NK cell activity in tuberculosis is associated with impaired CD11a and ICAM-1 expression: a regulatory role of monocytes in NK activation

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Summary

Although the role of natural killer (NK) cells in mycobacterial infections is unclear, it has been postulated that they contribute to protective immunity through the production of interferon (IFN)-γ. In this study, we evaluate the effect of interleukin (IL)-10, IL-15 and IL-18 on NK lytic activity through the expression of CD16, CD11a and CD69 molecules and the induction of IFN-γ production in patients with tuberculosis (TB) and healthy individuals (N). Our results showed an impairment of NK lytic activity and a gradual down-regulation of costimulatory and adhesion molecules on NK cells which were dependent on the severity of the disease. NK lytic activity was increased by exogenous IL-15 and IL-18 in both TB and N, and by neutralization of endogenous IL-10 only in TB; IL-15 and IL-18 increased CD69 receptor expression, while anti-IL-10 up-regulated CD16 and CD11a expression in TB. *Mycobacterium tuberculosis* reduced the number of intracellular adhesion molecule (ICAM)-1$^+$ CD14$^+$ cells, but in the presence of IL-15, IL-18 and anti-IL-10 its expression was up-regulated. In cells from TB patients, the observed effects of IL-15 and IL-18 on NK function were not dependent on IL-10 modulation of the surface expression of activator/adhesion molecules. In the absence of monocytes, IL-10 activated NK cells, suggesting an indirect effect on their function. Furthermore, in TB patients the depletion of monocytes increased the production of IFN-γ by NK cells. Therefore, monocytes from TB patients regulated the NK function involving IL-10 which, through an indirect mechanism, led to the down-regulation of costimulatory/adhesion molecules and/or IFN-γ production.

Keywords: natural killer cells; cytotoxicity; receptors; monocytes; tuberculosis

Introduction

A protective immune response against *Mycobacterium tuberculosis* depends on interferon (IFN)-γ production by CD4$^+$ and CD8$^+$ T cells.[1] However, the early production of IFN-γ by cells of the innate immune response at inflammatory sites can regulate innate resistance by activating phagocytic cells and priming antigen-presenting cells (APCs) for interleukin (IL)-12 production, thus shaping adaptive immunity towards the T helper type 1 (Th1) response necessary for elimination of many intracellular pathogens. Natural killer (NK) cells are critical components of the innate immune response that lack expression of the T-cell receptor (TCR)–CD3 complex and surface immunoglobulins but express CD56 antigen. NK cells are characterized by potent cytotoxic activity against tumours, virus-infected cells and intracellular parasites.[2] Resting NK cells circulate in the blood and, once activated, they are able to migrate to and infiltrate sites of infection where target cells are localized. The interaction of NK with target cells is mediated by various cell surface molecules, some involved in cell adhesion, others activating the NK cytolytic programme, and others inhibiting this activation by negative signalling.[2] The best studied activation receptor is the FcγRIII (CD16) molecule through which NK cells mediate antibody-dependent cellular cytotoxicity (ADCC) against target cells coated with immunoglobulin G (IgG).[2] Although not restricted to NK cells, integrins [CD11a/CD18, lymphocyte function-associated antigen (LFA)-2 and LFA-3]

which bind to intracellular adhesion molecule (ICAM)-1 (or CD54), ICAM-2 and ICAM-3 ligands have also been implicated in NK cell adhesion to target cells, degranulation and cytokine production.[3,4] Also, the CD69 molecule, which is rapidly acquired following activation and belongs to the family of C-type lectin receptors bearing strong similarity to the NK receptor CD94,[5,6] has been implicated in the cytotoxic activity and costimulation of cytokine production of activated NK cells.[7] It is unclear which mechanisms contribute to the priming phase of NK cell activation, but these cells can be rapidly activated in the periphery by chemokines in conjunction with IL-2 and macrophage or dendritic cell (DC)-derived cytokines such as IL-12, IL-15 and IL-18.[8,9]

Bacterial products activate macrophages to produce IL-12, IL-15 and IL-18, playing a central role in the type 1 cytokine response and NK activity.[8,10,11] NK cell cytokine production is induced by IL-12 in synergy with IL-15 and IL-18,[8,12,13] which stimulates IFN-γ production by NK cells via different intracellular pathways.[13–15] Considering that protection against intracellular pathogens is critically dependent on the function of NK cells at early stages of the immune response and on type-1 cells at later stages, the aim of the present study was to investigate the costimulatory molecules involved in *M. tuberculosis*-induced NK function through IFN-γ production in patients with tuberculosis. The role of IL-15 and IL-18 was also investigated. Our results show that, in patients with tuberculosis, monocytes regulate NK activity.

Materials and methods

Patients

A total of 42 male patients with pulmonary tuberculosis (TB) were included in the study. Patients were diagnosed by the presence of recent clinical symptoms of TB, a positive sputum smear test for acid-fast bacilli confirmed by a positive culture of TB bacilli and an abnormal chest radiography. Informed consent was obtained from patients according to the Hospital Francisco J. Muñiz Ethics Committee. All patients had active TB and were under multidrug treatment at the time of study (0–15 days). Pulmonary disease was classified according to the extent and type of X-ray findings as mild (M) or advanced (A) TB according to the American Tuberculosis Society criteria. Patients with diabetes, chronic renal failure or malignant diseases, as well as those who tested positive for human immunodeficiency virus (HIV) or other concurrent infectious diseases, were excluded. Patients were classified into two groups: (i) patients with moderate tuberculosis (M-TB; $n = 22$; age range 25–55 years) and (ii) patients with advanced tuberculosis (A-TB; $n = 20$, age range 22–60 years). Fourteen healthy individuals (age range 25–60 years) were included as controls.

Mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation[16] and then suspended in RPMI 1640 (Gibco Laboratory, New York, NY) containing gentamycin (85 μg/ml) and 15% heat-inactivated fetal calf serum (FCS) (Gibco) (complete medium).

Monocyte-depleted PBMC

In order to obtain monocyte-depleted cell suspensions, PBMC (5×10^6 cells/well) were incubated on the bottoms of 24-well Falcon plates for 2 hr at 37° to allow cells to adhere to the plastic (85–95% of monocytes). After removal of adherent cells, non-adherent lymphocytes were washed and suspended in complete medium. $CD14^+$ monocyte-depleted cell suspensions were obtained by magnetic methods by treatment of PBMC with anti-CD14 monoclonal antibody (Immunotech, Marseille, France) for 30 min at 4° and then with goat anti-mouse IgG-coated beads (Dynal, Oslo, Norway). Monocyte-depleted cell suspensions (both adherent-depleted and CD14-depleted populations) were suspended in complete medium, ensuring that the proportion of each lymphocyte subset was the same as in total cultured PBMC in order to allow comparison of NK lytic activities. The proportion of contaminated monocytes in cell suspensions depleted by adherence was 1–2% and that in cell suspensions depleted by magnetic methods was 0·5–0·6%.

Antigen

The γ-irradiated *M. tuberculosis* H37-Rv strain used in this study was kindly provided by Dr J. T. Belisle (Colorado University, Denver, CO). Mycobacteria were suspended in phosphate-buffered saline (PBS) free of pyrogen, sonicated and adjusted at a concentration of 1×10^8 bacteria/ml.

PBMC culture

PBMC (2×10^6 cells/ml) or monocyte-depleted PBMC ($1{\cdot}9 \times 10^6$ cells/ml) were cultured in Falcon 2063 tubes (Becton Dickinson, Lincoln, NJ) at 37° in a humidified 5% CO_2 atmosphere, in complete medium with or without *M. tuberculosis* (1×10^6 bacteria/ml), IL-15 (10 ng/ml), IL-18 (20 ng/ml), IL-10 (10 ng/ml) or a monoclonal antibody specific for human IL-10 (10 ng/ml) (Peprotech, Rocky Hill, NJ). After 24 hr of incubation, *M. tuberculosis*-stimulated and/or cytokine-treated and control cells were washed three times with RPMI 1640, suspended in complete medium (2×10^6 cells/ml) and tested for cytotoxic activity, expression of surface antigens and IFN-γ production by flow cytometry. An isotype-matched non-relevant

control IgG1/κ antibody for anti-IL10 was also tested and was found to have no significant effect on cytotoxicity, cytokine production or surface antigen expression.

Cytotoxic assay

Freshly isolated (*ex vivo*) and cultured PBMC or monocyte-depleted PBMC were added in triplicate at different effector to target cell ratios, in a final volume of 200 μl, to ^{51}Cr-labelled K562 target cells (5×10^3 cells seeded into each well of 96-well microtitre plates; Coming incorporated (NY) Corning, USA). Plates were centrifuged at 50 ***g*** for 5 min and incubated at 37° in 5% CO_2 for 4 hr. After centrifugation at 200 ***g*** for 5 min, 100 μl of supernatant was removed from each well. The radioactivity of supernatants and pellets was measured in a gamma counter. Results were expressed as percentage of cytotoxicity (% Cx):

$$\%\ Cx = [(\text{c.p.m. experimental release} - \text{c.p.m. spontaneous release})/(\text{c.p.m. total release} - \text{c.p.m. spontaneous release})] \times 100,$$

where c.p.m. is counts per minute. The radioactivity released from target cells incubated with complete medium alone was considered to represent spontaneous release. It ranged from 5 to 10%.

Results expressed in lytic units (LU) for 10^7 effector cells were calculated by defining 1 LU of cytotoxic activity as the number of effector cells required to lyse 30% of K562 target cells. These cell numbers were readily obtained from the dose–response curves.

Immunofluorescence analysis

Expression of CD16, CD11a and CD69 on CD3$^-$ CD56$^+$ lymphocytes and ICAM-1 on CD14$^+$ cells. In order to evaluate the expression of CD16, CD11a and CD69 antigens on CD3$^-$ CD56$^+$ NK cells, freshly isolated (*ex vivo*), cultured or monocyte-depleted PBMC were incubated for 30 min at 4° with the following anti-human antibodies: Cy5PE-CD3 (*e*Bioscience, San Diego, CA), PE-CD56 (Immunotech), FICT-CD16 (Ancell, Bayport, MN), FICT-CD11a (Caltag, Burlingam, CA) or FICT-CD69 (Ancell). ICAM-1 was evaluated in control and *M. tuberculosis*-stimulated PBMC with or without IL-15, IL-18 and anti-IL-10 for 18 hr and stained with PE-CD54 (*e*Bioscience) and FITC-CD14 (Ancell). PECy5-, FITC- or PE-labelled-isotype matched antibodies were also tested to evaluate non-specific staining. Stained cells were analysed by flow cytometry by acquiring 10 000–20 000 events, and gates were set to forward and side-scatter to exclude cell debris and apoptotic cells. Results are expressed as the percentage of positive cells or mean fluorescence intensity (MFI).

Measurement of IFN-γ$^+$ cells

To determine the expression of intracytoplasmic IFN-γ in control and *M. tuberculosis*-stimulated CD3$^-$ CD56$^+$ cells, PBMC or monocyte-depleted PBMC were cultured for 24 hr with or without *M. tuberculosis* and/or IL-15 or IL-18. Brefreldin A (5 μg/ml; Sigma, St Louis, MO) was added for the final 4 hr to block IFN-γ secretion. Cells were washed, and 5×10^5 cells suspended in 100 μl of PBS-azide were incubated with anti-CD3 and anti-CD56 MoAb (Ancell) for 15 min at room temperature. Thereafter, the cells were fixed according to the manufacturer's instructions (IntraPrep^{TM} permeabilization reagent; Immunotech), washed with PBS supplemented with 1% FCS and 0·01% azide (PBS-FCS-azide) and suspended in 100 μl of PBS-FCS-azide. Fluorescein-conjugated antibody for IFN-γ (Caltag) was added together with 100 μl of permeabilizing solution (IntraPrep^{TM}) and incubated for 30 min at 4°, washed with PBS-FCS-azide, suspended in Isoflow^{TM} (Becton Dickinson) and analysed by flow cytometry. 20 000 events were acquired for each sample and results are expressed as the percentage of IFN-γ-positive cells in the CD3$^-$ CD56$^+$ population.

Statistics

Comparisons of TB and N were performed using Student's *t*-test. Cytoxicity values or flow cytometry data obtained from the different subsets of effector cells of each individual were compared using the Wilcoxon signed rank test.

Results

CD3$^-$ CD56$^+$ cells in patients with tuberculosis showed reduced cytotoxicity against K562 target cells

Human NK cells are defined phenotypically by their expression of CD56 and their lack of expression of CD3, and two subsets of NK cells with different levels of CD56 expression have recently been identified.[17] The majority of cytotoxic human NK cells have low expression of CD56 (CD56^{dim}) and high expression of Fcγ receptor III (CD16$^+$), and have potent cytotoxic activity against tumour cells and cells infected with virus or bacteria. A minor population of NK cells, expressing the CD56^{bright} CD16^{dim} or CD56^{bright} CD16$^-$ phenotype, produce cytokines following activation of monocytes. Therefore, we analysed the proportions of CD3$^-$ CD56$^+$ CD16$^{+/-}$ cells and of these two subsets of cells (CD3$^-$ CD56^{dim} CD16$^+$ and CD3$^-$ CD56^{bright} CD16$^{dim/-}$) in peripheral blood mononuclear cells (PBMC) and 24-hr-cultured PBMC from M-TB and A-TB patients and healthy (N) controls by flow cytometry. As shown in Table 1, a larger proportion of CD3$^-$ CD56$^+$ CD16$^{+/-}$ cells was observed in recently isolated (*ex vivo*) and *M. tuberculosis*-stimulated

Table 1. Percentage and lytic activity of $CD3^-$ $CD56^+$ cells from patients with tuberculosis (TB) and healthy controls (N)

	M-TB			A-TB			N		
	Ex vivo	Control	*M.tb*	*Ex vivo*	Control	*M.tb*	*Ex vivo*	Control	*M.tb*
% total NK cells	20 ± 3*	21 ± 2*	20 ± 2*	17 ± 3*	17 ± 3	18 ± 3*	7 ± 2	8 ± 2	8 ± 3
% $CD56^{dim}$ $CD16^+$	16 ± 1	15 ± 2*	15 ± 3*	18 ± 2*	18 ± 3*	18 ± 2*	8 ± 2	8 ± 2	8 ± 2
% $CD56^{bright}$ $CD16^-$	0·6 ± 0·2	0·7 ± 0·1	0·6 ± 0·2	1·0 ± 0·5	1·3 ± 0·5	1·0 ± 0·5	0·7 ± 0·1	0·6 ± 0·1	0·6 ± 0·1
NK Cx (LU_{30})	43 ± 2^1	45 ± 3^1	58 ± 4^{*1}	24 ± $2^{2,3}$	25 ± $3^{2,3}$	29 ± $3^{1,3}$	57 ± 2	58 ± 4	71 ± 5*

Peripheral blood mononuclear cells (PBMC) were isolated from 22 patients with moderate tuberculosis (M-TB) and 20 patients with advanced tuberculosis (A-TB) and 14 healthy controls (N). The percentages of $CD3^-$ $CD56^+$ $CD16^{+/-}$, $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ $CD16^-$ cells from *ex vivo* and 24-hr-cultured PBMC in complete medium (control) or *Mycobacterium tuberculosis* (*M.tb*) were determined. The cytotoxic assay was performed employing K562 as target cells at different effector to target cell ratios, and lytic units (LU) for each individual were calculated. Results are expressed as mean ± standard error of the mean (SEM). Statistic differences: *M. tuberculosis*-stimulated PBMC versus control PBMC: $^*P < 0{\cdot}05$; patients versus N: $^1P < 0{\cdot}05$, $^2P < 0{\cdot}02$; A-TB versus M-TB: $^3P < 0{\cdot}05$.
NK, natural killer; Cx, cytotoxicity.

PBMC from TB patients, which also showed an increased proportion of the $CD3^-$ $CD56^{dim}$ $CD16^+$ subset relative to N controls. Moreover, *M. tuberculosis* stimulation did not modify the proportion of either subset in TB patients or N controls. Thus, *M. tuberculosis* stimulation did not modify the percentage of $CD3^-$ $CD56^{dim}$ $CD16^+$ or $CD3^-$ $CD56^{bright}$ $CD16^-$ cells found in PBMC from TB patients and N controls.

To assess whether the larger number of $CD56^{dim}$ $CD16^+$ cells could be related to higher cytotoxic activity, *ex vivo*, control and *M. tuberculosis*-stimulated PBMC were tested for their ability to lyse the NK-sensitive cell line K562. As shown in Table 1, the lowest lytic activity was observed in cells from A-TB patients, while in M-TB patients cytotoxicity was slightly diminished with respect to the N controls. In addition, in TB patients, NK lytic activity did not correlate with the days of treatment (data not shown). *M. tuberculosis* induced an increase in lytic activity in M-TB patients and N controls but not in A-TB patients. Thus, the high proportion of $CD56^{dim}$ $CD16^+$ cells observed in TB patients was associated with a progressive loss of NK lytic activity as the disease became more severe. Furthermore, NK cells from A-TB patients were not able to respond to *M. tuberculosis* stimulation as did cells from M-TB and N individuals.

Low cytotoxic activity in A-TB patients was related to low expression of adhesion/activation molecules on NK cells

The activation of NK cells required for target cell lysis is mediated by a balance of inhibitory and activatory NK receptors as well as various adhesion and costimulatory molecules.[2] Among these molecules, the CD16 antigen is one of the most extensively studied activating receptors in the signalling of NK cells. Signalling via CD16 results in the production of cytokines and several chemokines, and causes degranulation of NK cells.[18] In addition, it is known that conjugate formation between NK and target cells is a prerequisite for target cell lysis and is critically dependent on engagement of the β_2 integrin LFA-1 to its ligands on APCs.[19] Although constitutively expressed on few cell types, CD69 is rapidly acquired following *in vitro* activation.[6] Thus, we determined by flow cytometry the percentages and expression levels of CD16, CD11a and CD69 molecules on $CD3^-$ $CD56^+$ cells stimulated or not stimulated with *M. tuberculosis* for 18 hr.

As shown in Table 2, no differences in CD56 expression between TB patients and N controls were observed, but the expression of the CD16 molecule was reduced in $CD3^-$ $CD56^+$ cells from A-TB patients compared with those from M-TB patients and N controls. Furthermore, *M. tuberculosis* induced a slight down-regulation in CD16 expression in A-TB patients. The CD11a molecule was expressed in NK cells from patients and N controls (100% in all groups), although at low levels in cells from A-TB patients, and was not modified by *M. tuberculosis* stimulation. However, similar percentages of $CD69^+$ NK cells were found in control PBMC cultures from TB patients and N individuals [% $CD69^+$ cells in $CD3^-$ $CD56^+$ cells; mean ± standard error of the mean (SEM): M-TB: 28 ± 4; A-TB: 18 ± 4; N: 24 ± 3]. However, *M. tuberculosis* induced the activation of the CD69 receptor in NK cells from M-TB and N controls (% $CD69^+$ NK cells; mean ± SEM: M-TB: 46 ± 5, $P < 0{\cdot}05$; N: 67 ± 7, $P < 0{\cdot}05$) but not in A-TB patients (28 ± 5). Furthermore, the lowest expression of CD69 was observed in control and *M. tuberculosis*-stimulated NK cells from A-TB patients (Table 2). Taken together, these results show a gradual down-regulation of costimulatory and adhesion molecules on NK cells correlated with the severity of the disease, which could explain the impairment in lytic activity.

Table 2. Expression of CD56, CD16, CD11a and CD69 molecules on $CD3^-$ $CD56^{dim}$ natural killer (NK) cells from patients with tuberculosis (TB) and healthy controls (N)

	CD56		CD16		CD11a		CD69	
PBMC from	Control	*M.tb*	Control	*M.tb*	Control	*M.tb*	Control	*M.tb*
M-TB	305 ± 25	299 ± 31	265 ± 34	291 ± 41	271 ± 17	250 ± 48	134 ± 26*	189 ± 26
A-TB	292 ± 29	281 ± 18	132 ± 22*,2	110 ± 23*,2	139 ± 38*,1	109 ± 26**,1	75 ± 6***,2	75 ± 5***,3
N	282 ± 37	258 ± 40	254 ± 62	268 ± 62	256 ± 22	236 ± 17	189 ± 20	238 ± 21

Peripheral blood mononuclear cells (PBMC) from 22 patients with moderate tuberculosis (M-TB), 20 patients with advanced tuberculosis (A-TB) and 14 healthy controls (N) were cultured without or with *Mycobacterium tuberculosis* (control and *M.tb*, respectively) for 24 hr and the expression of CD56, CD16, CD11a and CD69 molecules was determined. Results are expressed as mean fluorescence intensity (MIF), and the mean ± standard error of the mean (SEM) is shown. Statistical differences: patients versus N: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}02$, $^{***}P < 0{\cdot}005$; A-TB versus M-TB: $^1P < 0{\cdot}05$, $^2P < 0{\cdot}02$, $^3P < 0{\cdot}005$.

IL-18 and IL-15 enhanced NK cell activity and up-regulated the expression of CD69, CD16 and CD11a molecules on NK cells from TB patients

It is well known that NK cells constitutively express several monocyte cytokine receptors, including those for IL-10, IL-12, IL-15 and IL-18, and thus receive some of the earliest activation signals from monocytes during the innate immune response. As IL-15 and IL-18 induce NK lysis and IFN-γ production by NK cells,[8,13,14] we analysed their role in the regulation of NK cell activation in TB patients. Thus, IL-15 and IL-18 were added to PBMC stimulated or not stimulated with *M. tuberculosis* for 18 hr and their lytic activity was tested. As shown in Table 3, addition of IL-15 or IL-18 enhanced NK activity in control or *M. tuberculosis*-stimulated PBMC from M-TB patients and N controls. However, IL-15 but not IL-18 increased NK activity in A-TB patients.

Given that IL-15 and IL-18 increased NK activity, we investigated whether the observed effect was also related to the modulation of CD69, CD16 and CD11a molecules on $CD3^-$ $CD56^+$ cells. Therefore, PBMC were stimulated

Table 3. Effect of interleukin (IL)-15 and IL-18 on CD16, CD69 and CD11a molecules and natural killer (NK) cell lysis

				MFI	
PBMC from	PBMC incubated with	% cytotoxicity	% $CD69^+$ NK cells	CD16	CD11a
M-TB	–	23 ± 3	32 ± 5	234 ± 45	245 ± 53
	IL-15	38 ± 2*	43 ± 6	235 ± 53	245 ± 37
	IL-18	35 ± 3	43 ± 6	238 ± 45	251 ± 49
	M.tb	33 ± 2	48 ± 5	255 ± 43	265 ± 37
	M.tb + IL-15	48 ± 3**	77 ± 3*	281 ± 34	307 ± 43
	M.tb + IL-18	42 ± 2*	65 ± 3*	284 ± 36	285 ± 43
	–	12 ± 2	17 ± 3	141 ± 27	140 ± 26
A-TB	IL-15	22 ± 3*	25 ± 4	133 ± 32	165 ± 45
	IL-18	17 ± 2	19 ± 4	135 ± 29	168 ± 36
	M.tb	16 ± 2	20 ± 3	134 ± 20	109 ± 26
	M.tb + IL-15	32 ± 2*	83 ± 4*	195 ± 21*	190 ± 35*
	M.tb + IL-18	23 ± 3	45 ± 3*	163 ± 34	168 ± 26*
	–	31 ± 3	22 ± 2	254 ± 56	256 ± 52
N	IL-15	41 ± 3*	31 ± 3	247 ± 48	260 ± 48
	IL-18	46 ± 4*	25 ± 4	250 ± 53	250 ± 47
	M.tb	46 ± 4	65 ± 9	268 ± 63	246 ± 28
	M.tb + IL-15	62 ± 4*	74 ± 3	269 ± 49	250 ± 43
	M.tb + IL-18	59 ± 4*	70 ± 5	273 ± 61	259 ± 41

Peripheral blood mononuclear cells (PBMC) from 14 patients with moderate tuberculosis (M-TB), 16 patients with advanced tuberculosis (A-TB) and 12 healthy controls (N) were incubated with or without *Mycobacterium tuberculosis* (*M.tb*), in the presence or absence of interleukin (IL)-15 or IL-18, for 24 hr and then used as effector cells (E) against K562 target cells (T) (E:T ratio 40 : 1) and the percentage of cytotoxicity was determined. CD16 and CD11a expression [mean fluorescence intensity (MIF)] and the percentage of $CD69^+$ on $CD3^-$ $CD56^+$ cells were also evaluated by flow cytometry. Results are expressed as mean ± standard error of the mean (SEM). Statistical differences: PBMC + IL-15 or IL-18 versus PBMC without cytokines: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}02$.

with *M. tuberculosis* in the presence or absence of IL-15 or IL-18 for 18 hr, and cell surface expression of CD69, CD16 and CD11a on $CD3^-$ $CD56^+$ cells was examined by flow cytometry. As shown in Table 3, the proportion of $CD69^+$ NK cells in TB patients was increased by *M. tuberculosis* stimulation in the presence of IL-15 and IL-18. In addition, in cells from A-TB patients, IL-15 modified CD16 and CD11a expression, while IL-18 up-regulated only the expression of the CD11a molecule. However, neither IL-15 nor IL-18 modified these molecules in N control cells. Taken together, these results suggest that these cytokines modulate the activation of NK cells in TB patients.

IL-10 down-regulated NK lytic activity and the expression of CD16, CD11a and CD69

It has been demonstrated that lipopolysaccharide (LPS)-stimulated monocytes can down-regulate the proliferation of $CD3^-$ $CD56^+$ NK cells through IL-10 secretion.[20] In addition, increased numbers of $CD14^+$ $IL\text{-}10^+$ cells in response to *M. tuberculosis* stimulation and low expression of costimulatory molecules on monocytes from A-TB patients have been demonstrated.[21] Thus, to investigate the role of IL-10 in NK activation, PBMC were incubated with IL-10 (TB patients or N controls) or anti-IL-10 (TB patients) for 18 hr and their lytic activity was tested. As shown in Table 4, exogenous IL-10 inhibited control and *M. tuberculosis*-induced NK activity in N controls. In contrast, the neutralization of endogenous IL-10 enhanced NK cytotoxicity in TB patients, suggesting a role of IL-10 in NK function.

In order to investigate whether the low expression of CD69, CD16 and CD11a molecules on NK cells could be ascribed to IL-10 production by cells from A-TB patients, anti-IL-10 was added to PBMC cultures from four A-TB patients and, after 18 hr, the cells were tested for surface expression of those molecules. As shown in Table 5, the neutralization of endogenous IL-10 enhanced the expression of CD16 and CD11a molecules, whereas the number of $CD69^+$ cells increased in *M. tuberculosis*-stimulated NK cells. In the presence of *M. tuberculosis*, the addition of IL-15 increased the number of $CD69^+$ NK cells, and neither IL-18 nor the neutralization of IL-10 modified the proportion of $CD69^+$ NK cells or the expression of CD16 and CD11a molecules. Overall, these results indicate that the effects of IL-15 and IL-18 on NK cells from A-TB patients are not dependent on IL-10 modulation of the surface expression of activator/adhesion molecules.

Table 4. Interleukin (IL)-10 modulated natural killer (NK) cell cytotoxicity

	% cytotoxicity		
PBMC treated with	M-TB	A-TB	N
–	23 ± 3	12 ± 2	31 ± 3
IL-10	19 ± 2	10 ± 2	19 ± 5*
anti-IL-10	27 ± 5	16 ± 2	–
M.tb	33 ± 2	16 ± 2	46 ± 4
M.tb + IL-10	22 ± 4	15 ± 3	30 ± 4*
M.tb + anti-IL-10	46 ± 1*	42 ± 3*	–

Peripheral blood mononuclear cells (PBMC) from 10 patients with moderate tuberculosis (M-TB), 12 patients with advanced tuberculosis (A-TB) and seven healthy controls (N) were incubated with or without *Mycobacterium tuberculosis* (*M.tb*) in the presence or absence of interleukin (IL)-10 or anti-IL-10 for 18 hr and then used as effector cells against K562 target cells (E:T ratio 40 : 1). Results are expressed as the percentage of cytotoxicity [mean ± standard error of the mean (SEM)]. Statistical differences: IL-10/anti-IL-10-treated cells versus untreated cells: $*P < 0{\cdot}05$.

Table 5. Neutralization of interleukin (IL)-10 modified stimulatory molecules on $CD3^-$ $CD56^+$ cells from patients with advanced tuberculosis (A-TB)

	MFI		
PBMC incubated with	CD16	CD11a	% $CD69^+$ cells
–	178 ± 27	190 ± 48	14 ± 5
anti-IL-10	232 ± 30*	315 ± 40*	14 ± 6
M.tb	165 ± 36	162 ± 31	16 ± 5
M.tb + anti-IL-10	206 ± 47*	290 ± 46*	30 ± 6*
M.tb + IL-15	219 ± 50	250 ± 35	56 ± 6
M.tb + IL-15 + anti-IL-10	204 ± 31	310 ± 57	55 ± 6
M.tb + IL-18	171 ± 36	250 ± 38	25 ± 5
M.tb + IL-18 + anti-IL-10	166 ± 26	270 ± 46	28 ± 6

Peripheral blood mononuclear cells (PBMC) from four patients with advanced tuberculosis (A-TB) were incubated in complete medium (–) or *Mycobacterium tuberculosis* (*M.tb*) in the presence or absence of interleukin (IL)-15 or IL-18, with or without anti-IL-10, for 24 hr and cell surface expression of CD16, CD69 or CD11a on $CD3^-$ $CD56^+$ cells was analysed by flow cytometry. Mean fluorescence intensity (MFI) for CD16 and CD11a molecules and the percentage of $CD69^+$ cells are shown. Results are expressed as mean ± standard error of the mean (SEM). Statistical differences: PBMC + anti-IL10 versus PBMC without anti-IL-10: $*P < 0{\cdot}05$.

M. tuberculosis down-regulates ICAM-1^+ $CD14^+$ cells

Considering that CD11a (LAF-1) is an adhesion molecule implicated as an early signal for NK cytotoxicity,[4,19] and given that its expression is down-regulated in A-TB patients, we evaluated whether its ligand on monocytes, ICAM-1, may be modulated by *M. tuberculosis* and/or IL-15 or IL-18, or by neutralization of IL-10. Thus, PBMC from six TB patients (two M-TB and four A-TB) were incubated at different PBMC:*M. tuberculosis* ratios (1 : 1, 1 : 3 and 1 : 5) in the presence or absence of IL-15, IL-18 and anti-IL-10 for 18 hr, and ICAM-1 expression was determined on $CD14^+$ cells. As shown in Fig. 1(a), the number of ICAM-1^+ $CD14^+$ cells decreased by between

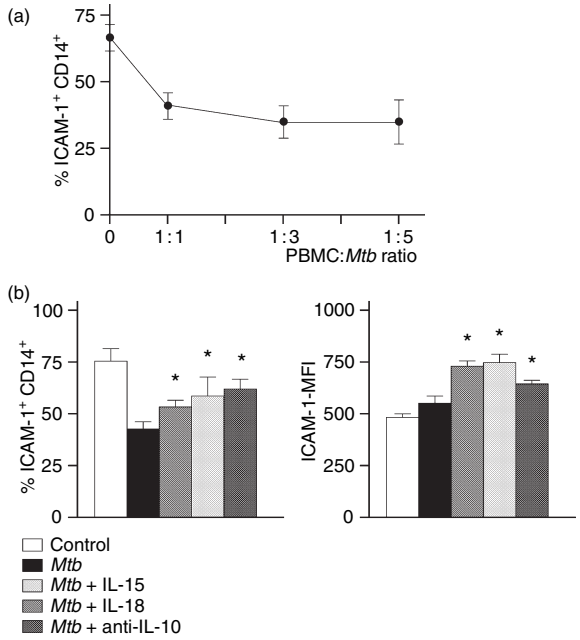

Figure 1. *Mycobacterium tuberculosis* (*Mtb*) regulated the number of intracellular adhesion molecule (ICAM)-1$^+$ CD14$^+$ cells and ICAM-1 expression. (a) Peripheral blood mononuclear cells (PBMC) from two patients with moderate tuberculosis (M-TB) and four patients with advanced tuberculosis (A-TB) were incubated for 18 hr at different PBMC to *M. tuberculosis* ratios and the percentage of ICAM-1$^+$ CD14$^+$ cells was determined. Results are expressed as mean ± standard error of the mean (SEM). Statistical differences: PBMC + *M. tuberculosis* versus non-stimulated PBMC: $^*P < 0{\cdot}01$. (b) PBMC from six A-TB patients were incubated in complete medium (control) or *M. tuberculosis* in the presence or absence of interleukin (IL)-15, IL-18 or anti-IL-10 for 18 hr. The percentage of ICAM-1$^+$ CD14$^+$ cells and ICAM-1 expression [mean fluorescence intensity (MFI)] were then analysed by flow cytometry. Results are expressed as mean ± SEM. Statistical differences: PBMC + IL-15, IL-18 or anti-IL-10 versus PBMC without cytokines or anti-IL-10: $^*P < 0{\cdot}05$.

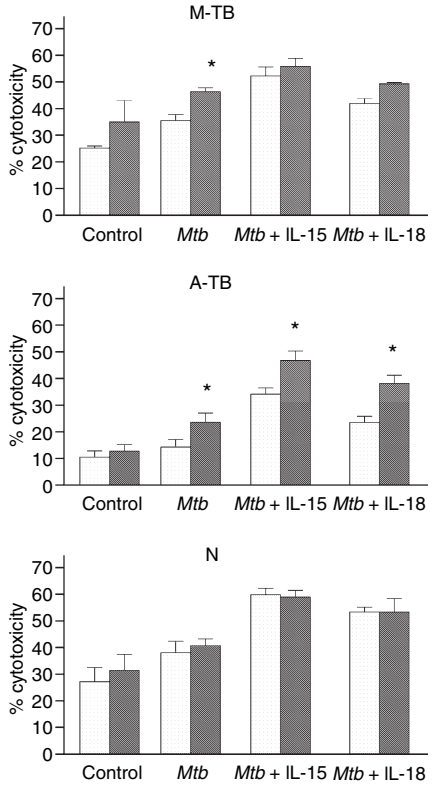

Figure 2. Monocytes from patients with tuberculosis (TB) down-regulated natural killer (NK) activity. Peripheral blood mononuclear cells (PBMC) (grey bar) or monocyte-depleted mononuclear cells (black grey bar) from 12 patients with moderate TB (M-TB), 15 patients with advanced TB (A-TB) and 12 healthy individuals (N) were cultured in complete medium (control), *Mycobacterium tuberculosis* (*Mtb*) alone or *M. tuberculosis* plus interleukin (IL)-15 or IL-18 for 24 hr and tested for their lytic activity against K562 target cells. Results are expressed as percentage of cytotoxicity [mean ± standard error of the mean (SEM)]. Statistical differences: % cytotoxicity from monocyte-depleted cells versus % cytotoxicity from PBMC: $^*P < 0{\cdot}05$.

25 and 40% at the 1 : 1 ratio and was not modified by higher bacterial concentrations. In addition, the number of ICAM-1$^+$ CD14$^+$ cells and ICAM-1 expression were increased by IL-15 or IL-18 or by neutralization of IL-10 in the presence of *M. tuberculosis* (Fig. 1b). Taken together, these results suggest that the decrease in the number of ICAM-1$^+$ CD14$^+$ cells produced by *M. tuberculosis* may modulate NK activation and lytic activity by affecting adhesion to NK cells.

NK cell activity is down-regulated by monocytes in TB patients

Given that monocytes play an important role in NK activation, not only through the release of cytokines but also by signalling through costimulatory molecules, we investigated whether monocytes could modulate *M. tuberculosis*-induced NK activation. Thus, PBMC depleted of monocytes were stimulated with *M. tuberculosis* in the presence or absence of IL-15, IL-18 and IL-10 for 18 hr and tested for NK activity and CD16, CD11a and CD69 expression. As shown in Fig. 2, an increase in *M. tuberculosis*-induced NK lytic activity in cells from TB patients was detected by depletion of monocytes, and furthermore, in the presence of IL-15 or IL-18, an up-regulation of NK function was observed in cells from A-TB patients. However, *M. tuberculosis*-induced NK function in N controls was not affected by monocyte depletion. Taken together, these results suggest a regulatory role of monocytes in NK activation in TB patients.

As shown in Fig. 3, monocyte depletion did not modify the proportion of CD69$^+$ NK cells in control cells from TB patients or healthy individuals. However, *M. tuberculosis* stimulation in the absence of monocytes induced a

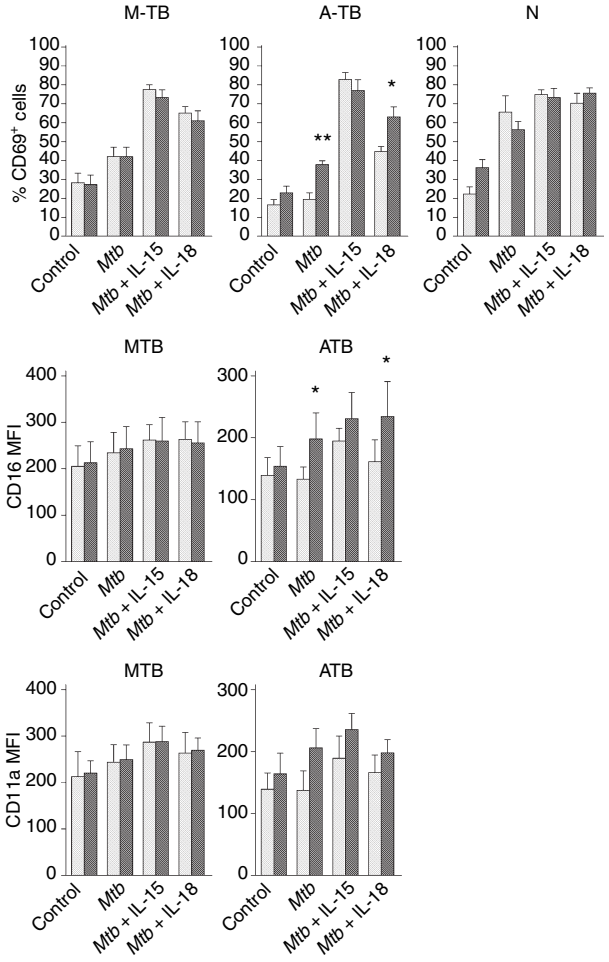

Figure 3. Monocytes down-regulated activation molecules on natural killer (NK) cells from patients with advanced tuberculosis (A-TB). Peripheral blood mononuclear cells (PBMC) (grey bar) and monocyte-depleted mononuclear cells (grey black bar) from 10 patients with moderate tuberculosis (M-TB), 13 patients with advanced tuberculosis (A-TB) and eight healthy controls (N) were incubated in complete medium (control), *Mycobacterium tuberculosis* or *M. tuberculosis* plus interleukin (IL)-15 or IL-18 for 24 hr. The expression of CD16, CD69 and CD11a molecules on $CD3^-$ $CD56^+$ cells was then determined by flow cytometry. Results are expressed as mean fluorescence intensity (MFI) for CD16 and CD11a molecules and percentage of $CD69^+$ cells [mean ± standard error of the mean (SEM)]. Statistical differences: monocyte-depleted versus PBMC: $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

decrease in the number of $CD69^+$ NK cells from N controls and an increase in that from A-TB patients, and did not modify that from M-TB patients. Furthermore, while IL-15 had no effect on the number of $CD69^+$ NK cells in TB and N controls in the absence of monocytes, the addition of IL-18 increased the percentage of $CD69^+$ NK cells and enhanced the expression of the CD16 molecule in *M. tuberculosis*-stimulated cells from A-TB patients.

To assess whether IL-10 directly modulates the activation of NK cells, PBMC were depleted of $CD14^+$ cells by magnetic methods and stimulated or not stimulated with

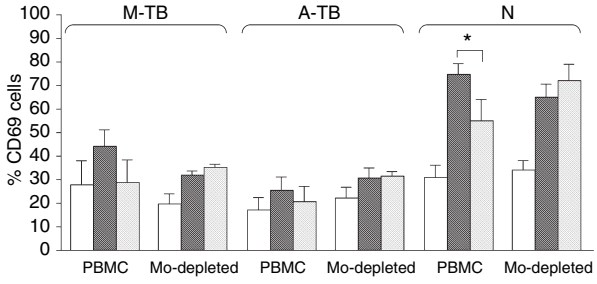

Figure 4. Effect of interleukin (IL)-10 in the monocyte-depleted (Mo-depleted) $CD69^+$ $CD3^-$ $CD56^+$ population. Peripheral blood mononuclear cells (PBMC) or monocyte-depleted mononuclear cells from six patients with moderate tuberculosis (M-TB), six patients with advanced tuberculosis (A-TB) and five healthy individuals (N) were cultured in complete medium (white bar), *Mycobacterium tuberculosis* (grey black bar) or *M. tuberculosis* + IL-10 (grey bar) for 24 hr and the percentage of $CD69^+$ on $CD3^-$ $CD56^+$ cells was evaluated by flow cytometry. Results are expressed as mean ± standard error of the mean (SEM). Statistical differences: N-PBMC (Mtb vs Mtb + IL-10): $^*P < 0{\cdot}05$.

M. tuberculosis in the presence or absence of IL-10 for 18 hr. Thereafter, the percentage of $CD69^+$ cells (as a measurement of activation) and CD16 expression on $CD3^-$ $CD56^+$ cells were determined by flow cytometry. As shown in Fig. 4, while in the presence of monocytes exogenous IL-10 down-regulated the activation of NK cells in M-TB patients and N controls, it had no effect on NK activation in the absence of monocytes. In addition, CD16 expression was not modified in NK cells by the depletion of $CD14^+$ cells (data not shown). With regard to NK function, *M. tuberculosis*-induced NK lytic activity was increased by monocyte depletion in M-TB patients [PBMC (mean ± SEM): 33 ± 5; CD14-depleted: 49 ± 6, $n = 3$] and A-TB patients (PBMC: 10 ± 2; CD14-depleted: 24 ± 5, $n = 3$). Moreover, the addition of IL-10 to monocyte-depleted PBMC increased NK lytic activity in cells from M-TB patients (PBMC: 29 ± 4; CD14-depleted: 50 ± 6, $n = 3$) and did not modify that in cells from A-TB patients (PBMC: 10 ± 2; CD14-depleted: 16 ± 6, $n = 3$). Overall, these data indicate that IL-10 does not exert a direct effect on NK activation, suggesting an indirect effect through the down-regulation of costimulatory molecules on monocytes necessary for the activation of NK cells.

Numbers of *M. tuberculosis*-induced IFN-γ^+ NK cells were increased by IL-15 and IL-18 in TB patients

In the light of the known roles of IL-15 and IL-18 in the induction of IFN-γ production by NK cells, we tested whether the observed effect of these monokines on NK activity could be ascribed to IFN-γ production. For this purpose, PBMC from TB patients and N controls were stimulated or not stimulated with *M. tuberculosis* for

Table 6. *Mycobacterium tuberculosis*-induced interferon (IFN)-γ^+ natural killer (NK) cells

	% IFN-γ^+ NK cells	
PBMC from	Control PBMC	*M.tb*-stimulated PBMC
M-TB ($n = 5$)	1·0 ± 0·5	5·8 ± 0·8*
A-TB ($n = 5$)	0·4 ± 0·3	2·5 ± 0·9
N ($n = 5$)	0·6 ± 0·3	7·5 ± 0·7*

Peripheral blood mononuclear cells (PBMC) from patients with moderate tuberculosis (M-TB) and advanced tuberculosis (A-TB) and healthy controls (N) were incubated in complete medium (control) or with *M. tuberculosis* (*M.tb*) for 24 hr and then the percentage of IFN-γ^+ cells on $CD3^-$ $CD56^+$ cells was evaluated by flow cytometry. Results are expressed as mean ± standard error of the mean (SEM). Statistical differences: control versus *M. tuberculosis*-stimulated PBMC: $^*P < 0{\cdot}05$.

24 hr and IFN-γ production by $CD3^-$ $CD56^+$ cells was analysed by flow cytometry. As shown in Table 6, no differences in IFN-γ production by non-stimulated (control) NK cells from TB patients and N controls were observed. However, upon *M. tuberculosis* stimulation, an increase in the proportion of IFN-γ^+ $CD3^-$ $CD56^+$ cells was detected for M-TB patients and N controls, while for A-TB patients no such increase was detected. Considering that IL-15 and IL-18 increased the number of $CD69^+$ cells, up-regulated expression of the CD16 molecule and enhanced NK function in both PBMC and monocyte-depleted PBMC from A-TB patients, we assessed whether these findings could be ascribed to enhanced IFN-γ production. As shown in Fig. 5, the proportion of IFN-γ^+ NK cells upon *M. tuberculosis* stimulation was increased by IL-15, even in the presence of monocytes, while IL-18 produced no such effect. However, IL-18 up-regulated the number of NK cells producing IFN-γ in *M. tuberculosis*-induced monocyte-depleted PBMC. Taken together, these data indicate that monocytes have a down-regulatory effect on IFN-γ-producing NK cells from A-TB patients.

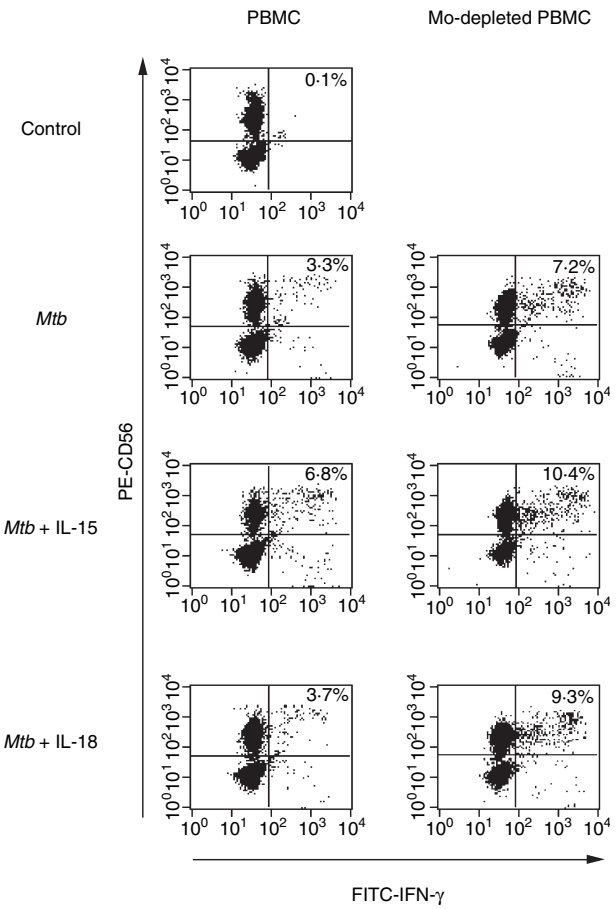

Figure 5. Monocytes from patients with advanced tuberculosis (A-TB) modulated interferon (IFN)-γ^+ production by $CD3^-$ $CD56^+$ cells. Peripheral blood mononuclear cells (PBMC) and monocyte-depleted mononuclear cells (Mo-depleted) from three A-TB patients were incubated in complete medium (control), *Mycobacterium tuberculosis* (*Mtb*), or *M. tuberculosis* plus interleukin (IL)-15 or IL-18 for 24 hr and IFN-γ production by $CD3^-$ $CD56^+$ cells was determined by flow cytometry. Results are expressed as the percentage of positive cells (data in the panels). A representative example is shown. FITC, fluorescein isothiocyanate.

Discussion

Although the role of NK cells in mycobacterial infections is unclear, *in vitro* studies have demonstrated that peripheral blood NK cells may contribute to protective immunity through the production of IFN-γ to maintain the frequency of *M. tuberculosis*-responsive $CD8^+$ IFN-γ^+ cells and expand their cytotoxic activity.[22,23] In addition, NK cells mediate early killing of intracellular *M. tuberculosis* via granule-independent mechanisms.[24]

Our study demonstrating a higher proportion of NK cells in TB patients is in contrast with data previously reported.[25,26] However, the decrease in lytic activity observed in NK cells from TB patients is in accordance with the results of studies employing either *M. tuberculosis*-infected macrophages or the K562 cell line as target cells.[25,26] Moreover, a major reduction in NK lytic activity is observed in severe pulmonary involvement, indicating that the microenvironment might affect NK activation and/or function. *In vitro* studies have demonstrated that human NK cells can be activated either by mycobacterium (*M. bovis*) bacille Calmette–Guérin (BCG)[27] or by lysed human monocytes infected with live *M. bovis* BCG[28] or *M. tuberculosis*.[29] The ability of NK cells to interact with target cells depends on the expression of cell surface receptors and adhesion molecules capable of binding to ligands on target cells. Herein, we have demonstrated that *M. tuberculosis* stimulates NK cells from healthy individuals, as shown by high expression of CD69 antigen and increases in natural cytotoxicity and IFN-γ production. However, *M. tuberculosis* stimulation of NK cells from TB

patients showed impairment of their function, with the severity of pulmonary disease, with low numbers of $CD69^+$ NK cells and an absence of *M. tuberculosis*-induced lysis and IFN-γ production, as was observed in A-TB patients. CD69 is a costimulatory molecule transiently expressed on activated lymphocytes,[6] and the mechanism regulating its expression on NK cells is obscure. It has been demonstrated that CD69 is up-regulated after activation of CD16, LFA-1/CD11a, IL-2 or IFN-α receptors which signal through protein tyrosin kinase (PTK) phosphorylation and Vav/extracellular regulated kinase (Vav/ERK).[7,30,31] Binding of the β2-integrin LFA-1 on NK cells to ICAM-1 on target cells initiates triggering signal transduction pathways[3,4,32] and, furthermore, LFA-1 ligation costimulates the CD16 receptor for cytokine production.[33] In addition to the role of CD16 in the killing of target cells by NK cells, its cross-linking initiates signalling events inducing granule exocytosis and cytokine production.[18,34] Herein, we have demonstrated that the expression of CD11a and CD16, molecules implicated in the lysis of target cells, is down-regulated in cells from A-TB patients and, furthermore, that *M. tuberculosis* reduces the numbers of $ICAM\text{-}1^+$ $CD14^+$ cells in TB patients. However, in the presence of inflammatory cytokines or neutralization of IL-10, the number of $ICAM\text{-}1^+$ $CD14^+$ cells and ICAM-1 expression are enhanced, even in the presence of *M. tuberculosis*, along with an increase in NK activity. Given that conjugate formation between NK and target cells is a prerequisite for target cell lysis and is critically dependent on engagement of CD11a with its ligands on APCs, ICAM-1 regulation on monocytes from TB patients could be a mechanism used by *M. tuberculosis* to reduce NK activity.[3,4,19,21,32] The finding that the expression of CD11a and CD16 molecules was associated in our study with the severity of the disease suggests that, in TB patients, the expression of costimulatory/activatory molecules and their biological functions could be conditioned by circulating cytokines and by those secreted upon *M. tuberculosis* stimulation by accessory cells.[35,36] Therefore, the low expression of CD11a on NK cells, together with ICAM-1 down-regulation on monocytes by *M. tuberculosis*, may contribute to the impairment of NK activity in TB patients by diminishing the adhesiveness necessary for triggering signalling pathways downstream of CD11a/ICAM engagement. Although, to date, no receptor has been shown to be either necessary or sufficient in signalling for NK cell cytotoxicity, other receptors on NK cells, such as NKG2D and NKp46, and their ligands on target cells could be involved in the regulation of NK cell effector function, indicating a possible redundancy of activation receptors and activation pathways.[2,37] In this context, natural cytotoxicity receptors may also be involved in the impairment of NK activity, as demonstrated for NKp46 in TB patients.[25]

It is well known that cytokines produced by accessory cells play an essential role in NK development and activation. IL-15 promotes *in vitro* NK cell development, mediates LFA-1 expression,[38] and enhances the lytic activity and proliferation of resting NK cells as well as the production of cytokines and chemokines.[13] While IL-18 increases the lytic activity of NK cells by up-regulating perforin-dependent cytotoxic pathways and Fas ligand (FasL) expression,[39,40] the role of IL-10 in NK activation is not clear.[41,42] Our results showed that, in effect, IL-15 enhanced *M. tuberculosis*-induced NK cytotoxicity in healthy individuals, inducing CD69 expression on NK cells just as *M. tuberculosis* does. However, IL-15 also up-regulated the expression of CD16 and CD11a on *M. tuberculosis*-stimulated NK cells from A-TB patients, in addition to increasing NK cytotoxicity in both groups of TB patients in association with a high number of $CD69^+$ NK cells. In contrast to IL-15, IL-18 did not modify NK cytotoxicity in A-TB patients but slightly up-regulated the expression of CD69 and CD11a molecules. IL-10 neutralization in PBMC from TB patients increased *M. tuberculosis*-induced NK cytotoxicity and up-regulated CD16 and CD11a expression, suggesting that endogenous IL-10 could inhibit NK activation and function through the down-regulation of ICAM-1 on monocytes. Therefore, the increase in costimulatory molecules could enhance the efficiency of NK lysis by increasing the interaction with target cells and triggering granule exocytosis.

IL-10 can boost IFN-γ production by isolated NK cells,[41,43] but in the presence of APCs it inhibits IFN-γ production.[20,42] In this context, our data for monocyte-depleted populations demonstrated that *M. tuberculosis* directly triggered the activation of NK cells from both TB patients and healthy controls, as previously reported with pathogenic and non-pathogenic bacteria.[27,44] Moreover, in the absence of monocytes, *M. tuberculosis*-induced NK cell activity was increased in TB patients while in N controls it was not. In addition, CD69 and CD16 expression on *M. tuberculosis*-stimulated NK cells was also increased in cells from A-TB patients, and IL-18 up-regulated their expression while IL-15 did not, demonstrating the different roles of these two cytokines in NK function.[38–40] Furthermore, in TB patients, the modulatory effect of monocytes was also observed in the production of IFN-γ by *M. tuberculosis*-stimulated NK cells, where the lowest levels detected in A-TB patients were increased by the depletion of monocytes. Although IFN-γ is not required for NK cytotoxicity and does not mediate the killing of intracellular *M. tuberculosis*,[24,38] it may contribute to switching the immune response towards type 1 by enhancing the production of IL-12 and IL-18 in response to *M. tuberculosis*.[47,48] Taken together, these results confirm a regulatory role of monocytes in NK cell activation and function in TB patients.

In conclusion, we have demonstrated that monocytes from TB patients regulate NK function involving IL-10, which acts through an indirect mechanism leading to down-regulation of costimulatory/adhesion molecules

and/or the IFN-γ production necessary for the activation of NK cells. Therefore, our findings demonstrate an underlying role of monocytes in NK function in TB patients.

Acknowledgements

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