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# Reduced NK activity correlates with active disease in HIV$^-$ patients with multidrug-resistant pulmonary tuberculosis

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## SUMMARY

There has been a global increase in the incidence of multidrug-resistant pulmonary tuberculosis (TB). As there are no previous reports of immune function in HIV$^-$ patients with multidrug-resistant pulmonary TB, a comprehensive assessment of cellular immunity in this setting was undertaken. This involved a prospective, case-controlled study which included five patients with active multidrug-resistant pulmonary TB and five matched controls with active non-resistant infection, and documented the changes in immune parameters which occurred upon clinical resolution. Patients with multidrug-resistant TB had significantly lower fresh natural killer (NK) cell activity than matched controls with non-resistant pulmonary TB ($P < 0.05$). This was a specific abnormality, as there were no significant differences in antigen-specific cytotoxicity or lymphocyte proliferation in the case-controlled study. Follow-up assessment of the patients with multidrug-resistant infections indicated that clinical improvement correlated with a moderate increase in NK cell activity. Impaired NK cell function may be involved in the pathogenesis of multidrug-resistant TB.

**Keywords** tuberculosis drug-resistance natural killer cells

## INTRODUCTION

The global incidence of multidrug-resistant pulmonary tuberculosis (MDRPTB) is increasing, especially in regions where pulmonary tuberculosis (PTB) is endemic and treatment inadequate [1]. In the Western Cape province of South Africa where this study was performed, TB has been endemic for several decades, with an estimated incidence at present of 709 cases per 100 000 population [2]. Multidrug resistance is defined as the culture of *Mycobacterium tuberculosis* resistant to two or more first-line anti-tuberculous agents from clinical isolates, and has been classified into primary and secondary occurrences. Primary MDRPTB involves culture of a multi-drug-resistant strain of *Myco. tuberculosis* from pre-treatment specimens. Secondary (acquired) drug-resistance, as indicated by the emergence of resistant strains of *Myco. tuberculosis* following therapy for a drug-sensitive infection, is more common than primary drug-resistance, and often associated with poor compliance [3]. In countries where the prevalance of TB is low, primary MDRPTB has been associated with HIV infection [4], perhaps implicating a role for defective host immunity in its pathogenesis [5]. To date, the focus of investigation in MDRPTB has been the organism, and host immune factors that may favour the emergence of resistant strains in HIV$^-$ individuals, or that may predispose to the development of primary multidrug-resistant disease, have not previously been defined.

A case-controlled and longitudinal study was undertaken to investigate cellular immune function in HIV$^-$ patients with MDRPTB, with particular emphasis on the evaluation of cellular cytotoxic function in primary MDRPTB. Given the ability of mycobacteria to survive within macrophages, it has been suggested that lysis of infected macrophages forms an essential component of protective cellular immunity, by exposing the organisms to alternate defence mechanisms [6]. Recent reports have emphasized the importance of antigen-specific cytotoxicity, mediated by CD4$^+$ lymphocytes, in TB [7,8]. In addition, there is experimental evidence that natural killer (NK) cells have a significant function in overcoming mycobacterial infection [9,10].

We present evidence of defective NK function in patients with multidrug-resistant pulmonary tuberculosis. We provide information indicating the specificity of this finding, and results of follow-up study which reveal significant improvement in NK activity upon clinical resolution and clearing of the drug-resistant organisms.

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**Table 1.** Clinical data of five patients with multidrug-resistant pulmonary tuberculosis and their matched controls

| Patient | Age, years | Sex | Body mass index, kg/m$^2$ | Serum albumin, g/*l* | Disease duration, months | Sputum AFBs | Drug resistance* |
|---|---|---|---|---|---|---|---|
| 1a | 22 | M | 13·9 | 35 | 44 | Positive | Primary (Rif, INH, Strep) |
| 2a | 42 | M | 16·5 | 29 | 14 | Positive | Primary (Rif, INH, Thia, Etham) |
| 3a | 35 | M | 17·6 | 37 | 20 | Positive | Primary (Rif, INH, Thia, Cyclo) |
| 4a | 45 | M | 20·1 | 36 | 6 | Positive | Primary (Rif, INH, Strep) |
| 5a | 19 | F | 13·3 | 36 | 12 | Positive | Secondary (Rif, INH, Etham) |
| Mean (±s.e.m) | 32·6 ± 5·2 | | 16·3 ± 1·2 | 34·6 ± 1·4 | 19·2 ± 6·6 | | |
| 1b | 31 | M | 13·6 | 37 | 18 | Positive | Non-resistant |
| 2b | 26 | M | 16·8 | 36 | 8 | Positive | Non-resistant |
| 3b | 35 | M | 15·1 | 33 | 28 | Positive | Non-resistant |
| 4b | 42 | M | 21·4 | 29 | 7 | Positive | Non-resistant |
| 5b | 23 | F | 17·8 | 26 | 14 | Positive | Non-resistant |
| Mean (±s.e.m.) | 31·4 ± 3·4 | | 16·9 ± 1·3 | 32·2 ± 2·1 | 15 ± 3·8 | | |
| † Wilcoxon rank sum test | $P = 0·855$ | | $P = 0·50$ | $P = 0·34$ | $P = 0·79$ | | |

* Rif, rifampicin; INH, isoniazid; Strep, streptomycin; Etham, ethambutol; Thia, thiacetazone; Cyclo, cycloserine.

† Comparison of multidrug-resistant group to non-resistant control group.

In addition to the data shown, patients and controls were matched for extent of chest radiographic changes. Multidrug-resistant patients (a) and their matched controls (b) are indicated by corresponding numbers.

## PATIENTS AND METHODS

*Patients*

A case control study was undertaken involving five patients with documented MDRPTB and five matched controls with non-resistant PTB. Eighteen healthy laboratory and medical staff were used as normal controls. At the time of the baseline study all the patients in both groups were in-patients at the same tuberculosis hospital, and all patients were sputum-positive for acid-fast bacilli and HIV$^-$. Patients and controls were closely matched according to clinical criteria (Table 1) and extent of chest radiographic involvement.

*Mycobacterium tuberculosis* isolated from the sputum were cultured according to standard techniques [11]. All five patients in the multidrug-resistant group had isolates that were resistant to at least three anti-tuberculosis agents at the time of the initial study (Table 1). Four of these patients had primary resistance as evidenced by the isolation of resistant organisms from pretreatment cultures. One patient developed a multidrug-resistant infection while receiving standard first line anti-tuberculous therapy for a sensitive infection (secondary resistance). This ratio of primary to secondary drug-resistance is the converse of that found in the population as a whole, and results from selection criteria. We specifically selected for patients with primary MDRPTB, as we reasoned they were more likely to have an underlying immune disorder. Mycobacteria cultured from the control group were sensitive to all first-line anti-tuberculous drugs.

Four of the multidrug-resistant patients underwent repeat evaluation of NK activity and flow cytometric analysis of peripheral blood mononuclear cells (PBMC) at an interval of 11–16 months after the initial study. At this point all patients were undergoing ambulatory therapy and were sputum-negative for *Myco. tuberculosis*.

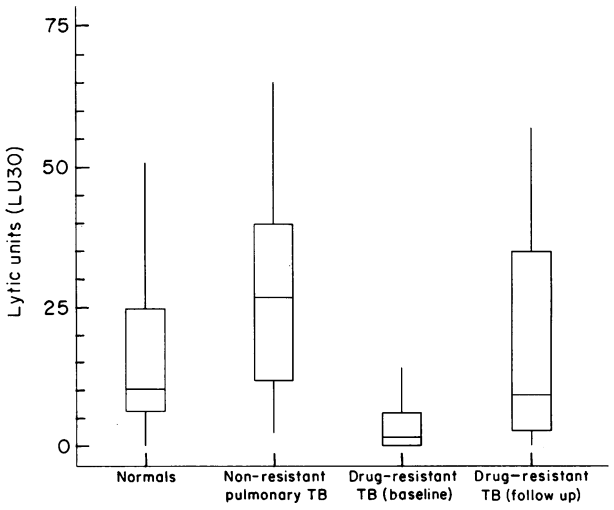


**Fig. 1.** Box and whisker plot of fresh natural killer (NK) activity in five patients with active multidrug-resistant pulmonary tuberculosis and matched controls with non-resistant pulmonary tuberculosis, four of the multidrug-resistant patients after 11–16 months of treatment, and 18 healthy laboratory workers. There was a significant reduction in fresh NK activity in the drug-resistant group compared with controls at the time of initial study ($P < 0·05$).

*Cellular immune function*

All baseline assays were performed without knowledge of the clinical diagnosis. Samples from each drug-resistant patient were processed simultaneously with their matched control. PBMC were isolated from 50 ml fresh venous blood by density centrifugation (Lymphoprep; Nycomed, Oslo, Norway) [8]. Following the final wash, cells were resuspended at $10^6$/ml in RPMI 1640 (Flow Labs, Mclean, VA) containing 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% pooled AB human serum (complete medium). For the NK and lymphokine-activated killer (LAK) assays 10% fetal calf serum (FCS) was used in place of the human serum. An aliquot of cells was cryopreserved for phenotypic analysis at a later stage.

PBMC proliferation in response to mitogen and antigens was analysed in microculture as previously described [8]. The mitogen phytohaemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, UK) was used at a final concentration of $5{\cdot}75 \times 10^{-3}$ mitogenic units/ml. The antigens included purified protein derivative of *Myco. tuberculosis* (PPD; Connaught Labs, Willodale, Ontario, Canada), a recombinant 65-kD heat-shock protein (hsp) of *Myco. bovis* (kind gift of Dr J. D. A. van Embden, Bilthoven, The Netherlands [12]) and streptokinase–streptodornase (SK–SD; Lederle Laboratory, Wayne, NJ; 250 U/ml streptokinase, 62·5 U/ml streptodornase). These antigens were used at the following final concentrations: PPD 3 $\mu$g/ml, 65 kD 5 $\mu$g/ml, and SK–SD at a final dilution of 1:40. Cultures were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 3 days (mitogen) or 6 days (antigens). Tritiated thymidine (Amersham International, Aylesbury, UK; specific activity 5 Ci/mmol) was added (2 $\mu$Ci/well) for the final 18 h of incubation. Means of triplicates were calculated and results expressed as a stimulation index (SI), where SI = mean ct/min in stimulated wells/mean ct/min in background (unstimulated) wells.

To assess cytotoxic cell activity, fresh NK activity was evaluated in a standard 4-h $^{51}$chromium ($^{51}$CR) release assay against the K562 erythroleukaemic cell line, as previously described [13]. Target cells ($3 \times 10^3$, labelled with 250 $\mu$Ci sodium chromate$^{51}$, specific activity 3·7–13 GBq/mg; Amersham) were added to wells of a 96-well U-bottomed tissue-culture plate (Flow) in 100 $\mu$l culture medium supplemented with 10% FCS. Mononuclear effector cells were serially diluted to give final effector : target (E/T) ratios of 90:1, 30:1, 10:1 and 3:1 and added to triplicate wells. Plates were centrifuged at 200 *g* for 5 min and incubated for 4 h at 37°C in a humidified atmosphere containing 5% $CO_2$. From each well 130 $\mu$l of supernatant were transferred to disposable counting tubes (Greiner, Nurtingen, Germany). Radioactivity of samples was measured in a gamma counter (auto-gamma scintillation spectrometer; Packard). To avoid diurnal variation all NK assays were performed at the same time of day. The percentage of specific $^{51}$Cr release (% cytotoxicity (Cx)) for the mean of triplicate wells at each E/T ratio was calculated as follows: $\%Cx = (E - S)/(W - S) \times 100$, where $E$ is the mean ct/min for the three experimental microwells, $S$ is the mean ct/min for spontaneous $^{51}$Cr release, and $W$ is the mean ct/min for maximal $^{51}$Cr release. Results are shown as the percentage specific cytotoxicity at each E/T ratio (Fig. 2) or expressed as lytic units (LU). This figure is calculated from the linear portion of the dose-response curve [14], where 1 LU is defined as the number of effector cells required to cause 30% specific cytolysis of $3 \times 10^3$ target cells (LU30).

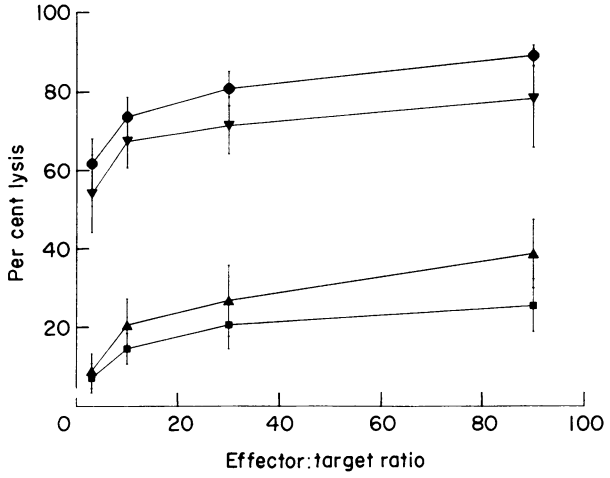


**Fig. 2.** Fresh natural killer (NK) activity and lymphokine-activated killer (LAK) in multidrug-resistant pulmonary tuberculosis. An improvement in fresh NK activity with clinical resolution is shown ($P = 0{\cdot}04$ at 90:1 effector:target ratio). High levels of LAK against both target cell lines were found in the follow-up study. ■, Baseline fresh NK activity *versus* K562; ▲, follow-up fresh NK activity *versus* K562; ▼, follow-up LAK *versus* Daudi; ●, follow-up LAK *versus* K562.

For measurement of LAK activity effector cells were generated by culturing $6–10 \times 10^6$ PBMC at $10^6$/ml in complete medium with 100 U/ml recombinant IL-2 (rIL-2; Cetus Corporation, Emeryville, CA) in 25 cm$^2$ tissue culture flasks for 7 days. The remainder of the assay was performed as for the fresh NK cytotoxicity assay described above, with the additional inclusion of the NK-resistant but LAK-sensitive Daudi cell line as a second target cell.

Antigen-specific cytotoxicity was measured in a 15 h $^{51}$Cr release assay as previously reported [8]. Effector cells were generated from PBMC by culturing $6–10 \times 10^6$ PBMC at $10^6$/ml in complete medium with 3 $\mu$g/ml PPD in 25-cm$^2$ tissue culture flasks for 6 days. Target cells were autologous adherent PBMC that were plated at the initiation of the assay. On day 5 of culture, target cells were pulsed with antigens (PPD 10 $\mu$g/ml; SK–SD 1:10 dilution, or medium alone) and labelled with 6 $\mu$Ci/well $^{51}$Cr for 24 h. Effectors were added to give final E/T ratios of 10:1, 3:1 and 0·3:1. Adherent target cell numbers were estimated as 10% of mononuclear cells plated. After 15 h the total content of each well was transferred to a disposable counting tube, and 100 $\mu$l of 5% Triton X added to the remaining adherent cells for the determination of maximum release. After 3 h incubation at 37°C the total volume of Triton X was transferred to similar tubes and radioactivity counted. The percentage specific cytotoxicity for the mean of triplicate wells was calculated as follows: percentage specific lysis = (mean test ct/min/(mean test + mean ct/min after Triton X treatment of the same triplicate wells)) × 100% − percentage spontaneous release. The percentage spontaneous release was calculated as follows: mean ct/min in spontaneous release wells/(mean ct/min in spontaneous release wells + mean ct/min after Triton X treatment of the same triplicate wells) × 100%. For

the results in Table 2, lytic units were calculated as described above, except in this instance 1 LU is the number of effector cells required to cause specific lysis of $1 \times 10^4$ adherent monocyte target cells.

*Skin tests*

Skin reactivity to a panel of seven antigens was determined in the five patients with MDRPTB and three of the matched, non-resistant controls using a multiple skin test applicator (Multitest CMI; Institut Mérieux, Lyon, France). Induration was measured after 48 h.

*Flow cytometric analysis*

MoAbs used were CD3-RD1, CD4-FITC, CD8-FITC, NKHI-RD1 (anti-CD56), B4-FITC (anti-CD19) and T11-RD1 (anti-CD2) (Coulter Immunology, Hialeah, FL). Dual parameter flow cytometric analysis was performed on an Epics Profile II (Coulter). PBMC were analysed at $10^6$/ml in complete medium, using 100 $\mu$l per assay. Histograms generated were (i) forward scatter (FS) *versus* log side scatter (LSS), (ii) log fluorescence 1 (LF1, green fluorescence) *versus* log fluorescence 2 (LF2, red fluorescence), (iii) and (iv) single parameter histograms of LF1 and LF2.

Lymphocytes were bitmapped on histogram 1 to visually exclude debris and monocytes; where this proved difficult the quality of the lymphocyte gate was assessed using CD45 and CD14 (KC56-FITC, Mo2-RD1; Coulter). Non-specific antibody binding was evaluated using the relevant mouse isotypic controls, allowing for 2% false positives. Histogram data was generated for $10^4$ events.

*Statistical analysis*

The Mann–Whitney *U*-test and, where appropriate, the Wilcoxon rank sum test for paired data were employed for statistical comparison using the software program Statgraphics (STSC Inc, Rockville, MD).

**Table 2.** Peripheral blood mononuclear cell proliferation and purified protein derivative (PPD)-specific cytotoxicity of five patients with active multidrug-resistant pulmonary tuberculosis and five matched controls with non-resistant infection

| | Resistant | Controls |
|---|---|---|
| PHA* | 421·0 ± 133·9 | 346 ± 110·9 |
| PPD* | 63·4 ± 18·5 | 108·0 ± 57·8 |
| 65-kD–hsp* | 2·0 ± 0·4 | 14·1 ± 11·0 |
| SK–SD* | 50·4 ± 36·7 | 80·2 ± 55·9 |
| PPD cytotoxicity† | 28·0 ± 21·7 | 33·2 ± 16·9 |

* Stimulation index (SI): mean ct/min in stimulated wells/mean ct/min in background (unstimulated) wells.

† Lytic units (LU) at 30% cytolysis (LU30): 1 LU is defined as the number of effector cells required to cause 30% specific cytolysis of $10^4$ target cells.

PHA, Phytohaemagglutinin; SK–SD, streptokinase–streptodornase.

Means ± s.e.m. are indicated. There were no statistically significant differences between the two groups in any of these parameters.

## RESULTS

*Patient population*

Patients with MDRPTB and controls with non-resistant disease were closely matched for age, sex, disease duration and severity, nutritional status (height/weight ratio and serum albumin) (Table 1), and extent of chest radiographic changes. There were no statistically significant differences between the two groups in any of the matching parameters. All patients were sputum-positive for acid-fast bacilli at the time of the baseline study. All patients in the multidrug-resistant group had isolates from their sputa that were resistant to at least three anti-tuberculous drugs; four of these patients had primary resistance. Although clustering of primary MDRPTB in families has been noted in this region previously, none of these patients had a significant family history of PTB.

*Impaired NK activity in MDRPTB*

Fresh NK activity, measured in a 4-h $^{51}$Cr release assay against the K562 target cell line, was significantly lower in the multidrug-resistant group compared with their matched, non-resistant case-controls at the time of initial study ($P < 0{\cdot}05$) (Fig. 1). This difference was present at each of the four E/T ratios tested and was consistent for all five pairs of patients. In addition, the drug-resistant group showed evidence of reduced NK activity compared with healthy laboratory normals ($P < 0{\cdot}05$). Conversely, the non-resistant controls exhibited a tendency to higher NK activity than healthy laboratory normals, but this was not statistically significant.

*Increase in NK activity following therapy in MDRPTB*

The impaired NK activity of the MDRPTB patients may have preceded infection, i.e. the result of a primary immune defect, or may have been a direct consequence of active infection by a multidrug-resistant strain. To distinguish between these possibilities, four patients with multidrug-resistant TB were followed up for a period of between 11 and 16 months after the initial study, at which point all patients had been sputum-negative for acid-fast bacilli for at least 6 months, and complete clinical resolution had occurred. According to treatment protocols, these patients were still receiving ambulatory therapy at this stage. Repeat assays showed levels of fresh NK activity within the range of laboratory normals (Fig. 1). When compared with the initial assessment, this increase was statistically significant (Fig. 2) ($P < 0{\cdot}05$). However, some diversity was noted in this follow-up assessment: one of the four patients demonstrated a marked increase in NK activity of 14·0–57·1 LU; the same patient had the highest NK activity of this group at baseline study. Two patients showed moderate improvement. One patient, who showed no change at all, had the lowest baseline NK activity of the group (LU30 < 1·0). Of note, this particular patient had developed MDRPTB while receiving appropriate treatment for a drug-sensitive infection with no prior history of PTB, and no other factors commonly associated with the emergence of secondary drug-resistance.

*Generation of LAK activity by* in vitro *culture with recombinant IL-2*

To investigate whether the impaired NK function noted in the initial study was the result of an intrinsic defect in NK effector lytic capability, mononuclear cells from the four patients

included in the longitudinal study were stimulated *in vitro* with rIL-2 for 7 days. Markedly increased lysis of both K562 and Daudi target cells was found (Fig. 2). Even the patient with the lowest baseline fresh NK activity, which showed no increase following therapy, demonstrated high levels of cytotoxicity in this assay (94% specific lysis against both target cell lines at 90:1 E/T ratio).

*Lymphocyte proliferation and antigen-specific cytotoxicity*

As part of a comprehensive assessment of cellular immune function in the case controlled study the ability of PBMC to mount *in vitro* proliferative and cytotoxic responses to recall antigens, both relevant and irrelevant, was assessed. There was no significant difference in lymphocyte proliferative response to mitogen or antigen between the drug-resistant group and non-resistant control patients (Table 2). A stimulation index (SI) of greater than 20 in response to PPD was found in all patients. Only three patients (two non-resistant and one multidrug-resistant) showed significant responses (SI > 3) to the recombinant 65-kD hsp of *Myco. bovis*. Similarly, no significant difference in PPD-specific cytotoxicity between the multidrug-resistant and their matched non-resistant controls was found (Table 2). Lymphocytes from all patients were capable of at least moderate cytolytic activity against PPD-pulsed autologous monocytes (20–40% lysis at an E/T ratio of 10:1), but considerable inter-patient variation was noted.

*Skin reactivity*

*In vivo* proliferative responses to a panel of seven recall antigens were measured in a conventional skin test. There was no significant difference in the composite score of skin reactivity between the drug-resistant patients and their controls at the time of baseline study. Apart from one patient with drug-resistant TB, all patients reacted to tuberculin.

*Lymphocyte subset phenotypic analysis*

Apart from one patient with primary MDRPTB, the percentage of lymphocytes expressing CD3, CD4, CD8 and CD56 antigens from both patient groups in the baseline assessment, and three of the four patients with MDRPTB included in the follow-up study, were in the normal range (Table 3). The single MDRPTB patient with abnormal phenotypic analysis had a profound reduction in the percentage of $CD3^+$ lymphocytes at both initial and follow-up assessments. This was associated with an inverted CD4:CD8 ratio of 0·15 at initial assessment, which increased to 3·0 with clinical resolution. These abnormally low percentages of $CD3^+$ lymphocytes were considered a real finding, as the total of $CD3^+$, $CD56^+$ and $CD19^+$ percentages was within acceptable limits on both occasions (100 ± 10%). Interestingly, despite abnormally low percentages of $CD4^+$ lymphocytes, this patient had normal *in vitro* proliferative and cytotoxic responses to recall antigens.

The case-controlled study revealed a statistically significant decrease of the CD4:CD8 ratio in the drug-resistant patients, but not the matched controls, compared with normals. Given the small numbers included in the analysis and the strong influence of a single abnormal patient (2a) on this result, it is of doubtful biological significance. There was, however, a tendency to increased percentages of $CD56^+$ (NK) cells in both the multidrug-resistant and non-resistant control groups compared with laboratory normals, but this was not statistically significant. Both patient groups had similar percentages of $CD56^+$ cells, and there were no significant differences in the

**Table 3.** Phenotypic analysis of peripheral blood mononuclear cells

| Patient | CD3 (baseline) | CD3 (follow up) | CD4 (baseline) | CD4 (follow up) | CD8 (baseline) | CD8 (follow up) | CD56 (baseline) | CD56 (follow up) | CD4:CD8 (baseline) | CD4:CD8 (follow up) |
|---|---|---|---|---|---|---|---|---|---|---|
| *MDRPTB* | | | | | | | | | | |
| 1a | 81·9 | 78·4 | 51·2 | 38·1 | 26·6 | 32·9 | 11·8 | 15·7 | 1·92 | 1·15 |
| 2a | 23·6 | 21·1 | 3·0 | 15·0 | 20·0 | 5·0 | 46·0 | 25·5 | 0·15 | 3·00 |
| 3a | 60·2 | 67·3 | 38·7 | 40·1 | 25·4 | 23·7 | 17·8 | 15·7 | 1·52 | 1·69 |
| 4a | 66·8 | ND* | 38·4 | ND | 24·3 | ND | 17·9 | ND | 1·58 | ND |
| 5a | ND* | 80·6 | ND | 44·8 | ND | 24·0 | ND | 7·8 | ND | 1·87 |
| Mean (±s.e.m.) | 58·1 ± 12·4 | 62·0 ± 13·8 | 32·8 ± 10·4 | 34·5 ± 6·7 | 24·1 ± 1·5 | 21·4 ± 5·9 | 23·4 ± 7·7 | 13·6 ± 4·6 | 1·29 ± 0·39† | 1·93 ± 0·39 |
| *Controls* | | | | | | | | | | |
| 1b | 56·4 | | 30·3 | | 23·3 | | 39·6 | | 1·30 | |
| 2b | 67·9 | | 40·6 | | 23·2 | | 34·1 | | 1·75 | |
| 3b | 66·8 | | 34·8 | | 40·2 | | 21·5 | | 0·87 | |
| 4b | 86·8 | | 73·5 | | 15·1 | | 7·6 | | 4·86 | |
| 5b | 76·0 | | 57·2 | | 13·8 | | 4·1 | | 4·14 | |
| Mean (±s.e.m.) | 70·8 ± 5·1 | | 47·3 ± 8·0 | | 23·1 ± 4·7 | | 21·4 ± 7·0 | | 2·54 ± 0·44 | |
| Normals ($n = 8$) Mean (±s.e.m.) | 69·3 ± 2·0 | | 45·9 ± 2·4 | | 21·0 ± 1·1 | | 11·3 ± 1·5 | | 2·25 ± 0·20† | |

* Analysis not done.

† $P < 0·05$.

Repeat analysis of the drug-resistant group was performed after 11–16 months of treatment. Values shown are percentages of lymphocytes as determined by standard flow cytometric techniques. Multidrug-resistant patients (a) and their matched controls (b) are indicated by corresponding numbers.

percentages of the $CD8^+$ $CD56^+$ subpopulation of NK cells (data not shown).

## DISCUSSION

The increasing emergence of multidrug-resistant strains of *Myco. tuberculosis* is of considerable concern. In addition to the high levels of morbidity and mortality associated with MDRPTB [16], new anti-mycobacterial drugs require prolonged courses of treatment and are often associated with new resistant strains [17]. The capacity of mycobacteria to mutate continuously [18], and the selection pressures that new drugs provide, would suggest that alternative therapeutic strategies are required. Stimulation of host immunity may be one such strategy, but requires the identification of a relevant immune parameter that could be the target of therapeutic modulation. Although secondary drug-resistance is more common, we reasoned that analysis of immune function of $HIV^-$ patients with primary MDRPTB was more likely to reveal such an immune parameter, especially given the epidemiological evidence for an association of immune impairment with primary MDRPTB [4,16,19]. Our in-depth analysis of cellular immune function in a selected group of patients with MDRPTB has revealed a significant impairment in fresh NK activity associated with active disease. This is the first indication of immune dysfunction in patients with MDRPTB where the effects of HIV infection have been excluded.

Increased NK cell number with enhanced activity has previously been described in active PTB [20,21], and also occurred in our drug-sensitive control group. By contrast, although there was an increase in the percentage of circulating NK cells in the drug-resistant group at the time of initial assessment, this was associated with reduced lytic capability. This reduction in lytic capability was not a result of generalized anergy due to extensive disease in the drug-resistant group, as other parameters of cell-mediated immunity (proliferation, PPD-specific cytotoxicity and skin tests) were within normal limits. Similarly, non-specific immunosuppression associated with mycobacterial infection cannot be involved, as this defect was confined to patients with MDRPTB and was not a feature of matched controls with active but non-resistant TB. Thus reduction in NK activity may suggest a role for impaired NK function in the pathogenesis of MDRPTB in $HIV^-$ patients.

The increase in NK activity following treatment in three of the four patients included in the longitudinal study suggests that in these patients the impairment of NK function was secondary to the disease, and possibly the result of the use of less effective second- and third-line anti-tuberculous agents. Effective bactericidal therapy would be expected to be associated with the release of mycobacterial antigens, immune activation and cytokine release, with resultant augmentation of NK activity [22]. Thus it is possible that such immune activation fails to occur in the patients with active MDRPTB, where the mycobacteria remain sequestered within the intracellular compartment due to ineffective treatment. This hypothesis could be tested by comparing NK function in newly diagnosed patients with drug-sensitive and MDRPTB before therapy. If the hypothesis was correct, similar NK function would be observed in both groups at this assessment, and effective bactericidal therapy should result in a significant increase in NK function confined to the patients with drug-sensitive infections.

One patient, however, failed to show any improvement in NK cell activity 9 months after clearing the multidrug-resistant organisms, indicating the possibility of a primary immune defect. Most interestingly, the impairment in NK function of this patient was associated with the development of multidrug-resistant disease while receiving appropriate therapy for a drug-sensitive infection, in the absence of other risks for developing secondary resistance (poor compliance, prior infection, family contact, alcoholism or drug abuse). This may suggest a role, in this patient, for NK cells in preventing the emergence of new resistant strains, perhaps by reducing the bacterial load [10] and thereby the risk of mutation to drug-resistance [23].

Our conclusions must be considered tentative given the small size of the current study. NK function in MDRPTB needs to be evaluated in a larger series. However, confirmation of the association of impaired NK activity in the pathogenesis of multidrug-resistant pulmonary tuberculosis may provide the rationale for the use of cytokines to stimulate NK activity [24,25], as an adjunct to conventional anti-tuberculous therapy. With the progressive impairment of NK function in HIV infection, and the high incidence of MDRPTB, cytokine augmentation of NK function may also be a useful adjunct to current prophylaxis and treatment in $HIV^+$ patients.

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