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Onchocerciasis modulates the immune response to mycobacterial antigens

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SUMMARY

Chronic helminth infection induces a type-2 cellular immune response. In contrast to this, mycobacterial infections commonly induce a type-1 immune response which is considered protective. Type-2 responses and diminished type-1 responses to mycobacteria have been previously correlated with active infection states such as pulmonary tuberculosis and lepromatous leprosy. The present study examines the immune responses of children exposed to both the helminth parasite *Onchocerca volvulus* and the mycobacterial infections, *Mycobacterium tuberculosis* and *M. leprae*. Proliferation of peripheral blood mononuclear cells (PBMC) and production of IL-4 in response to both helminth and mycobacterial antigen (PPD) decreased dramatically with increasing microfilarial (MF) density. Although interferon-gamma (IFN-γ) production strongly correlated with cellular proliferation, it was surprisingly not related to MF density for either antigen. IL-4 production in response to helminth antigen and PPD increased with ascending children's age. IFN-γ and cellular proliferation to PPD were not related to age, but in response to helminth antigen were significantly higher in children of age 9–12 years than children of either the younger age group (5–8 years) or the older group (13–16 years). Thus, there was a MF density-related down-regulation of cellular responsiveness and age-related skewing toward type 2 which was paralleled in response to both the helminth antigen and PPD. This parasite-induced immunomodulation of the response to mycobacteria correlates with a previous report of doubled incidence of lepromatous leprosy in onchocerciasis hyperendemic regions. Moreover, this demonstration that helminth infection in humans can modulate the immune response to a concurrent infection or immunological challenge is of critical importance to future vaccination strategies.

Keywords *Onchocerca volvulus* helminth mycobacterium PPD immune response

INTRODUCTION

The filarial nematode *Onchocerca volvulus* is a large multicellular parasite that can cause persistent and debilitating disease in individuals from endemic areas. The adult worms can reside in the dermis for up to 15 years [1] and a single female may produce $0{\cdot}7–1{\cdot}5 \times 10^3$ microfilariae per day [2]. The success of this physically conspicuous parasite relies upon its ability to modulate the host immune response to its favour. The peripheral blood mononuclear cells (PBMC) of onchocerciasis patients with generalized microfiladermia show minimal responsiveness to parasite antigens [3–6], suggesting specific T cell tolerance to parasite antigens. It has also been suggested that patent *O. volvulus* infection induces a parasite-specific immune response which is biased to a type-2 pathway [7,8]. However, the kinetics of the cellular response with respect to parasite intensity and host age has not been previously elucidated. To resolve this we examined the *in vitro* responses of PBMC from children resident in the Sanaga valley of Cameroon. This region is hyperendemic for onchocerciasis and the intensity of infection in such regions is known to increase from early childhood well into adulthood [9]. Thus, these children, aged 5–16 years, provided a key age profile for the examination of the dynamics of the immune response through the early development of *O. volvulus* infection. The study region is also endemic for the mycobacterial diseases tuberculosis and leprosy, and so provided the opportunity

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to examine how *O. volvulus*-driven immunomodulation affects the immune response to unrelated but co-endemic infectious agents.

SUBJECTS AND METHODS

Study population

In vitro cellular immune responses of PBMC from children ($n = 50$), aged 5–16 years, resident in or near Ntsan-Mendouga in the Sanaga valley of Cameroon were examined. This region is hyperendemic for onchocerciasis and is co-endemic for the mycobacterial diseases tuberculosis and leprosy, caused by *Mycobacterium tuberculosis* and *M. leprae*. All of the study individuals have lived in this hyperendemic area for their entire lives and none had previously received chemotherapy for onchocerciasis. Levels of microfilariae were assessed by bilateral standardized skin-snip taken with a sclerocorneal punch at the iliac crests. Examination of medical records and vaccination scars demonstrated that the study population was heterogeneous in terms of bacille Calmette–Guérin (BCG) vaccination status, with 46% vaccinated, 18% non-vaccinated and 36% of unknown status. All participants were offered appropriate ivermectin treatment.

Antigens and mitogen

A saline extract of adult female *O. volvulus* antigens (OvAg) was prepared as previously described [3]. *Mycobacterium tuberculosis* PPD was obtained from Connaught Laboratories (Willowdale, Ontario, Canada) and phytohaemagglutinin (PHA) from Burroughs Wellcome (Research Triangle Park, NC).

Cell proliferation and cytokine production

Whole blood (10–20 ml) was drawn from each individual and PBMC were separated on a Ficoll–diatrizoate gradient and cultured at 2×10^5 cells/0·2 ml per well essentially as described [7]. Cells were stimulated with OvAg 5 μg/ml, PPD 10 μg/ml, PHA 1:100 or medium alone for 5 days and proliferation was measured by ^{3}H-thymidine incorporation [7]. Values of proliferation are expressed as Δct/min obtained by subtraction of ^{3}H-thymidine incorporation of cells in medium alone from incorporation in stimulated cells.

For cytokine analysis, PBMC were cultured at 2×10^5 cells/0·2 ml per well with OvAg, PPD, PHA or medium alone and supernatants were harvested at 2 days for IL-4 and 5 days for interferon-gamma (IFN-γ). Measurement of IL-4 and IFN-γ levels was by capture ELISA on cell supernatants as described [10].

Statistical analysis

The data were found to be over-dispersed, which means the underlining error structure was not strictly Poisson. Consequently we assumed an error structure with an empirical scale parameter. The scale parameter is the ratio of residual deviance to the number of residual degrees of freedom. The scale parameter was approximately 70 with the residual deviance being in the order of 3500 with between 46 and 48 degrees of freedom depending on the model. Terms fitted to the model were host age, sex, BCG vaccination status (positive, negative and unknown) and mean number of skin microfilariae (MF) per snip. Age was considered in three ways: as a linear variate, a quadratic variate and a categorical variable (three levels: 5–8 years, 9–12 years and 13–16 years). No model could include age as both a continuous variable and a categorical variable. Age was considered as a categorical variable as well as a continuous variable in order to highlight any non-linear age effects. The levels of the categorical variable were chosen by fitting all ages as a categorical variable and then grouping ages by examination of the regression estimates. Up to second order interaction terms were included in the analyses with terms remaining in the model if significant at $P < 0{\cdot}05$. The resultant predicted values have been displayed graphically as two-dimensional plots when no interaction terms were significant and three-dimensional plots when interaction terms were significant. Error bars represent s.e.m. around the predictions.

RESULTS

Proliferative responses

The proliferative response of PBMC stimulated with OvAg was influenced by both age and level of MF (Fig. 1a). Children of age 9–12 years had significantly higher proliferative responses than children of either the younger age group (5–8 years) or older group (13–16 years). There was also a down-regulatory effect of MF intensity on OvAg-stimulated proliferation and this was evident throughout the age range. Proliferation in response to PPD was not influenced by age, but it showed a strong inverse relationship to the level of MF (Fig. 1b). Mitogen-stimulated proliferative responses significantly increased with age but were unaffected by MF intensity (Fig. 1c), thus demonstrating the antigen-specific nature of the OvAg and PPD MF-related down-regulation.

Neither BCG vaccination status nor sex had any significant influence on proliferative responses to any of the antigens/mitogen or on MF levels.

IL-4 and IFN-γ responses

IL-4 production by PBMC in response to OvAg was significantly influenced by age and MF intensity (Fig. 2a). IL-4 production was positively related to age group with no detectable production by children of the youngest age group (5–8 years) but detectable IL-4 production in age group 9–12 years and significantly higher production in the oldest group (13–16 years). There was a significant negative relationship between IL-4 production and level of MF. PPD-stimulated IL-4 production showed similar patterns of influence to OvAg-specific IL-4. Age as a linear variate had a positive relationship with PPD-specific IL-4 production but a negative relationship with MF intensity (Fig. 2b). Neither BCG vaccination status nor sex had any significant influence on OvAg- or PPD-stimulated IL-4 production. PHA-stimulated IL-4 was not influenced by age, MF or BCG vaccination status, but males produced significantly more IL-4 than females ($\chi^2 = 5{\cdot}17$, d.f. $= 1$; $P < 0{\cdot}05$).

Age was influential to OvAg-stimulated IFN-γ production, and like the proliferative response to OvAg children of age 9–12 years

Fig. 1. Proliferation of peripheral blood mononuclear cells (PBMC) from children resident in a region hyperendemic for onchocerciasis. PBMC were stimulated with: (a) adult female *Onchocerca volvulus* antigen (OvAg); (b) *Mycobacterium tuberculosis* PPD; (c) phytohaemagglutinin (PHA). (a) OvAg-stimulated cells were significantly influenced by age category and microfilariae (MF) ($\chi^2 = 4{\cdot}6$, d.f. $= 2$ and $\chi^2 = 4{\cdot}5$, d.f. $= 2$, respectively; $P < 0{\cdot}05$ in both cases). (b) PPD-stimulated cells were significantly influenced by MF ($\chi^2 = 4{\cdot}18$, d.f. $= 1{\cdot}48$; $P < 0{\cdot}05$). (c) PHA-stimulated cells were influenced by age as a linear variate ($\chi^2 = 4{\cdot}96$, d.f. $= 1$; $P < 0{\cdot}05$). Mean ct/min in medium alone $= 652$, s.e.m. $= 98$.

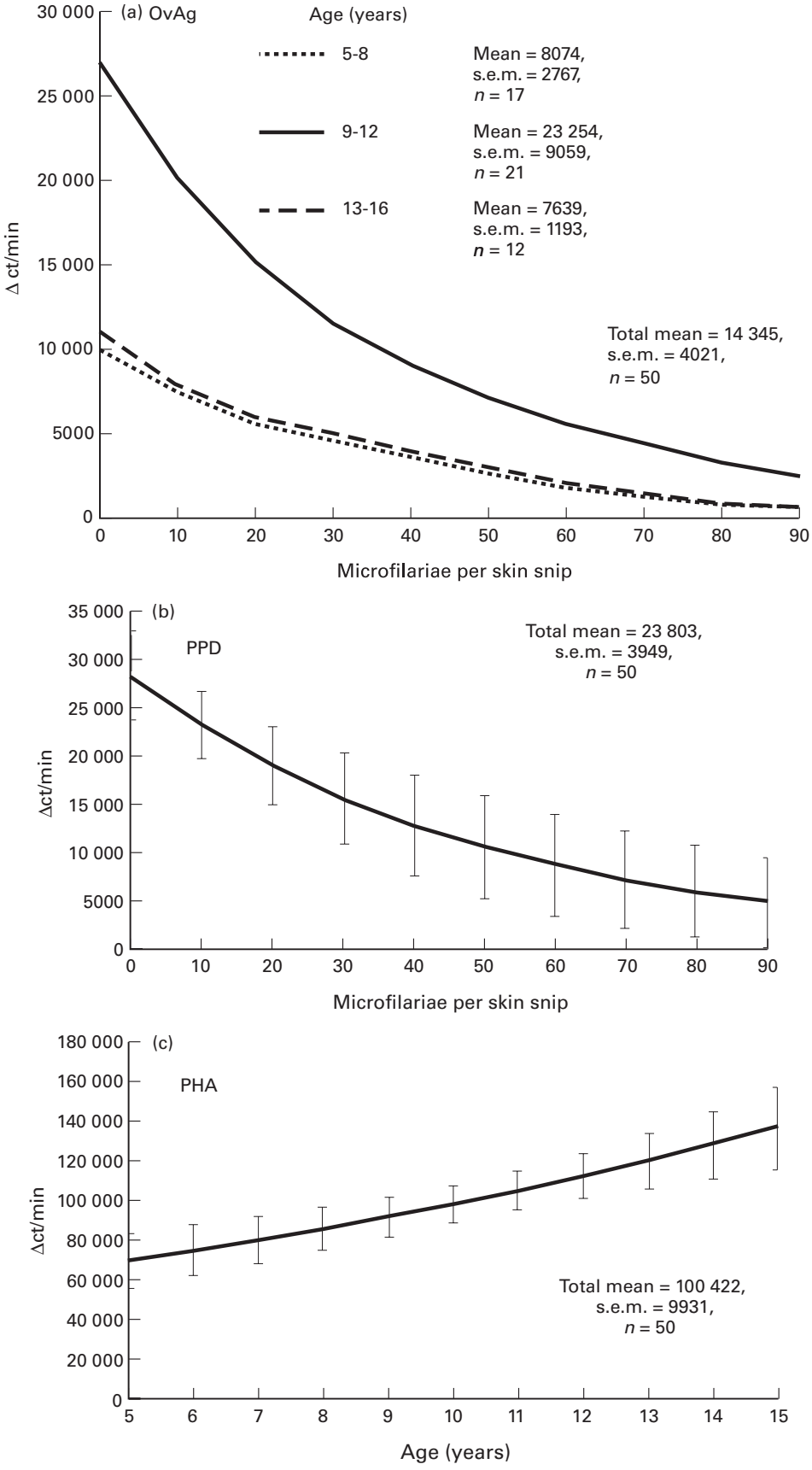
(a) OvAg
Age (years)
5-8
Mean = 8074,
s.e.m. = 2767,
n = 17
9-12
Mean = 23 254,
s.e.m. = 9059,
n = 21
13-16
Mean = 7639,
s.e.m. = 1193,
n = 12
Total mean = 14 345,
s.e.m. = 4021,
n = 50
Δ ct/min
Microfilariae per skin snip
(b)
PPD
Total mean = 23 803,
s.e.m. = 3949,
n = 50
Δct/min
Microfilariae per skin snip
(c)
PHA
Total mean = 100 422,
s.e.m. = 9931,
n = 50
Δct/min
Age (years)

Fig. 1.

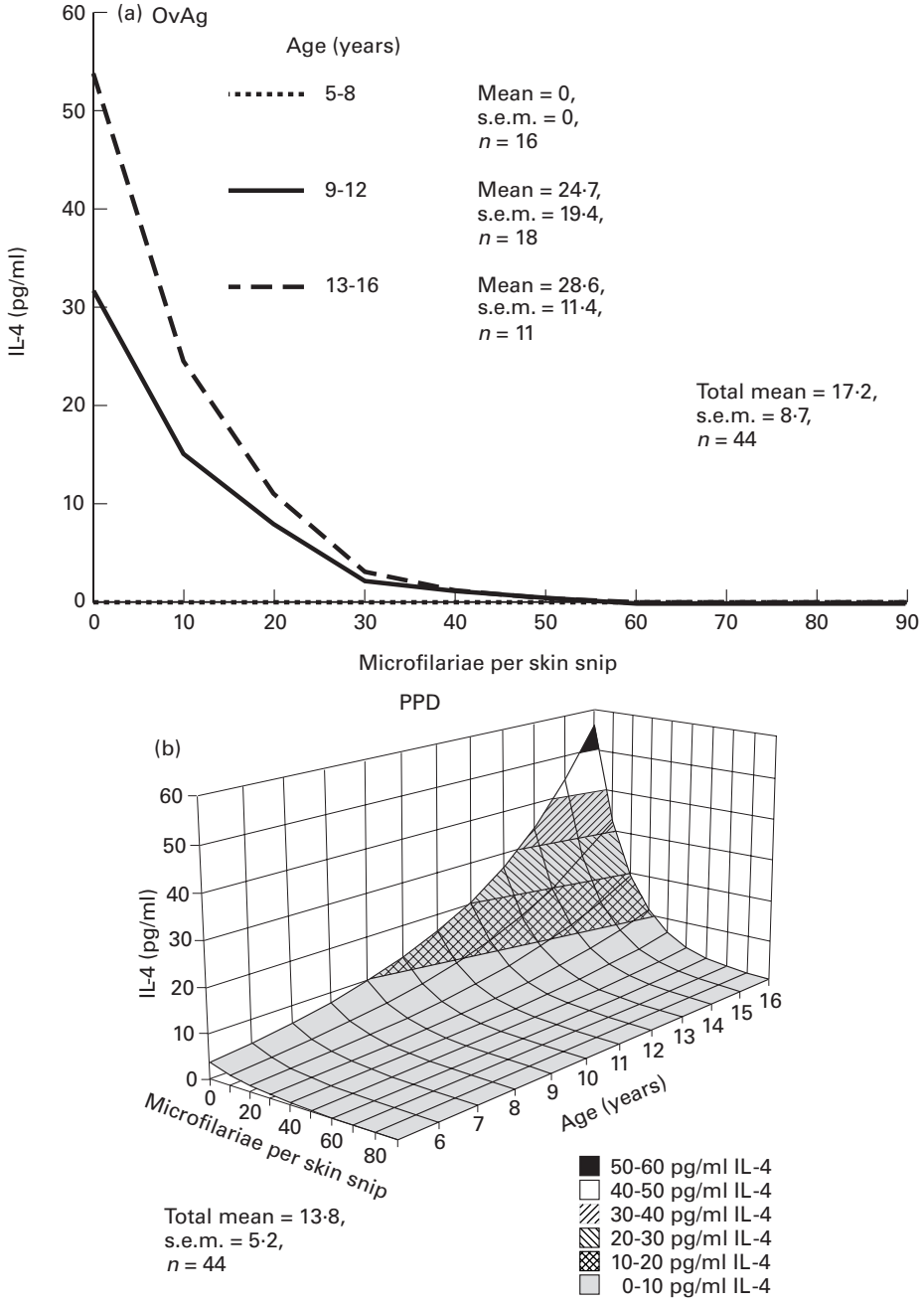

Fig. 2. IL-4 production by peripheral blood mononuclear cells (PBMC) stimulated with: (a) adult female *Onchocerca volvulus* antigen (OvAg); (b) mycobacterial antigen (PPD). (a) Age as a categorical variable and intensity of microfilariae (MF) significantly influenced OvAg-stimulated IL-4 production ($\chi^2 = 5{\cdot}1$, d.f. $= 2$ and $\chi^2 = 7{\cdot}3$, d.f. $= 2$, respectively; $P < 0{\cdot}05$ in both cases). (b) Both age as a linear variate and intensity of MF influenced PPD-stimulated IL-4 production ($\chi^2 = 7{\cdot}2$, d.f. $= 2$ and $\chi^2 = 5{\cdot}3$, d.f. $= 2$, respectively; $P < 0{\cdot}05$ in both cases).

had significantly higher responses than children of either the younger age group (5–8 years) or the older group (13–16 years) (Fig. 3a). BCG-vaccinated individuals produced higher levels of PPD-specific IFN-γ than non-vaccinated or unknown status children (Fig. 3b), but surprisingly there was no significant MF-related decrease in either PPD- or OvAg-specific IFN-γ. However, there was a downward trend in response to both antigens ($0{\cdot}05 < P < 0{\cdot}1$) and IFN-$\gamma$ production positively correlated with proliferative responses for both worm and mycobacterial antigens ($P < 0{\cdot}01$ in both cases; $r^2 = 0{\cdot}9$ and $0{\cdot}39$, respectively). Males produced more PPD-specific IFN-γ than females. PHA-stimulated IFN-γ was not influenced by age, MF, BCG vaccination status or sex.

DISCUSSION

This study utilizes powerful generalized linear modelling [11] to analyse the immune responses of children infected with onchocerciasis and exposed to the mycobacterial infections tuberculosis

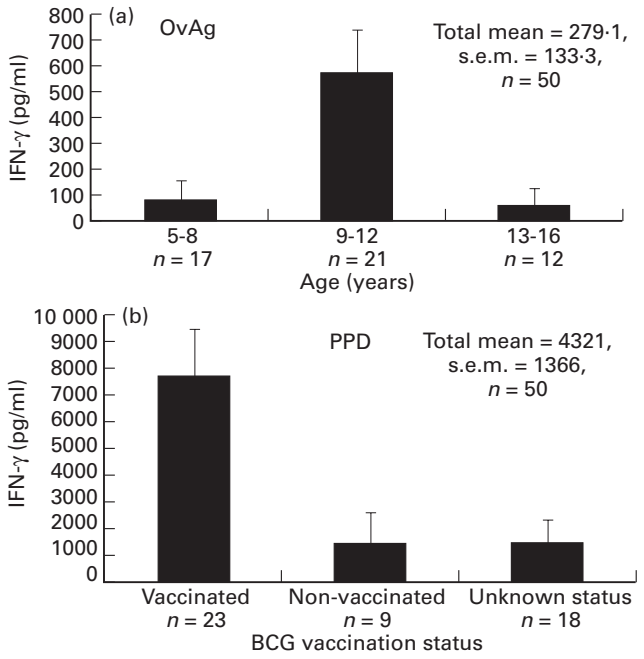

Fig. 3. IFN-γ production by peripheral blood mononuclear cells (PBMC) stimulated with: (a) adult female *Onchocerca volvulus* antigen (OvAg); (b) mycobacterial antigen (PPD). (a) Age was significantly influential to OvAg-stimulated IFN-γ production both as a quadratic variate ($\chi^2 = 6{\cdot}7$, d.f. = 2, $P < 0{\cdot}05$) and a categorical variable ($\chi^2 = 6{\cdot}4$, d.f. = 2, $P < 0{\cdot}05$). (b) PPD-stimulated IFN-γ production was significantly influenced by bacille Calmette–Guérin (BCG) vaccination status, *b* ($\chi^2 = 4{\cdot}2$, d.f. = 1, $P < 0{\cdot}05$), and also by sex with males producing higher levels of IFN-γ ($\chi^2 = 7{\cdot}3$, d.f. = 1, $P < 0{\cdot}05$). Error bars = s.e.m.

and leprosy. It has previously been shown that individuals with patent onchocerciasis or lymphatic filariasis show cellular hyporesponsiveness to both parasite and non-parasite antigens in proliferative assays and defects in DTH skin tests [12–17]. Our findings concur with this, showing that in children with *O. volvulus* microfiladermia, the proliferation of PBMC in response to stimulation with both adult female parasite antigen (OvAg) and mycobacterial antigen (PPD) was down-regulated with increasing intensity of skin MF. It is of particular note that, although BCG-vaccinated individuals tended to have higher PPD-specific proliferative responses, the MF-related down-regulatory effect was independent of BCG vaccination status.

The present study dissected this down-regulation further by examining IL-4 and IFN-γ production in response OvAg and PPD stimulation. Upon antigen stimulation, murine [18,19] and human [20,21] $CD4^+$ T cells polarize into subpopulations with characteristic profiles of cytokine production. Type-1 cells produce IFN-γ and IL-2 but little or no IL-4 or IL-5, whereas type-2 cells produce mainly IL-4 and IL-5. *Onchocerca volvulus,* like other helminth infections [22–24], will commonly induce an immune response to parasite antigens which is biased towards a type-2 response [7,8]. Areas that are endemic for onchocerciasis are often co-endemic for the mycobacterial infections tuberculosis and leprosy, which commonly induce a type-1 immune response [20,21]. However, it has been previously observed that the presence of a type-2 response to mycobacterial antigens and a diminished type-1 response is associated with the development of lepromatous leprosy [25,26] and pulmonary tuberculosis [27–29]. It is thus important to examine what happens when the two infections occur together, because evidence exists from murine models that a helminth-induced type-2 response can alter the nature of immune response to non-parasite or 'third-party' antigens. Indeed, murine infections of both *Schistosoma mansoni* and *Brugia malayi* induce a skewing of the immune response towards type 2 to antigens that normally promote a type 1 response [30–32], and *S. mansoni* infection induces an infection-resolving type-2 response to concomitant *Trichuris muris* infection [33]. In humans, however, the only evidence for similar immunomodulation of 'third-party' antigens is in *S. mansoni*-infected individuals who, following tetanus toxoid (TT) vaccination, produced less TT-specific IFN-γ [34].

The MF-related down-regulation of proliferation to PPD was paralleled by a significant decrease in both OvAg- and PPD-stimulated IL-4 production. There was no statistically significant MF-related decrease in either PPD- or OvAg-specific IFN-γ, but there was a downward trend in response to both antigens and IFN-γ production positively correlated with the down-regulated proliferative responses for both worm and mycobacterial antigens. This suggests that the cellular hyporesponsiveness may extend to both types 1 and 2 T cell subsets.

Concurrent with the MF-related cellular down-regulation was a host age effect on the balance of T cell subsets responding to both the parasite and mycobacterial antigens. OvAg-stimulated IFN-γ production reflected the proliferative response, with highest values in the middle (9–12 years) age group and negligible responses in the oldest (13–16 years) age group. This, in conjunction with the marked increase in OvAg-dependent IL-4 production through ascending age groups, is indicative of a down-regulation of type-1 responses in the older children accompanied by an age-related increase in type-2 responses. In the context of this study, the term age comprises two possible influences on the immune response, namely maturation of the childhood immune system, and duration of exposure to the parasite. However, as it is known that the adult female worms of *B. malayi* are the driving force of type 2 polarization in mouse infections [35], it seems likely that the age-related parasite-specific type 1 to type 2 shift is a function of duration of exposure to the female parasite.

Neither proliferative responses nor IFN-γ production by PBMC stimulated with mycobacterial antigen showed any relationship to age but IL-4 production increased markedly, with no difference between individuals of different BCG vaccination status. Unlike the response to parasite antigen, this suggests that there is no age-related down-regulation of the type-1 response to PPD, but there is an up-regulation of the PPD-stimulated type-2 cell subset. Although we do not have data relating to the immune response to PPD in children not exposed to *O. volvulus*, such a shift in the T cell subset bias could be caused by the parasite-induced IL-4-rich environment favouring the development of mycobacterial-specific T cells from a type-0 to a type-2 phenotype. In agreement with this, PPD immunization of *B. malayi*-infected mice produced a similar skewing of PPD-specific responses towards type 2 but with no change in IFN-γ production [31]. However, recent studies demonstrated that adult human infection with *B. malayi* [36] and *in utero* exposure to helminth antigens [37] did not influence the T helper cell response to PPD. This absence of an observed alteration in the response to PPD may simply reflect differences between human infection with *O. volvulus* and other helminth infections, or may result from methodological differences between the studies. Also, it may be that the parasite-induced up-regulation of type-2 responses to PPD is only evident in the developing immune system of children,

and/or it may be that what are relatively subtle, yet meaningful changes in cellular responsiveness and cytokine production can only be revealed by the power of generalized linear modelling such as used in this study.

In mice infected with *S. mansoni,* a parasite-stimulated down-regulation of type-1 cytokine secretion and simultaneous increase in type-2 cytokines has been shown to extend not only to non-parasite antigen but also to mitogen, demonstrating a generalized imbalance in the T cell population [30,32,38]. In the present study, mitogen-specific proliferative responses significantly increased with age and were unaffected by MF intensity, and neither IFN-γ nor IL-4 production showed any relationship to MF intensity or age. This demonstrates that the parasite intensity-driven down-regulation of cellular responsiveness and the skewing of responses to type 2 are antigen-specific for OvAg and PPD and that the T cell population as a whole remains unaffected. To define further any changes in T cell subsets we had examined production of IL-2, IL-5 and IL-10. However, with the small sample size and low numbers of responders we were unable to reveal any significant relationships between production of these cytokines and age or MF.

The balance of type 1 and type 2 responses has a striking effect on the clinical manifestations of many diseases, including tuberculosis and leprosy. Tuberculin-positive healthy individuals have high PBMC proliferative responses to PPD and a strong PPD-specific type-1 cytokine profile [27,29]. Patients with pulmonary tuberculosis, however, exhibit low proliferative responses to PPD and show diminished production of type-1 cytokines but unaltered or elevated type-2 cytokine production [27–29]. Similarly, tuberculoid leprosy patients, who mount a resistant response, produce a type-1 cytokine expression pattern in skin lesions, whereas lepromatous leprosy patients express a type-2 cytokine profile [25,26]. Thus, in onchocerciasis patients we envisage that the down-regulation of cellular responsiveness to mycobacterial antigens and age-related ascension of a type-2 response observed in this study will affect the clinical manifestation of mycobacterial infection. Indeed, a previous study reported that the incidence of lepromatous leprosy in onchocerciasis hyperendemic communities was double that in areas with little or no onchocerciasis but a similar overall prevalence of leprosy [12]. To our knowledge there have been no controlled epidemiological studies to examine the incidence of active tuberculosis in onchocerciasis regions. The observed modulation of the immune response to mycobacterial antigens occurred irrespective of BCG vaccination status, suggesting that the efficacy of BCG vaccination may be altered. Indeed, it has been previously shown that PPD skin test-negative onchocerciasis patients are less likely than uninfected individuals to convert to skin test positivity following BCG vaccination [39]. Our study exposes the underlying mechanisms of this poor response to BCG and, further, may implicate for the first time the powerful immunomodulatory effect of helminthic infection in general as a contributory factor in the variable efficacy of BCG vaccination worldwide. It is notable that BCG offers the lowest protective efficacy in tropical regions [40], the very areas where helminth infections are most prevalent. It should also be considered that helminth infection may alter the response to other vaccines and the outcome of other concurrent infections.

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