Low-dose IL-2 in children with recently diagnosed type 1 diabetes: a Phase I/II randomised, double-blind, placebo-controlled, dose-finding study

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4514 Words

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Abstract

Aims/hypothesis Low-dose interleukin 2 (ld-IL2) selectively activates and expands regulatory T cells (Tregs) and thus has the potential to skew the regulatory/effector T (Treg/Teff) cell balance towards improved regulation. We investigated which low doses of IL-2 would more effectively and safely activate Tregs during a 1-year treatment in children with recently diagnosed type 1 diabetes.

Methods DF-IL2-Child was a multicentre, double-blinded, placebo-controlled, dose-finding phase 1/2 clinical trial: 24 children (7–14 years old) with type 1 diabetes diagnosed within the previous 3 months were randomised leading to a 7/5/6/6 patient distribution of placebo or IL-2 at doses of 0.125, 0.250, or 0.500 MIU/m^2, given daily for a 5-day course and then fortnightly for 1 year. The primary outcome was change in Tregs expressed as a percentage of CD4$^+$ T cells at day 5. It pre-specified that a ≥60% increase in Tregs from baseline would identify Treg high-responders.

Results There were no serious adverse events. Non-serious adverse events (NSAEs) were transient and mild to moderate. In treated patients vs placebo, the commonest NSAE was injection site reaction (37.9% vs 3.4%), whereas other NSAEs were at the same level (23.3% vs 19.2%). Ld-IL2 induced a dose-dependent increase in the mean proportion of Tregs, from 23.9 ± 11.0% at the lowest to 77.2 ± 44.8% at the highest dose, which was significantly different from placebo for all dose groups. However, the individual Treg responses to IL-2 were variable and fluctuated over time. Seven patients, all among those treated with the 0.250 and 0.500 MIU/m^2/day doses, were Treg high-responders. At baseline, they had lower Treg

proportions in CD4+ cells than Treg low-responders, and serum sIL-2RA and VEGFR2 levels predicted the Treg response after the 5-day course. There was no significant change in glycaemic control in any of the dose groups compared to placebo. However, there was an improved maintenance of induced C-peptide production at one year in the 7 Treg high-responders as compared to low responders.

Conclusion/interpretation The safety profile at all doses, the dose-dependent effects on Tregs and the observed variability of the Treg response to ld-IL2 in newly diagnosed type 1 diabetes children call for use of the highest dose in future developments. The better preservation of insulin production in Treg high-responders supports the potential of Tregs in regulating autoimmunity in type 1 diabetes and warrants pursuing the investigation of ld-IL2 for its treatment and prevention.

Trial registration ClinicalTrials.gov, NCT01862120

Funding Assistance Publique-Hôpitaux de Paris, Investissements d'Avenir programme (ANR-11-IDEX-0004-02, LabEx Transimmunom and ANR-16-RHUS-0001, RHU iMAP) and European Research Council Advanced Grant (FP7-IDEAS-ERC-322856, TRiPoD).

Key words: Immunotherapy, Tolerance, Autoimmunity, autoimmune diseases, T1D

Abbreviations

IDAA1c: insulin-dose-adjusted A1c

ld-IL2: low-dose interleukin 2

ITT: intention to treat

Tregs: regulatory T cells

MIU: million international units

MMTT: mixed meal tolerance test

Research in context

What is already known about this subject?

- Type 1 diabetes could benefit from ld-IL2 therapy because the disease is linked to abnormalities in the IL-2 pathway and regulatory T cells.
- Proof-of-concept clinical trials have reported the safety and efficacy of ld-IL2 in auto-immune diseases, including type 1 diabetes.
- The incidence of type 1 diabetes is increasing in children, so it is important to assess immunotherapies in this population and to define safe dosing.

What is the key question?

- What is the optimal dose of IL-2 for safe expansion of Tregs during one-year treatment of children with recently diagnosed type 1 diabetes?

What are the new findings?

- Ld-IL2 in children was safe at all doses.
- Treg low- and high-responders were identified.
- Treg high-responders showed a better preservation of stimulated insulin secretion at 1 year compared to Treg low-responders.
- Baseline serum sIL-2 and VEGFR2 predicted the Treg response after the 5-day ld-IL2 course

How might this impact on clinical practice in the foreseeable future?

- These results support the investigation of chronic regimens of 0.5 MIU/m²/day of IL2, with a maximum of 1 MIU/day, to study the clinical benefit for children and adolescents with recently diagnosed type 1 diabetes in a fully powered efficacy study.

Introduction

Since the recognition that type 1 diabetes is an autoimmune disease, clinical trials have tested therapies to control or suppress islet autoimmunity. Trials began in the 1980s with the use of classical immunosuppressive agents, including cyclosporine, at the time of diagnosis. This efficiently controlled the autoimmune process, with some patients being insulin-free two years after diagnosis[1–4]. While these results further demonstrated the importance of autoimmunity in the pathogenesis in type 1 diabetes and provided proof of concept that immunotherapy could be effective, the drugs used had an unfavourable risk/benefit ratio[2]. Stem cell transplantation, by resetting the immune system, could also stop the autoimmune process, although with significant side effects and expense[5, 6].

The discovery that Treg cells control effector T cells has changed the paradigm from immune suppression to immune regulation to treat autoimmune diseases, including type 1 diabetes [7]. Attempts to stimulate antigen-specific Tregs with appropriate antigens to induce antigen specific tolerance are actively pursued[8, 9], and so are Treg cell therapies [10]. The expansion and reinjection of large amounts of polyclonal Tregs have been shown to be safe, and to preserve C-peptide production in several individuals[11].

The recognition that IL-2 when given at low doses can selectively stimulate Tregs has offered novel means for harnessing Tregs for type 1 diabetes treatment[12–17]. IL-2 is used at a high dose (18-60 MIU/injection) as a marketed drug designed to stimulate Teffs for treating cancer[18]. Although IL-2 was the first effective immunotherapy of cancer, with >5% long duration complete response, the severe adverse effects of the drug at high doses precluded its large use[19]. The recognition that, unlike other T cells, Tregs express constitutively the high-affinity receptor for IL-2 led us to hypothesise that low-dose IL-2 might preferentially activate Tregs over Teffs. We showed that this was indeed the case and that at low dose (1.5-3 MIU/injection) IL-2 was well tolerated[20]. This opened the path to investigate ld-IL2 in type

1 diabetes, a disease which is associated with low IL-2 production and Treg insufficiency[21, 22]. We first conducted a dose-finding study in adult patients with established type 1 diabetes in order to determine a dose that would safely activate Treg cells. A 5-day course of daily IL-2 injections led to a dose-dependent increase in Tregs over a dose range of 0.33 to 3 MIU/day. In another study, IL-2 was administered in combination with rapamycin with the aim of apoptosing diabetogenic effector T cells[23]. Such treatment actually led to a Treg increase and to a transient decrease of C-peptide production [24] which has been attributed to the direct toxic effects of rapamycin on pancreatic beta cells[25].

As the treatment of type 1 diabetes with IL-2 is likely to be of long duration, we next aimed at investigating the lowest dose that would stimulate Tregs over a one-year treatment. Type 1 diabetes is very commonly diagnosed in children, in whom disease progression and response to immunotherapies may differ from those of adult patients[26]. Therefore, we conducted a dose-finding study with ld-IL2 in children with recently diagnosed type 1 diabetes. Treating children with ld-IL2 appeared possible because of the safety profile of the drug when given at low dose[12, 27]; moreover, even foetuses can be safely exposed to increased IL-2 concentrations during a normal pregnancy[28]. In adults we observed a good safety profile up to the dose of 3 MIU/injection, but better tolerance at 1 MIU/injection[12]. The primary objective of the study was thus to determine the optimal dose of IL-2 for safe expansion of Tregs in children with recently diagnosed type 1 diabetes.

Methods

Study design and participants

This was a multicentre, randomised, double-blind, parallel-group study of three doses of IL-2 (0.125, 0.25 or 0.5 MIU/m^2/day). Patients were recruited, randomised, treated, and followed up at three centres at the Assistance Publique-Hôpitaux de Paris (Kremlin Bicêtre, Robert-Debré and Necker Hospitals) and one in Nîmes (Nîmes Hospital). Patients were eligible if

they were aged 7 to 13 years for females and 7 to 14 years for males; had a diagnosis of type 1 diabetes confirmed by the presence of at least one of the following diabetes-related autoantibodies: ICA, GAD, PTPRN (IA2), or SLC30A10 (ZnT8); had been treated with insulin for less than 3 months; had no history of or current cardiopathy; and had no clinically significant abnormal value in haematological, biochemical, hepatic and renal assessments, and had lymphocyte counts in the normal range.

Exclusion criteria were a known contraindication to aldesleukin; a documented history of other autoimmune diseases (except stable thyroiditis); acidosis, $HbA_{1c} \geq 119$ mmol/mol (13%) and weight loss $\geq 10\%$ at diagnosis; continuous nocturnal polyuria ≥ 3 months; positive autoantibodies to 21-hydroxylase or stage 2 obesity. Moreover, patients were not included if they had positive serology (IgM) indicating recent exposure to Epstein-Barr virus and/or cytomegalovirus, and if they had received a vaccination with live attenuated virus in the previous 4 weeks. Immunomodulators, cytotoxic and modifying plasma glucose drugs were not accepted during treatment (electronic supplementary material [ESM] Table 1).

The study was approved by the institutional review board of Pitié-Salpêtrière Hospital and was conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines. Written informed consent was obtained from all participants before enrolment in the study.

Dose, randomisation and masking

As children aged 7-14 may vary considerably in size and weight, we adjusted the dose used per square meter, approximating that adults receiving 1 MIU/injection have a body surface area of 1.8 square meters. Patients were randomised in a 1:1:1:1 ratio to placebo or IL-2 at one of the 3 targeted doses: 0.125, 0.250, or 0.500 MIU/m^2/day. Patients with a body surface area ≤ 1.1 m^2 received 0.125, 0.25 or 0.5 MIU/day and those with body surface area >1.1 m^2 received 0.2, 0.4 or 0.8 MIU/day.

The randomisation list was generated by computer (block size of 4), stratified by body surface area (≤1.1 m^2 or >1.1 m^2), and forwarded to the pharmacist in charge of preparing the drug. A study number was attributed to patients by an investigator unaware of the randomisation list, according to the patient's stratum of body surface area and order of entry in the centre. For each patient number and at each patient visit with drug administration, a pharmacist prepared the treatment according to the randomisation list, with labels bearing only the study number of the patient. Placebo- and IL-2-containing syringes had the same appearance and were labelled according to good manufacturing practice for traceability and accountability purposes. All investigators remained blinded until the end of the study. The randomisation list was stored at the local pharmacy in each centre.

Procedures

Aldesleukin (Proleukin®18 mIU, Novartis) was purchased by the Central Pharmacy of the AP-HP. For each patient, clinical trial units were prepared at the pharmacy of the centre. Syringes each containing 0.5 (body surface area ≤1.1 m^2) or 0.8 mL (body surface area >1.1 m^2) of either a solution of aldesleukin at the required IL-2 dosage (0.125, 0.25, 0.50 MIU/m^2) or vehicle only (glucose 5% water) used as placebo, were prepared according to the randomisation list. The experimental treatment was administered subcutaneously in a day-care ward or at home by a qualified nurse. The treatment was administered according to two periods: (i) an induction course of once daily administration for 5 days [day 1 - day 5]; (ii) a maintenance course with fortnightly injections for 12 months [day 15 - day 337] (appendix). According to the study protocol, a "prior single administration" was given at day minus 7, followed by blood sampling at day minus 6 and day zero. This was done to measure the biological effects of a single injection at 24 h and one week post injection. The core treatment was then initiated and the monitoring of Tregs (primary outcome) performed at day 8. Thus, the protocol called for 8 visits within the first 15 days of the treatment and was not easy to

implement because of poor acceptance by patients. To reduce patient burden, after the first 5 patients were recruited, the steering committee decided to remove this "prior single administration" and at the same time to switch the Treg evaluation for the primary outcome from day 8 to day 5, just prior to the last treatment injection (ESM Fig. 1). These modifications were approved by the ethics committee and the regulatory agency.

Blood samples were obtained for specific immunological tests including assessment of Treg and lymphocyte subsets at day 1 (baseline), day 5/8, day 15, day 30, day 45, day 99, day 183, day 267, day 351 and for follow-up at day 436. Blood samples for assessment of diabetes parameters (fasting blood glucose and C-peptide, HbA_{1c}) were obtained on day 1, day 99, day 183, day 267, day 351 at day 436 of the follow-up; a mixed meal tolerance test (MMTT) was performed at day 1, day 183, day 351 and day 436. Routine laboratory assessments were (1) biochemistry, including blood glucose, blood electrolytes; lactate dehydrogenase; C-reactive protein, procalcitonin, blood calcium, hepatic and renal functions 2) haematology assessments included haemoglobin, haematocrit, white blood cell count, red blood cell count, and platelets and were performed at day 0, day 15, day 99, day 183, day 267, day 351 and day 436. Immunoglobulins and specific auto-antibodies for thyroiditis (anti-thyroperoxidase and anti-TSH receptor), Addison's (anti-21 hydroxylase) and celiac disease (anti-transglutaminase) were evaluated at the screening visit, day 183, day 351 and day 436. Serology for cytomegalovirus and Epstein Barr virus were evaluated at the screening visit, day 99, day 183, day 267, day 351 and day 436.

The primary endpoint was the increase in the relative concentration of Treg cells, measured by flow cytometry as $CD3^{+}CD4^{+}CD25^{hi}CD127^{-/lo}FoxP3^{+}$ cells among the $CD4^{+}$ T cells (ESM Fig. 2), at the end of the induction period compared to baseline. The baseline sample was obtained immediately prior to the first treatment administration (day 1). The post-treatment sample was obtained at day 5, except for the first 5 patients who received the "prior single

administration" for whom it was performed at day 8. The immunological secondary endpoint was the Treg response during the maintenance period compared to the baseline expressed as the area under the curve (AUC) of the changes from day 15 to day 351. All the immunomonitoring procedures (flow cytometry and quantification/analysis of cytokine and chemokine expression levels) are described in the ESM.

Diabetes secondary endpoints were: change in C-peptide (fasting C-peptide and C-peptide AUC response to an MMTT), HbA_{1c} and IDAA1C score during the maintenance period compared to the baseline (ESM Table 2).

Safety was assessed with vital signs (temperature, weight, blood pressure, heart rate); adverse events were reported at each visit, with a systematic assessment of the most commonly reported reactions to IL-2 during hospital visits at day 1 to day 5, day 15, day 99, day 183, day 267, day 351 and day 436. Adverse events were graded according to the WHO Common Toxicity Criteria (version 3.0). A safety committee of five independent experts was established to review all serious adverse events. Records of insulin intake and of hypoglycaemic episodes during the treatment period were recorded by the patients and collected by during visits.

Statistical analysis

Power calculations[29] determined that 6 patients per arm would provide 80% power in detecting a difference between active drug and placebo corresponding to an effect size equal to 1.8 for the main criterion of the study. Such an effect size has been anticipated using data from a previous study[20].

All outcomes were analysed in the intention to treat (ITT) population with the exception of variables linked to the MMTT since some patients exhibited major deviations in this test that prevented interpretation of their exams (ESM Table 3).

Since the main objective of the study was to identify the lowest active dose of IL-2 on Tregs, we analysed the dose-response relationship for the main criterion by the Jonckheere-Terpstra test and compared each dose vs placebo by the non-parametric Shirley-Williams test.

A similar method was used to compare groups for AUC during the maintenance phase. In addition, we compared the time-dependent profile of changes in Tregs during the maintenance phase by ANOVA on ranks and tested the significance of the increase in Tregs during the maintenance phase by calculating the AUC of the difference of each time from baseline and testing that this difference was significantly different from zero. According to their statistical distribution, quantitative secondary criteria were compared among the four groups by ANOVA (after log-transformation if required) or the Kruskal-Wallis test and between high- and low-responders by the t-test (after log-transformation if required) or Mann-Whitney test. Number of episodes of hypo/hyperglycaemia were compared using generalised estimating equations for Poisson regression.

Role of the funding source

The sponsor of the study had no role in study design. MR, RL, CB, EV, and DK had access to the raw data. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Patients were enrolled between June 2013 and January 2016 (Fig. 1). Twenty-four patients were randomised leading to a 7/5/6/6 patient distribution for the 0, 0.125, 0.25 and 0.5 IL-2 doses, respectively. One patient, in the 0.5 MUI/m² group, dropped out of the study after 270 days because of a grade 2 abdominal pain (Fig. 1). No major deviations were observed during the study. Minor protocol deviations included out of window visits (n=110/576; 19%) or drug

administration not performed because of intercurrent diseases (n=2) during the maintenance period. Some deviations in the MMTT have been reported (ESM Table 3). Diabetes secondary outcomes were analysed in the ITT population and in the per protocol (PP) population, which excluded patients with major deviations in the MMTT.

There was no difference between groups at baseline for demographic and laboratory characteristics, including diabetes parameters (fasting glycaemia, fasting C peptide and C-peptide AUC) (Table 1), nor for biological/immunological parameters including Tregs (Fig. 2a). According to the inclusion criteria, all patients had at least one positive type 1 diabetes-associated autoantibody. Anti-GAD and anti-IA2 antibodies were the most frequent such autoantibodies, in accordance with the literature.

Safety

Clinical safety was satisfactory at all doses; no serious adverse events occurred during the treatment and off-treatment follow-up periods (Table 2). Over the entire observation period, non-serious adverse events (NSAEs) were all transient and mild to moderate. During the treatment period, there was a dose-effect relationship for all NSAEs taken together. Local reactions at the injection site accounted for most of the common NSAEs, with a dose-effect relationship from 3.4% of administration for placebo-treated patients to 26.2%, 36.9% and 47.7% at the 0.125, 0.25 and 0.5MIU/m^2/day doses, respectively. The other non-serious adverse events (headache, gastrointestinal symptoms, transient asthenia and fever) had the same frequency in the different therapy groups, including placebo. Importantly, the one-year treatment period covered the seasons with a high rate of infections. Four upper respiratory tract infections were noted and all resolved rapidly without complications (Table 2).

Two patients had hypereosinophilia during the maintenance period, but no concomitant allergic disease or other symptoms related to hypereosinophilia were observed (ESM Fig. 3). One patient had anti-TPO antibodies at baseline that doubled at month 6 with normal thyroid

function throughout the treatment and follow-up periods. No adverse events were reported concerning other laboratory parameters.

Primary efficacy criteria: IL-2 effects on Treg cells during the induction course

The mean (95% CI) baseline percentage of Tregs in patients was 5.5% (5.0; 6.1) of $CD4^+$ T cells (Table 1 and Fig. 2a). At the end of the induction period, a significant dose-response relationship between Treg increase and IL-2 dose (p=0.0002) was observed as the primary efficacy endpoint. The mean relative change in Tregs was -0.2% (-30.4; 30.0) in the placebo group and 23.9% (-11.8; 59.6) (p=0.02), 54.2% (21.6;86.8) (p=0.007) and 77.2% (44.7;109.8) (p=0.0002) for the 0.125, 0.25 and 0.5 MIU/m^2/day doses, respectively (Fig. 2b, 2c and ESM Table 4). Although mean Treg values were significantly different from those of placebo at all IL-2 doses, the individual Treg response to IL-2 appeared variable.

As pre-specified in the protocol, an individual was defined as a Treg high-responder (H-Treg patients) if his/her Treg response showed a ≥60% increase over baseline at day 5. According to this criterion, 7 patients were H-Treg, 3 and 4 of whom received the 0.250 and 0.500 MIU/m^2/day doses, respectively (Fig. 2b). The other patients had a low Treg response (L-Treg patients) (Fig. 2b). This heterogeneity of the Treg response to IL-2 was reminiscent of what we observed in our previous trial in adults with established type 1 diabetes (Fig. 3) [12].

Immunological secondary efficacy criteria

Time-dependent changes in Tregs during the maintenance course differed between groups. While the mean Treg values rapidly returned to baseline after the induction course for patients receiving the lowest dose, they remained elevated over the baseline during the entire maintenance course for the two highest doses, with a significant effect only for the highest (p=0.02 for 0.5 MIU/m^2/day) (Fig. 2c and ESM Table 4 & 5). The increased percentage of $CD4^+$ Tregs was associated with an increase in the Treg/Teff ratio (Fig. 2d). There were no

statistically significant changes during induction and maintenance periods in activated $CD25^+$ Teffs (ESM Fig 4), B cells or natural killer (NK) cells (ESM Fig 5) in any of the dose groups.

As a mean, the H-Treg patients maintained a 50% increase of Tregs throughout the treatment period (Fig. 4b). However, there were individual variations (Fig. 4c) that we did not see in other clinical trials of ld-IL2[12, 27]. In contrast, the L-Treg patients had Treg values that never exceeded the threshold of a 60% increase. As a mean, L-Treg patients (treated with IL-2 or placebo) maintained Treg levels around baseline values (Fig. 4d).

Metabolic secondary efficacy criteria

We found no deleterious effects of ld-IL2 on blood glucose levels. In the ITT population, there was no significant difference between the 4 treatment groups in any parameters including plasma C-peptide iAUC response during a MMTT, HbA_{1c}, fasting glycemia, fasting C-peptide levels and insulin requirements (Fig. 2e, ESM Fig. 6 & Table 2).

There were, however, differences between H-Treg and L-Treg patients in plasma C-peptide iAUC response during an MMTT. Both groups showed an initial similar decrease from baseline to month 6, after which the C-peptide remained stable in the H-Treg group, whereas it decreased further in L-Treg patients. At days 351 and 436, changes from baseline were significant for L-Treg patients ($p<0.001$), but not for H-Treg patients. No difference in HbA_{1c} and IDAA1C scores was observed (ESM Table 2).

Identification of potential biomarkers of patients' responses

We first looked at Treg levels at baseline. H-Treg patients had a lower level of Tregs compared to L-Treg patients (4.3 ± 1.0 vs 6.1 ± 1.1, $p= 0.018$) (Fig. 4a). There were no differences between the H- and L-Treg groups in Teffs, B or NK cells.

We then analysed whether the expression levels of 61 serum cytokines/chemokines at baseline were correlated with Treg increase at day 5 relative to baseline. We found a positive correlation between sIL2Ra ($p=0.0004$), VEGFR2 ($p=0.0063$), IL22 ($p=0.0207$), IL27

(p=0.0137) and IL28A (p=0.0183) (Fig. 5a-5e). However, at baseline, sIL2Ra and VEGFR2 were the only cytokines statistically differentially expressed between H- and L-Treg patients (p=0.0202 and p=0.0211, respectively) (Fig. 5f, 5g).

To evaluate the potential of these biomarkers to predict the Treg response, we constructed a regression model using the multivariate adaptive regression spline method[30] (Fig. 5h, 5i). The generated model was able to correctly predict the percentage of Tregs at day 5 relative to baseline (Pearson coefficient of correlation=0.84 and p=2.078e-07). The expression levels of sIL2Ra and VEGFR2 were the only contributors to this regression model. The generalised cross-validation coefficient used to estimate the importance of each variable in the model showed a dominant importance of sIL2Ra compared to VEGFR2. sIL2RA does not have any clear biological function and is viewed as a surrogate marker of Treg activation[27]. In addition, polymorphism of sIL2Ra and VEGFR2 have been described in T1D [31] and other autoimmune diseases [32–34]. Altogether, this warrants further evaluation of these markers in future studies.

Conclusion

Immunotherapy holds great promise in the treatment of autoimmunity in type 1 diabetes. An extreme modality is allogeneic hematopoietic stem cell transplantation, which has been reported to induce long-term complete remission (insulin independence) in patients with recently diagnosed type 1 diabetes [35]. However, as for the use of cyclosporine, its side effects do not allow its broad use. Targeting the regulatory arm of the immune response may offer efficacious and safer means to treat type 1 diabetes Results from our trial emphasize the safety profile of ld-IL2 in type 1 diabetes children 7-14 years old. The main adverse event was a reaction at the injection site; the frequency was dose-related, but reactions were mild to moderate and did not require medication. Since the treatment lasted for one year, all patients

went through the cold months in which infections are more prevalent. There were very few infectious episodes reported and all showed a normal course. These results add to the expanding clinical experience showing a very good safety profile of ld-IL2.

As this trial was a dose-finding one, the main primary outcome was the Treg response after the 5 daily consecutive IL-2 injections. In our previous trial in adults with established type 1 diabetes, we reported at the same time point a dose-dependent and significant increase in Tregs at all doses (3, 1 and 0.33 MIU/injection). Due to the large variation in body surface area in children, we adapted our injected dose of IL-2 according to this parameter, approximating the body surface area of adults to around 1.8 m^2. In line with our previous results, we observed a dose-dependent increase of Tregs that was significant at all doses, including the lowest dose of 0.125 MIU/m^2. We had noticed some variability in the Treg response in our trial in adults, with some patients receiving the highest dose who responded less than others receiving the lowest dose. We had thus pre-specified the threshold of a ≥60% increase in Tregs to define a robust response to IL-2. According to this criterion, no patient had such a response at the lowest dose, while 3/6 and 4/6 had it in the two highest dose groups; thus, 58% of the type 1 diabetes children treated with the two highest doses had a high Treg response. For reference, we had 35/46 (76%) high responders in our TRANSREG trial of adult patients with one of 11 autoimmune diseases receiving 1 MIU/injection[27]. It is noteworthy that, at baseline, the H-Treg responders had a lower proportion of peripheral blood Tregs than L-Treg responders, and also had higher plasma sIL2RA levels, which are known to reflect Treg activation[16, 36, 37]. These observations suggest that H-Treg responders have actively engaged Tregs that may not be receiving the amount of IL-2 they need for optimal efficacy. This is further supported by the capacity to predict the Treg response based on plasma sIL2RA levels at baseline. Altogether, the dose of 1 MIU/injection, adjusted to body surface area in the case of children, appears to be optimal regarding our

administration scheme. Indeed, it is safe and the only one that maintained a significant increase of Tregs throughout the maintenance period. This dose is close to the 260,000 IU/m^2 every 3 days proposed by others [17, 38, 39]

We noticed a greater variability of the response in the high Treg responders (Fig. 3c), not previously seen in other patients treated with IL-2. It remains to be seen whether these peculiar responses to IL-2 are related to age or to the fact that we treated patients with recent-onset type 1 diabetes, which may correspond to a period of instability of the immune response. We also need to consider that fluctuations in proportions of Tregs in the circulation may also reflect recruitment to the pancreas or lymphoid tissues, or other tissues, which could be beneficial. Obviously, this hypothesis is not possible to test without access to tissue or advanced imaging to track Treg cells.

There were no noticeable differences in diabetes outcome in the different dose groups. All patients showed a decrease in C peptide production over time with a progressive decrease in the C-peptide AUC during an MMTT. However, when comparing the H- to the L-Treg responders, the former group showed a clear trend to improved preservation of stimulated insulin secretion, the decrease of stimulated C-peptide from baseline being significant for L-Treg patients ($p<0.001$), but not for H-Treg patients.

In most studies reporting some preservation of insulin secretion after treatment there was mostly a delay in C-peptide decline, but afterwards the treated and placebo groups had a similar slope for their C-peptide decline[40, 41]. We observed the contrary in this study: the C-peptide declined initially with the same slope in H-Treg and L-Treg patients, but after 6 months of follow-up the H-Treg group exhibited less decline in C-peptide, which continued to be higher until the end of the follow-up, about 3 months after the one-year treatment. In addition, there was also a trend to less increase in fasting glycemia in H- vs L-Treg

responders (Table 4). While these findings are exploratory, concern a small number of patients and so are not statistically significant, they suggest that Treg regulation may require some time to show benefit. As therapies that debulk/deplete effector T cells (cyclosporine[1, 4, 42], thymoglobulin[43] anti-CD3[40], anti-memory T cell agents[43]) may allow early preservation of C-peptide, this suggests that combination with such agents could help maintain and enhance preservation of insulin secretion.

Overall, this study provides novel insights into the use of ld-IL2 therapy for type 1 diabetes (and beyond). First, it confirms the good safety profile over a one-year treatment period, in children. Second, it provides more data about individual responsiveness to ld-IL2 doses; the primary outcome at 5 days, as implemented in this trial, could be further investigated as a biomarker of response that could guide dose adjustment to uniformly achieve a 60% increase in Tregs. Future trials could validate this outcome as a biomarker for early prediction of responders to personalised dosing regimens. While the study was not formally powered to assess impact of the therapy on insulin secretion, the potential effects on preservation of insulin secretion in those with a higher Treg response provide an initial signal of clinical benefit that supports further investigation. We are currently completing enrolment of a ld-IL2 phase-IIb trial in Europe (DIABIL-2, NCT02411253). In this trial, 138 patients with recently diagnosed type 1 diabetes, 6-35 years old, are being treated for one year with 1 MIU/day for adults and 0.5 MIU/m²/day of IL2 with a maximum of 1 MIU/day for children and adolescents, or placebo, according to 2 arms in which IL-2 is given once a week or fortnightly during the maintenance period. This treatment scheme is fully supported by the current study.

We envision that ld-IL2 could be beneficial not just at onset, but even later in patients with more established type 1 diabetes, a notion that will be tested in a planned trial (NCT03243058). Finally, the recent milestone results showing that it is possible to delay type 1 diabetes onset by a single injection of teplizumab[44] should also prompt the use of ld-IL2

in disease prevention. The good safety profile of ld-IL2 and the fact that it does not induce anti-drug antibody should make it an excellent candidate for this indication, alone or after a first teplizumab injection.

Acknowledgements: We thank the patients and the members of the DF-CHILD-study group for their participation. We thank all the personnel of the Clinical Investigation Center for Biotherapies (CIC-BTi), and in particular Michèle Barbié, Cornélia Degbé, Natalie Féry, Anne Laure Raveu, Fabien Pitoiset and Karim El Soufi for their excellent help, and of the Clinical Investigation Center of the Robert-Debré Hospital. We thank Olivia Tran for the monitoring of the study, Gilbert Bensimon for his initial contribution to the trial design and Anne Filleron and Renaud Cezar for help in Treg analyses at the Nîmes Hospital.

Data availability

Individual participant data from this study (after de-identification) will be available from the publication date of this manuscript on a collaborative basis for individual participant data meta-analyses. Proposals should be directed to David Klatzmann (david.klatzmann@sorbonne-universite.fr).

Duality of interest: DK, MR and CB are co-inventors of a patent entitled "IL 2 based therapy" (OEB 11 305269.0) and DK and MR are shareholders in ILTOO pharma, the exclusive licensee of this patent.

Contribution Statement:

PB, JCC, MP & T-AT were the principal clinical investigators of the study and participated to the acquisition of data in their study sites in the Paris area and Nîmes.

RS, CBi, JB & CS, were clinical investigators of the study and participated to the acquisition of data.

RL, CA and AH contributed to data analyses.

NT performed the analysis and modelling of cytokine/chemokine expression levels.

MR supervised immunomonitoring and analysed results.

AR and PC performed the immunomonitoring and analysed results.

CBe participated in the study design.

EV performed the statistical analyses and interpretation of the data.

DK conceived the study, analysed the results and wrote the article.

All authors edited the article and approved the final version.

DK is the guarantor of this work.

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FIGURE LEGENDS

Figure 1: Trial profile

Twenty-four patients were assessed for eligibility and randomised. Seven were assigned to placebo, five to 0.125 MIU/m^2/day, six to 0.25 MIU/m^2/day and six to 0.5 MIU/m^2/day. In the initial version of the protocol, the first five patients (2 placebo and 1 for each dose) received a prior single administration of IL-2 one week before the induction course and their Treg response was measured at day 8. In a modified version of the protocol aimed at facilitating recruitment, this single injection was eliminated and Tregs were determined at day 5 just prior to the IL-2 injection. One patient dropped out of the study at day 270 because of grade 2 abdominal pain. All patients were analysed for primary and secondary endpoints

Figure 2: Treg and C-peptide dynamics in patients treated with ld-IL2

(a) Data represent Tregs as a percentage of CD4+ T cells in the different groups of patients at baseline; (b) Representation of the primary outcome: individual change in Tregs at day 5 (dot)

or day 8 (triangle) compared to baseline per IL-2 dose; (c) Secondary outcome: mean ± sd changes in Tregs over the whole treatment period and follow-up per IL-2 dose. (d) Data represent changes in Treg/Teff ratio defined as the percentage of Tregs divided by the percentage of $CD4^{+}CD25^{lo/+}Foxp3^{-}$ T cells; mean ± sd changes in Treg/Teff ratio over the whole treatment period and follow-up per IL-2 dose. (e) Changes in C-peptide AUC from baseline to day 436 per IL-2 dose. Each colour corresponds to an IL-2-dose: grey for placebo, blue for 0.125 MIU/m^2/day, red for 0.25 MIU/m^2/day and black for 0.5 MIU/m^2/day. Data were normalised by baseline values for each patient at the different time points and are represented as fold change, but all statistics were calculated using the raw data.

Figure 3: Comparison of the Treg increase per IL-2 dose in newly diagnosed paediatric T1D (DF-IL2-CHILD trial) and in adults with established T1D (DF-IL2 trial). Representation of individual changes in Tregs after the induction period compared to baseline per IL-2 dose in DF- IL2-CHILD (black dots) and in DF-IL2 (empty squares). Data were normalised by baseline values for each patient at the different time points and are represented as fold change, but all statistics were calculated using the raw data.

Figure 4: Treg and C-peptide dynamics in L-Treg and H-Treg patients

(a) Data represent Tregs as a percentage of CD4+ T cells in L-Treg and H-Treg patients at baseline; (b-d): Mean ± sd (B) and individual changes in Treg cells from day 1 to day 436 in H-Treg (c) and L-Treg (d) groups, respectively. (e) Changes in C-peptide AUC in H-Treg vs L-Treg patients. H-Treg group is represented as black squares and L-Treg group as empty triangles. Data were normalised by baseline values for each patient at the different time points and are represented as fold change, but all statistics were calculated using the raw data.

Figure 5: Biomarkers of Treg response to ld-IL2.

(a-e) Dotplot representations showing statistically significant correlations between the expression levels of soluble proteins and the percentage of Treg FoxP3+ at day 5 relative to baseline in patients. The regression lines are indicated in blue and the confidence intervals are indicated with grey ribbons. The Spearman coefficients of correlation and the associated p-values are indicated for each cytokine. (f,g) Boxplot and jitter representations showing the

expression levels for cytokines found to be statistically different between the groups of low-responders (grey dots) and high-responders (black dots) to ld-IL2 treatment. The p-values obtained by the Wilcoxon rank-sum test are indicated for each cytokine (h,i) Barplot and dotplot representations showing the estimated variable importance, quantified using the generalised cross-validation coefficient, and the prediction capacity of the regression model.

Table 1: Baseline demographic and laboratory characteristics of patients (intention to treat population): Data are mean ± SD or Number of patient (n).

	Placebo	**0.125 MUI/m^2**	**0.25 MUI/m^2**	**0.5 MUI/m^2**	***p-value***	**L-Treg**	**H-Treg**	***p-value***
	(n = 7)	**(n=5)**	**(n=6)**	**(n=6)**		**(n=17)**	**(n=7)**	
Demographics								
Sex (Male/Female)	5/2	1/4	2/4	4/2	*0.2748‡*	8/9	4/3	*1.000‡*
Age (years)	9.3 ± 1.4	10.6 ± 1.1	9.7 ± 1.6	10.2 ± 2	*0.4985††*	10 ± 1.6	9.6 ± 1.6	*0.5586†*
BMI (kg/m2)	16.4 ± 2	19.4 ± 2.9	16.5 ± 1.8	18.2 ± 2.3	*0.1100***	17.7 ± 2.4	17.1 ± 2.6	*0.6176**
Body surface area (BSA) (m2)	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.3	1.2 ± 0.3	*0.8574***	1.2 ± 0.2	1.1 ± 0.2	*0.1614**
BSA>1.1m^2 % (n)	57.1% (4)	80% (4)	50% (3)	66.7% (4)	*0.8434‡*	64.7% (11)	57.1% (4)	*1.0000‡*
BSA≤1.1m^2 % (n)	42.9% (3)	20% (1)	50% (3)	33.3% (2)		35.3% (6)	42.9% (3)	
Glucose metabolism								
Daily insulin dose (UI/Kg/day)	0.5 ± 0.3	0.5 ± 0.1	0.3 ± 0.2	0.6 ± 0.5	*0.5267***	0.5 ± 0.2	0.4 ± 0.4	*0.7669**
Fasting glycemia (mmol/L)	5.6 ± 1.6	5.7 ± 0.7	5.5 ± 1.2	5.8 ± 1.8	*0.9796***	5.7 ± 1.4	5.5 ± 1.1	*0.6498**
Fasting C-peptide (nmol/L)	0.23 ± 0.10	0.33 ± 0.07	0.30 ± 0.10	0.33 ± 0.13	*0.2668***	0.30 ± 0.13	0.30 ± 0.10	*0.9986**
C- peptide AUC (nmol h/L)	0.96 ± 0.49	1.23 ± 0.13	1.13 ± 0.49	1.19 ± 0.56	*0.7810***	1.13 ± 0.46	1.13 ± 0.43	*0.9332**
HbA_{1C} (mmol/mol) (%)	61.6 ± 14.6 (7.8 ± 1.3)	50.6 ± 3.5 (6.8 ± 0.3)	64.7 ± 21.3 (8.1 ± 1.9)	57.4 ± 9.2 (7.4 ± 0.8)	*0.1794††*	58.0 ± 10.8 (7.5 ± 1.0)	61.4 ± 21.2 (7.8 ± 1.9)	*0.6558†*
IDAA1C	9.8 ± 1.2	8.9 ± 0.7	9.3 ± 2.5	9.5 ± 2.5	*0.5934††*	9.4 ± 1.1	9.6 ± 2.9	*0.2703†*
Auto antibodies (positive/patients tested)								
Islet cell autoantibodies (ICA)	3/3	1/3	0/2	2/3	*0.4728‡*	4/9	2/2	*0.4030‡*
Antibodies to insulin (IAA)	1/3	1/4	1/4	1/5	*0.8946‡*	4/11	0/5	*0.3687‡*
Antibodies to glutamic acid decarboxylase (GAD)	5/7	3/5	6/6	4/6	*0.5070‡*	13/17	5/7	*1.0000‡*
Antibodies to protein tyrosine phosphatase (IA2)	6/7	3/5	5/6	3/6	*0.5161‡*	13/17	4/7	*0.3742‡*
Zinc transporter 8 autoantibodies (ZnT8)	1/2	0/0	0/1	0/1	*1.000‡*	1/2	0/2	*0.4401‡*
Immunocytometry								
Treg cells (% of CD4+T cells)	6.4 ± 1.1	5.8 ± 0.9	4.9 ± 1.9	4.9 ± 0.7	*0.1232***	6.1 ± 1.1	4.3 ± 1.0	*0.0018*
CD4+ T cells (cells per mm3)	778 ± 146	931 ± 271	1133 ± 427	731 ± 190	*0.0977***	884 ± 309	877 ± 299	*0.9607*
CD8+ T cells (cells per mm3)	500 ± 194	522 ± 123	738 ± 339	542 ± 296	*0.3610***	556 ± 256	620 ± 274	*0.5894*
CD19+ B cells (cells per mm3)	357 ± 116	320 ± 131	344 ± 174	268 ± 151	*0.7132***	328 ± 138	314 ± 151	*0.8244*
CD56+CD3- NK cells(cells per mm3)	149 ± 146	99 ± 59	127 ± 86	99 ± 68	*0.8141††*	116 ± 106	131 ± 75	*0.3248*

** ANOVA, †† Kruskal Wallis test, ‡ Fisher's exact test, * Two-Sample T-test, † Mann Whitney U test/Wilcoxon Sum Rank test

Table 2: Summary of adverse events (intention-to-treat population)

	Placebo	0.125 MUI/m^2	0.25 MUI/m^2	0.5 MUI/m^2	L-Treg	H-Treg
	(n = 7)	(n=5)	(n=6)	(n=6)	(n=17)	(n=7)
Number of treatment administered (per patient 29)	**203**	**145**	**174**	**174**	**493**	**203**
Induction (per patient 5)	35	25	30	30	85	35
Maintenance (per patient 24)	168	120	144	144	408	168
Serious adverse events	**0**	**0**	**0**	**0**	**0**	**0**
Non serious adverse events	**46**	**76**	**102**	**124**	**211**	**137**
% administrations	22.7%	52.4%	58.6%	71.3%	42.8%	67.5%
Injection site reaction	**7**	**38**	**66**	**83**	**108**	**86**
Number of patient	4	4	5	6	12	7
% administrations	3.4%	26.2%	36.9%	47.7%	21.9%	42.4%
Induction period	3	7	8	20	17	21
number of patient	1	3	3	5	6	6
% administrations	8.6%	28.0%	26.7%	66.7%	20.0%	60.0%
Maintenance period	4	31	58	63	91	65
number of patient	3	4	6	6	11	8
% administrations	2.4%	25.8%	40.3%	43.8%	22.3%	38.7%
Other non serious adverse events	**39**	**38**	**36**	**41**	**103**	**51**
number of patient	7	5	6	6	17	7
% administrations	19.2%	26.2%	20.7%	23.6%	20.9%	25.1%
Other non serious adverse events related to treatment	**2**	**8**	**2**	**11**	**13**	**10**
number of patient	1	5	2	6	8	6
% administrations	1.0%	5.5%	1.1%	6.3%	2.6%	4.9%
Headache	2	3	1	2	6	2
GI symptoms	0	3	0	4	2	5
Asthenia	0	1	0	2	1	2
Upper respiratory tract infections	0	1	1	2	3	1
Fever	0	0	0	1	1	0