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Cytokine Profiles in Human Immunodeficiency Virus-Infected Children Treated With Highly Active Antiretroviral Therapy

Brian M Jones*¹, Susan SS Chiu², Wilfred HS Wong³, Wilina WL Lim⁴ and Yu-lung Lau⁵

Address: ¹Head of Division of Clinical Immunology, Department of Pathology, University of Hong Kong, Hong Kong, PR China, ²Associate Professor, Department of Pediatrics and Adolescent Medicine, University of Hong Kong, Hong Kong, PR China, ³Senior Technician, Department of Pediatrics and Adolescent Medicine, University of Hong Kong, Hong Kong, PR China, ⁴Head of Unit, Government Virus Unit, Department of Health, Hong Kong, PR China and ⁵Professor and Chair, Department of Pediatrics and Adolescent Medicine, University of Hong Kong, Hong Kong, PR China

Email: Brian M Jones* - bmjones@ha.org.hk

* Corresponding author

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Abstract

Context: There have been few longitudinal studies of cytokine production in neonatally acquired HIV-1 infection and none in Asian or Chinese children.

Objective: To determine whether monitoring cytokine production could contribute to the better management of pediatric patients with HIV-1 infection.

Setting: Clinical Immunology Laboratory and Pediatrics Department, University Hospital, Hong Kong.

Patients: Ten Asian and 2 Eurasian children infected with HIV-1 by mother-to-child transmission were followed for up to 5 years while on treatment with highly active antiretroviral therapy (HAART).

Main Outcome Measures: Numbers of unstimulated and mitogen-activated cytokine-secreting cells (IFN-gamma, interleukin [IL]-2, IL-4, IL-6, IL-10, IL-12, and TNF-alpha) were measured by ELISPOT assay at frequent intervals, and correlations were sought with CD4+ and CD8+ cell counts and viral loads.

Results: Mitogen-stimulated IL-2-secreting cells were directly associated with recovery of CD4+ cells. Correlations with viral load were found for Con A-induced IFN-gamma, Con A-induced IL-4, and unstimulated IL-10, suggesting that these cytokines were either suppressed by high virus levels or that higher cytokine levels suppressed virus. IFN-gamma, IL-2-, IL-4-, and IL-12-secreting cells induced by PHA, Con A, and/or SAC tended to increase for the first 34 years of treatment but declined thereafter.

Conclusion: Alterations in cytokine profiles were not associated with adverse clinical events and there was little evidence to indicate that monitoring cytokine enzyme-linked immunospots (ELISPOTs) could contribute to pediatric patient management.

Introduction

With the advent of highly active antiretroviral therapy (HAART), human immunodeficiency virus type 1 (HIV-1) can be controlled for prolonged periods,[1] although the virus cannot be eliminated[2] and treatment failures occur due to development of drug-resistant mutations.[3] Chronic immune hyperactivation and raised T-cell turnover due to continued viral replication and antigenic stimulation are present even after HAART has decreased the viral load to undetectable levels.[4]

Both proinflammatory and regulatory cytokines are produced during chronic immune stimulation. Proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-alpha), contribute to tissue pathology, especially in the brain,[5] and can induce transcription of latent HIV-1.[6,7] Type 2 or regulatory cytokines, such as IL-4, IL-6, and IL-10, can suppress type 1 cytokines and induce polyclonal B-cell activation,[8] lymphomagenesis,[9] autoantibody production,[10] and manifestations of allergy.[11] Type 1 cytokines, such as IL-12, interferon (IFN) gamma, and IL-2, are important for antiviral cell-mediated immunity.[12] During the long course of HIV-1 infection, type 2 cytokines gradually come to predominate over type 1 cytokines,[13-16] although this finding is not universally accepted.[17]

There have been few studies of in vitro cytokine production in neonatally acquired HIV-1 infection in Asian or Chinese children. The enzyme-linked immunospot (ELISPOT) system for measuring unstimulated or mitogen-activated cytokine secreting cells has not been evaluated in this context. We wished to know whether monitoring cytokine production in addition to CD4+ cell counts and viral load could provide additional useful information in pediatric patients with HIV-1 infection being treated with HAART. We hoped to identify cytokine profiles that are characteristic of either clinical improvement or disease progression, so that manipulation towards the desirable profile might be attempted.

Materials and methods

Patients

This study was approved by the Institutional Review Board of Hong Kong West Hospital Cluster and The University of Hong Kong, and informed consent was obtained from the parents of all subjects. Clinical findings in 8 of the patients have been described previously.[18] Ten Asian and 2 Eurasian children, 4 girls and 8 boys, were infected by mother-to-child-transmission of HIV-1. They were initially diagnosed between 1996 and 2002 at age 364 (median, 32) months and they have been followed for 961 (median, 44) months (Table). At the time of diagnosis, 9 children had low CD4+ cell counts (compared with the age-specific normal range[19]) and the median

plasma HIV-1 RNA level was 500,000 copies/mL (110,000-1,300,000). All children had lymphadenopathy and/or hepatosplenomegaly at diagnosis. One girl (patient 3) developed NKT-cell lymphoma which caused her death, the only fatality during the study period. Most of the patients had infectious complications, including *Pneumocystis carinii* pneumonia (1), viral pneumonia (1), disseminated *Penicillium marneffeii* (1), thrush (4), tinea capitis (1), and herpes simplex (1). Other complications included neutropenia in 1 patient, hepatitis and anemia in 1, and asthma and/or rhinitis in 3. Patients were started on HAART immediately after confirmation of HIV-1 infection and were treated with 2 nucleoside reverse transcriptase inhibitors (zidovudine, lamivudine, didanosine, stavudine, and/or abacavir) plus 1 protease inhibitor (indinavir, nelfinavir, *Kaletra* (lopinavir + ritonavir), ritonavir, or amprenavir) or the nonnucleoside reverse transcriptase inhibitor nevirapine. Details are given in the Table. Patients were examined and blood for hematologic, virologic, and immunologic evaluation was taken every 26 months. The first cytokine evaluation was performed within 1 month of starting HAART in 7 patients, within 24 months in 3 patients, and after 16 and 19 months in 2 patients.

ELISPOT Assay

Numbers of cytokine-secreting cells in unstimulated cultures or cultures stimulated with T-cell activators phytohemagglutinin (PHA), Concanavalin A (Con A), or monocyte activator *Staphylococcus aureus* Cowan I (SAC) were determined using ELISPOT assays.[20,21] Details of our adaptation of this method and its specificity and reproducibility (intra- and interassay CVs $8.8 \pm 5.8\%$ and $13.2 \pm 4.9\%$, respectively) have been reported.[22-26] Results for normal controls evaluated over the study period remained stable within our established reference ranges.

Briefly, peripheral blood mononuclear cells (PBMCs) were separated over *Lymphoprep* (Nycomed; Oslo, Norway) within 1 hour of blood collection and added to 96-well *Multiscreen* plates (Millipore; Bedford, Massachusetts, USA) which had previously been coated overnight at 4 °C with cytokine capture antibodies (Pharmingen; San Diego, California, USA) at 2 (IL-4, IL-6, IL-10), 4 (IL-12, TNF-alpha), or 8 (IFN-gamma, IL-2) mcg/mL in 0.1 M NaHCO₃, pH 8.2, and blocked with 5% fetal calf serum (FCS) in culture medium RPMI 1640 for at least 1 hour at 37 °C. Duplicate cultures of 10⁴ (for IL-6 and TNF-alpha) or 10⁵ (for IFN-gamma, IL-2, IL-4, IL-10, and IL-12) viable cells/well in RPMI + 5% FCS with or without PHA at a final concentration of 10 mcg/mL, Con A at 20 mcg/mL, or SAC at 0.001% v/v were incubated for 22 hours at 37 °C in 5% CO₂. Cells were then washed out with 0.01 M phosphate-buffered saline containing 0.05% Tween 20 (PBS-

T) and plates incubated sequentially with biotinylated detection anti-cytokine antibodies (Pharmingen), 0.5 mcg/mL in PBS-T for 90 minutes, streptavidin-alkaline phosphatase (Sigma; St. Louis, Missouri, USA), 1/400 v/v in PBS-T for 60 minutes and 5-bromo-4-chloro-3-indolyl-phosphate-nitroblue tetrazolium (Calbiochem; La Jolla, California, USA) for 20 minutes, all at room temperature. Plates were washed extensively with PBS-T between each incubation and with saline to remove phosphate prior to addition of phosphatase substrate. Color development was stopped and pathogens inactivated by immersion in 2% Clorox bleach followed by rinsing under the tap and allowing plates to dry for 1 hour. Blue spots corresponding to each cytokine-secreting cell were counted by microscopy and results expressed as ELISPOTS/ 10^6 PBMCs.

Flow Cytometry

CD3+4+ T-helper cells and CD3+8+ T-cytotoxic cells were enumerated using commercial monoclonal antibodies (Beckman Coulter; Miami, Florida, USA) by dual color flow cytometry (EPICS XL-MCL, Coulter). White cell and differential counts were performed by standard methods.

Virus Load Measurement

HIV-1 RNA quantitation was by Amplicor HIV-1 Monitor (Roche Diagnostics Corporation; Branchburg, New Jersey, USA). The standard method, performed according to the manufacturer's recommendations, has a measuring range of 400750,000 RNA copies/mL.

Statistical Analysis

Correlations between numbers of cytokine-secreting cells and proportions and absolute numbers of CD4+ and CD8+ cells, CD4:CD8 ratios, and virus load were evaluated by multiple regression analysis, with or without log-

arithmic transformation, and linear regression lines were plotted. Parametric rather than nonparametric statistics were used, despite small numbers of patients, because we wished to derive formulae for estimation of cytokine levels predictive of viral load or lymphocyte subset count. Log transformation was performed for viral load data because skewness and kurtosis of raw data were 3.7 and 12.8, respectively; these became 1.2 and 0.5, respectively, after log transformation. Curves of numbers of cytokine-secreting cells plotted against length of treatment with HAART were fitted by nonlinear regression. The statistical software used was GraphPad Prism Version 4.00 for Windows (GraphPad Software; San Diego, California, USA, www.graphpad.com).

Results

Twelve Asian or Eurasian children infected with HIV-1 by mother-to-child transmission were treated with HAART from the time of diagnosis. They were 360 (median, 25) months old when initially diagnosed and were therefore heterogeneous with regard to immunologic maturity, duration of infection with HIV-1, and extent of immunodeficiency due to HIV-1. They all had high viral loads and most had low CD4+ cell counts when diagnosed and entered into the study. One child died of lymphoma at age 29 months after she had received HAART for 9 months, during which time her CD4+ cells increased and the viral load decreased to undetectable. At the end of the study, the 11 surviving patients were well and thriving. Seven had normal or higher-than-normal circulating CD4+ cells/mL, but 4 patients still had reduced numbers and/or percentages. Plasma HIV-1 RNA was consistently below the level of detection in all but 1 of the surviving children when the study closed. Undetectable plasma

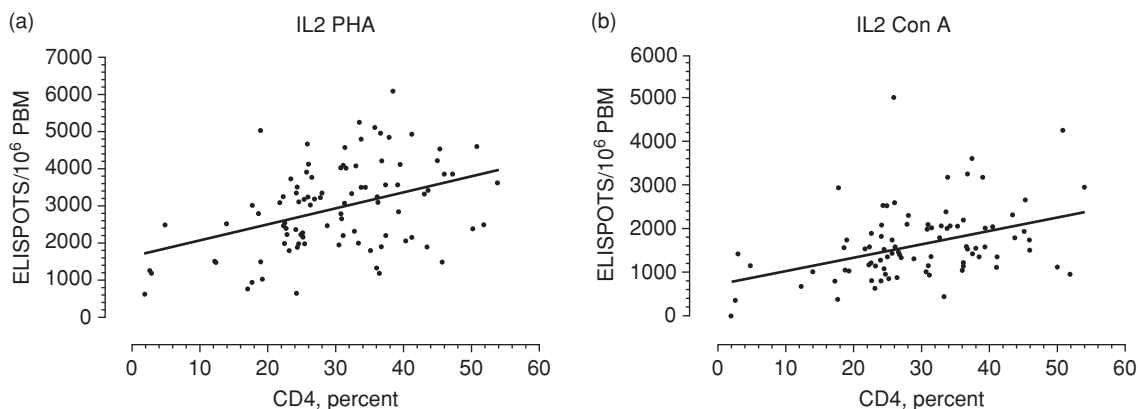


Figure 1
Multiple regression correlation of numbers of IL-2-secreting cells/10⁶ PBMCs with CD4+ and CD8+ T-cell counts in 12 pediatric patients treated with HAART. (A) IL-2 PHA vs CD4%, P = .0149; (B) IL-2 Con A vs CD4%, P = .0109

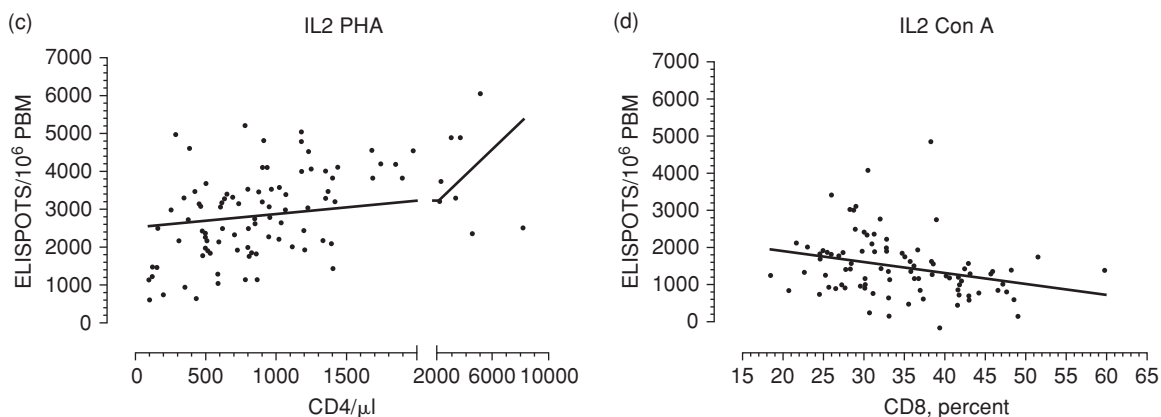


Figure 2
Multiple regression correlation of numbers of IL-2-secreting cells/106 PBMCs with CD4+ and CD8+ T-cell counts in 12 pediatric patients treated with HAART. (C) IL-2 PHA vs CD4/mcL, P = .0008; (D) IL-2 Con A vs CD8%, P = .0196

HIV-1 RNA was achieved in 255 months (median, 9.5 months).

For each cytokine and culture condition studied while patients were receiving HAART, 112 corresponding values of CD4+ and CD8+ cells and 96 corresponding values of plasma HIV-1 RNA copies/mL were available. All of these data were used to examine whether cytokine production correlated with disease progression.

IFN-gamma, IL-2, and IL-4 ELISPOTs were undetectable in unstimulated PBMCs, as reported previously.[22-26] Numbers of PHA- or Con A-stimulated IL-2-secreting cells increased during recovery from CD4 deficiency and correlated directly with CD4 and CD8 absolute counts, CD4

percentages, and CD4:CD8 ratios and inversely with CD8 percentages by multiple regression analysis. The data could be described by the following equations: $CD4\% = 0.00234 (IL-2 PHA) + 0.00355 (IL-2 Con A) + 17.47$; $CD4/mcL = 0.3852 (IL-2 PHA) + 1084.8$; $CD8\% = 40.650.0025 (IL-2 Con A)$; $CD8/mcL = 0.2083 (IL-2 PHA) + 1211.9$; $CD4:CD8 = 0.00018 (IL-2 Con A) + 0.478$. IFN-gamma, IL-4, IL-6, IL-10, IL-12, and TNF-alpha-secreting cells induced under any of the culture conditions employed did not correlate with T-cell subsets. See Figures 1, 2 and 3.

There were no significant correlations of cytokine-producing cells with virus load by multiple regression analysis of untransformed data, but log-transformed Con A-induced

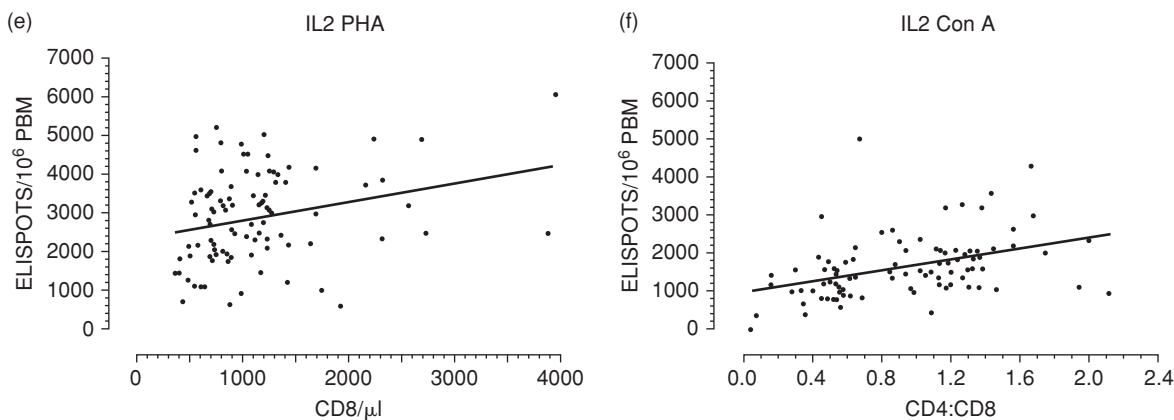


Figure 3
Multiple regression correlation of numbers of IL-2-secreting cells/106 PBMCs with CD4+ and CD8+ T-cell counts in 12 pediatric patients treated with HAART. (E) IL-2 PHA vs CD8/mcL, P = .0008; (F) IL-2 Con A vs CD4:CD8, P = .0035

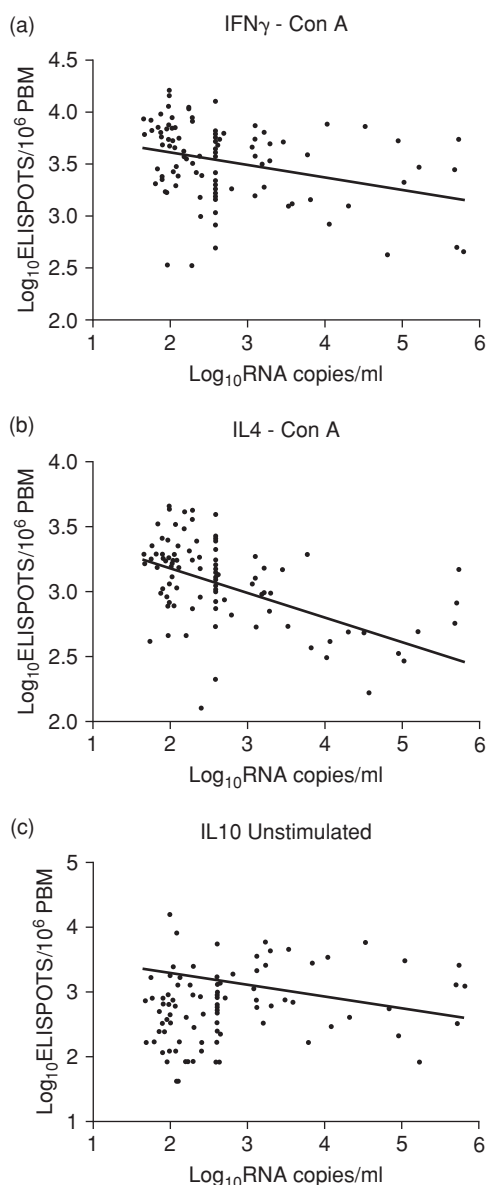


Figure 4
Multiple regression correlations of virus load with numbers of (A) Con A-induced IFN-gamma-secreting cells/10⁶ PBMCs, P = .0231; (B) Con A-induced IL-4-secreting cells/10⁶ PBMCs, P = .0294; (C) unstimulated IL-10-secreting cells/10⁶ PBMCs, P = .0015. The data were log-transformed.

IFN-gamma-, Con A-induced IL-4- and unstimulated IL-10-secreting cells increased significantly as virus load fell (Figure 4). The data were described by the following equation: \log_{10} viral load = 7.4530.6207 (\log_{10} IL-4 Con A) 0.9504 (\log_{10} IL-4 Con A) + 0.5434 (\log_{10} IL-10 unstimulated).

All of the data from ELISPOT assays were plotted against duration of HAART. Numbers of IFN-gamma, IL-2, IL-4, and IL-12-secreting cells tended to increase for the first 34 years of treatment but declined thereafter. Changes in IL-6, IL-10, and TNF-alpha-secreting cells over time were less apparent. See Figures 5, 6, 7, 8 and figures 9, 10 and 11.

Discussion

The effect of HIV-1 on maturation of the immune system in general and of cytokine production in particular is not well understood, especially in the context of treatment with HAART. We wished to know whether regular monitoring of mitogen-induced cytokine production in addition to CD4+ cell counts and virus load would be a valid measure of immunologic competence and therefore a useful additional parameter for clinical monitoring. We also looked for correlations between cytokine production, viral load, and CD4+ cell numbers in the hope of identifying cytokine profiles associated with favorable outcome. However, we were limited to only 12 HIV-infected children available for study in Hong Kong, and statistical bias could have occurred due to heterogeneity with regard to immunologic maturity at the time of diagnosis, duration of infection with HIV-1, and extent of immunodeficiency when starting HAART.

IL-2 was the only cytokine of those studied that correlated positively with increasing CD4+ T-cell percentage and absolute number and increasing CD4:CD8 ratios. Treatment with exogenous IL-2 has been shown to increase peripheral expansion of CD4+ cells.[27] IL-2 production also correlated with CD8+ T-cell increases but, surprisingly, because this population includes the major cytotoxic effector cells against HIV, it did not correlate with viral load.

HIV-1 RNA copies/mL correlated inversely with Con A-induced IFN-gamma, Con A-induced IL-4, and unstimulated IL-10, suggesting that these cytokines might be involved in the control of HIV-1 levels. It is impossible to distinguish between the possibility that high levels of virus suppressed production of these cytokines and/or that virus survived better when production of these cytokines was limited. In contrast to our findings, a previous study reported that plasma IL-10 declined during adequate virologic and immunologic responses in HAART-treated adults.[28] Differences in race and age of patients in the 2 studies may have contributed to these conflicting findings. Also in contrast to our study, IFN-gamma[29] and TNF-alpha[29,30] declined during adequate virologic and immunologic responses in HAART-treated adults. However, Reuben and colleagues[31] found *increased* plasma IFN-gamma after virus suppression in pediatric patients and Resino and coworkers[32] found lower PHA-induced TNF-alpha and IFN-gamma in

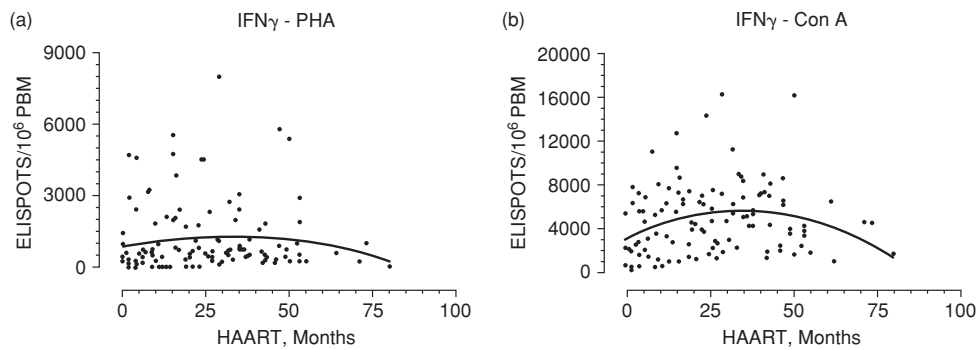


Figure 5
Numbers of IFN-gamma, IL-2, IL-4, and IL-12-secreting cells in 12 pediatric patients treated with HAART.
 Cytokine-secreting cells tended to increase for the first 34 years of treatment but declined thereafter. Curves were fitted by nonlinear regression.

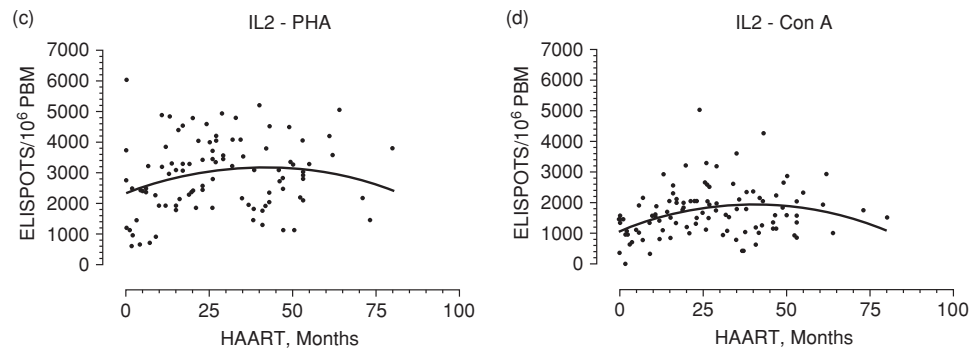


Figure 6
Numbers of IFN-gamma, IL-2, IL-4, and IL-12-secreting cells in 12 pediatric patients treated with HAART.
 Cytokine-secreting cells tended to increase for the first 34 years of treatment but declined thereafter. Curves were fitted by nonlinear regression.

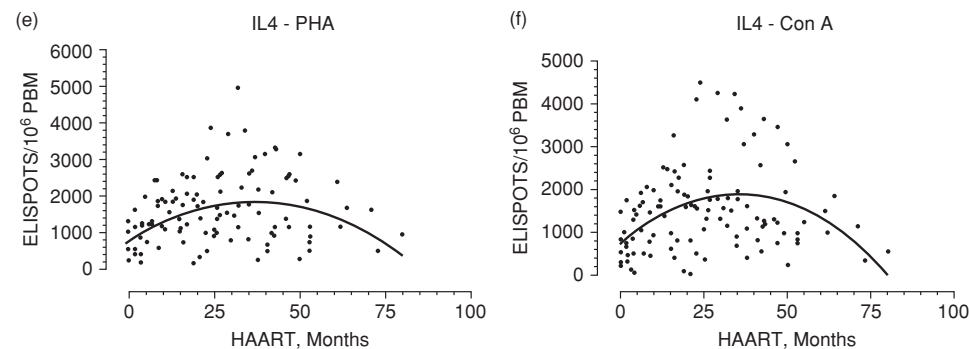


Figure 7
Numbers of IFN-gamma, IL-2, IL-4, and IL-12-secreting cells in 12 pediatric patients treated with HAART.
 Cytokine-secreting cells tended to increase for the first 34 years of treatment but declined thereafter. Curves were fitted by nonlinear regression.

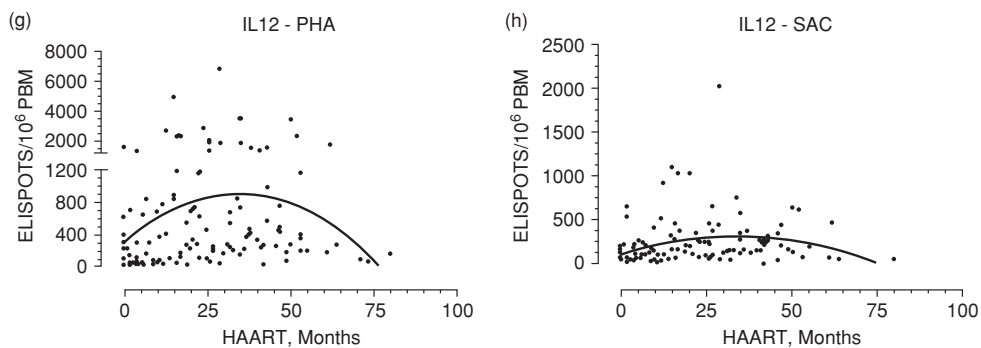


Figure 8
Numbers of IFN-gamma, IL-2, IL-4, and IL-12-secreting cells in 12 pediatric patients treated with HAART. Cytokine-secreting cells tended to increase for the first 34 years of treatment but declined thereafter. Curves were fitted by nonlinear regression.

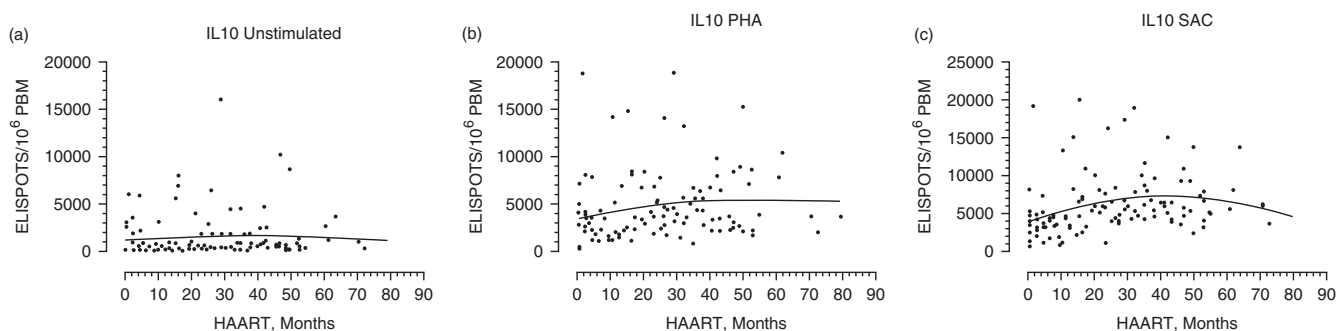


Figure 9
Numbers of IL-6, IL-10, and TNF-alpha-secreting cells in 12 pediatric patients treated with HAART. Cytokine-secreting cells tended to remain stable over the study period. Curves were fitted by nonlinear regression.

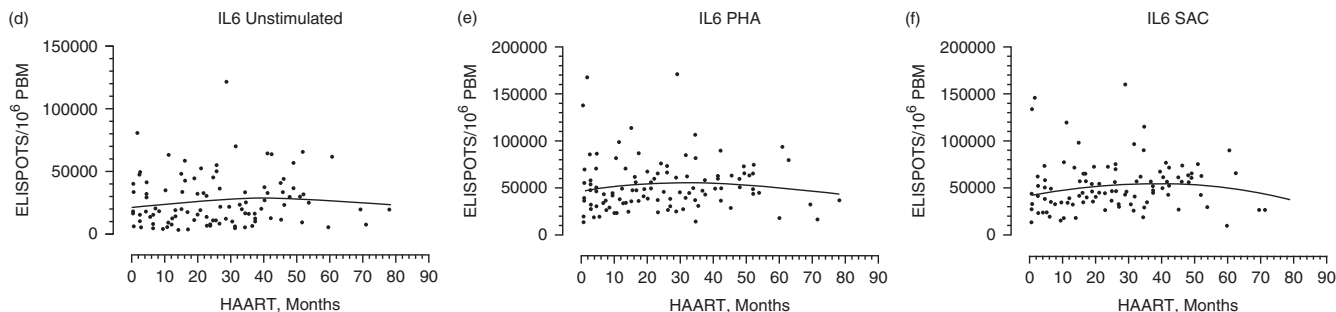


Figure 10
Numbers of IL-6, IL-10, and TNF-alpha-secreting cells in 12 pediatric patients treated with HAART. Cytokine-secreting cells tended to remain stable over the study period. Curves were fitted by nonlinear regression.

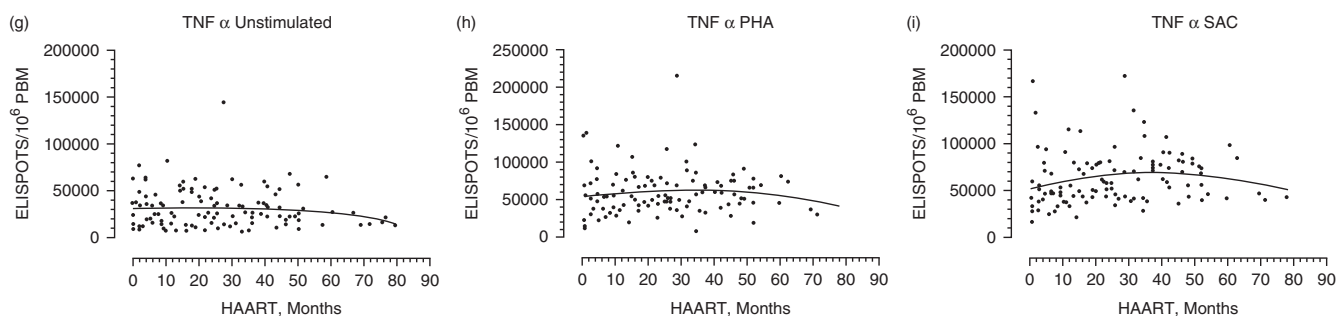


Figure 11
Numbers of IL-6, IL-10, and TNF-alpha-secreting cells in 12 pediatric patients treated with HAART. Cytokine-secreting cells tended to remain stable over the study period. Curves were fitted by nonlinear regression.

rapid-progressor children than in those who were long-term asymptomatic. It is therefore possible that these cytokines may interact differently with HIV in children and adults. It should also be borne in mind that enumeration of cytokine-secreting cells following in vitro mitogen stimulation of isolated PBMCs is unlikely to compare directly with cytokine quantitation in plasma.

Our novel finding that Con A-induced IL-4 was negatively correlated with viral load is in line with its ability to inhibit phorbol ester-stimulated HIV-1 expression in chronically infected promonocytic U1 cells.[33] The effect of IL-4 on HIV in culture merits further study.

We did not observe changes over time that suggested that type 2 cytokine production was tending to predominate over type 1 cytokines, as was described in some[13-16] but not all[17] reports. Instead we observed that the presumably desirable increase in numbers of both type 1 (IFN-gamma, IL-2, and IL-12) and type 2 (IL-4) ELISPOTs/10⁶ PBMCs during the first 34 years of treatment with HAART was not maintained beyond this time (Figure 3). It is not known whether a reducing trend of this nature presages failing immune protection or, more hopefully, lessening of HIV-1-induced immune hyperactivation. Continued observation of this small cohort of patients should allow us to determine whether these changes in cytokine production are related to the eventual clinical outcome.

The ELISPOT assay used in this investigation has been optimized for reproducibility and sensitivity. It does not require specialized equipment and is relatively easy to perform and inexpensive (approximately US\$32 per patient for 7 cytokines and the different activating conditions). We have previously used this system to investigate in vitro cytokine production in a number of clinical situations.[22-26] The assay performed favorably when data from groups of patients were pooled for statistical comparison, but there was wide variation in values for differ-

ent subjects and day-to-day variability due to factors such as subclinical illness, mild tissue injury, and possibly variable stress levels. It was not ethically feasible to have either a healthy matched pediatric control group or an untreated pediatric HIV control group in the present study, so we were limited to a comparison of cytokine profiles in individual patients at times of relatively good and poor health and of improving or worsening CD4+ cell counts or viral loads. We were unable to identify cytokine profiles that were associated with or predictive of HIV-related clinical events. Cytokine profiling using mitogen-stimulated ELISPOT assays is therefore unlikely to be an important clinical measure that could influence or improve the accuracy of patient management decisions.

Authors and Disclosures

Brian M. Jones, PhD, has disclosed no relevant financial relationships.

Susan S.S. Chiu, MD, has disclosed no relevant financial relationships.

Wilfred H.S. Wong, MMedSci, has disclosed no relevant financial relationships.

Wilina W.L. Lim, MD, has disclosed no relevant financial relationships.

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