

Heterogeneity in Diphtheria-Tetanus-Acellular Pertussis Vaccine-Specific Cellular Immunity during Infancy: Relationship to Variations in the Kinetics of Postnatal Maturation of Systemic Th1 Function

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Cellular immunity to vaccines is highly variable during infancy. This study addressed the hypothesis that these responses are governed by the pace of maturational changes in adaptive immune competence, in particular, cellular functions that underlie the postnatal transition from Th2 to Th1 "bias." Tetanus-specific cytokine responses were tracked in peripheral blood mononuclear cells collected from infants at months 2, 4, 6, 12, and 18. These were compared with polyclonal responses. Results show that the Th2 component of the vaccine response develops rapidly and remains stable, unlike interferon (IFN)- γ production, which also is initiated early but commonly declines after the final priming dose at 6 months. However, between 12 and 18 months, the IFN- γ component of the vaccine-specific response has a spontaneous resurgence that coincides with a parallel increase in overall IFN- γ production capacity. The Th2 component of vaccine-specific responses was more prominent in children with atopic family history.

Reduced infant mortality and morbidity resulting from mass vaccination against infectious diseases represent one of the most significant achievements of modern medicine, and efforts continue worldwide to broaden the spectrum of available vaccines and to improve their efficiency, particularly in the very young. The long-term goal of much of this research is the development of multivalent vaccines that will provide long-lasting protection against the widest possible range of pathogens from as early as possible during infancy. While issues related to vaccine formulation are central to this development process, it is also important to gain a clearer understanding of how the infant immune system responds to parenteral vaccination and, in particular, to identify any developmental-related deficiencies in these responses that potentially limit their efficiency in host protection.

Until comparatively recently, interest in this question focused principally on humoral aspects of the host response. Specific IgG antibody titers currently represent the only accepted *in vitro* correlates of vaccine immunity that enable discrimination between responders and nonresponders. However, it is becoming increasingly evident that humoral responsiveness does not necessarily

equate directly with protection, given that vaccines such as acellular pertussis appear to provide relatively long-lasting immunity in children, despite the early waning of serum antibody titers [1–3]. Accordingly, attention is shifting toward cellular immune responses to vaccines in childhood [4–9], particularly in view of the results in animal models that show the important contributions of Th1 and Th2 cytokines to vaccine responses in the neonatal period [10]. Other studies also have shown the apparent capacity of cellular immunity to protect against infection with organisms such as *Bordetella pertussis*, even in the absence of antibody [11, 12]; however, a covert contribution from B memory cells could not be ruled out in these models.

Recent studies indicate that, analogous to findings in experimental animals, the induction and expression of cellular immunity in human infants differ both quantitatively and qualitatively from those in adults (reviewed in [13–15]), and full functional competence is commonly not attained until the end of the preschool years. The contributions of cytokines such as interleukin (IL)-5 and -13 to vaccine responses in infants are relatively more prominent than at later ages [16–18], reflecting the overall "Th2 bias" that is characteristic of the neonatal immune system [19].

T cell cytokine responses during this early life period also appear to be highly variable and are frequently transient. These variations within the pediatric population may be significant etiologic factors in certain diseases. A notable example is atopy, in which high risk for disease development has been linked to delayed postnatal maturation of Th1 function, in particular, the capacity to secrete the cytokine interferon (IFN)- γ [20, 21].

The development of cellular immunity to infant vaccines has

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not been systematically investigated in this context. However, from recent reports it is clear that T cell responses to tetanus and/or pertussis vaccination are highly variable among infants [17, 18], and the basis for this heterogeneity is unknown. In this study, we addressed this issue, focusing on the relationship between age-related changes in systemic Th1 and Th2 cytokine function during infancy and the maturation of specific cellular immune responses to a vaccine antigen. We report on the post-natal development of Th cell responses to the tetanus toxoid (TT) component of the diphtheria-tetanus-acellular pertussis (DTaP) vaccine in a cohort of 132 infants aged 2–18 months.

Materials, Subjects, and Methods

DTaP vaccine. The DTaP vaccine (Infanrix; SmithKline Beecham) used in this study contained 25 Lf of diphtheria toxoid, 10 Lf of TT, 25 µg of pertussis toxoid, 25 µg of filamentous hemagglutinin, and 8 µg of pertactin adsorbed on 0.5 mg of aluminum (aluminum hydroxide).

Subjects. We recruited 132 healthy 2-month-old infants for this study, who were classified as having a positive atopic family history ($n = 83$; AFH⁺) or negative atopic family history ($n = 49$; AFH[−]) on the basis of a standardized questionnaire answered by the parent. AFH⁺ was defined as having ≥ 1 first-degree relative with a positive allergy history [22].

At ages 2, 4, and 6 months, the infants received DTaP in addition to oral polio (SmithKline Beecham) and intramuscular *Haemophilus influenzae* b (HibTITER; Wyeth-Lederle) vaccines. A measles-mumps-rubella vaccine (Merck Sharp & Dohme) was administered at age 12 months; hepatitis B vaccine (Engerix-B; SmithKline Beecham) was given at ages 12 and 15 months at parent request. Peripheral blood was obtained before immunization at 2, 4, 6, 12, and 18 months; samples were obtained from $\sim 77\%$ of the group on each occasion. Blood was collected into an equal volume of RPMI 1640 (Cytosystems) containing preservative-free heparin. As described elsewhere [23], peripheral blood mononuclear cells (PBMC) were isolated and were cryopreserved at collection for subsequent batch analysis.

Cell preparation and culture. As described elsewhere [17], PBMC that were cryopreserved at collection were batch analyzed in groups of 10 within a short period and with identical reagents. Cryopreserved PBMC were thawed and were resuspended at 1×10^6 viable cells/mL in either RPMI 1640 supplemented with 5% pooled human AB serum (for cultures with TT or respiratory syncytial virus [RSV] F/G protein), or AIM-V serum-free medium (Gibco Life Technologies) supplemented with 2-mercaptoethanol (4×10^{-5} M final concentration; Sigma) for cultures with phytohemagglutinin (PHA). Aliquots of 0.5–1.0 mL from each child were cultured for 48 h at 37°C with 5% CO₂, as follows: medium alone or medium containing TT (0.5 Lf/mL; CSL), PHA (HA16; 1 µg/mL; Murex), or RSV F/G protein (1 µg/mL; SmithKline Beecham). After culture, the cells were collected by centrifugation and were used immediately for RNA extraction. Supernatants were stored at -20°C for ELISAs. Previous studies from our laboratory [23, 24] and elsewhere [1] have shown that cryopreservation and thawing do not distort PBMC cellular immune responses.

Semiquantitative reverse transcriptase-polymerase chain reaction

(RT-PCR) detection of cytokine-specific mRNA. Total RNA from the cell pellets was obtained by using RNeasy B extracting solution (Bresatec), as described elsewhere [25]. cDNA was transcribed in a total volume of 25 µL at 42°C, using 250 ng of Oligo (dT) 15 (Biotech International) and 4.5 U of avian myeloblastosis virus RT (Promega) in the presence of 10 U of ribonuclease inhibitor (RNaseOUT; Biotech). cDNA was amplified for β -actin, IL-4, and IL-9. The PCR reaction mixture contained 1 µL of cDNA, 50 ng of the specific primers (Gibco), $1 \times$ PCR reaction buffer (Gibco), each dNTP (Biotech) at 0.2 mM, 1.5 mM MgCl₂ (Gibco), and 0.5 U of platinum Taq DNA polymerase (Gibco) in a total volume of 12.5 µL overlaid with mineral oil.

The PCR was done as follows, with an initial denaturation step of 94°C for 3 min: denaturation at 94°C for 1 min, annealing at the respective temperatures for 1 min, and extension at 72°C for 1 min. All reactions were done in a programmable thermocycler (Perkin-Elmer). A positive cDNA control was always amplified in parallel in all PCRs. As described elsewhere [17], the sequences for the primers were as follows for primer sequence (5'→3'), annealing temperature, and product size: β -actin forward (F) and β -actin reverse (R), CGT GAC ATT AAG GAG AAG CTG TGC and CCT AGG AGG AGC AAT GAT CTT GAT, 58°C, 375 bp; IL-4 F and IL-4 R, CAA GTG CGA TAT CAC CTT ACA GG and CCT TCA CAG GAC AGG AAT TCA AGC, 58°C, 306 bp; and IL-9 F and IL-9 R, GGG ATC CTG GAC ATC AAC TT and CAG AAG ACT CTT CAG AAA TG, 56°C, 307 bp.

Rigorous cycle analyses were performed with each primer set, to ensure that we had reached detection levels and that the reaction remained in the linear phase (30 cycles for β -actin, 43 cycles for IL-4, and 40 cycles for IL-9). In each case, PCR products of the expected size were obtained, as verified by analysis in 1.5% agarose gel and staining with ethidium bromide of a subset of samples and a positive control.

Slot blot, hybridization, and detection. We analyzed the PCR products by use of slot blot (Hoefer Scientific Instruments), with a modification of methods described elsewhere [25]. In brief, double-stranded probes were produced by PCR reactions by using biotin-16-dUTP (Boehringer Mannheim) at a ratio of 5:1. The template for probe synthesis was cDNA obtained from adult PBMC stimulated with PHA (1 µg/mL) for 24 h at 37°C. After overnight hybridization with biotinylated probes, the binding was visualized by chemiluminescence, using a commercial kit (ECL; Amersham) according to the manufacturer's instructions. The membranes were exposed to hyperfilm (Amersham), and the intensity of each dot was determined by densitometer (Scan Analysis 2.02; Biosoft). We expressed the results as a ratio of cytokine to β -actin density.

ELISAs for detection of cytokine protein. The levels of IL-13 and IFN- γ in the culture supernatants were determined by commercial ELISA kits (PeliKine Compact; CLB). The sensitivity of the assay was 3 pg/mL for IL-13 and 4 pg/mL for IFN- γ . IL-5 protein was measured by an in-house ELISA, using rat IgG1 anti-human IL-5 monoclonal antibody (clone TRFK5; PharMingen) for capture and biotinylated rat IgG2a anti-IL-5 monoclonal antibody (clone JES1-5 A10; PharMingen) for detection. The standard curve was generated by using serial dilutions of recombinant human IL-5 (PharMingen). The limit of detection was 6 pg/mL.

Statistical analysis. Cytokine responses induced by TT and PHA were analyzed by the Wilcoxon matched pairs signed-rank test for

paired responses. Differences between TT- or PHA-stimulated cytokine production for 2 groups of subjects (i.e., those with a positive cellular response vs. those with a negative cellular response to RSV) were determined by the Mann-Whitney *U* test for unpaired responses. The significance of correlations was analyzed by Fisher's *r*-to-*z* transformation with the StatView 5.0.1 software package.

Results

Vaccine-specific cytokine responses. Figure 1 illustrates in vitro responses to the TT component of the DTaP vaccine. Responses in blood at 2 months, before vaccination, were not present or were extremely low. At the 4-month bleed, 2 months after the initial priming dose, significant responses were detected for IL-9 and IFN- γ , and especially for IL-4, by the sensitive semi-quantitative RT-PCR technique, and by 6 months, IL-5, IL-13, and IFN- γ protein were detectable in about half the study population. TT-specific production of all Th2 cytokines (IL-4, -5, -9, and -13) remained at or above levels recorded at 6 months through the final observation at 18 months. The IFN- γ protein component of the response was unique in that mean production levels among the positive responders and the frequency of responders within the overall population declined between 6 and 12 months ($P < .002$ for difference in response frequency between 6 and 12 months, χ^2 test), followed by a resurgence at 18 months ($P < .001$ for difference in response frequency between 12 and 18 months, χ^2 test). A similar biphasic pattern of IFN- γ production was observed when the analyses were restricted to subjects from whom samples were available at all 3 time points.

As shown in figure 1, the maximum response frequency for any cytokine up to age 18 months was 63% of the population (see IL-4). However, this underestimates overall cellular immune responsiveness within the vaccinated population, as shown in figure 2. In this analysis, the presence of ≥ 1 positive cytokine response to TT, with a 2-fold increase above background (unstimulated culture) used as a cutoff, was considered to be indicative of underlying cellular immunity at each observation point. By this criterion, >80% of the study population were responsive from 6 months onward.

We examined the relationship between vaccine-specific Th1 and Th2 cytokine production throughout the study period, focusing on the cytokines measured as protein. Relevant correlation coefficients were determined and were tested for significance by using Fisher's *r*-to-*z* transformation. Highly significant correlations ($P < .001$ – $P < .004$) were observed between the Th2 cytokines IL-5 and IL-13 from 4 months onward. In contrast, production of these cytokines correlated weakly or not at all with IFN- γ (data not shown).

Cytokine responses to viral infection. At the 6-month time point, a transient peak of responsiveness to RSV was observed in the study population, consistent with the seasonal nature of the infection. PBMC from 51% of the subjects showed evidence of cellular immunity to RSV, as demonstrated by production of ≥ 1 cytokine after culture with RSV F/G antigen. Half of

this group displayed RSV-specific IFN- γ responses (mean $\pm$ SE, 22.0 ± 8.2 pg/mL of culture fluid), while the rest responded via low-level production of ≥ 1 Th2 cytokine in the absence of IFN- γ . The most prominent were IL-5 (7.3 ± 3.8 pg/mL) and IL-13 (6.0 ± 7.1 pg/mL). Cytokine responses (including IFN- γ) in all but 2 of these subjects had disappeared by 12 months.

Systemic (polyclonal) cytokine responses. The experiments shown in figure 3 examined the kinetics of postnatal development of capacity to secrete the Th2 cytokines IL-5 and IL-13 versus the Th1 cytokine IFN- γ in response to the polyclonal T cell mitogen PHA. The Th2 cytokines had an upsurge in production after the 4-month blood sample; overall production levels peaked at age 12 months. In contrast, postnatal development of the capacity to secrete the Th1 cytokine IFN- γ remained stable at levels equivalent to those of cord blood cells (data not shown) up to age 12 months, before a late upsurge at 18 months.

Vaccine-specific versus systemic Th1 function. We observed that the upsurge in IFN- γ production in response to PHA stimulation beyond the 12-month observation point, shown in figure 3, was not universal throughout the study population. Having observed that an upsurge in TT-specific IFN- γ production also occurred in a subset of the population (figure 1), we accordingly questioned whether these increases were related. We therefore segregated the study population shown in figure 1 into those who showed a resurgence of TT-specific IFN- γ responsiveness between 12 and 18 months versus those who did not and asked what had occurred with respect to their overall (polyclonal) capacity to produce IFN- γ over the same period (data from figure 3). As illustrated in figure 4, a resurgence in TT-specific IFN- γ responses between 12 and 18 months was strongly correlated with a parallel increase in overall (polyclonal) IFN- γ production capacity during this period, presumably reflecting systemic maturation of this latter function.

Th1/Th2 polarization of vaccine responses and atopy. As noted in the introductory section, slow postnatal development of IFN- γ production capacity is linked to genetic risk for atopy, a syndrome that affects up to 40% of the pediatric population. The maturation deficiency in Th1 function in these subjects is associated with a bias of their immune responses to environmental allergens toward the Th2 cytokine phenotype. We therefore questioned whether children at risk of atopy would display a similar bias in their responses to the vaccine.

We used standardized techniques to identify subjects at high risk of atopy development on the basis of positive atopic family history (≥ 1 first-degree relative) versus those at low risk (no family history). We then compared the Th1/Th2 balance within the polyclonal and TT-specific in vitro responses, expressed as ratios of IFN- γ to IL-5 and to IL-13 (table 1). The relative contribution of the Th1 component in the vaccine responses was consistently higher at 6 and 12 months among the group with no atopic family history; this difference had disappeared by 18 months, as the response matured.

Postnatal maturation of IFN- γ function and susceptibility to

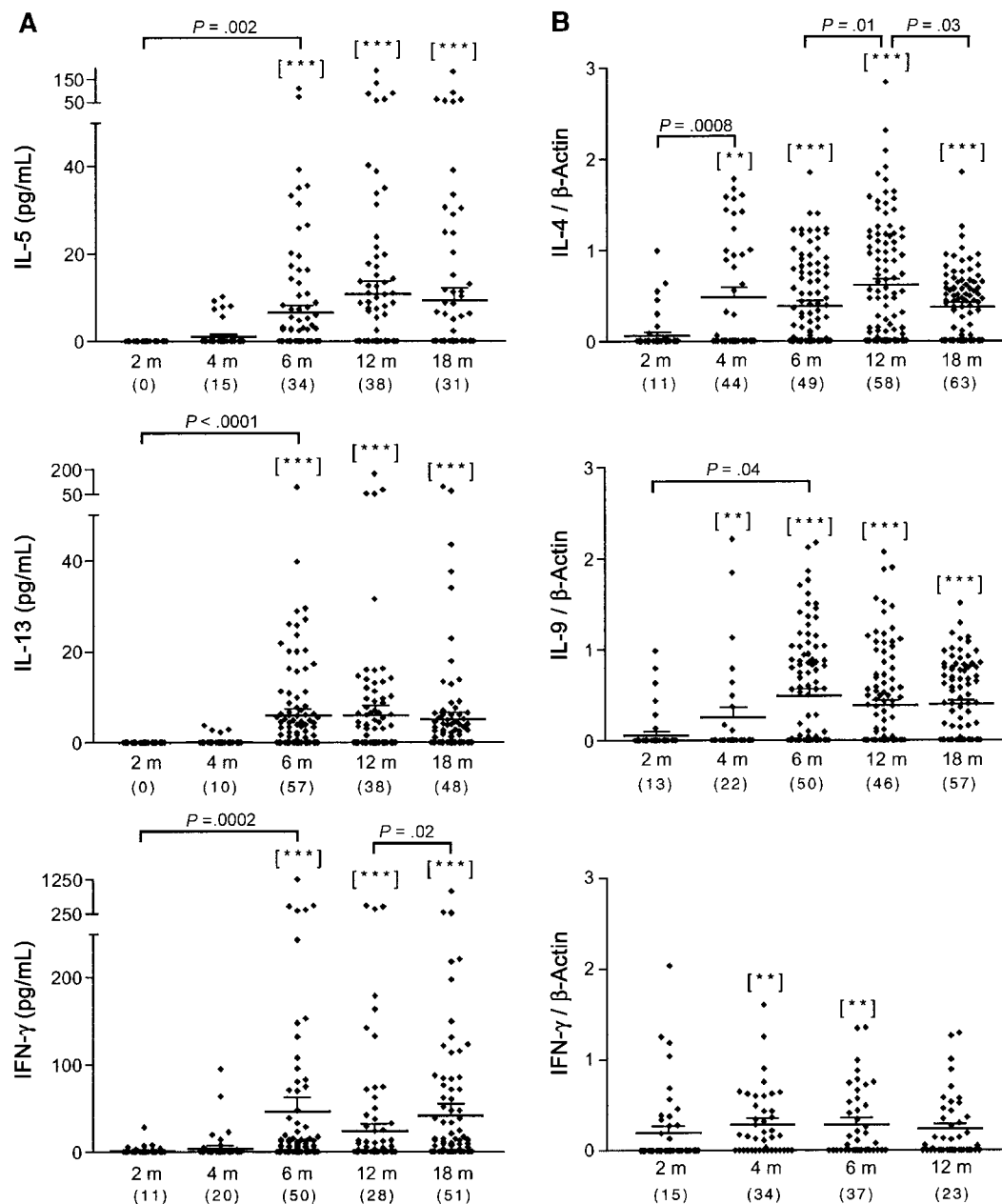


Figure 1. Vaccine-specific cytokine production by peripheral blood mononuclear cells (PBMC). PBMC from infants at ages 2, 4, 6, 12, and 18 months (m) were cultured for 48 h alone or with tetanus toxoid (TT; 0.5 Lf/mL). In panel *A*, supernatant protein content was determined by ELISA (data expressed as pg/mL). In panel *B*, total RNA was extracted, transcribed into cDNA, and amplified by using primers for the respective cytokines. Polymerase chain reaction products were analyzed by slot blot. Data are expressed as arbitrary activity units after normalizing the scanned area value for each slot blot against the respective value for β -actin in the same culture. *A* and *B*, Data are expressed as scatter plots comprising Δ values (treatment – control) for each subject. Solid bar, mean + SE for the whole population ($n = 50$ for each age group for detection of interferon [IFN]- γ mRNA). Values in parentheses are percentages of infants positively responding to TT (positive cytokine response = 2-fold increase above control production); nonresponders are tightly clustered on the *X*-axis. Significant differences were determined by the Wilcoxon matched pairs signed-rank test for paired responses. Significant differences between TT-stimulated and control cultures for each subject are indicated by asterisks (* $P < .05$; ** $P < .01$; *** $P < .0001$). *P* values shown on the figure represent significant differences between group mean TT response at different ages. IL, interleukin.

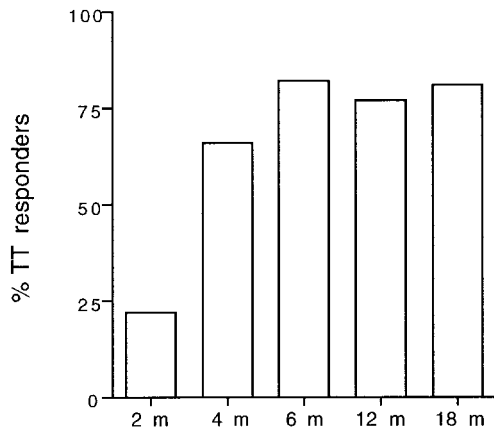


Figure 2. Percentages of children with positive cellular responses (for each age group) to tetanus toxoid (TT), as shown in figure 1, were analyzed, using ≥ 1 positive cytokine response (2-fold increase above control production) as the cutoff. m, month.

viral infection. We then asked whether an additional consequence of slow postnatal maturation of Th1 function during early infancy may be increased susceptibility to viral infection. RSV is one of the most common infections during infancy and is a major cause of hospitalization during winter in Perth. No direct virus isolation data for individual subjects in the study cohort were available to us. As an alternative, we took the expression of positive cellular immunity to RSV by individual study subjects (as defined by *in vitro* production of ≥ 1 cytokine by PBMC in response to RSV F/G protein) as a surrogate marker of clinical or subclinical infection. Focusing on the 6-month sampling point, by which time all subjects would have experienced 1 RSV "season," we segregated the subjects into those who demonstrated positive or negative RSV responses and replotted the data from figure 3 for the 2 subgroups. As shown in figure 5, priming for positive cellular immunity to RSV was most frequent in the subgroup in whom Th1 function failed to display evidence of significant postnatal maturation.

Discussion

Cytokines secreted as part of the immune response to pathogens play a key role in host defense and also represent a potential source of tissue damage if their production is not sufficiently well controlled. An important example of the latter can be seen in the consequences of infection-induced release of the Th1 cytokine IFN- γ at the fetomaternal interface, which can result in placental detachment [19, 26]. To minimize the frequency of such occurrences, and also to guard against the sequelae of induction of 1- or 2-way mixed lymphocyte reactions in this microenvironment as a result of the major histocompatibility complex disparity between mother and fetus, a series of regulatory mechanisms have developed, to selectively dampen Th1 functions in the fetal compartment [19]. Studies

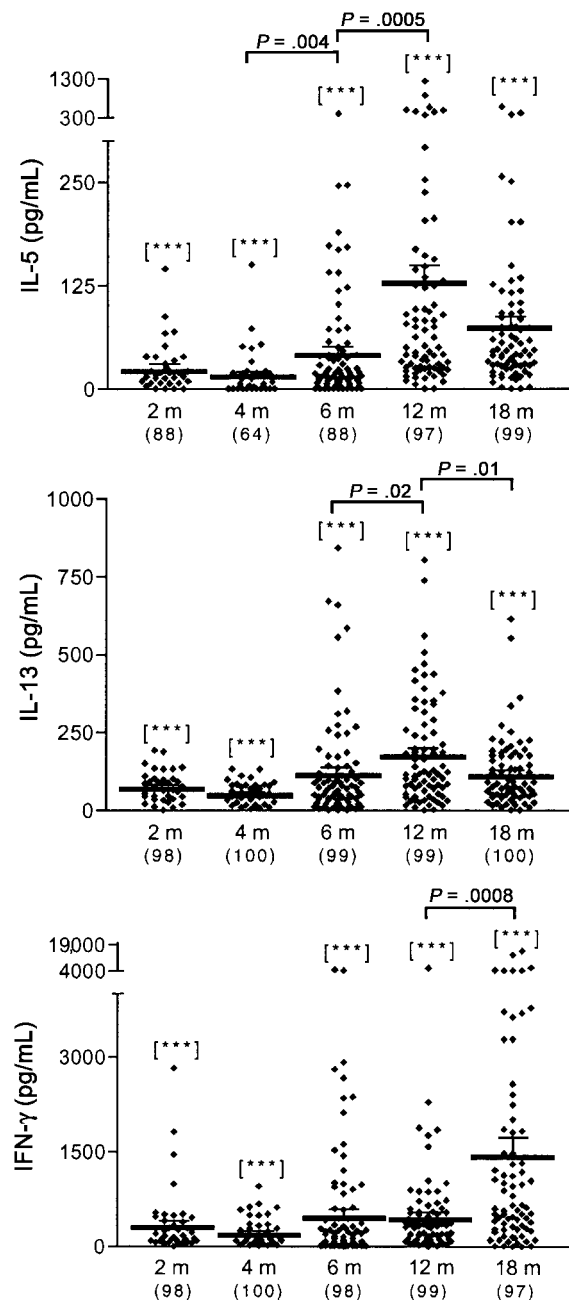


Figure 3. Polyclonal cytokine production by peripheral blood mononuclear cells collected from infants at ages 2, 4, 6, 12, and 18 months (m) and cultured for 48 h alone or with phytohemagglutinin (PHA; $1 \mu\text{g/mL}$). Supernatant protein content was determined by ELISA; Δ values (treatment – control) are given for each child. Solid bars, mean $\pm$ SE of the whole population. Values in parentheses are percentages of infants with a positive cytokine response (2-fold increase above control production) to PHA. Significant differences were determined by the Wilcoxon matched pairs signed-rank test for paired responses. Asterisks indicate significant differences between PHA-stimulated and control cultures for each child ($*P < .05$; $**P < .01$; $***P < .0001$). P values shown on the figure represent significant differences between group mean PHA responses at different ages. IFN, interferon; IL, interleukin.

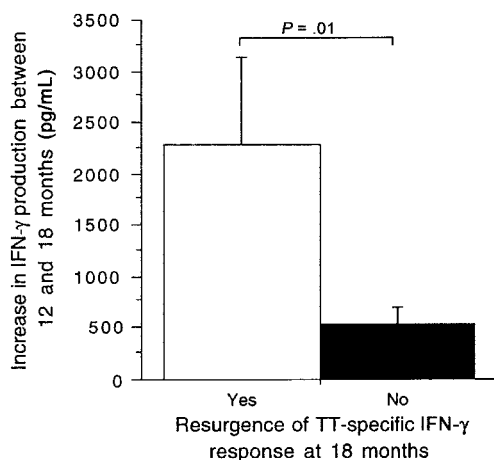


Figure 4. Vaccine-specific vs. polyclonal Th1 function at age 18 months. As shown in figures 1 and 3, peripheral blood mononuclear cells were cultured for 48 h alone or with tetanus toxoid (TT; 0.5 Lf/mL) or phytohemagglutinin (PHA; 1 μ g/mL). Supernatant protein content was determined by ELISA. Infant data were segregated between those with a resurgence of TT-specific interferon (IFN)- γ responsiveness between 12 and 18 months (resurgence = TT-specific IFN- γ levels at 18 months ≥ 2 times those at 12 months) vs. those with no resurgence. The magnitude of the difference between the groups in PHA-stimulated IFN- γ production between 12 and 18 months was determined. The significance of the difference between the 2 groups was assessed by the Mann-Whitney *U* test for unpaired responses.

in mice indicate that the effects of these (largely uncharacterized) mechanisms persist for variable periods postnatally, and, as a result, T cell responses during infancy are commonly Th2 biased [27–29].

The situation in humans is less well understood, but it is evident that similar general mechanisms are operative. In particular, capacity to produce the Th1 cytokine IFN- γ in response to polyclonal T cell stimulation is diminished in human infants [20, 30, 31]. However, limited information is available on the Th1/Th2 balance in vaccine-specific responses during infancy, aside from small studies on the acute response to the DTaP vaccine directly after completion of the initial priming schedule [5, 9]. We recently tracked DTaP responses up to age 12 months [17]. The present report extends the latter study in an expanded group of subjects followed to age 18 months (i.e., 1 year after completion of the 2-month/4-month/6-month DTaP priming schedule).

As illustrated in figure 1, TT-specific cytokine responses appear rapidly after the commencement of priming. Whereas the Th2 component of these responses persisted over the 1-year observation period after completion of priming at 6 months, the IFN- γ component waned after priming. This disparity in the apparent stability of the Th1 versus Th2 components of vaccine response in human infants, which is similar to that reported in mice [10, 32], suggests that the generation of Th1 memory after neonatal priming is delayed relative to Th2 memory.

This difference between the Th1 and Th2 cytokine com-

partments was also evident when overall (polyclonal) cytokine production capacity was tracked. As shown in figure 3, Th2 cytokine production (IL-5 and IL-13) peaks at age 12 months and then declines, and this decline continues at least to age 6 years [16]. In contrast, IFN- γ production remains at the low levels characteristic of neonates through age 12 months. The decline in vaccine-specific IFN- γ responses between 6 and 12 months suggests that ≥ 1 developmental deficiencies in the Th1 compartment serve to limit the maturation of IFN- γ -producing effector cells into long-lived memory cells and/or limit the level of activation they can attain during *in vitro* challenge.

Any explanation for these age-related changes in IFN- γ production must also account for the late and apparently vaccination-independent response to TT shown in figures 1 and 4, which is similar to that reported recently by Ausiello et al. [1] in relation to pertussis-specific responses. It is pertinent to note in this context the results of earlier investigations of postnatal maturation of polyclonal IFN- γ -producing capacity in T cells from infants. In particular, studies on PBMC [30, 31] and on isolated T cell clones [20] have demonstrated that, although part of the maturational deficiency in PHA-induced IFN- γ secretion is intrinsic to the responding T cells, a significant part is due to a parallel deficiency in the function(s) of accessory cells that provide the necessary costimulation signals required for complete T cell activation [30, 31].

A substantial proportion of the peripheral CD45RA⁺ T cell compartments in infants are functionally immature relative to their adult counterparts [13–15]. With increasing age, these are progressively replaced with functionally mature naive T cells [14]. Given that no further TT-specific antigen stimulation was provided in this study beyond the final 6-month priming dose, it seems unlikely that the late upswing in TT-specific responses between ages 12 and 18 months is due to the recruitment of more mature naive T cells into the antigen-specific response.

Table 1. Polyclonal and tetanus toxoid (TT)-specific Th1/Th2 balance in infants with ≥ 1 first-degree relative with a positive allergy history (positive atopic family history; AFH⁺) or with none (negative atopic family history; AFH⁻).

Ratio	6 months		12 months		18 months	
	AFH ⁻	AFH ⁺	AFH ⁻	AFH ⁺	AFH ⁻	AFH ⁺
TT						
IFN- γ :IL-5	4.7	2.0 ^a	2.5	0.4 ^a	2.3	3.8 ^b
IFN- γ :IL-13	6.6	1.6 ^c	5.9	0.8 ^c	2.5	7.9 ^b
PHA						
IFN- γ :IL-5	11.4	6.3 ^b	4.9	4.2 ^b	14.4	13.9 ^b
IFN- γ :IL-13	2.2	2.6 ^b	1.9	2.4 ^b	9.6	7.0 ^b

NOTE. Data are median values by infant age. Ratios of interferon (IFN)- γ :interleukin (IL)-5 and IFN- γ :IL-13 were calculated for each infant. Differences between AFH⁻ and AFH⁺ groups were assessed for significance by using the Mann-Whitney *U* test for unpaired responses. Individuals in whom both IFN- γ :IL-5 and IFN- γ :IL-13 cytokine production were undetectable were not included. PHA, phytohemagglutinin.

^a *P* < .05.

^b Not significant.

^c *P* < .01.

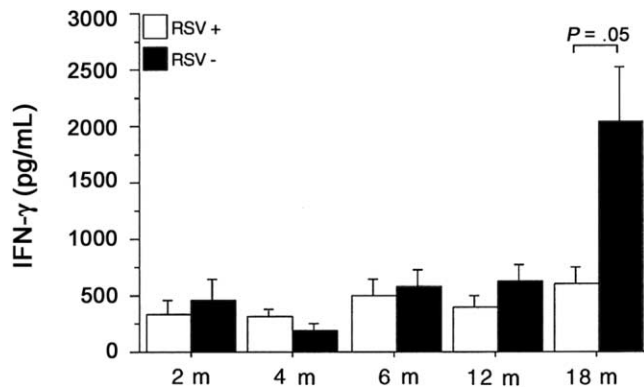


Figure 5. Responsiveness to respiratory syncytial virus (RSV) F/G protein and postnatal maturation of interferon (IFN)- γ function. Peripheral blood mononuclear cells collected at 6 months (m), when all subjects would have experienced only 1 RSV season, were cultured for 48 h alone or together with RSV F/G protein (1 μ g/mL). Infants were classified as having a positive cellular response to RSV if ≥ 1 positive cytokine response to RSV F/G protein was detected in vitro; unresponsive subjects (solid bars) produced no detectable cytokines. Data from figure 3 were then replotted for these 2 groups. For each group, data are mean + SE. The significance of differences between the groups was determined by the Mann-Whitney *U* test for unpaired responses.

As noted, a similar delayed response in pertussis-specific cellular immunity in recipients of DTaP vaccine has been attributed to boosting via asymptomatic infection with *B. pertussis*. Although this explanation is plausible, given the extent of *B. pertussis* carriage in the community, it appears less likely in relation to TT-specific immunity, although it may be possible that some degree of boosting occurs via environmental antigens that cross-react with TT.

Two additional possibilities are as follows. First, the capacity to detect Th memory cells is dependent on their presence in sufficient numbers in the peripheral circulation. It is possible that the trafficking properties of Th1 and Th2 cells per se differ at this age; for example, Th1 memory cells in infants may recirculate less efficiently than their Th2 counterparts. Although there is no direct evidence to support this concept, recent studies suggest that trafficking of Th1 and Th2 cells may be independently regulated [33]. A second and, in our view, more likely explanation is that other cell types that participate in Th1 cell activation are involved. Evidence from a number of laboratories has established that accessory cell functions [34], especially those associated with expression of Th1 immunity [35], are deficient during the neonatal period and mature slowly during infancy. In human infants, if peripheral blood T cells are provided with a source of fully functional accessory cells (maternal monocytes), their IFN- γ responses to polyclonal mitogen can be boosted to approximate those of adults [30, 31].

In relation to the findings shown in figure 4, it is conceivable that the upswing in polyclonal IFN- γ -secreting capacity in a subgroup of infants between ages 12 and 18 months is a result

of parallel maturational changes in accessory cell function as opposed to changes within the T cells themselves and that these maturational changes may, in turn, permit more efficient in vitro expression of IFN- γ recall responses by previously primed TT-specific Th1 memory cells. In this context, it is pertinent also to note that recent studies in infant mice have demonstrated that the use of appropriate adjuvants (e.g., complete Freund's adjuvant), which are known to up-regulate accessory cells, can redress much of the Th2 imbalance in responses to vaccine antigens via selective stimulation of Th1 immunity [32] and, furthermore, that potent mycobacterial vaccines with intrinsic adjuvant properties can stimulate vigorous vaccine-specific Th1 responses in many human infants [36].

In addition to the core analyses detailed above, we addressed 2 peripheral questions that may provide additional perspectives on some of the vaccine-related issues, in particular in relation to the significance of findings shown in figure 3 on the kinetics of postnatal maturation of Th1 function. The first concerns the relationship to atopy, a syndrome that has increased markedly in prevalence in First World countries over the last 10–20 years, especially in children. Recent research from a number of groups, including ours (reviewed in [21]), suggests that, intrinsic to the etiology of this disease, is an “exaggeration” of the Th2 skew that is characteristic of the neonatal immune system (i.e., children at high genetic risk of this disease tend to be at the lower extremity of the normal range for Th1 function at a given age throughout infancy and early childhood and, as a consequence, are more prone to the development of allergy-associated Th2-polarized immunologic memory against environmental allergens encountered in early life).

Some evidence suggests that an additional consequence of the delayed maturation of Th1 function in this large subgroup may be reduced capacity to respond efficiently to vaccination in infancy, notably with bacille Calmette-Guérin (BCG), DTaP, and pneumococcal vaccines. In relation to BCG, a retrospective study in Japanese schoolchildren showed that failure to develop long-lasting delayed-type hypersensitivity to tuberculin in response to infant BCG vaccination is associated with a markedly elevated risk for atopy at age 12 years ([37]; see also [21]). As for DTaP, in a small pilot study from our group, vaccine antigen-specific lymphoproliferative responses at age 2 years were inversely related to expression of the atopic phenotype [38]. A recent report also indicates reduced responsiveness to pneumococcal vaccine in children with atopic eczema [39].

The data in table 1 are consistent with the findings that the Th1/Th2 balance in developing TT-specific responses remains consistently more Th2 skewed in atopic family history-positive infants up to age 12 months. This disparity is transient and is not evident in these study subjects at age 18 months (table 1). Nor is it found in 6-year-olds who have completed the standard priming/boosting schedule [16]; however, the possibility remains that current vaccines may provide a lower level of protection

against pathogens to infants manifesting this early Th1 maturational deficit as a result of the early Th1/Th2 imbalance.

The results shown in figure 5 suggest that natural host defense mechanisms in children with delayed maturation of Th1 competence may be less effective than in the rest of the population. Such children appear more likely to contract an RSV infection of sufficient intensity to prime for RSV-specific cellular immunity. In indirect support of this conclusion, it was recently reported that the expression of severe bronchiolitis during RSV infection is associated with diminished IFN- γ function [40] and, furthermore, that diminished capacity to secrete Th1-tropic IL-12 at birth is associated with enhanced risk for subsequent viral bronchiolitis [41]. In this context, recent findings in a mouse model emphasize the key role of IL-12 in sustaining ongoing Th1 responses [42]. More detailed follow-up studies are required to elucidate the relationship between postnatal maturation of Th1 function and the capacity to mount primary and secondary immune responses to viruses such as RSV during infancy.

In conclusion, this comprehensive analysis of the postnatal development of DTaP vaccine-specific cellular immunity during human infancy demonstrates the importance of immune maturational processes unrelated to vaccination per se in the early outcome of the vaccination program. One implication of these findings is that, analogous to the situation in the mouse, the efficient induction of a persistent Th1 component in vaccine-specific immunity during early infancy in humans may require the use of novel delivery systems designed specifically to compensate for maturational deficiencies in immune function, which currently limit the early effectiveness of conventional vaccines in a significant subset of subjects during this period of high infection risk.

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