Functionally relevant decreases in activatory receptor expression on NK cells are associated with pulmonary tuberculosis \textit{in vivo} and persist after successful treatment

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Abstract

Correlates for the initiation of \textit{Mycobacterium tuberculosis hominis} (Mth) replication from latency are needed in order to improve Mth control. In order to analyze if perturbations of peripheral NK cells may be associated with exit from Mth latency, sequential patients with newly diagnosed lung tuberculosis (TB) were studied. Peripheral NK cells were analyzed by cytofluorometry, \textit{in vitro} culture and functional assays. At the onset of lung TB, imbalances in NK cell subsets were evident. Decreased CD56\(^{bright}\)CD16\(^{-}\) subsets with significantly compromised NKp30 and NKp46 expression and with specifically decreased \(\gamma\)-IFN production upon triggering were evident. These features were not completely restored when purified NK cells were cultured \textit{in vitro}. Culture supplementation with \(\alpha\)-IFN increased only NKp30 expression in TB and healthy donors. Extensive peripheral NK cell triggering was evident in these patients, as shown by the expression of NK cell activation markers and of the lymph node-homing chemokine receptor CCR7 on CD16\(^{-}\)CD56\(^{dull}\) cells. Significant persistence of decreased NKp30 and NKp46 after successful treatment with a standard four-drug regimen was detected after full recovery. NK cell function is deeply affected in patients at the onset of pulmonary TB. The involvement of multiple activatory receptors may provide a relevant contribution to the spread of mycobacteria exiting from latency.

Introduction

\textit{Mycobacterium tuberculosis hominis} (Mth) infects one-third of the world population and represents the leading cause of death due to infectious diseases exacting an annual global toll of 8.8 million new cases and 1.6 million deaths (1). While only 5–10\% of newly infected patients will develop active disease within 2 years, latent infection will eventually determine active disease at a rate of 0.1–0.5\% per year (1, 2). Several immune mechanisms contribute to the specific control of Mth after infection and during the latent phase of the disease including responses mediated by CD4\(^+\)TCR\(\beta\)\(^+\), CD8\(^+\)TCR\(\beta\)\(^+\) and TCR\(\gamma\)\(^+\) T cells (3–6). However, the mechanisms underlying loss of Mth control in patients developing pulmonary tuberculosis (TB) after initial invasion or after a latent phase are largely unexplored.

Cells of the innate immune compartment contribute to host defenses against intracellular pathogens including human immunodeficiency virus type1 (HIV-1) and hepatitis C virus (HCV). In these infections, functional defects of monocyte-derived dendritic cells (mDCs) and of plasmacytoid dendritic cells (pDCs) are described and may persist also during specific treatment (7–10). A reciprocal interaction occurs between NK and dendritic cells (DC). Immature dendritic cells (iDCs) activate resting NK cells. NK cells in turn provide support for iDC maturation while at the same time they kill iDC but spare...
mDC, thus performing DC ‘editing’ on the basis of their degree of maturation (11, 12). A critical feature in the interaction of NK cells with DCs is represented by the expression of NKp30 and DNAM-1-activating NK receptors (12, 13). Lysis of iDC occurs through the interaction of these receptors with their ligands on DCs. Clinical conditions characterized by decreased expression of NKp30 on peripheral NK cells are associated with decreased NK cell function, as in the case of chronic HIV-1 and acute myelogenous leukemia (14–16). Correspondingly, iDCs are subjected to a decreased NK-mediated lysis and editing, contributing to altered shaping of adaptive immune responses (17, 18). Also during other chronic infections including HCV and cytomegalovirus, altered NK cell function is associated to poor pathogen control (13, 19).

Different sets of observations support the notion that NK cells may be involved in the control of mycobacterial infections. First, NK cells from healthy donors (HDs) can directly respond to products via toll-like receptor 2 (20). Moreover, NK cells respond to mycobacteria-infecting mDCs (21). A direct link between NK cells and Mtb-infected cells has been shown to be mediated by the interaction of NKp46 and NKG2D-activating receptors on NK cells with infected monocytes (22). Evidences for NK-mediated control of Mtb replication are provided by experiments using cells from HDs showing their promotion of intracellular killing of mycobacteria (23, 24) and by their ability to lyse monocytes infected with Mtb, Mycobacterium tuberculosis bovis Bacille Calmette–Guerin or Mycobacterium avium intracellulare (25–27).

In addition, in patients with latent TB, NK cells have been reported to play a fundamental role in the maintenance and regulation of mycobacteria-specific CD8+ CTLs (28). Decreased antigen-specific CTL responses have been observed in patients with latent TB after NK cell depletion (28). Remarkably, CD8+ CTLs are critical for the maintenance of latency, as shown by the results of a depletion experiments in mice (29). In addition, the recent description of reduced mDC and pDC numbers and function in patients with TB (30) suggests that this, in turn, may impair NK cell function (e.g. through impaired production of cytokines promoting NK cell activation such as IL-12 and IL-15) contributing to reduced lysis of Mtb-infected macrophages which are subjected to the activation of anti-apoptotic pathways by Mtb (22, 31).

So far, evidences describing the role of NK cells in the containment of mycobacterial disease derive largely from in vitro studies on cells obtained from HDs (22–27). NK cells have been studied in districts where they accumulate during TB serositis using non-specific markers (32). There is however no study on NK cell phenotype and function at the very onset of disease, at or around the moment when mycobacteria are thought to exit latency and begin active replication with tissue spread and disease manifestations.

In the present study, we analyzed the possibility that defects in NK cell function might be associated with the loss of control of Mtb replication by the immune system leading to overt disease in patients with recently diagnosed pulmonary TB. We show for the first time that specific NK cell defects are indeed present in TB patients at onset of the disease. These findings document alterations of innate immune mechanisms that could provide a clue to understand the immunological mechanisms responsible for the loss of Mtb control and the development of overt disease.

Methods

Patients

Patients were admitted to Respiratory Unit, University of Genoa or at Infectious Diseases Unit, S. Martino Hospital of Genoa due to pulmonary infiltrates of recent onset, cough of chronic duration and deteriorating clinical conditions. In all cases, lung TB diagnosis was based on positive sputum, culture for Mtb with concordant lung radiography or computed tomography (CT) and symptoms. Patients with concomitant conditions including autoimmune disease, HIV infection, cancer, pregnancy, extrapulmonary TB and other systemic disease were excluded from the present analysis. Informed consent was obtained for blood sampling. Fifteen consecutive patients diagnosed with pulmonary TB were studied at the moment of diagnosis. Twelve patients had drug-sensitive Mtb infection, while three patients were excluded when anti-mycobacterial testing results became available, on the basis of multi-drug resistant Mtb infection (one case) and extended-drug resistant Mtb infection (two cases). Patients were Hispanic (four cases) or Caucasian (eight cases), with a median age of 46 years. In all cases, lung infiltrates with variable degrees of cavitation were present at the time of diagnosis, without extensive or predominant serositis. All the patients were treated according to the current guidelines with four drugs during the first 2 months (isoniazid, rifampin, pyrazinamide and ethambutol) and two drugs for other 4 months (isoniazid and rifampin). All patients were treated successfully and recovered fully. Peripheral blood (20 ml) was drawn for the purpose of the study at the beginning of anti-mycobacterial treatment and after 6 months of treatment. All patients were treated successfully according to the guidelines (33). Absence of symptoms, absence of signs of systemic inflammation and improvement of chest CT were verified after 6 months of treatment and after 6 months of stopping treatment. The control group consisted of 10 healthy uninfected age and sex-matched volunteer HDs from the local blood bank.

Cell cultures

PBMCs were obtained by gradient centrifugation (Ficoll-Hypaque) and cryopreserved until processed. NK cells were isolated from PBMCs by negative immunomagnetic separation using NK isolation Kit II (Miltenyi, Bergisch Gladbach, Germany). As determined by cytofluorimetric analysis, using anti-CD3 mAb (IgG1, UCHT-1), NK cell population purity was >95%.

After purification, NK cells were immediately analyzed or used to obtain in vitro-activated NK cell populations in the presence of irradiated mononuclear cells (5000 rad) rIL-2 (100 U ml⁻¹; Proleukin, Chiron Corp., Emeryville, CA, USA) and 1.5 ng ml⁻¹ PHA (Sigma, St Louis, MO, USA).

In some experiments, polyclonal NK cell populations were cultured in the presence of additional α-IFN 100 U ml⁻¹ (PeproTech, London, UK).
Culture medium was RPMI 1620 (BioWhittaker, Lonza) supplemented with 10% FCS, l-glutamine (2 mM) and 1% antibiotic mixture (Penicillin–Streptomycin 5 mg ml⁻¹).

Antibodies

The following panel of mouse anti-human mAbs was used: anti-CD3APC, anti-CD19APC, anti-CD14APC, PE and FITC conjugate anti-CD16 (BD Pharmingen, San Jose, CA, USA), anti-CD56PC7 and anti-CD83FITC (ImmuneTech-Coulter, Marseille, France), anti-CCR7APC, anti-NKG2C and anti-CCR3 (R&D Systems, Inc., Minneapolis, MN, USA), Anti-NKp46 (IgG1) BAB281, anti-NKp30 (IgG1) AZ20, anti-NKp44 (IgG1) ZIN231, anti-NKG2D (IgG1) BAT, anti-DAAM-1 (IgG1) F22, anti-LIR-1 (CD85j) (IgG1) F278, anti-KIR2DL2/ S2 (CD158b1/CD158j), anti-KIR3DL1 (CD158e1), anti-KIR2DL1/S1 (CD158b/CD158h) (mixture GL183, Z27, 11pb6, IgG1), anti-NKG2A (IgG2b) Z199, anti-CD69 (IgG1) c227 and anti-CXCR3 (IgG1) are all produced in our laboratory. Anti-HLA-DR (IgG2a) D1.12 was kindly provided by Dr R. S. Accolla (University of Insubria, Varese, Italy).

FITC-conjugated (Southern Biotechnology, Birmingham, AL, USA) and PE-conjugated goat anti-mouse isotype antibodies (BD Pharmingen) were purchased. For intracellular staining, anti-γ-IFNAPC and anti-CD107a-PE (BD Pharmingen) were used.

Immunofluorescence analysis

Samples were analyzed by four-color cytofluorometry on PBMCs and two- or three-color cytofluorimetric assay on purified NK cells or activated NK cell population. Briefly, 3 × 10⁵ PBMCs were incubated with primary mAbs, followed by PE- or FITC-conjugated anti-isotype-specific goat anti-mouse secondary reagents. Fluorochrome-conjugated mAbs were used after the indirect staining procedures. All cells were gated by forward and side scatter parameters and on exclusion of CD3+ CD14+ and CD19+ cells, analyzed on FACSCalibur (Becton Dickinson, Mountain View, CA, USA) with Cell Quest Pro program for fluorescence analysis. Mean fluorescence intensity (MFI) represents a linear measure and is represented with logarithmic scale. To reduce inter-assay variability, the MFI sample:MFI control ratio was applied to compare TB and HD patients.

Intracellular production of γ-IFN

PBMCs were stimulated using FcyR⁺ mouse P815 target cells at 1:10 E:T ratio in complete medium in the absence or presence of anti-NKp30 and anti-NKp46 mAbs mixture. Alternatively, purified NK cells were co-cultured with monocytes (E:T 40:1).

Phorbol myristate acetate (PMA) (25 ng ml⁻¹) plus ionomycin (1 μg ml⁻¹) (Sigma) were used for maximal γ-IFN production. After overnight incubation, Golgi Plug (BD Pharmingen) was added for 4 h at 37°C. Anti-CD3FITC and anti-CD56PC7 mAbs were used for surface staining followed by permeabilization fixation according to the Citofix/Citoperm™ protocol (BD Pharmingen) and subsequently intracellular staining for γ-IFN in the presence of permeabilizing solution (0.1% saponin in PBS) was performed. Cells were analyzed on a FACSCalibur (Becton Dickinson) counting 10 000 events in CD3⁺ gated lymphocytes.

Cytotoxicity assay

NK cell cytolytic activity was determined in 4-h ⁵¹Cr release assay as previously described (15). For mAb-mediated masking experiments, the FO-1 human melanoma cell line was used alone or in the presence of anti-natural cytotoxicity receptor (NCR)-specific mAbs (μ isotype) at E:T ratios of 5:1, 2:1 and 1:1 in duplicate wells. In the redirected killing assay, the FcyR⁺ murine mastocytoma P815 cell line was used as target in the absence or in the presence of different mAbs in duplicate wells at E:T ratios 2:1 and 1:1. In this assay, mAb heavy chains are bound to P815 cells via FcyR and trigger NK cells via specific interaction with NCRs. The mAb concentrations were 10 μg ml⁻¹ for the masking experiments and 0.5 μg ml⁻¹ for the redirected killing assay.

Statistical analysis

To compare the results obtained for molecule expression on NK cells or for γ-IFN production, the Mann–Whitney U-test for unpaired data analysis was employed to analyze the relationship between the proportion of γ-IFN-producing NK cells and CD16⁻/CD56⁺ NK cells. Statistical analysis was performed using the StatView 4.2 program (Abacus Concepts, Berkeley, CA, USA).

Results

Analysis of peripheral NK cell phenotype in TB patients

Analysis of PBMCs obtained from the patients at the onset of pulmonary TB was performed by four-color cytofluorimetry. A group of HD was used for comparative analysis. CD5⁺ CD19⁺ CD14⁻ CD56⁺ PBMCs were operationally defined as NK cells. Analysis was initially performed on NK cells with regard to the expression of CD56 and CD16. Decreased proportions of CD16⁻/CD56⁺ NK cells were observed in TB patients compared with HD (mean ± SD 8.5 ± 4.6% versus 16.2 ± 10.2%) with correspondingly increased CD16⁺/CD56⁺ NK cells (60.4 ± 17.6 versus 39.4 ± 18.9) (Fig. 1A and B). As a consequence, a decrease of peripheral NK cell ratio (CD16⁺ CD56⁺/CD16⁺CD56⁺) expressed as values × 100) was observed in TB patients (Fig. 1B) without increases of CD16⁻CD56⁻ cells.

Analysis of the expression of HLA-DR on purified NK cells showed ~20% of CD56⁻ NK cells are activated in TB patients (Fig. 1C) and that HLA-DR is expressed predominantly on CD16⁻CD56⁺ NK cells. Analysis of CD69, another molecule whose expression reflects cell activation, confirmed the presence of activated peripheral NK cells (Fig. 1, panel D). In order to further characterize whether activation of NK cells during TB could be associated to perturbations of tissue homing, the expression on peripheral NK cells of activation markers and of a lymph node-homing chemokine receptors (i.e. CCR7 and CXCR3) (33, 34) was next considered. Interestingly, CCR7 expression on NK cells of TB patients was present on a detectable proportion of NK cells or for γ-IFN production, the Mann–Whitney U-test for unpaired data sets was employed. Results are expressed as box plots indicating median values with interquartile ranges to graphically represent the proportion of cells expressing any given receptor or cytokine in the patient groups and in healthy controls. Spearman's rank correlation test for non-parametric data was employed to analyze the relationship between the proportion of γ-IFN-producing NK cells and CD16⁻/CD56⁺ NK cells. Statistical analysis was performed using the StatView 4.2 program (Abacus Concepts, Berkeley, CA, USA).

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Fig. 1. Cytofluorometric analysis of peripheral NK cells. (A) Representative cytofluorometric analysis of NK cells in TB and HDs. Dot plots show CD3/CD14+/CD19- gated PBMC expressing CD16 and CD56. Representative of 12 experiments. The dotted line indicates the gate used to identify CD16+/CD56bright NK cells. (B) Distribution of NK cells subsets in TB (12 patients) and HD (10 subjects). Box plot shows (CD16+CD56bright/CD16+CD56dull × 100) NK cells. The line indicates median expression; the boxes indicate 25–75 percentiles; vertical lines express standard deviation; **P < 0.01. (C) Cytofluorometric analysis of the expression of HLA-DR on peripheral CD56+ NK cells from a representative TB patient.
CD16<sup>+</sup>CD56<sup>dull</sup> NK cells (8%) (Fig. 1D). Expression of CXCR3, which represents another lymph node-targeting receptor, was in line with the expression of CCR7 in NK cells from TB patients, while in HD it was expressed predominantly on CD16<sup>+</sup>-CD56<sup>bright</sup> cells, as described (13). Analysis of purified CD56<sup>+</sup> NK cells for the simultaneous expression of CCR7 and HLA-DR showed that only a minor proportion of HLA-DR<sup>+</sup> NK cells (≤30%) in TB patients co-express CCR7 (Fig. 1D). Cytocentrifugation for CD83 expression was negative in all cases (data not shown).

Since peripheral NK cells may show selective NCR defects during chronic infections (e.g. HIV-1 and HCV) (13) and may also display expression of activation markers (15), we next studied NCR expression on NK cells in these TB patients. Analysis of activating receptor expression showed a selective reduction in NKp46 and NKp30 and DNAM-1, without significant involvement of NKG2D or NKG2C/CD94 (Fig. 2, panels A and B) and without NKp44 expression (18). As far as HLA class I-specific inhibitory NK cell receptors are concerned, no overall perturbations were detected as regards LIR1/ILT2 (CD85j), NKG2A/CD94, and overall killer inhibitory receptor (KIR) expression when investigated with a mixture of mAbs specific for KIR2DL2/S2 (CD158b1/CD158j), KIR2DL1 (CD158e1) and KIR2DL1/S1 (CD158b/CD158h) (Fig. 2, panel B).

In TB patients, the decrease in NKp30 and NKp46 expression was evident both as a reduced proportion of NK cells expressing each NCR (mean 8.1% ± 7.3 versus 35.8% ± 19.7 and 33.7% ± 14.5 versus 51.0% ± 17.5, respectively) and as a significant reduction in the molecule density on cells expressing each NCR, as determined by MFI ratio (Fig. 2B). The reduction of NKp30 expression was not different between CD16<sup>+</sup>-CD56<sup>dull</sup> and CD16<sup>+</sup>-CD56<sup>bright</sup> subsets at the time of disease onset (mean expression 1.63% ± 1.62 versus 2.32% ± 3.6).

Thus, a reduction of NKp46, NKp30 and DNAM-1 is present on peripheral NK cells in patients at the onset of pulmonary TB and is associated with a relevant degree of systemic NK cell activation on both CD16<sup>+</sup>-CD56<sup>dull</sup> and CD16<sup>+</sup>-CD56<sup>bright</sup> NK cells and with expression of lymph node-homing receptors also on CD16<sup>+</sup>-CD56<sup>dull</sup> NK cells.

Functional analysis of fresh NK cells

Specific functional triggering of freshly purified NK cells was studied against FcγR<sup>+</sup> P815 cells using redirected activation in the presence of anti-NKp30 mAb. This assay allows the study of functional characteristics of NK cells mediated by specific triggering of surface molecules. A reduction of intra-cellular γ-IFN production was observed in NK cells from TB patients in the presence of NKp30-mediated triggering (Fig. 3, upper row). Similar results were obtained in the presence of anti-NKp46 mAbs (Fig. 3, lower row). On the contrary, PMA/ ionomycin-mediated γ-IFN production (i.e. bypassing the need for receptor-mediated triggering) was comparable with HD (data not shown) suggesting a conserved overall functional potential for γ-IFN production. In line with this finding, no differences between TB patient and HD were observed for NKG2D (data not shown). Data of the whole cohort of patients and of HDs are shown in Fig. 6(B) represented graphically by box and whiskers graph where the line represents median values of γ-IFN<sup>+</sup> NK cells, the upper and lower sides of the box represent 75th and 25th percentiles and the vertical lines show the standard deviation. Thus, at TB onset, the reduced NKp30 surface expression translates in a reduced functional activity upon NCR triggering while the γ-IFN functional potential is not decreased.

NKp30 engagement plays a relevant role in the interaction and killing of iDC by NK cells. Inhibition of NKp30–NKp30 ligand interaction prevents iDC killing and the production of γ-IFN and tumor necrosis factor-α (TNF-α) by NK cells (35, 36). Failure to interact via NKp30 (and DNAM-1) results in reduced editing of iDC and this may favor survival of DC undergoing incomplete maturation that may be tolerogenic (37, 38). Therefore, these results suggest that the observed reduction in NKp30 expression on circulating NK cells in TB patients could impact on appropriate editing of DC and on Mth antigen presentation.

Phenotypic and functional NK cell defects are partially restored in TB patients in the presence of α-IFN

To evaluate whether the apparent defect in NCR expression in TB patients could be restored once cells are removed from the in vivo environment and are cultured in vitro with conditions that support NK cell activation and cytotoxic activity, we purified peripheral NK cells, derived activated NK cell populations from TB patients was increased compared with peripheral blood NK cells from the same patients (32.8 versus 7.5 MFI), but failed to reach the same mean level of expression of NK cell populations derived from HD. As shown in Fig. 4(A), molecule density of NKp30, expressed as MFI, was 53.3% lower compared with HD (32.8 versus 59.3 MFI, P < 0.05).

In view of this incomplete recovery of NKp30 expression in the presence of rIL-2, we studied whether additive effects could be obtained with the supplementation to rIL-2 cell cultures of α-IFN, which is known to increase NK cell cytotoxicity (39).

To this end, peripheral NK cells were purified from TB patients and cultured in vitro in the presence of either rIL-2 alone or of increasing α-IFN concentrations (10–1000 U ml<sup>−1</sup>). These concentrations reflect a range of concentrations around those attained in vivo by patients treated with α-IFN (100 U ml<sup>−1</sup> h<sup>−1</sup>) (40). Culture supplementation with α-IFN led to increased...
NKp30 expression already at low concentrations (10 U ml\(^{-1}\)), with maximal effect at 1000 U ml\(^{-1}\). As shown in Fig. 4B, \(\alpha\)-IFN supplementation (100 U ml\(^{-1}\)) led to an increased proportion of cells expressing NKp30 compared with rIL-2 alone (68.4 ± 23.6% versus 51.2 ± 19.4%) with increases in NKp30 molecule density. This effect of \(\alpha\)-IFN on triggering molecule expression was selective for NKp30 since NKp46 and NKp44 were not affected. Increased NKp30 expression upon \(\alpha\)-IFN supplementation was not restricted to patients with TB, as HD NK cell populations displayed a similar selective dose-dependent increase in NKp30 expression while none was observed for NKp46 and NKp44 (Fig. 4B). The modulation of NKp30 expression by \(\alpha\)-IFN supplementation of IL-2-containing medium could not be reproduced to the same level in NK cells from HIV-infected patients who have been described to express low levels of NKp30 (14). We performed

Fig. 2. Cytofluorometric analysis of NCRs on peripheral NK cells. (A) Cytofluorometric analysis of the expression of NKp46, NKp30, NKp44 NCRs and NKG2D, DNAM-1, NKG2C/CD94 on peripheral NK cells from a representative TB patient and an HD. Staining of the indicated mAbs is shown on CD3\(^{-}\)CD14\(^{-}\)CD19\(^{-}\)-gated PBMC. Open profiles represent negative-control mAbs. Representative of 12 experiments performed. (B) Box plot analysis of activatory and inhibitory NK cell receptor expression on peripheral NK cells. Molecule densities were determined by MFI. MFI sample/MFI control ratios are shown. Representative of 12 TB patients and 10 HDs. *P < 0.05, **P < 0.01.
the analysis on purified NK cells from three viremic-untreated patients presenting for the first time to clinical observation. α-IFN supplementation to IL-2 activated NK cell cultures from HIV-1-infected patients resulted in increases in NKp30 expression that are much more limited (Fig. 4B).

Thus, NK cells from TB patients partially recover NCR expression in vitro upon activation and culture, but maintain a reduced NKp30 expression also after in vitro activation with rIL-2. This defect in NKp30 expression can be at least partially restored with α-IFN supplementation.

Supplementation with α-IFN restores NCR-mediated cytolytic function in NK cells from TB patients

Given the restoration of NKp30 expression observed after α-IFN supplementation in vitro, we asked whether this could also bear favorable consequences on the function of NK cells purified from these patients. To this end, cytotoxic NK cell activity was evaluated by molecule-specific assays using classic NK cell target cells (FO-1 and FcγR+ P815) exploring killing specificity with NCR-specific mAbs that could either mask receptor-ligand interactions and thus reduce target cell lysis (FO-1) or mediate redirected triggering or antibody-dependent cell cytotoxicity and thus induce specific killing of P815 targets.

Purified rIL-2 activated NK cell populations derived from TB patients had significantly lower cytolytic activity against FO-1 cells compared with HD. This is in line with the reduced NKp30 and NKp46 expression observed on patient NK cells (Fig. 2A and B). NCR masking confirmed the comparable specific reduction of lysis in HD and TB patients (Fig. 5A). Inhibition of target cell lysis with mAb-mediated lysis in TB patients showed that residual baseline FO-1 cytotoxicity is NCR dependent. Experiments of mAb-mediated NCR masking using individual mAbs revealed that both NKp30 and NKp46 contribute to FO-1 lysis in HD and TB patients (data not shown). In the redirected killing assay using NKp30 mAb and FcγR+ P815 cells, lower NKp30-mediated cytolytic activity is observed in TB patients as compared with HD (Fig. 5B, left panel). Similar findings were observed using NKp46 mAb (data not shown). These findings are in line with the significantly lower NKp30 molecule density on NK cell populations from TB patients (Fig. 2B). Once supplemented in vitro with α-IFN 100 U ml−1 (in addition to rIL-2), NK cells displayed an increased NKp30-mediated FcγR+ P815 killing both in TB patients and in HD (Fig. 5B, right panel). Supplementation of TB patient NK cells with α-IFN resulted in lower cytotoxicity levels compared with HD under the same conditions. There was also a lower fractional increase in cytotoxic activity in TB patients (Fig. 5B, closed triangles in the left panel versus closed triangles in the right panel). With α-IFN supplementation, TB patient NK cells reached cytotoxicity levels approaching basal cytotoxicity of HD (Fig. 5B, compare closed triangles left panel with dashes in the right panel). Interestingly, this is obtained with α-IFN supplementation at concentrations that are in line with those obtained with in vivo administration (40).

These results therefore show that persistently decreased expression of NKp30 on NK cells from TB patients cultured in vitro determines reduced NK cell receptor-mediated cytotoxic activity. α-IFN supplementation results more restricted increases in NKp30-mediated cytotoxicity compared with what observed in HD. These levels however...
approach at least those observed in (α-IFN) untreated HD NK cells, thus providing a rationale for its in vivo administration (41).

**NK cell phenotype and function after 6 months antimycobacterial treatment**

According to the standard regimens in uncomplicated conditions (42), treatment for pulmonary TB requires at least 6 months of therapy with good adherence.

In order to understand the course of NK cell perturbations that were found at the onset of disease and before antimycobacterial treatment, phenotypic and functional analysis was repeated at the end of therapy. Analysis of the proportion of peripheral CD16$^{+}$/CD56$^{bright}$ NK cells showed that successful treatment of TB led to a normalization of this NK cells subset (Fig. 6A). The detection of γ-IFN-producing NK cells by cytofluorimetry also showed an improvement to levels comparable to HD (Fig. 6B) and fits with the general notion that CD16$^{+}$/CD56$^{bright}$ NK cells are the NK cell subset with more active cytokine-producing activity (TNF-α and γ-IFN). An increase in CD16$^{+}$/CD56$^{bright}$ NK cells during treatment in TB patients would be therefore expected to be paralleled by increases in γ-IFN-producing NK cells. Both these observations may represent redistribution of NK cells sequestered at sites of inflammation and are in line with and extend previous reports showing that NK cell compartmentalization at sites of mycobacterial replication may contribute to skewing peripheral blood NK cell subsets (32).

However, analysis of NCR expression revealed the persistence at the 6th month of the selective decrease of NCR (NKp30 and NKp46) expression observed at disease onset (Fig. 6C). All the other NK cell-triggering receptors were not differently expressed compared with HD. This finding therefore suggests that low level NCR expression may represent a feature of these patients irrespective of NK cell compartment redistribution.

As shown in Fig. 6(A and B), there was some variance in the degree of recovery of both γ-IFN production and CD16$^{+}$/CD56$^{bright}$ NK cells. These two parameters were significantly correlated ($P < 0.05$, Spearman’s correlation test) and patients showing higher degrees of recovery of γ-IFN production upon NCR-mediated stimuli after successful treatment were those with higher proportions of CD16$^{+}$/CD56$^{bright}$ NK cells. Therefore, functional NK cell recovery in TB patients is correlated to the actual recovery of CD16$^{+}$/CD56$^{bright}$ NK cells.

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**Fig. 4.** Phenotypic and functional NK defects are partially restored in TB patients in the presence of α-IFN. (A) NCRs MFI in rIL-2 (100 U ml$^{-1}$) activated NK cells derived from TB and HD. Bars represent mean MFI + SD from TB patients (filled bars) and HD (open bars) in six experiments, *$P < 0.05$. (B) Cytofluorometric analysis of in vitro-cultured NK cells to verify α-IFN dose–response relationship of NCRs expression in a representative HD, TB and HIV-infected patients. Histograms indicate the reactivity of the indicated mAbs. Purified NK cells were activated with rIL-2 (100 U ml$^{-1}$) (open profiles) or rIL-2 (100 U ml$^{-1}$) + α-IFN (at indicated concentrations) (filled profiles). Numbers in histograms indicate the proportion of positive cells. Representative of six experiments.
In view of this finding, it was important to verify whether failure to recover NCR expression after treatment could depend on lower recovery of CD16+/CD56bright cells. To this end, NKp30 and NKp46 expression was compared on CD16+/CD56bright and CD16+CD56dull NK cells. As shown in Fig. 3D, both the proportion of NK cells expressing NKp30 and molecule density (MFI) were significantly reduced on both CD16+/CD56bright and CD16+CD56dull NK cells, not only at the onset of TB but also after treatment.

Thus, NKp30 and NKp46 expression is persistently low, does not correlate with compartment redistribution of CD16+/CD56bright NK cells and persists after successful treatment even in the presence of recovery of NK cell distribution and function.

Discussion

Little information is available regarding innate immunity and NK cell function in patients exiting from Mtb control at the onset of overt clinical lung disease presentation. This work provides first time evidence that relevant perturbations of peripheral blood NK cell phenotype and function are present at the time of diagnosis of pulmonary TB in patients, even without serosal involvement. The findings confirm and extend relevant initial observation of NK cell involvement during TB infections that were collected before availability of specific mAb tools (22, 32).

Lymphocyte redistribution at serosal sites of Mtb replication has been previously observed for CD4+ T cells and also for NK cells. An enrichment of CD16+CD56bright NK cells in peripheral districts (e.g. pleural fluid and pleural biopsy) has been recently described in patients with tubercular pleural effusions without additional information on NCR expression (32). Similar Ag-specific CD4+ compartmentalization has been shown in patients with tubercular serosal effusions leading to cutaneous test anergy (43). Our finding of reduced CD16+/CD56bright/CD16+CD56dull ratio in the peripheral blood is in line with these observations. None of the patients included in the present study, however, had serosal disease involvement (43). Possible mechanisms explaining this observation in patients who lack serosal tubercular effusions could be represented by accumulation of CD16+/CD56bright NK cells, not only at the onset of TB but also after treatment.

At the time of clinical TB reactivation in the present study, both NKp30 and NKp46 expression was selectively reduced,
with a correspondingly depressed γ-IFN production and decreased cytolytic activity after in vitro activation. These findings bear similarity to HIV-1 infection, where a selective and profound decrease in NKp30 and NKp46 expression is observed (14) together with signs of NK cell activation (15) and failure of peripheral NK cells to recover a ‘healthy’ phenotype and function after in vitro activation (19). These features of NK cell derangement could represent a common pathway of immune evasion that is exploited by both HIV-1 and by Mth. Pathogen-targeting NK cells and innate immune mechanisms that regulate DC editing may favor pathogen-favorable shaping of adaptive responses, as shown by severe impairment of CD8+ CTLs in patients with latent TB after NK cell depletion (28).
expected by faulty editing of iDC by NK cells in HIV-1-infected patients (17) and in experimental allergic encephalomyelitis (47). We suggest that the present findings could represent an additional mechanism contributing to the strict reciprocal potentiating effect of HIV and Mth replication. In addition to HIV-1 infection of Ag-specific (Mth specific) activated CD4+cells (48) and to direct induction by Mth or PPD of HIV-long terminal repeat driven transcription in chronically (49) or acutely (50) infected monocytes, the decreased expression of NKP30 and NKP46 together with NK cell activation (15) observed during HIV-1 infection would reproduce the presently described derangement, leading to accelerated and early loss of Mth control in HIV-infected patients. Once established in HIV-1 patients, TB could then lead to additional homologous NK cell derangement with increased loss of HIV-1 control and viral escape mutants in areas of simultaneous HIV and Mth replication (51). The impaired γ-IFN production by NK cells described here would independently contribute to crippled control of Mth replication (52).

Regarding the possible mechanisms leading to reduced NCR (NKP30 and NKP46) expression in TB patients, preliminary analysis shows that this is not related to decreased transcription as determined by real-time PCR (data not shown). In addition, supplementation with α-IFN restored NCR expression and function to levels comparable to NK cell populations from HD cultured with rIL-2 alone. This is in agreement with a relative α-IFN deficiency and decreased pDC numbers that are described in TB patients (33). Previous work has shown successful therapy of TB patients with α-IFN (53, 54) and showed an increase of perforin-positive NK cells after α-IFN treatment in vivo (55, 56). Accordingly, the addition of α-IFN on NKP30 in vitro represents a mechanism of NK cell functional rescue. In addition, the activity of α-IFN already at concentrations of 10 U ml−1 [i.e. <1 log of those attained during systemic IFN treatment for chronic HCV (53)] and previous satisfactory experiences with low-dose aerosolized α-IFN during pulmonary TB (41) support the possible adjuvant use of α-IFN and the need for additional trials at dosages that entail considerably reduced costs and side-effects.

We were surprised to detect persistently decreased expression of NKP30 and NKP46 at the end of successful anti-mycobacterial treatment. In a condition where NCR expression is crippled, as with HIV-1, anti-retroviral treatment results in considerable restoration of NK cell function (17). Here, although overall NK cell function improved at the end of treatment with increases in CD56bright and in γ-IFN-producing NK cells, decreased NCR expression persisted. The finding of decreased NKP30 and NKP46 expression at the onset of TB may be ascribed to either a pre-existing reduced expression in these patients, but could also be secondary to the reactivation of Mth replication. The persistently decreased expression of these NCRs at the end of successful treatment, when CD56brightCD16+ NK cells and γ-IFN production have recovered, would support the hypothesis of constitutive low basal NCR expression in at least a fraction of latently infected subjects that will eventually develop TB reactivation. Whether transcriptional of post-transcriptional NCR regulation differs in these subjects remains to be elucidated.

In conclusion, Mth appears to target efficiently this arm of the innate immune system these effects may be countered by α-IFN supplementation. Specific NCR modulation on NK cells provides a useful tool to interpret some of the known derangements of failing immune surveillance during TB and may represent a key element in the transition from TB latency to overt pulmonary TB.

**References**


NK cells at onset of clinical TB


