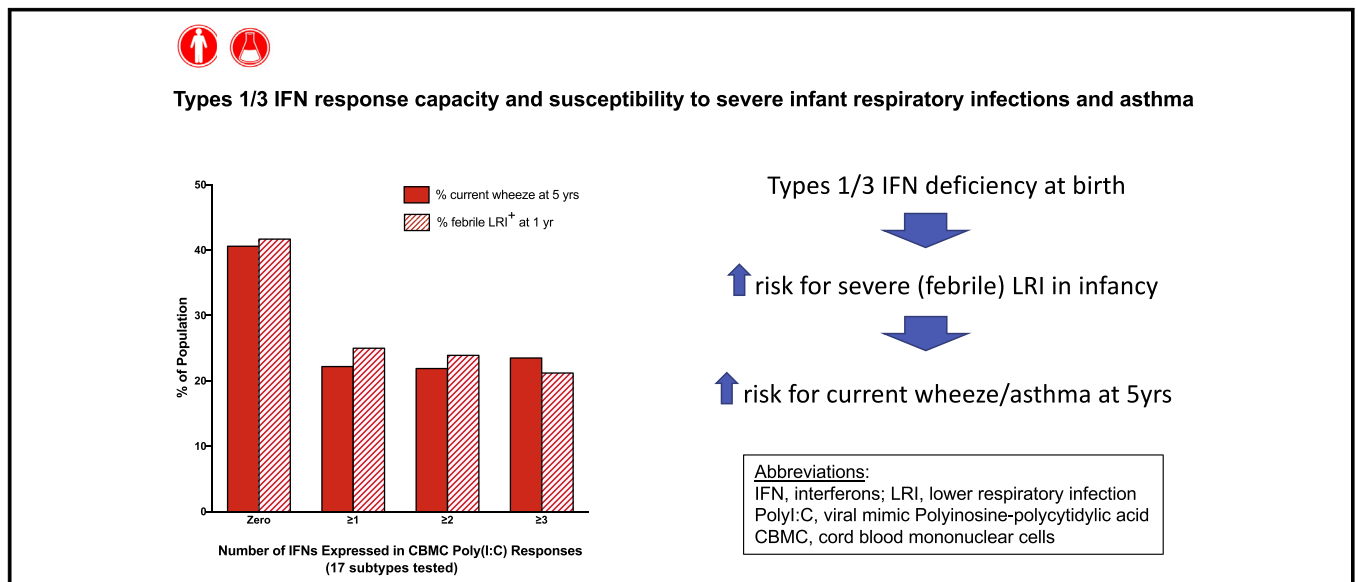


Developmental regulation of type 1 and type 3 interferon production and risk for infant infections and asthma development



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GRAPHICAL ABSTRACT



Background: Virus-associated febrile lower respiratory tract infections (fLRIs) during infancy have been identified as risk factors for persistent wheeze development. We hypothesized that variations in innate immune defense capacity during this

period, as exemplified by production of type 1 and 3 interferons (T1/3IFNs), might be an underlying determinant of risk.

Objective: We sought to investigate relationships between postnatal development of innate interferon response capacity and susceptibility to early infections and persistent wheeze.

Methods: We studied a subset of subjects from a birth cohort at high risk for asthma/allergy and determined the capacity of cord blood cells (n = 151) to produce any of a panel of 17 T1/3IFNs in response to the viral mimic polyinosinic-polycytidylic acid using a sensitive PCR assay. We investigated relationships between neonatal interferon responses and lower respiratory tract infection history during infancy, wheezing history to 5 age years, and ensuing maturation of innate immune capacity by age 4 years (n = 160) and 10 years (n = 125).

Results: Although cohort subjects produced an average of 2.6 ± 0.3 of the 17 innate interferons tested at birth, 24% showed no T1/3IFN production. This nonproducer subgroup showed increased risk for infant fLRIs (odds ratio, 2.62; 95% CI, 1.14-6.06; $P = .024$) and persistent wheeze (odds ratio, 4.24; 95% CI, 1.60-11.24; $P = .004$) at age 5 years relative to those producing 1 or more T1/3IFNs, whereas risk for infant wheezy lower respiratory tract infections or “transient early wheeze”

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was unaffected. Moreover, infants who experienced fLRIs subsequently demonstrated accelerated development of T1/3IFN response capacity between 1 and 4 years of age. **Conclusions:** T1/3IFN response capacity appears strongly developmentally constrained at birth. Infants in whom this negative regulation is strongest manifest increased risk for severe respiratory tract infections during infancy and subsequent persistent wheeze. (J Allergy Clin Immunol 2019;143:1176-82.)

Key words: Type 1 and type 3 interferons, persistent wheeze, asthma development, infant respiratory tract infections, febrile lower respiratory tract infections, hygiene hypothesis, innate immune development

Severe viral lower respiratory tract infections (LRIs) represent a major cause of hospitalization among infants and preschoolers,¹⁻³ and findings from a number of independent prospective birth cohort studies⁴⁻⁷ have implicated such infections as risk factors acting independently and/or in combination with atopy in the subsequent development of asthma-associated wheezy phenotypes. The increased susceptibility of the very young to these infections and their sequelae is widely attributed to delayed postnatal maturation of immune function, particularly those that direct innate antimicrobial defense pathways.^{8,9} However, functional innate immunity requires coordination of multiple immunoinflammatory mechanisms, and the identity of the precise steps that are rate limiting in these pathways during early postnatal life remains unclear. Identification of these critical steps might contribute to the future design of new therapeutic strategies for asthma prevention, and this prospect provided the impetus for the present study.

Prominent among front-line innate defense mechanisms is the capacity to produce T1/3IFNs¹⁰⁻¹² in response to microbial challenge. We present the first comprehensive data from a prospective birth cohort characterizing intrapopulation variations in the maturation of capacity to express a broad spectrum of T1/3IFN genes, and we demonstrate that this capacity is associated with susceptibility to severe LRIs during infancy and the development of persistent wheeze. We additionally present evidence that the “drive” for postnatal maturation of interferon response capacity attributed through the hygiene hypothesis to early postnatal microbial infections can be restricted to those that elicit febrile responses.

METHODS

Cohort details

Subjects were part of the Perth Childhood Asthma Study (CAS) initiated in 1997, which comprised a birth cohort of infants at high risk of atopy based on at least 1 parent with a doctor's diagnosis of allergy or asthma, as described previously.¹³ Initial cord blood collections were carried out over 1997-1998, and follow-ups relevant to this study comprising additional blood collections and clinical assessments were carried out as close as possible to the subjects' 4th, 5th, and 10th birthdays. Details of standard clinical definitions used in this cohort for acute respiratory tract infection, upper respiratory tract infection versus LRI, wheeze, transient early wheeze, and wheezy lower respiratory tract infection (wLRI) versus febrile lower respiratory tract infection (fLRI) are described elsewhere,^{5,13-15} with the main outcomes of interest being current wheezing status at year 5 (positive parental answer to “Has your child wheezed in the past year?” at the 5th birthday) and fLRI in year 1 (episode of fever $\geq 38^{\circ}\text{C}$ associated with rattly chest or wheeze during the first 12 months).

Abbreviations used

CAS: Perth Childhood Asthma Study
CBMC: Cord blood mononuclear cell
fLRI: Febrile lower respiratory tract infection
ISG: Interferon-stimulated gene
LRI: Lower respiratory tract infection
OR: Odds ratio
poly(I:C): Polyinosinic-polycytidylic acid
qPCR: Quantitative PCR
T1/3IFN: Type 1 and 3 interferon
TLR: Toll-like receptor

Cell preparation and culture

Cord blood mononuclear cells (CBMCs) and PBMCs were processed. Replicate samples were cryopreserved within approximately 8 to 12 hours of collection, as previously described,¹⁶ and held in a centrally controlled, alarmed, high-security liquid nitrogen storage facility equipped with an automatic backup system to ensure continuity of nitrogen supply. The samples were held in cryo-storage for approximately 15 years before performance of the analyses described here. Before culture, the cells were thawed and resuspended to 1×10^6 cells/mL in RPMI 1640 (Gibco, Thermo Fisher Scientific, Scoresby, Australia) containing 10% non-heat-inactivated FBS (Serana Australia, Fisher Biotech, Wembley, Australia). Microcultures using 96-well round-bottomed plates (Nunc, Thermo Fisher Scientific, Scoresby, Australia) were performed by using a modification of high-throughput techniques previously developed by our laboratory for cohort studies, which have been demonstrated to yield high-fidelity cellular immune function data with sample sizes up to 1340.^{16,17}

To minimize batch variation, samples were cultured sequentially by the same operators in randomized blocks using the same stocks of growth medium and immunostimulants. Cells were seeded in triplicate wells at 250,000 cells/well and cultured for 24 hours at 37°C in a 5% CO_2 atmosphere in medium alone or stimulated with 50 $\mu\text{g/mL}$ polyinosinic-polycytidylic acid (poly [I:C]; InvivoGen, San Diego, Calif). Plates were then centrifuged, and resultant cell pellets were resuspended in RNeasy Protect Cell Reagent (Qiagen, Chadstone Centre, Australia). Plates containing cell pellets were sealed and stored at -80°C before extraction.

RNA extraction and interferon quantitative PCR for type I and III interferons

Total RNA was extracted from cell pellets by using the Direct-zol 96-well Plate kit (Zymo Research, Irvine, Calif) and then reverse transcribed with the Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Mass). To detect the genes for the 12 IFN- α subtypes, cDNA was preamplified with a mix of 384 degenerate forward primers for a noncoding sequence in the 5' untranslated region (MTKRTGGYCSTVSTVSTGGTRC) and a reverse primer for a shared sequence at the 3' 120 tail of the coding region for the mature peptide (TCTCATGATTCTGCTCTGACAACC). Expression of the IFNA subtypes IFNB1, IFNW, and IFNL1 to IFNL3 using quantitative PCR (qPCR) was performed, as previously described.¹⁸ As reported, we used linearized plasmids with cDNA for each interferon to demonstrate that this qPCR assay has high sensitivity and low interplate variability (range of coefficient of variation, 0.4% to 3.7%).

Each PCR plate can analyze 16 samples and has 2 steps for quality control. The first step is inclusion on each plate of 4 dilutions of each linearized plasmid encoding for each interferon (total, 68 wells). Second, each PCR plate includes a positive control that consists of cDNA from PBMCs stimulated with multiple Toll-like receptor (TLR) agonists (LPS, imiquimod, CpG oligonucleotides, and poly [I:C]) and is spiked with plasmid cDNA of the IFNA subtypes expressed by the stimulated PBMCs.

Analysis of qPCR data was performed by using the comparative cycle threshold ($2^{-\Delta\Delta\text{Ct}}$) method,¹⁹ with ubiquitin C (UBC) as the reference gene, and data are expressed as detailed in the statistical methods section below.

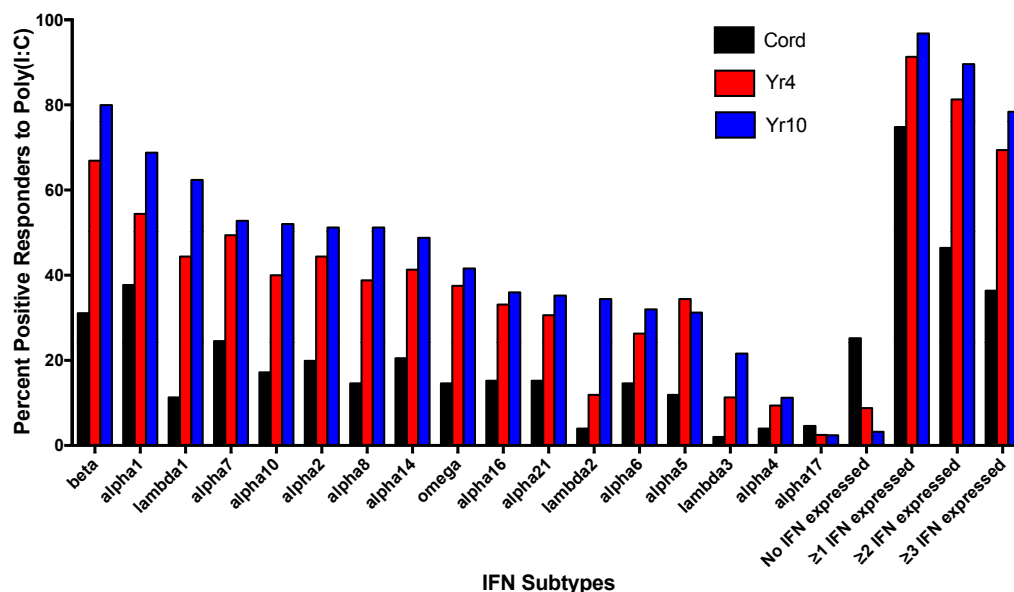


FIG 1. Postnatal development of T1/3IFN production capacity in the CAS cohort. Data shown are percentages of subjects' PBMC samples exhibiting interferon subtype-specific gene expression to greater than detection limits stratified by subject age. Sample numbers: cord blood, $n = 151$; age 4 years, $n = 160$; and age 10 years, $n = 125$.

A single sample failed to produce a signal for the reference gene, and this was removed from the analysis.

Because the control (unstimulated) and treatment (poly(I:C)) samples for any given subject are separate test samples, a type I error can occur when neither group expresses the target gene because of differences in expression levels of the reference gene in each sample. To eliminate this error, when this result was encountered, the sample was assigned a $2^{-\Delta\Delta C_t}$ value of 1, indicating no change in expression of the target gene when stimulated with poly(I:C).

Statistical methods

Statistical analyses were conducted with IBM SPSS Statistics software (version 24; IBM, Armonk, NY) for MacOS and STATA software (version 14; StataCorp, College Station, Tex) for Windows. Interferon RNA expression derived from RT-qPCR $2^{-\Delta\Delta C_t}$ values was compared based on clinical outcomes, with the latter being treated as categorical variables. The interferon data were used both as a continuous variable and converted into categorical variables for a positive (upregulated) response to poly(I:C) treatment (ie, $2^{-\Delta\Delta C_t}$ values of ≤ 1 was designated as 0, whereas all values > 1 were designated as 1) in our analyses. Univariate analyses with Mann-Whitney U tests were carried out to compare clinical outcomes and T1/3IFN-specific RNA expression. Categorical T1/3IFN variables were assessed to determine their expression frequencies with respect to clinical outcomes by using Pearson χ^2 or Fisher exact tests, where applicable. Logistic regression was used with current wheeze at year 5 and fLRI in year 1 as response variables to estimate the effect of progressively increasing numbers of interferon subtypes detected by using the suggested ordered category coding of Walter et al²⁰ and to estimate the best fitting fractional polynomial model for the continuous variable of each subject's sum of all positive T1/3IFNs.^{1-3,21}

RESULTS

Previous studies involving the CAS cohort^{5,14,15} have identified symptomatic LRIs during infancy and early atopic sensitization as important determinants of risk for subsequent development of persistent wheeze by age 5 years. The present study used a subset of these cohort subjects (interferon subset), selected on the basis of availability of both cryobanked CBMCs and year 5 wheezing outcomes for follow-up investigations on the role of

postnatal maturation of innate immune response capacity in disease pathogenesis. As shown in Table E1 in this article's Online Repository at www.jacionline.org, this subset does not differ from the overall CAS population with respect to outcomes of interest in this study, notably frequency/type of LRI and/or atopic sensitization during year 1 and persistent wheeze at year 5. Moreover, consistent with earlier findings in the overall cohort,^{5,14,15} fLRIs during year 1 (with or without adjustment for concurrent sensitization) were associated with risk for development of persistent wheeze in this subset, with a similar trend seen for wLRIs (see Table E2 in this article's Online Repository at www.jacionline.org). It should be noted that approximately half of the wLRI events in year 1 also involved fever, but further stratification was not feasible because of limiting numbers.

We modified a recently developed quantitative RT-PCR assay system to assess the capacity of CBMCs and PBMCs from study subjects to respond to the viral mimic poly(I:C) through production of mRNA specific to any of the panel of 17 T1/3IFNs, as detailed in the Methods section. Because of the low frequency of positive responders (Fig 1), it was not feasible to pursue group analyses related to levels of production of the individual interferon subtypes, and therefore we instead focused on responses as binary variables. T1/3IFN response capacity is strongly negatively regulated at birth, with the average infant producing 2.6 ± 0.3 T1/3IFNs in response to poly(I:C) (Fig 2), with 24% of CBMC samples producing none of these 17 T1/3IFN subtypes at birth (Fig 1). The proportion of producers increased with age for each of the interferon subtypes, except *IFNA17* (Fig 1), which might be due to the poor sensitivity of this primer/probe set rather than differences in expression of this IFN- α subtype. This is reflected in the average number of T1/3IFN genes expressed per subject, which increases progressively with age (Fig 2). In contrast, all PBMC samples from the 3 ages produced type 2 interferons in response to poly(I:C), as determined by using Luminex assays on culture supernatants (data not shown).

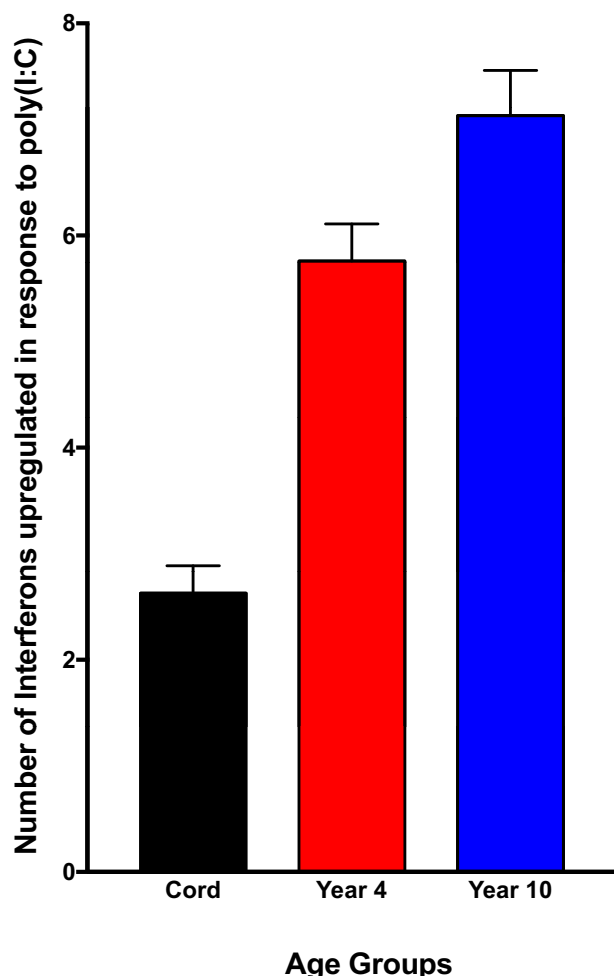


FIG 2. Age-dependent increase in T1/3IFN response capacity across the CAS cohort. Data show mean $\pm$ SEM numbers of interferons produced per subject at each age. Sample numbers are as per Fig 1.

We next tested for associations between the capacity to produce T1/3IFNs in CBMCs in response to poly(I:C) and clinical outcomes in infancy and beyond. As shown in Table 1, logistic regression analyses demonstrated that the capacity to produce 1 or more T1/3IFNs was associated with a lower incidence of early fLRI and persistent wheeze at age 5 years, and these relationships (particularly the latter) were strengthened by adjustment for early atopic sensitization. These associations remained after further adjustment for a series of potential confounders (maternal age, smoking in pregnancy, parity, caesarean delivery, prematurity [gestation <37 weeks], birth weight, and sex); moreover, relative to Table 1, adjustment for the number of days antibiotics were given during year 1 increased the strength of the association between reduced T1/3IFN response capacity and risk for both year 1 fLRI (OR, 3.90; 95% CI, 1.39–10.94; $P = .010$) and year 5 wheeze (OR, 7.38; 95% CI, 2.10–25.91; $P = .002$).

Additionally, we tested these same factors (excluding antibiotic exposure) as potential predictors of low T1/3IFN response capacity at birth, but no significant associations were found. It is noteworthy that the trend for reduced likelihood of fLRI in year 1 among subjects who expressed at least 1 T1/3IFN was consistent across all subtypes, except *IFNA17* (see Fig E1 in this article's Online Repository at www.jacionline.org), and a

TABLE 1. T1/3IFN response capacity at birth and risk for asthma-associated outcomes: logistic regression analyses

Outcome	OR	95% CI	P value
fLRI, year 1 (n = 38)	2.25*	1.02–4.99	.046
	2.62†	1.14–6.06	.024
wLRI, year 1 (n = 33)	1.21*	0.52–2.85	.657
Transient early wheeze (n = 30)	1.35*	0.55–3.31	.517
Persistent wheeze, year 5 (n = 35)	2.40*	1.02–5.60	.044
	4.24†	1.60–11.22	.004

Data shown are ORs for outcomes in nonproducers relative to those producing more than 1 T1/3IFNs, either unadjusted (*) or adjusted (†) for sensitization at age 1 year.

comparable pattern was observed for persistent wheeze (data not shown). In contrast, the risk for wLRI in year 1 or transient early wheeze that resolves by year 3 were not affected by T1/3IFN response capacity.

Fig 3 explores these associations in more detail. Fig 3, A, demonstrates an approximately 50% reduction in the prevalence of both year 1 fLRI and ensuing wheeze at year 5 in subjects capable of producing at least 1 T1/3IFN subtype relative to nonproducers ($P = .014$ and $P = .037$, respectively, Fisher exact test). However, this plot also suggests that the level of protection associated with the capacity to produce T1/3IFNs does not improve as the number of expressed T1/3IFN genes increases.

To confirm that subjects who express more than 2 T1/3IFN genes are no more protected from wheeze at year 5 than subjects who express 1 T1/3IFN gene, we performed additional logistic regression analyses to compare the effects of 1 or more, 2 or more, and 3 or more T1/3IFN subtypes detected and also to estimate the best fitting fractional polynomial model for the continuous variable of each subject's sum of all positive T1/3IFNs by using both persistent wheeze at year 5 (Fig 3, B) and fLRI in year 1 (data not shown) as response variables. Analyses using the fractional polynomial procedure in Stata 14 identified the best fitting polynomial model for wheeze at 5 as follows:

$$\log(\text{Odds}) = -1.924891 + 0.0142293 * [(N + 1)/10]^{-2} - 7.07 - 0.3916448 * [(N + 1)/10]^3 - 0.0532.$$

Although the odds for both fLRI by year 1 and wheeze at year 5 decreased significantly with detection of 1 or more interferons (OR, 0.41 [$P = .03$] and 0.24 [$P = .004$], respectively), neither the addition of variables for 2 or more T1/3IFNs nor the use of the polynomial terms resulted in an improvement in model fit for either outcome ($P \geq .68$ in each case).

As described above in Fig 2, T1/3IFN response capacity increased sharply between birth and year 4, with further but modest progression by year 10. Of note, the number of T1/3IFNs expressed by year 4 did not differ between subjects with or without persistent wheeze at year 4 and likewise T1/3IFN capacity/wheeze at age year 10 (see Fig E2 in this article's Online Repository at www.jacionline.org), indicating that this developmental deficiency operates only within a restricted temporal window during early life.

We questioned whether maturation of T1/3IFN response capacity beyond infancy can differ between subjects as a function of their infection history during infancy. Fig 4, A, indicates that T1/3IFN response capacity at year 4 is enhanced in subjects who experienced fLRIs in year 1 relative to those who had wLRIs. In this regard a subset of the wLRI group in Fig 4, A, also had fever, and restratification of the wLRI group (as with or without fever) confirmed that the surge in T1/3IFN capacity beyond

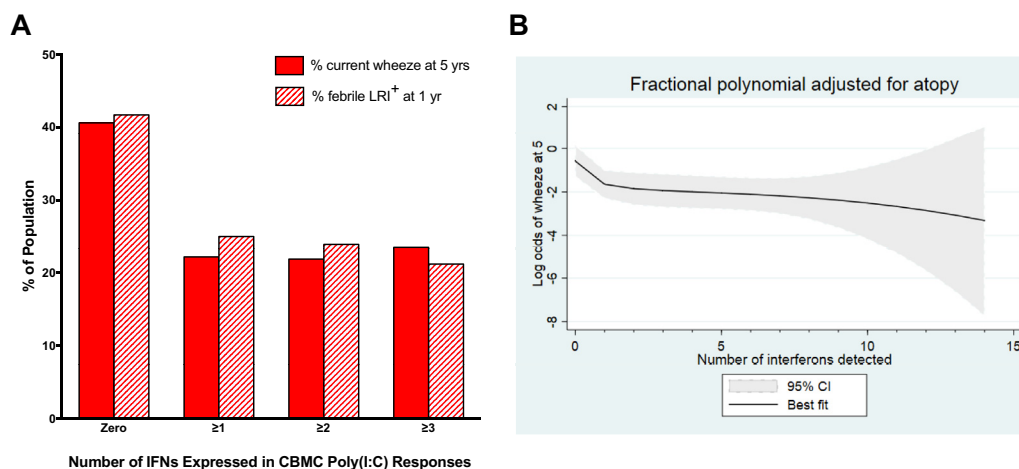


FIG 3. Susceptibility to early fLRIs and wheeze at year 5 in relation to interferon production capacity. **A**, Data shown were derived by means of cross-tabulation in SPSS software. **B**, Risk for development of wheeze at year 5 as a function of the number of interferons expressed in CBMCs. The fractional polynomial model is detailed in the Methods section. Sample sizes for analyses relating to fLRIs and wheezing outcomes were 144 and 132, respectively.

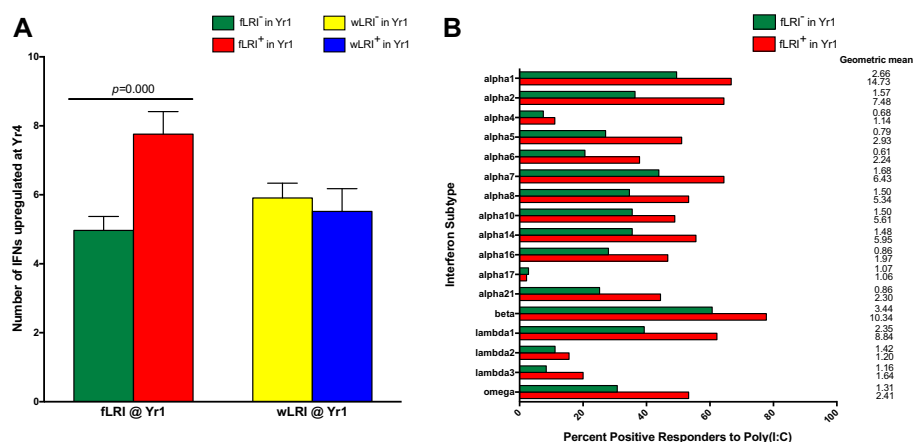


FIG 4. Influence of infection type or types experienced in year 1 on subsequent maturation of T1/3IFN response capacity. **A**, Number of interferons expressed by year 4 in subjects stratified by experience of fLRIs or wLRIs in year 1. **B**, Comparison of expression levels of individual interferons between groups who did ($n = 45$) versus those who did not ($n = 107$) experience fLRIs in year 1 by using the Mann-Whitney test. The study sample used all subjects with relevant data available.

infancy was confined to the fever-positive subgroup (data not shown). At the group level, this effect of fLRIs is highly consistent across the spectrum of individual T1/3IFNs (Fig 4, B), and comparable effects were not observed after stratification by wLRI (see Fig E3 in this article's Online Repository at www.jacionline.org), by "any" LRI, by febrile or nonfebrile upper respiratory tract infection, or by "any" febrile acute respiratory tract infection (data not shown).

DISCUSSION

Forerunner studies using a series of prospective birth cohorts⁴⁻⁷ have identified severe virus-associated respiratory tract infections during infancy as risk factors for the subsequent development of persistent wheeze and asthma. More recent studies suggest additional contributions from bacterial pathogens, particularly those that colonize the upper airways during the first weeks to months

of life.^{22,23} It is evident that the infections posing the greatest risk for subsequent wheezing development are those that spread from the upper to the lower respiratory tract, and findings from the present CAS cohort, which (uniquely among reported cohort studies) collected temperature data during every reported acute infection event during infancy, suggest that the most significant of these in relation to asthma pathogenesis were fLRIs.^{5,14,23} Why fever is a critical factor is incompletely understood, but fever is recognized to be a marker for production of a variety of inflammatory mediators, including the triad of IL-1, IL-6, and TNF- α ,²⁴ and hence these febrile responses might be a surrogate for underlying high-level inflammation that causes the bystander tissue damage associated with asthma pathogenesis.

As noted above, susceptibility to LRI is maximal during infancy, and although this is generally attributed to developmental delays in immune maturation, the precise mechanistic details of this deficiency are unclear. In the absence of a fully functional

adaptive immune system to protect against pathogen invasion,^{9,25} infants must rely more heavily on first-line innate immune defense mechanisms than older age groups. However, innate immune competence exemplified by TLR signaling is also compromised during this period,⁸ and as shown in Fig 1, this developmental deficiency also extends to T1/3IFNs. The innate interferon system comprises 21 different subtypes with multiple overlapping functions, and such extreme redundancy points to the likely importance of T1/3IFN-dependent functions to survival. Consistent with this premise, our data demonstrate that inability to produce any T1/3IFNs in the neonatal period in response to poly(I:C), which is observed in 24% of our high-risk cohort subjects, is associated with increased risk for both infant fLRIs and for subsequent development of persistent wheeze. This risk-associated deficiency appears limited to a specific temporal window during infancy because follow-up analyses in the cohort indicated that by age 4 years, T1/3IFN production capacity had matured equivalently in wheezing and nonwheezing children (Fig 2). It is also pertinent to note that this “interferon-null” risk phenotype does not extend to T2IFN (IFN- γ), which was produced in 100% of samples in response to poly(I:C), and further that variations in IFN- γ production levels were not predictive of risk for either fLRIs or persistent wheeze (data not shown).

Our data also show that expression of a single T1/3IFN is protective and that there is no additional protection when more than 1 T1/3IFN gene is expressed. Although T1/3IFNs each have distinct receptors (*IFNAR1/2* and *IL28R/IL10RB*, respectively), they all activate canonical signaling in which the interferon-stimulated growth factor 3 complex induces expression of a set of interferon-stimulated genes (ISGs) that have direct antiviral function (eg, *MX1* and *OAS1*). These ISGs are categorized as “robust” because their expression is exquisitely responsive to receptor activation, and comparatively low interferon concentrations are required to induce maximal expression.²⁶ The sensitivity of the canonical pathway accounts in large part for the redundancy of T1/3IFNs and might explain why expression of a single T1/3IFN is protective and there is no additional protection from expression of more than 1 interferon genes. However, a limitation to this conclusion is sample size, given that, as shown in Fig E1, many of these subtypes were produced by less than 20% of the neonates in the study population. The pattern we observed might look different in a larger cohort. In particular, the size of our cohort does not allow us to address important questions relating to comparative potencies or potential nonredundant functions of different T1/3IFNs that might be reflected by expression of “tunable” ISGs, which include downstream immune modulators and transcription factors, such as interferon regulatory factor 1, that can be differentially expressed in response to T1/3IFNs.²⁶

Additional questions arising from these analyses relate to the kinetics of maturation of T1/3IFN function beyond infancy, in particular questions about what drives this process and how it relates to subsequent expression of persistent wheeze. The data in Fig 2 show a clear developmental pattern, particularly across the preschool years, and this age-dependent increase in the number of potentially expressible T1/3IFNs in a poly(I:C) response matures fastest in the subgroup of children who experienced early fLRIs as opposed to any other categories of infection. Viewed through the prism of the hygiene hypothesis,²⁷ this suggests that the effectiveness of postnatal “education/training” of the innate immune system through experience of microbial infection during infancy can vary as a function of either the pathogen itself or the mix of

immunoinflammatory mediators it triggers in the infected host. In this cohort the marker of infection-related events that is associated with effective immune training would appear to the same factor that identifies wheeze/asthma risk: the accompanying febrile response (Fig 4, A). The presence of overt fever as part of the host immune response designates the operation of underlying inflammatory networks,²⁴ and the detailed characterization of these in infants experiencing fLRIs might enable the identification of network components responsible for immune training that are distinct from those driving bystander tissue damage. If successful, this could inform future efforts to develop therapeutics that can reproduce the positive immune-training effects of early febrile infections while avoiding their negative long term sequelae exemplified by asthma. A potential caveat to this approach, however, is the finding from other study settings that very severe acute viral bronchiolitis requiring hospitalization can be associated in some circumstances with exaggerated type 1 interferon production,^{11,28} suggesting that unrestrained bypassing of the developmental constraints on the innate interferon system during infancy can also entail covert disease risks.

There are several limitations to this study that should be acknowledged. First, the study cohort was restricted to subjects at high atopic risk, which in Australia constitute 45% to 50% of the overall population on the basis of family history. Second, because of the wide variability in expression in early life among the different interferon genes, subject numbers were insufficient to directly compare respective potencies in relation to the clinical phenotypes of interest. Third, PBMC stimulation was restricted to a single TLR stimulus, the viral mimic poly(I:C), and it would be of major interest to extend this to whole virus and especially to include bacterial stimuli and also to map the relevant T1/3IFN signals to specific PBMC subsets.

Notwithstanding these limitations, the study provides clear evidence of a relationship between the strength of developmental (negative) regulation of the innate interferon response capacity and susceptibility to both severe (febrile) LRIs during infancy and subsequent asthma-associated persistent wheeze, which merits follow-up in larger cohort studies.

Key messages

- Risk for severe LRIs during infancy and subsequent development of persistent wheeze are associated with reduced capacity of CBMCs to respond to virus-related stimuli through activation of T1/3IFN genes.
- At birth, the average number of T1/3IFN genes transcribed in these responses is less than 3, and this increases progressively to age 10 years.
- Children who experience fLRIs during infancy have accelerated kinetics of maturation of T1/3IFN response capacity, but this hygiene hypothesis effect was not observed in relation to LRIs associated with wheeze alone.

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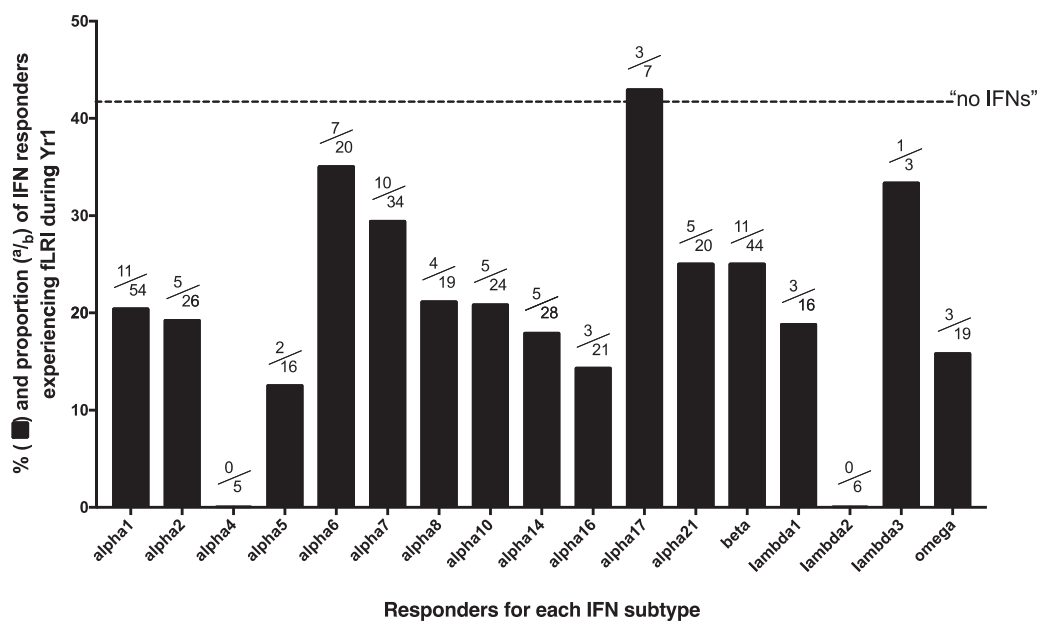


FIG E1. Data shown represent percentages/proportions of subjects producing the different interferon subtypes in response to poly(I:C) who experienced fLRIs in year 1 relative to subjects who produced no interferons (dotted line).

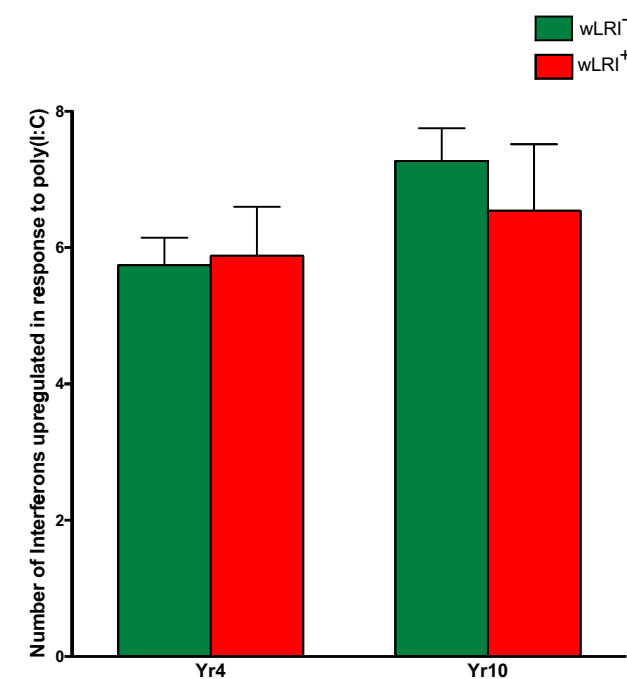


FIG E2. Cross-sectional relationships between wheezy phenotype and interferon production capacity at 4 and 10 years of age. Number of interferons upregulated in response to poly(I:C) in PBMCs of 4- and 10-year-olds stratified by current wheeze at respective ages.

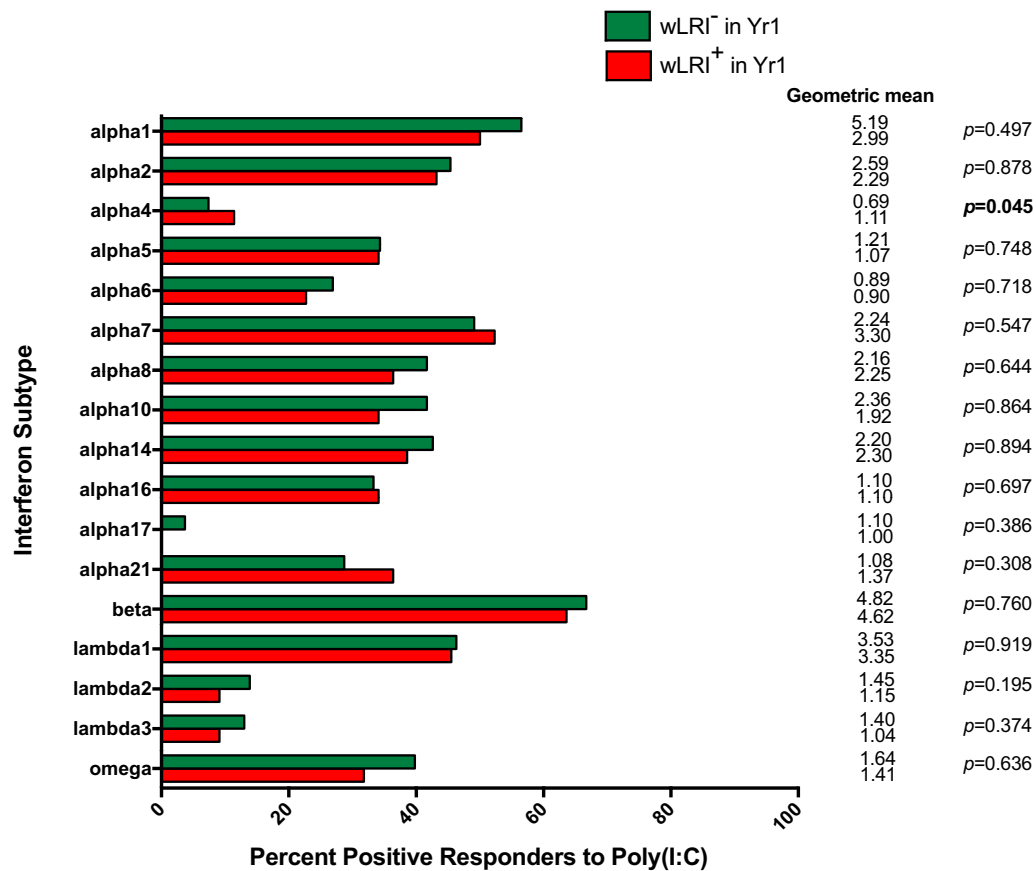


FIG E3. Influence of wLRIs in year 1 on maturation of T1/3IFN production capacity by year 4. Comparison of expression levels of individual interferons between groups by using the Mann-Whitney test.

TABLE E1. Wheeze-associated outcomes in overall CAS population versus the interferon subcohort

Outcomes	Whole population (n = 153)	Interferon subcohort (n = 132)
	Population prevalence (%)	
Wheeze, 5 y	26.8	26.5
fLRI, year 1	28.1	8.8
wLRI, year 1	25.5	25.0
Sensitization (any), year 1	17.6	17.4
Transient early wheeze	22.8	22.7

One hundred fifty-three subjects had wheeze outcomes available at age 5 years, but only 132 of those (the interferon subcohort) also had interferon data available.

TABLE E2. Year 1 risk factors for development of persistent wheeze at age 5 years: Logistic regression analysis

Risk variables	Whole population (n = 153)			Interferon subset (n = 132)		
	OR	95% CI	P value	OR	95% CI	P value
fLRI, year 1	2.80	1.21–6.49	.016	2.96	1.27–6.89	.012
wLRI, year 1	2.65	1.13–6.20	.024	2.31	0.98–5.43	.056
Any sensitization, year 1	8.00	2.88–22.20	<.001	8.38	3.01–23.35	<.001

The interferon subset is broadly representative of the overall cohort.