

# Cytokine profile and insulin antibody IgG subclasses in patients with recent onset Type 1 diabetes treated with oral insulin

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## Abstract

**Aims/hypothesis.** Tolerance to orally administered antigens may be generated through the induction of T helper cell type 2 and 3 (Th2/Th3) regulatory cells. We previously reported that treatment of recent onset Type 1 diabetes with oral insulin had no effect on residual beta cell function. The aim of this study was to evaluate whether this treatment produces a deviation in the immune response, with polarisation of the cytokine pattern and the induction of a Th2-like antibody response.

**Methods.** Mononuclear cells were collected from a total of 20 patients with Type 1 diabetes before and after 12 months of treatment with oral insulin ( $n=11$ ) or placebo ( $n=9$ ). Following stimulation of the cells with insulin or phytohaemagglutinin, levels of Th2 and Th3 cytokines (including TGF- $\beta$ , IFN- $\gamma$ , IL-4 and IL-5) in the culture supernatants were assessed by ELISA. In addition, levels of total and specific insulin antibody IgG subclasses were measured by radioimmunoassay in serum samples drawn from 33 patients with Type 1 diabetes before and after 3, 6 and 12 months of therapy with oral insulin ( $n=18$ ) or placebo ( $n=15$ ).

**Results.** After 12 months of treatment, the release of TGF- $\beta$  was significantly higher in patients who re-

ceived oral insulin compared with those who received placebo ( $p=0.025$  and  $p=0.006$  for lymphocytes challenged with insulin and phytohaemagglutinin respectively). The two groups had similar levels of IL-4 and IL-5 both at baseline and after 12 months of treatment. The release of IFN- $\gamma$  was markedly reduced in patients treated with oral insulin compared with those who received placebo at the 12-month follow-up. Circulating levels of IgG1 and IgG3 subclasses directed against insulin were significantly lower in the oral insulin group than in the placebo group after 12 months of treatment ( $p=0.05$  for IgG1 and  $p=0.014$  for IgG3).

**Conclusions/interpretation.** The increased TGF- $\beta$  release observed in patients treated with oral insulin suggests that a regulatory response can be induced in vivo by this treatment. The lower levels of insulin antibody IgG1 and IgG3 subclasses present in patients exposed to oral insulin are consistent with a Th2 deviation of the immune response. The failure of oral insulin treatment to provide any measurable clinical benefit may be due to the timing of treatment initiation.

**Keywords** Cytokines · IgG insulin antibodies · Oral insulin · Type 1 diabetes

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## Introduction

The induction of antigen-specific immune tolerance by the oral administration of soluble antigen has been widely investigated as a novel therapeutic approach in

**Abbreviations:** IA, insulin antibody · IMDIAB, immunotherapy diabetes · NOD, non-obese diabetic · PHA, phytohaemagglutinin · PBMC, peripheral blood mononuclear cell · Th, T helper cell

the treatment of autoimmune diseases [1]. The oral administration of low doses of auto-antigen modulates the immune system; however, the mechanism involved in this process has not been elucidated. One hypothesis is that oral administration of disease-associated antigen may act on gut immune function by up-regulating regulatory immune cells, which in turn suppresses the inflammatory response [2].

In Type 1 diabetes, beta cell destruction is thought to be mediated by a T helper cell type 1 (Th1) immune response in which pro-inflammatory Th1 cytokines (IL-1, IFN- $\gamma$  and TNF- $\alpha$ ) that are actively associated with 'destructive' insulinitis are released by islet-infiltrating immunocompetent cells [3]. Animal models have been used to assess the ability of islet cell antigens administered via different routes (oral, intravenous, subcutaneous or nasal) to modulate the immune response and prevent or delay beta cell loss [4, 5, 6, 7, 8]. Animal models of Type 1 diabetes have demonstrated that the administration of diabetes-associated antigens, such as insulin or GAD, is effective in reducing or delaying the onset of disease [8, 9]. These studies aimed to induce immune tolerance to beta cell antigens by promoting a deviation from a destructive Th1 response to a non-invasive/protective Th2/Th3 response. Regulatory Th3 cells secrete TGF- $\beta$ , which is thought to be involved in the development of oral tolerance [10].

Immunoglobulin production is also regulated by cytokines. Although the underlying mechanisms associated with this are well understood in mice, they are still unclear in humans. In mice, IL-4 and TGF- $\beta$  induce the production of IgG1 and IgG2b antibodies respectively [11], whereas IFN- $\gamma$  stimulates the production of IgG2a and IgG3 antibodies. In humans, IL-4 is thought to be responsible for switching to IgG4 and IgE, whereas IFN- $\gamma$  probably induces the production of IgG1 and IgG3 antibodies. It is known that TGF- $\beta$  stimulates the production of IgA in both humans and mice [12, 13].

Based on these observations, two double-blind trials were designed to assess the effect of oral insulin administration on the induction of immune tolerance in patients with recent onset Type 1 diabetes [14, 15]. In our own trial [14], as well as in the French trial of Chaillous et al. [15], the administration of oral insulin in addition to standard treatment with subcutaneous insulin failed to demonstrate a beneficial effect on residual beta cell function as assessed by C-peptide secretion.

The aim of the present study was to assess whether the administration of oral insulin in addition to intensive insulin therapy promotes immunomodulation by inducing a Th2/Th3 polarisation of the cytokine pattern. Since immunoglobulin isotype switching is also controlled by cytokines [16], the effect of study treatment on the antibody response to insulin was also evaluated.

## Subjects and methods

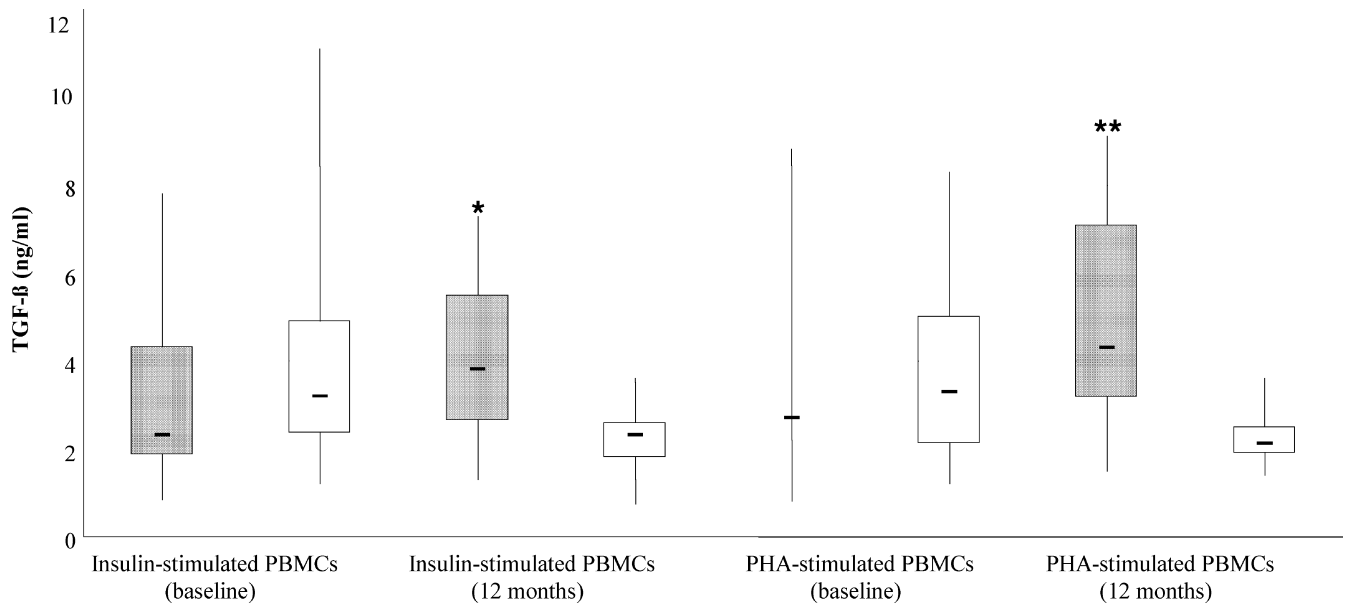
**Subjects.** Cytokine and/or insulin antibody measurements were studied in a total of 36 patients with Type 1 diabetes who were participating in the Immunotherapy Diabetes (IMDIAB) VII trial [14]. Venous blood samples were collected for assessment of cytokines from 20 of these patients at entry and after 12 months of treatment with either oral insulin ( $n=9$ , mean age  $13.5\pm 6.1$  years) or placebo ( $n=9$ , mean age  $16.2\pm 10.4$  years) [11]. Insulin antibody assessments were performed in 33 of the 36 patients prior to randomisation and after 3, 6 and 12 months of treatment with oral insulin ( $n=18$ , mean age  $12.7\pm 6.6$  years) or placebo ( $n=15$ , mean age  $11.2\pm 8.4$  years). Seventeen patients had both cytokine and insulin antibody levels assessed (nine treated with oral insulin and eight treated with placebo). The study was approved by the Ethical Committee of the University of Rome, La Sapienza, and was carried out in accordance with the Declaration of Helsinki. All patients gave their written informed consent to participate in the study.

**Measurement of cytokines.** Venous blood for cytokine assessment was drawn at diagnosis (before randomisation to either placebo or oral insulin) and after 12 months of treatment. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation at 1800 rev/min (800 g) for 30 min at room temperature. Cells were collected at the interface, washed twice and cultured at a concentration of  $2\times 10^6$ /ml in 24-well plates in the presence of phytohaemagglutinin (PHA, 10  $\mu$ g/ml), insulin (200 IU/ml) or medium alone for 48 h at 37 °C and 5% CO<sub>2</sub>. Culture supernatants were then collected and frozen at -20 °C until cytokine detection.

All cytokine measurements were performed by the same operator in a blinded manner. IL-4, IL-5, IFN- $\gamma$  and TGF- $\beta$  levels were measured by ELISA using commercially available antibodies. IL-5, IFN- $\gamma$  and IL-4 antibodies were obtained from Pharmingen (San Diego, Calif., USA) and DNAX (Palo Alto, Calif., USA), and TGF- $\beta$  antibodies were obtained from R&D Systems (Minneapolis, Minn., USA). Briefly, plates were coated with antibodies (100  $\mu$ l per well) diluted in PBS overnight at 4 °C and washed twice with a solution of PBS containing 0.05% Tween 20 (PBS/Tween). Non-specific binding was blocked by the addition of a solution of PBS containing 0.05% BSA (PBS/BSA) for 30 min. This and all subsequent steps were performed at room temperature. Appropriate dilutions of standards and samples were added in duplicate to the plates and left for 2–3 h. After washing with PBS/BSA the biotinylated detecting antibody (1:1000 dilution in PBS/BSA) was added and plates were incubated for 2 h. After further washing with PBS/Tween, a streptavidin-horseradish peroxidase conjugated antibody (1:1000 dilution in PBS/BSA) was added and plates were incubated for 1 h. After a final wash with PBS/Tween, 3,3',5,5'-tetramethylbenzidine substrate was added, and the reaction was stopped after 10–15 min by the addition of 50  $\mu$ l of 2 mol/l H<sub>2</sub>SO<sub>4</sub> to each well. Plates were subsequently read at a wavelength of 450 nm.

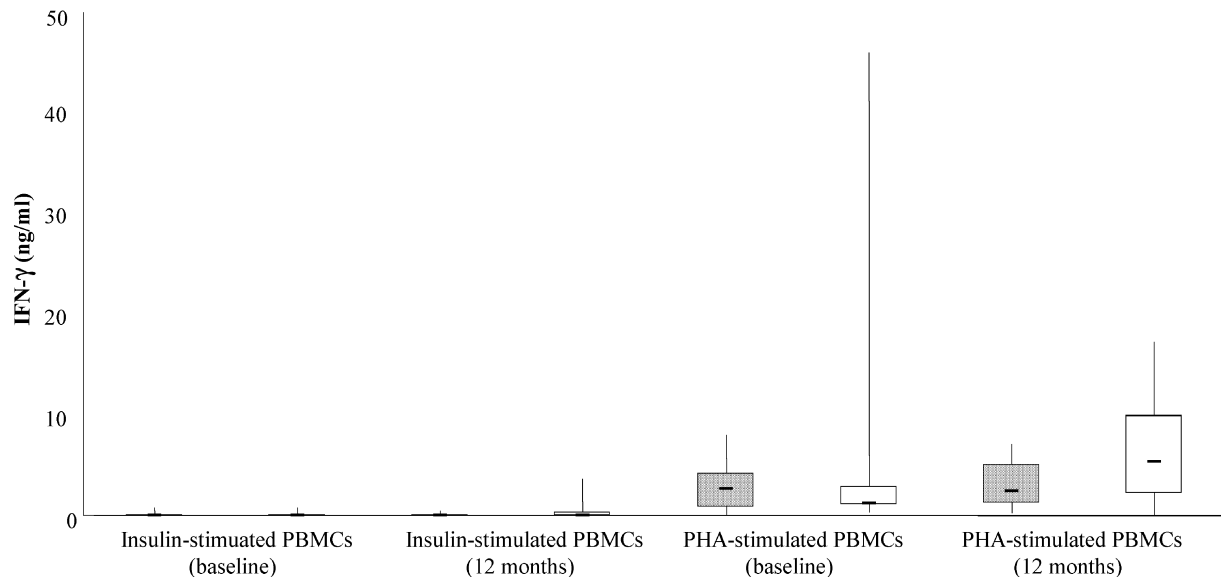
For TGF- $\beta$  detection, supernatants were activated prior to assay by the addition of an equal volume of 2.5 mol/l acetic acid in 10 mol/l urea at room temperature for 10 min and then neutralised to pH 7 with 1 mol/l Hepes in 2.7 mol/l NaOH. Results are expressed as ng/ml of culture supernatant.

**Measurement of IgG subclasses.** IgG subclasses of insulin antibodies (IA) were measured using the protein A/G radiobinding assay, which was modified such that protein A/G sepharose was substituted with IgG subclass-specific antibody-bound sepharose beads as previously described [17]. Insulin antibody concentrations were expressed as nU/ml of serum as previously described [14].



**Fig. 1.** TGF- $\beta$  production by insulin- or PHA-stimulated PBMCs from patients with Type 1 diabetes treated with oral insulin ( $n=11$ , grey boxes) or placebo ( $n=9$ , white boxes) at baseline

and after 12 months of treatment. Values are shown as box and whisker plots indicating the median, range and the 25th/75th percentiles. \*  $p=0.025$  vs placebo; \*\*  $p=0.006$  vs placebo



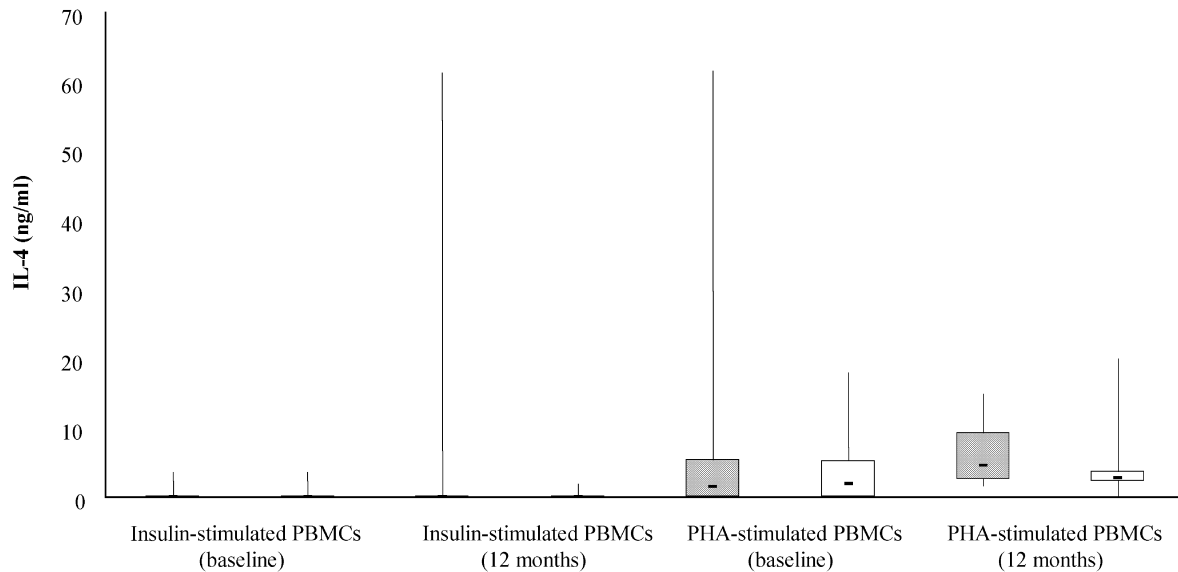
**Fig. 2.** IFN- $\gamma$  production by insulin- or PHA-stimulated PBMCs from patients with Type 1 diabetes treated with oral insulin ( $n=11$ , grey boxes) or placebo ( $n=9$ , white boxes) at baseline and after 12 months of treatment. Values are shown as box and whisker plots indicating the median, range and the 25th/75th percentiles. No significant differences were observed under the different experimental conditions

**Statistical analysis.** The Mann–Whitney U test for non-parametric values was used to compare median values for measurements of cytokines and IgG antibodies to insulin between the two groups. Results are shown as medians (ranges) unless stated otherwise. Any  $p$  value less than 0.05 was considered to be statistically significant.

## Results

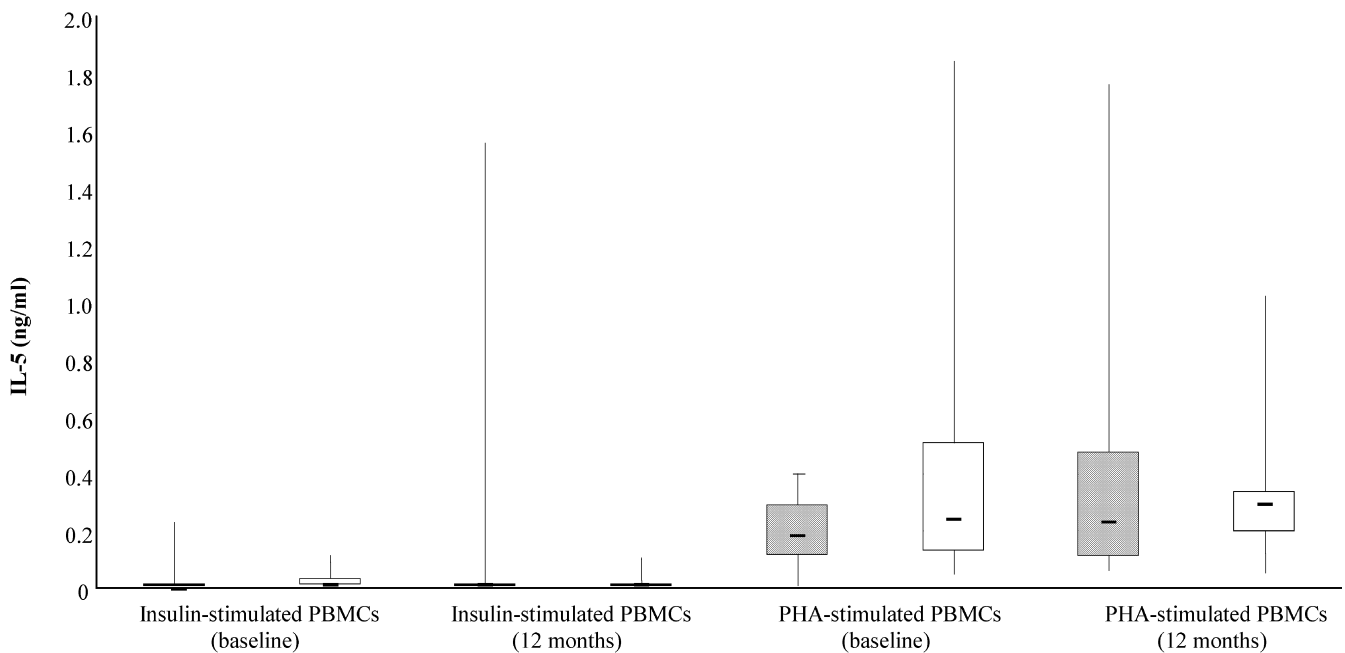
**Measurement of cytokines.** Cytokine levels were measured in the culture supernatant of unstimulated (data not shown) and/or antigen-stimulated PBMCs from patients with Type 1 diabetes treated with oral insulin or placebo both at baseline and after 12 months of treatment. At diagnosis, the oral insulin and placebo groups had similar levels of cytokines (Figs. 1, 2, 3, 4).

TGF- $\beta$  levels were similar in the two groups at diagnosis (3.50 ng/ml [1–9.5] for oral insulin patients vs 3.31 ng/ml [1.1–6.2] for placebo). After 12 months of treatment, spontaneous TGF- $\beta$  release was higher



**Fig. 3.** IL-4 production by insulin- or PHA-stimulated PBMCs from treated with oral insulin ( $n=11$ , grey boxes) or placebo ( $n=9$ , white boxes) at baseline and after 12 months of treatment. Values are shown as box and whisker plots indicating the

median, range and the 25th/75th percentiles. No significant differences were observed under the different experimental conditions

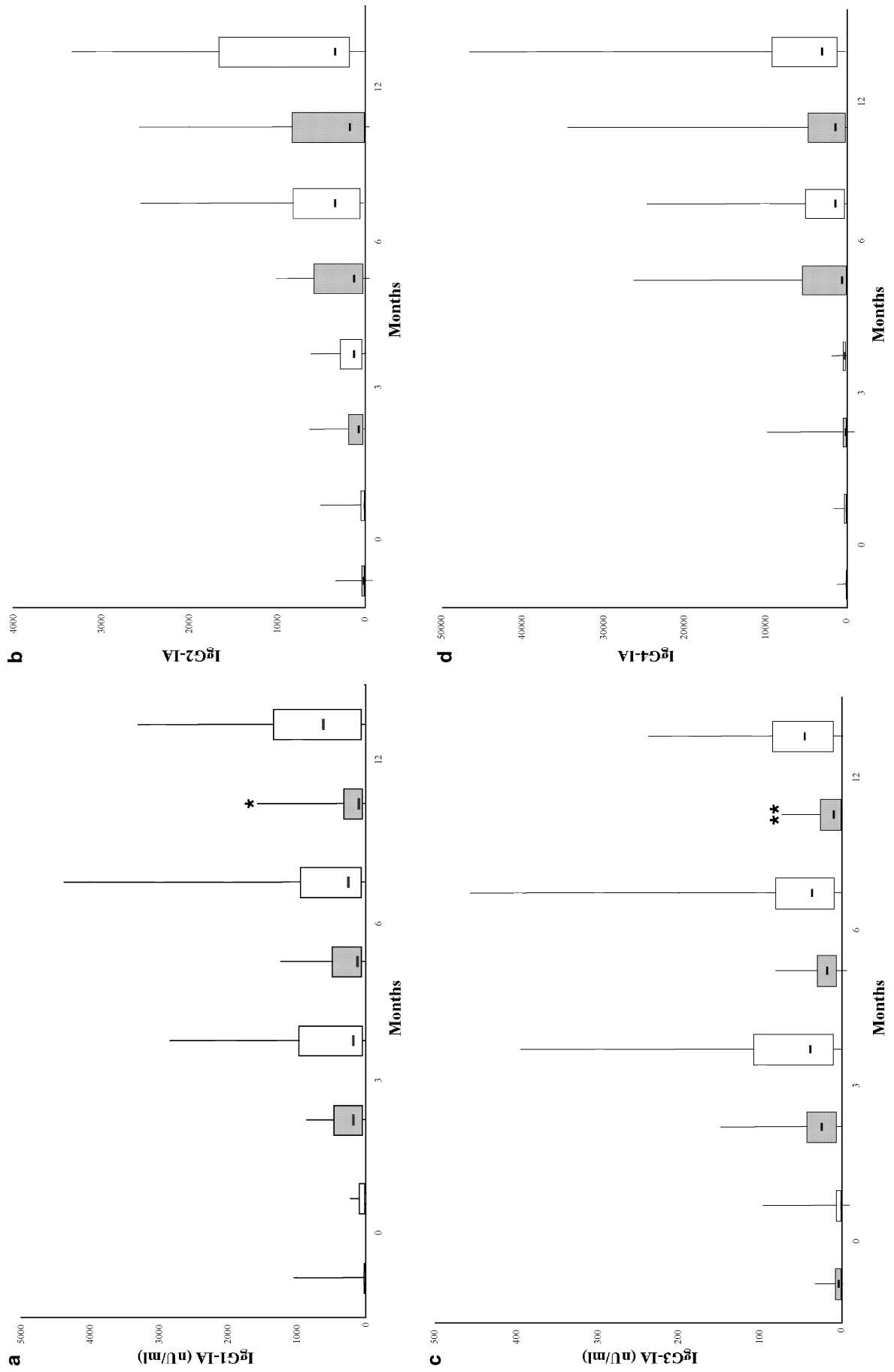


**Fig. 4.** IL-5 production by insulin- or PHA-stimulated PBMCs from patients with Type 1 diabetes treated with oral insulin ( $n=11$ , grey boxes) or placebo ( $n=9$ , white boxes) at baseline and after 12 months of treatment. Values are shown as box and

whisker plots indicating the median, range and the 25th/75th percentiles. No significant differences were observed under the different experimental conditions

in the oral insulin group than in the placebo group (4.5 ng/ml [1.7–8] vs 1.90 ng/ml [1.1–6.2];  $p=0.05$ ). At this time, a significant increase in TGF- $\beta$  was also detected in the PBMC culture supernatants of the oral insulin group compared with the placebo group following insulin or PHA stimulation ( $p=0.025$  and

**Fig. 5.** Levels of IgG1-IA (a), IgG2-IA (b), IgG3-IA (c) and IgG4-IA (d) subclasses in patients treated with oral insulin ( $n=18$ , grey boxes) or receiving placebo ( $n=15$ , white boxes) at entry and after 3, 6 and 12 months of treatment. Values are shown as box and whisker plots indicating the median, range and the 25th/75th percentiles. \*  $p=0.05$  vs placebo; \*\*  $p=0.014$  vs placebo



$p=0.006$  for lymphocytes challenged with insulin and PHA respectively) (Fig. 1).

No differences were observed with respect to IFN- $\gamma$  levels between the oral insulin and placebo groups either at entry (0.03 ng/ml [0.006–0.246] vs 0.04 ng/ml [0.006–0.55] respectively) or at the 12-month follow-up (0.02 ng/ml [0.006–1.1] vs 0.04 ng/ml [0.01–0.246] respectively). Interestingly, PHA-stimulated IFN- $\gamma$  release was reduced in patients receiving oral insulin compared with those receiving placebo after 12 months of treatment. This difference was not statistically significant (Fig. 2); however, the variation in IFN- $\gamma$  levels between 0 and 12 months was significantly higher in patients receiving placebo ( $p<0.05$ , data not shown). There were no differences in insulin-stimulated IFN- $\gamma$  production between the groups either at baseline or after 12 months of treatment (Fig. 2).

Levels of insulin- or PHA-stimulated IL-4 and IL-5 release were similar in the two groups at both time points tested (Figs. 3, 4).

No differences were observed in culture supernatant of PBMC not stimulated with antigen between the groups (data not shown).

**Measurement of IgG subclasses.** Prior to the initiation of treatment with either oral insulin or placebo, no statistically significant differences were observed between the two randomised groups with respect to insulin antibody levels. At inclusion, 54.5% (18 of 33) of patients tested positive for IA; IgG1 was observed to be the prevalent isotype at this time (data not shown). At 12 months, the frequency of IA-positive patients had increased up to 93.9% (31 of 33). Levels of the different IA IgG subclasses were not significantly different between the two groups at inclusion or after 3 or 6 months of treatment. However, serum levels of IgG1-IA and IgG3-IA were significantly lower in the oral insulin group than in the placebo group at the 12-month follow-up ( $p=0.05$  and  $p=0.014$  for IgG1-IA and IgG3-IA respectively) (Fig. 5). No statistically significant differences in IgG2-IA and IgG4-IA levels were detected between the two groups during follow-up (Fig. 5).

No correlations were observed between TGF- $\beta$  and any of the IgG-IA subclasses; however, oral-insulin-treated patients with higher TGF- $\beta$  levels had lower serum levels of IgG1-IA and IgG3-IA than placebo-treated patients at 12 months (data not shown).

## Discussion

This is the first study to investigate whether oral administration of insulin can modulate antigen-specific cellular and humoral immune responses in patients with recent onset Type 1 diabetes. The data presented show that after 1 year of treatment with oral insulin, relevant immunological changes are observed in terms of a Th2/Th3 polarisation of the cytokine pattern and

a Th2-like antibody response. However, previously reported metabolic data from the IMDIAB VII trial showed that this treatment has no effect on residual beta cell function, since C-peptide levels, insulin requirements and glycated haemoglobin levels were comparable during follow-up in patients receiving oral insulin or placebo in addition to intensive insulin therapy [14]. A different study also found no differences between treatment groups in terms of C-peptide levels and antibody responses to different antigens, including insulin, islet cell cytoplasmic antigen, GAD and islet antigen 2 [15].

Oral treatment with auto-antigens such as GAD65, either alone or in combination with insulin, produced encouraging results in terms of delaying and/or blocking the development of diabetes in animal models [4]. Exposure to antigens by oral administration seems to act on gut immune function by activating local regulatory T cells to release specific cytokines with immunosuppressive functions [6]. One of these cytokines is TGF- $\beta$ , which has previously been implicated in the induction of oral tolerance [10]. Several studies have discussed the potential of TGF- $\beta$  in the prevention of a number of autoimmune diseases, including rheumatoid arthritis, experimental allergic encephalomyelitis and Type 1 diabetes [18]. TGF- $\beta$  is secreted by Th3 lymphocytes and is known to exert its immunosuppressive role by regulating the proliferative responses of B lymphocytes, Th1 lymphocytes and cytotoxic T cells. It has been shown that TGF- $\beta$  suppresses IL-2-dependent T cell proliferation by interfering with signal transduction [19]. Furthermore, TGF- $\beta$  controls immunoglobulin secretion and isotype switching [20]. Taken together, these findings suggest that TGF- $\beta$  plays a key role in the regulation of the immunoinflammatory response.

In the present study, the up-regulation of TGF- $\beta$  production and concomitant down-regulation of IFN- $\gamma$  secretion in PHA-stimulated lymphocytes from patients treated with oral insulin suggests the occurrence of a shift from a potentially destructive Th1 response to a 'protective' Th2/Th3 reaction. In addition, a Th2 antibody response is indicated by the observed decrease in IgG3-IA levels after 12 months of treatment with oral insulin. Moreover, levels of IgG1-IA were lower in the oral insulin group than in the placebo group. This observation is in agreement with a previous study which showed that oral insulin administration is associated with lower levels of IgG1-IA after 3 months of treatment [15]. Conversely, subcutaneous insulin administration has been shown to induce high levels of IgG1-IA and a Th2-associated IgG4 response against insulin after 12 months of treatment [21].

Although our results are consistent with a deviation from a Th1-like to a Th2-like immune response, this did not delay disease progression in the patients treated with oral insulin. In other words, the shift in immune response was not associated with a clinical ben-

efit in this case. One possibility is that the timing of treatment implementation—after disease onset—was too late to have a demonstrable effect on residual beta cell function [22]. However, in a recent trial performed on a large population of first- and second-degree relatives of Type 1 patients with diabetes, treatment with subcutaneous insulin also failed to either delay or block diabetes onset [23]. Data from animal models and trials in humans indicate that even if a shift from a Th1 to a Th2 immune response does occur, the development of resistance to Type 1 diabetes is not clearly associated with these immunological events in terms of a cause–effect relationship [24, 25, 26, 27]. In studies on non-obese diabetic (NOD) mice genetically deficient in IFN- $\gamma$ , IL-4 or IL-10, the rate of spontaneous development of diabetes was not altered by the absence of these cytokines [26]. Furthermore, the results of additional experiments in the same study suggested that protection from disease conferred by complete Freund's adjuvant or bacillus Calmette-Guerin vaccine was independent from the Th1/Th2 balance. In another study, transgenic mice overexpressing TGF- $\beta_1$  and TNF- $\alpha$  in pancreatic beta cells developed diabetes by the age of 4 months [27]. Thus, the clinical benefits of the immune modulation afforded by the oral administration of auto-antigens have yet to be determined.

Using animal models, many studies have indicated that cytokines play a key role in the development and progression of autoimmune diseases, including Type 1 diabetes, and may therefore prove useful in delaying and/or monitoring the disease process. It has recently been reported that progression to Type 1 diabetes correlates with an increase in Th1-like soluble cytokines in the peripheral blood of NOD mice [28].

In conclusion, our data suggest that a regulatory immune response can be generated by the oral administration of insulin in recent onset Type 1 diabetes; however, this effect has no clinically relevant beneficial impact on disease progression.

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