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Psychological Stress in Children May Alter the Immune Response

Emma Carlsson,^{*,†} Anneli Frostell,^{‡,§} Johnny Ludvigsson,^{‡,¶} and Maria Faresjö^{*,†}

Psychological stress is a public health issue even in children and has been associated with a number of immunological diseases. The aim of this study was to examine the relationship between psychological stress and immune response in healthy children, with special focus on autoimmunity. In this study, psychological stress was based on a composite measure of stress in the family across the domains: 1) serious life events, 2) parenting stress, 3) lack of social support, and 4) parental worries. PBMCs, collected from 5-y-old high-stressed children ($n = 26$) and from 5-y-old children without high stress within the family ($n = 52$), from the All Babies In Southeast Sweden cohort, were stimulated with Ags (tetanus toxoid and β -lactoglobulin) and diabetes-related autoantigens (glutamic acid decarboxylase 65, insulin, heat shock protein 60, and tyrosine phosphatase). Immune markers (cytokines and chemokines), clinical parameters (C-peptide, proinsulin, glucose), and cortisol, as an indicator of stress, were analyzed. Children from families with high psychological stress showed a low spontaneous immune activity (IL-5, IL-10, IL-13, IL-17, CCL2, CCL3, and CXCL10; $p < 0.01$) but an increased immune response to tetanus toxoid, β -lactoglobulin, and the autoantigens glutamic acid decarboxylase 65, heat shock protein 60, and tyrosine phosphatase (IL-5, IL-6, IL-10, IL-13, IL-17, IFN- γ , TNF- α , CCL2, CCL3, and CXCL10; $p < 0.05$). Children within the high-stress group showed high level of cortisol, but low level of C-peptide, compared with the control group ($p < 0.05$). This supports the hypothesis that psychological stress may contribute to an imbalance in the immune response but also to a pathological effect on the insulin-producing β cells. *The Journal of Immunology*, 2014, 192: 2071–2081.

Stress is defined as a state of threatened homeostasis or disharmony, and is counteracted by a complex repertoire of physiologic and behavioral adaptive responses in order to establish homeostasis (1). Stressful experiences may affect both physical and psychological well-being, as well as immune functions in humans. A prolonged exposure to extreme stress is shown to be harmful, possibly leading to cell and tissue death. Confronted with a stressful condition, the nervous system and the immune system initiate a coping process to keep homeostasis in the body. The major neuroendocrine response to stress is via activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (2), where stimulation of the HPA axis increases the glucocorticoid (cortisol) secretion (3). It has been shown that childhood adversities are associated with alterations in

the stress response later in life (4). Today psychological stress is considered a public health issue even in children.

Stress may be induced by various physical or psychological factors (stressors) such as marital conflict in the family or loss of a loved one. The effect of a particular stressor on immune functions varies according to the previous stress experience of the individual, whereas various stressors may act in the same or in opposite ways on the same immune parameter. The kind and the magnitude of alterations of immune response depend on several factors, including the severity and the duration of the stressor, and the ability of the individual to cope (5). In 2004, a large meta-analysis of almost 300 independent studies over 30 y indicated that psychological stress was associated with suppression of the immune system (6) and with a number of immunological diseases such as inflammatory bowel disease (7), allergic disease (8), atopic dermatitis (9), and celiac disease (4). A number of studies have also found that serious life events during the first 2 y of a child's life increases the risk for type 1 diabetes (T1D) (10–12).

The consequences of stress on the immune system are generally adaptive in the short run but can be damaging when stress becomes chronic. Immune cells contain receptors for and respond to neurotransmitters, neuropeptides, and hormones secreted by sympathetic neurons and/or adrenal medulla. At the molecular basis, these effects involve a network of multidirectional signaling and feedback regulations between mediators of neuroendocrine and immune cells. Studies over the last decades have demonstrated a profound relationship between the nervous system and the immune system. Because the two systems are said to regulate each other reciprocally, findings have created the fairly new field of study known as psychoneuroimmunology. The impact of psychological stress on immune function has been the subject of extensive research efforts (13–15). Previous studies have shown that stress, for example, influences the Th1/Th2 balance (16). Therefore, IFN- γ versus IL-5 and IL-13 have in our study been used as markers of activated Th cells of Th1 versus Th2 nature and, furthermore, IL-17 as an indicator of acti-

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Abbreviations used in this article: ABIS, All Babies in Southeast Sweden; GAD65, glutamic acid decarboxylase 65; HPA, hypothalamic-pituitary-adrenal; HSP60, heat shock protein 60; IA-2, tyrosine phosphatase; β LG, beta-lactoglobulin; T1D, type 1 diabetes; Treg, regulatory T cell; TT, tetanus toxoid.

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vated Th17 cells. The activity of Th1, Th2, and Th17 cells can effectively be downregulated by regulatory T cells (Tregs), among them IL-10-producing Tr1 cells, which have potent immunosuppressive properties (17–19). Interestingly, a decrease of CD4⁺ FOXP3⁺ Tregs in peripheral blood of human subjects undergoing a mental stressor has been found (20). We therefore also analyzed IL-10 as an indicator of activated Tr1-cells and an important signal substance for immune cells with regulatory features.

In this study, we focus on four domains concerning psychological stress within the family situation: serious life events, parenting stress, lack of social support, and parental worries. A composite measure of these four domains of psychological stress was estimated to evaluate overall stress experienced in the family with focus on 5-y-old children (21). Exposure of stressors, in the absence of pathogenic challenge, can stimulate a systemic inflammatory response including, for example, cytokines and chemokines. In a rat model, it has been shown that an acute severe stressor could induce increased concentrations of inflammatory cytokines and chemokines (e.g., IL-1 β , IL-6, and monocyte chemoattractant protein-1 [CCL2]) (22). The proinflammatory cytokines IL-6 and TNF- α are also known to be enhanced by psychological stressors such as depression and abuse in childhood (23). Schizophrenia patients, asked about childhood trauma (e.g., death of a family member, exposure to physical and/or sexual abuse or significant illness or injury in childhood), also show increased plasma levels of both IL-6 and TNF- α (24). In contrast, higher perceived self-efficacy has been associated with significantly lower plasma concentration of IL-6 in children aged 7–13 y (25). Thus, we analyze IL-6 and TNF- α , as well as the chemokines CCL2, macrophage inflammatory protein-1 α (MIP-1 α ; CCL3), and IFN- γ -induced protein 10 (CXCL10), as markers of inflammation.

Solid evidence indicates that factors acting during fetal life, childhood, and adolescence have substantial effect on health and well-being on the individual throughout life (26). However, limited research has examined childhood adversity and stressors in relation to cell-mediated immune function. Therefore, we have examined the relationship between psychological stress and immune response in healthy children, with special focus on autoimmunity. Immune markers and clinical parameters are measured in 5-y-old children with high psychological stress (a composite measure of stress in the family across the domains: serious life events, parenting stress, lack of social support, and parental worries) and compared with a group of children without high psychological stress in the family.

Materials and Methods

Study population

This study is part of the prospective, longitudinal All Babies in Southeast Sweden (ABIS) project, involving 17,000 families who agreed to participate. The study presented in this article is based on data provided by the families at the 5- to 6-y follow-up. The follow-ups entailed extensive questionnaires and biological samples (urine, hair, and blood). We compared immune markers in prospectively collected blood samples from children with high psychological stress in the family and from children with low psychological stress in the family. High psychological stress in the family was defined as being exposed to stressors in three or four of the domains assessed (i.e., serious life events, parenting stress, lack of social support, and parental worries, as described later). PBMCs were available from 26 children (6 girls, 20 boys) exposed to high psychological stress. To evaluate the association between psychological stress and immune function, we selected a control group (C) consisting of 26 children (13 girls, 13 boys). The control group had no exposure in any of the four stress domains, irrespective of heredity or signs of autoimmunity. To examine the association between psychological stress and, more specifically, the autoimmune process associated with T1D, we chose a second control group (C1) consisting of 26 children (13 girls, 13 boys) who were not exposed to factors in any of the four stress domains and had no autoimmune disease, no diabetes-related autoantibodies, no T1D in the family, and no current infection. Demographics- and diabetes-related variables of the children ex-

posed to high psychological stress and the two control groups are presented in Table I.

Definition of stress

Four domains concerning psychological stress in the family were assessed in the 5- to 6-y questionnaire. First, *serious life events* were assessed with the following yes and no questions: “Have you been exposed to something which you perceive as a serious life event since the birth of your child?” and “Has the child been exposed to something which you perceive as a serious life event since the birth of the child?” Examples given were death of a relative, serious disease in the family, serious accident in the family, divorce, and exposure to violence or unemployment. Second, *parenting stress* was assessed with three subscales from the Swedish Parenting Stress Questionnaire (27), assessing the dimensions “incompetence” (11 items), “spouse relationship problems” (5 items), and “role restriction” (7 items). Six-point Likert scales ranging from “strongly disagree” to “strongly agree” were used. Children to parents with a calculated mean value over the 95th percentile (i.e., 3.87) were considered exposed to parenting stress. Third, *lack of social support* was assessed with 10+10 items (derived from Crnic et al. [28] and used by Ostberg and Hagekull [29]), including items such as “How many times do you meet your friends/relatives and/or keep in contact via telephone per week?” For each of the 10 quantitative questions, the parents were asked to rate how satisfied they were with the amount of support received, on 5-point Likert scales running from “very satisfied = 1” to “very dissatisfied = 5.” Mean values higher than the 95th percentile (i.e., 3.10) on the 10 qualitative items were defined as “lack of support.” Fourth, *parental worries* was assessed with seven items involving a potential risk for the child (i.e., that the child will fall seriously ill, get a serious or chronic disease, is going to be harmed, is going to be handicapped, is not developing normally, is going to be exposed to abuse, or is not going to survive). The parents estimated each item on a six-point Likert scale ranging from “very calm” to “very worried” concerning how worried they were that their child would get affected. Mean values higher than the 95th percentile (i.e., 4.55) were defined as “exposure to parental worries.”

A composite measure of psychological stress was calculated to evaluate the overall stress experienced in the family. This was done by counting the number of times the child was exposed in any of the four measured domains (*four* being the highest score and *zero* the lowest score; composite measure of this kind has been used by Ostberg [30] and Wekerle et al. [31]). Subjective scores indicating severe stress in three or four of these domains is supposed to reflect high stress in the family, with a likely negative impact on for example, the family climate, sleep patterns, parental tolerance, and relationship quality, all of which would be stressful for the child. Hence, children exposed to high psychological stress in their families are called the high-stress group (HS).

Separation and cryopreservation of PBMCs

Sodium heparinized blood samples were transported within 24 h to the research laboratory in Linköping, Sweden. PBMCs were separated with Ficoll Paque density centrifugation (Pharmacia Biotech, Sollentuna, Sweden). Because blood samples were taken at different occasions, PBMCs were cryopreserved in liquid nitrogen accordingly; PBMCs were centrifuged for 10 min at 400 $\times$ g. Freezing medium at a temperature of +4°C, containing 50% RPMI 1640 without glutamine (Life Technologies, Stockholm, Sweden), 40% FCS (Life Technologies), and 10% dimethyl sulfoxide (Me₂SO; Sigma-Aldrich, Stockholm, Sweden) was added dropwise while the tubes were agitated. PBMCs (concentration 5–10 $\times$ 10⁶ PBMCs/ml) were then moved to cryo vials. The vials were placed in a precooled (+4°C) Cryo 1°C Freezing Container (Nalge Nunc International, Rochester, New York) containing isopropanol. The container was placed in –70°C, and the freezing rate was –1°C/min. The vials were then transferred to liquid nitrogen (–196°C) on the following day and stored frozen until analysis (32).

In vitro culturing of PBMCs

PBMCs were quickly thawed from –196°C to +37°C in a water bath. Cell suspension was immediately washed, by adding RPMI 1640 containing 10% FCS dropwise, and then centrifuged at 400 $\times$ g for 10 min (32). One million PBMCs (viability >85% was considered acceptable) were diluted in 1000 μ l AIM V supplemented with 2 mM L-glutamine, 50 μ g/l streptomycin sulfate, 10 μ g/l gentamicin sulfate (Invitrogen Corporation, Carlsbad, CA), and 2 $\times$ 10^{–5} M 2-ME (Biochemica, Steinheim, Germany). PBMCs were then incubated at 37°C, in a humidified atmosphere with 5% CO₂ with medium exclusively (spontaneous secretion), or stimulated with Ag or autoantigen (Table II). All seven Ags have been explored in previous studies (32, 33), and order of priority in case of inadequate cell count was spontaneous, glutamic acid decarboxylase 65 (GAD₆₅) protein, heat shock

Table I. Background variables in children exposed to high psychological stress in the family, control group, and control group 1

Background Variables	HS (n = 26)	C + C1 (n = 26 + 26)	C (n = 26)	C1 (n = 26)	p
Demographic variables					
Education: mother					0.115 ^a
Low (elementary school = 1)	2	4	0	4	
Higher (all other education = 0)	24	48	26	22	
Education: father					0.183 ^a
Low (elementary school = 1)	4	9	2	7	
Higher (all other education = 0)	20	43	24	19	
Age					
Mother	30.80 (4.11) ^b	29.98 (3.16) ^b	29.00 (2.39) ^b	30.96 (3.59) ^b	0.106 ^c
Father	33.90 (3.77) ^b	31.65 (3.92) ^b	31.57 (4.10) ^b	31.79 (3.90) ^b	0.113 ^c
Foreign origin					0.054 ^a
One/both parents born outside Sweden = 1	4	1	1	0	
Both parents born in Sweden = 0	21	51	25	26	
Number of siblings	1.36 (0.76) ^b	1.47 (0.97) ^b	1.38 (0.90) ^b	1.54 (1.07) ^b	0.752 ^c
Marital status					0.004 ^a
Single = 1	8	2	1	1	
Living with partner = 0	18	50	25	25	
Body mass index child	16.53 (1.89) ^b	15.79 (1.74) ^b	15.40 (1.25) ^b	16.15 (2.10) ^b	0.075 ^c
Diabetes-related variables					
First-degree relative with T1D					0.128 ^a
Yes = 1	2	0	0	0	
No = 0	24	52	26	26	
Glutamic acid decarboxylase autoantibodies					0.128 ^a
>95th percentile					
Positive = 1 (>63.8 U/ml)	2	0	0	0	
Negative = 0	24	52	26	26	
Insulin autoantibodies over 95th percentile					0.289 ^a
Positive = 1 (>2.4 U/ml)	1	2	2	0	
Negative = 0	19	45	19	26	
Tyrosine phosphatase autoantibodies					
Positive = 1 (>9.9 U/ml)	0	0	0	0	
Negative = 0	26	52	26	26	

Background variables (demographical and T1D related) in children exposed to high psychological stress in the family (HS), control group (C), and control group 1 (C1).

^aStatistical analysis: χ^2 test.

^bStatistical analysis: mean (SD).

^cStatistical analysis: ANOVA.

protein 60 (HSP60) peptide, tetanus toxoid (TT), tyrosine phosphatase (IA-2) peptide, GAD₆₅ peptide, insulin peptide, β -lactoglobulin (β LG), and finally, PHA. PBMCs were harvested after 72 h of stimulation. The culture supernatants were saved and stored at -70°C for further analysis of cytokines and chemokines. By measuring the spontaneous secretion of cytokines and chemokines, and then subtracting these values from those obtained from the stimulated PBMC secretion, it was possible to measure the secretion that depended only on Ag stimulation.

Detection of cytokines and chemokines by multiplex fluorochrome technique (Luminex)

Cytokines and chemokines in cell-culture supernatants were analyzed with multiplex fluorochrome technique (Luminex, Bio-Rad). Cytokines (IL-5, IL-6, IL-10, IL-13, IL-17, IFN- γ , and TNF- α) and chemokines (CCL2, CCL3, and CXCL10) were analyzed in the high-stress group and in the two control groups using a Human Cytokine 10-Plex Group I assay (Bio-Rad Laboratories, Hercules, CA). A Bio-Plex 200 system (Luminex xMAP

Technology, Austin, TX) was used for identification and quantification of each cytokine/chemokine, and the threshold was set to a minimum of 50 individual microspheres per region. Raw data (median fluorescence intensity) for each reaction were analyzed using Bio-Plex Manager Software 5.0. To obtain sample concentration values, we used a five-parameter logistic equation to calculate each standard curve. The cutoff value enforced for minimum detectable concentrations for each immunological marker was as follows: IL-5 (0.59 pg/ml), IL-6 (0.47 pg/ml), IL-10 (0.42 pg/ml), IL-13 (0.37 pg/ml), IL-17 (0.39 pg/ml), IFN- γ (0.53 pg/ml), TNF- α (1.26 pg/ml), CCL2 (0.39 pg/ml), CCL3 (0.36 pg/ml), and CXCL10 (0.69 pg/ml).

Cortisol

Cortisol in sera was determined with chemoluminescence using Advia Centaur XP (Siemens, Erlangen, Germany). The method is competitive, where cortisol in the sample competes with marked cortisol in solution, to bind to polyclonal Abs. In a secondary step, another mAb, covalently bound to paramagnetic particles, binds to form a signal proportional to cortisol

Table II. Concentration and source of Ags used for stimulation of PBMCs

Ag	Protein or Peptide	Concentration	Source
β LG	Protein	50 $\mu\text{g/ml}$	Sigma-Aldrich, Stockholm, Sweden
GAD65	Protein, 65-kDa isoform	5 $\mu\text{g/ml}$	Diamyd, Medical AB, Stockholm Sweden
GAD65	Peptide, aa 247–279	5 $\mu\text{g/ml}$	Department of Medical and Physiologic Chemistry, University of Uppsala, Uppsala, Sweden
HSP60	Peptide, aa 473–460	5 $\mu\text{g/ml}$	The Weizmann institute of Science, Rehovot, Israel
Insulin β -chain	Peptide, aa 9–23	0.5 $\mu\text{g/ml}$	New England Peptide, Gardner, MA
PHA	Protein	5 $\mu\text{g/ml}$	Sigma-Aldrich, Stockholm, Sweden
TT	Protein	20 Lf/ml	SBL, Stockholm, Sweden
IA-2	Peptide, aa 853–872	10 $\mu\text{g/ml}$	New England Peptide

concentration. The detection limit for the assay is 5.5 nmol/L, and the reference value is 200–700 nmol/L between 07.00 and 09.00 AM.

C-peptide, proinsulin, and glucose

C-peptide was determined with a sandwich assay using the Cobas e602 (Roche, Basel, Switzerland). The sample was incubated with a biotinylated C-peptide-specific Ab, a C-peptide-specific Ab coated with an uthenium complex and streptavidin to form a complex, measured at 620 nm. The detection limit for this assay was 0.003 nmol/L, the fasting reference value for C-peptide being 0.12–1.2 nmol/L.

Proinsulin was determined using ELISA technique (DRG diagnostics, Marburg, Germany), according to the supplier's recommendation. In brief, serum was incubated in coated wells with enzyme conjugate, thereafter substrate solution was added, and the plate read at 450 nm. The range of the standard curve was 0–66 pmol/L.

Glucose was determined with a spectrophotometric method, using Advia 1200/1650/1800 (Siemens, Erlangen, Germany). In brief, glucose was phosphorylated in the presence of ATP and glucose hexokinase, thereafter oxidized in the presence of NAD, and the end product, NADH, measured at 340 nm. The detection limit for this assay was 0.2 mmol/L, the reference value being 4.2–6.0 mmol/L.

Statistics

Because the secretion of immunological markers was not normally distributed, Kruskal–Wallis test for unpaired observations was used as a pre-test, for comparison of three groups, and if significant, two groups were further analyzed with the Mann–Whitney *U* test for unpaired observations. Spearman's rank correlation coefficient (ρ) was used when comparing paired nonparametric variables. Concerning background variables, χ^2 test was used for categorical variables and ANOVA was used to compare mean values across the groups. A *p* level <0.05 was considered to be statistically

significant. Calculations were performed using the statistical package IBM SPSS 19.0 (New York, NY).

Ethical considerations

All appropriate approvals for the ABIS study were given by the research ethics committees of the Faculty of Health Science at the University of Linköping, Sweden, and the Medical Faculty at the University of Lund, Sweden. Parents gave their consent after oral and written information. All questionnaire data and biological samples are safely kept in a registered bio bank.

Results

Low spontaneous secretion but increased TT- and β LG-induced response in children exposed to high psychological stress

Spontaneously secreted IL-5 ($p < 0.001$; Fig. 1A), IL-10 ($p < 0.001$; Fig. 1B), IL-13 ($p < 0.001$; Fig. 1C), IL-17 ($p = 0.001$; Fig. 1D), CCL3 ($p < 0.001$; Fig. 1E), CCL2 ($p < 0.001$; Fig. 1F), and CXCL10 ($p < 0.01$; Fig. 1G) were lower in high-stressed children (composite measure of the domains: serious life events, parenting stress, lack of social support, and parental worries) compared with the control group (Table III). Exclusively, spontaneously secreted IFN- γ was higher among high-stressed children compared with the control group ($p < 0.01$; Fig. 2, Table III).

In contrast, high-stressed children showed increased levels of the cytokines IL-5 ($p < 0.001$; Fig. 3A), IL-6 ($p < 0.001$; Fig. 3B), IL-10 ($p < 0.001$), IL-13 ($p < 0.001$; Fig. 3C), IL-17 ($p < 0.001$; Fig. 3D), IFN- γ ($p < 0.001$; Fig. 3E), and TNF- α ($p < 0.001$;

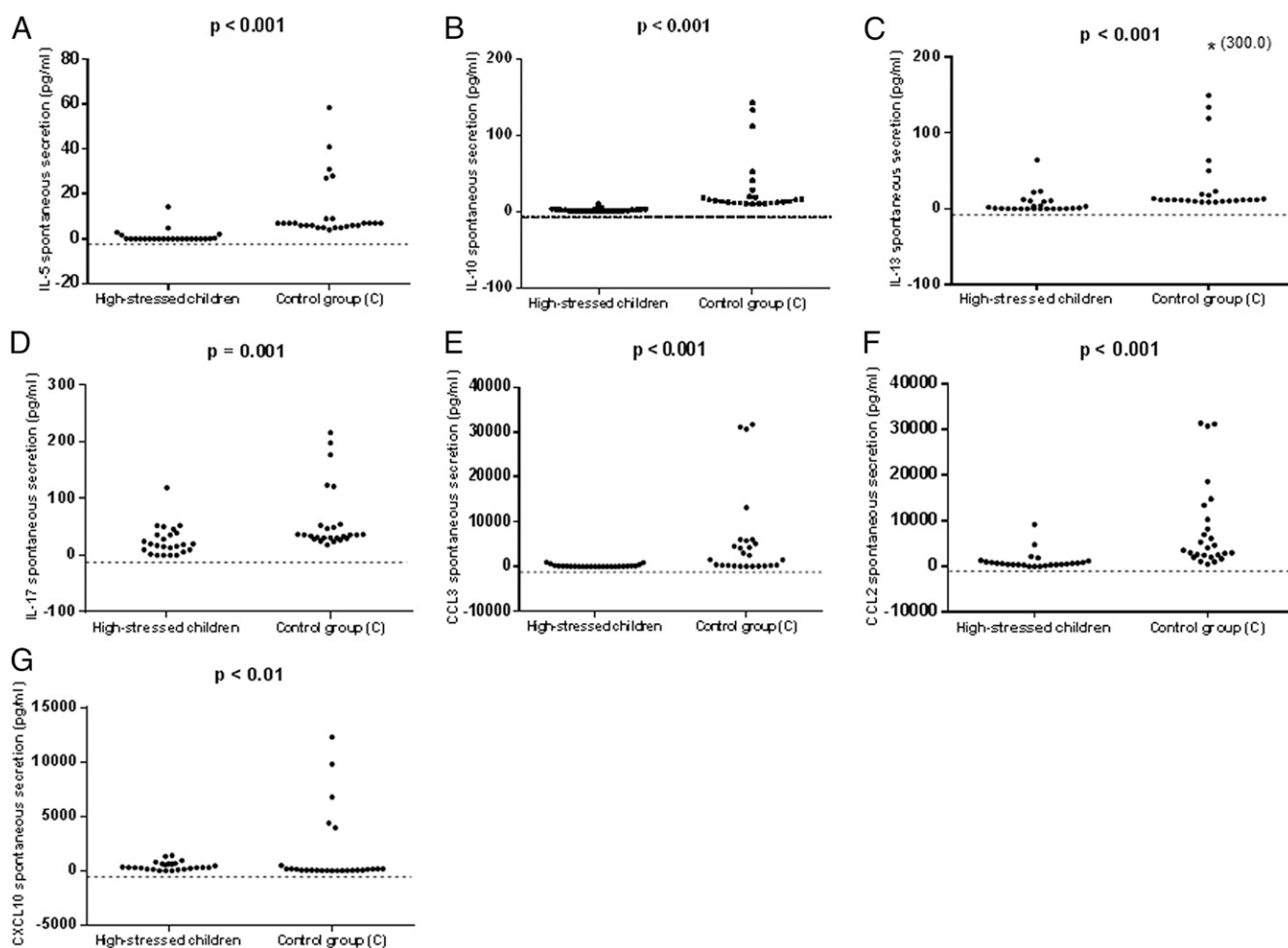


FIGURE 1. Low spontaneous secretion of cytokines and chemokines in children exposed to high psychological stress. Spontaneous secretion of cytokines and chemokines; IL-5 (A), IL-10 (B), IL-13 (C), IL-17 (D), CCL3 (E), CCL2 (F), and CXCL10 (G), analyzed by fluorochrome (Luminex) technique (pg/ml), were lower in high-stressed children compared with control (C) children (Mann–Whitney *U* test).

Table III. Summary of immunological changes by exposure to high psychological stress: immune imbalance hypothesis

	Th1-like		Th2-like		Tr1-like		Th17-like	
	IFN- γ		IL-5		IL-10		IL-17	
	High Stress	Control	High Stress	Control	High Stress	Control	High Stress	Control
Spontaneous secretion								
Mann-Whitney <i>U</i> test	166.0 ^{a,***}	25	5.0 ^{b,***}	5	10.5 ^{b,***}	11	31.0 ^{b,***}	31
Median	52	6-563	0	0-59	1	0-300	19	0-216
Min-max	0-823		0-14		0-65		0-119	
TT induced								
Mann-Whitney <i>U</i> test	3.0 ^{a,***}	3	2.4 ^{a,***}	2	21.5 ^{a,***}	20	9.0 ^{a,***}	4
Median	389	-32 to 1200	61	-10 to 2159	311	-86 to 7756	53	-49 to 107
Min-max	-714 to 1978		-11 to 516		-43 to 2463		-115 to 186	
β LG induced								
Mann-Whitney <i>U</i> test	3.0 ^{a,***}	3	1.0 ^{a,*}	1	5.0 ^{a,***}	5	2.0 ^{a,***}	2
Median	489	-27 to 5399	8	-2 to 47	76	-31 to 418	27	-26 to 163
Min-max	124-1123		0-88		1-229		2-48	
Proinflammatory								
Chemokines								
IL-6			TNF- α			CCL2		
High Stress	Control	High Stress	Control	High Stress	Control	High Stress	Control	High Stress
Spontaneous secretion								
Mann-Whitney <i>U</i> test	68.0 ^{a,***}	13	20.0 ^{a,***}	17	157.4 ^{b,***}	252	1905.4 ^{b,***}	453.3 ^{b,***}
Median	1035	-5471 to 11,611	235	-679 to 985	73	0-31,717	644	300
Min-max	-6457 to 13,437		-560 to 853		0-978		4-9217	0-1401
TT induced								
Mann-Whitney <i>U</i> test	733.8 ^{a,***}	734	33.5 ^{a,***}	32	34.6 ^{a,***}	47	116.1 ^{a,***}	8
Median	5431	35-5,174,169	389	-40 to 11,130	278	-12,929 to 5894	739	-7574 to 6604
Min-max	-22 to 34,462		57-1490		-9217 to 6393		-1247 to 4910	

Secretion of cytokines and chemokines, spontaneously, in vitro Ag-induced (TT and β LG), in high-stress children and in control groups (C and CI) detected by fluorochrome (Luminex) technique (pg/ml). Median and range (min-max) are presented for the high-stressed and control group, respectively.

^aIncreased secretion of cytokines and chemokines.

^bDecreased secretion of cytokines and chemokines.

Mann-Whitney *U* test value presented together with *p* values that represent differences in cytokine and chemokine secretion between high-stress children and control groups (C and/or CI): **p* < 0.05, ***p* $\leq$ 0.01, ****p* < 0.001.

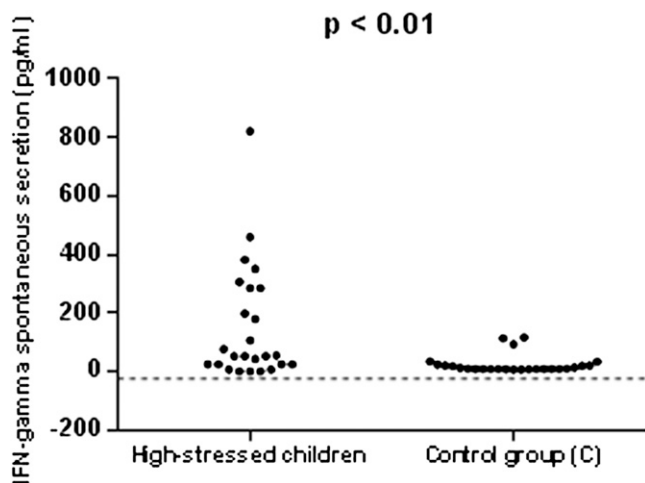


FIGURE 2. High spontaneous secretion of IFN- γ in children exposed to high psychological stress. Spontaneous secretion of IFN- γ , analyzed by fluorochrome (Luminex) technique (pg/ml), were higher in high-stressed children compared with control (C) children (Mann-Whitney *U* test).

Fig. 3F), as well as the chemokines CCL2 ($p < 0.01$, Fig. 3G) and CXCL10 ($p < 0.001$, Fig. 3H), compared with the control group when stimulated in vitro with TT, as well as β LG (Table III).

Increased proinflammatory response to the autoantigens GAD₆₅, HSP60, and IA-2 in children exposed to high psychological stress

Assessing the composite measure of stress, we found that high-stressed children showed increased levels of the chemokines CCL2 ($p < 0.01$; Fig. 4A) and CXCL10 ($p < 0.01$; Fig. 4B) from in vitro stimulation with the HSP60 peptide, increased levels of CCL2 ($p < 0.01$) from in vitro stimulation with the IA-2 peptide, and increased levels of IL-6 ($p < 0.05$) and CCL3 ($p < 0.05$) from in vitro stimulation with GAD₆₅ compared with the control groups (C and C1; Table IV).

The domain *serious life events* was reported regarding the child by 17 of 26 families. In 13 of these 17 families, *lack of social support* was also indicated as a stressor. This specific group of children showed increased levels of IL-5 ($p < 0.05$; Fig. 5A), IL-6 ($p < 0.05$; Fig. 5B), IL-10 ($p = 0.01$; Fig. 5C), IL-17 ($p < 0.01$; Fig. 5E), and IFN- γ ($p < 0.05$; Fig. 5F) in comparison with the control group (C1), and also increased levels of IL-6 ($p = 0.01$) and IL-13 ($p < 0.05$; Fig. 5D) compared with the control group (C) after in vitro stimulation with GAD₆₅.

β cell stress/function versus immune markers

Cortisol was significantly higher ($p = 0.01$; Fig. 6A) in high-stressed children compared with the controls (C1). In contrast, secretion of C-peptide was lower ($p < 0.05$; Fig. 6B) in high-stressed children compared with the controls (C1), and inversely

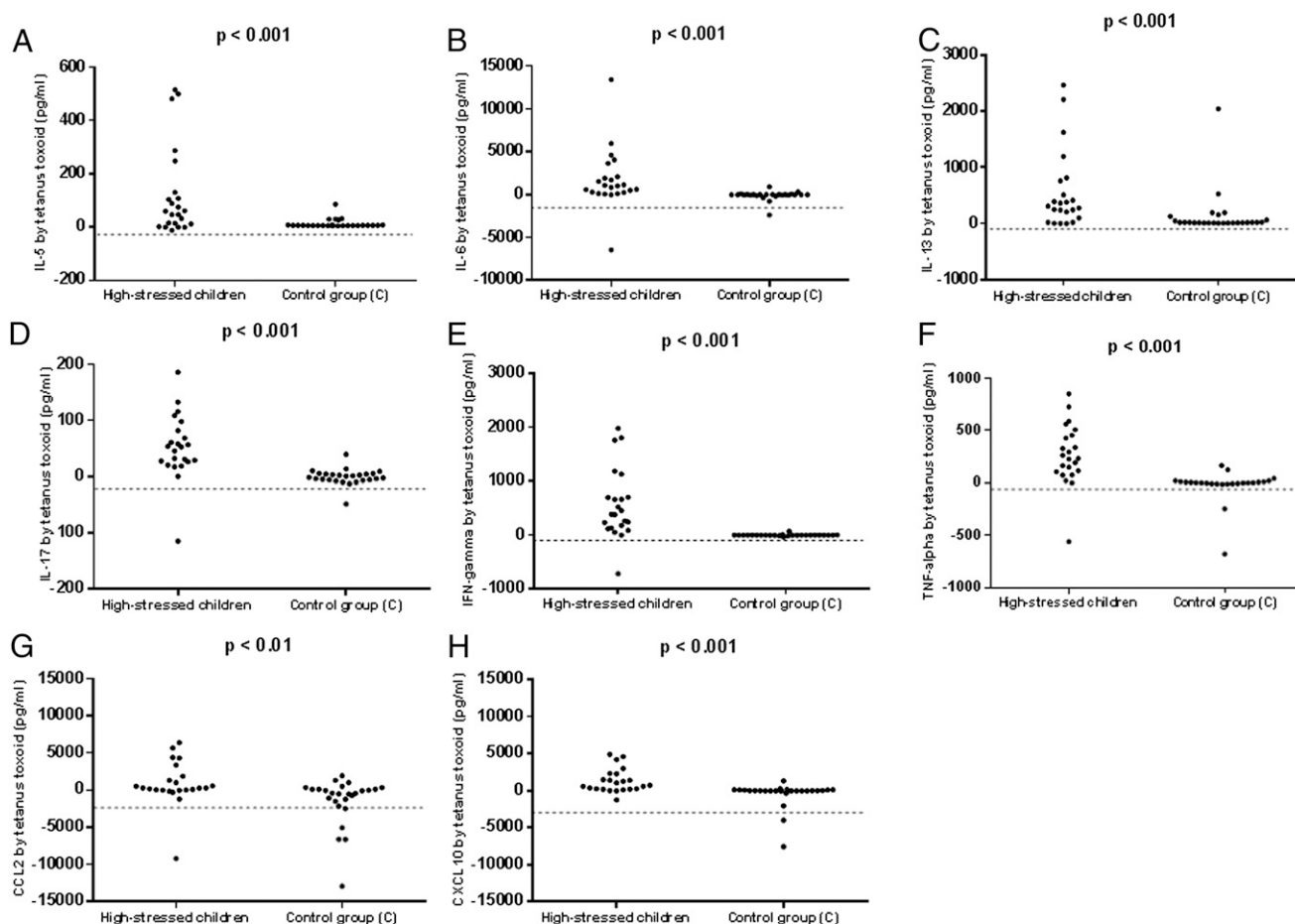


FIGURE 3. High TT-induced secretion of cytokines and chemokines in children exposed to high psychological stress. Secretion of cytokines and chemokines; IL-5 (A), IL-6 (B), IL-13 (C), IL-17 (D), IFN- γ (E), TNF- α (F), CCL2 (G), and CXCL10 (H) from PBMCs, in vitro stimulated with TT and analyzed by fluorochrome (Luminex) technique (pg/ml), were higher in high-stressed children compared with control (C) children (Mann-Whitney *U* test).

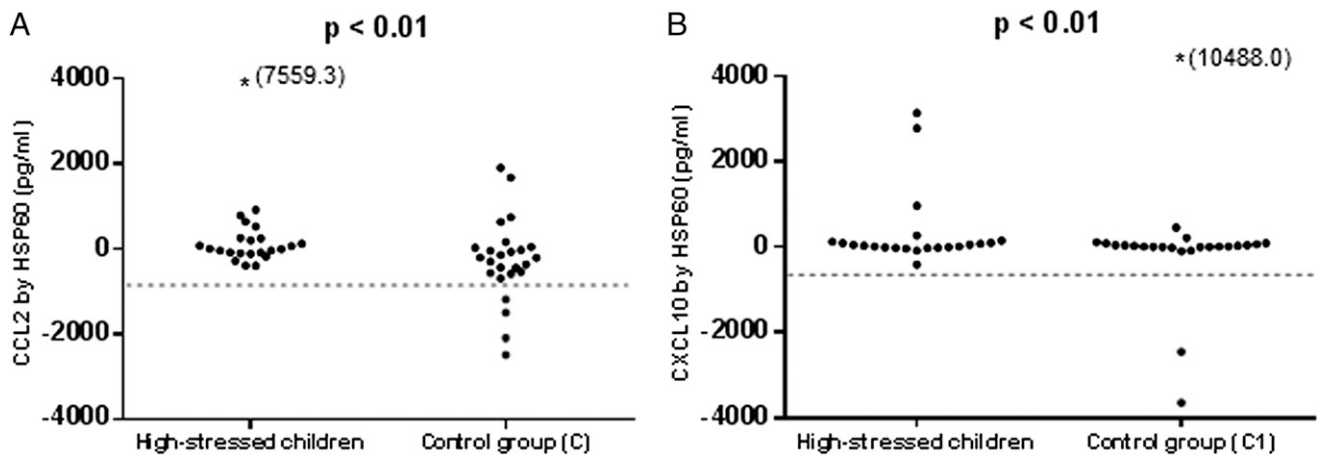


FIGURE 4. High HSP60 peptide-induced secretion of chemokines in children exposed to high psychological stress. Secretion of chemokines; CCL2 (**A**) and CXCL10 (**B**) from PBMCs, in vitro stimulated with the HSP60 peptide (aa 473–460) and analyzed by fluorochrome (Luminex) technique (pg/ml), were higher in high-stressed children compared with control (C and C1) children (Mann–Whitney *U* test).

correlated to spontaneously secreted IL-5 in high-stressed children ($r = -0.446$, $p < 0.05$).

There were no significant differences in glucose or proinsulin levels between the high-stressed children and the two control groups. However, glucose was inversely correlated to cortisol ($r = -0.434$, $p < 0.05$) but positively correlated to spontaneously secreted IL-10 ($r = 0.492$, $p = 0.01$; Fig. 7A), IL-17 ($r = 0.421$, $p < 0.05$; Fig. 7B), and CXCL10 ($r = 0.597$, $p < 0.01$; Fig. 7C) in high-stressed children.

Discussion

In this study, we aimed to examine the association between high psychological stress in the family (measured as the four domains: serious life events, parenting stress, lack of social support, and parental worries) and immune response. Two key results, regarding immune response in relation to psychological stress, emerged from this study. First, our results indicate that children exposed to high psychological stress in the family have a low spontaneous response in contrast with an increased TT- and β LG-induced response. Second, our results indicate that children exposed to high psychological stress have an increased response to the diabetes-associated autoantigens GAD₆₅, HSP60, and IA-2.

In more detail, high-stressed children showed a decreased spontaneous secretion of a number of different cytokines (IL-5, IL-10, IL-13, and IL-17) and chemokines (CCL2, CCL3, and CXCL10). This indicates that high-stressed children have a low spontaneous activity of Th2, Tr1, and Th17 cells, which suggests a suppressive immune response. Several studies have shown that stress exposure is associated with impaired cell-mediated acquired immunity (20, 34–39). In 2010, Freier et al. (20) showed that Tregs are downregulated during stress and concluded that this might result in an exacerbation of inflammatory conditions such as an autoimmune condition. Further, a recent study showed that chronic exposure to stress shifts the Th1/Th2 balance toward a proinflammatory Th1/Th17 dominance versus Th2 response and also downregulates Tregs in mice (40).

Several studies have shown that Th1 cells play a crucial role in the development of autoimmune diseases, for example, T1D (41, 42). We observed that exclusively the Th1-associated cytokine IFN- γ was elevated in high-stressed children. This is of interest, because we, as well as others, have shown a Th1-like dominance by high IFN- γ secretion in children with high risk for development of T1D (33, 43–45). Previous studies have shown that IFN- γ inhibits proliferation of Th2 and Th17 (46, 47), which correlates

well with our results of low levels of Th2 and Th17 versus high levels of Th1.

In contrast, in vitro stimulation with the Ags TT and β LG showed an increased secretion of several cytokines (IL-5, IL-6, IL-10, IL-13, IL-17, IFN- γ , and TNF- α) and chemokines (CCL2 and CXCL10) in high-stressed children. Thus, in vitro stimulation with both TT and β LG induced activity of Th1, Th2, Th17, Tr1 cells, as well as increased release of proinflammatory cytokines. The increased activity of both Th1 and Th2 cells might reflect an imbalance in the immune system. Th2 cells are supposed to have a protective effect against β cell destruction (45, 48, 49). However, higher modes of activation of Th2 can also lead to activation of B cells and Ab production, which can lead to inflammatory conditions such as allergy and asthma.

The high activity of Th1 and Th2 cells combined with a high activity of Tr1 cells might indicate failure to regulate the proliferation of these immune cells. We also observed a high activity of Th17 cells, which are thought to play a key role in autoimmune diseases such as diabetes, rheumatoid arthritis, and Crohn's disease (50–52). Studies have also shown increased levels of Th17 cells in children with new-onset T1D (53). Moreover, IL-17 stimulates the expression of inflammatory cytokines and chemokines such as TNF- α , IL-6, and CCL2 (41), strengthening our own results of an increased proinflammatory response. It has also been shown that childhood adversities can cause heightened IL-6 and TNF- α levels in later adult life (23).

Previous studies have found that adverse life events, such as divorce, loss of a close relative, and serious illness, are considered risk factors for T1D (12, 54). In 2005, Sepa et al. (10) showed similar results, indicating that maternal experience of serious life event was associated with diabetes-related autoimmunity in their children. It is well-known that children are very sensitive to parental signals, behavior, and moods (55), and that adverse childhood experiences later can lead to age-related diseases (16, 56, 57). In 2001, Wilkin (58) suggested the “accelerator hypothesis,” with three processes that accelerate the loss of β cells: constitution, insulin resistance, and autoimmunity. The β cell stress hypothesis (10, 11, 59) is an extension of the accelerator hypothesis, stating that a number of different factors, for example, psychological stress, reduced physical activity and rapid weight gain, cause β cell stress by an increase in insulin demand. This hypothesis has gained some empirical support in the ABIS project, where stress in the family (e.g., high parenting stress and serious life events such as divorce) was associated with

Table IV. Summary of immunological changes by exposure to high psychological stress: β cell stress hypothesis

	Proinflammatory				Chemokines					
	IL-6		TNF- α		CCL3		CCL2		CXCL10	
	High Stress	Control	High Stress	Control	High Stress	Control	High Stress	Control	High Stress	Control
GAD65 (protein) induced										
Mann-Whitney <i>U</i> test	15.0 ^{a,*}				-37.0 ^{a,*}	-93				
Median	33	7			-19					
Min-max	-381 to 9946	-6682 to 1646			-438 to 1693	-6293 to 1597				
HSP60 (peptide) induced										
Mann-Whitney <i>U</i> test							-72.5 ^{a,***}	-73	-8.66 ^{a,**}	-1
Median							1		29	
Min-max							-392 to 7559	-7712 to 3260	-416 to 3134	-6722 to 10,488
IA-2 (peptide) induced										
Mann-Whitney <i>U</i> test							7.5 ^{a,***}	-12		
Median min-max							218			
Min-max							-594 to 14,638	-12348 to 4627		

Secretion of cytokines and chemokines, in vitro autoantigen-induced (GAD65 and peptides of HSP60 and tyrosin phosphatase [IA-2]), in high-stress children and in control groups (C and CI) detected by fluorochrome (Luminex) technique (pg/ml). Median and range (min-max) are presented for the high-stressed and control group, respectively.

^aIncreased secretion of cytokines and chemokines.

Mann-Whitney *U* test value presented together with *p* values that represent differences in cytokine and chemokine secretion between high-stress children and control groups (C and/or CI): **p* < 0.05, ***p* ≤ 0.01.

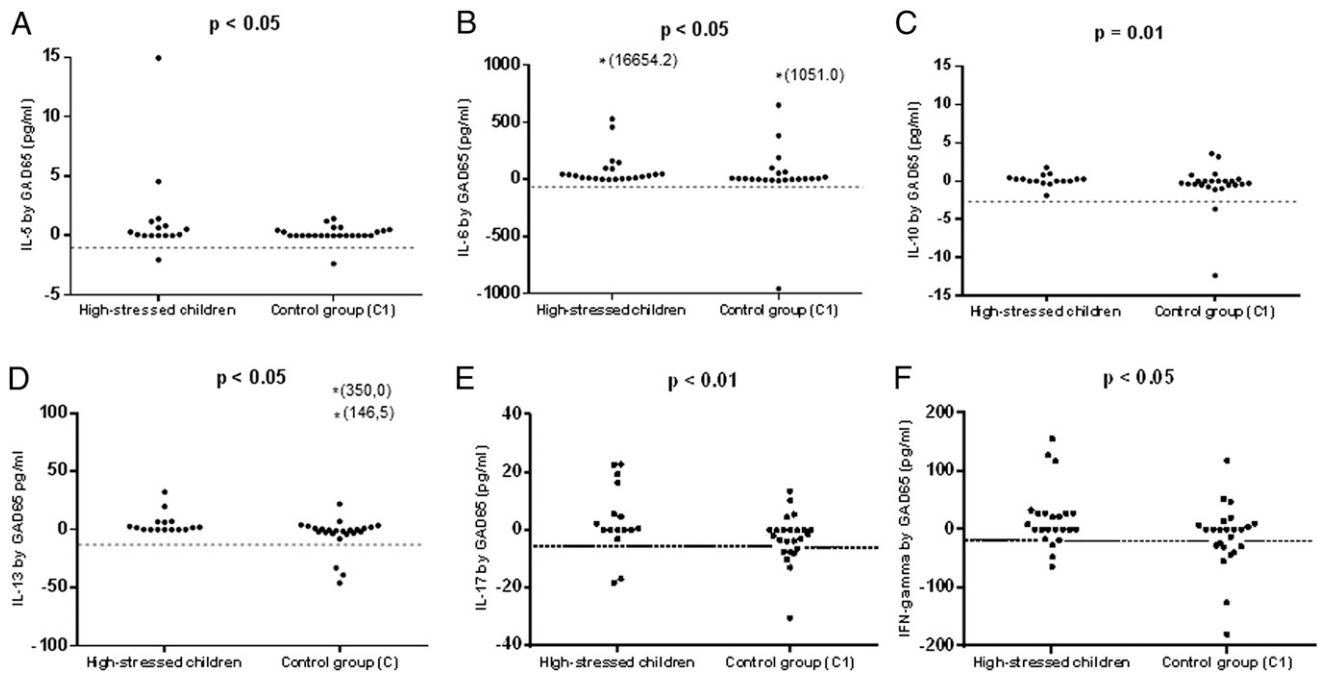


FIGURE 5. High GAD₆₅-induced secretion of cytokines and chemokines in children who experienced especially serious life events and lack of social support. Secretion of cytokines and chemokines; IL-5 (A), IL-6 (B), IL-10 (C), IL-13 (D), IL-17 (E), and IFN-γ (F) from PBMCs, in vitro stimulated with GAD₆₅ and analyzed by fluorochrome (Luminex) technique (pg/ml), were higher in children who experienced especially serious life events and lack of social support compared with control (C1) children (Mann–Whitney *U* test).

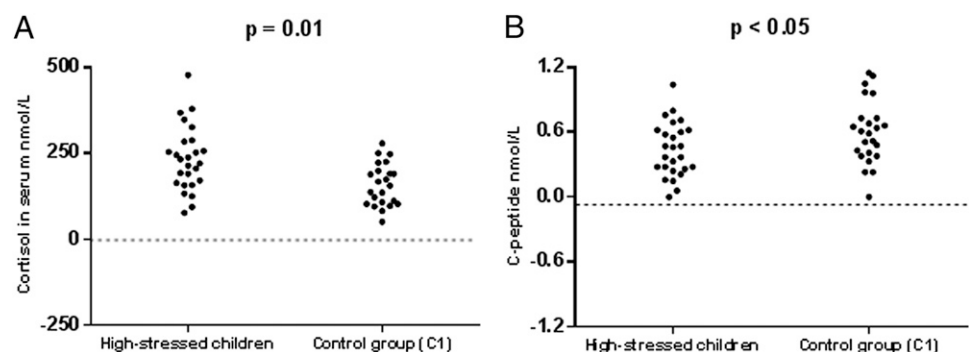
the occurrence of diabetes-related autoantibodies in 1- and 2.5-y-old children from the general population (8, 58).

In our cohort, the children in control group 1 (C1) were selected as follows: no autoantibodies, no autoimmune diseases in the family, and no stressors. Thus, this control group can specifically be compared with immune response against diabetes-related autoantigens in high-stressed children. Our results indicate that high-stressed children have an increased response to diabetes-related autoantigens compared with this specific control group (C1). Projecting the immune response on one single domain, serious life event (reported for the child by the parent), together with the domain lack of social support, an increase of a number of different cytokines (IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ, and CCL3) was observed after in vitro stimulation with GAD₆₅. Peptides of the T1D-associated autoantigens, HSP60 and IA-2, also caused an increased secretion of chemokines (CCL2, CCL3, and CXCL10). This result indicates that children exposed to, especially, serious life events have an increased inflammatory response to the T1D-associated autoantigen GAD₆₅, one of several autoantigens associated with the destruction of the β cells and suggested to cause proliferation of Th1-like lymphocytes in T1D development (60–63). Because lymphocytes specific for GAD₆₅ are among the first

to enter inflamed islets in T1D (60), this autoantigen has, in recent clinical trials, been used in attempts to preserve β cell function in T1D (64, 65). Children exposed to serious life events also showed an increased inflammatory response to the T1D-associated autoantigen IA-2. Previous studies have demonstrated that T1D patients, or those at risk for development of the disease, display a cellular response toward IA-2 (66), indicating the importance of IA-2 as an autoantigen involved in the autoimmune process preceding the development of T1D.

Children in high-stressed families were found to secrete high levels of cortisol but low levels of C-peptide compared with the children in the control group (C1). High levels of cortisol are considered a marker for a stress-induced activation of the HPA axis, whereas low levels of C-peptide are considered a marker for exhausted β cells. An inverse correlation between IL-5 and C-peptide was also found in the high-stressed children. Low levels of spontaneously secreted IL-5 indicate decreased protective properties known to be carried out by Th2 cells. We can speculate that the results presented in this article show a stress-related pressure on the β cells and a low protective immune response in children from families with high stress. This may induce an autoimmune reaction leading to progression toward T1D.

FIGURE 6. High cortisol and low C-peptide in children exposed to high psychological stress. Children exposed to high psychological stress secreted higher levels of cortisol (A), analyzed by chemoluminescence, but lower levels of C-peptide (B), analyzed by sandwich assay, compared with control (C1) children (Mann–Whitney *U* test).



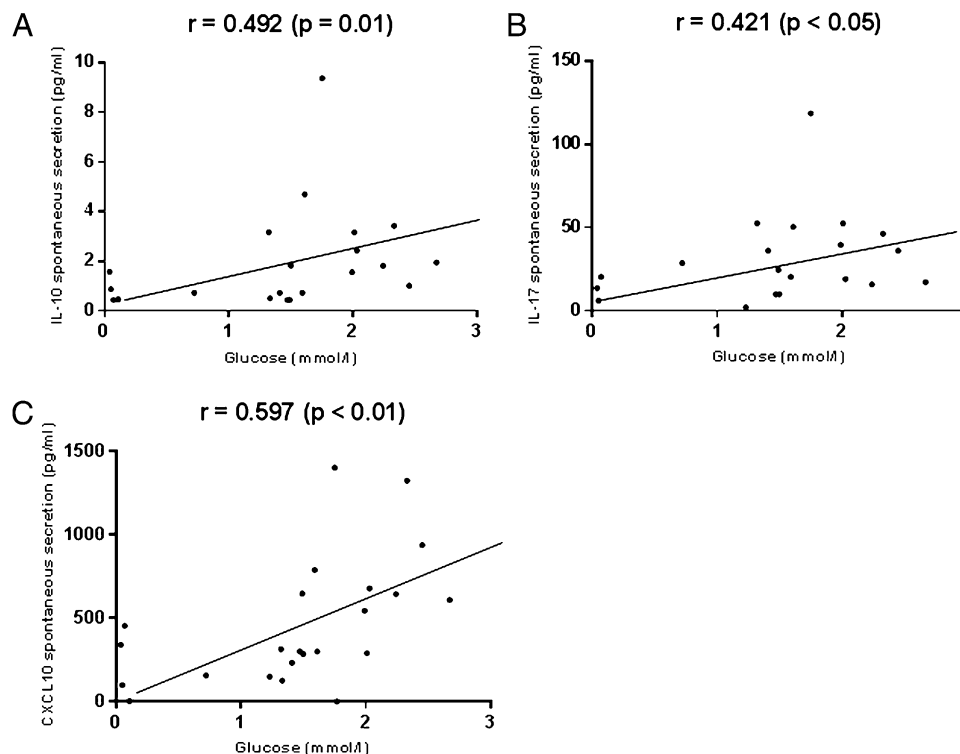


FIGURE 7. Spontaneous secretion of cytokines and chemokines are related to glucose level in children exposed to high psychological stress. Spontaneous secretion of cytokines and chemokines; IL-10 (A), IL-17 (B), and CXCL10 (C), analyzed by fluorochrome (Luminex) technique (pg/ml), were correlated to glucose concentration (mmol/l) in high-stressed children (Spearman's rank correlation coefficient).

Surprisingly, we found a negative correlation between cortisol and glucose, but a positive correlation between glucose and the proinflammatory cytokines IL-10, IL-17, and CXCL10 in high-stressed children. One could speculate that the high levels of cortisol in high-stressed children puts pressure on the β cells to produce more insulin, which decreases the glucose levels and thereby decreases the levels of spontaneously secreted IL-10, IL-17, and CXCL10. It is possible that the low level of the regulatory cytokine IL-10, as a direct cause of the high cortisol level, fails to suppress Th1 proliferation, and thereby induces a possible autoimmune reaction initiating a prediabetic phase in high-stressed children.

More importantly, the background variable *single parent* showed that high-stressed children to a greater extent live with a single parent, compared with children in the control groups. This may indicate that children living with a single parent are more exposed to, or less protected from, psychological stress than children living with more than one caregiver. Other background factors such as body mass index and foreign origin showed no significant difference between the groups of high-stressed and control children. Thus, we believe these background factors to be of minor importance for the outcome of immune responses observed in our cohort.

To our knowledge, there is no published evidence that sex is affecting the stress-induced immune reactivity in children. Actually, Mills et al. (67) suggested that traditional epidemiologic characteristics, such as sex, have limited influence on lymphocytosis. Rather, interindividual differences in sympathetic nervous system characteristics play a more prominent role in acute cellular immune system activation. Nevertheless, we have chosen an equal distribution of sex in both control groups to give, as far as possible, a general picture of the immune profile in healthy children not exposed to high stress in the family. However, the children constituting our group of high-stressed children were unfortunately not equally distributed in respect to sex. Anyhow, we found the same differences in immune response, regardless of sex.

Thus, in our cohort, sex did not affect the outcome of stress-induced immune reactivity in high-stressed children.

In conclusion, the results indicate that psychological stress may have a general effect on the immune system, causing not only an immune suppression, but also an imbalance that may possibly contribute to an autoimmune reaction against β cells. Moreover, children exposed to psychological stress, especially serious life events, induce an immune response against diabetes-related autoantigens. This may indicate an autoimmune reaction against the insulin-producing β cells, which, in some individuals, initiate progression toward T1D.

Disclosures

The authors have no financial conflicts of interest.

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