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The Immunological Effect of Photopheresis in Children with Newly Diagnosed Type 1 Diabetes

MARIA KARLSSON FARESJÖ, JAN ERNERUDH, GÖSTA BERLIN, JORGE GARCIA, AND JOHNNY LUDVIGSSON

Division of Pediatrics and Diabetes Research Centre [M.K.F., J.G., J.L.], Division of Clinical Immunology [J.E.], Division of Transfusion Medicine [G.B.], Department of Molecular and Clinical Medicine, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden

ABSTRACT

Photopheresis has been claimed to have immune-modulating effects, but the mechanisms of action are unknown. This study investigated the immune effect of photopheresis in children with type 1 diabetes, with a focus on the balance of Th1- and Th2-like cytokines. Ten children with newly diagnosed type 1 diabetes (10–17 y) were treated with five double treatments of photopheresis and 10 children matched for disease, age, and gender were given placebo tablets and sham pheresis. Expression of IFN-γ and IL-4 mRNA was determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and secretion of IFN-γ, IL-10, and IL-13 in cell-culture supernatants by ELISA after stimulation with glutamic acid decarboxylase (GAD_{65}) (a.a. 247–279), the ABBOS peptide (a.a. 152–169), insulin, phytohemagglutinin (PHA), and keyhole limpet hemocyanin (KLH). Photopheresis changed antigen-stimulated immune balance in line with a Th2-like shift. Thus, the ratio of IFN-γ/IL-4 mRNA expression after *in vitro* stimulation with a peptide of the autoantigen GAD_{65} was reduced after treatment in the photopheresis group. The IFN-γ/IL-4 mRNA expression ratio after *in vitro* stimulation with insulin was also lower in children treated with photopheresis compared with the placebo group. Photopheresis has an immune-modulating effect in children with type 1 diabetes, causing a Th2-like deviation. (***Pediatr Res* 58: 459–466, 2005**)

Abbreviations

GAD_{65}, glutamic acid decarboxylase 65
ICA, islet cell antibody
KLH, keyhole limpet hemocyanin
MOP, methoxypsoralen
PBMCs, peripheral blood mononuclear cells
PHA, phytohemagglutinin

Type 1 diabetes, characterized as an autoimmune disease, has been shown to be transferable by $CD4^+$ T cells expressing a Th1-like cytokine profile in neonatal NOD mice (1). Th1 responses, of which IFN-γ is a signature cytokine (2,3), stimulate phagocytose-dependent cellular immune responses that are detrimental in several organ-specific autoimmune diseases (4,5) as well as in transplantation reactions (6). Th1-responses are counteracted by Th2 cytokines, including IL-4 and IL-13. IL-13, shown to be produced mainly by activated Th2-like lymphocytes and natural killer cells (7), shows functions similar to those of IL-4 (8). In addition, IL-10 is a potent antiinflammatory cytokine, with down-regulating effects on Th1 responses (9). Evidence that β-cell destruction in type 1 diabetes has a Th1-like origin is accumulating, and it has also been suggested that Th2-like lymphocytes are protective for the disease (10,11).

It has been claimed that photopheresis has immune-modulating effects (12) and is a treatment of great interest for several immunological diseases including cutaneous T-cell lymphoma (13,14), acute and chronic graft versus host disease (15), cardiac allografts rejection (16), rheumatoid arthritis (RA) (17), systemic lupus erythematosus (SLE) (18) and psoriatic arthritis (19). However, the mechanisms of action are not well understood. It is known that 8-MOP on activation by UVA light, interacts with bases of DNA (20) and binds to sites on the cell surface and/or in the cytoplasm of the target cells (21). It has also been suggested that photopheresis is effective through dendritic-cell/T-cell interactions in stimulating T-cell responses or down-regulating a preexisting T-cell response (22,23). In a photopheresis trial of children with newly diagnosed type 1 diabetes, we noticed a moderate clinical effect with lower insulin requirement and slightly better residual insulin secretion in the treatment group (24). The double-blind,

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Correspondence: Maria Karlsson Faresjö, Ph.D., Clinical Research Center, Division of Pediatrics, Faculty of Health Sciences, S-581 85 Linköping, Sweden: e-mail: maria.faresjo@imk.liu.se
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placebo-controlled (placebo tablets and sham pheresis), randomized design allowed us to investigate short-term effects on the immune system and dissect them from the natural course or placebo effect. Our focus was the balance between Th1- and Th2-like cytokines, and we hypothesized that photopheresis could induce a shift in the balance between Th1 and Th2 cytokines.

METHODS

Treatment with photopheresis. Twenty children with newly diagnosed type 1 diabetes (10–17 y, 90% carrying the HLA-type DR3/4), a random subsample of a larger study (24), were included in the present detailed study on autoantigen-induced cytokine profiles. Ten children were randomly selected from the group of active treatment of photopheresis, consisting of an oral dose of 8-MOP and a subsequent apheresis procedure. Plasma and buffy coat cell solution concentrations of 8-MOP were followed (25). The mean plasma concentration was 320 ng/mL (range 101–466) and the mean 8-MOP concentration of the cell solution during irradiation was 106 ng/mL (range 30–186). The buffy coat cells were then exposed to UVA light (2 J/cm^2) for 90 min and returned to the patient's circulation. The control group, comprising 10 children with type 1 diabetes, matched by age and gender, received placebo tablets and sham treatment (similar procedure but without passage of blood cells through the photopheresis machine). The apheresis procedure (active photopheresis or sham pheresis) was administrated on two consecutive days (double treatment). The first treatment was given approximately 5–6 d after the diagnosis of type 1 diabetes and then repeated after 14, 28, and 42 d and 3 mo.

Peripheral blood mononuclear cells. Peripheral blood was collected right before start of each treatment at d 1, 14, and 28 and finally after 3 mo, the first sample thus representing status before any treatment (S1), and the remaining samples representing status after 1 (S2), 2 (S3), and 4 (S4) double-treatment procedures, respectively. PBMCs were isolated by Ficoll Paque density gradient centrifugation (Pharmacia, Biotech, Sollentuna, Sweden) from sodium heparinized venous blood samples. As blood samples were taken at different occasions, PBMCs were cryopreserved in liquid nitrogen until use.

Peptides and antigens. The synthetic peptide of human GAD_{65}) (a.a. 247–279) and the synthetic milk-derived BSA-peptide ABBOS (a.a. 152–169) (26) (both purchased from the Department of Medical and Physiologic Chemistry, University of Uppsala, Sweden), insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark), KLH (Calbiochem, Lab Kemi, Stockholm, Sweden), and PHA (Sigma Chemical Co., Stockholm, Sweden) were used for stimulation of PBMCs.

PBMCs and stimulation procedures. Frozen mononuclear cells were thawed, directly from –196°C to +37°C, in a water bath during addition of RPMI-1640 supplemented with 10% FCS; 1×10^6 PBMCs (viability approximately 90% for each population) were diluted in 500 μL AIM V serum-free medium (GIBCO, Täby, Sweden) supplemented with 2 mM L-glutamine, 50 μg/L streptomycin sulfate, 10 μg/L gentamicin sulfate and 2×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.). Then 1×10^6 PBMCs were stimulated with GAD_{65} (a.a. 247–279), the ABBOS peptide, PHA or KLH, all at a concentration of 5 μg/mL, or with insulin at a concentration of 50 μg/mL (27,28) for 48 h at 37°C in 5% CO_2 for further analysis of the expression of cytokine-specific mRNA.

For analysis of cytokines in the cell-culture supernatants, 1×10^6 PBMCs were stimulated with the same concentration of PHA or KLH, whereas 50 μg/mL was used for GAD_{65} (a.a. 247–279) and the ABBOS peptide (29) and insulin was used at a concentration of 500 μg/mL for 96 h at 37°C in 5% CO_2. PBMCs, incubated without antigen but otherwise under the same conditions, were used for determination of spontaneous expression of mRNA and secretion of cytokines. In samples with a limited number of cells, the order of priority for stimulation with antigens was GAD_{65} (a.a. 247–279), the ABBOS peptide, insulin, PHA, and KLH.

RNA isolation, cDNA synthesis, and real-time RT-PCR. Total RNA was isolated from PBMCs as described previously (29). Using equal amounts of total RNA (7 ng/μL) from PBMCs stimulated under various conditions, mRNA was marked with complementary random hexamers and cDNA was synthesized as described elsewhere (29). PCR reaction mixture contained specific target primers and probes for IFN-γ, IL-4, and endogenous reference rRNA (TaqMan cytokine gene expression kit, Perkin Elmer). The reaction mixture was amplified with ABI Prism 7700 Sequence Detector (Perkin Elmer) for 40 cycles with an annealing temperature of 60°C for IL-4 and IFN-γ according to previous description (29). In all experiments, controls without template as well as control RNA (Perkin Elmer) were included.

Calculation of relative quantification values. The relative quantification values for the cytokine gene expression assays were calculated from the accurate C_T, according to the manufacturer's description (protocol P/N 4304671, Perkin Elmer). The accurate C_T represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected, obtained from both dye layers in the assay. C_T value for rRNA (VIC) was subtracted from the specific cytokine C_T value (FAM for IFN-γ and IL-4) to calculate Δ-C_T for the calibrator and samples in each cytokine gene expression assay. Δ-C_T values for duplicate wells of the calibrator sample for each cytokine were averaged. The average delta (calibrator) value was subtracted from the Δ-C_T values of samples to calculate their $\Delta\Delta$-C_T (sample) value. The $\Delta\Delta$-C_T value for duplicate wells of each cytokine sample was averaged. This operation normalizes the number of target mRNA molecules to the number of rRNA molecules. Relative quantification values were obtained after calculating the 2-average $\Delta\Delta$-C_T.

ELISA. IFN-γ at the level of protein was measured in cell-culture supernatant by ELISA (30) in microtiter wells (Costar 3690, Life Technologies, Stockholm, Sweden) coated with 2 μg/mL monoclonal mouse anti-human antibody (clone 2571811, R&D Systems, Abingdon, U.K.) in coating buffer (CLB, Amsterdam, Netherlands) overnight at room temperature. Wells were blocked with 2% milk in PBS for 1 h at room temperature. Cell supernatants and standard (recombinant human IFN-γ, R&D) were applied in duplicates and incubated for 1 h at room temperature. IFN-γ was detected by 0.20 μg/mL goat anti-human biotinylated polyclonal antibody (R&D) diluted in high-performance ELISA dilution buffer (CLB) for 1 h at room temperature and further incubated for 30 min at room temperature with horseradish peroxidase–conjugated to polystreptavidin (0.1 μM, CLB). A colored product was formed in proportion to the amount of IFN-γ, after incubation with 3,3′,5,5′-tetramethylbenzidine liquid substrate (Sigma Chemical Co.-Aldrich) for 30 min in the dark and thereafter with 1.8 M H_2SO_4 to stop the reaction. The sensitivity for IFN-γ was 12 pg/mL, according to the manufacturer.

IL-10 and IL-13 were measured by commercially available ELISA (CLB Pelikine Compact, Amsterdam, The Netherlands). In brief, microtiter wells (Costar 3690, Life Technologies, Stockholm, Sweden) were coated with a monoclonal anti-human antibody in coating buffer overnight at room temperature, whereas nonbound material was removed by washing. Wells were blocked with 5% milk in PBS for 1 h at room temperature. Cell supernatants and standards (recombinant human IL-10/IL-13) were applied in duplicates and incubated for 1 h at room temperature. Cytokine was detected by anti-human biotinylated polyclonal antibody diluted in high-performance ELISA dilution buffer for 1 h at room temperature and further incubated for 30 min at room temperature with horseradish peroxidase–conjugated to polystreptavidin. The sensitivity was 2.35 pg/mL for IL-10 and 1.0 pg/mL for IL-13, according to the manufacturer.

Antigen-induced cytokine response was presented as pg/mL after subtraction of spontaneously secreted cytokine from PBMCs incubated without antigen but otherwise cultured under the same conditions.

C peptide. C peptide was determined with a RIA technique based on the original assay developed by Heding (31). The detection limit for the assay is 0.03 nmol/L, and our local reference value among fasting healthy children and adolescents is 0.18–0.63 nmol/L (32).

ICAs. ICA was detected with immunofluorescence on human pancreas sections according to Bottazzo *et al.* (33). A monospecific anti-IgG, conjugated with fluorescein-isothiocyanate was used for detection. Our method was standardized according to International Juvenile Diabetes Foundation (IJDF) standards for ICA determination. Our laboratory has participated in an international ICA workshop, with a specificity as well as sensitivity of 100%.

GAD autoantibodies (GADAs). GADAs were determined according to Grubin *et al.* (34) and Petersen *et al.* (35), using recombinant human ^{35}S-GAD_{65} as tracer according to Falorini *et al.* (36). Serum was immunoprecipitated with human ^{35}S-GAD_{65} in duplicates. Included in each assay was one strongly positive control (international JDF standard) and three negative samples (sera from three healthy controls). Our laboratory participated in the second GADA proficiency test 1996 and reached a sensitivity and a specificity of 100%. The cutoff level for positivity was mean + 3 SD for healthy controls (= 0.055).

Insulin antibodies (IAs). IAs were detected with a radioligand competition assay according to Palmer *et al.* (37). Included in each assay were a positive control (pool of sera from three IA-positive patients) and a negative control (with undetectable IA). Results were calculated as the difference between incubation with ^{125}I-insulin and incubation with ^{125}I-insulin and excess of unlabeled antigen. Cutoff was set at mean + 2 SD for healthy controls (= 99 nU/mL).

Statistics. Treatment *versus* placebo groups were compared by Mann-Whitney *U* test and paired groups (pre- *versus* posttreatment) by Wilcoxon signed rank test. Three or more groups were compared with the Kruskal-Wallis test for unpaired observations. Spearman's rank correlation was used when

comparing two variables nonparametrically. A probability level of <0.05 was considered to be statistically significant, whereas $p \leq 0.1$ was regarded as a tendency. Calculations were performed with a statistical package StatView 5.0.1 for Macintosh (Abacus Concepts Inc., Berkeley, CA)

Ethics. The study was approved by the Research Ethics Committee of the Faculty of Health Sciences, Linköping University, and all parents or responsible guardians gave their informed consent on behalf of the children.

RESULTS

Short-term immunological effects of photopheresis treatment. Photopheresis caused changes in the immune balance of children with type 1 diabetes in a number of parameters (Table 1 *a, b*). Spontaneously secreted IL-10 increased during the whole period of treatment ($p < 0.05$) (Table 1*b* shows changes in cytokine profile within the group of children treated with photopheresis).

GAD_{65} peptide-induced expression of IFN-γ mRNA decreased during treatment with photopheresis ($p < 0.05$) (Table 1*a*). In contrast, IL-4 mRNA expression tended to increase during treatment with photopheresis ($p = 0.07$), although expression of IL-4 mRNA was lower in these children before treatment ($p < 0.01$) (Table 1*b*). Thus, the ratio of expressed IFN-γ/IL-4 mRNA decreased significantly among children treated with photopheresis ($p < 0.01$) (Fig. 1*a*), whereas the IFN-γ/IL-4 mRNA ratio in fact increased in the sham pheresis group ($p < 0.05$) (Table 1*a* and Fig. 1*b*).

In children treated with photopheresis, insulin-induced expression of IL-4 mRNA increased ($p < 0.05$) (Table 1*b*) in relation to decreased IFN-γ mRNA expression ($p = 0.01$) (Table 1*a*) at the end of treatment. Thus, the ratio of IFN-γ/IL-4 mRNA decreased during active treatment ($p < 0.01$) (Fig. 2*a*). Insulin also decreased the secretion of IL-10 in children with type 1 diabetes during treatment with photopheresis ($p < 0.05$) (Table 1*b*).

The only significant effect from *in vitro* stimulation with the ABBOS peptide was observed as an increased secretion of IFN-γ in children treated with photopheresis ($p < 0.05$) (Table 1*a*).

PHA always induced prominent mRNA expression and cytokine secretion. However, the expression of both IFN-γ mRNA ($p < 0.05$) (Table 1*a*) and IL-4 mRNA ($p < 0.05$) (Table 1*b*) decreased during photopheresis treatment. Thus, the ratio of PHA-induced IFN-γ/IL-4 mRNA expression ($p = 0.01$) (Table 1*a*) as well as secretion of IL-10 ($p < 0.05$) (Table 1*b*), decreased after 3 mo of treatment with photopheresis.

Immunological effects in children treated with photopheresis compared with placebo-treated children. Spontaneous secretion of IFN-γ tended to be lower after 2 wk of treatment in the photopheresis group compared with the placebo group ($p = 0.08$). However, IFN-γ secreted spontaneously and was higher in the actively treated group at 3 mo compared with the sham pheresis group ($p < 0.05$) (Table 1*a*, photopheresis *versus* placebo).

The GAD_{65} peptide–induced secretion of IFN-γ tended to be lower among children treated with photopheresis when compared with the placebo group 1 mo after initial treatment ($p = 0.07$). In contrast, GAD_{65} peptide–induced secretion of IL-13 was higher after 2 wk of treatment with photopheresis compared with the levels in the placebo group ($p < 0.05$) (Table 1*b*).

The expression of IFN-γ mRNA ($p < 0.05$) and secretion of IFN-γ ($p < 0.05$), after *in vitro* stimulation with insulin, was significantly lower in the photopheresis group compared with the placebo group after 3 mo of treatment (Table 1*a*). Thus, the ratio of IFN-γ/IL-4 mRNA was lower in children treated with photopheresis compared with children in the sham pheresis group after 3 mo ($p = 0.001$) (Fig. 2*b*).

Exposure to PHA, regardless of treatment, caused increased secretion of IFN-γ, which tended to be correlated with the expression of IFN-γ mRNA during the first month of treatment ($\mathbf{r} = 0.62$, $p = 0.08$). The expression of IFN-γ mRNA ($p < 0.01$) (Table 1*a*) and IL-4 mRNA ($p < 0.05$) (Table 1*b*) was higher 2 wk after initial photopheresis treatment of the children with diabetes compared with the placebo group. In contrast, in the negative control, KLH did not cause any pronounced immune response in PBMCs from children with type 1 diabetes (data not shown). Further, the response from stimulation with the ABBOS peptide, both at the level of transcription and after translation into cytokines, did not differ between children treated with photopheresis or placebo.

Effect of photopheresis on C peptide and antibodies. C peptide increased during the first 3 mo in all children regardless of treatment with photopheresis or placebo (Table 2). The level of islet cell antibodies (ICA) was significantly lower at onset and at 1 mo among children receiving photopheresis treatment compared with children treated with sham pheresis ($p < 0.05$) (Table 2, photopheresis *versus* placebo). The frequency of children positive for GAD_{65} autoantibodies (GADA) was equal in children treated with photopheresis or placebo (seven of 10 children in each group). The numbers of insulin antibody (IA)-positive children treated with photopheresis or placebo during the first 2 wk were seven of 10 and nine of 10, respectively. Thereafter, all children regardless of treatment produced IAs and the concentration of IA increased at 3 mo in children treated with either photopheresis or sham pheresis ($p < 0.05$) (Table 2, changes in secretion). At 3 mo, there was a positive correlation observed between the GAD_{65} peptide–induced ratio of IFN-γ/IL-4 and GADA ($r = 0.87$, $p = 0.01$) but an inverse correlation between insulin-induced IFN-γ/IL-4 and IA ($r = -0.61$, $p = 0.07$).

DISCUSSION

We have previously reported that photopheresis has a slight short-term clinical effect on children with type 1 diabetes (24). Although there is little known of how photopheresis exerts its effects, we present cytokine data that give insight into immune mechanisms. The most prominent effects were found at the level of cytokine mRNA expression in the response against two putative autoantigens, a GAD_{65}-derived peptide, and insulin. Children treated with photopheresis responded to GAD_{65} with decreased expression of IFN-γ mRNA, in combination with increased expression of IL-4 mRNA. Thus, photopheresis induced a shift toward a Th2-like cytokine pattern. A Th2-like

Table 1a. *Th1-like cytokines*

		Photopheresis					Placebo					
Cytokines	Spontaneous/ *in vitro* stimulated	S1	S2	S3	S4	Changes in cytokine expression & secretion	S1	S2	S3	S4	Changes in cytokine expression & secretion	Photopheresis vs placebo
IFN-γ	Spontaneous	41	0	19	106	S2–S4 (+)	19	117	52	16	n.s.	S4 (+)
		0–453	0–82	0–592	35–265	$p < 0.05$	0–998	0–482	0–251	0–15		$p < 0.05$
IFN-γ	GAD_{65}	5.3	0	0	52	n.s.	0	14	48	25	n.s.	S3 (−)
	(a.a. 247–279)	−23–210	−15–35	−75–107	−5–249		−295–178	−71–128	0–284	−3–1,030		$p = 0.07$
		0.9	1.1	1.3	0.8	S2–S4 (−)	1.1	0.8	1.4	1.6	n.s.	n.s.
IFN-γ mRNA	(a.a. 247–279)	0.4–1.5	0.8–2.1	0.6–30	0.2–1.5	$p < 0.05$	0.4–3.4	0.5–2.2	0.5–2.6	0.2–4.1		
		1.3	0.9	1.2	0.7	S1–S4 (−)	0.9	0.9	1.1	1.0	S2–S3 (+)	n.s.
IFN-γ/IL-4 mRNA	(a.a. 247–279)	0.7–3.1	0.2–3.2	0.2–198	0.1–1.8	$p < 0.01$	0–4.2	0.2–2.9	0.6–2.8	0.2–8.8	$p < 0.05$	
IFN-γ	Insulin	−41	−7.3	−38	−160	n.s.	−26	−66	−52	0	n.s.	S4 (−)
		−444–14	−82.0	−592–0	−265–−43		−50–0	−162–0	−234–0	−51–0		$p < 0.05$
IFN-γ mRNA	Insulin	0.7	0.6	0.6	0.4	S3–S4 (−)	0.8	0.7	0.9	1.4	S1–S4 (+)	S4 (−)
		0.3–0.9	0.4–1.2	0.4–1.4	0.1–1.3	$p = 0.01$	0.5–1.4	0.2–3.5	0.4–2.5	0.4–4.8	$p < 0.05$	$p < 0.05$
		0.7	1.2	0.8	0.2	S2–S4 (−)	0.8	1.0	1.4	1.0	n.s.	S4 (−)
IFN-γ/IL-4 mRNA	Insulin	0–2.0	0.3–3.1	0.1–5.8	0–1.0	$p < 0.01$	0.2–2.4	0.1–4.6	0.7–3.0	0.4–8.2		$p = 0.001$
IFN-γ	ABBOS	−19	0	1.4	24	S1–S3 (+)	−12	−26	23	0	n.s.	n.s.
		−277–0	−54–13	−20–129	−52–16	$p < 0.05$	−26–0	−61–106	−50–51	−30–1,263		
		0.8	0.8	1.1	0.7	n.s.	1.0	0.7	0.9	1.4	S2–S4 (+)	n.s.
IFN-γ mRNA	ABBOS	0.4–1.2	0.5–1.7	0.2–26	0.3–2.2		0.5–2.4	0.2–1.6	0.4–1.4	0.2–5.0	$p < 0.05$	
		1.2	1.0	1.2	0.5	n.s.	0.7	0.8	1.0	1.2	n.s.	n.s.
IFN-γ/IL-4 mRNA	ABBOS	0.3–2.1	0.3–4.9	0.1–2.1	0.2–1.4		0.1–1.5	0.1–1.9	0.4–1.8	0.1–17.1		
IFN-γ	PHA	7,169	6,348	12,382	25,544	n.s.	5,283	4,093	7,410	23,606	n.s.	n.s.
		1,241–76,087	2,524–10,173	1,040–59,730	5,420–45,667		2,494–12,225	2,831–6,339	3,187–43,990			
		475	59	56	32	S1–S4 (−)	26	17	30	35	S2–S3 (+)	S2 (+)
IFN-γ mRNA	PHA	10–1,728	12–108	12–256	2–159	$p < 0.05$	56–379	3–46	1–261	3–993	$p < 0.05$	$p < 0.01$
IFN-γ/IL-4 mRNA	PHA	3.0	0.5	2.0	0.5	S1–S4 (−)	0.5	0.4	1.3	1.5	S1–S3 (+)	n.s.
		0.1–13.5	0.1–1.8	0.4–5.3	0–2.7	$p = 0.01$	0–9.2	0.1–2.2	0.4–2.4	0.2–12.7	$p < 0.05$	

The immunological effect of photopheresis in children with type 1 diabetes S, sample (S1, d 1; S2, d 14; S3, d 28; S4, mo 3); (+), increase; −, = decrease; n.s., not significant. Expression and secretion of cytokines, spontaneously and after *in vitro* stimulation with antigens, in children treated with photopheresis or placebo (sham pheresis), where the *p* value represents significantly up- or down-regulation. Photopheresis *versus* placebo represent differences in cytokine expression and secretion between children treated with photopheresis or placebo. Expression of IFN-γ (*a*) and IL-4 mRNA (*b*) detected by real-time RT-PCR, from stimulation with antigens after subtraction of spontaneous expressed mRNA is calculated as the relative amount of expressed cytokine in relation to rRNA and illustrated as median and range (in brackets). Secretion of the antigen-induced cytokines IFN-γ (*a*) and IL-10 and IL-13 (*b*), after subtraction of spontaneous secreted cytokine, detected at pg/mL by ELISA are illustrated as median and range (in brackets), where negative values still represent measurable concentrations.

Table 1b. *Th1-like cytokines*

Cytokines	Spontaneous/ *in vitro* stimulated	Photopheresis					Placebo					Photopheresis vs placebo
		S1	S2	S3	S4	Changes in cytokine expression & secretion	S1	S2	S3	S4	Changes in cytokine expression & secretion	
IL10	Spontaneous	21 0–118	34 0–215	51 4–155	87 3–313	S1–S4 (+) $p < 0.05$	29 0–311	89 0–736	58 0–360	134 0–573	S3–S4 (+) $p < 0.05$	n.s.
IL-13	Spontaneous	33 0–114	0 0–162	46 18–82	80 19–137	n.s.	13 0–156	115 9–317	85 5–207	19 0–278	n.s.	n.s.
IL-4 mRNA	GAD_{65}	0.9 0.2–1.9	1.4 0.3–3.5	1.1 0.4–9.8	1.0 0.7–3.6	S1–S4 (+) $p = 0.07$	1.8 0.8–22	1.1 0.3–2.4	1.2 0.5–2	0.9 0.3–1.8	S1–S4 (−) $p < 0.05$	S1 (−) $p < 0.01$
IL-10	(a.a. 247–279)	−0.6 −9.8–29	−4.7 −68–6	1.3 −88–23	−1.2 −167–10	n.s.	0.4 −72–24	−0.7 −62–12	1.9 −11–145	−8.0 −95–6	n.s.	n.s.
IL-13	(a.a. 247–279)	3.6 15–87	0 0–160	−7.1 −25–196	2.5 −51–118	n.s.	4.3 −82–119	−24 −122–2	39 −16–130	30 −5–126	S1-2 (−)S2–3 (+) $p < 0.05$	S2 (+) $p < 0.05$
IL-4 mRNA	Insulin	1.1 0.3–5.7	0.9 0.4–3.6	1.2 0.4–17.5	2.1 0.8–6.2	S3–S4 (+) $p < 0.05$	1.0 0.3–6.8	0.7 0.3–7.8	0.7 0.5–0.9	0.9 0.4–2.9	n.s.	n.s.
IL-10	Insulin	−8.8 −77–19	−24 −137–0	−33 −71–0	−51 −258–1	S1–S4 (−) $p < 0.05$	−7.8 −154–0	−50 −471–0	−9.2 −212–82	−45 −415–0	n.s.	n.s.
IL-13	Insulin	−33 −111–−10	−36 −162–0	−53 −82–−32	−81 −137–−19	n.s.	−26 −108–0	−100 −205–−9	−85 −207–5	−18 −56–0	n.s.	n.s.
IL-4 mRNA	ABBOS	0.9 0.2–4.8	1.4 0.3–4.6	1.2 0.5–4.6	1.2 0.6–6.2	n.s.	1.9 0.7–5.3	1.2 0.4–2.1	0.8 0.2–2.3	0.9 0.3–3.5	n.s.	n.s.
IL-10	ABBOS	−1.1 −11–57	−4.0 −19–73	−4.3 −21–14	−3.6 −178–159	n.s.	0 −23–154	−9.4 −30–4	1.5 −24–183	−14 −134–3	S1–S4 (−) $p < 0.05$	n.s.
IL-13	ABBOS	−12 −45–0	0 −54–29	−13 −75–34	−1.0 −14–19	n.s.	−3.5 −24–10	−12 −46–23	−18 −43–4	13 −35–81	S3–S4 (+) $p < 0.05$	n.s.
IL-4 mRNA	PHA	85 12–582	134 32–528	62 15–164	29 4–572	S1–S4 (−) $p < 0.05$	81 3–503	28 3–175	37 3–326	14 3–149	S1–S3 (−) $p = 0.01$	S2 (+) $p < 0.05$
IL-10	PHA	1774 217–4658	1156 204–6307	1746 552–2632	879 119–1957	S1–S4 (−) $p < 0.05$	1395 492–4081	1971 691–4040	2177 539–3898	2009 19–4177	n.s.	n.s.
IL-13	PHA	1008 273–2157	1260 916–1604	969 263–2542	1256 1234–1277	n.s.	1685 1579–3531	1407 869–1792	1836 841–2441	1120 692–1869	n.s.	S1 (−) $p < 0.05$

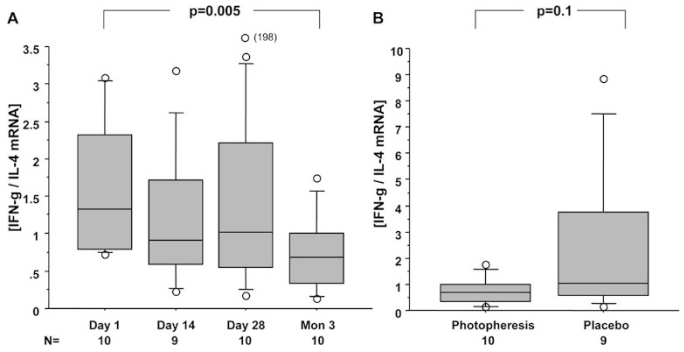

Figure 1. GAD_{65} (a.a. 247–279) induced IFN-γ/IL-4 mRNA, expressed as a ratio, decreased in children with type 1 diabetes during treatment with photopheresis ($p = 0.005$) (*a*) and tended to be lower compared with children treated with sham pheresis after 3 mo ($p = 0.1$) (*b*). Illustrated by box plots (10th, 25th, 50th, 75th, and 90th centiles, and outliers are indicated).

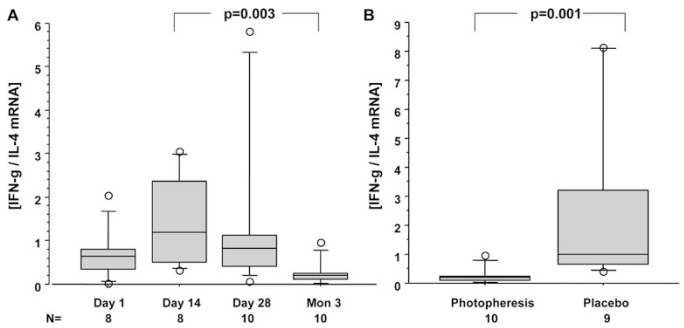

Figure 2. Children with type 1 diabetes treated with photopheresis responded with a decreased IFN-γ/IL-4 mRNA expression ratio to *in vitro* stimulation with insulin ($p = 0.003$) (*a*). The ratio was lower after 3 mo of treatment in the photopheresis group compared with the placebo group ($p = 0.001$) (**b**).

pattern, in the form of increased IL-4 mRNA expression, has previously been found after *in vitro* stimulation with GAD_{65} peptides in healthy, high-risk, first-degree relatives of patients with type 1 diabetes (28), indicating that this pattern has a protective role in the development of type 1 diabetes.

An immune-modulating effect by photopheresis could also be demonstrated after *in vitro* stimulation with insulin, which caused an inhibition of the cytokine response. Expression of Th1-associated IFN-γ mRNA decreased during treatment, implying a shift in favor of Th2. Deviation of the immune system toward Th2-like immunity after *in vitro* stimulation with insulin has been suggested to have an immunosuppressive effect in healthy, high-risk individuals (28). However, cytokine response by insulin at the level of translation into protein was very low. In fact, several recent studies have failed to detect any T-cell proliferation against insulin or insulin peptides in both diabetic and prediabetic patients (39–41). We have also previously reported about the phenomenon of a low IFN-γ response against insulin in both high-risk individuals and in children newly diagnosed with diabetes, in comparison with other antigens *e.g.*, the GAD_{65} peptide (28).

The mitogen PHA induced increased expression and secretion of both IFN-γ and IL-4 in all children studied. Expression of IFN-γ and IL-4 mRNA and also secretion of IL-10 did, however, decrease during the period of photopheresis treatment. Changes in cytokine response to PHA have also been observed in PBMCs from patients treated with photopheresis

Table 2. *C peptide and antibodies*

C-peptide and Auto a.b.	Photopheresis					Changes in secretion	Placebo					Changes in secretion	Photopheresis vs placebo
	S1	S2	S3	S4	S5		S1	S2	S3	S4	S5		
C-peptide	0.23 (0.08–0.58)	0.32 (0.07–0.83)	0.51 (0.11–0.99)	0.65 (0.20–1.39)	n.a.	S1–S4 (+) $p < 0.05$	0.30 (0.04–1.05)	0.33 (0.12–0.69)	0.44 (0.12–0.99)	0.48 (0.25–1.05)	n.a.	S2–S4 (+) $p < 0.05$	n.s.
ICA	50 (0–80)	60 (60–80)	40 (0–80)	n.a.	40 (0–80)	n.s.	80 (40–80)	80 (80–80)	80 (60–80)	n.a.	60 (20–80)	n.s.	S1 (−) $p < 0.05$ S3 (−) $p < 0.05$
GADA	0.392 (0.017–0.982)	0.291 (0.008–1.053)	0.268 (0.007–1.136)	0.260 (0.009–1.088)	0.419 (0–1.071)	n.s.	0.087 (0–1.018)	0.100 (0–1.010)	0.065 (0–0.979)	0.130 (0–0.999)	0.056 (0–1.003)	n.s.	S5 (+) $p < 0.05$
IA	2.7 (0–5.2)	2.6 (0.3–8.3)	4.2 (1.3–12.5)	7.0 (1.5–25.6)	n.a.	S1–S4 (+) $p < 0.05$	4.3 (0.8–19.7)	2.9 (0.2–11.2)	3.2 (1.7–18.6)	9.1 (0–41.4)	n.a.	S2–S4 (+) $p < 0.05$	S1 (−) $p < 0.05$

S, sample (S1, d 1; S2, d 14; S3, d 28; S4, mo 3; S5, mo 6); (+), increase; (−), decrease; n.s., not significant; n.a., not analyzed. Secretion of C peptide, islet cell antibodies (ICA), GAD_{65} autoantibodies (GADAs), and insulin antibodies (IAs) are illustrated as median and range (in brackets). *p* Value represents significant up- or down-regulation in children treated with photopheresis or placebo (sham pheresis) as well as comparison between the groups.

after heart transplantation (42). Secretion of IL-4 was found to increase, reaching a peak soon after initial treatment. IL-4 then progressively decreased together with decreased secretion of IFN-γ and IL-2 during continuous treatment with photopheresis (42). In contrast, KHL did not cause any pronounced immune response in PBMCs from children with type 1 diabetes, which indicates a selective and specific response of diabetes-associated antigens.

No differences in humoral or cellular response to BSA have been observed between patients newly diagnosed with diabetes and healthy adults (43,44). Treatment with photopheresis had no clear influence on mononuclear cells after *in vitro* stimulation with ABBOS, which is in agreement with our previous findings showing a modest increase in IFN-γ and IL-4 mRNA expression from ABBOS in both children with diabetes and healthy children (29). It is possible that the lack of response to this peptide can be influenced by the uptake of BSA and also the ABBOS peptide from FCS during cryopreservation.

We observed, not on an individual basis but in the group of children type 1 diabetes treated with photopheresis, a change mainly toward a Th2-like profile. These findings strengthen the importance of this kind of treatment, which obviously can affect the immune system. However, our treatment comes late in the disease process, when most of the β-cell function already is lost. Although our results clearly indicate an effect on autoreactive cytokine-secreting cells, the precise mechanisms for induction of this response are unknown. C peptide increased during the treatment period in all children regardless of treatment. Thus, we cannot show that the positive effect by photopheresis on β-cell function reported earlier (24) depends on the identified changes in immune balance.

The previously reported slight clinical effects and the immunological effects reported here correspond to the fact that only a minority of leukocytes are directly affected during each photopheresis procedure. It has previously been shown that reinfused treated cells stimulate an autologous suppressor response toward T cells of similar clones not reached by active photopheresis treatment (45), suggesting that resident cells are affected indirectly. This could be one reason why the immunological effect is seen after repeated treatments and especially during partial remission. Another possible mechanism would be that tolerized autoreactive T cells by bystander activation *e.g.*, in the target organ or in adjacent lymph nodes could induce down-regulating cytokines like IL-4, IL-10, and transforming growth factor β (46). Although speculative, another tempting explanation would be the induction of immune regulatory populations, as has been described in experimental models of autoimmunity (47).

In summary, our results show that photopheresis seems to have an immune-modulating effect on Th1/Th2-like cytokines, inducing a Th2-like shift in the response against a GAD_{65} peptide and insulin. Thus, treatment with photopheresis may act as an immunological modifier that can regulate a Th1/Th2-like imbalance toward normalization. This sheds light on the thus far obscure immunological effect of photopheresis treatment.

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