

# High Prevalence of Humoral and Cellular Immunity to Influenza Viruses in Preschool Children Living in Addis Ababa, Ethiopia

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**Background.** Influenza in children who reside in tropical and subtropical regions has until recently been regarded as insignificant. However, new evidence suggests that it significantly impacts hospitalization and promotes secondary bacterial coinfections. Ethiopia is situated in a subtropical area where influenza viruses are likely to circulate year round.

**Methods.** Clinical data were recorded in a cohort of 103 healthy preschool children recruited in Addis Ababa, Ethiopia. Humoral and cellular immune responses to influenza virus were determined by hemagglutination inhibition (HI) and interferon- $\gamma$  enzyme-linked immunospot assays.

**Results.** Ninety-six percent of the children (2–5 years old) had pre-existing HI antibody responses to 1 or more of the circulating influenza A subtypes, H1N1 (51%), H3N2 (86%), or influenza B (51%) strains. At the age of 4, all children had been infected with at least 1 strain, and 75% had been infected with 2–4 different viral strains. CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses against conserved viral antigens increased with repeated exposures, indicating boosting of cross-reactive cellular immunity. Malnutrition did not seem to affect these immune responses to influenza.

**Conclusions.** Influenza is highly prevalent among children in this area of Ethiopia. Due to the risk of secondary bacterial pneumonia, increased influenza awareness might benefit child health.

**Keywords:** cell-mediated; children; immune responses; influenza; humoral.

Influenza is responsible for 3 to 5 million cases of severe illness and approximately 250 000 to 500 000 deaths yearly [1]. In addition to the seasonal circulating strains, new pandemic strains arise occasionally, with 3 pandemics occurring in the 20th century [2], and most recently, the 2009 H1N1 pandemic [3]. In temperate regions where influenza has clear seasonality, most evident during the winter months, influenza viruses infect approximately 20% of the population. Of those infected, 75% represent subclinical infections [4]. In tropical and subtropical regions, however, influenza exposure is believed to occur more or less throughout the year [1, 5, 6]. The impact of influenza on

morbidity in these regions is less characterized and has previously been considered as insignificant [6], although studies from Hong Kong, another subtropical city, show that influenza is an important reason for hospitalization among children, with rates exceeding 10-fold those reported for temperate regions [7].

Severe clinical outcomes of influenza may be due to secondary bacterial infections; therefore, hospitalizations and deaths may not be attributed directly to influenza. In fact, influenza has been significantly associated with superinfections due to *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*, thereby leading to increased mortality [8–10]. Nasopharyngeal colonization with *S pneumoniae* in children with influenza is linked to invasive pneumococcal pneumonia [11], which may explain why pneumonia is the number one cause of death in children under 5 worldwide [12–14].

Antibody-mediated protection against influenza is conferred mainly through the dominant neutralizing epitopes, which are found in the major surface glycoprotein hemagglutinin. In contrast, cell-mediated immunity (CMI) is mainly directed against the conserved internal antigens of the virus and may thus be able to cross-protect against heterologous viral strains.

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Evidence supporting the role of CMI in influenza viral clearance and host survival is well documented in mouse models and increasingly so in humans [15–18]. Cross-subtype reactive CD8<sup>+</sup> T cell responses may clear virus infection even in people who lack virus-specific antibodies [19, 20].

CD4<sup>+</sup> T cells play an important role by providing help to B cells for antibody production and directing effector functions of CD8<sup>+</sup> T cells. In recent studies, it has been shown that CD4<sup>+</sup> T cells act not only by orchestrating the immune response, but also by directly killing infected cells. Furthermore, pre-existing influenza-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been associated with decreased viral shedding and reduced severity of illness [17, 18, 20].

In this study, we have analyzed both the humoral and cellular immunity to influenza in a cohort of healthy children under 5 years old and living in the Kolfe Kerano subcity of Addis Ababa in Ethiopia, an area with a subtropical climate. We found that a high percentage of children had been exposed to multiple influenza A and B strains, indicating that influenza is widespread in the area.

## METHODS

### Study Population

The study was approved by both the Regional Committee for Medical and Health Research Ethics South East, Norway (reference number 2012/2183) and AHRI/ALERT and National Health Research Ethics Review Committee of Ethiopia (reference numbers PO32/13 and 3.10/447/06). Written informed consent was obtained from the parents or next of kin of all participants. The children were recruited between March and September 2014. One hundred three children, aged 2–5 (23–60 months) years, were selected using a systematic sampling procedure from all the healthy children residing in Woreda 01, Kolfe Kerano subcity of Addis Ababa: chronically ill children or children who tested positive for human immunodeficiency virus were excluded. They were unvaccinated against influenza or *S pneumoniae*. At inclusion, a structured questionnaire, which collected information about weight, height, medical history, and socio-demographic parameters, was completed for each volunteer, and a blood sample was obtained.

### Sample Collection

Venous blood (up to 9 mL) was collected in acid citrate dextrose tubes (BD Vacutainer). Peripheral blood mononuclear cells (PBMC) ( $n = 95$  children with enough cells for analysis) and plasma ( $n = 103$ ) were isolated by Ficoll-Paque density gradient centrifugation (Ficoll-Paque Premium 1.077; GE Healthcare) using SepMate 50-mL tubes (Stemcell Technologies) following the manufacturer's instructions. Cells were cryopreserved at  $-150^{\circ}\text{C}$  in 25% fetal calf serum/10% dimethyl sulfoxide (DMSO)/65% AIM-V media (Gibco; Thermo Fisher Scientific, Waltham, MA), and plasma was stored at  $-80^{\circ}\text{C}$  until used.

### Peptides and Viruses

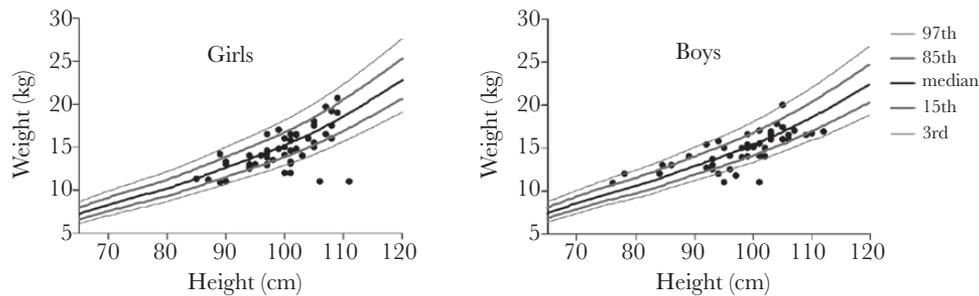
Peptides containing an optimal combination of human leukocyte antigen (HLA) class I- and II-restricted T cell epitopes from viral proteins conserved among influenza strains circulating between 1934 and 2009 were selected and chemically synthesized by Mimotopes (Clayton, Australia). The peptides were selected according to prevalence, conservancy, and HLA supertype coverage [21]. This approach circumvents the need for individual HLA typing. Influenza-specific CD4 and CD8 conserved peptides were pooled and used as antigen stimulants in interferon (IFN) $\gamma$  enzyme-linked immunospot (ELISpot) assays [21], in addition to stimulation with whole inactivated viruses (A/California/7/2009(H1N1)pdm09 and A/Brisbane/10/2007(H3N2) [22]. The inactivated influenza antigens A/California/7/2009(H1N1)pdm09, A/Perth/16/2009(H3N2), A/Victoria/361/2011-like(H3N2), B/Brisbane/60/2008, and B/Wisconsin/1/2010 (Influenza Reagent Resources, Centers for Disease Control and Prevention) were used in hemagglutination inhibition (HI) assays.

### Hemagglutination Inhibition Assay

All plasma samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) and heat inactivated before use. The HI assay was performed in duplicate for all RDE-treated plasma samples at the same time using 0.7% turkey red blood cells with 8 hemagglutinating units (HAU) of  $\beta$ -propiolactone-inactivated influenza A and ether-extracted influenza B strains, as previously described [23]. The HI antibody titer was determined as the reciprocal of the highest plasma dilution causing 50% inhibition of hemagglutination. Negative titers ( $<10$ ) were assigned a value of 5 for calculation purposes. Intermediate values represent geometric mean titers of repeated testing.

### Ex Vivo Interferon- $\gamma$ Enzyme-Linked Immunospot Assay

The influenza-specific IFN $\gamma$  T cell responses were measured by ex vivo ELISpot assay according to the manufacturer's instructions (CTL, Bonn, Germany) [21]. In short, 200 000 PBMC in AIM-V medium (Gibco) were added to each well in 96-well plates precoated with IFN $\gamma$  antibodies. Antigenic stimulants used were as follows: negative control (0.28% DMSO in AIM-V to match the final DMSO concentration in each of the epitope libraries), positive control (5  $\mu\text{g}/\text{mL}$  Concanavalin A), and CD4 and CD8 epitope libraries (2  $\mu\text{g}/\text{mL}$  final concentration per well of individual peptides in DMSO/AIM-V). In addition, 75 HAU of whole inactivated viruses (A/California/7/2009(H1N1)pdm09 and A/Brisbane/10/2007(H3N2)) were used as antigenic stimulants [22]. Plates were incubated for 20 hours at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  and developed the following day. The plates were read using a CTL S6 UltraV ImmunoSpot analyzer (Cellular Technology Limited, Shaker Heights, OH). Data were analyzed using Microsoft Excel and GraphPad Prism software (GraphPad Software, Inc.). Background values from



**Figure 1.** Study demographics. Weight-for-height percentiles in preschool (2–5 years old) children ( $n = 53$  girls and 50 boys), living in Addis Ababa, Ethiopia, using World Health Organization growth charts.

the negative control were subtracted from the values achieved after antigen stimulation. Two standard deviations (SD) above background were regarded as a positive response. Zero values were set to 1 for calculation purposes.

### Statistics

Statistical analyses were performed by the Mann-Whitney  $U$  test or linear regression using either GraphPad Prism version 5.04 or STATA version 13.  $P < .05$  was considered significant.

## RESULTS

### Study Population

Of the 103 children included, 53% were female. The average age was 51 months, 39 of whom were  $<49$  months old (only 2 children were  $<36$  months old), and 64 were  $\geq 49$  months of age. **Figure 1** shows the distribution of girls and boys according to the World Health Organization (WHO) standard weight-for-height growth curves for children 2–5 years old [24]. Most children fell within the normal range, but 7 of the children (4 girls, and 3 boys) were underweight, i.e., fell below the 3rd percentile, suggesting malnutrition.

### High Prevalence of Influenza Exposure in Preschool Children

Because no routine influenza surveillance occurs in Ethiopia, the influenza A and B strains expected to have circulated between 2009 and 2014 were selected from the annual WHO reports and vaccine recommendations [25]. Using results from the HI assay, we found that 86% of the children had positive responses ( $HI > 10$ ) to the A/Perth/16/2009(H3N2) strain, and 45% had positive responses to the A/H3N2/361/2011-like strain, whereas 50% were positive for the A/California/7/2009(H1N1) pdm09. With respect to the B strains, 37% were positive for B/Brisbane/60/2008, and 19% were positive for B/Wisconsin/1/2010 (**Figure 2**; Supplementary Table 1).

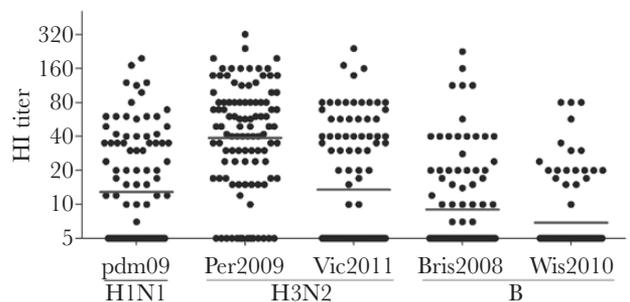
Ninety-six percent of the children had antibodies against influenza, and the number of influenza A and B strain exposures correlated with increasing age (**Figure 3**). Only 4% of the children lacked antibodies to any of the viral strains, and these children were among the younger ones. On the other hand, approximately 5% of the children displayed evidence

of a previous exposure to all 5 viruses tested. Children at or above 49 months of age were significantly more frequently exposed to the A/California/7/2009(H1N1)pdm09 and A/Victoria/361/2011-like(H3N2) strains of viruses than those below 49 months (Supplementary Table 1).

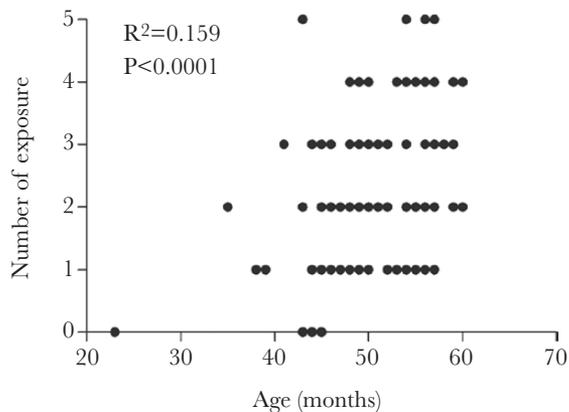
### Repeated Influenza Infections Boosts Cross-Reactive $CD4^+$ and $CD8^+$ T Cell Responses

Robust Th1 responses were observed after stimulation with the inactivated whole viruses, with 35% of the children displaying positive responses, i.e., 2 SD above background, to the H1N1 and 57% to the H3N2 viruses, respectively. A small but statistically significant increase in the mean  $IFN\gamma$  response towards the H3N2 virus was seen (**Figure 4A**).

Compared with whole virus stimulation, a considerable lower response was detected when using individual peptide pools as antigens (**Figure 4**). However, there was a positive correlation between T cell responses to peptide epitopes from conserved influenza antigens and whole virus used in the ELISpot assays. This correlation was present for both  $CD4^+$  and  $CD8^+$  T cell responses (data not shown). The number of  $CD4^+$  T cells recognizing internal antigens was slightly higher than



**Figure 2.** Humoral immune responses to influenza. Hemagglutination inhibition (HI) antibody titers to influenza A/H1N1/7/California/2009 (H1N1/pdm09), A/Perth/16/2009(H3N2) (H3N2/Per2009), A/H3N2/361/Victoria/2011-like (H3N2/Vic2011), B/Brisbane/60/2008 (B/Bris2009), and B/Wisconsin/1/2010 (B/Wis2010) viruses, in plasma from 103 preschool (2–5 years old) children living in Addis Ababa, Ethiopia. Hemagglutination inhibition values  $< 10$  were considered negative and assigned a value of 5 for calculation purposes. Horizontal lines represent geometric mean titers.



**Figure 3.** Exposure frequency to influenza increases with age. Number of influenza A and B virus exposures relative to age among 103 preschool children living in Addis Ababa, Ethiopia, measured as detectable hemagglutination inhibition antibody titers >10 to influenza A/California/7/2009(H1N1)pdm09, A/Perth/16/2009(H3N2), A/Victoria/361/2011-like(H3N2), B/Brisbane/60/2008, and B/Wisconsin/1/2010 viruses, with linear regression values.

those recognizing external antigens, although not significantly (Figure 4B). With respect to CD8 epitopes, the number of T cells recognizing internal and external antigens were approximately equal (Figure 4C).

The level of IFN $\gamma$  expressing T cells recognizing internal epitope sets increased with number of viral exposures (Figure 5A and B). After 1 exposure, the IFN $\gamma$  response to the internal and external CD4 epitope sets were equal, but after 3 exposures, the response to the internal, conserved epitope sets were significantly boosted above the response level detected with the external epitope set (Figure 5A). On the other hand, elevated CD8 $^+$  T cell responses were found for both internal and external antigens after 3 exposures, but this difference was significant only for external antigens.

Children who were underweight had immune responses in the same range as in the normal weight children. It is interesting to note that the underweight children had CMI responses against both H1 and H3 viruses (Supplementary Figure 1B and

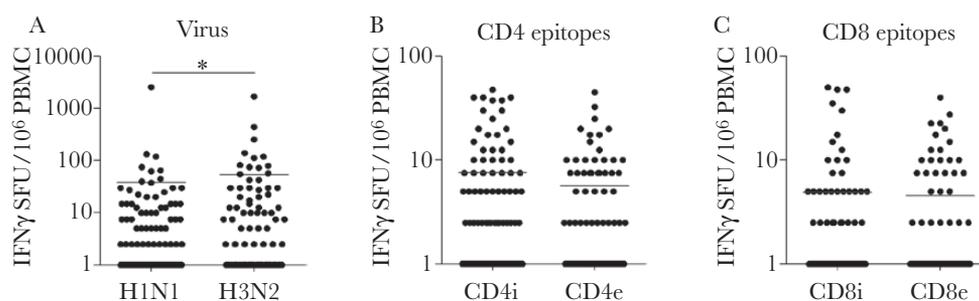
D) but only humoral responses against the H3 virus (except for 1) (Supplementary Figure 1A and C), indicating that these children were previously exposed only to an H3 virus. We found no correlation between humoral (HI titer) and cellular immune responses (IFN $\gamma$  ELISpot) in the children.

## DISCUSSION

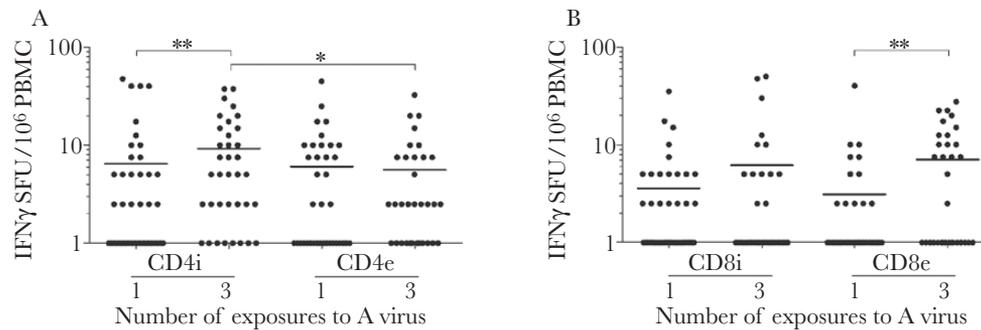
In this study, we examined immune responses to influenza viruses in healthy preschool children living in a city with a subtropical climate, allowing insight into the range of influenza incidence in the area and the associated disease burden it may cause. We found that almost all of the children in our study had antibodies against at least 1 influenza strain, which may not be completely unexpected, when compared with the seasonal infection rate of 20% in temperate regions [4]. However, the fact that 67% of the children had antibodies specific to 2–4 different influenza virus isolates, and 5% had antibodies to 5 different influenza strains, was surprising. Almost all of the children in our study were infected during the 2-year period between the age of 3 and 5. A large proportion of these children were infected with more than 1 influenza strain annually.

Eighty-six percent of the children tested positive for influenza A/H3N2/16/Perth/2009; this was equally frequent among the older and younger children, and it is therefore likely that this strain had circulated throughout the whole period during our study. On the other hand, 64% of the older children had antibodies to A/California/7/2009(H1N1)pdm09, compared with 28% of younger children, indicating that this strain was more prevalent during previous years. A similar pattern was observed with the A/H3N2/316/Victoria/2011-like and the B strains.

Correspondingly, 57% of the children developed influenza-specific IFN $\gamma$ -secreting T cell responses to the H3N2 virus, as opposed to only 35% towards the H1N1 virus. Moreover, these children had cross-subtype CD4 $^+$  and CD8 $^+$  T cell responses to antigens that are highly conserved among influenza A strains, a finding that has recently been shown to correlate with protection against symptomatic and culture confirmed influenza [17,



**Figure 4.** Cellular immune responses to influenza. Cellular immune responses, measured as spot-forming units (SFU), indicating the number of interferon (IFN) $\gamma$ -producing cells per million peripheral blood mononuclear cells (PBMC) from 95 preschool (2–5 years old) children living in Addis Ababa, Ethiopia, after stimulation with (A) whole inactivated influenza A/California/7/2009(H1N1)pdm09 or A/Brisbane/10/2007(H3N2) viruses or with (B) influenza-specific CD4 and (C) CD8 epitopes, derived from internal (i) or external (e) antigens. Zero values were set to 1. Horizontal lines represent mean values. Statistical significance was determined by Mann-Whitney *U* test; \**P* < .05.



**Figure 5.** Cellular immune responses are boosted with exposures. Cellular immune responses, measured as spot-forming units (SFU), indicating the number of interferon (IFN) $\gamma$ -producing cells in peripheral blood mononuclear cells (PBMC) from 95 preschool (2–5 years old) children living in Addis Ababa, Ethiopia, after stimulation with (A) influenza-specific CD4 and (B) CD8 epitopes, derived from internal (i) or external (e) antigens. The results were grouped according to the number of previous influenza A virus exposures, measured as hemagglutination inhibition antibody titers  $\geq 10$ . Zero values were set to 1. Horizontal lines represent mean values. Statistical significance was determined by Mann-Whitney *U* test; \**P* < .05; \*\**P* < .01.

20, 26]. Even low numbers of spot-forming units, as a measure of IFN $\gamma$ -secreting T cell responses, have been linked to reduced viral shedding in symptomatic polymerase chain reaction-confirmed influenza disease [18].

In contrast to the antibody responses that are highly strain specific, repeated exposures to several influenza A strains resulted in boosting of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses. This is due to the nature of the conserved antigens among different influenza strains, which may additionally boost memory T cells specific for previously encountered strains. It is in accordance with previous work showing that children develop virus-specific CD8<sup>+</sup> T cell responses after infection, and boosting increases with age, likely correlating to the number of influenza virus exposures experienced [27, 28]. As shown previously [29], repeated exposures skewed the CD4<sup>+</sup> responses from external towards internal antigens, suggesting maturation of immune responses known to correlate with protection [17, 20]. However, the CD8<sup>+</sup> responses measured in this study were not found to be skewed in this fashion, as repeated exposures increased responses against the external antigens. This difference may be explained by the formulation of the CD8e peptide pool, because it consists of only 3 peptides with 1 originating from the M2 extracellular region of the virus, a region highly conserved among influenza A viruses [30].

Among the underweight children in our study, only 1 had an HI titer against the A/California/7/2009(H1N1)pdm09 strain. Nevertheless, these same children reacted with T cell responses after stimulation with an H1N1 virus, a finding indicating previous infection with a different influenza A strain capable of inducing cross-subtype immune responses. In fact, 6 of 7 children produced antibodies specific for a H3N2 strain. Thus, even the underweight children had the capacity of reacting with antibodies as well as T cell responses against influenza.

## CONCLUSIONS

Our unexpected finding, that several strains of influenza viruses may have infected the same preschool children living in a subtropical area of Ethiopia, is of major concern with regard to general health. Next-generation influenza vaccines, aimed at controlling the spread of viruses and reducing the severity of infections, should ideally be capable of inducing cross-protective immunity.

## Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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