Regulatory T cell-associated activity in photopheresis-induced immune tolerance in recent onset type 1 diabetes children

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Summary

Extracorporeal photochemotherapy (ECP) has demonstrated immunological effects. The proposed cytotoxic lymphocyte antigen 4 (CTLA-4) involvement, together with forkhead box P3 (FoxP3) and transforming growth factor (TGF)-β are associated with regulatory T cell activity. The aim of the study was to evaluate the regulatory T cell-associated effect of ECP in recent onset type 1 diabetic (T1D) children. Children ($n = 20$) with T1D received photopheresis 8-methoxypsoralen + ECP or placebo + shampheresis. Peripheral blood mononuclear cells (PBMC) collected pretreatment (day 1) and post-treatment (day 90) were stimulated with phytohaemagglutinin (PHA) and T1D-associated glutamic acid decarboxylase 65 (GAD_{65}) peptide a.a. 247–279. CTLA-4, sCTLA-4, FoxP3 and TGF-β mRNA transcription was quantified. Photopheresis-treated individuals' relative mRNA expression was generally maintained during the course of the study. Placebo individuals increased in spontaneous CTLA-4 mRNA ($P < 0.05$) but decreased in expression after stimulation with GAD_{65}-peptide ($P < 0.05$) and PHA ($P < 0.05$). Spontaneous TGF-β ($P < 0.05$) increased whereas PHA- ($P < 0.01$) and GAD_{65}-peptide ($P < 0.01$)-induced TGF-β expression decreased in the placebo group, whereas it was maintained in the treated group. Without intervention, expression of CTLA-4 and TGF-β, stimulated with PHA and GAD_{65} peptide, decreased with time, with a parallel reduction of GAD_{65}-peptide and PHA-stimulated TGF-β expression. These parameters were counteracted by ECP. In conclusion, our results indicate that ECP maintains regulatory T cell-associated activity in recent-onset T1D.

Keywords: children, extracorporeal photochemotherapy, T1D, T_{reg}

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the infiltration of lymphocytes in the pancreas causing destruction of insulin-producing beta-cells [1]. Genetic factors are known to predispose individuals for the disease, while the role of environmental factors still remains elusive [2]. Molecular mimicry between Coxsackie enteroviruses and glutamic acid decarboxylase (GAD_{65}), a.a. 247–279 has been suggested as one possible trigger mechanism [3]. GAD_{65} is considered to be one of the major autoantigens in T1D, and its administration is tested currently in T1D intervention trials [4,5]

Regulatory $CD4^+$ $CD25^+$ T cells (T_{reg}) cells are characterized by expression of CD25 at high intensities, and pinpointed further by the expression of the transcription factor forkhead box P3 (FoxP3) [6,7]. Vital for tolerance and the T_{reg} cells is the cytotoxic lymphocyte antigen 4 (CTLA-4) protein, expressed by the majority of T_{reg} cells [8]. CTLA-4 is also expressed on activated T cells and acts as a general threshold for the immunological activation signal [9–11]. A splice variant of CTLA-4 that lacks the transmembrane domain and generates a soluble form of CTLA-4 (sCTLA-4) has been detected in humans as well as in mice and rats [12]. Initial reports indicate that increased concentrations are associated with autoimmune disease and T1D [13,14]. Native CTLA-4 binds to B7/CD80/82 in the immunological synapse, producing a suppressive immune response [9,15]. sCTLA-4 shares these characteristics, but the immunological effects mediated by endogenous sCTLA-4 have not yet been clarified.

The soluble and membrane-bound cytokine transforming growth factor (TGF)-β1 is also suggested to play a pivotal

role in the function and development of T_{reg} cells [16–19]. In addition to T_{reg} there are other T cells with regulatory activities. The interleukin (IL)-10 generated T regulatory 1 (Tr1) cells secreting high levels of IL-10 and TGF-β [20] and high-secreting TGF-β T helper 3 (Th3) cells have both been shown to be essential in immunological tolerance development and function [18,21,22].

A method for extracorporeal photochemotherapy (ECP) that could inhibit harmful immune system activity was developed in the late 1980s. Lymphocytes are removed from the patient, treated with ultraviolet-sensitizing 8-methoxypsoralen (8-MOP) and UVA, and are then returned to the patient [23]. Reinfusion of the treated ECP cells is thought to induce tolerance via dendritic cell or T_{reg} activity [24]. It has also been suggested that the observed tolerance induction of ECP involves CTLA-4 activity [25].

A randomized, double-blind, placebo-controlled extracorporeal photopheresis (ECP) intervention trail of recent onset T1D was performed previously at the Linköping University Hospital that showed immunological effects. Although clinical results were minor [26], the method is still of interest as it might be a complement to other types of immune intervention.

We hypothesize that ECP induces regulatory T cell-associated activity and suggest that this activity explains the effects recorded in the preceding ECP-trial. Our aim of the current study is to investigate how T_{reg}-associated markers are affected by ECP treatment.

Materials and methods

Study population

Recent-onset T1D patients had participated previously in a randomized double-blind, placebo-controlled intervention trial (described in detail elsewhere [26]). Patients were assigned active treatment, 8-MOP and subsequent apheresis treatment ($n = 19$) or received placebo (placebo tablet and shampheresis, $n = 21$). The procedure was repeated on two consecutive days, the first performed approximately 5–6 days after T1D diagnosis. The double treatment was repeated at days 14, 28, 42 and 90. Coded blood and serum samples were collected before each double treatment session. Laboratory personnel were thus blinded for sample group and identity during analysis.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by Fiqoll Paque density gradient centrifugations (Pharmacia, Biotech, Sollentuna, Sweden) from sodium heparinized venous blood samples collected before (day 0) and after the total of five double treatments (day 90). The samples came from a random subsample of 10 photopheresis-treated patients and 10 shampheresis placebo controls (ages

10–17 years) matched by gender and age that had been frozen carefully directly after sample collection. Cryofrozen cells were now thawed in 37°C water and washed in RPMI-1640 supplemented with 10% fetal calf serum. PBMCs, 1×10^6 (viability > 90%), were resuspended in 500 μl AIM V serum-free medium (Gibco, Täby, Sweden) supplemented with 2 nM 1-glutamine, 50 μg/l streptomycin sulphate, 10 μg/l gentamycin and 2×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St Louis, MO, USA), stimulated immediately with GAD_{65} peptide (a.a. 247–279) and phytohaemagglutinin (PHA), both at a concentration of 5 μg/ml for 48 h at 37°C in 5% CO_2 [27].

RNA isolation and cDNA synthesis

Total RNA was isolated from PBMC as described previously [28]. Total RNA were added in equal final concentrations (3·5 ng/μl) and transcribed using high capacity cDNA Archive Kit (no. 4322171; Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. In addition to the manufacturer's protocol, RNAse inhibitor (no. N8080119; Roche, Basel, Switzerland) was added to a final concentration of 1 U/ml. Transcription reactions were run in a GeneAmp polymerase chain reaction (PCR) System 2700 (Applied Biosystems) at 25°C for 10 min followed by 37°C for 2 h.

For sCTLA-4 mRNA detection, trace genomic DNA was first removed by DNAse 1 treatment (no. 776785; Roche Diagnostics, Mannheim, Germany) at a concentration of 0·1 U/μl for 30 min at 37°C followed by 5 min at 95°C.

Multiplex real-time reverse transcription–PCR

cDNA was analysed with *Taq*Man gene expression assay (Applied Biosystems) with primers and 6-carboxyfluorescein-(FAM)-labelled probes for TGF-β (Hs00171257_m1), FoxP3 (Hs00203958_m1) and CTLA-4 (Hs00175480_m1), as well as primers and VIC®-labelled probes for rRNA (18S) as endogenous control (no. 4310893E; Applied Biosystems). Real-time (RT–PCR) was carried out in a final reaction volume of 25 μl containing *Taq*Man predeveloped assay reagents (PDAR) Universal MasterMix (no. 4304437; Applied Biosystems), $1 \times$ PDAR primers/probes for target genes, and template cDNA in MicroAmp Optical 96-well plates covered with optical caps (Applied Biosystems). The reaction was amplified with an initial step of 95°C for 10 min followed by 50 cycles of 95°C for 15 s, 60°C for 60 s and 72°C for 60 s in a ABI Prism 7700 Sequence detector (Applied Biosystems).

A custom-designed assay for sCTLA-4 mRNA was run separately at the same reaction conditions. Primers/probes were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi). sCTLA-4 forward primer 5′-CATCTGCAAGGTGGAGCTCAT, reverse primer 5′-GGCTTCTTTTCTTTAGCAATTACATAAATC and probe

sequence 5′-6-FAM-ACCGCCATACTACCTGGGCATAGG
CA-TAMRA (Applied Biosystems).

All RT–PCR plates were loaded with duplicate samples
and with an internal calibrator duplicate sample from an
adult donor with T1D as intra-assay control as well as a
duplicate no template control. Cells from the internal cali-
brator were lysed and total RNA was isolated according to
the manufacturer's protocol (Sigma GenElute Mammalian
Total RNA kit (Sigma Chemical Co.).

The delta cycle threshold, ΔC_T, was calculated for each
sample by subtracting the 18S C_T (VIC®) from the target
gene C_T (FAM). As samples were run in duplicates, the mean
ΔC_T of each duplicate was calculated. $\Delta\Delta C_T$ was determined
by calculating the ΔC_T difference between each stimulated
sample ΔC_T and its corresponding spontaneous sample ΔC_T.
For example, GAD_{65}-peptide stimulated FoxP3 expression
was compared with spontaneous FoxP3 expression in each
individual. Relative quantification was calculated as $2^{-\Delta\Delta CT}$.

Results were generated from spontaneous as well as stimu-
lated expression, and at disease onset (0) and after 90 days of
treatment (3).

Immunoassay sCTLA-4

Cell supernatants from 20 patients in the same time inter-
val as the cell samples were used for sCTLA-4 analysis.
Gyrolab Bioaffy platform was used for a sandwich immu-
noassay detection of sCTLA-4 (Gyros AB, Uppsala,
Sweden) (described in detail elsewhere [29]). Biotinylated
anti-human CD152 (AS33-B; Antibody Solutions, Palo
Alto, CA, USA) was used to capture and detection was
made with a complementary anti-human CD152 antibody
(BNI.3; BD PharMingen, San Diego, CA, USA) labelled
previously with Alexa Flour 647 (Molecular Probes,
Eugene, OR, USA), according to the manufacturer's
instructions. The matching CD152 antibodies have been
successfully implemented previously in sCTLA-4 sandwich
immunoassay [12].

Statistics

As data are not expected to be normally distributed (even
after logarithmic transformation), paired observations were
computed by Wilcoxon's signed ranks test. Two groups were
compared by Mann–Whitney U-test and three or more
groups with the Kruskal–Wallis test for unpaired
observations. Spearman's rank correlation test was used
when comparing two variables non-parametrically. A prob-
ability level of < 0·05 was considered to be statistically sig-
nificant, whereas a P-value of 0·05 < 0·1 was regarded as a
tendency. Calculations were performed with SPSS for
Windows version 11·5 and GraphPad Prism version 4·03.

Ethics

Informed consent was given by the participating children
and their parents. The study was approved by the Research

Ethics Committee of the Faculty of Health Sciences,
Linköping University.

Results

Expression of T_{reg}-associated markers pre-ECP treatment

We evaluated the effect of ECP on tolerance-associated
markers in samples obtained from a clinical trial. The
samples did not differ in mRNA levels of the T_{reg}-associated
markers FoxP3, CTLA-4, sCTLA-4 and TGF-β between the
treated and placebo groups prior to treatment, except for
PHA-stimulated FoxP3 mRNA expression, where the photo-
pheresis group tended to be elevated compared with the
placebo control group ($P = 0·06$) (data not shown).

Expression of T_{reg}-associated markers post-ECP treatment

Cytotoxic lymphocyte antigen 4 mRNA increased during the
course of the treatment in the ECP treated group ($P = 0·04$)
(Fig. 1). Similarly, T1D placebo controls showed increased
spontaneous CTLA-4 mRNA expression after 3 months of
treatment ($P = 0·02$) (Fig. 2).

Extracorporeal photochemotherapy treated children
showed few significant changes in the observed markers
during the course of the treatment; no detectable changes
could be observed in relative GAD_{65}-peptide or PHA-
induced CTLA-4 transcription. The same was true for spon-
taneous, GAD_{65}-peptide and PHA-induced sCTLA-4
transcription. The results were consistent in TGF-β expres-
sions, where neither spontaneous, GAD_{65}-peptide nor PHA
stimulation resulted in differences between the two studied
time-points in ECP-treated patients.

Children receiving exclusively placebo

Relative transcription of GAD_{65}-peptide- and PHA-induced
CTLA-4 was decreased in the placebo group after 3 months
($P = 0·02$ and 0·03 respectively) (Fig. 2). Additionally, spon-
taneous sCTLA-4 mRNA expression tended to increase in the
placebo group ($P = 0·08$) (Fig. 2). sCTLA-4 mRNA response
to the GAD_{65}-peptide did not yield differences in the placebo
group, whereas PHA-stimulated sCTLA-4 mRNA tended to
decrease in the placebo controls ($P = 0·08$) (Fig. 2).

Spontaneous TGF-β mRNA expression increased in the
group of children receiving placebo ($P = 0·01$) (Fig. 2). In
contrast, GAD_{65}-peptide and PHA-stimulated TGF-β expres-
sions were observed to decrease during the trial in the placebo
group ($P = 0·002$ and $P = 0·004$ respectively) (Fig. 2).

In addition, relative changes in transcription was signifi-
cantly greater in the placebo controls than actively photo-
pheresis treated in both GAD_{65}-peptide-stimulated CTLA-4
and TGF-β expressions ($P = 0·04$ for both) (Fig. 3a and b).
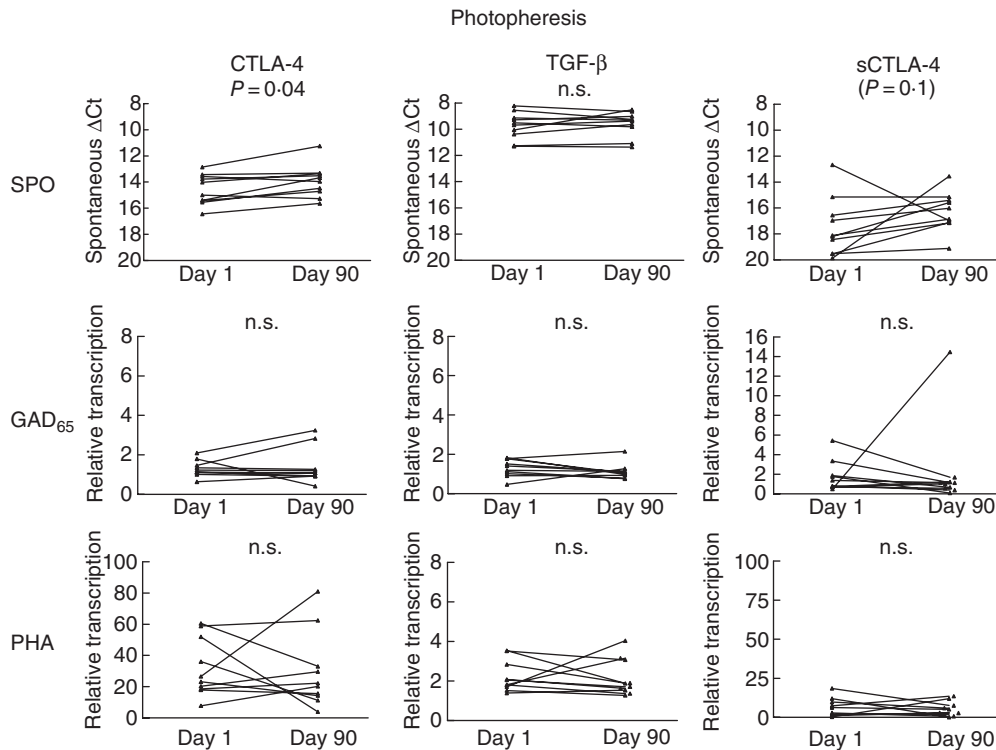
Fig. 1. Extracorporeal photochemotherapy (ECP)-treated children ($n = 10$) showed few changes in target gene transcription during the course of the study. mRNA transcription displayed at days 0–90. Spontaneous (unstimulated), glutamic acid decarboxylase (GAD$_{65}$)-peptide or phytohaemagglutinin (PHA) stimulated for the same target gene are organized horizontally. Target genes are displayed vertically. Note that spontaneous transcription is displayed at an inverted axis because of raw delta cycle threshold-values. Spontaneous expression of cytotoxic lymphocyte antigen 4 increased during treatment ($P = 0.04$). $n = 10$.

This relationship also tended to persist in spontaneous TGF-β mRNA expression ($P = 0.09$) (Fig. 3c). Relative change in transcription of remaining marker antigen responses did not reach statistical significance.

Expression of FoxP3 mRNA

Although no significant changes could be observed in FoxP3 expression in either group of treatment (data not shown), spontaneous TGF-β expression in the total group was correlated significantly with spontaneous FoxP3 both prior to ($R = 0.66$, $P = 0.002$) (Fig. 4a) and post-ECP treatment ($R = 0.7$, $P = 0.001$) (Fig. 4b).

Secretion of sCTLA-4

Low secretion of sCTLA-4 could be detected in unstimulated as well as PHA- and GAD$_{65}$-peptide 48 h-stimulated cell supernatants (data not shown). No significant differences were observed in sCTLA-4 levels between photopheresis-treated and placebo controls, neither pre- nor post-treatment. Secreted sCTLA-4 did not correlate with either spontaneous sCTLA-4 mRNA or GAD$_{65}$-peptide, or PHA-stimulated expression.

Discussion

Much research effort has been made to find an intervention for the autoimmune process in T1D. Although T1D can be intervened by several therapeutic strategies in animal models, successful clinical trials to preserve residual beta cell function have so far been achieved only partially in humans. Current trials of anti-CD3 [30–32] and GAD$_{65}$-'vaccination' [4,5] have shown promising results, but it may well be so that they need to be supported by other measures to regulate the immune system successfully. In this context photopheresis still is of interest.

Clinical outcome of T1D photopheresis treatment certainly showed very limited results. However, we find that a maintained immune regulation is induced by ECP treatment, and that it is active on GAD$_{65}$-peptide-specific stimulation. Thus, even if ECP alone is not successful as a T1D intervention therapy, it might be a way to boost effect of anti-CD3- or GAD$_{65}$-induced immune-modulated tolerance in recent-onset T1D individuals.

Recently it was discovered that CTLA-4 is active in the ECP-induced tolerance [25]. Our data support the theory that ECP-induced tolerance acts via regulatory T cells. It is a plausible mechanism, as only a small fraction of the total
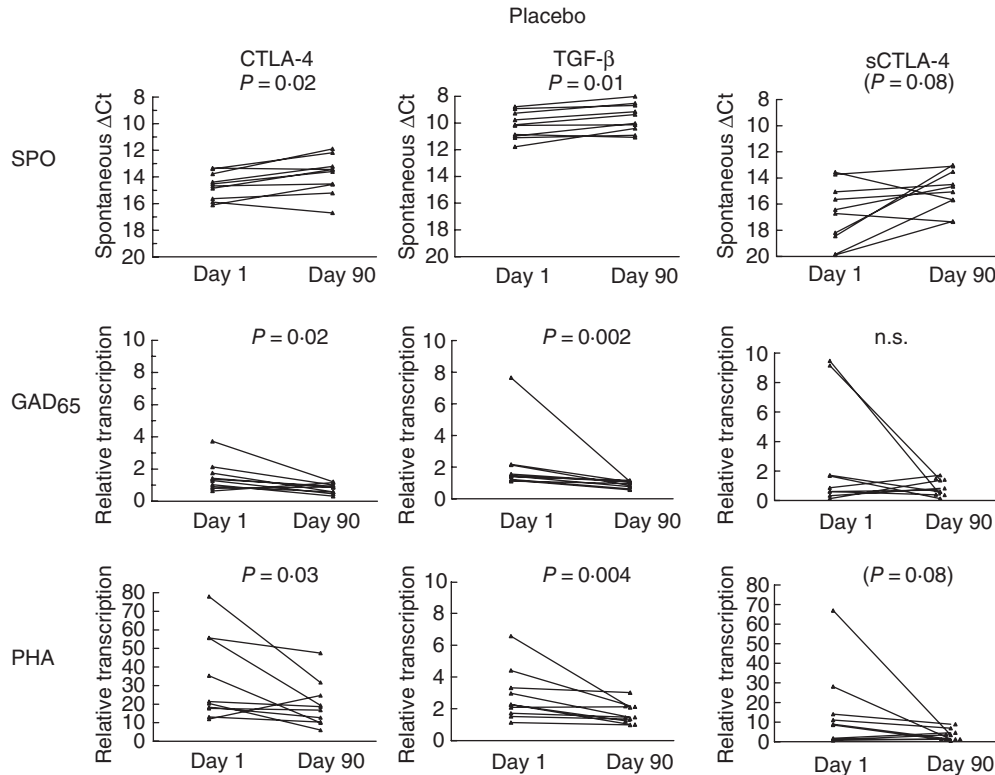
Fig. 2. Placebo controls ($n = 10$) showed several changes in target gene transcription. Transcription shown at days 0–90. Spontaneous (unstimulated), glutamic acid decarboxylase (GAD_{65})-peptide or phytohaemagglutinin (PHA) stimulated for the same target gene are organized horizontally. Target genes are displayed vertically. Note that spontaneous transcription is displayed at an inverted axis because of raw delta cycle threshold values. Spontaneous cytotoxic lymphocyte antigen 4 (CTLA-4) expression increased during the study ($P = 0.02$), whereas GAD_{65}-peptide and PHA-stimulated responses decreased ($P = 0.02$ and $P = 0.03$ respectively). Similarly, spontaneous transforming growth factor (TGF)-β increased ($P = 0.01$) and GAD_{65}-peptide and PHA stimulated responses decreased ($P = 0.002$ and $P = 0.004$ respectively). $n = 10$ except for in GAD_{65}-peptide stimulated sCTLA-4 ($n = 9$).

lymphocyte population needs to be treated in order for the ECP-treatment to have effect.

Our current results show a pre- to post-ECP treatment increase in spontaneous CTLA-4 mRNA expression in both treated and placebo control individuals. After stimulation of lymphocytes we detect disease progression-associated decreased specific CTLA-4 mRNA expression in response to GAD_{65}-peptide as well as PHA stimulation in placebo children. This was followed by a parallel reduction of TGF-β in response to the same antigen stimulations. Interestingly, this process of decreased antigen responses could not be observed in actively treated individuals. The GAD_{65}-peptide used in the study is not human leucocyte antigen (HLA)-restricted. There is, however, a minor overlap of five amino acids in a GAD_{65}-sequence that is HLA class II DRB1*0401-restricted [33]. This small overlap is not expected to influence the results.
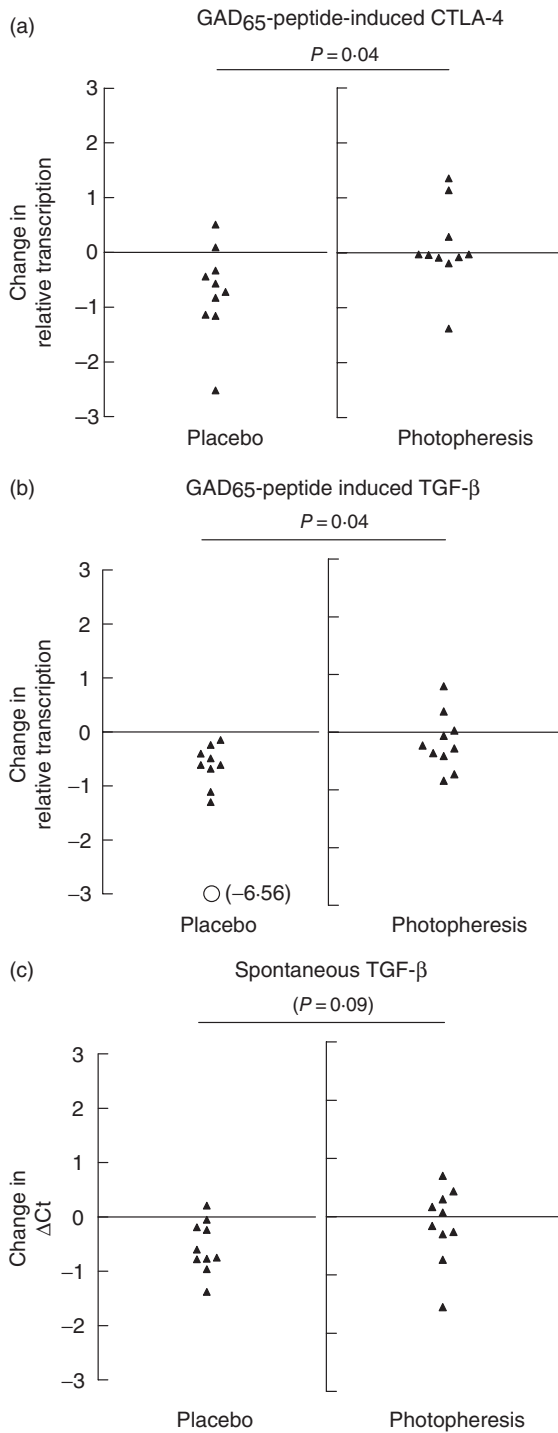
The disease-associated reduction in the studied markers was significantly greater in GAD_{65}-peptide responses of both CTLA-4 and TGF-β, suggesting that ECP has a greater effect on a T1D antigen-specific response rather than mitogen responses such as PHA. Another possibility is that the

GAD_{65}-peptide response drops more during the initial development compared with PHA, which results in a more clear ECP-induced effect.
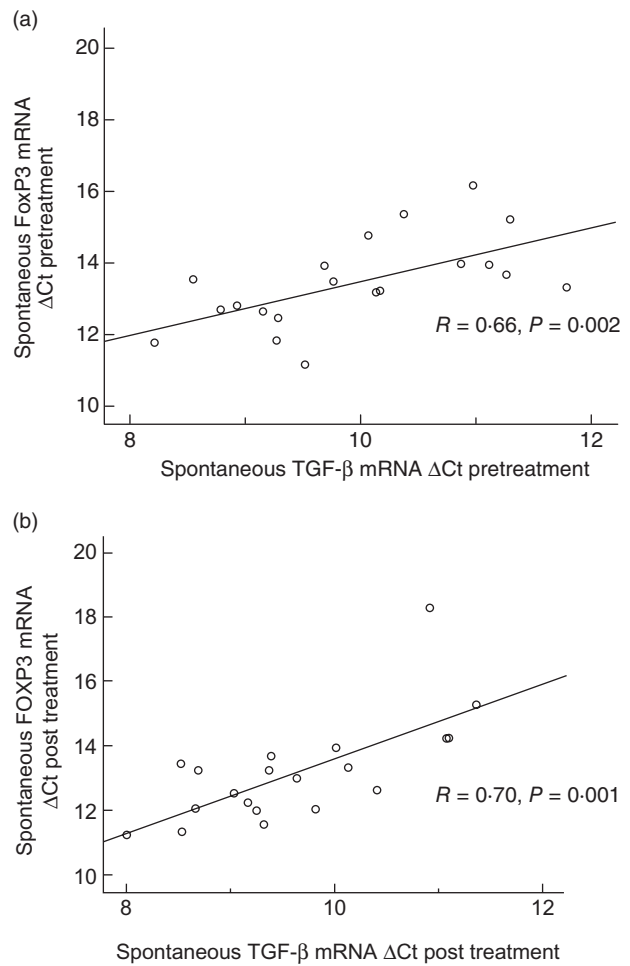
The collected results are consistent with the earlier finding regarding lymphocyte activation [34]. Maintained levels of TGF-β mRNA expression taken together with increased IL-4 and IL-10 expression makes it possible to assume that it could be Tr1 cells that are active in this tolerance maintenance.

Previous results from our ECP trial also showed that treated individuals had increased levels of IL-10 compared with placebo controls [27]. Additionally, there was an indication that activated $CD4^+$ and $CD8^+$ cells increased in the placebo controls during the course of the study, an increase that was not observed in the actively treated group [34].

The interaction of both TGF-β and FoxP3-expression is well documented in the field of T_{reg} cell research [17,25,35,36], and we now find further support of this relationship. The reason we fail to dissect the possible role of FoxP3 in ECP-induced tolerance could be because FoxP3 is part of many and complex immunological tolerance-associated interactions.

(a)

GAD$_{65}$-peptide-induced CTLA-4

$P = 0.04$

(b)

GAD65-peptide induced TGF-β

$P = 0.04$

(c)

Spontaneous TGF-β

$(P = 0.09)$

(a)

$R = 0.66, P = 0.002$

(b)

$R = 0.70, P = 0.001$

Fig. 4. Spontaneous mRNA transcription (delta cycle threshold) of transforming growth factor-β and forkhead box P3 correlated both prior to ($R = 0.66$, $P = 0.002$) (day 0) (a) and after ($R = 0.7$, $P = 0.001$) the study (day 90) (b) $n = 20$.

Fig. 3. Relative change in transcription from days 0–90. *P*-values indicating significant difference between relative change in target transcription in photopheresis *versus* placebo controls. Glutamic acid decarboxylase (GAD$_{65}$)-induced response of both cytotoxic lymphocyte antigen 4 (a) and transforming growth factor (TGF)-β (b) declined significantly more in placebo compared with photopheresis-treated type 1 diabetes patients ($P = 0.04$ for both). Spontaneous TGF-β (c) transcription tended to increase (note raw delta cycle threshold) more in the placebo group than the photopheresis-treated patients. $n = 10$ in each group.

The role and function of T$_{reg}$ cells in the pathogenesis of T1D is still subject to much debate. It has been shown that T$_{reg}$ in T1D individuals are unable to suppress cell proliferation associated with T1D, although the cell population numbers are the same as in healthy individuals [37]. These results contradict earlier results suggesting a reduced T$_{reg}$ number in T1D development [38]. Even though the mechanism is unclear, increasing evidence suggest a role for T$_{reg}$ cells in maintaining tolerance against T1D development [17,22,39,40]. What complicates the discussion is that we have limited understanding of how to separate the pathogenic mechanisms from the immune system's defence mounted to resist T1D pathogenesis.

We speculate that the observed antigen-specific parallel decrease in the immunoregulatory markers CTLA-4 and TGF-β mRNA expression represents the natural course of the T1D progression. No such changes were recorded in patients who received active treatment; rather, ECP-treated

patients maintain their regulatory T cell-associated activity towards T1D-specific autoantigen.

In conclusion, our results indicate that photopheresis acts to maintain immune regulation and thereby inhibit spontaneous islet autoimmunity.

Acknowledgements

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