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Age-related differences in cell-specific cytokine production by acutely ill Malawian patients*

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SUMMARY

Age-related changes in human cell-specific cytokine responses to acute illness have not been well examined. We therefore evaluated age-related differences in T, B and natural killer (NK) peripheral blood lymphocyte cytokine responses of 309 acutely ill hospitalized people in Malawi, Africa, <1 month–61 years of age. We used four-colour flow cytometry and performed Wilcoxon rank sum and Kruskal–Wallis tests, Pearson (r_p) and Spearman (r_s) correlations, and linear and logistic regression analyses to control for human immunodeficiency virus infection (HIV) status, the percentages of lymphocytes expressing CD4, and the nature of the acute infection. The percentages of $CD8^-$ and $CD8^+$ T cells producing induced IL-8 decreased with age ($r_s = -0{\cdot}44$ and $-0{\cdot}53$). The percentages of T cells producing TNF-α were higher, and the percentages producing IL-10 were lower, in those ≥13 than those <13 years old (medians: 17·7 *versus* 10·5 and 1·4 *versus* 3·0, respectively). The percentages of $CD8^-$ T cells producing IFN-γ were higher and stable in those ≥1 year old compared to infants (medians: 23·5 *versus* 10·4); the percentages of NK producing IFN-γ were higher post-infancy and then declined to relatively low levels with increasing age. The percentages of T cells producing IL-2 were highest in those 5–<31 years old (median 5·6) and lowest in those ≥31 years old (median 1·9). The ratios of the percentages of T cells producing IL-4 to those producing IL-8 and to those producing IL-10 both increased with age. These data suggest that innate immunity, represented by NK IFN-γ production, dominates in early life. A number of shifts occur after infancy and before adolescence, including a proinflammatory shift from IL-8 to TNF-γ and a type 2 shift from IL-10 to IL-4 dominance. These findings suggest distinct age-related differences in the human response to acute illness and may be useful in directing future efforts at immunomodulatory therapies.

Keywords age cytokine balance IFN-γ IL-8 immunosenescence T lymphocytes

INTRODUCTION

Much is known about the maturation, ageing and senescence of the human immune system and the clinical ramifications of these processes [1–3]. There is a large body of literature on age-related changes in the number, surface antigenic characteristics, and function of B lymphocytes, T lymphocytes, neutrophils and antigen-presenting cells, as well as about changes in antibody isotype, specificity, affinity, and avidity (for recent reviews, see [2,4–6]). For example, at one end of the age spectrum, neonates have relatively low levels of some complement components, associated with impaired opsonization, leucocyte function and vulnerability to bacterial infections. At the other age extreme, the elderly have relatively impaired T and immunoregulatory cell function, associated with heightened infectious disease mortality, poorer response to immunization, infection with intracellular organisms and reactivation of previously controlled infections [2]. Innate immunity, including natural killer cell (NK) function, has been reported to be fully functional at birth and remain relatively stable throughout life; however, adaptive immunity, especially associated with T cell and antigen-presenting cell-function, wanes with age [3,6].

Cytokines are central to immunoregulation, cellular interactions and immune function. Many studies have been carried out concerning changes in serum cytokine levels in people at various

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ages (for reviews, see [3,4]). Some of these shared carefully defined enrolment criteria to exclude the effects of malnutrition or underlying illness [7]. Despite shared criteria, these studies have disagreed with one another, sometimes markedly [4]. Further, very little is known about how cell-specific cytokine production changes with age, in part because the technology to efficiently examine cell-specific cytokines is relatively new [8]. Indeed, most of the cell-related studies performed thus far are of cytokines in cell culture supernatants, rather than of cell-specific production, and usually do not differentiate the cell types producing the cytokines. In one study, the peripheral blood cells of 16 elderly people ($\bar{\times}$79·6 ± 7·5 years) produced more tumour necrosis factor (TNF)-α with stimulation but less interleukin (IL)-2 than did the cells of 16 younger people ($\bar{\times}$24·6 ± 3·1 years) [9]. Similarly, in another study of 50 people 17–62 years old, the percentage of T cells producing interferon (IFN)-γ with *in vitro* stimulation increased with age, correlating highly with increases in the percentage of peripheral blood T cells expressing CD8 and CD57, but not CD28 [10]. In a third study, of 17 individuals 18–22-years old and 16 individuals 55–65 years old, a higher percentage of older peoples' peripheral blood $CD4^+$ T cells produced IFN-γ with stimulation and isolated $CD4^+$ T cells secreted higher levels of IFN-γ and lower levels of IL-4 than did those of the younger group [11]. The latter two of these studies suggest that a relative increase in the balance between type 1 cytokines, associated with cellular immunity (e.g. IFN-γ, IL-2) and type 2 cytokines, associated with humoral immunity (e.g. IL-4, IL-10), occurs with ageing.

The findings in two of these studies are somewhat contrary to the dogma that T cell immunity declines with age [3], the consensus that there is a shift toward type 2 immunity with ageing [3,4,12], and to the results of other recent studies [13–16]. Cakman *et al*. found that the peripheral blood cells of 21 people 70–90 years old produced less IFN-γ and soluble IL-2 receptors and more IL-10 *in vitro* than did the cells of 20 people 20–35 years old [13]. In another study, the production of IFN-γ by concanavalin-stimulated blood cells was associated inversely with the age of the donor [14]. Interesting, in light of the presumed stability of innate immunity with age, Krishnaraj determined that, although NK cytotoxicity was well preserved in the elderly, the amount of IFN-γ secreted by purified, activated NK cells of the elderly was only 25% that of the young (n = 16–25 per group for young and elderly); this could be counteracted by prolonged IL-2 stimulation [17]. Similar results by others have been reviewed recently [18]. Thus, studies concerning the effects of ageing on the cytokine network and profile involve few cytokines, few cell types or not even specific cells, adults and no children, and conflict with one another. Further, they often purposefully assess only healthy individuals, rather than examining the cytokine profile in a functional state, e.g. in response to acute infection or illness.

To clarify further the effects of age on the cytokine network and profile in response to acute illness, we examined the relationships between age and cell-specific cytokines in hospitalized people <1 month–61 years of age, in Malawi, Africa. In particular, we examined the percentages of peripheral blood lymphocytes producing a broad spectrum of type 1, type 2, proinflammatory and anti-inflammatory cell-specific cytokines (IFN-γ, IL-2, IL-4, IL-8, IL-10 and TNF-α), with *ex vivo* induction of cytokine production. We also examined the balances between type 1 and type 2 cytokines and between pro- and anti-inflammatory cytokines in these induced lymphocytes. In addition to evaluating $CD3^+CD8^+$, $CD3^+CD8^-$ and B lymphocytes, we examined cytokine production by NK cells, a cell type associated with both innate immunity and the regulation of acquired immunity.

METHODS

Study population. During three time periods (including both the wet and dry seasons) of 1997 and 1998, we enrolled 160 febrile (oral temperature ≥38°C) people ≥13-years old and 149 acutely ill people <13 years old, admitted to the Lilongwe Central Hospital, Malawi, Africa, into a study of the immune correlates of bloodstream infections [19]. All acutely ill people < 13 years old, rather than only those who were febrile, were enrolled because young children with bloodstream infections may not present with fever. These patients represent a random subset of and were comparable to all acutely ill, admitted patients [19]. For each patient at admission, blood samples, epidemiological data and a medical history were obtained and a physical examination was performed by one of the investigators. The study protocol was approved by the institutional review boards of the Centers for Disease Control and Prevention (CDC) and the Malawian Health Sciences Research Committee; informed consent for both the study and for HIV testing was obtained from all participants and/or their guardians. As in most developing nations, HIV-infected people in this study were neither receiving antiviral therapies nor being monitored for changes in $CD4^+$ cell counts or HIV plasma RNA levels.

Of those <13 years old, 44 were infants (i.e. <1 year old), 76 were 1–<5 years old (children) and 29 were 5–<13 years old (children). Of the older participants, 25 were 13–<21 years old (adolescents) and 135 were ≥21 years old (adults; mean 33, median 32 and range 21–61 years old). These age categories were chosen in order to evaluate changes occurring from infancy to childhood, to adolescence, to adulthood and through decades of adulthood, while maintaining an adequate number of persons in each category for subgroup analyses to be feasible.

Laboratory methods

(i) Microbiological. HIV antibody testing was performed at study enrolment, using enzyme-linked immunosorbent assay (ELISA) test kits (Murex Diagnostics Inc., Norcross, GA, USA). HIV-2 has not been reported in Malawi. Whole blood samples were cultured as described previously [20]. Plates were incubated at 35°C for 7 days, and the cultures were examined each day during incubation. These culture techniques detect pathogenic bacteria, fungi and mycobacteria species readily. Thick and thin malaria smears were performed at admission and read by a single, highly experienced individual not aware of the patient's clinical status or findings. A smear was considered positive if any *Plasmodium (falciparum)* asexual parasites were seen on examination of peripheral blood smears (thick films and the tails of thin films). For some participants, the degree of parasitemia was graded on a scale of 1+ to 4+, as described previously [21].

(ii) Stimulation of cytokine production. Blood was prepared for cytokine stimulation as described previously [21]. Blood was either stimulated for 5 h at 37°C with phorbol 12-myristate 13-acetate (PMA) (200 ng/ml) (Sigma Chemical Co., St Louis, MO, USA) and ionomycin (4 μg/ml) (Sigma) in the presence of brefeldin-A (40 μg/ml) (Sigma) and RPMI 1640 with L-glutamine

(induced or stimulated) or retained in identical medium without PMA and ionomycin but with brefeldin-A. No serum was added to the cultures. After being washed, the red blood cells were lysed with ammonium chloride solution and lymphocytes were permeabilized and fixed using Ortho Permeafix (Ortho Diagnostic Systems, Inc., Raritan, NJ, USA). After processing, samples were shipped at 4–8°C to CDC for further analysis. In multivariate analyses, the percentage of lymphocytes expressing $CD4^+$ was used as an index of HIV disease severity and to control for T cell subset differences in cytokine production [8]; it was based on data for unstimulated cells.

(iii) Flow cytometric reagents. The surface antigens assessed in this study were those shown in our laboratory to be stable with this permeabilization/fixation protocol, i.e. using these techniques, we had comparable results for the surface-related antigens when staining was performed either pre- or post-permeabilization. Fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated, peridinin chlorophyll protein (PerCP)-conjugated or allophycocyanin (APC)-conjugated murine monoclonal antibodies were obtained from Becton-Dickinson Immunocytometry Systems/PharMingen (BD/PMG; San Jose, CA, USA) (CD8-FITC and -PE [clone SK1], CD3-PerCP and -APC [clone SK7], CD4-APC [clone SK3], CD19-APC [clone SJ25C1], CD14-PE [clone MøP9], CD16-PE [clone B73·1] and CD56 [clone MY31], IL-4-PE [clone 8D4-8], IL-8-PE [clone G265-8] and IL-10-PE [clone JES3–9D7]) and Immune Source (Reno, NV, USA) (CD8-APC [clone KL.12], IL-2-APC [R-56·2], TNF-α-FITC [clone DTX.34], and IFN-γ-APC [clone 13.TR]). Isotype controls were obtained from BD/PMG. The specific four-colour staining panel used is provided elsewhere [22].

(iv) Flow cytometry. All staining was performed at room temperature for 30 min in the dark after permeabilization, fixation and shipment to CDC. Staining was followed by a buffered saline wash. Four-colour cytofluorometry was performed using a FACSort or FACSCalibur cytometer and CellQuest software (BD/PMG). Between 50 000 and 80 000 ungated events were collected from each tube in the panel.

Analytical techniques

For each participant, analyses were performed for various combinations of all lymphocytes, $CD3^+$ (T) lymphocytes, $CD3^+CD8^+$ lymphocytes, $CD3^+CD8^-$ lymphocytes, $CD3^+CD16/56^+$ lymphocytes (NT), $CD3^-CD16/56^+$ lymphocytes (NK) and/or $CD19^+$ (B) lymphocytes, depending upon the tube configuration. Cytokine profiles were represented by the ratios of the percentage of $CD3^+$ lymphocytes producing IL-10 or IL-4 to the percentage producing each of the other cytokines. IL-10 and IL-4 are both anti-inflammatory, regulatory and type 2 (humoral) cytokines; they induce antibody maturation and production, but also induce the production of other type 2 cytokines (e.g. IL-13) and inhibit the production of type 1 cytokines (e.g. IFN-γ). TNF-α and IL-8 are proinflammatory cytokines; IL-2, IFN-γ and, possibly, TNF-α are type 1 cytokines, inducing and involved in cellular immunity, essential for immunity to intracellular organisms.

Statistical techniques

Variability in demographic data among all age groups was assessed using χ^2 tests. Comparisons of continuous immune variables between ordered age categories were made using Kruskal–Wallis tests. Univariate analyses that were statistically significant for the entire study group were repeated for the following subgroups of participants: HIV-negative, blood culture-negative, malaria-negative, those with temperatures between 37·0 and 38·4°C, those who did not die, combinations of each of these subgroups and for those: without proven/suspected sepsis, with and without suspected pneumonia, without possible meningitis, without diarrhoea and without localized infections. Data are included only if trends similar to those for the entire group were present in each of these subgroups. Apparent differences between specific age categories were again tested using data dichotomized at that age cut-off and Wilcoxon rank sum tests. Comparisons of unimodal continuous immune variables were made by determining Spearman's (r_s) and Pearson's (r_p) correlation coefficients. Variables with unimodal variations with age were also assessed with linear regression analyses; data that appeared to have an age group at which a change occurred were tested using logistic regression analyses, with the dependent variable being age, dichotomized at that age break. For logistic and linear multivariate regression analyses, statistics for the final, reduced models containing only the significant variables are provided. For logistic regression analyses, the dichotomous dependent variable was age group, dichotomized at the age groups specified in the Results section. For linear regression analyses, the dependent variable was age. In the initial models, the following independent variables were included: the cytokine variable, gender, HIV serostatus, the percentage of cells expressing CD4 and a variable delineating the presence, type or absence of a bloodstream infection. This last variable included the following six categories: mycobacteria (n = 8), *Salmonella* spp. (n = 30), other Gram-negative organisms (n = 9), Gram-positive organisms (n = 12), fungi (n = 3), malaria (n = 36) or negative blood culture and malaria smear (n = 209). The significance level was set at $P < 0{\cdot}05$; data not provided herein did not reach that level of significance on any type of analysis.

RESULTS

Participant characteristics (Tables 1 and 2). Gender distribution did not vary significantly by age group but, as would be expected, HIV seropositivity rates increased with age until peaking among those ≥€31–<40 years old ($P < 0{\cdot}001$) and then declined ($P < 0{\cdot}001$)(Table 1). Those ≥31 years of age had higher rates of bacteraemia/mycobacteraemia; the rate and degree of malaria parasitaemia did not vary by age. Mortality rates were highest among adolescents and those ≥31 years old, but age-related differences did not reach significance when HIV serostatus was taken into account. The prevalence of three diagnoses varied by age group: suspected pneumonia (more common in older people), mild diarrhoea (more common in infants) and localized infections (more common in the young)(Table 2).

Significant relationships between immune parameters and age (Table 3)

All cytokine parameters that varied significantly by age in univariate, subgroup and appropriate multivariate analyses are listed in Table 3. Also provided are related parameters and CD4 lymphocyte data, since the latter are used in multivariate analyses (see Methods). Statistically, there were three general age-associated patterns: a linear decrease or increase in levels of the cytokine variable with increasing participant ages (Fig. 1); one level of the cytokine variable for younger individuals and another for older individuals (Fig. 2); and a linear, age-related increase in

Table 1. Characteristics of the study participants, by age group*

	Age in years							
	≥0–<1 (*n* = 44)	≥1–<5 (*n* = 76)	≥5–<13 (*n* = 29)	≥13–<21 (*n* = 25)	≥21–<31 (*n* = 61)	≥31–<41 (*n* = 48)	≥41–<61 (*n* = 26)	*P* value†
% Male	52	54	52	36	46	49	56	n.s.
% HIV-positive	20	31	17	40	69	94	64	<0·001
% Malaria-positive	7	11	7	24	20	8	16	n.s.
Degree of parasitaemia								n.s.
1 + (*n*/total)‡	1/5	3/10	0/2	2/6	1/11	3/3	1/4	
2 +	1/5	1/10	1/2	1/6	4/11	0/3	1/4	
3 +	0/5	4/10	0/2	1/6	3/11	0/3	2/4	
4 +	3/5	2/10	1/2	2/6	3/11	0/3	0/4	
% Blood culture-positive	9	18	10	0	21	40	42	<0·001
Mycobacteria (*n*)	0	0	0	0	1	5	2	
Salmonella spp. (*n*)	4	11	2	0	3	8	2	
Other Gram$^-$ bacteria (*n*)	0	3	1	0	2	0	3	
Gram$^+$ bacteria (*n*)	0	0	0	0	6	4	2	
Fungus/yeast (*n*)	0	0	0	0	0	2	1	
% Died	3	4	11	21	9	27	15	0·011
HIV + (number died/total)	2/18	4/28	1/5	8/27	12/97	33/123	9/37	0·114
HIV$^-$ (number died/total)	6/53	3/43	5/24	2/27	1/47	2/12	2/15	0·240

*Control participants ($n = 62$) not included. For patients, data are incomplete for various individuals: gender (two missing), HIV serostatus (three missing), malaria status (four missing), and mortality status (excludes 18 still in hospital at end of study phase, 12 who were signed out against medical advice, and 44 for whom mortality status was not recorded). †χ^2 tests. Not significant (n.s.): $P < 0{\cdot}05$. ‡Includes 41 people for whom degree of parasitaemia was assessed [21].

Table 2. Symptoms and diagnoses of the study participants, by age group*

	Age in years							
	≥0–<1 (*n* = 44)	≥1–<5 (*n* = 76)	≥5–<13 (*n* = 29)	≥13–<21 (*n* = 25)	≥21–<31 (*n* = 61)	≥31–<41 (*n* = 48)	≥41–<61 (*n* = 26)	*P* value†
% with proven or suspected sepsis‡	30	29	14	12	26	40	42	n.s.
% with suspected pneumonia	14	21	14	24	30	30	46	0·042
% with possible meningitis	9	0	7	4	8	10	4	n.s.
% with mild diarrhoea	13	9	4	0	0	0	0	0·016
% with severe diarrhoea	13	17	4	12	16	25	23	n.s.
% with localized infection	11	5	10	0	0	2	0	0·030

*Control participants ($n = 62$) not included. †χ^2 tests. Not significant (n.s.): $P < 0{\cdot}05$. ‡Positive blood culture or, for adults, leucocytosis and no identified source of infection or, for children, at least two of the following, without an identified source of infection: leukocytosis, abnormal temperature, and/or organomegaly.

levels for the cytokine variable until a peak or plateau was reached, followed by stable or declining levels (Fig. 3).

The first, simplest and statistically most hardy pattern was present only for variables involving IL-8 (Fig. 1); these findings remained highly significant on subgroup and linear regression analyses ($P \leq 0{\cdot}003$ for all analyses).

A number of IFN-γ variables had the second pattern. The percentage of CD8$^-$ T cells making induced IFN-γ was significantly higher in those ≥1 year old compared to infants and did not vary significantly thereafter. In logistic regression analyses, this change remained significant ($P = 0{\cdot}023$), as was the percentage of cells expressing CD4 ($P < 0{\cdot}001$); HIV serostatus and infection category did not contribute to the model. Similarly, the percentages of T cells making induced TNF-α was lower in infants than in older people and remained higher, but highly variable, thereafter. In logistic regression analyses, the difference between the infants and older people remained significant ($P < 0{\cdot}001$) and the percentages of cells expressing CD4 contributed to the model ($P < 0{\cdot}001$). For NK cells, the differences between each of the first three age strata were not significant, nor were the differences between each of the last four age strata (Fig. 2), but in logistic regression analysis the differences between those < 13 years old and ≥13-years old was significant ($P < 0{\cdot}001$), as were the infection category ($P = 0{\cdot}001$) and the percentage of cells expressing

Table 3. Median values for immune parameters associated with age, and related parameters, by age group*

		Median percentages or ratios of cells producing cytokine(s)							
		Age in years							
Cell type†	Cytokine(s) or CD4 antigen	≥0–<1 (n = 43–44)	≥1–<5 (n = 74–76)	≥5–<13 (n = 29)	≥13–<21 (n = 22–25)	≥21–<31 (n = 55–61)	≥31–<41 (n = 45–48)	≥41–<61 (n = 25–26)	P value‡
All $CD3^+$	IL-8	7·0	5·6	5·3	4·3	2·0	1·4	1·6	<0·001
$CD3^+8^+$	IL-8	8·1	6·2	6·9	3·6	1·9	1·5	1·9	<0·001
$CD3^+8^-$	IL-8	9·0	6·1	5·3	6·0	3·3	1·9	2·8	<0·001
All $CD3^+$	IL-4 ÷ IL-8	0·2	0·3	0·4	0·8	1·0	1·7	1·9	<0·001
$CD3^+8^-$	IFN-γ	10·4	20·3	19·2	16·1	28·5	18·5	21·6	0·013
NK	IFN-γ	13·7	21·1	14·1	3·6	4·5	3·8	3·7	<0·001
All $CD3^+$	IL-10	2·2	3·0	4·1	1·9	1·5	1·6	1·3	<0·001
All $CD3^+$	TNF-α	6·0	11·7	11·5	17·2	17·1	9·4	18·0	<0·001
All $CD3^+$	IL-2	3·5	3·3	6·4	6·3	5·1	1·8	1·9	<0·001
Lymphocytes	IL-2 and IFN-γ	0·2	0·5	0·7	0·2	0·3	0·1	0·2	<0·001
All $CD3^+$	IL-2 and IFN-γ	0·6	1·2	1·5	0·6	0·5	0·3	0·5	<0·001
All $CD3^+$	IL-4	2·1	1·7	3·5	3·2	2·9	2·2	2·4	0·288
All $CD3^+$	IL-4 ÷ IL-10	1·0	0·7	1·0	2·0	1·7	1·8	3·2	<0·001
Lymphocytes§	$CD4^+$, HIV^+	20·0	14·3	35·4	14·2	9·1	5·4	4·5	<0·001
	HIV^-	33·4	34·7	37·7	29·4	23·4	32·0	16·5	0·006

*Data incomplete for various individuals; therefore ranges of numbers used in analyses are provided. For those >0–<1 year old, for the following variables fewer patients were assessed, with the numbers for youngest to oldest age groups: the percentages of lymphocytes making both IL-2 and IFN-γ, n = 43, 75, 29, 12, 27, 26, 11; the percentages of T cells both IL-2 and IFN-γ, n = 44, 76, 29, 12, 27, 26, 11; the percentages of T cells making IL-4, n = 43, 74, 29, 25, 59, 48, 26; and the T cell IL-4 to IL-10% ratios, n = 43, 74, 29, 25, 59, 48, 25. †Natural killer lymphocytes (NK),$CD3^+CD8^-$ lymphocytes ($CD3^+CD8^-$), $CD3^+8^+$ lymphocytes ($CD3^+CD8^+$), and all $CD3^+$ lymphocytes ($CD3^+$). ‡Kruskal–Wallis tests. §For multivariate analyses, the percentages of T cells expressing CD4 and HIV status were included in the models. For HIV^+ people, n = 9, 23, 5, 10, 42, 15, 18, 3, 9; for HIV^- people, n = 35, 51, 24, 15, 18, 3, 9.

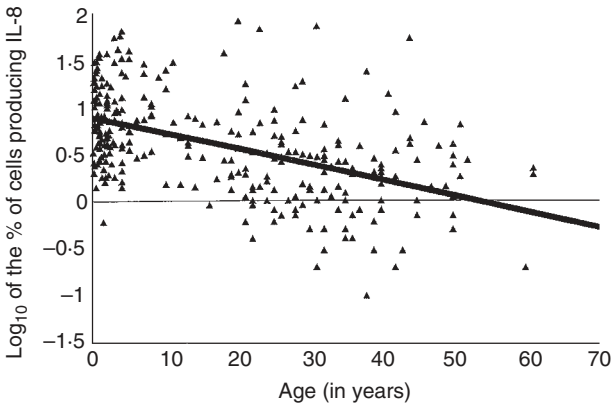

Fig. 1. Log_{10} of the percentages of $CD8^+$ T cells producing induced IL-8, by age. r_s = –0·53, P < 0·0001, n = 304.

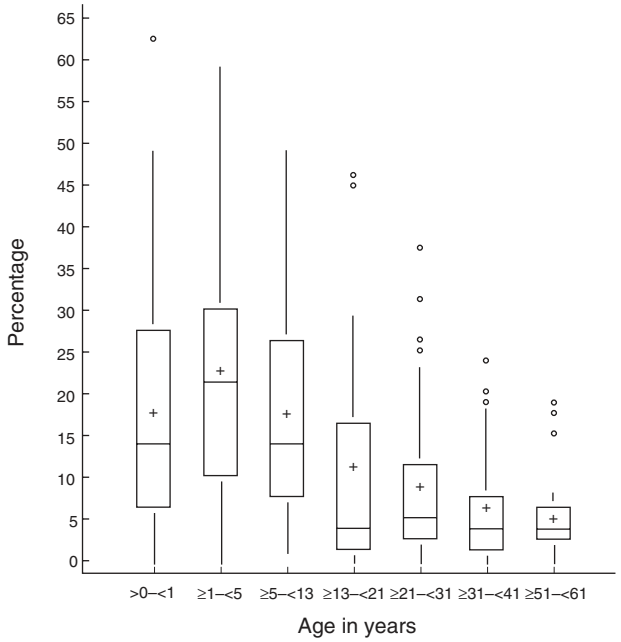

Fig. 2. Percentages of natural killer cells producing induced IFN-γ, by age group. n = 302. Boxes include medians (lines), means (crosses), and values between the 25th and 75th percentiles. Lines extend to the farthest value within ±1·5 × the interquartile range from the 25th and 75th percentiles; outliers are presented as circles.

CD4 (P < 0·001). Similarly, the percentage of T cells producing IL-10 was significantly lower for adolescents and adults than for children (P < 0·001, logistic regression analyses); again, the infection category (P < 0·001) and the percentage of cells expressing CD4 (P < 0·001) also contributed to the model.

Although the second and third patterns appear similar graphically, statistically the third pattern had an element of linearity, rather than the more precipitous changes present in the second

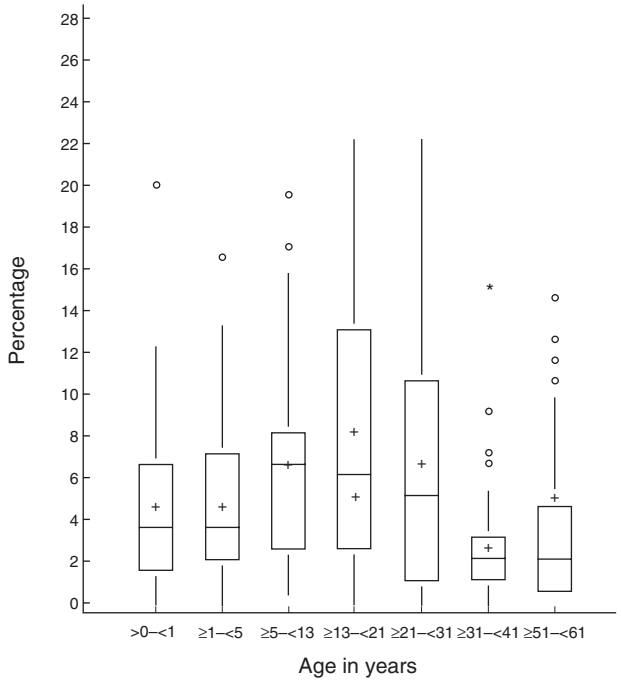

Fig. 3. Percentages of T cells producing induced IL-2, by age group. One outlier which would have increased the trend even further removed for purposes of scaling: a 20-year-old individual in whom 94% of peripheral blood T cells produced IL-2 with induction. Boxes include medians (lines), means (crosses) and values between the 25th and 75th percentiles. Lines extend to the farthest value within ±1·5 × the interquartile range from the 25th and 75th percentiles; outliers are presented as circles. n = 308 for calculations, n = 307 for graph.

pattern. For example, the percentages of T cells producing induced IL-2 increased significantly from the first to the third age strata, plateaued from the third to fourth age strata, and then declined significantly from the fourth through the seventh age strata (Fig. 3). The increases between the first and fourth strata were significant on linear regression analysis (P = <0·001); the decreases between the fourth and seventh strata were not (P = 0·127). Similarly, both the percentages of all lymphocytes and the percentages of T lymphocytes making both IL-2 and IFN-γ in the same cell increased until the third age stratum; these percentages declined significantly for adolescents (the fourth age stratum), and wavered at relatively low levels in the following age strata. As with IL-2, the increases between the first and third strata were significant on linear regression analysis (P = 0·002 for lymphocytes and P = 0·011 for T cells); later variations were not significant.

The ratio of the percentages of T cells making induced IL-4 to the percentages making IL-10 was lower in those < 13 than in those ≥13 years old (medians 0·7–1·0 *versus* 1·7–2·0) and highest in those ≥51 years old (median 3·2). This pattern did not permit multivariate analyses but was present in virtually all subgroups.

DISCUSSION

Malawi is not a country of healthy infants or elderly; its infant mortality rate is one of the highest in Africa and the projected life expectancy in those without HIV infection is only 57 years [23]. These patients would not have met the enrolment criteria of the SENIEUR protocol for studies designed to document the isolated effects of ageing on immunity [7]. Indeed, we expected acute and chronic illnesses to affect our study participants' cytokine patterns, since we have shown that malaria parasitaemia was associated with striking elevations in serum IL-10, especially in adults [21], and that TNF-α and IFN-γ production by natural T cells ($CD3^+$ $CD16/56^+$) was associated with HIV infection and mortality [19,24]. It is important to evaluate age-related cellular cytokine patterns during actual disease challenge, not only in healthy individuals, as is more commonly carried out. Although our patients were not comparable to healthy people enrolled into SENIEUR protocols, our study group was ideal for our purpose. To separate the effects of age from those of potentially confounding factors, we performed a number of subgroup and multivariate analyses taking into consideration the presence or absence of HIV infection, types of acute infection, presence or absence of extreme body temperatures, survival status and HIV disease severity, as represented by the percentages of lymphocytes expressing CD4. Because these participants were not healthy, these data should be extrapolated to healthy individuals only with caution. However, our data represent an in-depth assessment of differences in the patterns of cell-specific cytokine production by peripheral blood lymphocyte subsets of people from infancy to 61 years of age, taking into account their chronic and acute illnesses and symptoms.

Most of the literature on cytokines and ageing is based on serum cytokine and cell culture supernatant data. These studies generally suggest that infants and the elderly have a more type 2 immune profile than do individuals between those ages [12]; however, results concerning individual cytokines – including IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ– have varied from study to study [4,15,25–30]. Our findings herein differ from the literature in that we did not find distinctly higher cell-specific type 2–type 1 cytokine ratios in infants and older participants, based on ratios of the percentages of T cells making either IL-4 or IL-10 to the percentages making either IL-2 or IFN-γ. None of our participants were comparable to the 'elderly' in SENIEUR protocols, who are by definition healthy, well nourished and in their eighth to eleventh decades; it is rare for a Malawian to be well nourished or live to those ages. Further, we have shown previously that the correlation between serum and cellular cytokines is poor at best, even in patients with systemic symptoms [22]. It is unclear whether consistency between these studies and our own should even be expected. However, our findings are remarkably consistent with studies having experimental components similar to our own: *in vitro* cell stimulation, evaluation of specific cell types and subtypes, and participant ages overlapping with those in our study. Thus, although our IFN-γ findings differ from those in the studies of Cakman, Ouyang and Kita [13,14,16], in which whole blood was stimulated, our results are comparable to those in the publications by Bandrés, Sakata-Kaneko and Yen [10,11,30], in which IFN-γ assessments were cell-specific. Similarly, our findings concerning T cell production of TNF-α or IL-2 are consistent with those of other published studies [9,31–34]. In particular, our data concerning changes in NK cell production of IFN-γ with age are consistent with those of Krishnaraj, who used a technique somewhat similar to our own [17,18].

We report herein that several age-related patterns are remarkably consistent among all the subgroups analysed. The percentage of $CD8^-$ negative T cells making IFN-γ was signifi-

cantly lower in infants than in children and was stable among the older age groups. The percentage of NK making IFN-γ did not vary significantly between the infant and childhood age groups, was also stable among adult age groups, but was higher in childhood age groups than in adolescent and adult age groups, consistent with the findings of others [17,18]. The pattern for the percentage of T cells producing IL-2, another type 1 cytokine, was more complex than those for IFN-γ. The percentage of T cells producing IL-2 increased between the second and third age strata, plateaued at ≥5 to <31 years, declined between the fifth and sixth age strata, and was lowest for those ≥31 years old. Not surprisingly, the age-related pattern for the percentage of lymphocytes and T lymphocytes producing both IL-2 and IFN-γ was a compromise between the patterns for the two individual cytokines; the percentages increased until the adolescent age group, were lower in adolescents than in children, and were stable from adolescence to 61 years of age.

In a previous publication, we noted that a higher percentage of lymphocytes, but not monocytes, of children with malaria produced IL-8 than did those of adults [21]. Consistent with this, the most straightforward pattern found in the current analyses was that the percentages of both $CD8^-$ and $CD8^+$ T cells producing IL-8 decreased with age. This linear, age-related pattern held true for those with relatively normal temperatures (37·0–38·4°C) and the HIV^+ and HIV^- negative, blood culture- negative, malaria-negative and surviving people, based on both stratified and multivariate analyses. IL-8 is a proinflammatory, chemotactic chemokine for neutrophils and T cells and a neutrophil stimulant that is involved the outcome/severity of infectious diseases [35–37]. It activates neutrophils to release lysosomal enzymes and induces them to adhere to the vascular endothelium, all extremely useful effects in infants, whose leucocyte function is immature.

Most striking, we found strong age-related differences in the types of proinflammatory and type 2/anti-inflammatory cytokines produced by peripheral blood T cells, independent of HIV status and the nature of the acute illness. The ratio of the percentage of T cells making IL-4 to the percentage making IL-8 increased with age. These findings would be consistent with a shift from a proinflammatory towards a type 2 pattern with increasing age. However, this pattern was not found with IL-10 which is, like IL-4, both a type 2 and an anti-inflammatory cytokine. The percentages of T cells making IL-10 were higher in children than adults. IL-10 is a powerful inhibitor of IL-8 production [35] and is associated with a reduction in the production of oxygen radicals and nitric oxide intermediates created through leucocyte activation [38–40]. Thus, IL-10 production at early ages may counterregulate T cell IL-8 production. IL-10 also induces B cell proliferation, plasma cell differentiation and immunoglobulin production, all useful in infants, whose humoral immunity and complement systems also are immature. Further, the ratios of the percentages of T cells making IL-4 to those making IL-10 were higher in adults and highest in those ≥51 years old. Thus, although our data are consistent with many published studies, they provide a more extensive overview of the cytokine repertoire. By doing so, our results do not support a categorical cytokine shift with age. Rather, our data suggest that IL-10 may be the predominant type 2, anti-inflammatory cytokine and IL-8, the predominant proinflammatory cytokine in children. Conversely, IL-4 and TNF-α may predominate in older individuals.

In summary, we found that IL-8 was the predominant proinflammatory T cell cytokine in children, and IL-10 the predominant anti-inflammatory cytokine at these ages. Innate immune function, as reflected by the percentages of NK-producing IFN-γ production, is apparent at early ages. A number of shifts occur after infancy and before adolescence, including a proinflammatory shift from IL-8 to TNF-α dominance and a type 2 cytokine shift from IL-10 to IL-4 dominance. These findings suggest distinct age-related differences in the human response to acute illness and may be useful in directing future efforts at immunomodulatory therapies.

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