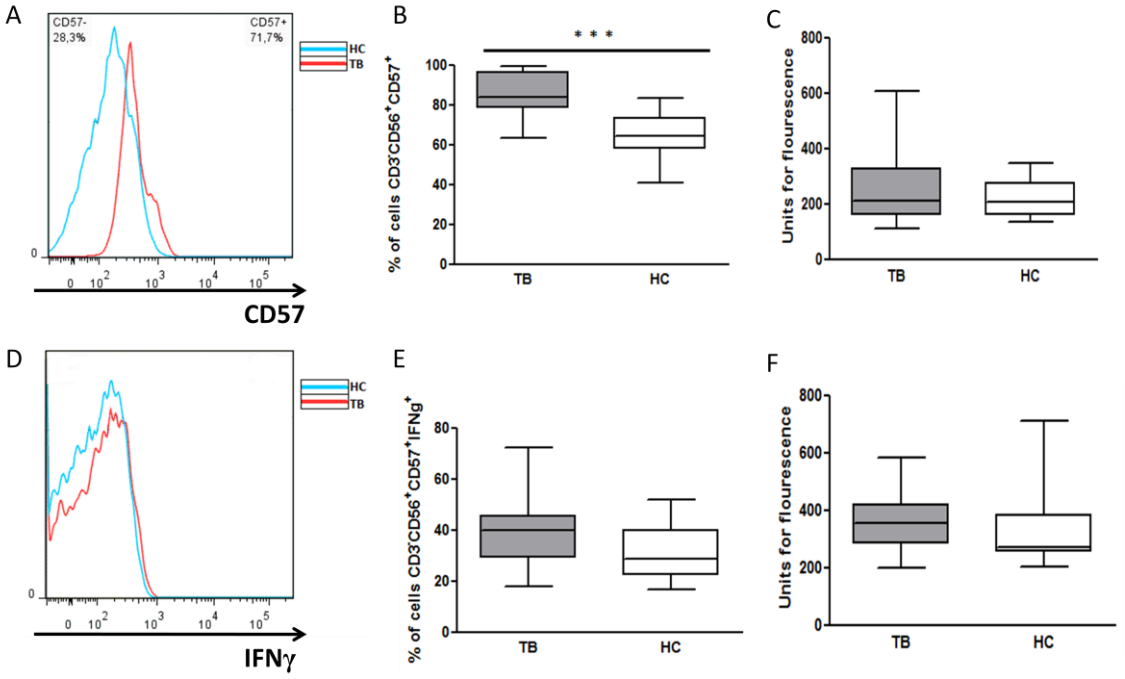

Figure 12 – A - Representative histogram of CD57 surface expression in NK cell ($CD3^-CD56^+$) of TB (red) and HC (blue). **B** – Analysis of CD57 expression on NK cell in peripheral blood of TB (gray, n=20) and HC (white, n=11). **C** – Mean fluorescence intensity of CD57 in NK cells of TB and HC. **D** – Representative histogram of IFNγ intracellular expression on NK cell $CD57^+$ for TB (red) and HC (blue). **E** – Anaysis of IFNγ intracellular expression on NK cells $CD57^+$. **F** – Mean fluorescence intensity of IFNγ in NK cells $CD57^+$ in TB and HC. Gated lymphocytes were used in analysis. ***p<0.001 compared to HC (Mann Whitney test).

The meaning of these results can be explained by the NK response to infection, once Lopez-Verges (35) explained that $CD57^+$ cells have more lytic activity and higher sensibility to stimulation. According to the same author, these cells also express higher levels of NCRs being well prepared to oppose infected cells with Mtb.

Table 7 – Percentage of cells expressing CD16 and in each CD27/CD11b subset for $CD3^{-}CD56^{+}CD57^{+}$ in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^{+}$	%	16.92 ±25.48	15.38 ±18.25	0.8688
	MFI CD16	600.27 ±512.45	560.05 ±454.17	0.5494
$CD11b^{-}$ $CD27^{+}$	%	0.01 ±0.03	0.00 ±0.00	-
	MFI CD27	-	-	-
	MFI CD11b	-	-	-
$CD11b^{+}$ $CD27^{+}$	%	1.54 ±1.00	1.52 ±1.50	0.5222
	MFI CD27	605.50 ±90.39	797.75 ±331.35	0.2020
	MFI CD11b	1904.90 ±1227.87	2302.60 ±1422.69	0.3442
$CD11b^{+}$ $CD27^{-}$	%	89.92 ±7.75	90.27 ±11.35	0.5084
	MFI CD27	245.91 ±46.15	332.70 ±130.77*	0.0475
	MFI CD11b	1706.91 ±1583.26	1823.65 ±1022.66	0.3529
$CD11b^{-}$ $CD27^{-}$	%	8.53 ±7.61	8.22 ±11.12	0.3957
	MFI CD27	122.97 ±49.24	127.71 ±27.72	0.2734
	MFI CD11b	315.00 ±183.85	273.43 ±46.70	0.3664

Values represent mean ±SE (HC n=20; TB n=11). *p<0.05 compared to HC (Mann Whitney test).

Surface expression of CD94 and NKG2D

CD94 is a Killer cell lectin-like receptor belonging to subfamily D, number 1 (also known as KLRD1). This receptor appears usually as heterodimers with elements of NKG2 family. NKG2D, a member of NKG2 family, is an activating receptor. CD94 can also appears linked with NKG2A (inhibitory receptor) and NKG2C (activatory receptor) as well as in form of oligodimer. Here as observed the co-expression of CD94 and NKG2D in TB and HC.

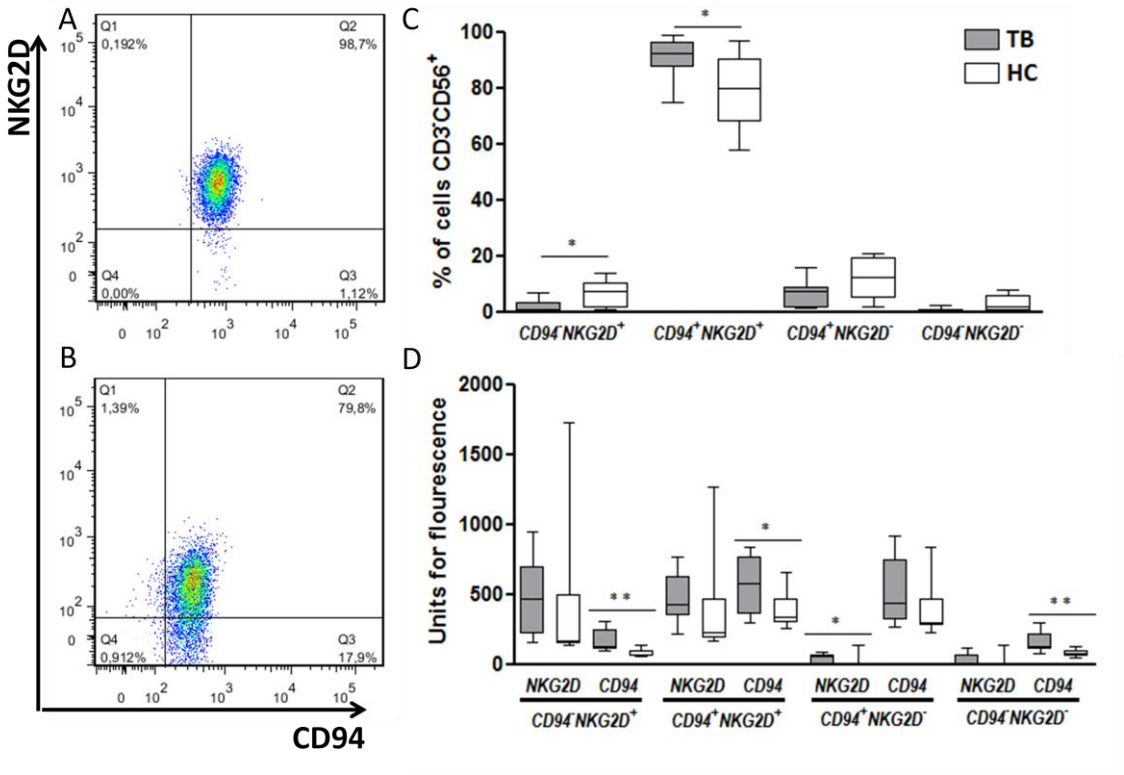

Figure 13 – A, B – Representative dot plots of NK cells and NK cell subsets according to CD94 and NKG2D surface expression in TB and HC, respectively. **C** – Analysis of NK cell subsets in peripheral blood of TB (gray, n=10) and HC (white, n=9). **D** – Mean fluorescence intensity for CD94 and NKG2D in NK cell subsets of TB and HC. Gated lymphocytes were used in analysis. *p<0.05, **p<0.01 compared to HC (Mann Whitney test).

Data presented in Figure 13 represents CD94/NKG2D populations in TB and HC. It can be observed a significant increase in double positive subset in TB patients as well as higher levels of CD94 expression in all subsets relatively to HC. NKG2D expression tends to increase in all subsets of TB patients.

Table 8 shows CD16 and intracellular IFNγ in each CD94/NKG2D subset. The significance of those NK cell subsets remains unclear. Further analysis dissecting NKG2A and NKG2C in those cells in needed.

Table 8 – Percentage of cells expressing CD16 and intracellular IFNγ in each CD94/NKG2D subset in TB and HC. Means of fluorescence intensity for CD16 and IFNγ.

		HC	TB	P
CD94$^-$ NKG2D$^+$	% CD16	32.91 ±33.56	50.89 ±31.31	0.3154
	MFI CD16	436.89 ±591.87	576.80 ±663.35	0.1207
	% IFNγ	33.21 ±15.13	44.42 ±14.49	0.1912
	MFI IFNγ	303.22 ±72.57	281.40 ±81.58	0.4470
CD94$^+$ NKG2D$^+$	% CD16	39.77 ±24.19	58.86 ±20.66	0.0653
	MFI CD16	306.22 ±325.78	423.40 ±440.73	0.0535
	% IFNγ	39.48 ±3.75	44.29 ±6.99	0.2528
	MFI IFNγ	271.78 ±44.67	277.00 ±57.82	0.8702
CD94$^+$ NKG2D$^-$	% CD16	54.17 ±10.25	53.15 ±22.65	0.8421
	MFI CD16	288.67 ±89.70	325.00 ±141.82	0.7197
	% IFNγ	40.08 ±10.02	40.71 ±9.40	0.7802
	MFI IFNγ	286.78 ±56.80	280.10 ±57.68	0.7802
CD94$^-$ NKG2D$^-$	% CD16	40.66 ±13.39	45.50 ±35.23	0.8380
	MFI CD16	345.78 ±106.31	225.88 ±93.59*	0.0384
	% IFNγ	37.74 ±15.15	32.78 ±29.21	0.4132
	MFI IFNγ	301.89 ±87.13	264.71 ±48.07	0.4698

Values represent mean ±SE (HC n=10; TB n=9). *p<0.05 compared to HC (Mann Whitney test).

Other Natural Cytotoxicity Receptors (NCRs)

Natural cytotoxicity receptors (NCRs) play a major role in NK cell cytotoxicity against transformed cells (47). NKp46, NKG2D and CD94 NCRs have already been discussed in the results. It was also analysed NKp44, NKp30 and NKp80 positive cells, expression, intracellular IFNγ, CD16 and CD27/CD11b positive cells and expression on TB and HC. In data it can be observed increased levels of positive cells for all of these NCRs as well as higher levels of intracellular IFNγ in TB patients (Figure 14). The most significant increase in NCRs was observed for NKp80 and this receptor is also the most expressed in NK cells in both groups. NKp30 is not only the least represented in

NK cells from two groups, but also the receptor that presented the least increase in TB patients. Although in terms of expression NKp30 was significantly increased in TB patients. NKp44 is present in a vast majority of NK cells in both groups however is increased in TB patients.

Intracellular IFNγ positive cells were significantly elevated in all NCRs positive cells and its expression tends to be elevated in TB patients.

NKp44^{+}CD16^{+} cells decreased in both groups regarding to expected for cells in high degree of maturation (Table 9).

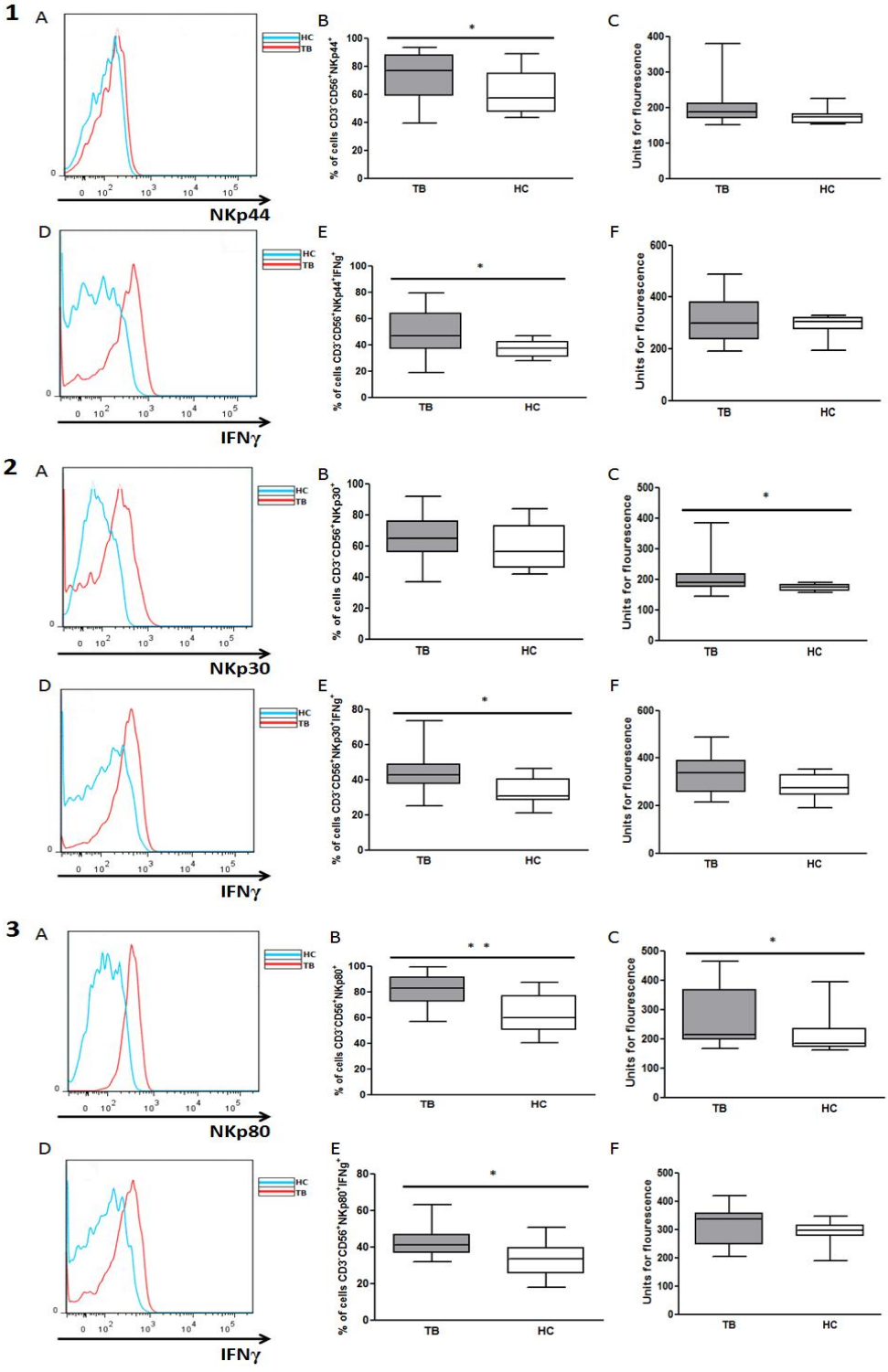

Figure 14 – 1 – Results for NKp44+ NK cells (TB n=23, HC n=10). **2** – Results for NKp30+ NK cells (TB n=19, HC n=9). **3** – Results for NKp80+ NK cells (TB n=16, HC n=10). **A** – Representative histogram of each NCR surface expression in NK cell ($CD3^-CD56^+$) of TB (red) and HC (blue). **B** – Analysis of each NCR expression on NK cell in peripheral blood of TB (gray) and HC (white). **C** – Mean fluorescence intensity of each NCR in NK cells of TB and HC. **D** – Representative histogram of IFNγ intracellular expression on NK cell positive for each NCR in TB (red) and HC (blue). **E** – Analysis of IFNγ intracellular expression on NK cells positive for each NCR. **F** – Mean fluorescence intensity of IFNγ in NK cells positive for each NCR in TB and HC. Gated lymphocytes were used in analysis. *p<0.05, **p<0.01 compared to HC (Mann Whitney test).

CD27/CD11b subsets of NCRs positive populations had no significant differences except for NKp80 in $CD11b^{+}CD27^{-}$ subset were can be observed an increase in TB patients (Table 11). But in general, almost all cells express CD11b and are negative for CD27 in both groups.

Table 9 – Percentage of cells $CD16^{+}$ and in each CD27/CD11b subset for $CD3^{-}CD56^{+}NKp44^{+}$ in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^{+}$	%	43.00 ±26.69	43.79 ±24.50	1.0000
	MFI CD16	313.80 ±280.27	409.26 ±573.55	0.6665
$CD11b^{-}$ $CD27^{+}$	%	0.00 ±0.01	0.00 ±0.01	0.1951
	MFI CD27	-	-	-
	MFI CD11b	-	-	-
$CD11b^{+}$ $CD27^{+}$	%	0.94 ±0.66	0.78 ±1.37	0.0575
	MFI CD27	716.80 ±107.19	908.78 ±302.37	0.1040
	MFI CD11b	2101.60 ±1067.03	2358.00 ±984.53	0.2992
$CD11b^{+}$ $CD27^{-}$	%	88.46 ±12.74	92.40 ±8.37	0.3177
	MFI CD27	285.60 ±32.81	368.65 ±110.48*	0.0243
	MFI CD11b	1936.60 ±1704.04	2435.91 ±2366.98	0.4220
$CD11b^{-}$ $CD27^{-}$	%	10.60 ±12.88	6.82 ±8.43	0.5054
	MFI CD27	132.58 ±41.36	209.36 ±101.21**	0.0090
	MFI CD11b	320.44 ±52.50	485.45 ±192.35**	0.0023

Values represent mean ±SE (HC n=23; TB n=10). *p<0.05 and **p<0.01 compared to HC (Mann Whitney test).

Table 10 – Percentage of cells each CD27/CD11b subset for $CD3^{-}CD56^{+}NKp30^{+}$ in TB and HC. Means of fluorescence intensity for CD27 and CD11b.

		HC	TB	P
$CD11b^{-}$ $CD27^{+}$	%	0.00 ±0.00	0.00 ±0.00	-
	MFI CD27	-	-	-
	MFI CD11b	-	-	-
$CD11b^{+}$ $CD27^{+}$	%	0.58 ±0.88	0.63 ±0.61	0.2476
	MFI CD27	786.50 ±106.59	862.58 ±257.60	0.7702
	MFI CD11b	2176.63 ±914.82	2548.58 ±2100.65	0.6517
$CD11b^{+}$ $CD27^{-}$	%	89.27 ±10.31	92.00 ±7.24	0.5063
	MFI CD27	280.22 ±47.76	355.42 ±95.16*	0.0237
	MFI CD11b	1861.56 ±1402.46	2315.89 ±2562.20	0.3252
$CD11b^{-}$ $CD27^{-}$	%	10.16 ±10.63	7.36 ±7.00	0.5882
	MFI CD27	143.00 ±67.12	182.00 ±74.96*	0.0270
	MFI CD11b	346.56 ±107.89	399.61 ±124.67	0.2688

Values represent mean ±SE (HC n=19; TB n=9). *p<0.05 compared to HC (Mann Whitney test).

Table 11 – Percentage of cells each CD27/CD11b subset for $CD3^{-}CD56^{+}NKp80^{+}$ in TB and HC. Means of fluorescence intensity for CD27 and CD11b.

		HC	TB	P
$CD11b^{-}$ $CD27^{+}$	%	0.00 ±0.01	0.00 ±0.01	-
	MFI CD27	-	-	-
	MFI CD11b	-	-	-
$CD11b^{+}$ $CD27^{+}$	%	0.45 ±0.88	0.41 ±0.54	0.3292
	MFI CD27	825.89 ±59.10	969.36 ±263.91	0.2703
	MFI CD11b	1997.00 ±989.74	2929.00 ±1397.04	0.0832
$CD11b^{+}$ $CD27^{-}$	%	89.13 ±8.38	94.81 ±6.25*	0.0287
	MFI CD27	277.10 ±46.22	397.88 ±108.91**	0.0013
	MFI CD11b	1715.70 ±1331.58	2679.25 ±2184.25	0.0775
$CD11b^{-}$ $CD27^{-}$	%	10.42 ±8.72	4.77 ±6.15	0.0775
	MFI CD27	127.86 ±61.08	241.50 ±114.56**	0.0017
	MFI CD11b	361.50±136.09	574.81 ±214.75**	0.0044

Values represent mean ±SE (HC n=16; TB n=10). *p<0.05 and **p<0.01 compared to HC (Mann Whitney test).

Inhibitory KIR: KIR2DL1, KIR2DL2 and KIR3DL1

Killer cell immunoglobulin-like receptors (KIRs) belong to a highly polymorphic family of receptors that recognise MHC-class I molecules. KIR acquisition is a late event during NK cell maturation. These receptors are mostly inhibitors but they may also have activating functions depending on the length of their cytoplasmic tail. Inhibitory KIRs recognise MHC-class I molecules and suppress the cytotoxic activity of NK cells.

Based on David G *et al.* (115) work descriptions, KIR2DL2 and KIR2DL1 phenotypes were analysed in TB patients and HC using a combination of mAbs (Figure 15 – 1, 2). Also KIR2DL2/DL3 and KIR3DL1 NK positive cells were analysed through the use of specific mAbs (CD158b and CD158e, respectively).

The percentage of KIR2DL2, KIR2DL1 and KIR3DL1 positive cells tends to decrease in TB patients and also their expression tends to be decreased. KIR3DL1 is significantly decreased in its expression in TB patients (Figure 15 – 1, 2 and 4).

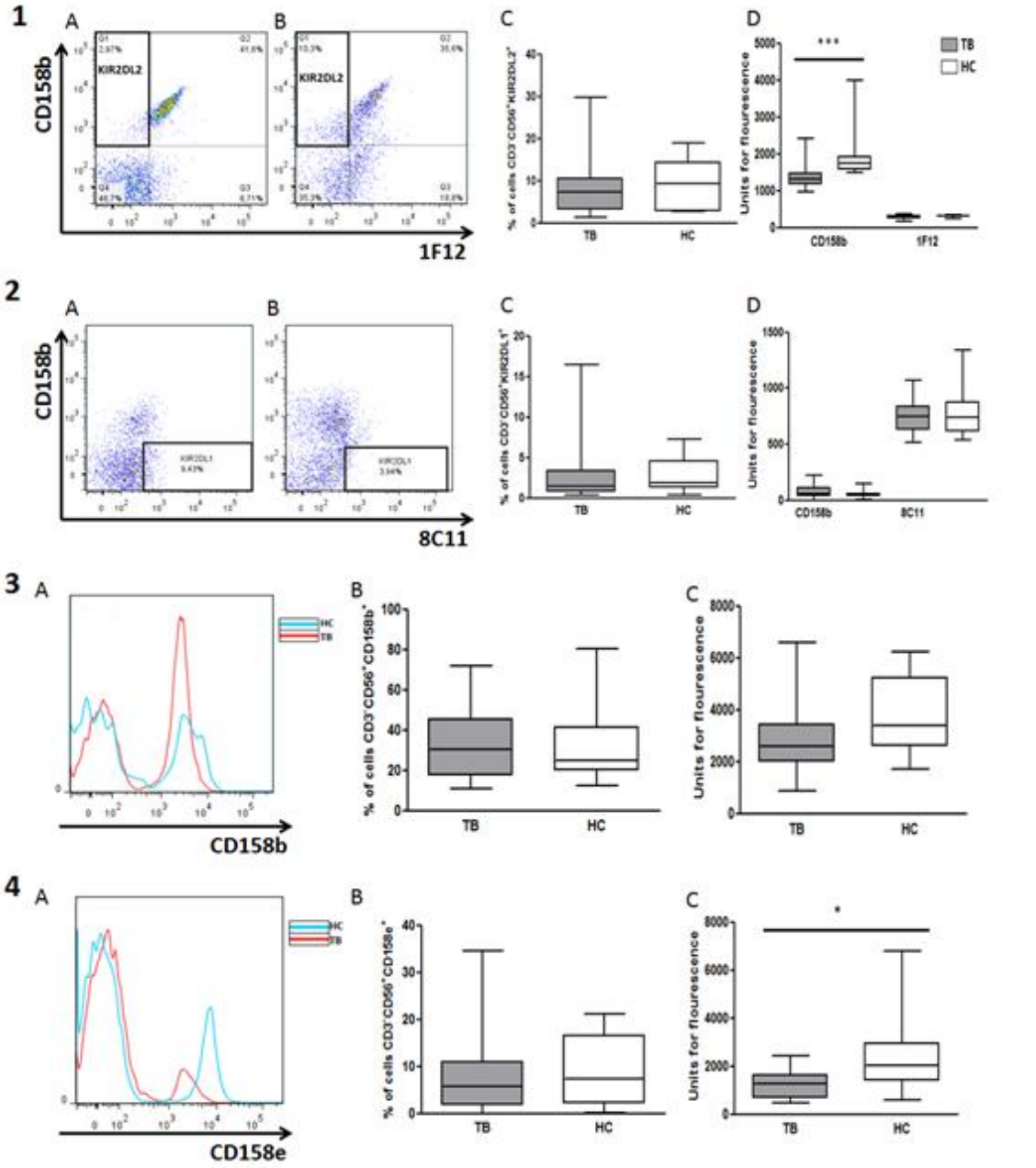

Figure 15 – 1-2 A-B – Representative dot plots of NK cells expressing KIR2DL2 (**1**) and KIR2DL1 (**2**) in TB and HC, respectively.**1-2 C** – Analysis of NK cell KIR2DSL2^{+} (**1**) (TB n=28; HC=11) and KIR2DL1^{+} (**2**) (TB n=29; HC n=11) in peripheral blood of TB and HC. **1-2 D** – Mean fluorescence intensity for CD158b and 1F12 in NK cell KIR2DL2^{+} (**1**) and for CD158b and 8C12 in NK cell KIR2DL1^{+} (**2**) of TB and HC. **3-4 A** – Representative histogram of CD158b (**3**) and CD158e (**4**) expression in NK cells from TB (red) and HC (blue). **3-4 B** – Analysis of CD158b (**3**) (TB n=28; HC n=11) and CD158e (**4**) (TB n=25; HC n=9) expression on NK cell in peripheral blood of TB and HC. **3-4 C** – Mean fluorescence intensity of CD158a/h (**3**) and CD158e (**4**) in NK cells of TB and HC. Gated lymphocytes were used in analysis. *p<0.05 ***p<0.001 compared to HC (Mann Whitney test).

Table 12 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $KIR2DL2^+$ cells in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	87.55 ±14.83	82.44 ±19.73	0.4261
	MFI CD16	1604.00 ±836.03	1337.32 ±810.45	0.2816
$CD11b^- CD27^+$	%	0.00 ±0.01	0.10 ±0.20	0.2239
	MFI CD27	537.00 ±0.00	2665.14 ±2036.33	-
	MFI CD11b	66.10 ±0.00	218.50 ±132.18	-
$CD11b^+ CD27^+$	%	0.77 ±0.93	1.15 ±1.26	0.5368
	MFI CD27	2851.25 ±1845.23	2990.47 ±1275.83	0.7300
	MFI CD11b	1760.13 ±513.90	1775.89 ±639.24	0.9365
$CD11b^+ CD27^-$	%	95.92 ±4.09	95.99 ±3.49	0.7667
	MFI CD27	39.86 ±13.29	41.68 ±21.01	0.7431
	MFI CD11b	2618.18 ±1056.50	2414.29 ±1056.85	0.4729
$CD11b^- CD27^-$	%	3.32 ±4.09	2.76 ±3.20	0.8508
	MFI CD27	41.71 ±30.21	84.98 ±145.84	0.5259
	MFI CD11b	256.36 ±74.37	287.83 ±62.65	0.5879

Values represent mean ±SE (HC n=28; TB n=11). Not significant data (Mann Whitney test).

Tables 12, 13, 14 and 15 show the results for CD16 and CD27/CD11b expression in NK cells expressing referred inhibitory KIRs. All cells present similar levels of differentiation as it can be observed by the surface expression of those markers.

Table 13 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $KIR2DL1^+$ cells in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	71.89 ±19.43	75.28 ±17.35	0.5547
	MFI CD16	1379.82 ±670.05	1027.66 ±467.92	0.1299
$CD11b^- CD27^+$	%	0.16 ±0.35	0.46 ±1.26	0.9271
	MFI CD27	2280.50 ±593.50	1117.60 ±591.01	0.3810
	MFI CD11b	412.00 ±39.00	278.80 ±26.81	0.0952
$CD11b^+ CD27^+$	%	10.36 ±9.59	9.08 ±10.55	0.6135
	MFI CD27	2140.63 ±539.81	1925.62 ±1360.13	0.3414
	MFI CD11b	2882.50 ±670.32	2893.71 ±1440.10	0.6783
$CD11b^+ CD27^-$	%	83.44 ±10.55	85.67 ±12.42	0.5148
	MFI CD27	58.61 ±31.44	85.44 ±56.64	0.2256
	MFI CD11b	2539.36 ±1347.64	2333.41 ±806.90	0.9156
$CD11b^- CD27^-$	%	6.04 ±8.96	4.79 ±6.40	0.6266
	MFI CD27	95.50 ±80.20	80.08 ±106.94	0.3511
	MFI CD11b	276.11 ±76.15	292.16 ±118.10	0.3787

Values represent mean ±SE (HC n=29; TB n=11). Not significant data (Mann Whitney test).

Table 14 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $CD158b^+$ cells in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	92.02 ±12.54	89.62 ±11.54	0.4260
	MFI CD16	1545.82 ±794.65	1291.04 ±888.48	0.2886
$CD11b^- CD27^+$	%	0.03 ±0.10	0.07 ±0.16	0.1404
	MFI CD27	4069.00 ±0.00	3660.80 ±3595.56	-
	MFI CD11b	127.00 ±0.00	154.31 ±57.28	-
$CD11b^+ CD27^+$	%	1.10 ±1.03	1.15 ±1.15	0.9251
	MFI CD27	3432.13 ±1129.40	2429.39 ±936.38	0.0610
	MFI CD11b	1962.38 ±474.60	2280.78 ±1471.33	0.8390
$CD11b^+ CD27^-$	%	95.89 ±4.98	97.50 ±2.56	0.3652
	MFI CD27	49.33 ±19.11	52.07 ±22.40	0.6209
	MFI CD11b	2410.27 ±899.16	2380.04 ±931.22	0.8883
$CD11b^- CD27^-$	%	2.97 ±5.13	1.29 ±1.83	0.1381
	MFI CD27	78.37 ±55.76	62.53 ±56.44	0.8367
	MFI CD11b	161.82 ±31.10	182.28 ±32.89	0.0714

Values represent mean ±SE (HC n=28; TB n=11). Not significant data (Mann Whitney test).

Table 15 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $CD158e^+$ cells in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	95.29 ±3.53	80.49 ±28.29	0.3564
	MFI CD16	1754.22 ±647.02	1144.57 ±527.56*	0.0263
$CD11b^-$ $CD27^+$	%	0.30 ±0.84	0.30 ±1.42	0.8119
	MFI CD27	2117.00 ±0.00	1862.00 ±378.00	-
	MFI CD11b	186.00 ±0.00	191.00 ±7.00	-
$CD11b^+$ $CD27^+$	%	0.98 ±1.39	12.19 ±26.69	0.2097
	MFI CD27	1634.67 ±573.95	1244.48 ±250.44	0.1797
	MFI CD11b	2556.00 ±1272.61	3322.10 ±1436.54	0.2938
$CD11b^+$ $CD27^-$	%	95.03 ±6.61	82.31 ±31.02	0.4121
	MFI CD27	4.39 ±8.79	96.61 ±194.48*	0.0237
	MFI CD11b	2683.11 ±576.38	2682.09 ±1005.39	1.0000
$CD11b^-$ $CD27^-$	%	3.69 ±6.63	1.19 ±2.07	0.1598
	MFI CD27	1.94 ±4.76	85.36 ±120.55**	0.0058
	MFI CD11b	176.66 ±65.75	211.79 ±73.88	0.2183

Values represent mean ±SE (HC n=25; TB n=9). Not significant data (Mann Whitney test).

Activating KIR: KIR2DS1

According to the length of the cytoplasmatic tail, KIR can be inhibitory or activating. The sort tail (S) has activation function. Here, once again methods and data analysis were based on David *et al.* (115) (Figure 16-1). CD158a/h correspond to KIR2DL1 (CD158a) – inhibitory receptor – and KIR2DS1 (CD158h) and the expression of these two KIR was analysed in TB and HC (Figure 16-2).

The percentage of NK cells expressing KIR2DS1 is significantly higher in TB patients and when the compare the mean of fluorescence intensity of the two markers that defines this population there's no differences to report.

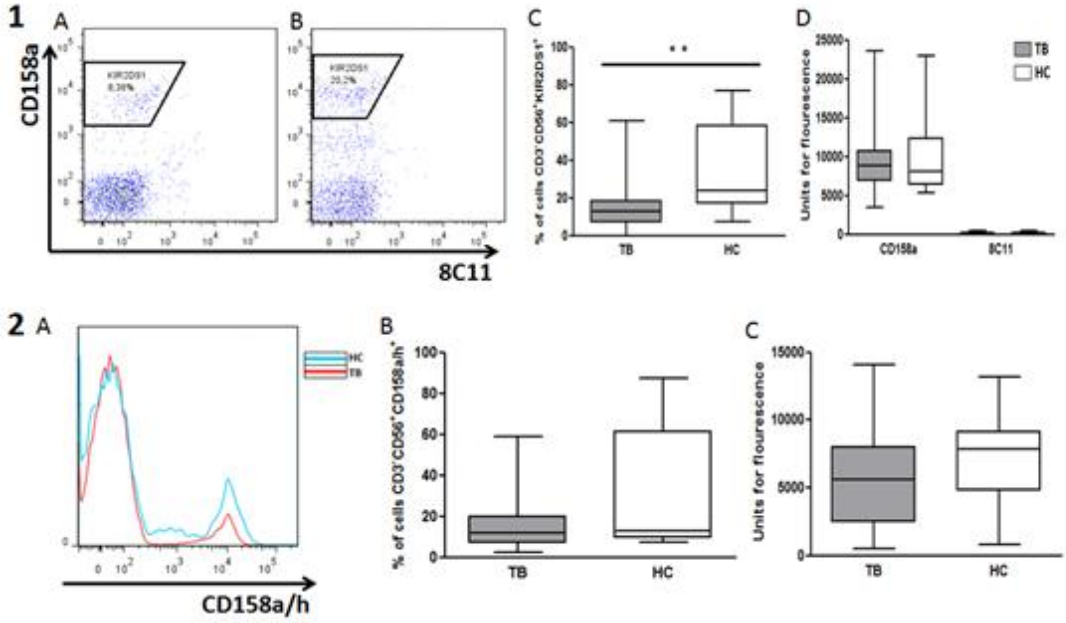

Figure 16 – **1A, 1B** – Representative dot plots of NK cells expressing KIR2DS1 in TB and HC, respectively.**1C** – Analysis of NK cell KIR2DS1$^+$ in peripheral blood of TB (gray, n=28) and HC (white, n=11). **1D** – Mean fluorescence intensity for CD158a/h and 8C11 in NK cell KIR2DS1$^+$ of TB and HC. **2A** – Representative histogram of CD158a/h expression in NK cells from TB (red) and HC (blue). **2B** – Analysis of CD158a/h expression on NK cell in peripheral blood of TB (gray, n=27) and HC (white, n=9). **2C** – Mean fluorescence intensity of CD158a/h in NK cells of TB and HC. Gated lymphocytes were used in analysis. **p<0.01 compared to HC (Mann Whitney test).

KIR2DS1$^+$ cells are greatly differentiated: high levels of CD16 and predominance of CD11b$^+$CD27$^-$ subset (Table 16) as expected for KIR$^+$ cells. There are significant reduction in two CD27/CD11b subsets in TB patients (CD11b$^-$CD27$^+$ and CD11b$^+$CD27$^+$), however the absolute numbers are similar.

Table 16 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $KIR2DS1^+$ cells in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	85.96 ±22.79	80.47 ±23.34	0.3572
	MFI CD16	1656.55 ±762.76	1332.07 ±671.50	0.2466
$CD11b^-$ $CD27^+$	%	0.25 ±0.51	0.06 ±0.14*	0.0301
	MFI CD27	3239.00 ±2845.02	2389.83 ±1466.43	0.8357
	MFI CD11b	193.31 ±157.31	264.83 ±203.31	0.8357
$CD11b^+$ $CD27^+$	%	0.69 ±0.56	0.33 ±0.44*	0.0451
	MFI CD27	2023.00 ±1314.96	2946.27 ±1530.93	0.1272
	MFI CD11b	2554.20 ±1632.15	1957.33 ±922.13	0.4212
$CD11b^+$ $CD27^-$	%	93.13 ±3.53	89.84 ±18.95	0.1978
	MFI CD27	74.66 ±40.83	60.68 ±38.50	0.2955
	MFI CD11b	2303.36 ±871.68	2475.15 ±954.72	0.7968
$CD11b^-$ $CD27^-$	%	5.94 ±3.24	6.19 ±7.88	0.3506
	MFI CD27	60.74 ±65.62	60.01 ±61.60	0.9230
	MFI CD11b	326.09 ±81.16	368.89 ±85.44	0.2337

Values represent mean ±SE (HC n=28; TB n=11). *p<0.05 compared to HC (Mann Whitney test).

The co-expressing cells for KIR2DS1 and KIR2DL1 ($CD158a/h^+$) are represented in histogram on Figure 13-2. Values are significantly different from values for isolated KIR2DS1. Levels of maturity are about the same as those observed for $KIR2DS1^+$ cells (Table 16).

These two KIR belongs to the same group, based on their structural characteristics (KIR2D type I) but belong to different haplotypes (KIR2DS1 – haplotype B and KIR2DL1 – haplotype A) (116).

Table 17 – Percentage of cells $CD16^+$ and in each CD27/CD11b subset for $CD158a/h^+$ cells in TB and HC. Means of fluorescence intensity for CD16, CD27 and CD11b.

		HC	TB	P
$CD16^+$	%	93.41 ±5.77	82.43 ±18.77	0.1119
	MFI CD16	1537.78 ±537.21	1123.30 ±536.24	0.0650
$CD11b^- CD27^+$	%	0.16 ±0.21	0.07 ±0.13	0.1662
	MFI CD27	2797.50 ±2611.08	1272.00 ±1744.33	0.1806
	MFI CD11b	97.37 ±64.81	183.73 ±73.99	0.0727
$CD11b^+ CD27^+$	%	3.99 ±4.46	21.73 ±30.40	0.1342
	MFI CD27	987.22 ±548.25	766.69 ±286.87	0.3551
	MFI CD11b	1938.67 ±656.11	2151.81 ±838.34	0.7771
$CD11b^+ CD27^-$	%	93.24 ±5.72	76.35 ±29.70	0.2893
	MFI CD27	16.22 ±45.88	35.89 ±61.52	0.3935
	MFI CD11b	2212.44 ±736.53	2313.15 ±1000.11	1.000
$CD11b^- CD27^-$	%	2.61 ±2.90	1.86 ±2.77	0.3509
	MFI CD27	18.99 ±27.06	37.56 ±61.53	0.7919
	MFI CD11b	101.40 ±31.32	116.66 ±73.22	0.3784

Values represent mean ±SE (HC n=27; TB n=9). Not significant data (Mann Whitney test).

CONCLUSION

Pulmonary tuberculosis still remains one of the greatest public health problems. In some research NK cells' importance have already studied, however the human NK cell phenotype in this pathology and in healthy contacts remains unclear. Several works have this NK cell characterization in animal models, and a few in human, but a wide research was needed.

Taken together the presented results for these NK cell characterization in both TB patients and healthy contacts, NK cells from TB patients suggest a higher maturation, a higher activation and also higher levels of cytokine (IFN-γ) production.

From the beginning, TB patients present a moderate lymphopenia ($p<0,05$) with a decreasing trend in all lymphocyte subsets. The exception was observed in B cells that tend to be increased and in T CD8+ cells that were significantly decreased ($p<0,05$).

As it was observed when lymphocyte subsets were evaluated and confirmed with other labeling, NK cells ($CD3^-CD56^+$) in TB patients tend to decrease, however, when $CD3^-NKp46^+$ NK cells were counted, it was observed a partial increase in TB patients. NKp46 was recently referred as better marker than CD56 for NK cells, however it was not found a correlation between this to markers nor in TB patients and in HC.

The classic markers for NK cells are CD56 and CD16. It is defined four subsets according the level of expression of these two markers in $CD3^-$ cells. Results suggest a higher percentage of NK cells in more mature stages in TB patients. The expression of CD27/CD11b in NK cells surface also define developmental subsets but presented data do not demonstrate differences between two groups.

CD57 is present in more mature NK cells, the expression of this marker were greatly increased in TB patients NK cells ($p<0.0005$). Despite CD27/CD11b, the higher

percentage of CD57+ cells and also the CD56/CD16 expression profile suggest a higher percentage of more mature NK cells in peripheral blood of TB patients.

It was observed a great increase in intracellular IFN-γ in total NK cells from TB patients ($p<0.01$). These increased values were also observed in NKp30, NKp44 and NKp80 positive subsets ($p<0.05$).

The co-expression of CD94 and NKG2D was also evaluated and a significant increase was observed in double positive subset ($p<0.05$) in TB patients. The meaning of these observations remains unclear.

Other NCRs and NKp80 (co-receptor) expression was increased (NKp44: $p<0.05$; NKp80: $p<0.01$; NKp30: n.s.). As referred above, it was observed an increase in intracellular IFN-γ^+ in all NK cells expressing these receptors and co-receptor.

Killer Immunoglobulin-like receptors expression was evaluated. Data demonstrate that three inhibitory KIRs (KIR2DL2, KIR2DL1 and KIR3DL1) tend to decrease, KIR2DL2/DL3 tend to increase and activating KIR2DS1 was significantly increased ($p<0.005$).

Despite the importance of the presented data, further research is needed. A general phenotyping of NK cells was performed, giving some highlights about the role of these cells in TB, opening new doors for the future work. KIR genotyping have to be done for the TB patients and HC for correlations to the phenotype results. Also the gene expression of KIR genes, cytokines, chemokines, as well as the SNP screening for NK cells receptors. Once NK cells crosstalk with other immune cells, the effect of NK cells on other cells (e.g. dendritic cells and monocytes) should be studied. Functional activation and inhibition tests should be performed to evaluate NK cells role in immune response against pulmonary TB.

Expansion and manipulation of NK cells for immunotherapy in infectious diseases is a promising field that remains to be explored.

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