# The Impact of Cytokine Levels in Young South African Children with and without HIV-associated Acute Lower Respiratory Infections

*Alicia A. Annamalay,[1] Salome Abbott,[2] Siew-Kim Khoo,[1,3] Julie Hibbert,[1,3] Joelene Bizzintino,[1,3] Guicheng Zhang,[1,4] Ingrid Laing,[1,3]Andrew Currie,[5,6] Peter N. Le Souëf[1] and Robin J. Green[3]*

[1]School of Paediatrics and Child Health, University of Western Australia, Perth, Australia

[2]Division of Paediatric Pulmonology, Steve Biko Academic Hospital, University of Pretoria, Pretoria, South Africa

[3]Telethon Kids Institute, The University of Western Australia, Perth, Australia

[4]School of Public Health, Curtin University, Perth, Australia

[5]Medical, Molecular & Forensic Sciences, Murdoch University, Perth, Western Australia

[6]Wesfarmers Centre of Vaccines and Infectious Diseases, Telethon Kids Institute, Perth, Western Australia.

## Abstract

Altered host immune responses are considered to play a key role in the pathogenesis of acute lower respiratory infections (ALRI). The existing literature on cytokine responses in ALRI is largely focussed on adults from developed countries and there are few reports describing the role of cytokines in childhood ALRI, particularly in African or human immunodeficiency virus (HIV)-infected populations. To measure systemic cytokine levels in blood plasma from young South African children with and without ALRI and with and without HIV to determine associations between cytokine responses and disease status and respiratory viral identification. Blood plasma samples were collected from 106 hospitalized ALRI cases and 54 non-ALRI controls less than 2

years of age. HIV status was determined. Blood plasma concentrations of 19 cytokines, 7 chemokines, and 4 growth factors (epidermal growth factor, fibroblast growth factor-basic, hepatocyte growth factor, and vascular endothelial) were measured using The Human Cytokine 30-Plex Panel. Common respiratory viruses were identified by PCR. Mean cytokine concentrations for G-CSF, interferon (IFN)-γ, interleukin (IL)-5, and MCP-1 were significantly higher in ALRI cases than in nonrespiratory controls. Within the ALRI cases, several cytokines were higher in children with a virus compared with children without a virus. Mean cytokine concentrations for IFN-α, IFN-γ, IL-4, IL-5, IL-13, tumour necrosis factor-α, and MIP-1α were significantly lower in HIV-infected cases than in HIV-uninfected cases, while IP-10 and monokine induced by interferon-γ were significantly higher in HIV-infected cases than in HIV-uninfected cases. Certain cytokines are likely to play an important role in the host immune response to ALRI. HIV-infected children have impaired inflammatory responses to respiratory infections compared with HIV-uninfected children..

**Keywords:** cytokines, disease control, human immunodeficiency virus, pathogenesis, respiratory tract,virus classification

## 1 Introduction

Acute lower respiratory infections (ALRI), caused primarily by viruses and bacteria, are the leading cause of childhood mortality worldwide.[1] Infections and their interaction with host immune system cytokine responses are considered to play a key role in the pathogenesis of ALRI. Cytokine type and levels indicate the nature of an immune response for a particular individual. Upon detection of a pathogen, immune cells initiate an immune response specific to that pathogen.[2]

Cytokines are often divided into proinflammatory cytokines (interleukin [IL]-1, IL-8, IL-12, IL-18, and tumour necrosis factor [TNF]-α) that stimulate the immune system and antiinflammatory cytokines (IL-1RA, IL-10, and IL-11) that suppress the immune system.[2] Some cytokines such as IL-6 can have either a proinflammatory or antiinflammatory action.[3] T helper (Th) cells or CD4+ T helper cells in particular play a key role in modulating immune responses. Th1-type cytokines (e.g., IL-12, and interferon [IFN]-γ) generate proinflammatory responses against intracellular pathogens such as viruses and bacteria.[4] Th2-type cytokines generate responses against extracellular parasites such as helminths and include ILs (e.g., IL-1, IL-4, IL-5, and IL-13) that are associated with the promotion of immunoglobulin E and eosinophilic responses in atopy as well as IL-10 which has more of an antiinflammatory response.[4] CD4+ cells not only regulate the immune system but also play a major role in inflammatory disease progression. Information on cytokine levels in children with and without ALRI in Africa has the potential to help elucidate the role of the immune system in the pathogenesis of childhood ALRI.

The existing literature on the pattern of cytokine responses in ALRI is largely focussed on only a select few, well-characterised cytokines including TNF-α, IFN-γ, IL-1β, IL-6, IL-8, and IL-10, and are mainly from adult studies.[5-9] There are few comprehensive reports on the role of cytokines in childhood ALRI. In one study of systemic responses of 15 cytokines in children with community-acquired pneumonia in the USA, IL-6 was associated with markers of disease severity.[10] The study also compared cytokine concentrations with different aetiologies (bacteria alone, viruses alone or mixed infections) and found different patterns of cytokine response in different infectious diseases, including IFN-α, IL-6, IL-17, GM-CSF, and TNF-α concentrations as well as higher concentrations of IFN-α, IL-6, GM-CSF, and TNF-α among children with mixed

infections compared with children with viruses or bacterial alone. Studies on cytokine responses in ALRI in Africa are lacking especially in paediatric populations. A study of infants with respiratory syncytial virus (RSV) in The Gambia reported an increase in IL-13 in response to RSV antigens.[11]

Human immunodeficiency virus (HIV) infection plays an important role in the frequency and outcome of ALRI[12] with pneumonia being the leading cause of morbidity and mortality in HIV-infected children.[13] HIV-infected individuals have a disordered immune system and disrupted function of the CD4+ T helper cells associated with decreased levels of the proinflammatory Th1 cytokines including IL-12, IL-2, and IFN-γ.[14] This may contribute to heightened disease responses to viruses and bacteria. Hence, HIV-infected individuals are more susceptible to infections including ALRI that are normally cleared by the immune system of a healthy individual. As HIV infection suppresses the immune response, both proinflammatory and antiinflammatory cytokines are likely to be altered in HIV-infected individuals with ALRI. Few investigations of cytokines in HIV-infected individuals with respiratory illness have been conducted in African countries. In African children, Green et al.[15] found that in HIV-infected infants with severe hypoxic pneumonia, IL-10 and IFN-inducible protein (IP)-10 levels were higher in infants with more severe lung disease. However, as only HIV-infected children were included in the study, the relative contribution of ALRI versus HIV to the cytokine changes was unknown. Another study of Malawian children 2 months to 16 years of age with pneumonia or meningitis found that in HIV-infected children, IL-1β, IL-6, and IL-10 levels were all significantly higher in HIV-infected nonsurvivors than in HIV-infected survivors but this difference was not significant among HIV-uninfected children.[16]

A wide range of respiratory viruses and bacteria are known to cause ALRI. However, no clinical symptom is pathognomonic of infection with a specific respiratory pathogen, and most pathogens are able to elicit a range of upper and lower respiratory tract symptoms and cause disease ranging from mild to severe.[17] Several studies have reported on cytokine levels associated with specific respiratory pathogens; human rhinovirus (HRV) infection has been associated with increased levels of Th2[18, 19] and Th17[20-22] cytokines and RSV infection has been associated with increased levels of Th2 cytokines.[11, 23] As evidence suggests that specific respiratory pathogens may elicit specific cytokine responses, for reasons that are not yet clear, host cytokine profiles could provide a biomarker for determining the aetiology of ALRI. The relative importance of individual pathogens and the mechanisms that lead to lower respiratory infection, remain poorly understood and an understanding of pathogen-specific cytokine profiles may provide insight into the pathogenesis of ALRI.

The aims of this study were to measure systemic cytokine (and chemokine and growth factors) in blood plasma from young South African children i) with and without ALRI, ii) with and without HIV and iii) with and without different respiratory viruses to determine associations between cytokine responses and disease status and respiratory viral identification. We hypothesised that there would be cytokine responses unique to ALRI, HIV-infection and specific viral infections.

## 2 Materials and Methods

### 2.1 Study Population

A prospective case-control study was conducted between July 2011 and November 2012 in Pretoria, South Africa. Children 0–2 years of age admitted to the Steve Biko Academic Hospital

or Tshwane District Hospital and diagnosed with ALRI (pneumonia or bronchiolitis) were enrolled as cases. A clinical diagnosis of pneumonia or bronchiolitis was determined by the treating physician; pneumonia was diagnosed in children with respiratory distress and either chest X-ray changes (e.g., consolidation or effusion), fever (defined as an axillary temperature of >37.5°C) or auscultatory findings (e.g., crepitations or bronchial breathing) while bronchiolitis was diagnosed in children with respiratory distress and at least one of the following; wheeze, chest X-ray changes (e.g., signs of hyperinflation) or Hoover's sign (inward movement of the lower rib cage during inspiration). Age-matched children presenting to the same hospitals over the same time period with a nonrespiratory illness or injury were enrolled as controls. Exclusion criteria for controls included current signs or symptoms of respiratory illness. Recruitment was conducted throughout the year regardless of season. This study was approved by the University of Western Australia Human Research Ethics Committee and University of Pretoria Ethics Committee before commencement. Written informed consent was obtained from parents or guardians before participation.

### 2.2 Data and Sample Collection

Information and samples were obtained from the enrolled cases and controls on the day of recruitment. One millilitre of whole blood was collected by venepuncture into ethylenediaminetetraacetic acid tubes which were centrifuged for 10 min at 1000–2000*g* within 1 h of blood draw to separate plasma from red blood cells. Nasopharyngeal aspirates (NPAs) were collected and respiratory viruses (HRV, adenovirus, RSV, bocavirus, coronavirus, parainfluenza viruses, influenza viruses, and metapneumovirus) were identified as described in Annamalay et al.[24] Plasma, red blood cells and NPAs were stored at 80°C until sent to Perth, Australia on dry ice for further analyses. HIV status was determined using HIV enzyme-linked immunosorbent assay

tests and confirmed with a PCR for children less than 18 months of age. A detailed questionnaire was administered by the study doctor to at least one parent or guardian of the enrolled children. Information on clinical symptoms (e.g., cough, wheeze, shortness of breath, fever, weak and tired, runny nose, congestion and sneezing), health history (e.g., antibiotic therapy and allergies) and past and present medications was collected. Demographic details including gender, age and ethnicity and environmental and socioeconomic information were also collected via the questionnaire.

### 2.3 Plasma Cytokine Concentrations

Plasma concentrations of 19 cytokines (G-CSF, GM-CSF, IFN-α, IFN-γ, IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, and TNF-α), 7 chemokines (Eotaxin, IP-10, MCP-1, monokine induced by interferon-γ (MIG), MIP-1α, MIP-1β, and RANTES) and 4 growth factors (epidermal growth factor, fibroblast growth factor-basic, hepatocyte growth factor [HGF], and vascular endothelial) were measured using The Human Cytokine 30-Plex Panel (Luminex). The Human Cytokine 30-Plex Panel is based on xMAP technology which uses a suspension array system with colour-coded beads that allow for the simultaneous detection of up to 100 cytokines in a single well of a microplate.[25] The assay was run according to The Human Cytokine 30-Plex Panel protocol of Novex by Life Technologies. Samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories). Analysis of experimental data was done using five-parametric curve fitting. All samples and standards were assayed in duplicate and samples with a coefficient of variation (CV) greater than 50% were excluded from analyses. The mean %CV for each cytokine ranged from 6.29% to 12.40%. Samples that were below the standard range or that were extrapolated below the standard

range were reported as the midpoint between the expected concentration (pg/ml) of the lowest standard and zero. A few samples were out of range (above) and were reported as the concentration (pg/ml) of the highest standard.

### 2.4 Statistical Analyses

Log-transformed cytokine concentrations were used for parametric analyses and the data are presented as geometric means with *SD*. Associations between plasma cytokine concentrations and ALRI, HIV, or HRV were first analysed using independent samples *t* test and if significant ($p < .05$), were then included in multiple linear regression analysis controlling for age. All analyses between ALRI cases and nonrespiratory controls were carried out only within the HIV-uninfected group. All analyses comparing HIV-infected and HIV-uninfected children were carried out only within the ALRI cases. Principal component analysis (PCA) was carried out on the panel of 30 cytokines. Principal components (PC) identified were retained for further analysis if their eigenvalues were above one. Varimax with the Kaiser Normalization rotation method was applied. PCA was employed to reduce the data dimension of the cytokine data. The score values of these PCA components were compared between groups using independent sample *t* tests. Statistical analyses were performed using SPSS version 22.0 (SPSS Inc.) and a $p < .05$ was considered statistically significant.

## 3 Results

### 3.1 Population Demographics

One hundred and six ALRI cases (67.0% males, median age 5.6 months, IQR 2.4-10.6 months) and 54 controls (57.4% males, median age 7.9 months, IQR 2.4-13.9 months) were enrolled

between July 2011 and November 2012. There were no significant differences in the population demographics (age, gender or ethnicity) between the ALRI cases and non-respiratory controls. Of the 106 ALRI cases, 58 were diagnosed with pneumonia (63.8% male) and 48 were diagnosed with bronchiolitis (70.8% male) ($p$=0.443). Seventeen children were HIV-infected, of which only 2 were non-respiratory controls ($p$=0.049). Of the 15 HIV-infected ALRI cases, only one was diagnosed with bronchiolitis whereas the rest were diagnosed with pneumonia ($p$=0.020).

### 3.2 Plasma cytokine concentrations in ALRI cases and controls

Cytokine concentrations were measured in plasma samples from 86 HIV-uninfected ALRI cases and 48 HIV-uninfected non-respiratory controls. Mean cytokine concentrations for G-CSF, IFN-γ, IL-5 and MCP-1 were significantly higher in ALRI cases than in non-respiratory controls (Figure 1, Supplementary Table 1). Between the ALRI diagnosis groups (pneumonia and bronchiolitis), mean hepatocyte growth factor (HGF) concentration was significantly higher in pneumonia cases (925.8 pg/ml, 95% CI: 685.7-1249.8) than in bronchiolitis cases (605.1 pg/ml, 95% CI: 494.6-640.4, p=0.020) and mean IL-13 concentration was significantly lower in pneumonia cases (70.4 pg/ml, 95% CI: 47.7-105.5) than in bronchiolitis cases (117.0 pg/ml, 95% CI: 88.9-154.0, p=0.040).

### 3.3 Cytokine concentrations in HIV-infected and HIV-uninfected ALRI cases

Cytokine concentrations were measured in plasma samples from 15 HIV-infected ALRI cases and 86 HIV-uninfected ALRI cases. Mean cytokine concentrations for IFN-α, IFN-γ, IL-4, IL-5, IL-13, TNF-α, and MIP-1α were significantly lower in HIV-infected cases than in HIV-uninfected cases, while IP-10 and MIG were significantly higher in HIV-infected cases than in HIV-uninfected cases (Figure 2 and Table S2).

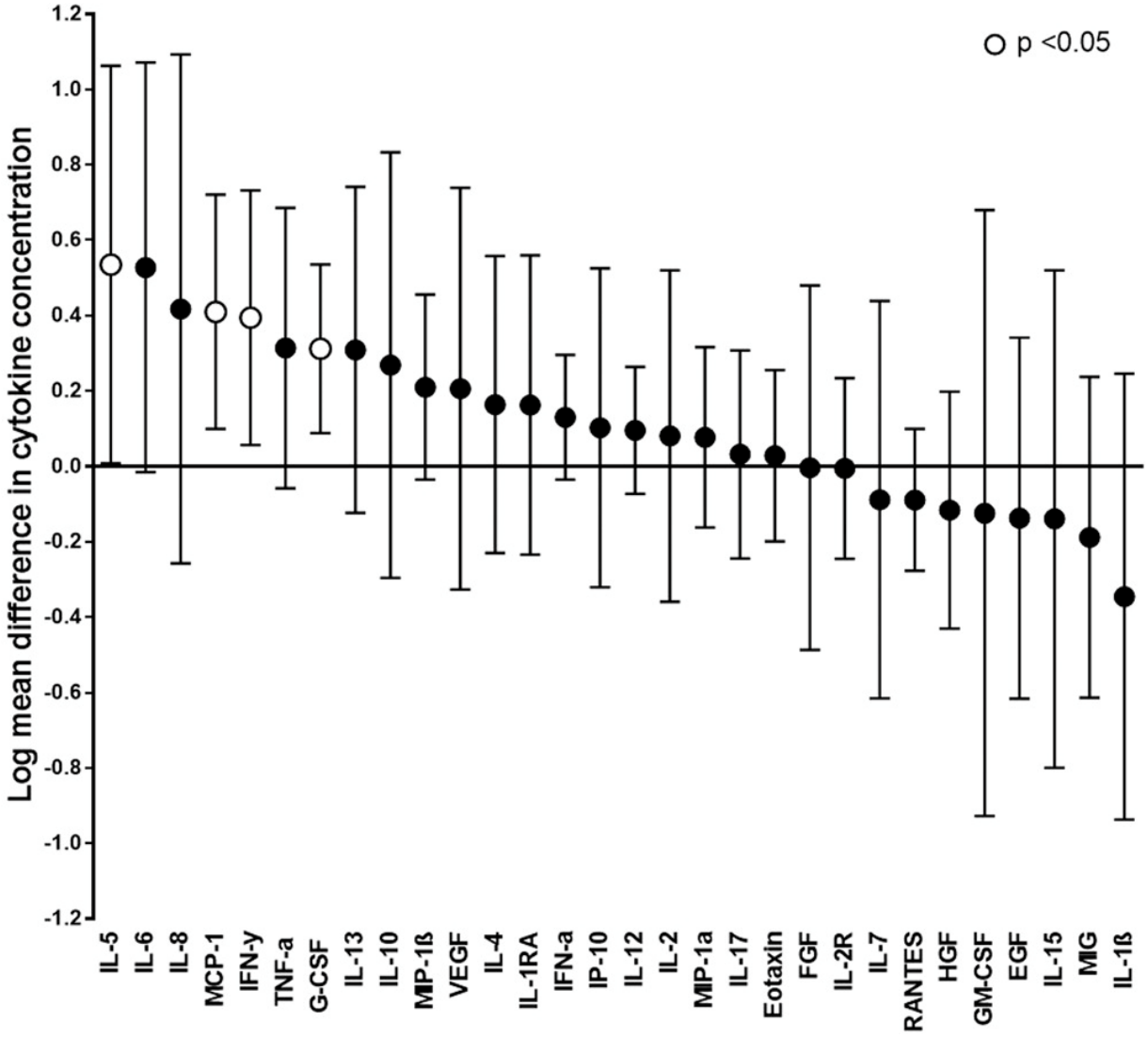


**Figure 1.** Logarithmic mean difference (and 95% CI) in plasma cytokine concentrations between (HIV-uninfected) ALRI cases and controls. Cytokines with mean difference values above 0 were higher in ALRI cases than controls. ALRI, acute lower respiratory infections; CI, confidence interval; HIV, human immunodeficiency virus

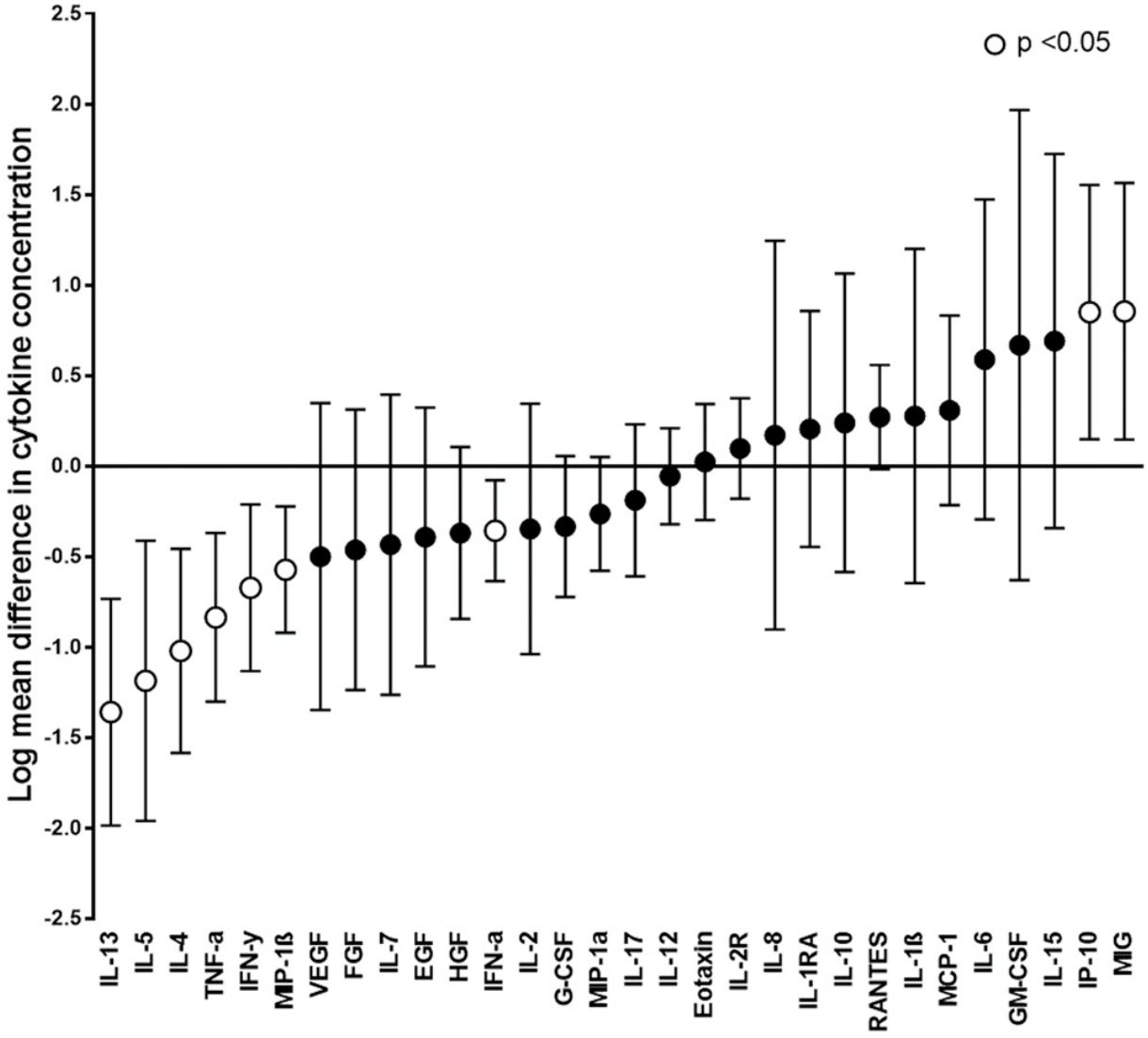


**Figure 2.** Logarithmic mean difference (and 95% CI) in plasma cytokine concentrations between HIV-infected and HIV-uninfected ALRI cases. Cytokines with mean difference values above 0 were higher in HIV-infected ALRI cases than HIV-uninfected ALRI cases. ALRI, acute lower respiratory infections; CI, confidence interval; HIV, human immunodeficiency virus

### 3.4 Cytokine concentrations specific for respiratory viruses

Plasma cytokine concentrations relating to respiratory viruses were investigated within HIV-uninfected ALRI cases and nonrespiratory controls. At least one respiratory virus was identified

in 72 (83.7%) ALRI cases and 33 (70.2%) nonrespiratory controls, of which, HRV was the most commonly identified virus (49.4% in cases and 36.2% in controls), as described in Annamalay et al.[24] This was followed by adenovirus (34.1% in cases and 29.8% in controls) and RSV (30.6% in cases and 17.0% in controls). For overall viral detection, there were no significant differences in cytokine concentrations between ALRI cases with at least one respiratory virus identified ($n = 72$) and ALRI cases with no respiratory virus identified ($n = 13$). One case was excluded as their cytokine concentration was above %CV. Among controls, however, IP-10, IL-8, and MCP-1 concentrations were significantly higher in children with at least one respiratory virus than in controls with no respiratory virus ($p = .008$, $p = .032$, and $p = .028$, respectively).

For HRV, mean cytokine concentrations for IL-5, TNF-α, IL-2, G-CSF, IL-7, and IL-17 were significantly higher in HRV-positive ALRI cases than in HRV-negative ALRI cases (Table 1). For controls, there were no significant differences in mean cytokine concentrations between HRV-positive and HRV-negative children. For RSV, mean cytokine concentrations for IL-12, IL-13, IL-2, and IFN-γ were significantly higher in RSV-positive cases than in RSV-negative ALRI cases while RANTES was significantly lower in RSV-positive ALRI cases than in RSV-negative ALRI cases (Table 1). Among controls, mean IL-12 concentration was significantly higher in RSV-positive children (600.6 pg/ml; 95% CI: 398.8–904.6) than in RSV-negative children (426.8 pg/ml; 95% CI: 375.1–485.6; $p = .039$). For adenovirus, the mean IL-6 concentration was significantly higher in adenovirus-positive ALRI cases (41.1 pg/ml; 95% CI: 20.9–80.9) than in adenovirus-negative ALRI cases (19.2 pg/ml; 95% CI: 13.2–28.1; $p = .035$) and there were no differences in mean cytokine concentrations between adenovirus-positive controls and adenovirus-negative controls.

**Table 1**. Mean concentrations (pg/ml) for cytokine, chemokine, and growth factors that were significantly different between virus-positive and virus-negative (HIV-uninfected) ALRI cases

| | Geometric mean plasma cytokine, chemokine, and growth factor concentrations (95% CI; pg/ml) | | |
|---|---|---|---|
| | HRV-positive ALRI cases *n* = 39 | HRV-negative ALRI cases *n* = 42 | *p* Value |
| IL-5 | 18.7 (12.2–28.5) | 8.12 (5.45–12.1) | .005 |
| TNF-α | 57.5 (45.8–72.2) | 35.9 (28.3–45.5) | .006 |
| IL-2 | 10.8 (7.09–16.5) | 5.69 (4.09–7.92) | .028 |
| G-CSF | 603.1 (500.6–726.7) | 457.9 (387.7–540.7) | .049 |
| IL-7 | 47.8 (29.2–78.3) | 23.9 (15.4–37.1) | .048 |
| IL-17 | 8.13 (6.01–11.0) | 5.69 (4.76–6.79) | .028 |

| | RSV-positive ALRI cases *n* = 26 | RSV-negative ALRI cases *n* = 58 | *p* Value |
|---|---|---|---|
| IL-12 | 629.5 (526.5–752.8) | 443.7 (390.4–504.1) | .001 |
| IL-13 | 155.9 (110.1–220.8) | 71.22 (52.1–97.3) | .004 |
| RANTES | 5088.8 (4342.7–5963.0) | 7370.7 (6374.2–8522.6) | .006 |
| IL-2 | 12.5 (6.95–22.5) | 6.36 (4.76–8.49) | .006 |
| IFN-γ | 91.6 (74.2–113.1) | 61.0 (49.0–75.9) | .029 |
| IP-10 | 275.7 (199.8–380.4) | 135.3 (92.1–198.8) | .021 |

Abbreviations: ALRI, acute lower respiratory infections; CI, confidence interval; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

### 3.5 Principal component analysis

Eight principal components with eigenvalues above one were identified and included in further analysis. Each principal component (PC1- PC8) was composed of cytokines that shared a correlation above 0.3 as presented in Table 2. The 8 principle components obtained cumulatively explain 74.2% of variation within the dataset.

**Table 2.** Cytokines in each of the eight rotated principal component matrix

| Principal components | Cytokines (correlation above 0.3) |
|---|---|
| 1 | IL-5, TNF-α, IL-4, IL-13, IFN-γ, IFN-α, IL-17, IL-7, G-CSF, MIP-1β, IL-2, MIP-1α, IL-12, Eotaxin, VEGF |
| 2 | IL-7, MIP-1β, IL-2, MIP-1α, VEGF, HGF, EGF, IL-15, FGF, IL-1β |
| 3 | G-CSF, VEGF, HGF, EGF, IL-8, IL-6, IL-1RA, IL-2R |
| 4 | IFN-α, MIP-1α, Eotaxin, VEGF, IL-15, IL-8, IL-6, MCP-1, IL-1RA |
| 5 | IL-2, IL-6, IL-10, GM-CSF |
| 6 | IL-12, Eotaxin, IP-10 |
| 7 | IFN-γ, MIG, IL-2R |
| 8 | Eotaxin, IL-2R |

*Note*: Principal components were composed of cytokines that shared a correlation above 0.3.

Abbreviations: ALRI, acute lower respiratory infections; CI, confidence interval; EGF, epidermal growth factor; FGF, fibroblast growth factor; IFN, interferon; IL, interleukin; MIG, monokine induced by interferon-γ; TNF, tumour necrosis factor; VEGF, vascular endothelial.

### 3.6 Principal component analysis of clinical groups

Associations between the eight PCs and different clinical groups were examined. A PC factor score is the composite measure created from the PCA and is referred to as either PC1-PC8 or the component of the most strongly associated cytokines. For examples, PC1 can be referred to as the IL-5 component. A lower PC factor score correlates to a lower plasma cytokine concentration. There were no differences in PC factor scores between HIV-uninfected ALRI cases and controls. Between ALRI diagnosis groups, PC3 factor score was significantly higher in HIV-uninfected pneumonia cases than in HIV-uninfected bronchiolitis cases.

Between HIV-infected and HIV-uninfected ALRI cases, the PC1 factor score was significantly lower for HIV-infected cases than for HIV-uninfected cases ($p < .001$) and PC6 and PC7 factor scores were significantly higher for HIV-infected cases than for HIV-uninfected cases ($p = .049$ and $p = .025$ for PC6 and PC7, respectively). We also compared HIV-infected ALRI cases ($n = 15$) with the control group ($n = 54$) and found PC1 was significantly lower in the HIV-infected ALRI cases than in the control group ($p = .004$) while PC4 and PC6 were higher in the HIV-infected ALRI cases than in the control group ($p = .013$ and $p = .018$, respectively).

### 3.7 Principal component analysis of identified respiratory viruses

We examined associations between PC factor scores and respiratory viral identification in ALRI cases and nonrespiratory controls. Among ALRI cases, there were no differences in PC factor scores for cases with at least one respiratory virus compared with cases with no respiratory virus. Among controls, PC4 and PC6 factor scores were significantly higher for children with at least

one respiratory virus than for children with no respiratory virus ($p = .005$ and $p = .003$, respectively).

For HRV-positive ALRI cases ($n = 39$), the PC1 factor score was significantly higher than for HRV-negative ALRI cases ($n = 42$, $p = .003$) and no differences were observed for factor scores between HRV-positive and HRV-negative controls. For RSV-positive ALRI cases ($n = 25$), PC6 and PC8 factor scores were significantly higher than for RSV-negative ALRI cases ($n = 56$; $p = .010$ and $p = .005$, respectively) and no differences were observed for factor scores between RSV-positive and RSV-negative controls. There were no differences in PC factor scores between adenovirus-positive and adenovirus-negative children for either the ALRI cases or controls group.

## 4 Discussion

Although other studies have examined cytokine levels in young children either with ALRI or with and without HIV, this is the first study to investigate cytokine levels in children with ALRI and with or without HIV. This comprehensive analysis included 30 cytokines, most of which had not been investigated anywhere in either adults or children with ALRI. We found significant associations between several cytokines and ALRI, HIV infection and respiratory virus identification, some of which support previous reports and others we report for the first time.

Comparing ALRI cases and nonrespiratory controls, all of whom were HIV-uninfected, the IFN-γ, IL-5, G-CSF, and MCP-1 levels were significantly higher in cases than controls. IFN-γ has been identified as an important cytokine in respiratory illness[26] and has been found to be elevated in both local and systemic samples from patients with community-acquired pneumonia compared

with healthy individuals.[9] However, there are limited data on the roles of IL-5, G-CSF, and MCP-1 in ALRI with scarce data demonstrating increased IL-5 in atopic asthmatics,[27-30] increased MCP-1 in patients with interstitial lung disease,[31] and increased G-CSF in mice induced with pneumococcal pneumonia.[32] This is the first study to find elevated levels of IL-5, G-CSF, and MCP-1 in young African children with ALRI.

Several studies in adults and children have reported increased IL-6 levels in individuals with pneumonia.[5, 7-10] Our findings support the current understanding that cytokines play an important role in the host immune response to ALRI by confirming known associations between cytokines and ALRI (i.e., IFN-γ and IL-6) as well as demonstrating novel associations (i.e., IL-5, G-CSF, and MCP).

Between HIV-infected and HIV-uninfected children with ALRI, IP-10 and MIG levels were significantly higher in HIV-infected children than in HIV-uninfected children, while IFN-α, IFN-γ, IL-4, IL-5, IL-13, TNF-α, and MIP-1α were significantly lower in HIV-infected children than in HIV-uninfected children. IP-10 and MIG are CXC chemokines that play an important role in innate and adaptive immunity.[33] Concordant with our findings, Green et al.[15] also found that IP-10 (and IL-10) were associated with more severe lung disease and that levels were higher in HIV-infected infants with severe hypoxic pneumonia than in HIV-infected infants with bronchiectasis.[15] As only HIV-infected children were included in Green et al.'s[15] study and as the control group consisted of children with bronchiectasis, the relative contribution of ALRI and HIV in that study is unknown. However, increased levels of IP-10 associated with HIV-infection have also been reported in adult populations outside of Africa.[33-36] Also, in agreement with our findings, a study

of adults with HIV-infection in the United States also found IFN-γ to be decreased in HIV-infected individuals compared with HIV-uninfected individuals.[37] Of the six cytokines tested in their study, Tudela et al.[37] reported three to be higher in the HIV-infected group than in the HIV-uninfected group. The three cytokines that were increased in the HIV-infected group (IL-6, IL-10, and TGF-β) were known anti-inflammatory cytokines, while the three cytokines that were decreased (IL-12, IL-2, and IFN-γ) were known as proinflammatory cytokines, which the authors hypothesized was a result of HIV's role in dampening the immune response to intracellular pathogens. In our study, we found that 7 of the 30 cytokines measured were significantly lower in HIV-infected children than in HIV-uninfected children (IFN-α, IFN-γ, IL-4, IL-5, IL-13, TNF-α, and MIP-1α) while only two cytokines were significantly higher in HIV-infected children than in HIV-uninfected children (IP-10 and MIG). Of the seven cytokines that were significantly lower in HIV-infected children, TNF-α is a known proinflammatory cytokine while IL-4 and IL-13 are known to be Th2 immunomodulatory cytokines. Hence, our findings challenge the proinflammatory versus antiinflammatory hypothesis put forth by Tudela et al.[37] However, unlike our study, the HIV-infected individuals in the study by Tuleda et al.[37] did not have an acute respiratory illness at the time of sampling. Our study examined HIV-infected and HIV-uninfected children with ALRI and hence, allowed us to elucidate how host immune responses to an ALRI were affected by HIV. For example, IFN-γ and IL-5 levels were significantly higher in ALRI cases compared with nonrespiratory controls. In the ALRI cases, IFN-γ and IL-5 levels were significantly lower among HIV-infected individuals than HIV-uninfected individuals. This suggests that HIV-infected children have impaired inflammatory responses to respiratory infections compared with HIV-uninfected children.

We also found associations between several cytokines and respiratory viruses. IL-5, TNF-α, IL-2, G-CSF, IL-7, and IL-17 levels were significantly higher in HRV-positive ALRI cases than in HRV-negative ALRI cases. HRV has been shown to generate strong Th2 and Th17 cytokine responses[18, 19] which are known to regulate airway inflammation during respiratory viral infections.[20-22] Indeed, we found elevated levels of the Th2 cytokine IL-5 and the Th17 cytokine IL-17 among ALRI cases with HRV infection when compared with ALRI cases without HRV infection. However, IL-2, which can be either a Th1 or Th2 cytokine, was also higher among the HRV-positive cases than the HRV-negative cases.

For RSV, cytokine levels of IL-12, IL-13, IL-2, and IFN-γ were significantly higher in RSV-positive ALRI cases than in RSV-negative ALRI cases while RANTES was significantly lower in RSV-positive ALRI cases than in RSV-negative ALRI cases. Among nonrespiratory controls, IL-12 was significantly higher in RSV-positive children than in RSV-negative children. Like HRV, there is evidence that RSV induces strong Th2 cytokines[38, 39] but there is conflicting evidence over the role of Th1 cytokines, with some studies reporting increased levels associated with RSV infection[38, 39] while others report reduced expression of Th1 cytokines (i.e., IFN-γ) among infants with more severe RSV bronchiolitis compared with those with mild disease.[40] For adenovirus, we found that IL-6 was significantly higher in adenovirus-positive ALRI cases than in adenovirus-negative ALRI-cases, which support the current literature on increased IL-6 levels in individuals with ALRI.[5, 7-10] In this study, the elevated cytokines among the virus-positive ALRI-cases identified belonged to Th1, Th2, and Th17 cytokines as well as to the proinflammatory and antiinflammatory groups. Hence, our data suggest that specific respiratory viral infections generate

different cytokine responses and collectively generate a wide range of cytokines that may not necessarily fit into the proinflammatory versus antiinflammatory or Th1 versus Th2 processes.

Cytokines are often functionally divided into proinflammatory versus antiinflammatory or Th1 versus Th2 cytokines. As we did not find any functional group to be associated with ALRI, HIV or respiratory viral identification, principle component analysis was carried out to determine if there were any other unique patterns of cytokine groupings associated with ALRI, HIV and respiratory viral identification. PC1 (IL-5 component) was significantly lower in HIV-infected ALRI cases than in both HIV-uninfected ALRI cases and nonrespiratory controls. The interaction between Treg cells, HIV-infection and ALRI is an interesting question, without any evidence in the literature. PC6 (IP-10 component) and PC7 (MIG component) were significantly higher in HIV-infected ALRI cases than in HIV-uninfected ALRI cases. When comparing findings between different diagnostic categories of ALRI, PC3 (IL-1RA component) was significantly higher in HIV-uninfected pneumonia cases than in HIV-uninfected bronchiolitis cases and we found different factors associated with the different respiratory viruses. These findings illustrate the usefulness of performing PCA to identify distinct cytokine patterns and inflammatory signatures associated with disease or specific respiratory pathogens.

One limitation of this study is the lack of information on bacterial infections. As bacteria is commonly identified in children with pneumonia, it is possible that bacterial aetiology may influence cytokine levels. Another limitation is the use of a hospital control group. Control children were hospitalized for nonrespiratory illnesses and hence, cytokine levels obtained for the control

group were not representative of healthy children. Furthermore, as systemic rather than local cytokine levels were measured, cytokine levels obtained may be influenced by different disease states among the control group. Nonetheless, comparison of ALRI cases with nonrespiratory controls allowed us to elucidate ALRI-specific systemic cytokine responses and show for the first time that IL-5, G-CSF, and MCP-1 cytokine levels are elevated in young South African children with ALRI.

## 5 Conclusion

Certain cytokines are likely to play an important role in the host immune response to ALRI. HIV-infected children have impaired inflammatory responses to respiratory infections compared with HIV-uninfected children. A number of cytokines are elevated in HIV-infected ALRI cases and many become reduced. Comparison of ALRI cases with nonrespiratory controls allowed us to elucidate ALRI-specific systemic cytokine responses and show for the first time that IL-5, G-CSF and MCP-1 cytokine levels are elevated in young South African children with ALRI.


## *Acknowledgements*

The authors thank all the children and families who participated in the study. This study resulted from the collaborative work of groups from the School of Paediatrics and Child Health, Faculty of Medicine, Dentistry and Health Sciences, University of Western Australia and the University of Pretoria, South Africa. This study was supported by the Alan King Westcare Project grant by the Lung Institute of Western Australia, a National Health and Medical Research Council (NHMRC) project grant (Peter Le Souëf) and the National Research Foundation South Africa (Robin Green).

## References


1. Walker CLF, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, et al. Global burden of childhood pneumonia and diarrhoea. Lancet. 2013;381(9875):1405-16.
2. Dinarello CA. Proinflammatory cytokines. Chest. 2000;118(2):503-8.
3. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2011;1813(5):878-88.
4. O'Garra A, Arai N. A. O'Garra, N. Arai. The molecular basis of T Helper 1 and T Helpercell differentiation. Trends Cell Biol. 2000;10:542-50.
5. Antunes G, Evans SA, Lordan JL, Frew AJ. Systemic cytokine levels in community-acquired pneumonia and their association with disease severity. Eur Resp J. 2002;20(4):990-5.
6. Bauer TT, Montón C, Torres A, Cabello H, Fillela X, Maldonado A, et al. Comparison of systemic cytokine levels in patients with acute respiratory distress syndrome, severe pneumonia, and controls. Thorax. 2000;55(1):46-52.
7. Fernández-Serrano S, Dorca J, Coromines M, Carratalà J, Gudiol F, Manresa F. Molecular Inflammatory Responses Measured in Blood of Patients with Severe Community-Acquired Pneumonia. Clin Diagn Lab Immunol. 2003;10(5):813-20.
8. Glynn P, Coakley R, Kilgallen I, Murphy N, O'Neill S. Circulating interleukin 6 and interleukin 10 in community acquired pneumonia. Thorax. 1999;54(1):51-5.
9. Paats MS, Bergen IM, Hanselaar WEJJ, Groeninx van Zoelen EC, Hoogsteden HC, Hendriks RW, et al. Local and systemic cytokine profiles in nonsevere and severe community-acquired pneumonia. Eur Resp J. 2013;41(6):1378-85.
10. Michelow IC, Katz K, McCracken GH, Hardy RD. Systemic cytokine profile in children with community-acquired pneumonia. Pediatr Pulmonol. 2007;42(7):640-5.
11. Van Der Sande MAB, Kidd IM, Goetghebuer T, Martynoga RA, Magnusen A, Allen S, et al. Severe respiratory syncytial virus infection in early life is associated with increased type 2 cytokine production in Gambian children. Clin Exp Allergy. 2002;32(10):1430-5.
12 Madhi SA, Venter M, Madhi A, Petersen K, Klugman KP. Differing manifestations of respiratory syncytial virus-associated severe lower respiratory tract infections in human immunodeficiency virus type 1-infected and uninfected children. Pediatr Infect Dis J. 2000;20(2):164-70.
13. Graham SM. HIV and respiratory infections in children. Curr Opin Pulm Med. 2003;9(3):215-20.
14. Noble A, Thomas MJ, Kemeny DM. Early Th1 / Th2 cell polarization in the absence of IL-4 and IL-12: T cell receptor signaling regulates the response to cytokines in CD4 and CD8 T cells. Eur J Immunol. 2001;31(7):2227-35.
15. Green RJ, Masekela R, Wittenberg D, Terblanche A, Rheeder P, Becker P, et al. Cytokine Profile and Clinical Correlates in Immune Deficient (HIV-infected) Infants with Severe (hypoxic) Pneumonia. J Allergy Clin Immun. 2013;131(2, Supplement):AB10.
16. Carrol ED, Guiver M, Nkhoma S, Mankhambo LA, Marsh J, Balmer P, et al. High pneumococcal DNA loads are associated with mortality in malawian children with invasive pnuemococcal disease Pediatr Infect Dis J. 2007;26(5):416-22.
17. Laham FR, Israele V, Casellas JM, Garcia AM, Lac Prugent CM, Hoffman SJ, et al. Differential Production of Inflammatory Cytokines in Primary Infection with Human Metapneumovirus and with Other Common Respiratory Viruses of Infancy. J Infect Dis. 2004;189(11):2047-56.
18. Schneider D, Hong JY, Popova AP, Bowman ER, Linn MJ, McLean AM, et al. Neonatal Rhinovirus Infection Induces Mucous Metaplasia and Airways Hyperresponsiveness. J Immunol. 2012;188(6):2894-904.
19. Hong JY, Bentley JK, Chung Y, Lei J, Steenrod JM, Chen Q, et al. Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness through IL-25 and type 2 innate lymphoid cells. J Allergy Clin Immun. 2014;134(2):429-39.e8.

20. Chesné J, Braza F, Mahay G, Brouard S, Aronica M, Magnan A. IL-17 in Severe Asthma. Where Do We Stand? Am J Respir Crit Care Med. 2014;190(10):1094-101.
21. Stoppelenburg AJ, de Roock S, Hennus MP, Bont L, Boes M. Elevated Th17 Response in Infants Undergoing Respiratory Viral Infection. Am J Pathol. 2014;184(5):1274-9.
22. Wiehler S, Proud D. Interleukin-17A modulates human airway epithelial responses to human rhinovirus infection. Am J Physiol. 2007;293(2):L505-L15.
23. Roman M, Calhoun W, Hinton K, AvendanO L, Simon V, Escobar A, et al. Respiratory Syncytial Virus Infection in Infants Is Associated with Predominant Th-2-like Response. Am J Respir Crit Care Med. 1997;156(1):190-5.
24. Annamalay A, Abbott S, Bizzintino J, Khoo SK, Green R, Le Souef P. Role Of Human Rhinovirus In Acute Lower Respiratory Infections In HIV-Infected And HIV-Uninfected South African Children. B63 GENETIC AND ENVIRONMENTAL EFFECTS ON LUNG GROWTH AND DEVELOPMENT. American Thoracic Society International Conference Abstracts: American Thoracic Society; 2013. p. A3251-A.
25. Vignali DAA. Multiplexed particle-based flow cytometric assays. J Immunol Methods. 2000;243(1–2):243-55.
26. Cazzola M, Matera MG, Pezzuto G. Inflammation – A New Therapeutic Target in Pneumonia. Respiration. 2005;72(2):117-26.
27. Broide DH, Paine MM, Firestein GS. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. J Clin Invest. 1992;90(4):1414-24.
28. Hamid Q, Azzaqi M, Ying S, Mogbel R, Wardlaw AJ, Corrigan CJ, et al. Interleukin-5 mRNA in mucosal bronchial biopsies from asthmatic subjects. Int Arch Allergy Appl Immunol 1991;94(1-4):169-70.
29. Fukuda T, Nakajima H, Fukushima Y, Akutsu I, Numao T, Majima K, et al. Detection of interleukin-5 messenger RNA and interleukin-5 protein in bronchial biopsies from asthma by nonradioactive in situ hybridization and immunohistochemistry. J Allergy Clin Immun. 1994;94(3):584-93.
30. Krishnaswamy G, Liu MC, Su S-N, Kumai M, Xiao H-Q, Marsh DG, et al. Analysis of Cytokine Transcripts in the Bronchoalveolar Lavage Cells of Patients with Asthma. Am J Respir Cell Mol Biol. 1993;9(3):279-86.
31. Suga M, Iyonaga K, Ichiyasu H, Saita N, Yamasaki H, Ando M. Clinical significance of MCP-1 levels in BALF and serum in patients with interstitial lung diseases. E Resp J. 1999;14(2):376-82.
32. Knapp S, Hareng L, Rijneveld AW, Bresser P, van der Zee JS, Florquin S, et al. Activation of Neutrophils and Inhibition of the Proinflammatory Cytokine Response by Endogenous Granulocyte Colony-Stimulating Factor in Murine Pneumococcal Pneumonia. J Infect Dis. 2004;189(8):1506-14.
33. Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, Heitman J, et al. Induction of a Striking Systemic Cytokine Cascade prior to Peak Viremia in Acute Human Immunodeficiency Virus Type 1 Infection, in Contrast to More Modest and Delayed Responses in Acute Hepatitis B and C Virus Infections. J Virol. 2009;83(8):3719-33.
34. Simmons RP, Scully EP, Groden EE, Arnold KB, Chang JJ, Lane K, et al. HIV-1 infection induces strong production of IP-10 through TLR7/9-dependent pathways. AIDS. 2013;27(16):2505-17.
35. Cozzi-Lepri A, French MA, Baxter J, Okhuysen P, Plana M, Neuhaus J, et al. Resumption of HIV replication is associated with monocyte/macrophage derived cytokine and chemokine changes: results from a large international clinical trial. AIDS (London, England). 2011;25(9):1207-17.
36. Stylianou E, Aukrust P, Bendtzen K, Müller F, Frøland SS. Interferons and interferon (IFN)-inducible protein 10 during highly active anti-retroviral therapy (HAART)—possible immunosuppressive role of IFN-α in HIV infection. Clin Exp Immunol. 2000;119(3):479-85.
37. Tudela EV, Singh MK, Lagman M, Ly J, Patel N, Ochoa C, et al. Cytokine levels in plasma samples of individuals with HIV infection. Austin Journal of Clinical Immunology. 2014;1(1):1-5.

38. Fullmer JJ, Khan AM, Elidemir O, Chiappetta C, Stark JM, Colasurdo GN. Role of cysteinyl leukotrienes in airway inflammation and responsiveness following RSV infection in BALB/c mice. Pediatr Allergy Immunol. 2005;16(7):593-601.
39. Han J, Jia Y, Takeda K, Shiraishi Y, Okamoto M, Dakhama A, et al. Montelukast during Primary Infection Prevents Airway Hyperresponsiveness and Inflammation after Reinfection with Respiratory Syncytial Virus. Am J Respir Crit Care Med. 2010;182(4):455-63.
40. Aberle J, Aberle S, Dworzak M, Mandl C, Rebhandl W, Vollnhofer G, et al. Reduced Interferon-γ Expression in Peripheral Blood Mononuclear Cells of Infants with Severe Respiratory Syncytial Virus Disease. Am J Respir Crit Care. 1999;160(4):1263-8.