Immunological markers of lung disease due to non-tuberculous mycobacteria

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Abstract. Lung disease due to non-tuberculous mycobacteria (NTM) is a poorly understood condition that is difficult to treat. Treatment remains problematic as few tools are available to help clinicians monitor disease progression or predict treatment outcome. In this study, plasma levels of several inflammatory molecules and the frequency of circulating T cell subsets were measured in patients with NTM lung disease and known treatment status, and compared with their adult offspring and with unrelated healthy controls. Plasma levels of the chemokine CXCL10 and IL-18 were assessed for associations with treatment efficacy. CXCL10 was higher in patients than adult offspring ($p < 0.001$) and unrelated controls ($p < 0.001$). Plasma CXCL10 was also lower in patients who responded well to therapy or who controlled their infection without requiring therapy, when compared to patients who did not respond to therapy ($p = 0.03$). Frequencies of activated (HLA-DR$^+$) CD4$^+$ T cells were higher in patients than adult offspring ($p < 0.001$) and unrelated controls ($p < 0.05$), with the highest frequencies in individuals who had completed at least 6 months of treatment. Frequencies of activated (CD38$^+$) CD8$^+$ T cells in most treatment responders were similar to unrelated controls. Low plasma levels of CXCL10 may reflect successful control of NTM lung disease with or without therapy. Compared with responders, patients who responded poorly to treatment generally had higher plasma levels of CXCL10 and IL-18, and higher frequencies of activated CD8$^+$ T cells.

Keywords: CXCL10, lung disease, non-tuberculous mycobacteria, T cells

1. Introduction

Non-tuberculous mycobacteria (NTM) comprise species of mycobacteria other than the *Mycobacterium tuberculosis* (MTb) complex and *M. leprae*. They are ubiquitous environmental organisms that rarely cause disease in immunocompetent hosts. NTM often cause extrapulmonary or disseminated disease in immuno-compromised individuals (including HIV-infected patients), but pulmonary infections are uncommon. The susceptibility of a subset of otherwise healthy adults to NTM lung disease (NTMLD) and the pathogenesis of this condition remain poorly understood.

Treatment of NTMLD and monitoring of treatment responses remains problematic. Cure rates are low and some NTM are resistant to antituberculous drugs. Other problems include poor adherence to treatment regimens (minimum 3 drugs for at least 18 months), side effects and adverse interactions with other medications. Relapse or reinfection is common after apparently successful therapy [1–3]. There are few tools to accurately predict treatment response or relapse, or to monitor disease progression.

In the present study, pathogenesis of NTMLD was investigated using simple tools appropriate for use in routine clinical laboratories. We enrolled patients with NTMLD and known treatment status, the offspring of some patients and unrelated healthy controls. The offspring (adult sons and daughters) control for past or current exposure to NTM. NTMLD has been associated with suboptimal T-helper 1 (Th1) cell-mediated immunity. Here we measured plasma levels of molecules associated with Th1, Th2 and Th17 inflammatory re-

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sponses to determine whether an immunological bias could be detected in the blood of NTM patients. IL-10, IL-18 and the chemokine CXCL10 were included as candidate regulatory and effector molecules. IL-18 induces IFNγ production in the presence of IL-12 [4]. CXCL10 is induced in several cell types including peripheral blood mononuclear cells (PBMC), fibroblasts and epithelium after exposure to IFNγ [5,6]. Levels of these molecules were considered in relation to NTMLD status and treatment outcome. Frequencies of activated, memory and regulatory T cells were measured in cryopreserved PBMC.

2. Methods

2.1. Study groups and sample collection

We enrolled 27 patients with NTMLD attending outpatient clinics at Royal Perth Hospital (Western Australia) between March 2007 – March 2009 (7 males, 20 females; median age and range = 66, 37–92), 15 adult offspring of 12 patients (7 males, 8 females; median age and range = 42, 15–67), and 28 unrelated healthy controls consisting predominantly of hospital and university staff (17 males, 11 females; median age and range = 55, 36–79). All subjects were Caucasian except for 3 patients and 1 adult offspring who were Asian. Offspring and unrelated controls had no histories of mycobacterial disease. NTMLD was diagnosed using guidelines of the American Thoracic Society [7]. Exclusion criteria were current smoking, alcohol excess, cystic fibrosis, HIV infection and use of immunosuppressive medications.

At the time of enrolment, patients provided a blood sample and were categorized by the treating physicians. Patients were categorized as 'early treatment' if they had received less than 6 months of therapy. Four patients were treatment-naïve when they were sampled – three never required treatment and the fourth was sampled prior to commencing treatment. Treatment 'responders' or 'non-responders' were sampled after a minimum 6 months of therapy or after completion of therapy (usually 18 months). Treatment responses were defined at the time of sampling by the treating physicians. Response required elimination of NTM from sputum (if present) as well as symptomatic improvement (weight gain and/or reduced cough). Informed written consent was obtained from all individuals. This study was approved by the Ethics Committee of Royal Perth Hospital and conforms with the Declaration of Helsinki.

Routine cultures for NTM in patient sputum samples were performed by the PathWest Mycobacterium Reference Laboratory using a Bactec MGIT 60 Mycobacterial Detection System (BD Microbiology Systems, San José, CA) and Gerloff's media at 37°C and 32°C. Colonies containing acid-fast bacilli were identified by an in-house multiplex PCR assay [8]. Identification was achieved through sequencing of the gene encoding 16S ribosomal RNA. Species with identical 16S sequences were differentiated by additional biochemical testing. Species isolated from these patients were *M. intracellulare* ($n = 14$), *M. avium* ($n = 5$) or dual infection with *M. intracellulare* and *M. avium* ($n = 8$).

Plasma was separated from heparinised whole blood and stored at $-80°$C. PBMC were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and cryopreserved in foetal calf serum with 10% dimethyl sulfoxide. Plasma was available from 24 patients, all offspring and 21 unrelated controls. PBMC were available from 16 patients, all offspring and 13 unrelated controls.

2.2. Plasma assays

To measure CXCL10, IL-4 and IL-10, plasma samples were diluted 1/5 and assayed by Cytometric Bead Array (CBA; BD Biosciences, San José, CA) according to the manufacturer's protocol. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure levels of IL-18 (Bender MedSystems, Burlingame, CA), IL-17A (eBioscience, San Diego, CA), IFNγ, TNFα and IL-5 (BD Biosciences) in plasma samples diluted 1/3 and 1/9. All ELISAs were performed according to the manufacturers' protocols, with the exception of the assay diluent, where 1% BSA/PBS was used throughout.

2.3. Flow cytometry

PBMC were thawed into warm RPMI and resuspended at a concentration of 1×10^7 cells/mL. Six-colour flow cytometry was performed on 5×10^5 cells stained with fluorochrome-conjugated monoclonal antibodies (mAb) to CD4 (clone L200), CD8 (SK1), CD45RO (UCHL-1), HLA-DR (L243), CD38 (HIT2) and FoxP3 (259D/C7). All mAb were purchased from BD Biosciences. Acquisition was performed immediately after staining on a FACSCanto II cytometer (BD Biosciences). The stopping gate was set at 100,000 lym-
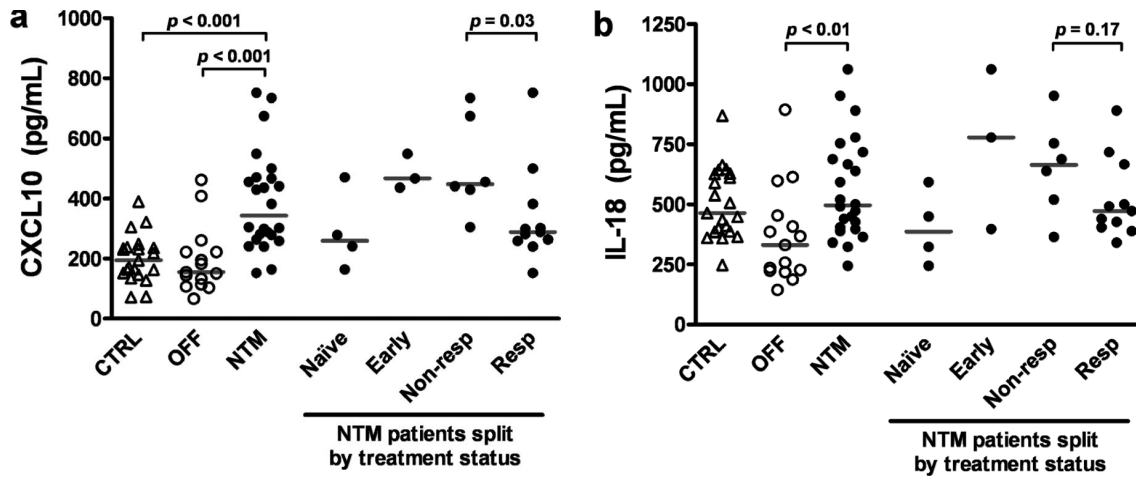
Fig. 1. Lower plasma levels of CXCL10 and IL-18 in patients with NTMLD may be associated with better control of NTM infection. Levels of CXCL10 (a) and IL-18 (b) were quantified in plasma from patients with NTMLD (NTM, $n = 24$), offspring of NTM patients (OFF, $n = 15$) and unrelated healthy controls (CTRL, $n = 21$). The patients were also divided into subgroups to assess whether levels of either or both molecules were associated with response to treatment. Dark gray horizontal lines represent the median value. Non-resp, non-responders to treatment; Resp, responders to treatment.

phocyte events as defined by forward scatter (FSC) and side scatter (SSC). Files were exported in FCS 3.0 format and visualised using FlowJo software v7.2.5 (Tree Star, Ashland, OR). Doublets were excluded using FSC-height vs. FSC-area parameters. $CD4^+$ and $CD8^+$ T cells were defined by high expression of CD4 or CD8 respectively against SSC-area [9].

2.4. Statistical analysis

All statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA) using tests for non-parametric data. Kruskal-Wallis tests with Dunn's multiple comparison test were used to compare patients, offspring and controls. Mann-Whitney tests were used to compare data between two groups where there were at least 6 subjects in both groups. P-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Patients with NTMLD display elevated plasma CXCL10 and IL-18 and successful control of disease is associated with lower levels of both molecules

IFNγ, TNFα, IL-4, IL-5, IL-17A and IL-10 could not be detected in plasma from any individuals tested. Levels of CXCL10 were higher in NTM patients compared to offspring or unrelated controls (Fig. 1a). Plas-

ma IL-18 was higher in NTM patients than offspring (Fig. 1b). NTM patients displayed a weak correlation between levels of CXCL10 and IL-18 ($r = 0.40$, $p = 0.05$).

As plasma levels of CXCL10 and IL-18 varied between patients, we hypothesised that levels may reflect treatment status or response to treatment. Patients were categorized as treatment-naïve ($n = 4$), early treatment ($n = 3$), treatment non-responders ($n = 6$) and treatment responders ($n = 11$). Plasma levels of CXCL10 were lower in responders than non-responders, but remained elevated compared with offspring ($p = 0.004$) or unrelated controls ($p = 0.002$) (Fig. 1a). Plasma IL-18 was also lower in responders than non-responders, but the difference was not significant (Fig. 1b). Plasma IL-18 was higher in responders compared with offspring ($p = 0.02$) and similar to unrelated controls ($p = 0.72$). The treatment-naïve patients had low levels of CXCL10 and IL-18, possibly reflecting their milder disease course (all remain healthy with or without treatment).

When NTM patients were divided by absence ($n = 17$) or presence ($n = 7$) of COPD, or divided into common clinical phenotypes of NTMLD (nodules only *or* bronchiectasis only, $n = 6$; nodules *and* bronchiectasis, $n = 13$; cavitation, $n = 5$), levels of plasma CXCL10 or IL-18 were similar ($p > 0.05$, data not shown).

Together, our data shows elevated plasma levels of CXCL10 and IL-18 in NTM patients compared with unrelated controls and/or offspring. Plasma levels of CXCL10 and IL-18 may be useful surrogate markers of

successful outcome in patients receiving treatment, or of well-controlled disease in treatment-naïve patients.

3.2. Patients with NTMLD exhibit higher frequencies of activated T cells in the circulation

Frequencies of $CD4^+$ and $CD8^+$ T cells with a memory, activated or regulatory phenotype were assessed in PBMC from 16 patients with NTMLD (3 treatment-naïve, 3 early treatment, 6 responders and 4 non-responders), 15 offspring and 13 unrelated controls. Patients with NTMLD exhibited lower frequencies of $CD4^+$ T cells compared with offspring but not with unrelated controls (Fig. 2a). No differences were observed in the frequency of $CD8^+$ T cells (Fig. 2b), memory ($CD45RO^+$) $CD4^+$ or $CD8^+$ T cells (Fig. 2c and d) or $FoxP3^+$ regulatory $CD4^+$ T cells (Fig. 2e). No remarkable differences were evident when patients were divided into treatment subgroups (data not shown).

The frequency of activated $CD4^+$ T cells (defined as $HLA-DR^+CD4^+$) was higher in both NTM patients and unrelated controls compared with offspring, in whom expression of HLA-DR displayed a narrow range (2–6% of $CD4^+$ T cells). Patients who had completed at least 6 months of therapy generally had higher frequencies of $HLA-DR^+CD4^+$ T cells (Fig. 2f). The frequency of activated $CD8^+$ T cells (defined as $CD38^+CD8^+$) was higher in both NTM patients and offspring compared with unrelated controls. The 4 non-responders exhibited higher frequencies of activated $CD8^+$ T cells (40–58%) than most responders ($< 40\%$ in 5 of 6 patients) (Fig. 2g).

Overall, most patients had elevated proportions of activated T cells. Activated $CD4^+$ T cells were lowest in patients' offspring and were clearest in patients on long-term (> 6 months) treatment, irrespective of their response to therapy. Activated $CD8^+$ T cells were evident in the patient's offspring and were greatest in patients failing therapy.

4. Discussion

Management of patients with NTMLD is hampered by a lack of diagnostic or prognostic markers [7]. This situation will likely remain whilst factors influencing susceptibility to NTMLD are unclear. We assessed T cell profiles and plasma levels of inflammatory molecules associated with Th1, Th2 and Th17 immunity and their use as simple assays that may identify an immunological bias towards or away from Th1 immunity and consequently may reflect treatment status.

We could not detect T cell-derived cytokines or the regulatory cytokine IL-10 in the plasma of any individuals with the commercial assays employed. This suggests that T cell-mediated inflammatory processes remain sequestered in the lung. CXCL10 and IL-18 are recognised as important mediators of the innate immune response against mycobacteria, and elevated levels of both molecules have been detected in patients with tuberculosis (TB) [10–12]. Previous studies of immune function associated NTMLD with poor IFNγ responses to mitogens and/or bacterial antigens [13–17]. However, elevated levels of CXCL10 reported here (Fig. 1a) indicate that ongoing IFNγ release is occurring *in vivo*. Furthermore, PBMC from patients with NTMLD demonstrate similar or higher *in vitro* production of IFNγ in response to mitogen or sensitin purified protein derivative compared with those from healthy control donors (Lim *et al.*, manuscript in press). Lower levels of CXCL10 in patients who responded to therapy may reflect lower mycobacterial burden. Plasma IL-18 was also slightly lower in the responders. Lower plasma levels of IL-18 in offspring compared to both NTM patients and unrelated controls suggest that IL-18 production may be intrinsically lower in these families, but increased by NTM infection. Further investigation of immunological pathways involving IL-18 is warranted.

CXCL10 is emerging as a tool for the diagnosis of TB and to assess the immune status of individuals in regions with high burdens of TB. Measurement of CXCL10 in whole blood cultures may discriminate between infected and uninfected individuals better than measurement of IFNγ from the same cultures or than performing tuberculin skin tests [18–21]. Our data suggest that plasma levels of both CXCL10 and IL-18 may correlate with NTM burden. Plasma CXCL10 displayed less variation than IL-18 in individuals without NTMLD. Low CXCL10 may be a useful surrogate marker for successful control of NTMLD (with or without therapy).

Activated and memory T cell subsets are routinely monitored for HIV care in diagnostic laboratories, and so can be adapted for use in patients with NTMLD. $CD4^+$ T cells comprise a lower proportion of lymphocytes in NTM patients compared to offspring. This may reflect migration of $CD4^+$ T cells from the bloodstream into the lung. Age-associated reductions in circulating $CD4^+$ T cells have also been described [22]. The frequencies of activated $HLA-DR^+CD4^+$ T cells were highest in NTM patients who had completed at least
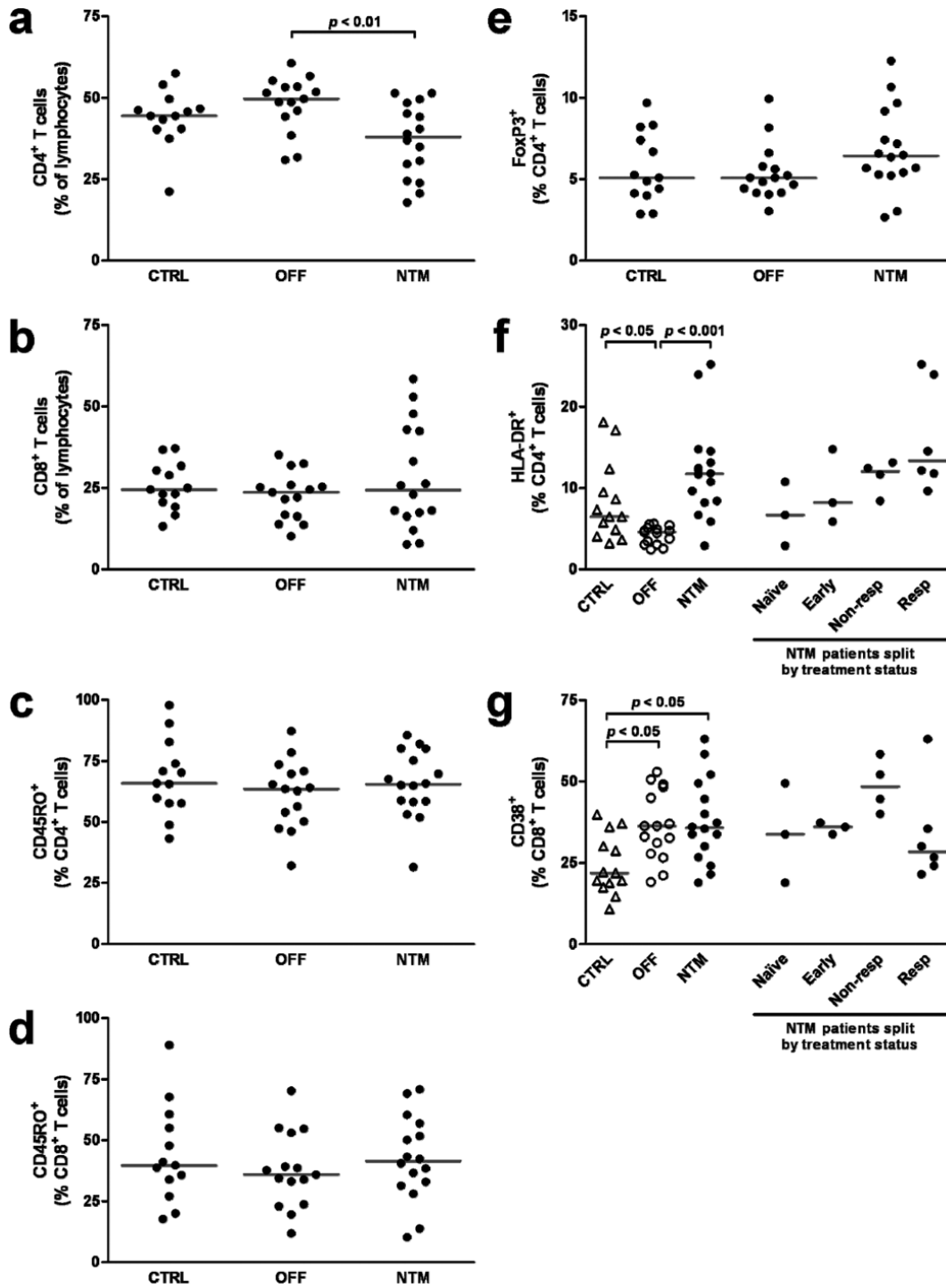
Fig. 2. Frequencies of activated CD4$^+$ and CD8$^+$ T cells are elevated in patients with NTMLD. The frequencies of total, memory, regulatory and activated CD4$^+$ T cells (a, c, e and f, respectively) and of total, memory and activated CD8$^+$ T cells (b, d and g, respectively) were quantified by 6-colour flow cytometry in cryopreserved PBMC from patients with NTMLD ($n = 16$), offspring of NTM patients ($n = 15$) and unrelated healthy controls ($n = 13$). Patients were also divided into subgroups to assess whether patterns of T cell activation were evident in relation to treatment status (f and g). Dark gray horizontal lines represent the median value. Non-resp, non-responders to treatment; Resp, responders to treatment.

6 months of therapy. These may represent a spillover of CD4$^+$ T cells responding to NTM antigens in the lung. Responders tended to have lower frequencies of activated CD38$^+$CD8$^+$ T cells than non-responders, though data from more individuals are needed.

Patients in the non-responder subgroup tended to display a more "activated" phenotype – higher plasma levels of CXCL10 and IL-18, and greater average frequencies of activated CD4$^+$ and CD8$^+$ T cell subsets – compared to patients in the other subgroups. An exception was the responder with the highest frequency of CD38$^+$CD8$^+$ T cells, who also had the highest frequency of HLA-DR$^+$CD4$^+$ T cells as well as the highest plasma levels of both CXCL10 and IL-18 of any patient. This profile is more consistent with most non-responders in our study. This patient successfully completed 18 months of therapy, but continues to have significant radiological abnormalities. It is possible that this immune profile may predict a high risk of clinical relapse in the near future due to subclinical disease. Further delineation of favourable and unfavourable immunological profiles will require long-term follow-up of these patients given the propensity for clinical relapse [23]. This patient also had type 2 diabetes mellitus and arthritis. Both may elevate markers of immune activation. Co-morbidities should be taken into account when assessing the markers described here.

Immunological markers that closely associate with treatment outcome and/or disease progression of NTMLD are lacking. Such markers could improve the ability of physicians to monitor disease, to determine which patients require therapy or possibly to predict which patients are likely to fail therapy or relapse. Here, plasma levels of CXCL10 and IL-18, and frequencies of activated T cells were quantified, as these can be implemented as rapid tests in a diagnostic laboratory. In patients who responded to therapy and in untreated patients who were successfully controlling disease, levels of CXCL10, IL-18 and activated CD8$^+$ T cells tended to be lower than in patients who did not respond to therapy. Hence, successful and unsuccessful control of NTMLD may be differentiated by using these markers in combination as an immunological profile or phenotype.

Acknowledgments

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