Changes in Cytokine and Cytokine Receptor Levels During Postnatal Development of the Human Dorsolateral Prefrontal Cortex

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Research Article

Keywords: BA46, IL1B, IL6, TNF, complement, adolescence

Posted Date: August 23rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1969039/v1

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Abstract

In addition to their traditional roles in immune cell communication, cytokines regulate brain development. Cytokines are known to influence neural cell generation, differentiation, maturation, and survival. However, most work on the role of cytokines in brain development investigates rodents or focuses on prenatal events. Here, we investigate how mRNA and protein levels of key cytokines and cytokine receptors change during postnatal development in the human prefrontal cortex. We find that most cytokine transcripts investigated (IL1B, IL18, IL6, TNF, IL13) are lowest at birth and increase between 2–5 years old. After 5 years old, transcriptional patterns proceeded in one of two directions: decreased expression in teens and young adults (IL1B, p = 0.002; and IL18, p = 0.004) or increased mean expression with maturation, particularly in teenagers (IL6, p = 0.004; TNF, p = 0.002; IL13, p < 0.001). In contrast, cytokine proteins tended to remain elevated after peaking significantly at age 5 (IL1B, p = 0.012; IL18, p = 0.026; IL6, p = 0.039; TNF, p < 0.001), with TNF protein being highest in young adults. Early developmental increases in cytokines were paralleled by increases in their receptor binding subunits, such as IL1R1 (p = 0.033) and IL6R (p < 0.001) transcripts. In contrast, cytokine receptor-associated signaling subunits, IL1RAP and IL6ST, did not change significantly between age groups. Of the two TNF receptors, the ‘pro-death’ TNFRSF1A and ‘pro-survival’ TNFRSF1B, only TNFRSF1B was significantly changed (p = 0.028), increasing first in toddlers and again in young adults. Finally, the cytokine inhibitor, IL13, was elevated first in toddlers (p < 0.001) and again in teenagers (p < 0.001). While the mean expression of interleukin-1 receptor antagonist (IL1RN) was highest in toddlers, this increase was not statistically significant. The fluctuations in cytokine expression reported here support a role for increases in specific cytokines at two different stages of human cortical development. The first is during the toddler/preschool period (IL1B and IL18) and the other at adolescence/young adult maturation (IL6 and TNF).

Introduction

Research into the role of immune signals like cytokines in brain development has been partly driven by their association with atypical brain development and with psychiatric disorders. Epidemiological work further suggests that infection during gestation and early childhood is associated with the occurrence of neurodevelopmental disorders (1–17). In particular, inflammation during prenatal and postnatal development is reportedly associated with the development of autism and schizophrenia (14, 18–23). However, activation of cytokine signaling alone, in the absence of infection, can induce pathological changes in the brain (24–26). Additionally, genetic variation in immune-associated signals can alter the expression and function of immune signals independent of pathogen exposure. These were described with the copy number variant of complement component C4 (27). Thus, genetic and epigenetic changes to immune signaling molecules have the potential to disrupt normal patterns of expression and may exhibit heightened responses during episodes of infection-associated and/or sterile inflammation. Furthermore, psychiatric disorders first become evident during postnatal development. Therefore, we aimed to characterize the developmental timing of typical cytokine signaling during the normal postnatal
period to determine when immune signaling perturbations would be expected to impact cortical development.

We previously investigated the trajectory of complement pathway members in neurotypical human prefrontal cortex across major periods of post-natal brain development. We found that the peak expression of many complement pathway members was in toddlers 2–5 years old (28). However, this study did not examine whether an increase in pro-inflammatory and anti-inflammatory cytokines occurs in tandem with complement pathway activation or whether the trajectory of cytokine change is distinct. Cytokines serve as intercellular messengers both inside and outside the immune system and regulate pro- and anti-inflammatory activity (29). Complement and cytokines are capable of reciprocal signaling between pathways, wherein cytokines can induce complement expression and vice versa (30–37). Therefore, given this existing association between complement and cytokine induction. We would expect pro-inflammatory cytokines and their associated receptors to coordinately regulated with complement during development.

Low levels of cytokines are detectable under normal conditions in the fetal and postnatal brain, and are believed to facilitate the development of the mammalian cortex. For instance, multiple cytokines can be detected during the first trimester in the human forebrain at the mRNA and protein level, including $IL1B$, $TNF$, $IFNG$, $IL6$, $IL4$, $IL10$, and $TGFB1$ (38). $IL6$, $TNF$, and $IL1B$ have also previously been implicated in some of the crucial processes involved in brain development, such as progenitor cell proliferation (39), neuronal migration (40, 41) and both glial (42, 43) and neuronal (42) differentiation. In addition, transcripts for $IL1B$, $IL6$, $TNF$, and $IL18$ (44–54), as well as their receptors (52, 53, 55–65) have been identified in the postnatal cortex of rats, mice, and humans. However, the time course of change in cytokine levels across human postnatal brain development remains unclear.

To date, the studies investigating cytokine expression in human postnatal brain have been predominantly restricted to adults. Therefore, there is a gap in our understanding of immune expression in the brain at earlier, more dynamic ages. These include age periods coinciding with the typical age of onset for neurodevelopmental disorders such as autism spectrum disorders (ASD) (66, 67), which is concomitant with a peak cortical expression of the complement pathway (28). Additionally, abnormal cytokine levels have been implicated in ASD (68–70). Aberrant cytokine signaling could disrupt numerous processes during postnatal brain development. These include interneuron migration (71, 72), developmentally programmed cell death (73–76), neuronal maturation (77–80), synaptogenesis (81, 82), synaptic pruning (83–87), and oligodendrocyte maturation/myelination (88–90). Therefore, for the first time, we quantified the normal human cortical expression of an array of pro-inflammatory and anti-inflammatory cytokines implicated in neurodevelopment and characterized their expression trajectory from near birth until early adulthood using both mRNA and protein-based methods. We hypothesized that the mRNA and protein of pro-inflammatory cytokines $IL1B$, $IL6$, $IL18$ and $TNF$, and their associated receptors, would follow a similar trajectory to that of complement, peaking during early postnatal human brain development. Additionally, we hypothesized that the expression of associated inhibitory cytokines $IL1RN$, $IL10$, and $IL13$ would increase from school-age children through young adult, comparable to complement inhibitors (28).
Methods

Subjects

Postmortem samples from Brodmann’s area 46 in the DLPFC were obtained from the University of Maryland Brain and Tissue Bank in conjunction with the National Institute of Child Health and Development (N=65). The individuals in this cohort ranged from 6 weeks old to 25 years old. Subjects (neonates, infants, toddlers, school-age children, teenagers, and young adults) were assigned to one of six age groups based on cognitive development stages in consultation with a pediatrician. These developmental groups roughly correspond with stages of sensory and cognitive development, i.e., visual acuity and grasping (Neonates: birth-3 months old); sitting upright and environmental engagement (Infants: 4 months-1 year old); preoperational, pre-conceptual rapid language development (Toddlers: 1.5-4 years old); concrete operations (School-age children: 5-12 years old); formal operations, reproductive competence and heightened peer interactions (Teenagers: 14-18 years old); social independence and early maturity (Young Adults: around 22 years old). Eight to thirteen samples were selected per developmental group as described: groups were established with care to balance male and female samples as best as possible, and groups were matched for RNA integrity number (RIN), pH, and postmortem interval (PMI) for most groups. However, PMI was significantly longer in neonates and infants than in older ages (ANOVA, F(5,51)=2.881, p=0.023). Age groups analyzed, and their respective demographic and tissue quality information, are reported in Table 1. We have also included several Sudden Infant Death Syndrome (SIDS) samples in our age groups under two years old. A role for inflammation in SIDS has been proposed (91). However, the syndrome remains poorly understood, with much potential for heterogeneous causes of death. We studied ages 25 and under to focus on developmental processes, rather than aging (92) – however, the time at which “aging” begins remains an area of debate (93-96). All subjects were free of neurological and psychiatric symptoms at the time of death. Individual sample information, including age, sex, RIN, pH, PMI, and cause of death, are in Supplementary Table 1.

Table 1. Summary of patient demographics. RIN, pH, and PMI are presented as mean ± standard deviation.
<table>
<thead>
<tr>
<th>Age Group</th>
<th>Age Range (years)</th>
<th># Male/Female</th>
<th>RIN</th>
<th>pH</th>
<th>PMI</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonate</td>
<td>0.11-0.24</td>
<td>5/5</td>
<td>7.48±1.36</td>
<td>6.52±0.20</td>
<td>22.3±5.36</td>
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<tr>
<td>Infant</td>
<td>0.25-0.91</td>
<td>8/5</td>
<td>7.58±0.59</td>
<td>6.61±0.16</td>
<td>17.46±6.36</td>
<td>13</td>
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<tr>
<td>Toddler</td>
<td>1.58-4.86</td>
<td>5/4</td>
<td>7.02±1.02</td>
<td>6.66±0.26</td>
<td>22.33±9.26</td>
<td>9</td>
</tr>
<tr>
<td>School-age</td>
<td>5.39-12.97</td>
<td>4/4</td>
<td>7.33±0.63</td>
<td>6.70±0.17</td>
<td>14.75±4.86</td>
<td>8</td>
</tr>
<tr>
<td>Teenager</td>
<td>15-17.82</td>
<td>6/2</td>
<td>7.45±0.71</td>
<td>6.75±0.09</td>
<td>15.5±5.26</td>
<td>8</td>
</tr>
<tr>
<td>Young Adult</td>
<td>20.14-25.38</td>
<td>6/3</td>
<td>7.53±0.79</td>
<td>6.67±0.23</td>
<td>13.67±8.26</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>0.11-25.38</td>
<td>34/23</td>
<td>7.41±0.87</td>
<td>6.64±0.20</td>
<td>17.82±7.31</td>
<td>57</td>
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</tbody>
</table>

**RNA extraction and cDNA synthesis**

Total RNA was extracted from 30-100 mg of frozen DLPFC tissue using the Trizol (Cat# 15596026, Invitrogen, Thermo Scientific, Waltham, MA, USA) reagent method. A tissue homogenizer (Model PT10/35, Brinkmann Instruments) was used to homogenize frozen tissues on wet ice. Chloroform was used to dissociate nucleoprotein complexes; then, soluble RNA was removed from each sample. RNA was precipitated using isopropyl alcohol and re-suspended in diethyl-pyrocarbonate-treated water (97). The yield and purity of the extracted RNA were determined via spectrophotometer analysis. Following extraction, the RIN was determined for each sample using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). All samples had 260/280 ratios >1.70 and RIN values >6. RNA (1.5 μg)/sample was converted into cDNA using Superscript IV First Strand Synthesis Kit (Cat # 18091200, Thermo Scientific, Waltham, MA, USA) as described (98).

**High-throughput quantitative PCR**

High-throughput quantitative PCR was run on a Biomark HD (Fluidigm, South San Francisco, CA, USA) using a 96.96 Dynamic IFC high-throughput qPCR chip (Cat# 100-6123, Fluidigm, South San Francisco, CA, USA), enabling simultaneous Taqman gene expression assays on all samples. All probes are included in Supplementary Table 2. The following transcripts passed quality control analysis: *IL6, IL1B, IL18, TNF, IL1R1, IL18R1, IL6R, IL6ST, TNFRSF1A, TNFRSF1B*. No signal in either the no-template control or the no-RT control exceeded cycle threshold levels. Therefore, no transcripts were excluded from further analysis based on a low expression. ‘Low expression’ here is categorized as having a detectable signal in less than five samples of any given age group or a detectable signal in less than three points of the standard curve.

**Single target quantitative RT-PCR**
Quantitative PCR was run on an Applied Biosystems 7900HT Fast Real-Time PCR System (Thermo Fisher, Waltham, MA, USA). Taqman gene expression assays were used (Supplementary Table 3). A 1:6 plate cDNA dilution was used for transcripts IL10, IL13, IL1RN, and IL1RAP, which were added for analysis following the high-throughput quantitative PCR. In addition, serial dilutions of pooled cDNA from these samples were used to establish a standard curve, with dilutions 1:1, 1:3, 1:9, 1:27, 1:81, 1:243, and 1:729. No signal in the no-template control or the no-RT control exceeded cycle threshold levels for any qPCR run.

**Protein extraction**

Total protein was extracted from 30-60 mg of fresh frozen human DLPFC tissue. We used neuronal protein extraction reagent (N-PER) (Fisher Scientific, Hampton, NH, USA, Cat# PI87792) and Halt protease and phosphatase inhibitor cocktail plus 0.5 mM ethylenediaminetetraacetic acid (0.5mM EDTA) (Fisher Scientific, Hampton, NH, USA, Cat# PI78440) at 10 µL N-PER per 1 mg tissue in 1.5mL Axygen centrifuge tubes (Cat# MCT-175-C, Axygen). Tissues were homogenized on wet ice with hand-held Axygen tissue grinders (Fisher Scientific, Hampton, NH, Cat# 14 222 358) for 10 min using continual manual agitation. The homogenate was then incubated on wet ice for an additional ten minutes. Microcentrifuge tubes of homogenate were then centrifuged for 10 min at 10,000g at 4°C to pellet tissue debris and insoluble material. The supernatant was removed and stored in 1.5 ml low-protein binding centrifuge tubes (Fisher Scientific, Hampton, NH, USA, Cat# E925000090) at -80°C. Before use, the protein was quantified by bicinchoninic acid (BCA) assay. All samples were then diluted in N-PER with Halt protease and phosphatase inhibitor cocktail to a concentration of 2 mg/mL.

**Multiplex immunoassay**

For cytokine protein expression, multiplex immunoassays were performed. Multiplex immunoassays were run on a BioRad BioPlex 200 System (Bio-Rad, Hercules, CA, USA, Cat# 171000201). A custom 4-Plex Human Cytokine Assay Kit, including two 96-well plates, was used. The custom-designed assay targeted human cytokines IL6, IL1B, IL18, and TNF used existing probe antibodies from a 48-plex kit (Bio-Plex Pro Human Cytokine Screening Panel, Bio-Rad, Hercules, CA, USA, Cat #12007283). The assay used the 100-region bead map with a bead count of 50 beads per region. The sample timeout was set to 60 seconds. Multiplex immunoassays were run in the Molecular Core of SUNY Upstate Medical University, following the manufacturer’s protocol. Before running experimental samples, the measurement error (variation in values for repeated measurement of the same sample for each of the cytokines) was estimated. Samples (100 µg) were run in triplicate, and pooled internal controls were included in triplicate on each plate to monitor plate-to-plate variability. The samples used are reported in Supplementary Table 1. The kit provided lyophilized standards that were reconstituted in 250 µL in Standard Diluent HB (Bio-Rad, Hercules, CA, USA). Standards were diluted to ratios of 1:4, 1:16, 1:64, 1:256, 1:1024, 1:4096, 1:16384, 1:65536, and 1:262,144. The coefficient of variation (% CV) was calculated for triplicate readings of each
Samples that exceeded ± 15% CV (30% CV total) had one “outlier replicate” removed, which resulted in <± 15% CV from replicate measures for each sample.

**Statistical analysis**

Statistical tests of qPCR and multiplex immunoassay results were performed using SPSS statistics (Version 25, OSX, IBM, Armonk, NY, USA). Outliers by age group were identified using the ROUT test for outliers using GraphPad Prism (GraphPad Prism version 9.3.1 for Windows, GraphPad Software, San Diego, CA, USA) and then removed (~ 2 outliers per transcript). For TNF mRNA, 5 out of 11 samples in the neonate and infant groups were below the level of detection. Since the visual inspection of the data suggested the neonates and infants had similar levels of TNF mRNA (when detectable), the neonate and infant groups were combined for this analysis. Outliers were then removed using ROUT analysis. Data were tested for normality using Kolmogorov-Smirnoff testing and for homogeneity of variance using Levene's test. If the data were non-normally distributed, data were logged, and Cox Box transformed (Real Statistics Excel plug-in, Release 5.4. Copyright 2013 – 2018 Charles Zaiontz. www.real-statistics.com). Data were tested for correlation with pH, RIN, and PMI.

Data without covariates that were normally distributed were analyzed using ANOVA, and non-normally distributed data were analyzed using the Kruskal-Wallis test. Normally distributed data with covariates were analyzed by ANCOVA. Non-normally distributed data with covariates were analyzed using Quade’s ranked test. A significant ANOVA, ANCOVA, Kruskal-Wallis, or Quade’s ranked test was followed by a Fisher’s Least Significant Difference (LSD) posthoc test. Welch’s ANOVA was used for normally distributed data exhibiting non-homogenous variance between groups (99, 100), followed by the Games-Howell posthoc test. Sex differences were analyzed by independent sample 2-tailed t-tests for each transcript but could not be compared between age groups due to sample size limitations. All transcripts were tested for correlation with age under the age of 5 years old using Spearman’s ρ. Statistical significance was set at p ≤ 0.05.

**Results**

**Increases in IL1B mRNA and protein early in life**

*IL1B* mRNA levels (Fig. 1a) were at a nadir just after birth in the neonatal prefrontal cortex and increased during postnatal life (F(5,46) = 4.451, p = 0.002). For *IL1B* mRNA, levels increased approximately 95% between neonates and infants (p = 0.038) and almost 380% between infants and toddlers (p = 0.006), ultimately peaking with an 830% net increase from the lowest age group (Neonates) to the highest (Toddlers) (Fig. 1a). When examining ages ranging from 2 months to 5 years old more specifically, *IL1B* mRNA was found to be significantly positively correlated with age (Spearman’s rho, ρ = 0.580, p < 0.001) (Table 2). Mean *IL1B* mRNA levels were then reduced back to roughly infant levels in school-age children but were still significantly elevated compared to neonates through school-age children (p = 0.022) and
adolescence ($p = 0.008$). *IL1B* mRNA levels in young adults were intermediate compared to earlier ages and not significantly different from any other age group.

Analysis of protein also revealed significant increases in IL1B (Fig. 1b) levels across postnatal human life ($F(5,52) = 3.265, p = 0.012$) and resembled mRNA expression patterns overall. IL1B protein was at its lowest level in neonates and showed an approximate 70% increase in levels between neonates and infants ($p = 0.008$) (Fig. 1b). IL1B subsequently remained elevated, with toddler ($p = 0.002$), teenager ($p = 0.004$), and young adult ($p = 0.005$) levels of IL1B protein significantly higher than those for neonates. A significant linear increase in IL1B protein was detected in earlier age groups (ages 2 months old-5 years old) (Spearman's rho, $\rho = 0.479$, $p = 0.006$) (Table 2). Overall, the magnitude of changes in protein across age was not as great as those in the mRNA, never exceeding a 140% increase in the mean between any two groups.
Table 2. Correlation of cytokine protein and transcript expression levels with age under 5. Bolded transcripts and proteins are significantly correlated with age under 5.

However, there were some interesting differences in the pattern of change between the IL1B protein and mRNA, in that the plateau seen between ages 5-18 years old in the mRNA seemed to extend in the IL1B protein into the young adult group, whereas the mean IL1B mRNA levels dropped in the young adult group. In fact, the young adult IL1B protein level was still significantly increased by 100% compared to the IL1B protein level in neonates (p=0.005).

IL1R1 transcript, like its ligand IL1B, started with low expression levels, with a sharp increase in toddlers before returning to around neonatal levels during the school-age period (F(5,47) = 2.683, p = 0.033). IL1R1
mRNA levels significantly increased by 90%-100% from neonates and infants to toddlers (Neonates, p = 0.007; infants, p = 0.003) (Fig. 1c). *IL1R1* mRNA was significantly correlated with age during the first 5 years of life (Spearman's rho, ρ = 0.509, p = 0.003) (Table 2); however, *IL1R1* mRNA demonstrated a delayed increase compared to *IL1B* mRNA and was not significantly increased in the first year of life. At later ages, mean *IL1R1* mRNA was slightly, but not significantly, elevated to about 60–70% above neonatal levels (School-age children, p = 0.168; teenager, p = 0.131; young adult, p = 0.454). However, the cytoplasmic receptor accessory protein for *IL1R*, *IL1RAP*, did not change significantly between age groups (ANCOVA, RIN, pH; F(5,42) = 2.090, p = 0.086) (Fig. 1d). Similarly, *IL1RAP* did not correlate significantly with age under 5 (Spearman's rho, ρ = 0.175, p = 0.363).

**Increases in IL6 mRNA and protein both early in life and at maturation**

Similar to *IL1B*, *IL6* mRNA increased gradually, starting with low levels in neonates, which stabilized in the toddler age group and were sustained later in development. The first significant increase in *IL6* mRNA by age group (F(5,52) = 3.265, p = 0.012) was a 380% increase from neonates to toddlers (p = 0.003) (Fig. 2a), and *IL6* mRNA was moderately correlated with age within the first 5 years of life (Spearman's rho, ρ = 0.419, p = 0.033) (Table 2). After this, *IL6* mRNA showed an additional significant 410% increase in the expression of teenagers compared to infants (p < 0.019) and a 520% increase compared to neonatal levels. There was a slight subsequent reduction in *IL6* mRNA in young adults, which returned the mean expression to intermediate levels, increased by only 140% compared to neonates (p = 0.010).

Like *IL6* mRNA, IL6 protein increased gradually early in life before plateauing in toddlers. However, IL6 protein had an overall greater magnitude of mean change than *IL6* mRNA. As with *IL6* mRNA, IL6 protein was first significantly elevated in the toddler age group (F(5,44) = 2.585, p = 0.039), with an approximately 680% increase from neonatal levels (p = 0.006). Mean IL6 protein levels did start to increase in infants, which is reflected in the positive correlation of IL6 protein with age between 2 months and five years old (Spearman's rho, ρ = 0.474, p = 0.011) (Table 2). While the early life *IL6* mRNA increase was reflected in the IL6 protein level, the second period, where we found the highest mean *IL6* mRNA in teenagers, was not. Mean protein level showed no significant difference between school-age children and teenagers (p = 0.514) (Fig. 2b). Thus, the highest IL6 protein levels were detected during the school-age period, which did increase by a large amount, 1200%, compared to neonates (p = 0.004).

*IL6R* mRNA increased in a steadier fashion after birth (F(5,49) = 6.067, p < 0.001) before peaking in toddlers, then stabilized at relatively elevated levels, similar to *IL6* mRNA. When analyzed by age group, *IL6R* mRNA increased significantly by approximately 160% from neonates to toddlers (p < 0.001) and 90% from infants to toddlers (p < 0.001) (Fig. 2c). *IL6R* mRNA was significantly correlated with age between 2 months old and 5 years old (Spearman's rho, ρ = 0.542, p = 0.001) (Table 2). The initial increase in *IL6R* expression resembles both *IL6* mRNA and protein, but this change is of a smaller magnitude. *IL6R* expression remained elevated compared to both neonates and infants and school-age children (Neonates, p = 0.003; infants, p = 0.015), teenagers (Neonates, p = 0.002; infants, p = 0.010), and young
adults (Neonates, p = 0.003; infants, p = 0.018). Furthermore, unlike IL-6 mRNA, we did not find a mean increase in IL6R mRNA in teenagers.

In contrast to IL1R1 and IL6R, IL6ST mRNA did not change significantly across development (F(5,49) = 2.284, p = 0.061) (Fig. 2d). In spite of the modest and non-significant changes in gene expression represented by the group means, IL6ST mRNA was significantly correlated with age under 5 years old, suggesting that there is an early steady increase in expression for all three of the IL1/IL6 receptors studied (ρ = 0.498, p = 0.004) (Table 2).

**Increases in IL18 mRNA and protein, but not IL18R, with age**

As with IL1B and IL6, IL18 mRNA levels were lowest just after birth in neonates and increased early in postnatal life (F(5,48) = 4.053, p = 0.004) (Fig. 3a). Like IL1B, IL18 mRNA then decreased in school-age children. IL18 mRNA decreased once more in young adults. Thus, IL18 mRNA peaked in toddlers with a significant 200% increase from neonates (p < 0.001), with mean IL18 mRNA levels in infants falling between neonates and toddlers (p = 0.229). IL18 mRNA was also significantly increased in toddlers by 90% when compared to infants (p = 0.001) (Fig. 3a). When focusing on the three youngest developmental groups, a fairly strong linear correlation between age and IL18 mRNA was detected between 2 months old and 5 years old (Spearman's rho, ρ = 0.580, p < 0.001) (Table 2). The mean level of IL18 mRNA then decreased slightly in school-age children where it was still significantly elevated by 130% compared to neonates (p = 0.025). Mean IL18 mRNA levels were reduced to approximately neonatal levels in the young adult group (p = 0.129).

IL18 protein levels followed a similar pattern as their encoding transcript, increasing during postnatal life (Fig. 3b) (F(5,49) = 2.825, p = 0.026). Unlike IL18 mRNA, IL18 protein remained stably elevated in the teenager and young adult groups compared to neonates and infants (all p > 0.05). IL18 protein was elevated in toddlers by 50% compared to neonates (p = 0.046) and by 130% compared to infants (p = 0.043). There was a slight mean decrease in the school-age group which was not significantly different from any other age group (all p > 0.05).

While mean levels of IL18R1 mRNA appeared to increase over the age period studied, the change in transcript levels did not differ significantly with age (F(5,42) = 1.957, p = 0.105) (Fig. 3c). The levels of IL18R1 mRNA were quite variable at every age. Unlike other cytokines and receptors, IL18R1 mRNA was not significantly correlated with age when only considering those under 5 years old (Spearman's rho, ρ = 0.254, p = 0.232) (Table 2).

**Increases in TNFA and TNFR2, but not TNFR1, with age**

As with the three other cytokines studied, TNF mRNA levels were lowest earlier in postnatal life and significantly increased during development (F(4,40) = 5.104, p = 0.002) (Fig. 4a). TNF mRNA increased 360% between the neonate + infant group and the toddler group (p = 0.044). When focusing on changes within the first few years of life, no significant correlation was found between TNF mRNA and age (Spearman's rho, ρ = 0.421, p = 0.057) (Table 2). Similar to IL18, TNF decreased by 64% from toddlers to
school-age children, where it was not significantly different from neonatal levels (p = 0.650). Then, in a pattern distinct from all three other cytokines investigated, TNF mRNA increased again in teenagers, exceeding even toddler levels by 100% (Fig. 4a) (p = 0.046).

Levels of TNF protein resembled TNF mRNA levels during post-natal life, increasing significantly in toddlers and remaining elevated into young adulthood (Fig. 4b) (F(5,50) = 7.722, p < 0.001). The fluctuations found in TNF mRNA expression levels between toddlers, school-age children, and teenagers could not be detected in TNF protein levels, but mean levels were elevated in toddlers, school-age children, teenagers, and young adults compared to neonates (All p < 0.001) and infants (Toddler, p = 0.007; school-age children, p = 0.008; teenager, p < 0.001, young adult, p = 0.004) Fig. 4b). When focusing on changes within the first few years of life, a strong positive correlation was found between TNF protein and age (Spearman's rho, p = 0.555, p < 0.001). In agreement with TNF mRNA, mean TNF protein levels peaked in teenagers, which were increased by almost 430% compared to neonates (p < 0.001).

TNFRSF1A mRNA did not show any significant differences when analyzed by age group (F(5,50) = 0.111, p = 0.989) (Fig. 4c). TNFRSF1B mRNA levels were increased beginning in toddlers and remained elevated throughout young adulthood (F(5,48) = 2.763, p = 0.028). TNFRSF1B mRNA levels were increased approximately 60% on average when comparing infants to toddlers (p = 0.045), school-age children (p = 0.023), teenagers (p = 0.019), and young adults (p = 0.002). Neither TNFRSF1A mRNA (Spearman's rho, p = 0.085, p = 0.648) nor TNFRSF1B mRNA (Spearman's rho, p = 0.208, p = 0.261) levels were correlated with age under 5 years old (Table 2). The absence of correlation in TNFRSF1B with age under 5 years old, combined with the age group data, suggests an abrupt increase and peak in expression between 2–5 years old, which persists into young adulthood (Fig. 4d).

‘Anti-inflammatory’ cytokines increased during later stages of brain development

Messenger RNA for IL1RN, the receptor antagonist of IL1B, was not significantly changed between age groups (Welch's ANOVA, F(5,21.645) = 2.534, p = 0.059) (Fig. 5a), and this was reflected by a lack of a significant correlation with age under 5 (Spearman's rho, p = 0.343, p = 0.059) (Table 2). IL13 mRNA, in contrast, did change significantly between age groups (Welch's ANOVA, F(5,19.561) = 10.9232, p < 0.001). IL13 mRNA first increased 164% in toddlers relative to neonates (p = 0.006) (Fig. 5b) and was significantly correlated with age under 5 years old (Spearman's rho, p = 0.528, p < 0.001) (Table 2). IL13 mRNA levels peaked in young adults, where it increased by 237% relative to neonates (p = 0.014) and 118% relative to infants (p = 0.036). In contrast, IL10 mRNA was 74% higher in teenagers (Welch's ANOVA, F(5,22.036) = 3.232, p = 0.024) than it was in young adults (p = 0.049) (Fig. 5c). No clear changes could be detected in IL10 mRNA in earlier age groups, though toddlers had the (statistically non-significant) greatest mean expression level of IL10 mRNA. IL10 mRNA was also not correlated with age under 5 years old (Spearman's rho, p = 0.221, p = 0.233) (Table 2).

**Discussion**
In this study, we have characterized changes in mRNA and protein expression of cytokine and cytokine receptors in the developing human prefrontal cortex, with sufficient power to allow a quantitative rather than qualitative analysis. All cytokines studied were expressed in the post-natal human brain as both mRNA and protein (excepting the very little to no expression of TNF mRNA in some neonatal prefrontal cortices). We found that both transcript and protein levels of almost all pro-inflammatory cytokines were characterized by a steady increase under age 5, which was consistent with complement pathway factors previously reported in the same cohort (28). Given what we know about the interplay between complement and cytokines, we interpret that this concomitant increase could mean that these components of the immune system operate together or in a coordinated fashion to regulate normal development. However, following the initial increase in toddlers, the four pro-inflammatory cytokines studied here diverged in expression during brain maturation. IL1B and IL18 decreased in transcription in the school-age children and young adult age groups as predicted, similar to the changes previously seen in complement expression (28). In contrast, IL6 and TNF expression, increased a second time during adolescence. Thus, we saw peaks in cytokine expression during two distinct periods of development.

Previously, we found that complement inhibitor expression increased after complement peaked in toddlers, and that complement expression subsequently decreased (28). In contrast, here we found that the inhibitory cytokines IL1RN or IL10 were not as dynamically expressed across development as the inhibitors of complement we previously characterized. The only cytokine inhibitor to show any change in expression was IL13. As IL13 inhibits proinflammatory cytokines (101–103), the increase in IL13 mRNA in adolescence may represent regulatory pressure on proinflammatory cytokine expression in that age group. However, IL13 can also regulate the effects of complement-mediated lysis in the periphery by inhibiting the downstream effects of the membrane attack complex (104), raising the possibility that elevated IL13 may be more involved in the regulation of complement pathway activity than in IL6 or TNF signaling.

As with cytokines, certain cytokine receptors varied across development. In particular, IL1R1, IL6R and TNFRSF1B expression changed between age groups. Notably, when changes were found in receptor expression, those changes approximated the pattern of their associated cytokine ligand. IL1R1, for instance, increased in toddlers and decreased in older age groups. IL6R and TNFRSF1B, like IL6 and TNF protein and mRNA, increased in toddlers and remained relatively elevated during late development, including adolescence. Other receptors, IL1RAP, IL18R, IL6ST, and TNFRSF1A remained stable throughout the first 2 decades of postnatal life. These results suggest that, like cytokines, the trajectory of cytokine receptor expression across development is not uniform, and that increases in ligand and receptor pairs are more often concomitantly increased when they activate signaling rather than inhibit it.

The evidence presented here suggests that cytokine expression is upregulated during two major periods in human development: early neurodevelopment, when processes such as interneuron migration (71, 72) and synaptogenesis (81, 82) dominate brain development; and late neurodevelopment, which is associated with synaptic pruning (81, 82), myelination (89, 90), and final GABAergic system maturation (105, 106). Cytokines have the potential to affect these processes based on both the timing reported here,
and on effects previously reported in the literature. For instance, IL1B has been previously associated with neuronal migration (41) and the survival of differentiating oligodendrocytes (107) in vitro and in vivo. Based on the timing of the peak in IL1B, it is interesting to consider whether IL1B during postnatal development may be associated with maturation from preoligodendrocyte to mature oligodendrocyte, which drops around age 5 in humans (108). Similarly, the adolescent peak in IL6 and TNF mRNAs could be associated with maturation of neurotransmitter systems, i.e. the GABAergic system. The development of GABAergic signaling is a known developmental process that is estimated to extend into adolescence (105, 106). Both IL6 and TNF are known regulators of GABA receptor levels through endocytosis of GABA receptors at the synapse (109, 110). It is interesting to consider whether increased expression in IL6 and/or TNF could contribute to the normal regulation of GABA receptor levels at the synapse in adolescents. We speculate that exaggerated increases in either IL6 or TNF during maturation may throw off normal homeostasis.

The adolescent increase in IL6 and TNF may be significant when considered in light of the age of onset for schizophrenia, which commonly occurs just after adolescence. Notably, increased IL6 mRNA and protein levels are one of the most robust changes found in the brain and blood of people with schizophrenia (111–121). There is increasing evidence of the role of the immune system in neurodevelopmental psychiatric disorders. Epidemiological work in humans suggests that immune activation during gestation and early childhood is associated with the occurrence of neurodevelopmental disorders, in particular the development of ASD, ADHD (Attention-decit/hyperactivity disorder), and schizophrenia (23, 122–126). Immune activation during development may have short- and long-term impacts on brain development, potentially by disrupting immune factor levels directly during development (127), by inducing changes that affect the systemic response to environmental triggers later in life (e.g. (128–130)), and/or by inducing long-term changes in immune signal expression (50, 113, 128, 131). In this study, we have found evidence that cytokine expression is also active and regulated well after the fetal period in humans, from birth through young adulthood, indicating that cytokines likely have a role in normal postnatal human cortical development. Thus, the dysregulation of cytokine expression during early postnatal life could similarly disrupt brain development through direct or indirect effects on developmental processes. We can use this new knowledge of regulated cytokine expression in the postnatal human prefrontal cortex to expand upon the interaction between cytokine expression, specific brain development processes, and behavioral outcomes using experimental data. In particular, future studies utilizing targeted knockdown, knockout, and/or overexpression of these cytokines with inducible rodent models may allow us to assess the contribution of specific cytokines to development more directly. For instance, by inhibiting a given cytokine during its period of elevated expression reported here, it may be possible to tie normal cytokine activity to particular processes in postnatal development. Characterizing the normal contribution of cytokines to development will help clarify both the effects of abnormal inflammation/cytokine expression on developmental processes, and whether those effects relate to distinct periods of vulnerability.

Unlike mRNA, cytokine proteins circulate freely in the blood serum under physiological conditions. Unfortunately, studies regarding the expression patterns of cytokines in the serum at different
developmental ages in humans are somewhat limited. In the case of IL1B, IL6, IL18, and TNF, a comparison of the protein levels detected in this study to serum protein levels reported under physiological conditions during childhood (132) suggests that the contamination of tissue with circulating cytokines would not fully explain the changes in cytokine protein expression seen in this study. The reported pattern of IL18 blood serum protein expression, for instance, found that IL18 was highest between ages 1–7 years old and lowest in samples over 18 years old (132). IL18 brain protein levels were lowest in neonates and infants, so a contribution from the serum would presumably artificially inflate apparent IL18 protein concentrations in this age group. If anything, excluding any serum protein contribution from the results in our study, would emphasize the difference between age groups by decreasing neonate and infant levels to diverge further from teenager and young adult levels. In short, serum protein levels may contribute to the total protein levels reported in this study; however, the patterns of protein expression reported herein were largely consistent with corresponding mRNA expression patterns and did not strongly resemble existing reports of serum cytokine levels during human development.

**Conclusion**

This work provides a foundation for understanding the normal changes in levels of cytokine, cytokine receptors, and inhibitors from early postnatal neurodevelopment to maturation in the human cortex. Our results suggest that there may be two distinct time windows (toddlers and teenagers) when cytokine signaling takes a more salient role in human cortical development, both of which are associated with an increased incidence of major psychiatric disorders. The increases seen in cytokine expression for *IL1B*, *IL6*, *IL13*, *IL18*, and *TNF* between birth and age 5 suggest a significant action around the time of onset of ASD. These results highlight toddlerhood not just as an overall transformative period in the brain but as a period of development strongly tied to immune-associated cytokine signaling as well as complement signaling. Given that late adolescence is considered a period of vulnerability to psychiatric disorders, especially schizophrenia, the role of IL6 and TNF in adolescent cortical development needs further consideration and study. Additionally, since adolescence is normally not associated with increased complement levels but rather is associated with increased complement inhibition, this suggests that increased cytokines may be acting independently of complement at maturation and without associated transcriptional increases in major cytokine inhibitors.

**Abbreviations**

ADHD
attention-deficit/hyperactivity disorder
ASD
Autism Spectrum Disorder
BCA
bicinchoninic acid
EDTA
ethylenediaminetetraacetic acid
N-PER
Neuronal Protein Extraction Reagent
LSD
Least Significant Difference
PMI
post-mortem interval
RIN
RNA integrity number
SIDS
Sudden Infant Death Syndrome

**Declarations**

_Ethical approval and consent to participate_

Experiments involving human tissue were approved by the University of New South Wales Human Research Ethics Committee (HC16441).

_Consent for publication_

All authors consent to publication.

_Availability of data and materials_

All data is available upon request.

_Competing interests_

The authors declare no competing interests.

_Funding_

The NSW Ministry of Health, Office of Health and Medical Research provided funding for this work. CSW is a recipient of a National Health and Medical Research Council (Australia) Principal Research Fellowship (2009237). SUNY Upstate Medical University provided stipend support (R.E.H.S) and their Hendricks Intramural Pilot Grant (C.S.W.).

_Authors’ contributions_

Acknowledgements

We would like to thank the University of Maryland Brain and Tissue Bank, and the families of donors for providing human samples.

Tables


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Figures
**IL6 and IL1B mRNA and protein expression**

*a.* *IL1B* mRNA was significantly different between age groups (ANCOVA, pH; $F(5,46)=4.451$, $p=0.002$). Post-hoc testing (Fisher’s LSD) found differences between neonates and infants ($p=0.038$); neonates and toddlers ($p<0.001$); neonates and school-age children ($p=0.022$); neonates and teenagers ($p=0.008$); infants and toddlers ($p=0.006$); and toddlers and young adults ($p=0.011$). pH contributed significantly to the model ($F(1,46)=5.850$, $p=0.020$). The variance between age groups was non-homogenous (Levene’s, $F(5,47)=4.172$, $p=0.003$). N=53; 4 outliers were removed for this transcript.
b. IL1B protein was significantly different between age groups (ANOVA, F(5,52)=3.265, p=0.012). Post-hoc testing (Fisher's LSD) found differences between neonates and infants (p=0.008); neonates and toddlers (p=0.002); neonates and teenagers (p=0.004); and neonates and young adults (p=0.005). N=58; 1 outlier was removed for this protein.

c. IL1R1 mRNA was significantly different between age groups (ANCOVA, PMI, RIN; F(5,47)=2.683, p=0.033). Post-hoc testing (Fisher's LSD) found significant differences between neonates and toddlers (p=0.007) and infants and toddlers (p=0.003). RIN did not contribute significantly to the model. pH contributed significantly to the model (F(1,47)=5.351, p=0.025). N=55; 2 outliers were removed for this transcript.

d. IL1RAP mRNA was not significantly different between age groups (ANCOVA, RIN, pH; F(5,42)=2.488, p=0.645). RIN contributed significantly to the model (F(1,42)=4.929, p=0.032). N=50. No outliers were removed for this transcript.

*≤0.05; **≤0.01; ***≤0.001 for all images. Colored asterisks denote comparisons to age groups with a shared color scheme.

The horizontal line represents the mean, and the bars represent SEM.
Figure 2

**Expression levels of IL6 and receptors**

a. *IL6* mRNA was significantly different between age groups (ANCOVA, RIN; F(5,40)=4.062, p=0.004). Post-hoc testing (Fisher's LSD) found differences between neonates and toddlers (p=0.003); neonates and school-age children (p=0.003); neonates and teenagers (p<0.001); neonates and young adults (p=0.010); and infants and teenagers (p=0.019). RIN contributed significantly to the model.
(F(1,40)=5.492, p=0.024). N=47; 4 samples were undetectable; 6 outliers were excluded from the full cohort for this transcript.

b. IL6 protein was significantly different between age groups (ANOVA, F(5,44)=2.585, p=0.039). Post-hoc testing (Fisher’s LSD) found differences between neonates and toddlers (p=0.006); neonates and school-age children (p=0.004); neonates and teenagers (p=0.031); and neonates and young adults (p=0.045). N=50; 9 outliers were removed for this protein.

c. IL6R mRNA was significantly different between age groups (ANCOVA, pH; F(5,49)=6.067, p<0.001). Post-hoc testing (Fisher’s LSD) found significant differences between neonates and toddlers (p<0.001); neonates and school-age children (p=0.003); neonates and teenagers (p=0.002); neonates and young adults (p=0.003); infants and toddlers (p>0.001); infants and school age (p=0.013); infants and teenagers (p=0.010); and infants and young adults (p=0.018). pH did not contribute significantly to the model. The variance between groups was non-homogenous (Levene’s, F(5,50)=4.319, p=0.002). N=56; 1 outlier was removed for this transcript.

d. IL6ST was not significantly different between age groups. (ANCOVA, PMI, pH; F(5,49)=2.284, p=0.061). PMI did not contribute significantly to the model. RIN contributed significantly to the model (F(1,49)=8.859, p=0.005). The variance was non-homogenous between age groups (Levene’s, F(5,51)=2.444, p=0.046). N=57; no outliers were removed for this transcript.

*≤0.05; **≤0.01; ***≤0.001 for all images. Colored asterisks denote comparisons to age group with a shared color scheme. The horizontal line represents the mean, and the bars represent SEM.
**IL18 mRNA, protein, and receptor expression**

*a. IL18 mRNA was significantly different between age groups (ANCOVA, pH; F(5,48)=4.053, p=0.004). Posthoc testing (Fisher’s LSD) found significant differences between neonates and toddlers (p<0.001); neonates and school-age children (p=0.030); neonates and teenagers (p=0.025); infants and toddlers*
(p=0.001), and toddlers and young adults (p=0.010). pH contributed significantly to the model (F(1,48)=10.318, p=0.002). The variance was non-homogenous between age groups (Levene's, F(5,49)=3.168, p=0.015). N=55; 1 sample was undetectable for this transcript; one outlier was removed.

b. IL18 protein was significantly different between age groups (ANCOVA, pH; F(5,49)=2.825, p=0.026). Posthoc testing (Fisher's LSD) found significant differences between neonates and toddlers (p=0.046); neonates and teenagers (p=0.005); neonates and young adults (p=0.047); infants and toddlers (p=0.043); infants and teenagers (p=0.004); and infants and young adults (p=0.044). N=56; 3 outliers were removed for this protein.

c. IL18R1 mRNA was not significantly different between age groups (ANCOVA, PMI; F(5,42)=1.957, p=0.105). PMI contributed significantly to the model (F(1,42)=4.856, p=0.033). N=49; 7 samples were undetectable for this transcript; 1 outlier was removed.

*≤0.05; **≤0.01; ***≤0.001 for all images. Colored asterisks denote comparisons to age group with a shared color scheme.

The horizontal line represents the mean, and the bars represent SEM.
Expression levels of *TNF* mRNA, protein, and receptors

**Figure 4**

**a.** *TNF* mRNA was significantly different between age groups (ANCOVA, RIN; F(4,40)=5.104, p=0.002). Posthoc testing (Fisher’s LSD) found significant differences between the neonate-infant combined group and toddlers (p=0.044); the neonate-infant combined group and teenagers (p<0.001); the neonate-infant combined group and young adults (p=0.025); toddlers and teenagers (p=0.046); school-age children and teenagers (p=0.015); and teenagers and young adults (p=0.047). RIN did not contribute significantly to the model. N=46; 7 samples were undetectable for this transcript; 4 outliers were removed.

**b.** *TNF* protein

**c.** *TNFRSF1A* mRNA

**d.** *TNFRSF1B* mRNA
b. TNF-α protein was significantly different between age groups (ANCOVA, pH; F(5,50)=7.722, p<0.001). Posthoc testing (Fisher’s LSD) found significant differences between neonates and toddlers (p<0.001); neonates and school-age children (p<0.001); neonates and teenagers (p<0.001); neonates and young adults (p<0.001); infants and toddlers (p=0.007); infants and school-age children (p=0.008); infants and teenagers (p<0.001); and infants and young adults (p=0.004). pH did not contribute significantly to the model. The variance was non-homogenous between groups (Levene’s, F(5,51)=3.019, p=0.018). N=57; 2 outliers were removed for this protein.

c. **TNFRSF1A** mRNA was not significantly different between age groups (ANOVA; F(5,50)=0.111, p=0.989). N=56; 1 outlier was removed for this transcript.

d. **TNFRSF1B** mRNA was significantly different between age groups (ANCOVA, RIN; F(5,48)=2.763, p=0.028). Posthoc testing (Fisher’s LSD) found significant differences between neonates and young adults (p=0.052); infants and toddlers (p=0.045); infants and school-age children (p=0.023); infants and teenagers (p=0.019); and infants and young adults (p=0.002). RIN contributed significantly to the model (F(1,48)=13.325, p=0.001). N=55; 2 outliers were removed for this transcript.

*≤0.05; **≤0.01; ***≤0.001 for all images. Colored asterisks denote comparisons to age group with a shared color scheme. Bars represent standard error of the mean. The horizontal line represents the mean, and the bars represent SEM.
Figure 5

Expression levels of cytokine inhibitors

a. *IL1RN*mRNA level was not significantly different between age groups (Welch's ANOVA; F(5,21.645)=2.534, p=0.059). The variance was non-homogenous between groups (Levene's, F(5,49)=3.257, p=0.013). N=55.
b. *IL13* mRNA was significantly different between age groups (Welch's ANOVA; F(5,19.561)=10.9232, p<0.001). Posthoc testing (Fisher's LSD) found significant differences between neonates and toddlers (p=0.006), neonates and young adults (p=0.014), infants and young adults (p=0.036). The variance was non-homogenous between groups (Levene's, F(5,49)=3.101, p=0.016). N=55; 2 outliers were removed for this transcript.

c. *IL10* mRNA was significantly different between age groups (Welch's ANOVA; F(5,22.036)=3.232, p=0.024). Posthoc testing (Fisher's LSD) found significant differences between teenagers and young adults (p=0.049). The variance was non-homogenous between groups (Levene's, F(5,49)=3.780, p=0.006). N=55; 2 outliers were removed for this transcript.

*≤0.05; **≤0.01; ***≤0.001 for all images. Colored asterisks denote comparisons to age group with a shared color scheme. The horizontal line represents the mean, and the bars represent SEM.

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