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Role of NK Cells in Tuberculous Pleurisy as Innate Promoters of Local Type 1 Immunity with Potential Application on Differential Diagnosis

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1. Introduction

An efficient host immune response against pathogens encompasses both fast acting innate immunity as well as slower, but more specific, adaptive immunity. The innate immune system is diverse and comprises a variety of cells including natural killer (NK) cells, neutrophils, macrophages, dendritic cells (DCs), as well as soluble factors such as complement. The adaptive immune response is typified by antigen-specific T and B lymphocytes that provide long-lasting protection known as immunological memory. While these two systems are often discussed separately, neither arm of the immune system works in isolation (Medzhitov & Janeway, 1999). The succession of cells interacting with *Mycobacterium tuberculosis* (*Mtb)* comprises tissue macrophages (MΦ) and dendritic cells (DC) followed by chemokine-attracted immigrating neutrophils and monocytes, and then activation and recruitment of natural killer (NK) and γδ T cells, followed by effector T lymphocytes primed in the draining lymph nodes (Ulrichs & Kaufmann, 2006).

Pleuritis is the most frequent clinical manifestation of extrapulmonary tuberculosis (TB) among young adults, and is normally considered a relatively benign form of disease since it may resolve without chemotherapy (Light, 2010). Tuberculous pleurisy is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of *Mtb* infection, but it may also be developed as a complication of primary pulmonary TB infection (Antoniskis et al., 1990). The presence of mycobacterial antigens in the pleural space elicits an intense cellular immune response, initially characterized by abundant neutrophils and macrophages, followed by interferon (IFN)-γ-producing T-helper cell (TH) type 1 lymphocytes, resulting in lymphocyte-predominant exudative effusions (Aleman et al., 2005; Mitra et al., 2005; Porcel, 2009). The cellular trafficking is facilitated by homing surface markers and chemokine gradients. This intense but poorly understood local immune response is synonymous of Koch phenomenon and normally prevents the caseous evolution of lesions. The inflammatory process results in an increased pleural vascular permeability leading to the accumulation of fluid enriched in proteins and the recruitment of specific leukocytes into the pleural space, making this biological sample a physiologically relevant model of human tuberculosis infection (Kroegel & Antony 1997).

Given this effective local resistance, the few bacilli that enter into pleural cavity are rapidly destroyed turning impracticable the rapid diagnosis by direct microscopic observation (Porcel, 2009). This fact often results in the requirement of alternative diagnostics strategies like *Mtb* identification through cultures or PCR amplification of pleural effusions, histopathological examination of pleural biopsies, inflammatory related enzymatic activities and immunological based methods (Liang et al., 2008; Trajman et al., 2008). Among the last ones, detection of mycobacterial antigens, antimycobacterial specific antibodies (Ab), TH1 related biomarkers and *in vitro* evoked T cell responses are matter of active current research (Steingart et al., 2007; Budak et al., 2008; Dheda et al., 2009a; Supriya et al., 2008). Regarding the performance of T and B cell based assays in detection of active forms of disease; the most important trouble is the immunological memory background as result of previous Ag exposition or BCG vaccination (Dheda et al., 2009b; Hooper et al., 2009; Salazar-Lezama et al., 1997). In this line, for high antigen experienced populations, we hypothesized that an innate immune cell based diagnostic assay may circumvent those issues.

Among innate lymphocytes, Natural Killer (NK) cells display an important number of effector functions, including recognition and lysis of infected, stressed, or transformed cells and production of immunoregulatory cytokines, particularly IFN-γ (Vivier et al., 2011). Human NK cells account for 10–20% of peripheral blood lymphocytes and are defined by the presence of the CD56 and NKp46 molecules and the lack of CD3 and CD19 expression. Their activity is regulated by both positive and inhibitory signals from a wide range of germ line encoded cell surface receptors. Two major subsets of NK cells have been identified in humans according to CD56 and CD16 intensity expression and also in terms of chemokine receptors and adhesion molecules expression that differ in phenotype and function (Caligiuri, 2008). Functionally, $CD56^{bright}$ cells are effective cytokine producers, whereas $CD56^{dim}$ cells are efficient effectors of natural and antibody-dependent target cell lysis (Hanna & Mandelboim 2007). Together with the classical NK functions (i.e. cytotoxicity and cytokine production), novel skills have recently been described in niche-specific and in vitro-activated human NK cells. These unconventional capabilities include angiogenesis and tissue remodeling, immunological memory, functional cross-talk with T cells and direct pathogen recognition (Cooper et al., 2009; Di Santo, 2008; Vivier et al., 2011).

During the last years we have begun to characterize the phenotype and function of pleural NK from TB patients. In contrast to peripheral blood (PB) counterpart that are mostly composed by $CD56^{dim}CD16^{+}$ resting cells, pleural NK population is enriched in activated $CD56^{bright}CD16^{neg}$ cells that quickly and strongly respond to *Mtb* stimulation by producing IFN-γ (Schierloh et al., 2005a, 2007). Besides, *Mtb* stimulated IFN-γ production by NK shows Ag-specific features, given that pleural NK cells derived from non TB patients' lack of this response. According to these findings, we realized that pleural NK cells properties could be utilized in an innate immune cell based assay for differential diagnosis of tuberculous pleurisy (Schierloh et al., 2008).

2. Phenotype of pleural NK cells: Activated $CD56^{bright}$ NK cells

Along several studies, phenotype of tuberculous pleural fluid derived NK cells have been extensively analyzed as starting point in the understanding of their role at the site of active *Mtb* infection (Alvarez et al., 2010; Fu et al., 2011; Okubo et al., 1986, 1987; Pokkali et al.,

2009; Schierloh et al., 2005a, 2007, 2009). The finding that immunoregulatory $CD56^{bright}$ NK subset showing cellular activation features are strongly enriched among pleural NK cells was interesting given that these cells are known to link innate with adaptative immunity in a number of intracellular infections (Artavanis-Tsakonas et al., 2003; Culley, 2009; Fehniger et al., 2003). Along the present section we summarize these findings.

2.1 Enrichment of $CD56^{bright}$ NK cells in tuberculous pleural fluid

In tuberculous patients, T helper ($CD4^+/CD3^+$) is the predominant cell population among pleural fluid derived mononuclear cells (PFMC), which has been associated with a selective recruitment of antigen specific TH1 effectors cells to the site of infection (Li et al., 2010; Mitra et al., 2005). This increased T helper abundance account for the reduced percentage of pleural fluid NK cells ($CD3^-/CD56^+$) close to 5% in TB patients (Schierloh et al., 2005a). In contrast, in pleural effusions caused by cancer or paraneumonic infections, the percentage of NK among PFMC tends to be constant compared to peripheral blood (Dalbeth et al., 2004). However, when we analyzed the composition of NK cell populations, a drastic change in the proportion of these subsets was found (Schierloh et al., 2005a). In the case of tuberculosis, the cytotoxic $CD16^+CD56^{dim}$ NK subset, which represents more than 95% in the circulation, is reduced to less than 50% in the pleural effusions with a concomitant enhancement of the immuneregulatory $CD16^{dim/-}CD56^{bright}$ NK subset.

Phenotypic differences between $CD56^{brigth}$ NK with $CD56^{dim}$ include higher expression of the C-type lectin CD94/NKG2 family, weak expression of killer cell immunoglobulin (Ig)–like receptors (KIRs) and high levels of L-selectin (CD62L) and CCR7, both of which are involved in trafficking of immune cells to lymph nodes (Caligiuri, 2008). In agreement, the levels of CD94/NKG2A, CD62L and CCR7 were all augmented in tuberculous pleural NK cells (Schierloh et al., 2005a). On the other hand, the percentages of NK cells expressing the fractalkine receptor (CX_3CR1), the cytotoxic granular protein perforin and the HLA-C2 receptor KIR2DL1/S1, which are all specific markers for the cytotoxic $CD16^+CD56^{dim}$ NK subset, are reduced in the tuberculous pleural effusions (Figure 1).

2.2 Pleural NK cells exhibit activated phenotype

NK cells activation can be triggered via two primary mechanisms: cytokine stimulation and engagement of activating NK receptors. Together or in isolation, both activation signals can result in NK cell responses (Vivier et al. 2011). Because tuberculous pleural microenvironment is plenty of soluble mediators and cells with stimulatory potential (Shimokata et al., 1991; Valdés et al., 2009; Vankayalapati et al., 2000), we hypothesized that NK cells arriving to this site may turn activated. Indeed, we found an elevated percentage of NK cells expressing the early activation markers CD69 and HLA-DR together with enhanced expression of the lymphocyte function-associated antigen 1a integrin (LFA-1/ CD11a) and its ligand, intercellular adhesion molecule-1 (ICAM-1, CD54) (Schierloh et al., 2005a, 2005b, 2009). Simultaneously, we observed a subpopulation of pleural NK cells expressing Toll-like Receptor 2 (TLR2), a molecule undetectable in resting NK cells but up-regulated in response to IL-12 and protozoan glycolipids (Becker et al., 2003; Lindgren et al., 2010; Schierloh et al., 2007). Furthermore, we and others recently identified a subpopulation of pleural NK that down-modulates the CD45RA and up-regulates CD45R0 isoform,

resembling the well known phenomenon that takes places during memory differentiation of T lymphocytes (Warren et al., 1994; Fu et al., 2011).

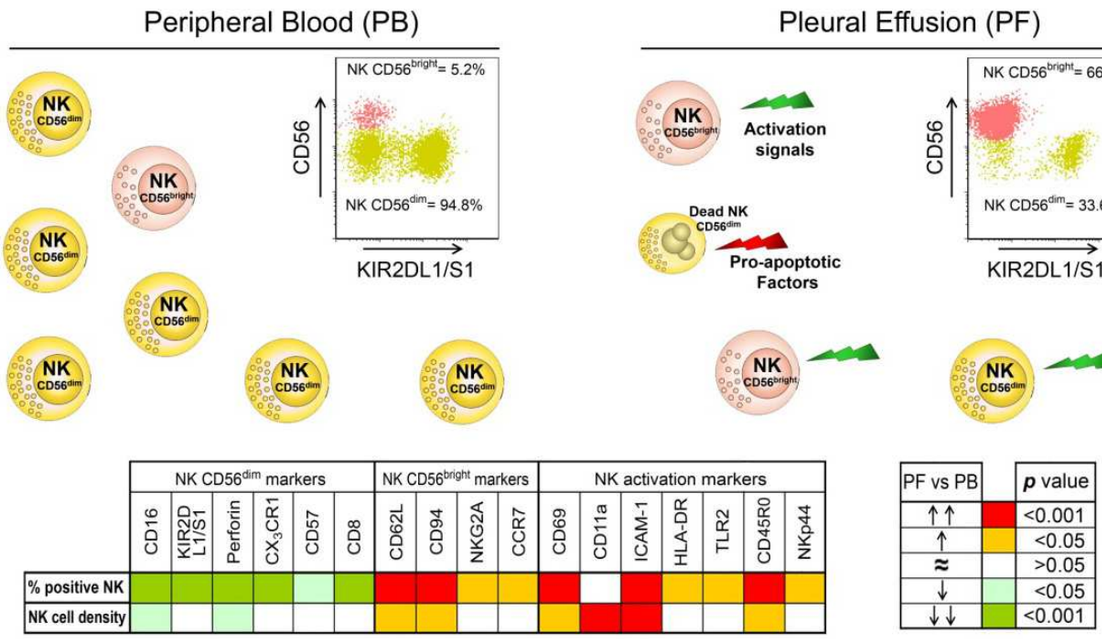


Fig. 1. Enrichment of NK CD56^{bright} cells with activated phenotype in tuberculous pleural effusions.

Immunophenotypic analysis of peripheral blood (PB) and pleural fluid (PF) NK cells (CD56^{+}/CD3^{-}). Dot plots are from one representative TB patient. Color scale indicates relative variations of several markers between PF and PB samples: invariant (white), augment (orange), strong augment (red), reduction (light green) and strong reduction (dark green).

2.3 Pleural fluid factors inducing CD56^{dim} apoptosis explain the altered NK subset ratio

In order to understand the causes of altered CD56^{bright}/CD56^{dim} subset ratio observed on pleural NK population, we hypothesize that soluble factors (i.e.: chemokines, cytokines, pathogen derived factors, immune complexes, etc.), present at the site of *Mtb* infection, may differentially affect the migration, the differentiation, the proliferation or the apoptosis of NK subsets. Our experimental approaches directed to test this were conducted by incubating peripheral resting NK cells with tuberculous cell-free-pleural fluid or purified factors (Schierloh et al., 2005a). Indeed, CD56^{dim}CD16^{+} cells show an increased susceptibility to pleural fluid induced caspase 9 dependent-apoptosis, explaining the predominance of the CD56^{bright} population. These finding were later confirmed in different experimental settings. These studies demonstrate that NK CD56^{bright} subset has larger resistance to oxidative stress (Harlin et al., 2007; Thorén et al., 2007). Our experiments directed to test if NK CD16^{+} cells could be differentiated to CD16^{-} NK cells by cytokines present at the site of infection gave negative results, in accordance with other group (Dalbeth et al., 2004). Additionally, a recent

report indicates that differential migration of NK cell subsets to the site of infection also take place (Pokkali et al., 2009).

3. Function of pleural NK cells: IFN-γ production and TH1 cell co-stimulation

Considering that type 1 cytokine and chemokine profile is a hallmark of tuberculous pleurisy (Dheda et al., 2009a; Kroegel & Antony 1997; Li et al., 2010; Mayanja-Kizza et al., 2009; Trajman et al., 2008), and given that pleural NK cells exhibit an endogenously induced activation state together with an enrichment of immunoregulatory NK CD56^{bright} subset, we asked whether these cells were polarized to the production of pro and/or anti-inflammatory cytokines. Also, we evaluated if these cells were capable to modulate other cells function. The answers to these questions gave us important clues for better understanding the immunopathogenesis of TB infection and will be discussed during the present section.

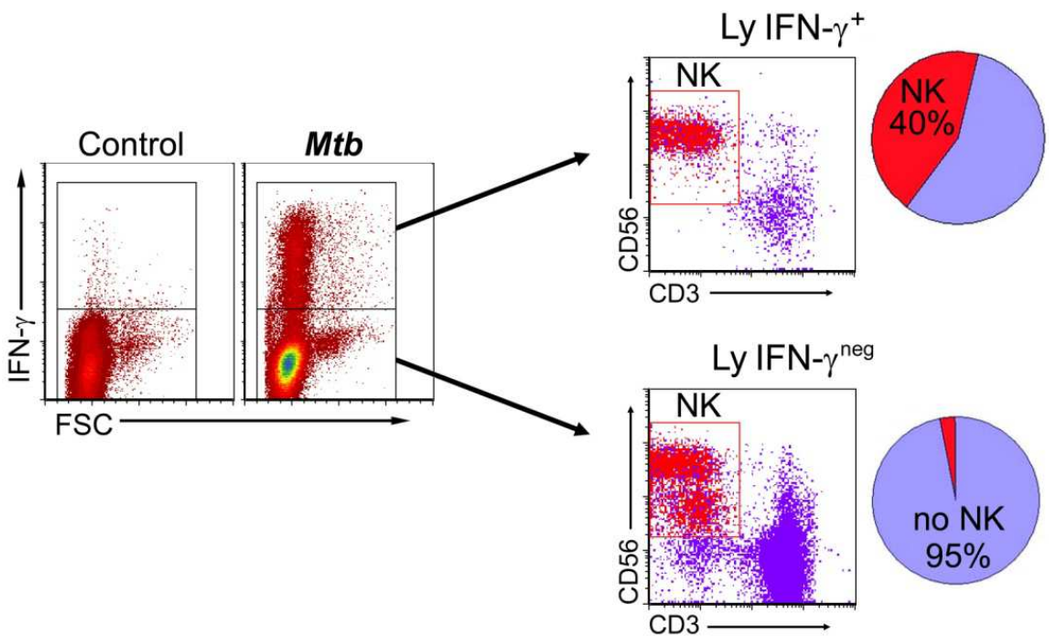


Fig. 2. Pleural NK cells are a major early source of IFN-γ upon ex vivo Mtb-stimulation.

PFMC were stimulated with *Mtb* for 24h .Then cells were gated on the basis of IFN-γ-positive (Ly IFN-γ^{+}) and IFN-γ-negative (Ly IFN-γ^{neg}) cells and their surface phenotype were determined according to CD56 and CD3 expression by flow cytometry. Dot plots are from one representative TB patient. NK cells are highlighted in red. Pie charts indicate mean values of 30 TB patients.

3.1 Pleural NK cells strongly produce IFN-γ after *Mtb* stimulation

Experiments varying the quality and quantity of signaling input for NK cell activation have revealed a hierarchy in requirements for induction of chemokines and cytokines (Fauriat et al., 2010). Furthermore, recent data indicate that anatomical niche and developmental stage of NK cells strongly determine its cytokine profile production giving rise to NK1, NK2 and

NK22 cells (Di Santo, 2008; Spits & Di Santo, 2011). In this sense, pleural fluid $CD56^{bright}$ NK did not produce significant levels of IL-10, IL-17A or TNF-α spontaneously or after γ-irradiated *Mtb*-stimulation. However, the percentage of IFN-γ^+ NK cells (NK IFN-γ+) was strongly increased under the same experimental conditions (Schierloh et al., 2005a). It is interesting to note that, in spite of its reduced numbers in TB pleural fluid (~5% of PF lymphocytes), NK constitute a major source of IFN-γ together with $CD3^+$ cells (Figure 2). *Mtb* derived culture filtrate proteins (CFP), TLR2 and 4 agonist and recombinant IL-12 also induced IFN-γ among pleural NK but to a lesser extent than *Mtb* does (Schierloh et al., 2007). Interestingly, pleural fluid NK cells derived from other etiologies (i.e: cancer, paraneumonic or helmintic infections) did not give *Mtb*-stimulated IFN-γ responses, suggesting a paradoxical "Ag specific response" induced in an innate immune cell (see Figure 4). This finding is in accordance with recent data provided by experimental mice models which clearly demonstrate the adaptative properties of NK cells (Cooper et al., 2009; Paust & von Andrian, 2011; Vivier et al., 2011). Instead, this apparent "Ag specific" NK cell responses may be produced by T cell-secreted IL-2 (Horowitz et al., 2010; Fehniger et al., 2003).

3.2 Pleural NK cells co-stimulate local TH1 response

Among novel skills described for human activated NK cells, it has been shown that they may stimulate T cells by cell contact-dependent mechanisms (Hanna & Mandelboim 2007). In this line, we were able to demonstrate that, in TB pleurisy, a functional ICAM-1-dependent cell to cell interaction among pleural fluid NK and T cells lead to T cell activation (Schierloh et al 2009). Likewise, peripheral blood human NK cells can instruct *in vitro* cytotoxic $CD8^+$ T cells from PPD responsive donors to lyse *Mtb*-infected monocytes (Vankayalapati et al., 2004). Taken together, these findings suggest a previously unappreciated role of NK cells in the maintenance and/or activation of T cell functions during the immune response in tuberculosis.

4. Cellular and molecular factors controlling pleural NK cells functions

Having observed that NK cells were the main early source of IFN-γ within the pleural space, we investigate extracellular events and signaling pathways that drive this process. The mechanisms involved reveal classical and particular ways of NK cell activation and signaling. Three environmental signals act in concert to fulfill IFN-γ response in pleural NK cells: cytokines, activation ligands expressed on accessory cells and direct *Mtb* recognition (Schierloh et al., 2007).

4.1 Pleural NK response involve Ca^{2+} influx and Calcineurin, ERK and p38 MAPK signaling pathways

NK cells express on their surface an array of germ-line encoded inhibitory and activating receptor as well as cytokine receptors that, upon activation, mediate intracellular signaling pathways for IFN-γ together with other functional responses. It is well known that interaction of NK receptors cells with their activating ligands on target and/or Ag presenting cells (APC) induce a quick increase of cytoplasmic calcium (Ca^{2+}) concentration, a universal second messenger (Maghazachi, 2005). Employing divalent cation chelators we

demonstrate a pivotal role for this process during *Mtb*-induced pleural NK IFN-γ response. Downstream the Ca^{2+} influx, signaling proteins and their target transcription factors are activated, including calcineurin, a calmodulin-dependent serine/threonine phosphatase, and its target NFAT (nuclear factor of activated T cells). By mean of Cyclosporin A treatment, we confirm the involvement of this pathway too. Similarly, using several protein kinase specific inhibitors and phospho-specific monoclonal antibodies, we also address the participation of other two phosphorylation cascades, p38 MAPK and ERK1/2 (Schierloh et al., 2007). Figure 3 summarize the most important events.

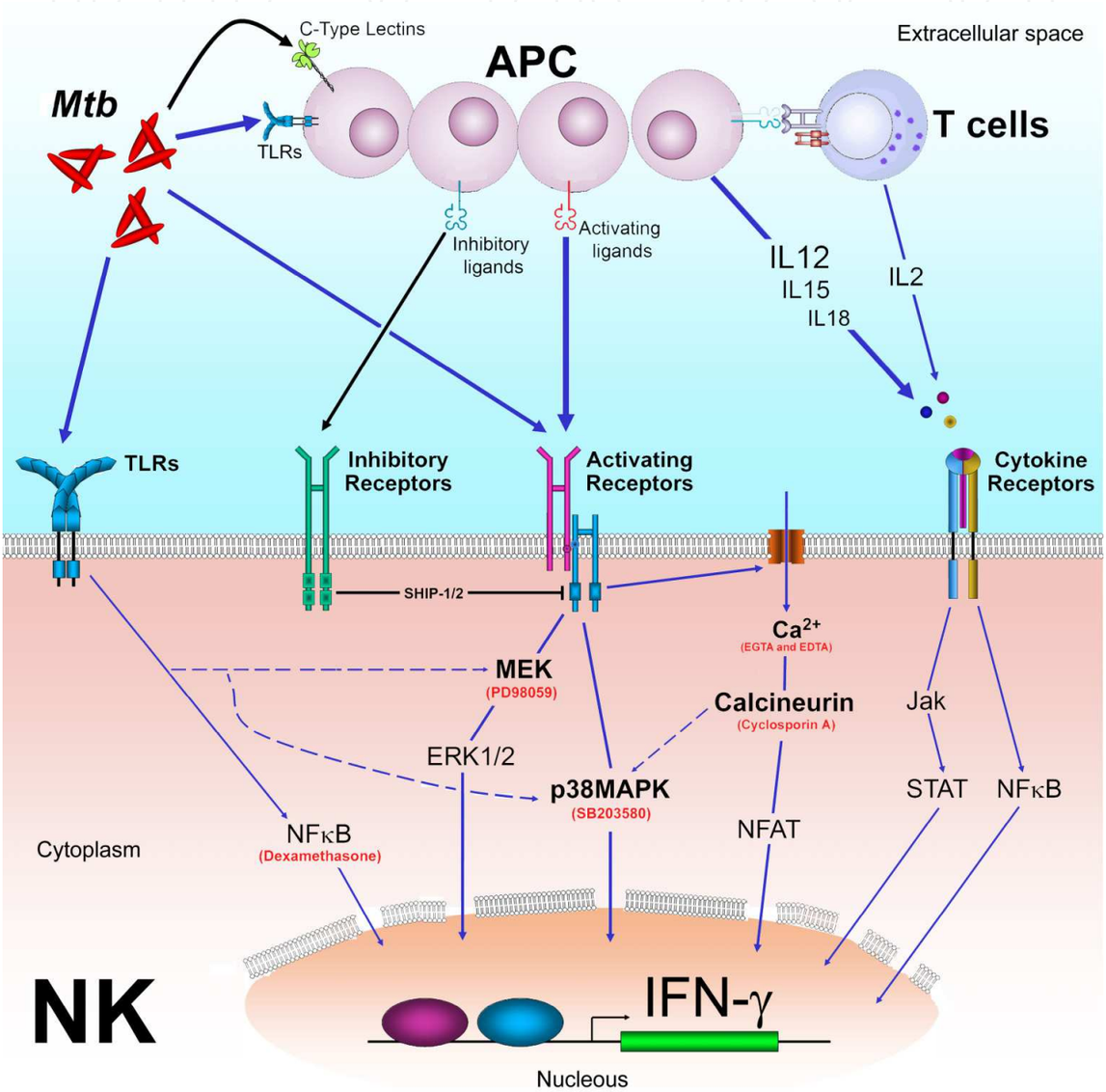


Fig. 3. Extracellular signals and intracellular pathways in IFN-γ production by pleural NK cells.

Picture describe interactions among activation signals (i.e: cytokines, APC expressed activating ligands and *Mtb*), modulating/inhibitory signals (i.e: APC expressed inhibitory

ligans and *Mtb* derived lipoglicans recognized by C-type lectins) and cellular receptors together with their corresponding intracellular signaling pathways. Blue arrows indicate positive interactions. Dotted blue arrows denote cross-talk between pathways. Black arrows denote inhibitory interactions. Pharmacological inhibitors used in our experiments are written in red within brackets under its target molecule.

4.2 Pleural NK response is dependent on IL-12 and accessory cell contact

Numerous *in vitro* and *in vivo* studies show that a wide variety of APC secreted cytokines, especially IL-12, IL-15, and IL-18, can activate NK cells to produce IFN-γ. By means of cytokine neutralization, we showed that IL-12 is necessary for *Mtb*-induced NK IFN-γ production in tuberculous pleurisy (Schierloh et al., 2007). This requirement is not surprising because IL-12 has been shown to mediate bystander activation of NK cells in response to a number of different pathogens (Artavanis-Tsakonas et al., 2007; Culley, 2009, Lindgren et al., 2011). However, IL-12 alone does not allow the same level of IFN-γ^+ NK cells than *Mtb* does. Indeed, costimulatory signals delivered by APC such as receptor-ligand interactions are required as demonstrated by Ab blockade and APC depletion experiments. Among activating ligands ICAM-1, CD86 and Vimentin has been shown to be engaged. At the same time, these signals could be counter-balanced by MHC class I, PD-L1 and PD-L2 inhibitory ligands (Alvarez et al., 2010; Garg et al., 2006; Schierloh et al., 2007).

At the level of pleural APC, we also observed that *Mtb* induced stimulatory signals mediated by TLR2 and TLR4 are counter-modulated by C-type lectin receptors like mannose receptor (MR) and DC-SIGN (Schierloh et al., 2007). Both molecules bind mycobacterial derived mannosilated lipoglycans and may indirectly inhibit NK cell response by limiting IL-12 production and/or down-modulating activating ligands expression (van Kooyk Y & Geijtenbeek, 2003).

4.3 Pleural NK cells directly recognize *Mtb*

Recent studies have pointed out the capacity of NK cells to bind and been directly activated by *Mycobacterium* species (Esin et al., 2004; Evans et al., 2011; Watkins et al., 2008). This recognition seems to be mediated, at least, by two putative activating receptors: TLR2 and NKp44 (Esin et al., 2008; Marcenaro et al., 2008). Interestingly, these receptors are both up-regulated among pleural NK cells (Figure 1). Consistently we observe that, compared with its PB counterparts, pleural NK cells have enhanced capacity to bind *Mtb* and that p38 MAPK phosphorilation on pleural NK occurs shortly after *Mtb*-NK coculture, independently on bystander cell derived signals (Schierloh et al., 2007). Furthermore, it has been observed that NK $CD56^{bright}$ cells are more reactive to direct BCG stimulation than $CD56^{dim}$ NK (Batoni et al., 2005). Altogether, these results strongly indicate that direct interaction between NK and *Mtb* play a significant role during functional response of pleural NK $CD56^{bright}$ cells.

5. Pleural NK application: Immunodiagnosis of tuberculous pleurisy

Conventional diagnostic tests for pleural TB include microscopic examination of pleural fluid for acid-fast bacilli and differential cytology; mycobacterial culture of pleural fluid, sputum or pleural tissue; pleural fluid Adenosin Deaminanse (ADA) activity as well as

determination and histopathological examination of pleural tissue looking for granulomatous inflammation (Porcel, 2009; Light, 2010). These tests have limitations for clinical use; however, in combination, they have been recognized as the best reference standard for evaluation of the accuracy of novel tests (Trajman et al., 2008). Although detection of serum antibodies against *Mtb* antigens is known to have poor and highly variable sensitivity and specificity, attempts have been made to detect antibodies in pleural fluid by ELISA. Even though, these tests show high specificity they are limited by the very poor sensitivity (Weldingh & Pai 2007). Several T cell based assays have been employed for diagnosis of TB pleurisy such as in vitro stimulation of lymphocytes with PPD or RD-1 encoded antigens leading to T-cell proliferation and/or IFN-γ release by ELISPOT and ELISA assays (TIGRAs) (Hooper et al., 2009). For example, using a commercially available *Mtb*-specific ELISPOT for peripheral blood mononuclear cells and pleural fluid mononuclear cells from patients with exudative pleurisies, its sensitivity in active tuberculosis was very high (95%); however, the specificity was suboptimal (76%) (Losi et al., 2007). The high coverage of BCG vaccination as well as the high prevalence of latent TB infection (PPD^+ individuals) at the population level might impair the results when employing T and B cell based immunodiagnostic methods. On the other hand, immunocompromised or HIV^+ infected patients may lead to false negative results due to the ablation or reduction of cellular immunity (Trajman et al., 2008). In this context, we thought that pleural IFN-γ^+ NK cells could be a promising target for immunodiagnostic method that circumvent these memory related problems in differential diagnosis of tuberculous pleurisy.

5.1 Preliminary trial for testing clinical value of NK cell based assay in Argentina

In order to provide evidence that support or reject the clinical diagnostic utility of NK IFN-γ^+ cell based assay in differential diagnosis of TB pleural effusion, we have performed a retrospective, single-center preliminary study in a reference center of the city of Buenos Aires, Argentina. To do this, PFMC were stimulated with *Mtb* and the percentage of NK and T cells expressing IFN-γ were determined by flow cytometry as described in Figure 2. In this trial we included 40 consecutive patients with profuse exudative pleural effusion (TB n=28 and No-TB n=12; Cancer=6, Paraneumonic infection=5, Helmintic infection=1) admitted and diagnosed at the Tisioneumonolgy service of the Hospital Muñiz during 2006-2009. According to epidemiological data provided by medical staff, all the patients were at high relative risk of TB infection. As can be observed in table 1 and Figure 4, pleural NK shows

	Median Δ % IFN-γ^+ (25–75% percentil)	**Cut off** (Max. likelihood ratio)	**Area under ROCurve** (95% CI)	**Sensitivity** (95% CI)	**Specificity** (95% CI)
NK assay	30.69% (9.23–42.77%)	> 1.555% (11.57)	0.9643 (0.9002–1.028)	96.43% (81.65–99.91%)	91.67% (61.52–99.79%)
T assay	2.14% (1.04– 4.63%)	> 1.230% (8.14)	0.9435 (0.8670–1.020)	67.86% (47.65–84.12%)	91.67% (61.52–99.79%)

Table 1. ROC analysis for NK cell and T cell assays. Data values summarized above are derived from ROC analysis depicted in Fig.4.

better performance than pleural T cell based assay in discriminating TB and no-TB pleural effusions. These differences may reflect the polyclonal vs oligoclonal nature of IFN-γ response in NK compared to T cells (Schierloh et al., 2008).

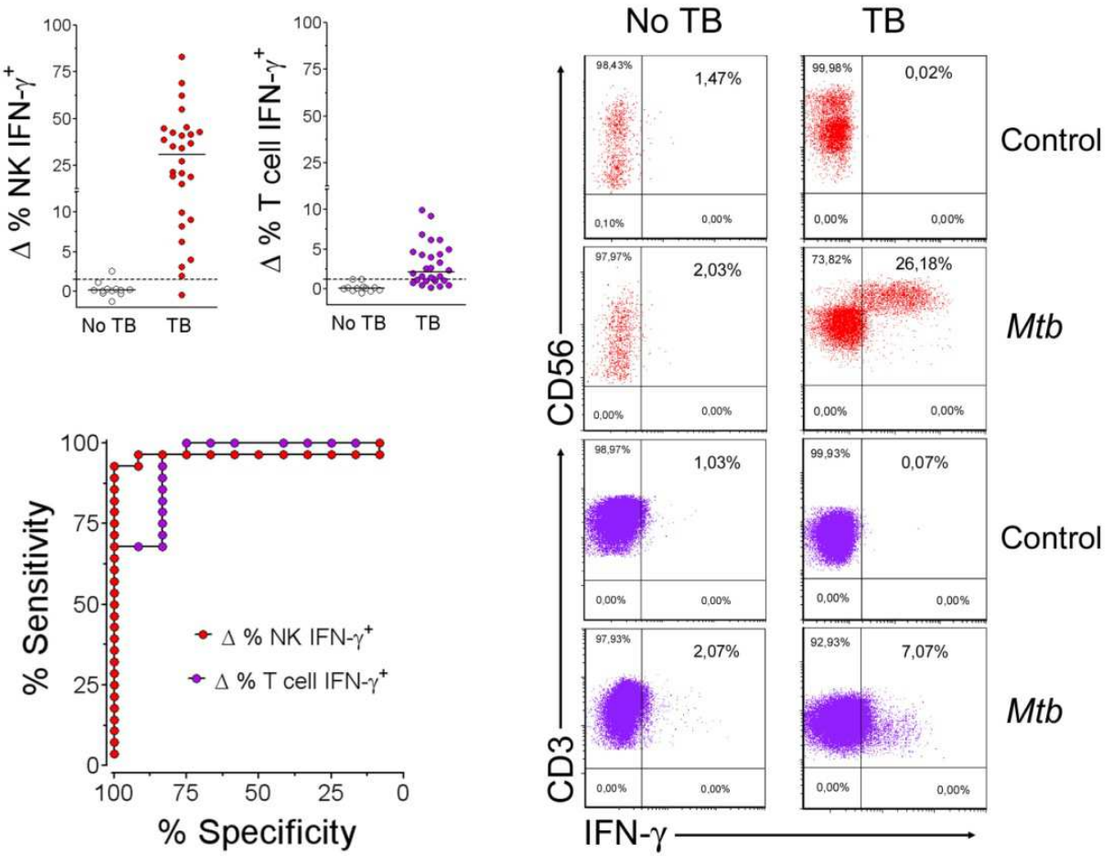


Fig. 4. Mtb-induced IFN-γ response by NK and T cells in the diagnosis of tuberculous pleurisy.

PFMC were incubated 24h with medium alone (Control) or stimulated with *Mtb*. Then, *Mtb* specific IFN-γ response was obtained from gated NK ($CD56^+/CD3^-$) and T cells ($CD3^+$) by subtracting the percentage of spontaneous production: **Δ% IFN-γ⁺= % IFN-γ⁺ *Mtb* - % IFN-γ⁺ control**. Upper left scatter plots depict the results of the study population (TB patients n=28; No-TB patients n=12). Lower graph present ROC analysis for NK and T cell based assays. Flow cytometry dot plots analyses are from one representative TB patient and one patient with helmintic (*Echinococcus granulosus*) infection (No TB). Red dots are gated NK ($CD56^+/CD3^-$) and purple dots are T cells ($CD3^+$).

5.2 Rational design for NK based IFN-γ release assay: "KIGRA"

Flow cytometers are sophisticated equipments that may be not available in almost all public health laboratories. Therefore, in order to make our NK derived IFN-γ based assay more applicable for common clinical settings, we attempt to introduce experimental modifications that could direct the development of a NK based IFN-γ release assay.

Unlike flow cytometry, cytokine release detection devises did not allow the identification of secreting cell. This fact represents an important challenge in our particular case, where the cellular source of IFN-γ is the basis of the diagnostic improvement. One way to avoid this issue is blocking IFN-γ production by pleural T cells, leaving NK as the main producer cells. To do so, we employed anti-HLA class I and class II monoclonal antibodies (mAbs), which block Ag presentation to $CD8^+$ and $CD4^+$ T cells. As can be observed in Figure 5, most T IFN-γ^+ cells were inhibited when both mAbs were present during *Mtb* stimulation; however, NK cells still remain expressing IFN-γ^+ under the same treatment. Similarly, when we analyzed IFN-γ release by ELISA, the presence of mAbs diminished but not abolished the secretion of this biomarker. This result, together with other adjustments that are under current testing, may constitute the rational for a more accurate assay.

Furthermore, we think that in the context of diagnosis of tuberculous pleurisy, commercially available IGRA, which employ ELISPOT (Losi et al., 2007), ELISA (Losi et al., 2011) or immunochromatography (Corstjens et al., 2008), could be easily adapted in order to obtain a Natural Killer IFN-gamma release assay or "KIGRA".

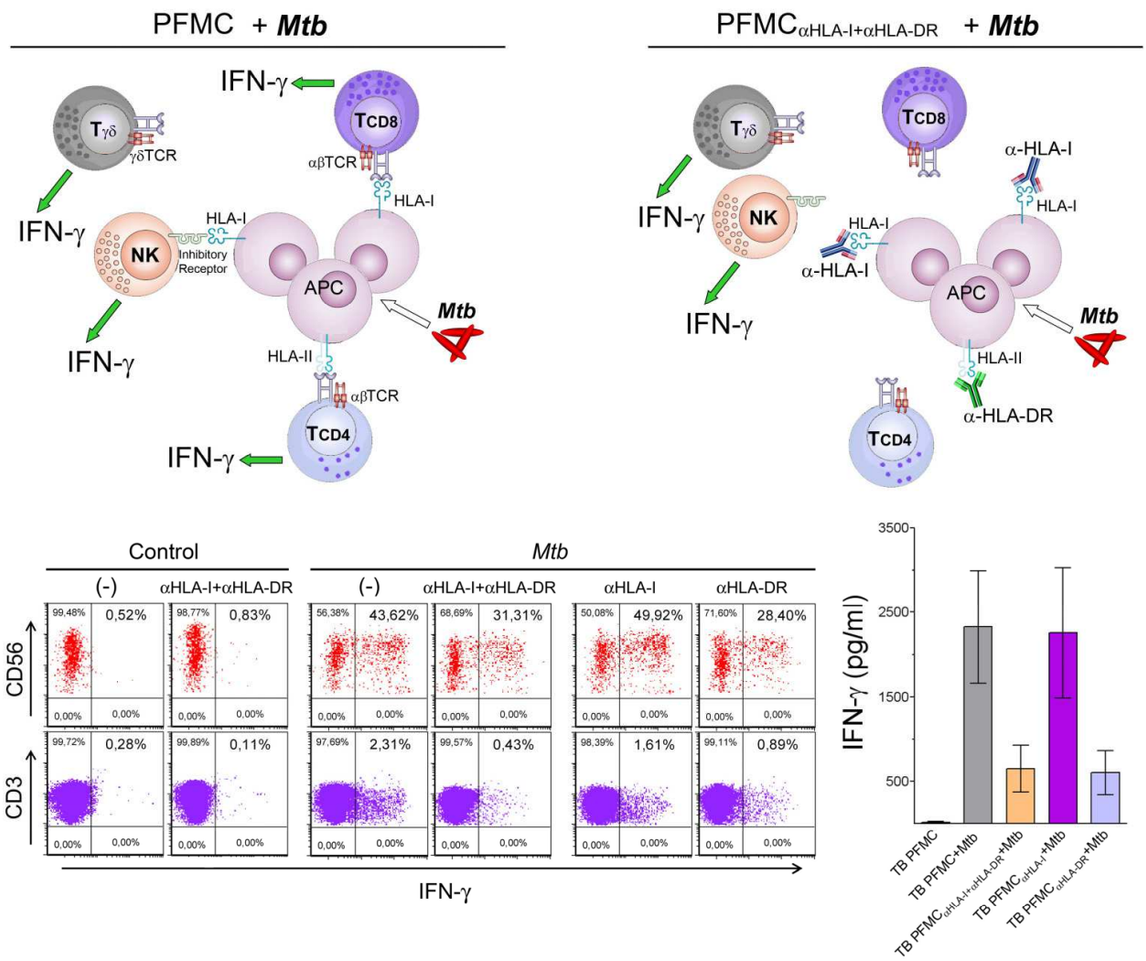


Fig. 5. Rational design for development of NK IFN-γ release assay.

PFMC were *Mtb*-stimulated as indicated in Fig.4 with or without the addition of anti-HLA-class I and/or anti-HLA-class DR mAbs. A schematic explanation of how mAbs interfere on antigen presentation to $CD4^+$ and $CD8^+$ T αβ cells without affecting NK or T γδ cells activation (upper cartoon). Flow cytometry dot plots analyses are from one representative TB patient. Red dots are gated NK ($CD56^+/CD3^-$) and purple dots are T ($CD3^+$) cells. Bar graph of IFN-γ ELISA assay (TB n=3).

6. Conclusion

Tuberculous pleurisy, one of the most common extrapulmonary manifestations of tuberculosis among young adults, is characterized by strong delayed type hypersensitivity reaction mediated by effector lymphocytes. Our data demonstrate that a substantial part of these cells are indeed NK cells. Herein, we have discussed phenotypic and functional features of this local innate immune cell population and the factors involved in its regulation. Furthermore, we identify NK cells as the main source of IFN-γ, the most widely used TB biomarker, in the context of tuberculous pleurisy.

In the context of high antigen experienced population the diagnosis that allow the discrimination between tuberculous pleurisy from other exudative pleural effusions remain as an unresolved clinical issue (Dheda et al., 2009b; Hooper et al., 2009; Salazar-Lezama et al., 1997). Hence, we propose an NK cell IFN-γ based assay as complementary procedure. To our knowledge, no previous NK inmunodiagnostic were reported for TB or any other infectious diseases. The advantageous characteristics of this functional assay are: (i) short time result output (1 day) and ii) very good performance in terms of specificity and sensitivity.

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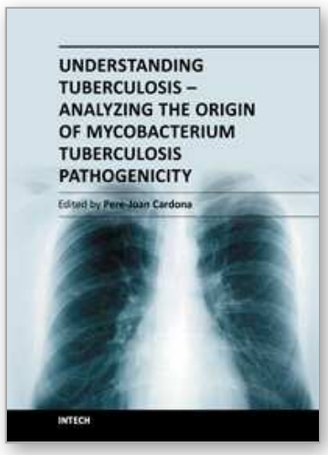
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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

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