INNATE IMMUNE SYSTEM REACTIVITY TO AN IMMUNE CHALLENGE: EFFECTS OF EARLY LIFE STRESS, ACUTE STRESSORS, PARENTAL RESPONSIVENESS, AND EARLY INTERVENTION

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology

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ABSTRACT

Early life stress is associated with numerous diseases in adulthood that may be caused by dysregulation of pro-inflammatory and anti-inflammatory immune responses. The current study investigated contributions of early life stress, acute stress, and parental nurturance on innate immune functioning after the administration of the influenza vaccine. The influenza vaccine produced an elevation in CRP and IL-10 levels in children aged 8 to 11 years, but no significant elevation in IL-6 levels was observed. Early life stress did not significantly affect the trajectory of any cytokine after the influenza vaccine. Likewise, acute stress, or daily hassles, did not significantly affect cytokine production following the vaccine among children regardless of exposure to early life stress. In addition, parental nurturance did not significantly alter the innate immune system response following the influenza vaccine for either risk group. Among children who experienced high levels of early life stress, cytokine trajectory after receiving the influenza vaccine did not differ significantly between children who received an attachment based intervention, Attachment and Biobehavioral Catch-Up, and those who received a control intervention.

Chapter 1

INTRODUCTION

Individuals who experience early life stress are at increased risk of developing various diseases in adulthood, including diabetes, obesity, autoimmune diseases and cardiovascular disease (Melchior, Moffitt, Milne, Poulton, & Caspi, 2007; Poulton et al., 2002; Repetti, Taylor, & Seeman, 2002; Shonkoff et al., 2012). Many of these diseases in adulthood are associated with atypical levels of immune system markers (Slopen, Koenen, & Kubzansky, 2012). Levels of these markers are related to retrospective reports of early life stress, suggesting that immune dysregulation in early childhood may lead to later disease states (Danese & McEwen, 2012; Fagundes, Glaser, & Kiecolt-Glaser, 2013). Although the effects of early stress on adult immune system functioning are well documented, there are fewer studies documenting the effects of early stress on immune system function during childhood. Developing an understanding of how early life stress affects immune system function during childhood represented the first aim of this study. Acute stress is also associated with adult immune system functioning (Steptoe, Hamer, & Chida, 2007). Early life stress may sensitize the immune system to dysregulation from acute stress (Miller & Chen, 2010). The second aim of this study was to investigate whether acute stressors in childhood interact with early life stress to affect immune system functioning during childhood. Other research indicates that that high-quality parenting can buffer children's physiological systems and processes from the effects of stress (Blair & Raver, 2012). The third aim examines whether high-quality parenting affects immune

system functioning in childhood among children who experienced early life stress. Lastly, Attachment and Biobehavioral Catch-up, an intervention that enhances parenting quality, regulates biological functioning in children who have experienced early adversity (Bernard, Dozier, Bick, & Gordon, 2015a). The final aim investigated whether this intervention also regulates immune system functioning in childhood.

1.1 Disease and Dysregulated Immune Function

Early life stress is associated with an increased risk for various diseases in later life (Poulton et al., 2002; Repetti et al., 2002; Shonkoff et al., 2012). Many of these diseases (i.e. diabetes, obesity, autoimmune diseases, and cardiovascular disease) are associated with dysregulation of typical immune responses. For example, autoimmune disorders, such as Type-1 diabetes, multiple sclerosis, rheumatoid arthritis and lupus, occur when the immune response attacks healthy cells within the body (Castano & Eisenbarth, 1990; Firestein, 2003; Wong, Ho, Li, & Lam, 2000). Autoimmune disorders are also associated with alterations in apoptosis mediated by the immune system, indicating that diseases are affected when the immune system does not properly regulate itself following an immune response (Thompson, 1995). Cardiovascular disease and atherosclerosis are also associated with higher levels of pro-inflammatory cytokines, including interleukin-6, tumor necrosis factor-alpha (TNF-α) and C-reactive protein (CRP) (Slopen et al., 2012). Abnormal levels of these immune markers are also associated with obesity (Black, 2003). In sum, many diseases associated with early life stress exhibit dysregulated immune systems in adulthood.

1.2 The Innate Immune System

The immune system can be conceptualized as two distinct systems: the adaptive immune system and the innate immune system. The adaptive immune system develops specific responses to distinct pathogens (Iwasaki & Medzhitov, 2010). The innate immune system is an evolutionarily conserved system that immediately reacts in a non-specific manner to the presence of an injury or pathogen (Beutler, 2004; Medzhitov & Janeway, 1997). Cells involved in the innate immune system release cytokines, or cell signaling proteins, that recruit other components of the innate and adaptive immune system (Iwasaki & Medzhitov, 2010; Medzhitov & Janeway, 1997). Pro-inflammatory and anti-inflammatory cytokines act in concert to respond to the presence of a pathogen (e.g. bacteria or virus). Pro-inflammatory cytokines upregulate processes leading to cell death, whereas anti-inflammatory cytokines resolve this immune response when appropriate (Dinarello, 2000). Additional biological systems, including the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system, also regulate the innate immune system's response to pathogens (Elenkov & Chrousos, 2002; Padgett & Glaser, 2003).

The innate immune system involves a complex, coordinated response between multiple cell types and many immune molecules. The current study examines the following immune molecultes: interleukin-6 (IL-6), interleukin-10 (IL-10), and C-Reactive Protein (CRP). IL-6 is a critical multifunctional, pro-inflammatory cytokine that regulates the acute phase response of the innate immune system (Akira, Hirano, Taga, & Kishimoto, 1990; Heinrich, Castell, & Andus, 1990; Kishimoto, 1989; Van Snick, 1990). IL-6 regulates the production of acute phase proteins, including CRP, serum amyloid A, and fibrinogen (Heinrich et al., 1990; Van Snick, 1990). IL-6 plays an important role in the development of adaptive immunity because it induces the

proliferation and differentiation of human T cells and immunoglobulins (Van Snick, 1990). Overproduction of IL-6 is associated with the production of myeloma and lymphoma cells, cardiovascular heart disease, hypertension and autoimmune diseases, such as rheumatoid arthritis (Cesari et al., 2003; Ishihara & Hirano, 2002; Kishimoto, 1989; Niskanen et al. 2004; Van Snick, 1990). Increased levels of IL-6 predicted mortality over 5 years in a sample of rural adults, independent of other risk factors, including vascular disease and smoking (Harris, et al. 1999). IL-6 has also been associated with mental health in adulthood, with circulating levels of IL-6 higher in adults with depression than adults without a mood disorder (Howren, Bryant, Lamkin, & Suls, 2009).

C-Reactive Protein (CRP) is an acute phase immune molecule that is produced rapidly after an immune challenge is detected (Black, Kushner, & Samols, 2004). Functionally, CRP has both pro-inflammatory and anti-inflammatory effects. CRP stimulates and increases the expression of other pro-inflammatory cytokines, including IL-1, IL-6, IL-18 and TNFα, and other anti-inflammatory cytokines, such as IL-10 (Black et al., 2004). In addition to cytokine expression, CRP is also noted to alter cellular processes associated with the pro-inflammatory response, such as enhancing phagocytosis and upregulating expression of adhesion molecules (Black et al., 2004). Elevated levels of CRP have specifically been associated with various disease states, including obesity, cardiovascular disease, and depression (Howren et al., 2009; Miller, Maletic, & Raison, 2005; Yudkin, Stehouwer, Emeis, & Coppack, 1999).

Interleukin-10 is an anti-inflammatory cytokine that regulates the innate immune response by inhibiting the production of pro-inflammatory cytokines, including IL-6 (Fiorentino, Zlotnik, Mosmann, Howard, & O'Garra, 1991). After an

ex vivo exposure of human monocytes to LPS, IL-10 was released hours after cytokines involved in the acute phase response, and inhibited production of IL-6 (de Waal Malefyt, Abrams, Bennett, Figdor, & de Vries, 1991). This delayed effect of IL-10 helps to limit the duration of inflammatory responses in general (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). Like IL-6, IL-10 is also involved in hematopoiesis of T cells and B cells (Moore et al., 2001). The association between IL-10 and disease is complex. The anti-inflammatory role of IL-10 has important implications for host defense. IL-10 deficient mice exhibit colitis, or inflammation of the bowel. However, IL-10 is upregulated in patients with rheumatoid arthritis and lupus (Ouyand, Rutz, Crellin, Valdez, & Hymowitz, 2011).

1.3 Approaches to Study the Innate Immune Response

Several methods have been used to study processes of adaptive and innate immunity in humans. Many research paradigms involve using a vaccine as an immune challenge in order to study developed, adaptive immunity (Carrat et al., 2008; Steptoe et al., 2007). After administering a vaccine, blood is typically drawn between two to eight weeks later, when the antibodies generated against the vaccine can be measured. In order to investigate innate immune function, which reacts in a much shorter timeframe, blood samples may be exposed to an immune challenge *ex vivo*. In such a design, a sample of blood may be exposed to lipopolysaccharide (LPS), a marker found on the cell wall of many bacteria (Steptoe et al., 2007). However, *ex vivo* studies cannot account for the effects of autonomic and neuroendocrine systems on the immune system (Sanders & Kavelaars, 2007). Thus, using an *in vivo* exposure to a pathogen provides a more comprehensive understanding of how the immune response of an organism reacts to a pathogen as a system.

The influenza vaccine serves as an immune challenge that activates the innate immune system *in vivo*. In healthy adults, exposure to the influenza vaccine is associated with a rise in IL-6, IL-10, and CRP one day after immunization (Tsai et al., 2005). Similarly, H1N1 influenza vaccinations were associated with increases in IL-10. Among individuals who naturally contracted HINI, levels of IL-6 and IL-10 were higher among those experiencing mild symptoms than among healthy individuals (Yu et al., 2011).

1.4 Early Life Stress, Acute Stressors and the Innate Immune System

Early life stress is associated with maladaptive changes in a variety of biological systems, including the HPA axis, brain structure, and function (Bernard, Butzin-Dozier, Rittenhouse, & Dozier, 2010; Glaser, 2000; Lupien, McEwen, Gunnar, & Heim, 2009; Tarullo & Gunnar, 2006; Tottenham et al., 2010). Early life stress is also associated with differences in the innate immune system across the life course (Baumeister, Akhtar, Ciufolini, Pariante, & Mondelli, 2016; Miller, Chen, & Parker, 2011a). In a sample of older adults undergoing current chronic stress (caregiving for a family member with dementia), those who had experienced maltreatment in childhood had higher levels of baseline circulating IL-6 than caregivers who had not experienced maltreatment (Kiecolt-Glaser et al., 2011). At age 32, adults who had experienced maltreatment in early childhood were more likely to have clinically significant CRP levels than adults who had not experienced maltreatment (Danese, Pariante, Caspi, Taylor, & Poulton; 2006). The association between early life stress and circulating markers of inflammation is also observable in infancy; socioeconomic disadvantage and maternal psychosocial stress were associated with elevated CRP levels among 17-month-old infants (David, Measelle, Ostlund, & Ablow, 2017).

Early life stress also alters the innate immune system's response to an immune challenge. Miller and colleagues (2009) stimulated peripheral mononuclear blood cells (PMBCs) with ligands of toll-like receptors (a variety of proteins found on cell surfaces of bacterial and viral cells) to study the effects of socioeconomic status (SES) on innate immune system response (Miller et al., 2009). In adults who were reared in low-SES households, IL-6 levels increased significantly more in response to stimulation than those of adults who were reared in high-SES households (Miller et al., 2009). In a population study of children in the Avon Longitudinal Study of Parents and Children, children who experienced stressors before age 8 (e.g., maltreatment, separation from a caregiver) had higher levels of circulating IL-6 and CRP at age 10 than children who had not experienced a stressor (Slopen, Kubzansky, McLaughlin, & Koenen, 2013). In late adolescence, retrospective reports of a harsh family climate in early life were associated with increasing LPS-stimulated IL-6 levels across the span of 2 years, indicating that dysregulation of the immune system due to early life stress may increase over time (Miller & Chen, 2010). This line of research suggests that the response of the immune system to an *in vivo* immune challenge may be altered by exposure to early life stress.

Acute stressors, or brief, naturalistic, non-catastrophic stressors, are also associated with immediate activation of the innate immune system (Steptoe et al., 2007). Levels of circulating IL-6 increase after adults complete public speaking tasks (Buske-Kirschbaum, Kern, Ebrecht, & Hellhammer, 2007; Segerstrom & Miller, 2004). IL-10 levels decrease after adults experience stressors like failing an exam (Segerstrom & Miller, 2004). Largely, acute psychosocial stress does not appear to alter CRP production in adulthood, with a meta-analysis showing only a marginal

effect of acute psychosocial stress altering circulating levels of CRP (Steptoe et al., 2007). However, greater interpersonal stress during a 2-week period was associated with higher levels of circulating CRP in late adolescence (Fuligni et al., 2009).

Exposure to chronic stress or early life stress may moderate the response of the innate immune system to an acute stressor. Among adults experiencing chronic caregiving stress, more frequent daily stressors are associated with higher circulating levels of CRP and IL-6, though daily stressors are unrelated to cytokine levels among adults who do not have caregiving stress (Gouin, Glaser, Malarkey, Beversdorf & Kiecolt-Glaser, 2012). Adults who reported experiencing childhood trauma had a greater IL-6 response to a public speaking task than adults who did not experience trauma during early childhood (Carpenter et al., 2010; Pace et al., 2006). Living in a harsh family climate in early life moderates the association between acute stressors and levels of IL-6 when PMBCs are exposed to LPS. IL-6 production was higher among young adults raised in harsh climates if they had experienced an acute stressor within the past 6 months than if they had not experienced such an event. Among young adults who were not raised in a harsh family climate, there was no association between acute stressors and stimulated IL-6 response (Miller & Chen, 2010). Research regarding acute stressors and the immune system primarily consists of studies using adult participants. However, among children aged 9 to 18 diagnosed with asthma, a "double exposure" of chronic family stress and acute stress was associated with increased stimulated production of IL-4, IL-5, and IFN-γ (Marin, Chen, Munch & Miller, 2009). Thus, levels of CRP, IL-6 and IL-10 in children may be similarly altered by acute stress. Further, as found by Marin and colleagues (2009), acute stress

may only affect cytokine levels among children who have experienced chronic forms of stress.

1.5 Positive Parenting and the Innate Immune System

Early life stress is an important predictor of biological dysregulation (Tarullo & Gunnar, 2006). However, positive parenting practices in early childhood and adolescence may serve as a buffer between the child and negative environmental events. In fact, responsive caregiving is associated with biological functioning across a number of indicators of stress during childhood, including morning cortisol value in low-risk infants, allostatic load among high-risk adolescents, and telomere length in high-risk children (Asok, Bernard, Roth, Rosen, & Dozier, 2013; Evans, Kim, Ting, Tesher, & Shannis, 2007; Spanglar, Schieche, Ilg, Maier, & Ackermann, 1994).

Retrospective reports of caregiving quality are associated with immune functioning in adulthood. Chen et al. (2011) assessed cytokine production in stimulated PMBC samples of adults who were raised in low-SES households. High maternal warmth in childhood, as reported by the participant, was associated with a lower stimulated IL-6 response than low maternal warmth (Chen, Miller, Kobor, & Cole, 2011). Adults reared in low-SES households who reported experiencing low levels of maternal nurturance had more indices of metabolic syndrome than adults who were reared in low-SES households who reported experiencing high levels of maternal nurturance and adults who were reared in high-SES households (Miller et al., 2011b). In a rodent model, enhanced maternal care in early life was associated with increased IL-10 production in the nucleus accumbens when compared to rodents that did not receive enhanced maternal care. Moreover, IL-10 production after exposure to morphine was decreased in the enhanced maternal care condition (Schwarz,

Hutchinson, & Bilbo, 2011). Among children, the parent-child relationship may alter functioning of the immune system. Parental psychosocial stress is associated with circulating levels of salivary CRP of infants, such that higher levels of parental psychosocial stress are associated with higher levels of infant salivary CRP (David, et al., 2017). Attachment relationships in early childhood are also associated with circulating CRP levels: children with disorganized attachments exhibit higher levels of circulating salivary CRP in infancy than children with secure or insecure attachments (Measelle, David & Ablow, 2017). Moreover, CPS-referred children who had insecure or disorganized attachments to their caregivers had higher levels of circulating CRP in childhood than securely attached, CPS-referred children and low-risk children (Bernard, Hostinar, & Dozier, under review). These findings suggest that maternal care early in life may alter cytokine levels in middle childhood. Rodent models and stimulation studies suggest that maternal care may also attenuate the response of the innate immune system during an immune challenge.

1.6 Increasing positive parenting through intervention: Attachment and Biobehavioral Catch-up

Given that parental warmth and responsiveness appear to buffer biological systems (including the innate immune system) from environmental stress, an intervention geared towards improving positive parenting characteristics may alter immune system functioning. Attachment and Biobehavioral Catch-up (ABC) is a 10 session intervention for families of children who have experienced various forms of early adversity, including maltreatment and institutional care (Dozier, Roben, Caron, Hoye, & Bernard, 2018). The ABC intervention focuses on increasing parents' nurturance, responsiveness, and decreasing frightening behaviors (Dozier & The

Infant Caregiver Project Lab, 2012). CPS-referred children who received the ABC intervention were more likely to develop secure attachments to their parents than children who received a control intervention (Bernard et al., 2012). In addition, children whose parents received ABC had more normalized diurnal cortisol patterns than children whose parents received a control intervention (Bernard et al., 2015a). This pattern of normalized cortisol was present at a follow-up visit conducted at age 5, indicating that an intervention delivered in early childhood may have pervasive effects on biological systems (Bernard, Hostinar, & Dozier, 2015b). As glucocorticoids play a regulatory role in the immune system, the ABC intervention was expected to be efficacious in normalizing cytokine responses to an immune challenge in addition to diurnal cortisol levels.

1.7 Present Study

The present study used an influenza vaccination as an *in vivo* immune challenge to assess the effects of (1) early life stress, (2) reported acute stressors, (3) observed parenting behaviors, and (4) early intervention on the function of the innate immune system in middle childhood. The first aim of the present study was to assess whether early life stress differentially affects the production of CRP, IL-6, and IL-10 caused by an immune challenge. I hypothesized that there would be differences in immune system responses of children who experienced early life stress and children who had not experienced early life stress. *Ex vivo* studies suggested that the immune systems of children who are exposed to early life stress would demonstrate a larger change in response to an immune challenge than children who had not experienced early life stress.

The second aim was to examine whether acute stress moderate the impact of early life stress on the response of CRP, IL-6 and IL-10 to the influenza vaccine. I predicted that exposure to acute stressors would moderate the effects of early life stress on cytokine response to an immune challenge, such that among children who experienced early life stress, acute stressors will be related to a greater increase in cytokine production compared to children who did not experience acute stressors. I predicted that acute stressors would not be associated with cytokine production among children who had not experienced early life stress. Alternatively, early life stress and acute stress might each have a main effect on immune response.

The third aim of the study was to evaluate whether maternal behavior moderates the association between early life stress and cytokine response to the flu vaccine. Based on research showing that positive parenting characteristics buffer biological systems from the effects of early life stress, I predicted that nurturance would moderate the association between early life stress and immune system response. Among children who experienced early life stress, nurturance was expected to affect cytokine response, but nurturance was not expected to affect cytokine response among children who had not experience early life stress.

The final aim was to investigate whether Attachment and Biobehavioral Catch-up is associated with lower cytokine production after the immune challenge among children who experienced early life stress. I hypothesized that children who received Attachment and Biobehavioral Catch-up in infancy would exhibit a more normalized change in cytokine levels following an immune challenge than children who received a control intervention.

Chapter 2

METHODS

2.1 Participants

The current sample included 62 children and 55 parents (7 sibling pairs completed this study); 47 children had a history of CPS involvement in infancy whereas 15 children had no previous involvement with CPS. Children with a history of CPS involvement constitute the High Early Life Stress (ELS) group and children with no previous CPS involvement constitute the Low ELS group. Participants were recruited from an ongoing longitudinal study examining the effectiveness of a parenting intervention for infants. Families with a previous history of CPS-involvement were recruited in infancy through the Department of Human Services (DHS) in Philadelphia. These families were part of a randomized clinical trial assessing an experimental intervention, Attachment and Biobehavioral Catch-up. After consenting, families were randomized to receive Attachment and Biobehavioral Catch-Up (ABC) or a control intervention, Developmental Education for Families (DEF). Following the intervention, families completed annual lab visits until children were five years of age. When children were eight years old, families were asked to participate in additional annual lab visits. A low-risk comparison sample of children without previous CPS involvement was recruited from community after school programs, announcements on a university website, and by word of mouth. Parents had been screened to ensure that there was no history of involvement with Child Protective

Services. In addition, parents had no history of homelessness, incarceration, or hospitalization due to mental illness or substance use.

At the time of the current study, children ranged in age from 8.04 to 10.96 years old (M=9.62 years, SD=0.78). Children identified as African-American (64.5%), Biracial (22.6%), Hispanic (9.7%) or Caucasian (3.2%). Fifty percent of children were female. Descriptive statistics for demographic data for each group are presented in Table 1. Household income and the composition of child ethnicity significantly differed between the High-ELS group and Low-ELS comparison group. Correlations between variables of interest are presented in Table 2.

Table 1: Demographic Information and Descriptive Statistics for High and Low Early Life Stress (ELS) Groups

Variable	**High-ELS Group (*n* = 47)**		**Low-ELS Group (*n* = 15)**	
	n	%	*n*	%
Child ethnicity*				
African American	35	74.5	5	33.3
White	1	2.1	1	6.7
Hispanic	3	6.4	3	20
Biracial	8	17.0	6	40
Child gender				
Male	25	53.2	6	40.0
Female	22	46.8	9	60.0
	M (SD)	Min - Max	M (SD)	Min - Max
Child age	9.65 (0.80)	8.04 – 10.96	9.53 (0.78)	8.50 – 10.85
Household income**	$21,316 (16,844)	$1,800 – 70,000	$57,785 (35,837)	$1,580 – 120,000

Table 1 continued

CRP level (mg/L)	0.34 (0.43)	0.01 – 2.65	0.44 (0.70)	0.04 – 3.70
Box-cox transformed CRP	0.15 (0.09)	0.03 – 0.28	0.14 (0.08)	0.00 – 0.28
IL-6 level (pg/mL)	6.13 (8.14)	0.89 – 41.91	4.53 (2.05)	1.02-7.78
Box-cox transformed IL-6	1.29 (0.54)	0.12 – 1.87	1.29 (0.70)	0.00 – 3.12
Il-10 level (pg/mL)	20.27 (11.68)	0.64 – 48.76	22.35 (12.42)	0.64 – 46.10
Temperature (°F)	98.13 (0.77)	96.4 – 99.70	98.29 (1.03)	96.0 – 100.0
BMI (z score)	0.69 (1.08)	-1.59 – 2.87	0.72 (1.04)	-0.86 – 2.72
PDS Score*	1.58 (0.47)	1.00 – 2.60	1.92 (.54)	1.20 – 3.00
CBCL Internalizing Items	5.29 (4.56)	0 – 21	5.20 (5.28)	0 – 18
CBCL Externalizing Items	7.41 (7.07)	0 – 27	4.93 (6.36)	0 – 22
Hours of sleep	9.94 (2.19)	4.75 – 17	8.69 (1.57)	5.00 – 10.50
Recent Stressors	16.41 (10.12)	0 – 50	12.80 (5.57)	5 – 25
Nurturance	3.59 (1.34)	1 - 5	4.08 (1.04)	2 – 5

Note: $^{\dagger}p < .10$, $^{*}p < .05$, $^{**}p < .01$.

Table 2: Bivariate Correlations Among Covariates and Primary Variables

Variable	1	2	3	4	5	6	7	8	9	10	11
1. Child Age											
2. Household Income	0.09										
3. Temperature	-0.07	0.06									
4. BMI (z score)	0.17	0.16	0.02								
5. PDS Score	0.19	0.14	-0.03	0.14							
6. CBCL Internalizing Score	0.11	-0.15	0.05	-0.24†	0.26†						
7. Hours of Sleep	-0.10	-0.23	-0.03	0.02	0.05	-0.08					
8. Recent Stressors	0.01	-0.25*	-0.12	0.22†	-0.08	0.05	0.01				
9. Nurturance	-0.02	0.09	0.00	0.09	-0.14	-0.02	-0.08	-0.31*			
10. Box-cox transformed CRP	0.15	-0.03	0.19†	0.37**	0.18†	0.22*	0.10	-0.01	-0.05		
11. Box-cox transformed IL-6	-0.09	-0.14	0.13	-0.10	-0.08	0.01	0.00	-0.09	0.01	0.13	
12. IL-10	-0.00	-0.01	-0.05	-0.11	-0.07	0.24†	-0.02	-0.08	0.03	0.03	0.47**

Note: $^{\dagger}p < .10$, $*p < .05$, $**p < .01$.

2.2 Study Protocol

Upon consent in infancy, children who experienced early life stress were randomly assigned to receive the experimental intervention, Attachment and Biobehavioral Catch-up (ABC) or a control intervention, Developmental Education for Families (DEF). Families completed post-intervention research visits 1 month after completing the intervention and continued to participate in yearly research visits until children were 4 years old, and again between the ages of 8-10. Parents actively completing research visits were contacted by phone to assess their interest in the

present study, which included two research visits in the family's home. At the first visit, a trained research staff member received parental consent and child assent. Children completed several questionnaires, provided a temperature reading, and a trained research staff member completed a brief finger prick to collect a blood sample for cytokine measurement (see below). Next, a research nurse administered an influenza vaccine while children watched a video clip of a children's movie with their parents. Parental behaviors during the administration of the vaccine were video recorded. Twenty-four hours after the administration of the vaccine, children provided additional blood samples via a finger prick and completed a brief questionnaire.

Four children were unable to be reached for the 24-hour follow-up visit. Their initial data were retained in the current dataset. Children were excluded from the present study for the following reasons: 1) used medication that altered their immune response (e.g. corticosterone, TNF-inhibitors), 2) had already received an influenza vaccine for the 2016-2017 flu season, 3) had phobia of needles or receiving shots, or 4) were diagnosed with Guillain-Barré Syndrome, an allergy to eggs, or an allergy to other ingredients of the vaccine. The research visit was discontinued if the child revoked assent or appeared overly distressed. One child revoked assent during the research visit prior to the finger prick. Approval for the conduct of this research was obtained from the University of Delaware Institutional Review Board.

2.3 Interventions

2.3.1 Attachment and Biobehavioral Catch-up

Attachment and Biobehavioral Catch-up is a 10-session intervention delivered in the family's home by a qualified parent coach. Though the aim of ABC is to

improve child outcomes, content is delivered to the parent. Sessions 1 and 2 address nurturing behaviors. Sessions 3 and 4 focus on "following the lead," or engaging in responsive parenting that is contingent on the child's cues. Sessions 5 and 6 focus on decreasing frightening behaviors, such as intrusive tickling or yelling. During sessions 7 and 8, parents are asked to reflect on their experiences of being parented. Parent coaches help parents to recognize how voices from their past may be influencing their own ingrained parenting practices towards their children. Sessions 9 and 10 provide additional time to consolidate gains made over the course of treatment (Dozier & The Infant Caregiver Project, 2012). Desired parenting behaviors are reinforced through "in-the-moment comments" wherein the parent coach may point out the child's behavior and parent's response, label the behavior, and relate the behavior to long-term outcomes (Bernard, Meade, & Dozier, 2013).

2.3.2 Developmental Education for Families

Developmental Education for Families is a time- and context- matched intervention; families complete 10 sessions within their own home and sessions are completed with a trained interventionist. Throughout the sessions, parents learn about children's cognitive and language development. This education is supported with weekly activities surrounding educational domains, such as colors, shapes, and instruments. Parent coaches do not comment on parent's behaviors. This intervention was adapted from the in-home component of an intervention shown to improve motor skills, cognition, and language abilities in early childhood (Brooks-Gunn, Klebanov, Liaw, & Spiker, 1993).

2.4 Research Measures

2.4.1 Blood Sampling

Blood samples were collected prior to immunization and approximately 24 hours afterwards. Prior to the finger prick, children were provided with a hand warmer. The child's finger was cleaned with an alcohol swab and allowed to dry. Children received a finger stick using a BD Microtainer® Contact Activated Lancet (BD Diagnostics, Franklin Lakes, NJ). The first drop of blood was discarded. For CRP analyses, five drops of whole blood were allowed to fall on specimen collection paper (Whatman 903 Protein Saver Card; GE Healthcare). Blood spot samples were stored in airtight bags with desiccant until assayed.

In order to maximize recovery of IL-6 and IL-10, whole blood was collected in a BD Microtainer® Tube coated with EDTA (BD Diagnostics, Franklin Lakes, NJ; de Jager, Bourcier, Rijkers, Prakken, & Seyfert-Margolis, 2009). Blood samples were stored on ice for transportation back to the laboratory. Samples were centrifuged at 700x g for 10 minutes to separate plasma from other blood components. Plasma was transferred to a clean polypropylene tube and stored at -80°C until processing.

2.4.2 Cytokine Measurement

Blood samples collected from finger pricks were used to assess concentrations of CRP, IL-6, and IL-10. CRP was analyzed from dried whole blood spots using the Human C-Reactive Protein/CRP Quantikine ELISA kit (R & D Systems, Minneapolis, MN). The intraassay coefficient of variance was 6.10%. Following Danese et al., (2012), CRP values were transformed using the equation for bloodspot efficiency determined by McDade et al. (2004) ($y=1.15x +0.13$). In the current sample, no values of CRP were elevated about 10mg/L, which is suggestive of an acute infection

(Pearson et al., 2003). Two children did not provide an adequate sample volume for CRP analyses. Furthermore, three CRP levels fell above three standard deviations above the mean and were removed from further analyses. Thus, the final sample includes data for 60 children, including pre-immunization and post-immunization data from 58 children, and pre-immunization data only for 2 children. Due to positive skew in the raw data, CRP values were normalized using a Box-Cox power transformation ($\lambda = -3.50$).

IL-6 and IL-10 were measured from plasma samples using a Milliplex® Multi-analyte Profiling kit (EMD Millipore, Billerica, CA) read via the Luminex® MagPix™ system (Luminex, Austin, TX). Participant samples were randomized across plates to ensure that experimental conditions (e.g. group and time) were not confounded with differences in plate measurement. Samples were not run in duplicate due to low sample volume. The intraassay coefficients of variance for IL-6 and IL-10 were 26.61% and 14.74%, respectively. Forty-six samples did not have enough volume to be run. Of the 74 remaining samples, 28 children had usable samples from both the pre-immunization and post-immunization visits (n=56 samples), 12 children had valid data from the pre-immunization visit only, and 6 children had valid data from the post-immunization visit only. Outliers, as defined as values falling three standard deviations above the mean, were removed from further analyses; three IL-6 outliers were removed and one outlying IL-10 value was removed. Four samples had IL-6 and IL-10 levels below the detectable limit of the assay; these samples were replaced with the value of 1.02 pg/mL and 0.64 pg/mL, respectively. IL-10 values were normally distributed. However, IL-6 values were positively skewed and were subsequently normalized using Box-Cox power transformation ($\lambda = -0.10$).

2.4.3 Acute Stressors

The presence of acute stressors was assessed using an adapted version of the Children's Hassles Scale (Kanner, Feldman, Weinberger, & Ford, 1987). This scale assessed a broad range of stressors related to family, peer, and school contexts (see Appendix A). In addition to the original items, two items were added for the current study: "You missed a day at school" and "You had a test or important project due." Language in the original scale was updated for the current population (e.g. "mother and father" was replaced with "mother and other caregiver"). In order to assess perceived stress, the Children's Hassles Scale was revised to mirror the response options included in the Adult Life Events Questionnaire, wherein a respondent can denote whether a certain event was good or bad, and respond as to whether he or she perceives the event to have had "no effect," "some effect," "moderate effect," or "great effect" on his or her life. The total intensity score is the sum of all perceived impact ratings, regardless of whether an event was considered to be a good or bad event. The Children's Hassles Scale demonstrated acceptable internal consistency for this sample ($\alpha = 0.77$).

2.4.4 Parental Nurturance

Parental nurturance was coded from video-recordings of parental behavior as the child received the influenza vaccination. Parents were asked to sit next to their child and to "provide support as you usually would during a doctor's visit." Parental behavior was coded after the injection until the nurturance episode was complete. Ratings for parental nurturance were made on a 5-point Likert scale using ratings based on the Qualitative Scales of the Observational Ratings of the Caregiving environment (ORCE; NICHD Early Child Care Research network, 1999, 2003) and

the Emotional Support Given Scale from The Supportive Behavior Task Coding Manual (Allen et al., 2012). At high levels of nurturance, parents appeared attuned to the child's emotional response and offered matched, appropriate levels of physical or verbal nurturance. At low levels of nurturance, parents dismissed the child's emotional response or appeared disengaged from the interaction. Nurturance scores ranged from 1.0 to 5.0 ($M = 3.70$, $SD = 1.28$). Nine videos (16.7%) were double coded by coders blind to group status; interrater reliability was excellent, $r = 0.91$.

2.4.5 Pubertal Development

Pubertal development is associated with decreases in adipokine levels as pubertal status advances (Martos-Moerno, Barrios, & Argente; 2006). Pubertal status was considered as a covariate in the current study. To assess pubertal development, we administered the Petersen Pubertal Development Scale (PDS) (Petersen, Crockett, Richards, & Boxer, 1988). Parents responded to this questionnaire at each participant's most recent yearly visit. This scale included seven questions regarding physical attributes associated with the onset of puberty, such as body hair and skin changes. The PDS has separate versions for each biological sex. The PDS-Girl includes questions related to breast development and menarche. The PDS-Boy includes questions related to voice changes and facial hair. Each attribute is rated as "not yet started," "barely started," "definitely started," or "seems complete." Scores across all items are averaged for an overall indicator of pubertal development. Internal consistency in the current sample was adequate for girls ($\alpha = 0.70$), though slightly lower for boys ($\alpha = 0.42$).

2.4.6 Body Mass Index

Subcutaneous adipose tissue secretes IL-6 and CRP, thus BMI levels are included in the analytic models as covariates (Fantuzzi, 2005; Yudkin et al., 1999). Children's height and weight were measured at their most recent annual lab visit (Mean age = 9.31 years, *SD* = 0.81). Body mass index (BMI) was calculated using the Center for Disease Control (CDC) formula. Children's weight in pounds was divided by height in inches, squared. This number was then multiplied by 703. Following Must and Anderson (2006), raw BMI scores were converted to standardized z-scores adjusted for child age and sex using growth charts from the CDC.

2.4.7 Child Behavior Checklist

As depression symptomatology is associated with increased levels of IL-6, IL-10 and CRP in adulthood, internalizing symptoms in childhood were included as a covariate in the current study (Dhabar et al., 2009; Howren et al., 2009; Zorilla et al., 2001). Children's internalizing symptoms were assessed using the Child Behavior Checklist for Ages 6-18 (CBCL, Achenbach & Rescorla, 2001). Parents completed this questionnaire at each participant's most recent visit. In order to reduce response errors associated with poor reading, researchers read the CBCL aloud to parents and recorded their responses. The CBCL consists of 118 items that assess emotional and behavioral problems in middle childhood. For each item, parents were asked to rate whether a statement about their child's behavior as 0 = Not True, 1 = Somewhat or Sometimes True, or 2 = Very True or Often True. The current study utilized the subscale for Internalizing Behavior (number of items in factor = 32). This subscale had good internal reliability within this sample ($\alpha = 0.87$).

2.4.8 Sleep

In adults, sleep duration is correlated with levels of circulating baseline IL-6 and IL-10 (Patel et al., 2009). Thus, sleep was assessed and included as a covariate in the current study. Children's sleep quality was assessed using parent and self-report. At the second research visit, children reported their sleep and wake time for the previous night's sleep. Parents confirmed or refined their child's report if necessary. The number of hours of sleep was included as a covariate.

2.5 Data Analytic Plan

Mixed models were utilized to estimate change in cytokine level over time because two observations were obtained for each participant. Accordingly, day was entered as a repeated measure (e.g. day 1 and day 2) within person and an unstructured variance-covariance matrix was fitted to estimate the error variance. Mixed models also allowed for the use of Restricted Maximum Likelihood Estimation in order to estimate model parameters using all available data. Using hierarchical linear, or mixed, models, multiple observations are treated as nested within persons, which allows models to include time-varying covariates, such as time and temperature. Time was included as a continuous level-1 variable. Time was entered as "0" on day 1 and the length of time between sample measurements (e.g. "23:50") on day 2. Each cytokine outcome was entered as the dependent variable. Covariates were selected based on their theoretical and empirical relationships to cytokine levels and variables of interest. Every model included the following covariates: age, gender, ethnicity, temperature at the time of blood draw, body mass index, pubertal development score, internalizing score, and overnight sleep. All models were analyzed using SPSS

Version 24 (IBM, New York, NY). Ancillary analyses examined each model without including siblings in the sample size. Discrepancies are noted below.

Chapter 3

RESULTS

3.1 Early Life Stress and Cytokine Response (Aim 1)

Models to assess whether early life stress altered cytokine response to an immune stressor were estimated using the equations below. These equations specify main effects and interaction effects of the primary variables of interest (e.g. Time, ELS Group, and Time by ELS Group). Covariates were also included in the model. Models were specified for Box-Cox transformed CRP, Box-Cox transformed IL-6, and IL-10 separately.

Level-1 Model: $\text{Cytokine}_{ti} = \pi_{0i} + \pi_{1i}*(\text{Time}_{ti}) + \pi_{2i}*(\text{Temperature}_{ti}) + e_{ti}$

Level-2 Model: $\pi_{0i} = \beta_{00} + \beta_{01}*(\text{ELS Group}_i) + \beta_{02}*(\text{Ethnicity}_i) + \beta_{03}*(\text{Gender}_i) + \beta_{04}*(\text{Income}_i) + \beta_{05}*(\text{Internalizing}_i) + \beta_{06}*(\text{BMI}_i) + \beta_{07}*(\text{Pubertal Development Score}_i) + \beta_{08}*(\text{Sleep}_i) + \beta_{09}*(\text{Age}_i) + r_{0i}$

$\pi_{1i} = \beta_{10} + \beta_{11}*(\text{ELS Group}_i) + r_{1i}$

Combined equation: $\text{Cytokine}_{ti} = \beta_{00} + \underline{\beta_{01}*(\text{ELS Group}_i)} + \underline{\beta_{10}*(\text{Time}_{ti})} + \underline{\beta_{11}*(\text{Time}_{ti}\ \text{ELS Group}_i)} + \pi_{2i}(\text{Temperature}_{ti}) + \beta_{02}(\text{Ethnicity}_i) + \beta_{03}(\text{Gender}_i) + \beta_{04}(\text{Income}_i) + \beta_{05}(\text{Internalizing}_i) + \beta_{06}(\text{BMI}_i) + \beta_{07}(\text{Pubertal Development Score}_i) + \beta_{08}(\text{Sleep}_i) + \beta_{09}(\text{Age}_i) + e_{ti}$

In the level-1 model, Cytokine_{ti} represents the cytokine outcome (Box-Cox transformed CRP, Box-Cox transformed IL-6, or IL-10) at time t for each child i. π_{0i} is the cytokine level for high-ELS, Hispanic, males at baseline when other covariates are held at average levels. Π_{1i} is the slope coefficient for the change in cytokine level over time high-ELS, Hispanic, males when other covariates are held at average levels. Π_{2i}

represents the added change in cytokine level at baseline due to temperature, a time-varying covariate. E_{ti} is the residual variance of child *i*'s cytokine level at time *t* from the predicted value. In the level-2 model, the within-person intercept, π_{0i}, is estimated by adding β_{00}, which represents the level of cytokine for the high-risk, Hispanic, males at baseline with additional β values associated with the effects of interest and covariates that do not vary with time, and r_{0i}, which represents the deviance for child *I* from the predicted baseline cytokine level. Specifically, β_{01} represents the difference between high- and low-ELS groups at baseline, β_{02} represents the difference in cytokine level between Hispanic and other ethnicity groups at baseline, β_{03} represents the difference in cytokine level between males and female at baseline, β_{04} represents added change in cytokine level due to income level at baseline, β_{05} represents added change in cytokine level at baseline due to internalizing symptoms, β_{06} represents added change in cytokine level due to BMI at baseline, β_{07} represents added change in cytokine level due to pubertal development at baseline, β_{08} represents added change in cytokine level due to sleep at baseline, and β_{09} represents added change in cytokine level due to age at baseline. The within-persons slope, $\pi_{1i,}$ is estimated by adding β_{10}, which represents change in cytokine level across Time for high-ELS children, β_{11}, which represents the difference between high-and low-ELS groups change in average cytokine level over time, and r_{1i}, which is the deviance for child *I* from the average slope. Within the combined equation, the main effects of interest (i.e. Time and ELS Group) and the cross-level interaction effect of Time by ELS Group are underlined.

3.1.1 Early Life Stress and CRP Response

The main effect of ELS Group on CRP level at baseline was not significant, $F_{1, 26.93} = 2.16$, $p = 0.15$. The main effect of Time on CRP level was significant, $F_{1, 34.52} =$

34.32, $p < 0.001$, indicating that CRP reliably increased over time (Table 3). However, the interaction between ELS Group and Time on CRP was not significant, $F_{1, 34.42} = 2.52$, $p = 0.12$, indicating that exposure to early life stress did not affect CRP-response over time. The statistical significance of effects of interest (i.e. Time, ELS Group, and Time by ELS Group) did not change when covariates were omitted from the model.

Table 3: Multilevel Model Predicting Box-Cox transformed CRP as a Function of Early Life Stress and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	0.08	0.03	$F_{1, 27.28} = 28.94$	0.00
ELS Group	-0.05	0.03	$F_{1, 26.93} = 2.16$	0.15
Time	0.07	0.02	$F_{1, 34.52} = 34.32$	0.00
ELS Group * Time	0.06	0.04	$F_{1, 34.42} = 2.52$	0.12
Ethnicity			$F_{3, 25.38} = 1.60$	0.21
Ethnicity = African American	0.00	0.03		
Ethnicity = Biracial	0.05	0.03		
Ethnicity = Caucasian	0.00	0.07		
Gender	0.06	0.02	$F_{1, 24.97} = 8.26$	0.01
Internalizing	0.01	0.00	$F_{1, 24.86} = 4.78$	0.04
BMI	0.04	0.01	$F_{1, 26.33} = 17.49$	0.00
Temperature	0.00	0.01	$F_{1, 51.72} = 0.01$	0.92
Pubertal Development Score	-0.02	0.02	$F_{1, 24.95} = 0.36$	0.55
Sleep	0.01	0.01	$F_{1, 24.84} = 1.91$	0.18
Age	0.01	0.00	$F_{1, 25.54} = 0.53$	0.12

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

Notably, several covariates were significantly associated with CRP level. Higher BMI scores were associated with higher levels of CRP levels at baseline, $F_{1, 26.33} = 17.49$, $p < 0.001$. Females had significantly higher levels of CRP levels at baseline than males, $F_{1, 24.97} = 8.26$, $p < 0.01$. At baseline, higher levels of internalizing symptoms were associated with higher levels of CRP, $F_{1, 24.86} = 4.78$, $p = 0.04$. When siblings were not included in the analysis, internalizing symptoms no longer predicted CRP at baseline, $F_{1, 22.11} = 3.99$, $p = 0.06$.

3.1.2 Early Life Stress and IL-6 Response

The main effect of ELS Group on IL-6 level at baseline was not significant, $F_{1, 26.41} = 0.29$, $p = 0.59$. Further, the main effect for Time on IL-6 yielded an F-ratio of $F_{1, 21.42} = 0.21$, $p = 0.66$, indicating that this cytokine did not significantly over time. The interaction effect of ELS Group by Time was not significant, $F_{1, 22.05} = 0.09$, $p = 0.77$. There was no difference in how IL-6 levels changed over time between children who experienced high levels of early life stress and those who experienced low levels of early life stress. Further, there were no significant associations between covariates included in the model and IL-6 (Table 4). Excluding siblings from the model did not change the statistical significance of any effects. The statistical significance of effects of interest (i.e. Time, ELS Group, and Time by ELS Group) did not change when covariates were omitted from the model.

Table 4 Multilevel Model Predicting Box-Cox transformed IL-6 Level as a Function of Early Life Stress and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	0.62	0.38	$F_{1, 25.35} = 20.71$	0.00
ELS Group	0.26	0.49	$F_{1, 26.41} = 0.29$	0.59
Time	0.19	0.25	$F_{1, 21.42} = 0.21$	0.66
ELS Group * Time	-0.15	0.51	$F_{1, 22.05} = 0.09$	0.77
Ethnicity			$F_{3, 13.94} = 0.30$	0.82
Ethnicity = African American	0.32	0.34		
Ethnicity = Biracial	0.23	0.43		
Ethnicity = Caucasian	0.18	0.55		
Gender	0.36	0.20	$F_{1, 14.00} = 3.30$	0.09
Income	0.00	0.00	$F_{1, 13.75} = 0.32$	0.58
Internalizing	-0.02	0.02	$F_{1, 14.53} = 0.51$	0.48
BMI	0.10	0.10	$F_{1, 13.40} = 0.99$	0.34
Temperature	-0.03	0.12	$F_{1, 24.22} = 0.08$	0.78
Pubertal Development Score	0.04	0.20	$F_{1, 17.73} = 0.32$	0.86
Sleep	0.00	0.06	$F_{1, 17.71} = 0.00$	0.99
Age	-0.17	0.13	$F_{1, 22.65} = 1.72$	0.20

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

3.1.3 Early Life Stress and IL-10 Response

In the full model, the main effect of ELS Group on IL-10 level at baseline was significant, such that children who experienced high levels of stress in early childhood had lower levels of IL-10 at baseline than those who experienced low levels of stress

in early childhood, $F_{1, 20.24} = 4.84$, $p = 0.04$ (Table 5). There was a main effect of Time on IL-10, indicating that IL-10 levels were significantly higher one day after receiving the vaccination than at baseline, $F_{1, 19.02} = 4.65$, $p = 0.04$. The interaction of ELS Group and Time was not significant, $F_{1, 18.91} = 0.71$, $p = 0.41$. There were no associations between any covariates and IL-10 level at baseline (Table 5). When covariates were not included in the model, the effect of ELS Group on IL-10 was no longer significant, but the main effect of Time remained significant. Excluding siblings from the model did not change the statistical significance of any effects.

Table 5: Multilevel Model Predicting IL-10 Level as a Function of Early Life Stress and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	5.70	4.97	$F_{1, 16.15} = 25.77$	0.00
ELS Group	15.84	7.20	$F_{1, 20.24} = 4.84$	0.04
Time	11.62	3.81	$F_{1, 19.02} = 4.65$	0.04
ELS Group * Time	-6.51	7.75	$F_{1, 18.91} = 0.71$	0.41
Ethnicity			$F_{3, 15.37} = 0.96$	0.44
Ethnicity = African American	4.49	4.87		
Ethnicity = Biracial	2.81	7.38		
Ethnicity = Caucasian	-9.15	8.86		
Gender	3.87	3.35	$F_{1, 15.92} = 1.34$	0.26
Income	0.00	0.00	$F_{1, 14.67} = 0.21$	0.65
Internalizing	-0.36	0.36	$F_{1, 16.26} = 0.97$	0.34
BMI	2.00	1.85	$F_{1, 16.47} = 1.17$	0.30
Temperature	-2.95	1.87	$F_{1, 24.30} = 2.49$	0.13
Pubertal Development Score	1.04	3.12	$F_{1, 15.00} = 0.11$	0.74
Sleep	1.52	0.98	$F_{1, 16.72} = 2.43$	0.14
Age	-0.46	1.91	$F_{1, 17.86} = 0.06$	0.81

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

3.2 Early Life Stress, Acute Stress, and Cytokine Response (Aim 2)

Models to assess whether early life stress or acute stress altered cytokine response to an immune stressor were estimated using the equations below. These equations specify main effects and interaction effects of the primary variables of interest (i.e. Early Life Stress, Acute Stress, and Time). Covariates were also included in the model. Models were specified for Box-Cox transformed CRP, Box-Cox transformed IL-6, and IL-10 separately.

Level-1 Model: $\text{Cytokine}_{ti} = \pi_{0j} + \pi_{1i}*(\text{time}_{ti}) + \pi_{2i}*(\text{Temperature}_{ti}) + e_{ti}$

Level-2 Model: $\pi_{0i} = \beta_{01}*(\text{ELS Group}_i) + \beta_{02}*(\text{Acute Stress}_i) + \beta_{03}*(\text{ELS Group}_i*\text{Acute Stress}_i) + \beta_{04}*(\text{Ethnicity}_i) + \beta_{05}*(\text{Gender}_i) + \beta_{06}*(\text{Income}_i) + \beta_{07}*(\text{Internalizing}_i) + \beta_{08}*(\text{BMI}_i) + \beta_{09}*(\text{Pubertal Development Score}_i) + \beta_{010}*(\text{Sleep}_i) + \beta_{011}*(\text{Age}_i) + r_{0i}$

$\pi_{1i} = \beta_{10} + \beta_{11}*(\text{ELS Group}_i) + \beta_{12}*(\text{Acute Stress}_i) + \beta_{13}*(\text{ELS Group}_i * \text{Acute Stress}_i) + r_{1i}$

Combined Equation: $\text{Cytokine}_{ti} = \beta_{00} + \underline{\beta_{01}*(\text{ELS Group}_i)} + \underline{\beta_{02}*(\text{Acute Stress}_i)} + \underline{\beta_{10}*(\text{Time}_{ti})} + \underline{\beta_{11}*(\text{Time}_{ti}*\text{ELS Group}_i)} + \underline{\beta_{12}*(\text{Time}_{ti}*\text{Acute Stress}_i)} + \underline{\beta_{03}*(\text{ELS Group}_i*\text{Acute Stress}_i)} + \underline{\beta_{13}*(\text{Time}_{ti}*\text{ELS Group}_i*\text{Acute Stress}_i)} + \pi_{2i}*(\text{Temperature}_{1i}) + \beta_{04}*(\text{Ethnicity}_i) + \beta_{05}*(\text{Gender}_i) + \beta_{06}*(\text{Income}_i) + \beta_{07}*(\text{Internalizing}_i) + \beta_{08}*(\text{BMI}_i) + \beta_{09}*(\text{Pubertal Development Score}_i) + \beta_{010}*(\text{Sleep}_i) + \beta_{011}*(\text{Age}_i) + e_{ti}$

In the level-1 model, Cytokine_{ti} represents the cytokine outcome (Box-Cox transformed CRP, Box-Cox transformed IL-6, or IL-10) at time t for each child i. π_{0i} is the cytokine level for high-risk, Hispanic, males at baseline when other covariates are held at average levels. π_{1i} is the slope coefficient for the change in cytokine level over

time for high-risk, Hispanic, males when other covariates are held at average levels. π_{2i} represents the added change in cytokine level due to temperature, a time-varying covariate. e_{ti} is the residual variance of child *i*'s cytokine level at time *t* from the predicted value. In the level-2 model, the within-person intercept, π_{0i}, is estimated by adding β_{00}, which represents the level of cytokine for the high-risk, Hispanic, males at baseline with additional β values associated with the effects of interest and covariates that do not vary with time, and r_{0i}, which represents the deviance for child *i* from the predicted baseline cytokine level. Specifically, β_{01} represents the difference between high- and low-ELS groups at baseline, β_{02} represents the added change in cytokine level at baseline due to Acute Stress, β_{03} represents the added change in cytokine level at baseline due to the interaction between ELS-group and Acute Stress, β_{04} represents the difference in cytokine level between Hispanic and other ethnicity groups at baseline, β_{05} represents the difference in cytokine level between males and female at baseline, β_{06} represents change in cytokine level due to income level at baseline, β_{07} represents change in cytokine level at baseline due to internalizing symptoms, β_{08} represents change in cytokine level due to BMI at baseline, β_{09} represents change in cytokine level due to pubertal development at baseline, β_{010} represents change in cytokine level due to sleep at baseline, and β_{011} represents change in cytokine level due to age at baseline. The within-persons slope, $\pi_{1i,}$ is estimated by adding β_{10}, which represents the slope of cytokine level across Time for high-ELS children, β_{11}, which represents the difference in slope between high-and low-ELS groups for change in average cytokine level over time, β_{12}, which represents the change in slope due to Acute Stress, β_{13}, which represents the change in slope due to the interaction between

ELS Group and Acute stress, and r_{1i}, which is the deviance for child *i* from the average slope.

Within the combined equation, the main effects of interest (i.e. Time, ELS Group, and Acute Stress), two-way interactions (Time by ELS Group, Time by Acute Stress, and ELS Group by Acute Stress) and the three-way interaction effect of Time by ELS Group by Acute Stress are underlined.

3.2.1 Early Life Stress, Acute Stress, and CRP Response

There was no main effect of ELS Group ($F_{1, 24.64} = 1.66$, $p = 0.21$) or Acute Stress on CRP at baseline, $F_{1, 25.79} = 0.10$, $p = 0.76$ (Table 6). Moreover, the interaction effect of ELS Group by Acute Stress on CRP level at baseline was not significant, $F_{1, 25.21} = 0.90$, $p = 0.76$. The main effect of Time on CRP was significant, such that as time increased, CRP level also increased, $F_{1, 32.86} = 24.66$, $p < 0.001$. There was no interaction effect of ELS Group by Time ($F_{1, 32.89} = 1.36$, $p = 0.25$) or Acute Stress by Time ($F_{1, 33.13} = 0.39$, $p = 0.54$), indicating that there were no significant differences in CRP increases related to exposure to early life stress or recent stress (Table 6). The interaction effect of ELS Group by Acute Stress by Time was also not significant ($F_{1, 33.00} = 0.28$, $p = 0.60$). The statistical significance of these effects did not change when covariates were omitted from the model.

Table 6: Multilevel Model Predicting Box-Cox transformed CRP as a Function of Early Life Stress, Acute Stress, and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	0.08	0.03	$F_{1, 24.77} = 29.22$	0.00
ELS Group	-0.04	0.03	$F_{1, 24.64} = 1.66$	0.21
Acute Stress	0.00	0.00	$F_{1, 25.79} = 0.10$	0.76
Time	0.07	0.02	$F_{1, 32.86} = 24.66$	0.00
ELS Group * Time	0.05	0.04	$F_{1, 32.89} = 1.36$	0.25
Acute Stress * Time	0.00	0.00	$F_{1, 33.13} = 0.39$	0.54
ELS Group * Acute Stress	0.00	0.00	$F_{1, 25.21} = 0.90$	0.35
ELS Group * Acute Stress * Time	0.00	0.00	$F_{1, 33.00} = 0.28$	0.60
Ethnicity			$F_{3, 23.91} = 2.07$	0.13
Ethnicity = African American	0.00	0.03		
Ethnicity = Biracial	0.07	0.04		
Ethnicity = Caucasian	0.01	0.07		
Gender	0.06	0.02	$F_{1, 23.81} = 8.10$	0.01
Internalizing	0.01	0.00	$F_{1, 23.24} = 6.14$	0.02
BMI	0.04	0.01	$F_{1, 24.69} = 17.69$	0.00
Temperature	0.00	0.01	$F_{1, 48.65} = 0.01$	0.91
Pubertal Development Score	-0.15	0.02	$F_{1, 23.38} = 0.51$	0.48
Sleep	0.01	0.01	$F_{1, 23.16} = 2.04$	0.17
Age	0.01	0.01	$F_{1, 23.90} = 0.39$	0.54

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Acute Stress, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

Notably, several covariates were significantly associated with CRP level at baseline. Females had significantly higher levels of CRP levels at baseline than males, $F_{1, 23.81} = 8.10$, $p = 0.01$. Higher BMI scores were associated with higher levels of CRP

at baseline, $F_{1, 24.69} = 17.69$, $p < 0.001$. At baseline, higher levels of internalizing symptoms were associated with higher levels of CRP, $F_{1, 23.24} = 6.14$, $p = 0.02$ (Table 6). When siblings were not included in the analysis, internalizing symptoms no longer predicted Box Cox transformed CRP at baseline, $F_{1, 22.11} = 3.99$, $p = 0.06$. Excluding siblings from the model did not change the statistical significance of any effects.

3.2.2 Early Life Stress, Acute Stress, and IL-6 Response

The main effect of ELS Group on IL-6 level at baseline was not significant, $F_{1, 22.44} = 0.10$, $p = 0.75$ (Table 7). In addition, there was no main effect of Acute Stress on IL-6, $F_{1, 22.89} = 0.88$, $p = 0.36$. The interaction effect of ELS Group by Acute Stress was not significant, $F_{1, 22.12} = 0.21$, $p = 0.65$. The main effect of Time on IL-6 was also not significant ($F_{1, 19.56} = 0.29$, $p = 0.60$), indicating that IL-6 did not increase significantly as a result of the vaccination. There was no interaction effect of ELS Group by Time ($F_{1, 19.44} = 0.06$, $p = 0.82$) or Acute Stress by Time ($F_{1, 18.68} = 0.22$, $p = 0.65$), indicating that the trajectory of IL-6 over time was not affected significantly by an individual's exposure to early life stress or acute stress (Table 7). Moreover, the three-way interaction between ELS Group by Acute Stress by Time was not significant ($F_{1, 18.52} = 0.21$, $p = 0.65$). The statistical significance of these effects did not change when covariates were omitted from the model. There were no significant effects between covariates included in the model on the level of IL-6 (Table 7). Excluding siblings from the model did not change the statistical significance of any effects.

Table 7: Multilevel Model Predicting Box-Cox transformed IL-6 as a Function of Early Life Stress, Acute Stress, and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	0.47	0.43	$F_{1, 24.75} = 4.91$	0.04
ELS Group	0.21	0.65	$F_{1, 22.44} = 0.10$	0.75
Acute Stress	-0.02	0.03	$F_{1, 22.89} = 0.88$	0.36
Time	0.33	0.32	$F_{1, 19.56} = 0.29$	0.60
ELS Group * Time	-0.20	0.85	$F_{1, 19.44} = 0.06$	0.82
Acute Stress * Time	0.03	0.04	$F_{1, 18.68} = 0.22$	0.65
ELS Group * Acute Stress	-0.05	0.10	$F_{1, 22.12} = 0.21$	0.65
ELS Group * Acute Stress * Time	0.00	0.12	$F_{1, 18.52} = 0.00$	0.98
Ethnicity			$F_{3, 13.32} = 0.34$	0.80
Ethnicity = African American	0.30	0.37		
Ethnicity = Biracial	-0.04	0.54		
Ethnicity = Caucasian	0.01	0.61		
Gender	0.45	0.23	$F_{1, 11.95} = 4.02$	0.07
Income	0.00	0.00	$F_{1, 13.75} = 0.05$	0.83
Internalizing	-0.02	0.02	$F_{1, 12.47} = 0.32$	0.58
BMI	0.12	0.12	$F_{1, 12.47} = 0.87$	0.37
Temperature	-0.02	0.14	$F_{1, 19.24} = 0.02$	0.91
Pubertal Development Score	0.04	0.22	$F_{1, 16.12} = 0.03$	0.86
Sleep	-0.02	0.07	$F_{1, 14.81} = 0.07$	0.80
Age	-0.17	0.14	$F_{1, 19.95} = 1.52$	0.23

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Acute Stress, Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

3.2.3 Early Life Stress, Acute Stress, and IL-10 Response

The main effect of ELS Group on IL-10 level at baseline was not significant, $F_{1, 18.53} = 3.95$, $p = 0.06$ (Table 8). When siblings were not included in the analysis, this effect became statistically significant, $F_{1, 18.15} = 5.05$, $p = 0.04$. However, without

covariates included in the model, the effect of ELS Group on Il-10 level was not significant, with siblings: $F_{1, 36.35} = 4.18$, $p = 0.05$, without siblings: $F_{1, 35.35} = 3.79$, $p = 0.06$.

Table 8: Multilevel Model Predicting IL-10 as a Function of Early Life Stress, Acute Stress, and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	5.63	5.41	$F_{1, 17.27} = 8.08$	0.01
ELS Group	16.66	8.39	$F_{1, 18.53} = 3.95$	0.06
Acute Stress	-0.09	0.33	$F_{1, 18.80} = 0.13$	0.72
Time	11.90	4.49	$F_{1, 15.45} = 2.29$	0.15
ELS Group * Time	-4.20	13.03	$F_{1, 15.25} = 0.10$	0.75
Acute Stress * Time	-0.13	0.53	$F_{1, 14.81} = 0.04$	0.85
ELS Group * Acute Stress	-0.30	1.28	$F_{1, 17.71} = 0.05$	0.82
ELS Group * Acute Stress * Time	0.61	1.79	$F_{1, 14.84} = 0.12$	0.74
Ethnicity			$F_{3, 15.25} = 0.64$	0.60
Ethnicity = African American	3.76	5.52		
Ethnicity = Biracial	-0.58	10.90		
Ethnicity = Caucasian	-10.20	10.38		
Gender	4.32	3.96	$F_{1, 13.22} = 1.19$	0.30
Income	0.00	0.00	$F_{1, 13.13} = 0.50$	0.49
Internalizing	-0.31	0.39	$F_{1, 13.23} = 0.65$	0.43
BMI	1.54	2.14	$F_{1, 14.91} = 0.52$	0.48
Temperature	-2.92	2.09	$F_{1, 18.40} = 1.95$	0.18
Pubertal Development Score	0.51	3.33	$F_{1, 12.47} = 0.02$	0.88
Sleep	1.33	1.17	$F_{1, 14.82} = 0.04$	0.85
Age	-0.47	2.04	$F_{1, 15.41} = 0.05$	0.82

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Acute Stress, Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

The main effect of Acute Stress on Il-10 was not significant, $F_{1, 18.80} = 0.13$, $p = 0.72$; IL-10 level at baseline was not altered significantly by exposure to recent stress. The interaction effect of Acute Stress by ELS Group was also not significant, $F_{1, 17.71} = 0.05$, $p = 0.82$. There was no main effect of Time on IL-10 level ($F_{1, 15.54} = 2.29$, $p = 0.15$), indicating that IL-10 levels did not significantly increase after the immunization (Table 8). There was no interaction effect of ELS Group by Time ($F_{1, 15.25} = 0.10$, $p = 0.75$) or Acute Stress by Time ($F_{1, 14.81} = 0.04$, $p = 0.85$), indicating that the trajectory of IL-10 over time was not affected significantly by an individual's exposure to early life stress or acute stress (Table 8). Lastly, the three-way interaction of ELS Group by Acute Stress by Time was not significant, $F_{1, 14.84} = 0.12$, $p = 0.74$. The statistical significance of these effects did not change when covariates were omitted from the model. There were no significant effects between covariates included in the model on the level of IL-10 (Table 8). Excluding siblings from the model did not change the statistical significance of any effects other than the ELS Group main effect.

3.3 Early Life Stress, Nurturance, and Cytokine Response (Aim 3)

Models to assess whether early life stress and parental nurturance altered cytokine response to an immune stressor were estimated using the equations below. These equations specify main effects and interaction effects of the primary variables of interest (i.e. Early Life Stress, Nurturance, and Time). Covariates were also included in the model. Models were specified for Box-Cox transformed CRP, Box-Cox transformed IL-6, and IL-10 separately.

Level-1 Model: $\text{Cytokine}_{ti} = \pi_{0j} + \pi_{1i}*(\text{time}_{ti}) + \pi_{2i}*(\text{Temperature}_{ti}) + e_{ti}$

Level-2 Model: $\pi_{0i} = \beta_{01}*(\text{ELS Group}_i) + \beta_{02}*(\text{Nurturance}_i) + \beta_{03}*(\text{ELS Group}_i*\text{Nurturance}_i) + \beta_{04}*(\text{Ethnicity}_i) + \beta_{05}*(\text{Gender}_i) + \beta_{06}*(\text{Income}_i) + \beta_{07}*(\text{Internalizing}_i) + \beta_{08}*(\text{BMI}_i) + \beta_{09}*(\text{Pubertal Development Score}_i) + \beta_{010}*(\text{Sleep}_i) + \beta_{011}*(\text{Age}_i) + r_{0i}$

$\pi_{1i} = \beta_{10} + \beta_{11}*(\text{ELS Group}_i) + \beta_{12}*(\text{Nurturance}_i) + \beta_{13}*(\text{ELS Group}_i * \text{Nurturance}_i) + r_{1i}$

Combined Equation: $\text{Cytokine}_{ti} = \beta_{00} + \underline{\beta_{01}*(\text{ELS Group}_i)} + \underline{\beta_{02}*(\text{Nurturance}_i)} + \underline{\beta_{10}*(\text{Time}_{ti})} + \underline{\beta_{11}*(\text{Time}_{ti}*\text{ELS Group}_i)} + \underline{\beta_{12}*(\text{Time}_{ti}*\text{Acute Nurturance}_i)} + \underline{\beta_{03}*(\text{ELS Group}_i*\text{Nurturance}_i)} + \underline{\beta_{13}*(\text{Time}_{ti}*\text{ELS Group}_i*\text{Nurturance}_i)} + \pi_{2i}*(\text{Temperature}_{1i}) + \beta_{04}*(\text{Ethnicity}_i) + \beta_{05}*(\text{Gender}_i) + \beta_{06}*(\text{Income}_i) + \beta_{07}*(\text{Internalizing}_i) + \beta_{08}*(\text{BMI}_i) + \beta_{09}*(\text{Pubertal Development Score}_i) + \beta_{010}*(\text{Sleep}_i) + \beta_{011}*(\text{Age}_i) + e_{ti}$

In the level-1 model, Cytokine_{ti} represents the cytokine outcome (Box-Cox transformed CRP, Box-Cox transformed IL-6, or IL-10) at time *t* for each child *i*. π_{0i} is the cytokine level for high-risk, Hispanic, males at baseline when other covariates are held at average levels. π_{1i} is the slope coefficient for the change in cytokine level over time for high-risk, Hispanic, males when other covariates are held at average levels. π_{2i} represents the added change in cytokine level due to temperature, a time-varying covariate. e_{ti} is the residual variance of child *i*'s cytokine level at time *t* from the predicted value. In the level-2 model, the within-person intercept, π_{0i}, is estimated by adding β_{00}, which represents the level of cytokine for the high-risk, Hispanic, males at baseline with additional β values associated with the effects of interest and covariates that do not vary with time, and r_{0i}, which represents the deviance for child *i* from the predicted baseline cytokine level. Specifically, β_{01} represents the difference between high- and low-ELS groups at baseline, β_{02} represents the added change in cytokine level at baseline due to Nurturance, β_{03} represents the added change in cytokine level at baseline due to the interaction between ELS-group and Nurturance, β_{04} represents the difference in cytokine level between Hispanic and other ethnicity groups at

baseline, β_{05} represents the difference in cytokine level between males and female at baseline, β_{06} represents change in cytokine level due to income level at baseline, β_{07} represents change in cytokine level at baseline due to internalizing symptoms, β_{08} represents change in cytokine level due to BMI at baseline, β_{09} represents change in cytokine level due to pubertal development at baseline, β_{010} represents change in cytokine level due to sleep at baseline, and β_{011} represents change in cytokine level due to age at baseline. The within-persons slope, $\pi_{1i,}$ is estimated by adding β_{10}, which represents the slope of cytokine level across Time for high-ELS children, β_{11}, which represents the difference in slope between high-and low-ELS groups for change in average cytokine level over time, β_{12}, which represents the change in slope due to Nurturance, β_{13}, which represents the change in slope due to the interaction between ELS Group and Nurturance, and r_{1i}, which is the deviance for child *i* from the average slope.

Within the combined equation, the main effects of interest (i.e. Time, ELS Group, and Nurturance), two-way interactions (Time by ELS Group, Time by Nurturance, and ELS Group by Nurturance) and the three-way interaction effect of Time by ELS Group by Nurturance are underlined.

3.3.1 Early Life Stress, Nurturance, and CRP Response

There was no main effect of ELS Group ($F_{1, 18.61} = 2.29$, $p = 0.15$) or Nurturance on CRP at baseline, $F_{1, 18.80} = 0.49$, $p = 0.49$ (Table 9). Likewise, the interaction effect of ELS Group by Nurturance on CRP level at baseline was not significant, $F_{1, 18.92} = 1.45$, $p = 0.24$. The main effect of Time on CRP was significant, such that as time increased, CRP level also increased, $F_{1, 23.33} = 21.56$, $p < 0.001$. There was no interaction effect of ELS Group by Time ($F_{1, 32.89} = 1.36$, $p = 0.25$) or

Acute Stress by Time ($F_{1, 33.13} = 0.39$, $p = 0.54$), indicating that CRP increases were not significantly related to exposure to early life stress or recent stress (Table 6). The interaction effect of ELS Group by Acute Stress by Time was also not significant ($F_{1, 33.00} = 0.28$, $p = 0.60$). The statistical significance of these effects did not change when covariates were omitted from the model.

Table 9: Multilevel Model Predicting Box-Cox transformed CRP as a Function of Early Life Stress, Nurturance, and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	0.05	0.03	$F_{1, 17.80} = 11.51$	0.00
ELS Group	-0.05	0.03	$F_{1, 18.61} = 2.29$	0.15
Nurturance	-0.01	0.01	$F_{1, 18.80} = 0.49$	0.49
Time	0.08	0.02	$F_{1, 23.33} = 21.56$	0.00
ELS Group * Time	0.03	0.04	$F_{1, 23.39} = 0.52$	0.48
Nurturance * Time	0.01	0.01	$F_{1, 23.84} = 1.96$	0.17
ELS Group * Nurturance	0.03	0.02	$F_{1, 18.92} = 1.45$	0.24
ELS Group * Nurturance * Time	0.03	0.03	$F_{1, 23.72} = 0.75$	0.40
Ethnicity			$F_{3, 14.40} = 3.23$	0.05
Ethnicity = African American	0.04	0.03		
Ethnicity = Biracial	0.08	0.03		
Ethnicity = Caucasian	-0.05	0.06		
Gender	0.06	0.02	$F_{1, 13.86} = 9.55$	0.01
Internalizing	0.00	0.00	$F_{1, 14.51} = 1.60$	0.23
BMI	0.04	0.01	$F_{1, 15.25} = 19.49$	0.00
Temperature	0.02	0.01	$F_{1, 43.09} = 2.95$	0.09
Pubertal Development Score	0.00	0.02	$F_{1, 13.91} = 0.00$	0.95
Sleep	0.01	0.01	$F_{1, 14.05} = 3.60$	0.08
Age	-0.01	0.01	$F_{1, 14.13} = 0.39$	0.54

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Nurturance, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

Similar to other models with CRP as the outcome variable, several covariates were significantly associated with CRP level at baseline. Females had significantly higher levels of CRP levels at baseline than males, $F_{1, 23.81} = 8.10$, $p = 0.01$. Higher BMI scores were associated with higher levels of CRP at baseline, $F_{1, 24.69} = 17.69$, $p < 0.001$. At baseline, higher levels of internalizing symptoms were associated with higher levels of CRP, $F_{1, 23.24} = 6.14$, $p = 0.02$ (Table 6). When siblings were not included in the analysis, internalizing symptoms no longer predicted Box Cox transformed CRP at baseline, $F_{1, 22.11} = 3.99$, $p = 0.06$. Excluding siblings from the model did not change the statistical significance of any effects.

3.3.2 Early Life Stress, Nurturance, and IL-6 Response

In this model, there was no main effect of ELS Group ($F_{1, 16.73} = 0.00$, $p = 0.95$) or Nurturance on IL-6 level at baseline, $F_{1, 15.70} = 0.01$, $p = 0.95$ (Table 10). In addition, the interaction effect of ELS Group by Nurturance was not significant, $F_{1, 14.04} = 0.20$, $p = 0.66$. There was also no main effect of Time on IL-6, $F_{1, 15.78} = 0.05$, $p = 0.82$. The interaction effects of ELS Group by Time ($F_{1, 16.25} = 0.02$, $p = 0.90$) and Nurturance by Time ($F_{1, 17.22} = 1.02$, $p = 0.33$) were not statistically significant, indicating that the trajectory of IL-6 over time was not affected significantly by exposure to early life stress or their experience of parental nurturance (Table 10). Lastly, the three-way interaction between ELS Group by Nurturance by Time was not significantly associated with IL-6 ($F_{1, 16.43} = 0.36$, $p = 0.56$). The statistical significance of these effects did not change when covariates were omitted from the

model. There were no significant effects between covariates included in the model on the level of IL-6 (Table 10). Excluding siblings from the model did not change the statistical significance of any effects.

Table 10: Multilevel Model Predicting Box-Cox transformed IL-6 as a Function of Early Life Stress, Nurturance, and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	1.23	0.49	$F_{1, 15.50} = 23.72$	0.00
ELS Group	0.03	0.55	$F_{1, 16.73} = 0.00$	0.95
Nurturance	0.11	0.28	$F_{1, 15.70} = 0.01$	0.95
Time	0.03	0.32	$F_{1, 15.78} = 0.05$	0.82
ELS Group * Time	0.07	0.58	$F_{1, 16.25} = 0.02$	0.90
Nurturance * Time	-0.12	0.34	$F_{1, 17.22} = 1.02$	0.33
ELS Group * Nurturance	-0.19	0.43	$F_{1, 14.04} = 0.20$	0.66
ELS Group * Nurturance * Time	-0.31	0.52	$F_{1, 16.43} = 0.36$	0.56
Ethnicity			$F_{3, 6.17} = 1.14$	0.40
Ethnicity = African American	-0.09	0.40		
Ethnicity = Biracial	-0.19	0.51		
Ethnicity = Caucasian	0.87	0.60		
Gender	0.23	0.20	$F_{1, 6.13} = 1.29$	0.30
Income	0.00	0.00	$F_{1, 6.48} = 0.01$	0.92
Internalizing	-0.03	0.03	$F_{1, 5.75} = 0.86$	0.39
BMI	0.09	0.11	$F_{1, 6.59} = 0.59$	0.47
Temperature	-0.12	0.12	$F_{1, 9.32} = 0.90$	0.37
Pubertal Development Score	0.02	0.21	$F_{1, 7.16} = 0.01$	0.92
Sleep	-0.03	0.06	$F_{1, 8.37} = 0.25$	0.63
Age	-0.11	0.14	$F_{1, 9.49} = 0.59$	0.46

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Nurturance, Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

3.3.3 Early Life Stress, Nurturance, and IL-10 Response

In this model, the main effect of ELS Group on IL-10 level at baseline was significant ($F_{1, 12.98} = 10.70$, $p = 0.01$), indicating that children who experienced low levels of early life stress had significantly higher levels of IL-10 at baseline than children who experienced high levels of early life stress. The main effect of Nurturance on IL-10 was not statistically significant, $F_{1, 14.74} = 4.10$, $p = 0.06$ (Table 11). However, there was a significant main effect of Time on IL-10 level, indicating that IL-10 increased as time progressed, $F_{1, 16.21} = 7.35$, $p = 0.02$.

Table 11: Multilevel Model Predicting IL-10 as a Function of Early Life Stress, Nurturance, and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	15.03	4.83	$F_{1,10.55} = 59.07$	0.00
ELS Group	18.26	5.58	$F_{1, 12.98} = 10.70$	0.01
Nurturance	-5.38	2.47	$F_{1, 14.74} = 4.10$	0.06
Time	16.50	4.47	$F_{1, 16.21} = 7.35$	0.02
ELS Group * Time	-9.76	8.58	$F_{1, 16.49} = 1.29$	0.27
Nurturance * Time	7.22	4.09	$F_{1, 16.43} = 0.00$	0.96
ELS Group * Nurturance	1.94	3.76	$F_{1, 12.91} = 0.27$	0.61
ELS Group * Nurturance * Time	-14.02	7.07	$F_{1, 17.20} = 3.93$	0.06
Ethnicity			$F_{3, 3.36} = 0.66$	0.63
Ethnicity = African American	-4.70	3.80		
Ethnicity = Biracial	-5.62	5.48		
Ethnicity = Caucasian	-1.42	5.58		
Gender	-0.22	2.49	$F_{1, 4.87} = 0.01$	0.93
Income	0.00	0.00	$F_{1, 3.57} = 3.39$	0.15
Internalizing	-1.01	0.30	$F_{1, 3.12} = 11.41$	0.04
BMI	3.31	1.26	$F_{1, 3.27} = 6.91$	0.07
Temperature	-3.85	1.96	$F_{1, 19.19} = 3.80$	0.07

Table 11 continued

Pubertal Development Score	2.30	2.20	$F_{1, 3.70} = 1.09$	0.36
Sleep	1.36	0.73	$F_{1, 11.05} = 3.46$	0.09
Age	3.34	1.58	$F_{1, 6.80} = 4.47$	0.07

Note: The ELS Group variable was coded such that the high-ELS group was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Nurturance, Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

In regards to interaction effects, the interaction effect of ELS Group by Nurturance on IL-10 level at baseline was not significant, $F_{1, 12.91} = 0.27$, $p = 0.61$ (Table 11). There was no interaction effect of ELS Group by Time ($F_{1, 16.49} = 1.29$, $p = 0.27$) or Nurturance by Time, $F_{1, 16.43} = 0.00$, $p = 0.96$. The increase of IL-10 over time was not altered by exposure to early life stress or nurturance. Further, the three-way interaction of ELS Group by Nurturance by Time was not statistically significant, $F_{1, 17.20} = 3.93$, $p = 0.06$ (Table 11). The statistical significance of the interaction effects and main effects of interest did not change when covariates were omitted from the model.

Within this model, internalizing symptoms were significantly associated with IL-10 level at baseline, $F_{1, 3.12} = 11.41$, $p = 0.04$, indicating that children with higher levels of internalizing symptoms exhibited lower levels of IL-10 at baseline (Table 11). All other covariates included in the model were not significantly associated with IL-10 level (Table 11). The model was run with and without siblings. When siblings were excluded from the model, the statistical significance of all effects remained the same as when all siblings were included in the model.

3.4 Early Intervention and Cytokine Response (Aim 4)

The last model assessed whether receiving an intervention in early childhood altered cytokine response to an immune stressor among children exposed to early life stress; children in the low-ELS group were not included in the following analyses. This model was estimated using the equations below. These equations specify main effects and interaction effects of the primary variables of interest (e.g. Time, Intervention, and Time by Intervention). Covariates were also included in the model. Models were specified for Box-Cox transformed CRP, Box-Cox transformed IL-6, and IL-10 separately.

Level-1 Model: $\text{Cytokine}_{ti} = \pi_{0i} + \pi_{1i}*(\text{Time}_{ti}) + \pi_{2i}*(\text{Temperature}_{ti}) + e_{ti}$

Level-2 Model: $\pi_{0i} = \beta_{00} + \beta_{01}*(\text{Intervention}_i) + \beta_{02}*(\text{Ethnicity}_i) + \beta_{03}*(\text{Gender}_i) + \beta_{04}*(\text{Income}_i) + \beta_{05}*(\text{Internalizing}_i) + \beta_{06}*(\text{BMI}_i) + \beta_{07}*(\text{Pubertal Development Score}_i) + \beta_{08}*(\text{Sleep}_i) + \beta_{09}*(\text{Age}_i) + r_{0i}$

$\pi_{1i} = \beta_{10} + \beta_{11}*(\text{Intervention}_i) + r_{1i}$

Combined equation: $\text{Cytokine}_{ti} = \beta_{00} + \underline{\beta_{01}*(\text{Intervention}_i)} + \underline{\beta_{10}*(\text{Time}_{ti})} + \underline{\beta_{11}*(\text{Time}_{ti}*\text{Intervention}_i)} + \pi_{2i}*(\text{Temperature}_{ti}) + \beta_{02}*(\text{Ethnicity}_i) + \beta_{03}*(\text{Gender}_i) + \beta_{04}*(\text{Income}_i) + \beta_{05}*(\text{Internalizing}_i) + \beta_{06}*(\text{BMI}_i) + \beta_{07}*(\text{Pubertal Development Score}_i) + \beta_{08}*(\text{Sleep}_i) + \beta_{09}*(\text{Age}_i) + e_{ti}$

In the level-1 model, Cytokine_{ti} represents the cytokine outcome (Box-Cox transformed CRP, Box-Cox transformed IL-6, or IL-10) at time *t* for each child *i*. π_{0i} is the cytokine level at baseline for Hispanic, males who received a control intervention (DEF) when other covariates are held at average levels. π_{1i} is the slope coefficient for the change in cytokine level over time for Hispanic, males who received DEF when other variables are held at average levels. π_{2i} represents the added change in cytokine level at baseline due to temperature, a time-varying covariate. e_{ti} is the residual variance of child *i*'s cytokine level at time *t* from the predicted value. In the level-2

model, the within-person intercept, π_{0i}, is estimated by adding β_{00}, which represents the level of cytokine at baseline for Hispanic, males who received DEF with additional β values associated with the effects of interest and covariates that do not vary with time, and r_{0i}, which represents the deviance for child *i* from the predicted baseline cytokine level. Specifically, β_{01} represents the difference between ABC and DEF groups at baseline, β_{02} represents the difference in cytokine level between Hispanic and other ethnicity groups at baseline, β_{03} represents the difference in cytokine level between males and female at baseline, β_{04} represents added change in cytokine level due to income level at baseline, β_{05} represents added change in cytokine level at baseline due to internalizing symptoms, β_{06} represents added change in cytokine level due to BMI at baseline, β_{07} represents added change in cytokine level due to pubertal development at baseline, β_{08} represents added change in cytokine level due to sleep at baseline, and β_{09} represents added change in cytokine level due to age at baseline. The within-persons slope, $\pi_{1i,}$ is estimated by adding β_{10}, which represents change in cytokine level across Time for children who received DEF, β_{11}, which represents the difference in slope between DEF and ABC groups, and r_{1i}, which is the deviance for child *i* from the average slope. Within the combined equation, the main effects of interest (i.e. Intervention and Time) and the cross-level interaction effect of Intervention by Time are underlined.

3.4.1 Early Intervention and CRP Response

The main effect of Intervention Group on CRP level at baseline was not significant, $F_{1, 20.51} = 1.45$, $p = 0.24$ (Table 12). In this model, the main effect of Time on CRP level was significant, $F_{1, 29.17} = 23.61$, $p < 0.001$, indicating that CRP reliably increased over time among high risk children. The interaction between Intervention

Group and Time on CRP was not significant, $F_{1, 29.24} = 0.00$, $p = 0.97$, indicating that the trajectory of CRP over time in children who received ABC did not differ significantly from that of children who received DEF. The statistical significance of effects of interest (i.e. Intervention Group, Time, and Time by Intervention Group) did not change when covariates were omitted from the model.

Table 12: Multilevel Model Predicting Box-Cox transformed CRP as a Function of Intervention Group and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	0.07	0.04	$F_{1, 21.28} = 69.36$	0.00
Intervention	-0.03	0.02	$F_{1, 20.51} = 1.45$	0.24
Time	0.07	0.02	$F_{1, 29.17} = 23.61$	0.00
Intervention * Time	0.00	0.03	$F_{1, 29.24} = 0.00$	0.97
Ethnicity			$F_{2, 21.01} = 2.95$	0.07
Ethnicity = African American	0.03	0.04		
Ethnicity = Biracial	0.09	0.04		
Gender	0.05	0.02	$F_{1, 20.31} = 4.74$	0.04
Internalizing	0.01	0.00	$F_{1, 20.09} = 5.26$	0.03
BMI	0.04	0.01	$F_{1, 21.02} = 17.46$	0.00
Temperature	0.01	0.01	$F_{1, 42.74} = 1.20$	0.28
Pubertal Development Score	-0.02	0.02	$F_{1, 20.19} = 0.41$	0.53
Sleep	0.01	0.01	$F_{1, 19.69} = 2.42$	0.14
Age	0.02	0.01	$F_{1, 20.32} = 1.26$	0.27

Note: The Intervention variable was coded such that DEF was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

Similar to other models with CRP as the outcome variable, several covariates were significantly associated with CRP level. Higher BMI scores were associated with

higher levels of CRP levels at baseline, $F_{1, 21.02} = 17.46$, $p < 0.001$. Females had significantly higher levels of CRP levels at baseline than males, $F_{1, 20.31} = 4.74$, $p = 0.04$. At baseline, higher levels of internalizing symptoms were associated with higher levels of CRP, $F_{1, 20.09} = 5.36$, $p = 0.03$. When siblings were not included in the analysis, gender no longer predicted CRP at baseline, $F_{1, 17.09} = 2.58$, $p = 0.13$. However, the statistical significance of other effects remained the same.

3.4.2 Early Intervention and IL-6 Response

The main effect of Intervention Group on IL-6 level at baseline was not significant, $F_{1, 16.74} = 0.01$, $p = 0.92$; children who received DEF and children who received ABC did not differ significantly in levels of IL-6 prior to receiving the vaccination (Table 13). There was no significant effect of Time on IL-6 level, $F_{1, 16.50} = 1.15$, $p = 0.30$. Moreover, the interaction effect of Intervention Group by Time was not significant, $F_{1, 17.60} = 0.27$, $p = 0.61$. Among both intervention groups, IL-6 levels did not increase as time increased. When covariates were excluded from the model, the statistical significance of these effects remained the same.

Table 13: Multilevel Model Predicting Box-Cox transformed IL-6 as a Function of Intervention Group and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	0.83	0.41	$F_{1, 20.72} = 12.77$	0.00
Intervention	-.04	0.41	$F_{1, 16.74} = 0.01$	0.92
Time	0.14	0.32	$F_{1, 16.50} = 1.15$	0.30
Intervention * Time	0.27	0.53	$F_{1, 17.60} = 0.27$	0.61
Ethnicity			$F_{2, 7.12} = 1.45$	0.30
Ethnicity = African American	0.00	0.30		
Ethnicity = Biracial	-0.47	0.36		

Table 13 continued

Gender	0.42	0.13	$F_{1, 6.05} = 10.36$	0.02
Income	0.00	0.00	$F_{1, 5.39} = 0.31$	0.87
Internalizing	-0.03	0.01	$F_{1, 6.15} = 5.33$	0.06
BMI	0.10	0.07	$F_{1, 6.15} = 2.50$	0.16
Temperature	-0.08	0.10	$F_{1, 8.09} = 0.62$	0.46
Pubertal Development Score	0.12	0.15	$F_{1, 7.28} = 0.60$	0.46
Sleep	0.00	0.04	$F_{1, 7.00} = 0.01$	0.92
Age	-0.27	0.10	$F_{1, 8.19} = 7.05$	0.03

Note: The Intervention variable was coded such that DEF was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

Of the covariates included in the model, gender and age were significantly associated with IL-6 level at baseline. Females had higher levels of IL-6 at baseline than males, $F_{1, 6.05} = 10.36$, $p = 0.02$. Older children had lower levels of IL-6 at baseline than younger children, $F_{1, 8.19} = 7.05$, $p = 0.03$. Excluding siblings from the model did not change the statistical significance of any effects.

3.4.3 Early Intervention and IL-10 Response

The main effect of Intervention Group on IL-10 level at baseline was not significant, $F_{1, 10.89} = 1.04$, $p = 0.33$ (Table 12). There was a main effect of Time on IL-10, indicating that as time increased, IL-10 levels increased as well, $F_{1, 14.63} = 10.89$, $p = 0.01$. The interaction effect of Intervention Group by Time was not significant, indicating that children who received ABC and those who received DEF did not differ significantly in IL-10 trajectories over time, $F_{1, 14.44} = 0.28$, $p = 0.61$.

When covariates were excluded from the model, the statistical significance of these effects remained the same. No covariates included in this model were associated with IL-10 level at baseline (Table 14). Excluding siblings from the model did not change the statistical significance of any effects.

Table 14: Multilevel Model Predicting IL-10 as a Function of Intervention Group and Time

	Coefficient	SE	*F-test*	*p*-value
Intercept	8.71	6.70	$F_{1, 18.19} = 3.78$	0.07
Intervention	-4.79	4.70	$F_{1, 10.89} = 1.04$	0.33
Time	11.02	4.79	$F_{1, 14.63} = 10.89$	0.01
Intervention * Time	4.22	7.98	$F_{1, 14.44} = 0.28$	0.61
Ethnicity			$F_{2, 13.29} = 0.15$	0.87
Ethnicity = African American	1.14	6.17		
Ethnicity = Biracial	-3.88	10.60		
Gender	6.07	3.40	$F_{1, 7.55} = 3.18$	0.12
Income	0.00	0.00	$F_{1, 6.01} = 0.06$	0.82
Internalizing	-0.72	0.36	$F_{1, 9.36} = 3.92$	0.08
BMI	3.53	1.78	$F_{1, 8.00} = 3.92$	0.08
Temperature	-3.70	2.32	$F_{1, 20.69} = 2.56$	0.13
Pubertal Development Score	1.74	3.29	$F_{1, 7.93} = 0.28$	0.61
Sleep	1.32	1.04	$F_{1, 9.97} = 1.62$	0.23
Age	-0.27	2.12	$F_{1, 11.25} = 0.02$	0.90

Note: The Intervention variable was coded such that DEF was the reference group. The reference group for the Ethnicity variable was Hispanic ethnicity. For Gender, Male was the reference group. Income, Internalizing, BMI, Temperature, Pubertal Development Score, Sleep and Age were grand-mean centered.

Chapter 4

DISCUSSION

This study aimed to determine whether and how stress impacts the innate immune system's response to an immune challenge. Chronic exposure to stress, including exposure to maltreatment in childhood, is associated with altered immune functioning (Kiecolt-Glaser et al., 2011; Miller et al., 2009; Slopen et al., 2013). Thus, exposure to early life stress was examined as a primary factor in the current study. Exposure to recent stressors also appears to alter cytokine production (Marin et al., 2009; Steptoe et al., 2007). In the current study, acute stressors were examined as a potential factor leading to the accumulation of abnormal cytokine response among individuals exposed to early life stress. Sensitive parental behaviors are associated with improved regulation of several biological systems (Asok et al., 2013; Evans et al., 2007; Spanglar et al, 1994). In this study, parental responsiveness to distress, or nurturance, was examined as a potential buffer to the dysregulation of the immune system caused by early life stress. Among children exposed to early life stress in infancy, the Attachment and Biobehavioral Catch-Up intervention is associated with the regulation of the HPA axis (Bernard et al., 2015a; Bernard et al., 2015b). The final aim of this study explored whether the ABC intervention is associated with the regulation of the innate immune system in middle childhood.

The present study did not provide evidence that exposure to early life stress significantly altered the innate immune system's response to the influenza vaccine. In addition, acute stress did not significantly alter cytokine response to the immune

stressor, regardless of exposure to early life stress. Similarly, parental nurturance did not significantly alter the innate immune system's response to the immune stressor. Lastly, there was no evidence that Attachment and Biobehavioral Catch-up altered cytokine response to the influenza vaccine among children exposed to early life stress.

Several common findings across models show that the influenza immunization challenge was successful in elucidating an *in vivo* response from the innate immune system. Across all models, time since vaccination was associated with a marked increase in CRP. In addition, increases in IL-10 were seen over time in most models. However, there was no significant effect of Time on IL-6, suggesting that IL-6 was not significantly increased 24 hours post-vaccination. This null finding may be due to small sample size, but may also be due to timing of measurement. IL-6 is one of the first cytokines produced when infection is detected. IL-6 goes on to produce CRP, and is down-regulated by the production of IL-10 (Heinrich et al. 1990; Fiorentino et al., 1991). The measurement window in the current study detected elevations in CRP and IL-10, and may have missed the increase in IL-6 caused by the vaccine.

In the first, second, and third aims, the effect of early life stress on levels of circulating, non-stimulated, cytokine levels, were estimated. Across models, baseline levels of CRP did not differ between children who were exposed to high levels of early life stress and those who experienced low levels of early life stress. The effect of childhood trauma on circulating levels of CRP is well established in adulthood (for a review see Baumeister, Akhtar, Ciufolini, Pariante, & Mondelli et al., 2016). In adolescence and infancy, elevated CRP is associated with stress exposure, including low parental education, poverty, and crime. The effect of early life stress on circulating levels of CRP specifically in middle childhood is not widely documented

in the literature and was not supported by the present study. Early life stress was also not associated with circulating levels of IL-6, though Slopen et al. (2013) determined that IL-6 levels were elevated in children who experienced maltreatment in childhood in comparison to children who did not experience maltreatment. However, the effect size documented by Slopen and colleagues was relatively small, and, if present in this study's population, was likely unable to be detected with the sample size in this study. In the current study, early life stress was associated with baseline levels of IL-10 in two out of three models; children who experienced early life stress had lower levels of IL-10 prior to receiving the vaccination than children who had not experienced early life stress. These lower levels of IL-10, an anti-inflammatory cytokine, in the high risk group appears consistent with the larger literature that exposure to early adversity is associated with an irregular, pro-inflammatory pattern (Ehrlich, 2019).

The first aim of the current study was to investigate whether exposure to early life stress altered the innate immune system's response to a challenge. C-Reactive Protein and IL-10 were both significantly elevated approximately 24 hours after vaccination, while IL-6 did was not significantly different from the pre-intervention measurement. However, this immune response did not differ between children who experienced early life stress and those who had not experienced early life stress. These findings did not support the hypothesis that children who experienced early life stress would display an aberrant innate immune response to the immunization. There are several possible explanations for the null finding in the current study. First, in previous studies investigating the effect of early life stress on immune response, blood samples were collected and stimulated *in vitro* using LPS, a molecule found on the gram negative bacteria (Steptoe et al., 2007). In vitro stimulation may differ from in vivo

stimulation, as the effects of other biological systems, such as the HPA axis, may alter the immediate immune response (Sanders & Kavelaars, 2007). It is also possible that there are subtle differences in how the innate immune system in middle childhood responds to a bacterial challenge (e.g. LPS stimulation) and a viral challenge (e.g. influenza) (Begitt et al., 2014). Second, the small sample size may not have had enough power to detect a relatively small effect. Third, the effect of early life stress on LPS-stimulated immune response has been reported in late adolescence (e.g. ages 15-23) and adulthood (Kiecolt-Glaser et al., 2011; Miller & Chen, 2010, Miller et al., 2009). The current study included children between the ages of 8 and 11. Dysregulation of the innate immune response due to early life stress may not be apparent in middle childhood.

In the second aim, the association between early life stress, acute stressors, and immune response was investigated. I hypothesized that among children who experienced early life stress, higher levels of acute stress would be associated with a greater increase in cytokine production, while levels of acute stress would not be associated with cytokine response among children who had not experienced early life stress. Acute stress was not significantly associated with any cytokine level at baseline, nor with the trajectory of cytokine production after exposure to the vaccination. This lack of association did not differ between children who experienced early life stress and those who had not. While acute stress is associated with altered IL-6 and IL-10 levels in adulthood, this association has not been documented in children (Buske-Kirschbaum et al., 2007, Segerstrom & Miller, 2004). It is possible that the effect of acute stress on IL-6 or IL-10 does not develop until adulthood, although other cytokines are affected by acute stress in childhood (Marin et al., 2009).

The third aim was to determine whether parental nurturance altered cytokine production after receiving the influenza vaccination. I hypothesized that high-ELS children who experienced high levels of parental nurturance would exhibit a similar cytokine response to the vaccination as low-ELS children, whereas high-ELS children who experienced low levels of parental nurturance would display an altered cytokine response. In the current study, nurturance was not associated significantly with circulating levels of IL-6, Il-10, or CRP at baseline, regardless of exposure to stress in early childhood. Further, parental nurturance did not significantly alter the trajectory of any cytokine after the delivery of the influenza vaccine. It is possible that the current study was not adequately powered to detect such effects. There was a marginally significant interaction of parental nurturance, early life stress, and time predicting the level of IL-10. With a larger sample size, this effect, if truly present, may have been detected. Previous research on parental behavior and the immune system in adults utilized participant reports of parental warmth and nurturance experienced in childhood (Chen et al., 2011; Miller et al. 2011b). It is possible that the measurement of perceived parental warmth or nurturance included in other studies is different, and perhaps more important to biological regulation, than the coded parental behavior in the current study. The current study employed one specific measure of parental behavior: responsiveness to physical distress. Other measures of parental behavior, such as responsiveness to non-distress or nurturance during emotional distress, may be associated with immune functioning and should be investigated in further studies.

The final aim examined whether Attachment and Biobehavioral Catch-up, an intervention for high-risk children, altered immune functioning among children who

experienced early life stress. As the ABC intervention is associated with the regulation of the HPA axis (Bernard et al., 2015a; Bernard et al., 2015b), I hypothesized that children who received ABC in infancy would display a normalized cytokine response similar to that of children who did not experience early life stress, while children who received a control intervention in infancy (Developmental Education for Families) would display an aberrant cytokine response following the influenza vaccination. Intervention type was not associated significantly with circulating levels of IL-6, IL-10, or CRP prior to receiving the intervention. Moreover, cytokine response to the influenza vaccine did not differ significantly between children who received ABC and those who received DEF. These results fail to provide evidence that ABC alters the function of the innate immune system during middle childhood.

The current study benefitted from several design strengths. First, the influenza vaccine was successfully administered in children's homes, and viable blood samples were collected and assayed in the laboratory. In addition, elevations in CRP and IL-10 were detected with this paradigm, indicating that the influenza vaccine elicits a measurable response from the innate immune system approximately 24 hours after administering the vaccine. Future research may alter this time window in order to determine whether an increase in IL-6 can be measured at a different time. This research paradigm may enable future studies to conduct research on immune function with hard to reach populations. Second, previous research on parental behavior and immune function has largely relied on retrospective reports of parent behavior (Chen et al., 2011; Miller et al. 2011b). The present study utilized observational coding of parent behavior as the child was receiving the vaccine to assess parental nurturance in a controlled task. Third, this study expanded research on the effect of acute stress on

the immune system by assessing acute stress among school-aged children. Lastly, this sample was recruited from an ongoing longitudinal, randomized control trial of Attachment and Biobehavioral Catch-Up, which allowed an assessment of whether early intervention altered immune function in middle childhood among children involved in the child welfare system.

It is important to note that this study also had several limitations that affect the generalizability of the findings. First, as noted above, the sample size may not have had adequate power to detect associations in the current study. This may be particularly true for the questions posed in the second and third aims, where three-way interaction terms were used to assess the interplay between early life stress, time, and nurturance/acute stress, as each additional term entered in the model decreases statistical power. Further, samples were not run in duplicate, and the intraassay coefficients were somewhat higher than typically seen in studies utilizing ELISA or multiplex assay techniques. This variability may mean that findings from this study should not be generalized to the larger population. However, the associations supported by this study (e.g. increases in CRP and IL-6 over time, associations between CRP and BMI) are well-documented in the existing literature.

Future studies should continue to assess the impact of various forms of early life stress on immune functioning. The field would benefit from understanding how various types of early stress, e.g. poverty and child maltreatment, independently cause variations in immune functioning. Such delineation would help guide policy and allocation of intervention resources. Future studies may also assess the impact of various types of parenting behavior, such as sensitivity to non-distress, or indicators of parent-child relationships quality in middle-childhood, on immune functioning. The

current study did not provide support for the hypothesis that parental nurturance in middle-childhood altered immune function. However, parent-child relationship quality, support, or sensitivity to non-distress may buffer the immune system from the effects of early life stress.

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Appendix A

IRB/HUMAN SUBJECTS APPROVAL

UNIVERSITY OF DELAWARE

RESEARCH OFFICE

210 Hullihen Hall
University of Delaware
Newark, Delaware 19716-1551
Ph: 302/831-2136
Fax: 302/831-2828

DATE:	October 13, 2016
TO:	Julie Hoye, M.A.
FROM:	University of Delaware IRB
STUDY TITLE:	[958181-1] Key Middle Childhood Outcomes Immune Function Substudy
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE:	October 13, 2016
EXPIRATION DATE:	September 20, 2017
REVIEW TYPE:	Full Committee Review

Thank you for your submission of New Project materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Full Committee Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

Please note that any revision to previously approved materials must be approved by this office prior to initiation. Please use the appropriate revision forms for this procedure.

All SERIOUS and UNEXPECTED adverse events must be reported to this office. Please use the appropriate adverse event forms for this procedure. All sponsor reporting requirements should also be followed.

Please report all NON-COMPLIANCE issues or COMPLAINTS regarding this study to this office.

Please note that all research records must be retained for a minimum of three years.

Based on the risks, this project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Nicole Farnese-McFarlane at (302) 831-1119 or nicolefm@udel.edu. Please include your study title and reference number in all correspondence with this office.

RESEARCH OFFICE

210 Hullihen Hall
University of Delaware
Newark, Delaware 19716-1551
Ph: 302/831-2136
Fax: 302/831-2828

DATE:	September 7, 2017
TO:	Julie Hoye, M.A.
FROM:	University of Delaware IRB
STUDY TITLE:	[958181-2] Key Middle Childhood Outcomes Immune Function Substudy
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	Approved for Data Analysis Only
APPROVAL DATE:	September 7, 2017
EXPIRATION DATE:	September 20, 2018
REVIEW TYPE:	Expedited Review
REVIEW CATEGORY:	Expedited review category # *(9)*

Thank you for your submission of Continuing Review/Progress Report materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

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Please note that all research records must be retained for a minimum of three years.

Based on the risks, this project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

RESEARCH OFFICE

210 Hullihen Hall
University of Delaware
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Ph: 302/831-2136
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DATE:	August 21, 2018
TO:	Julie Hoye, M.A.
FROM:	University of Delaware IRB
STUDY TITLE:	[958181-3] Key Middle Childhood Outcomes Immune Function Substudy
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	Approved for Data Analysis Only
APPROVAL DATE:	August 21, 2018
EXPIRATION DATE:	September 20, 2019
REVIEW TYPE:	Expedited Review
REVIEW CATEGORY:	Expedited review category # *(9)*

Thank you for your submission of Continuing Review/Progress Report materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

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