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Michelle Rosenzweig, Randa Salet, Roberta Lorenzon, Nicolas Tchitchek, Alexandra Roux, et al.. Low-dose IL-2 in children with recently diagnosed type 1 diabetes: a Phase I/II randomised, double-blind, placebo-controlled, dose-finding study. *Diabetologia*, 2020, 63 (9), pp.1808-1821. 10.1007/s00125-020-05200-w . hal-03345047

HAL Id: hal-03345047

<https://hal.umontpellier.fr/hal-03345047>

Submitted on 30 Jan 2023

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1 **Low-dose interleukin 2 in children with recently diagnosed type 1 diabetes: a phase 1/2**
2 **randomised, double-blind, placebo-controlled, dose-finding study**

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36 **4514 Words**

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4 **Abstract**

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

10 **Methods** DF-IL2-Child was a multicentre, double-blinded, placebo-controlled, dose-finding
11 phase 1/2 clinical trial: 24 children (7–14 years old) with type 1 diabetes diagnosed within the
12 previous 3 months were randomised leading to a 7/5/6/6 patient distribution of placebo or IL-
13 2 at doses of 0.125, 0.250, or 0.500 MIU/m², given daily for a 5-day course and then
14 fortnightly for 1 year. The primary outcome was change in Tregs expressed as a percentage of
15 CD4⁺ T cells at day 5. It pre-specified that a $\geq 60\%$ increase in Tregs from baseline would
16 identify Treg high-responders.

17 **Results** There were no serious adverse events. Non-serious adverse events (NSAEs) were
18 transient and mild to moderate. In treated patients vs placebo, the commonest NSAE was
19 injection site reaction (37.9% vs 3.4%), whereas other NSAEs were at the same level (23.3%
20 vs 19.2%). Ld-IL2 induced a dose-dependent increase in the mean proportion of Tregs, from
21 $23.9 \pm 11.0\%$ at the lowest to $77.2 \pm 44.8\%$ at the highest dose, which was significantly
22 different from placebo for all dose groups. However, the individual Treg responses to IL-2
23 were variable and fluctuated over time. Seven patients, all among those treated with the 0.250
24 and 0.500 MIU/m²/day doses, were Treg high-responders. At baseline, they had lower Treg

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

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

12 Trial registration [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01862120), NCT01862120

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

16

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

18

1 **Abbreviations**

2 IDAA1c: insulin-dose-adjusted A1c

3 ld-IL2: low-dose interleukin 2

4 ITT: intention to treat

5 Tregs: regulatory T cells

6 MIU: million international units

7 MMTT: mixed meal tolerance test

8

9 **Research in context**

10 **What is already known about this subject?**

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

17• **What is the key question?**

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

20• **What are the new findings?**

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

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

- 28 • These results support the investigation of chronic regimens of 0.5 MIU/m²/day of IL2,
29 with a maximum of 1 MIU/day, to study the clinical benefit for children and
30 adolescents with recently diagnosed type 1 diabetes in a fully powered efficacy study.
31

1 **Introduction**

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

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

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

1 1 diabetes, a disease which is associated with low IL-2 production and Treg insufficiency[21,
2 22]. We first conducted a dose-finding study in adult patients with established type 1 diabetes
3 in order to determine a dose that would safely activate Treg cells. A 5-day course of daily IL-
4 2 injections led to a dose-dependent increase in Tregs over a dose range of 0.33 to 3 MIU/day.
5 In another study, IL-2 was administered in combination with rapamycin with the aim of
6 apoptosing diabetogenic effector T cells[23]. Such treatment actually led to a Treg increase
7 and to a transient decrease of C-peptide production [24] which has been attributed to the
8 direct toxic effects of rapamycin on pancreatic beta cells[25].

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

20 **Methods**

21 *Study design and participants*

22 This was a multicentre, randomised, double-blind, parallel-group study of three doses of IL-2
23 (0.125, 0.25 or 0.5 MIU/m²/day). Patients were recruited, randomised, treated, and followed
24 up at three centres at the Assistance Publique-Hôpitaux de Paris (Kremlin Bicêtre, Robert-
25 Debré and Necker Hospitals) and one in Nîmes (Nîmes Hospital). Patients were eligible if

1 they were aged 7 to 13 years for females and 7 to 14 years for males; had a diagnosis of type
2 1 diabetes confirmed by the presence of at least one of the following diabetes-related
3 autoantibodies: ICA, GAD, PTPRN (IA2), or SLC30A10 (ZnT8); had been treated with
4 insulin for less than 3 months; had no history of or current cardiopathy; and had no clinically
5 significant abnormal value in haematological, biochemical, hepatic and renal assessments,
6 and had lymphocyte counts in the normal range.

7 Exclusion criteria were a known contraindication to aldesleukin; a documented history of
8 other autoimmune diseases (except stable thyroiditis); acidosis, $HbA_{1c} \geq 119$ mmol/mol
9 (13%) and weight loss $\geq 10\%$ at diagnosis; continuous nocturnal polyuria ≥ 3 months;
10 positive autoantibodies to 21-hydroxylase or stage 2 obesity. Moreover, patients were not
11 included if they had positive serology (IgM) indicating recent exposure to Epstein-Barr virus
12 and/or cytomegalovirus, and if they had received a vaccination with live attenuated virus in
13 the previous 4 weeks. Immunomodulators, cytotoxic and modifying plasma glucose drugs
14 were not accepted during treatment (electronic supplementary material [ESM] Table 1).

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

19 **Dose, randomisation and masking**

20 As children aged 7-14 may vary considerably in size and weight, we adjusted the dose used
21 per square meter, approximating that adults receiving 1 MIU/injection have a body surface
22 area of 1.8 square meters. Patients were randomised in a 1:1:1:1 ratio to placebo or IL-2 at
23 one of the 3 targeted doses: 0.125, 0.250, or 0.500 MIU/m²/day. Patients with a body surface
24 area ≤ 1.1 m² received 0.125, 0.25 or 0.5 MIU/day and those with body surface area >1.1 m²
25 received 0.2, 0.4 or 0.8 MIU/day.

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

11 **Procedures**

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

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

6 Blood samples were obtained for specific immunological tests including assessment of Treg
7 and lymphocyte subsets at day 1 (baseline), day 5/8, day 15, day 30, day 45, day 99, day 183,
8 day 267, day 351 and for follow-up at day 436. Blood samples for assessment of diabetes
9 parameters (fasting blood glucose and C-peptide, HbA_{1c}) were obtained on day 1, day 99, day
10 183, day 267, day 351 at day 436 of the follow-up; a mixed meal tolerance test (MMTT) was
11 performed at day 1, day 183, day 351 and day 436. Routine laboratory assessments were (1)
12 biochemistry, including blood glucose, blood electrolytes; lactate dehydrogenase; C-reactive
13 protein, procalcitonin, blood calcium, hepatic and renal functions 2) haematology assessments
14 included haemoglobin, haematocrit, white blood cell count, red blood cell count, and platelets
15 and were performed at day 0, day 15, day 99, day 183, day 267, day 351 and day 436.
16 Immunoglobulins and specific auto-antibodies for thyroiditis (anti-thyroperoxidase and anti-
17 TSH receptor), Addison’s (anti-21 hydroxylase) and celiac disease (anti-transglutaminase)
18 were evaluated at the screening visit, day 183, day 351 and day 436. Serology for
19 cytomegalovirus and Epstein Barr virus were evaluated at the screening visit, day 99, day
20 183, day 267, day 351 and day 436.

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

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

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

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

17 *Statistical analysis*

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

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

1 Since the main objective of the study was to identify the lowest active dose of IL-2 on Tregs,
2 we analysed the dose-response relationship for the main criterion by the Jonckheere-
3 Terpstra test and compared each dose vs placebo by the non-parametric Shirley-Williams test.
4 A similar method was used to compare groups for AUC during the maintenance phase. In
5 addition, we compared the time-dependent profile of changes in Tregs during the maintenance
6 phase by ANOVA on ranks and tested the significance of the increase in Tregs during the
7 maintenance phase by calculating the AUC of the difference of each time from baseline and
8 testing that this difference was significantly different from zero. According to their statistical
9 distribution, quantitative secondary criteria were compared among the four groups by
10 ANOVA (after log-transformation if required) or the Kruskal-Wallis test and between high-
11 and low-responders by the t-test (after log-transformation if required) or Mann-Whitney test.
12 Number of episodes of hypo/hyperglycaemia were compared using generalised estimating
13 equations for Poisson regression.

14 **Role of the funding source**

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

18

19 **Results**

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

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

5 There was no difference between groups at baseline for demographic and laboratory
6 characteristics, including diabetes parameters (fasting glycaemia, fasting C peptide and C-
7 peptide AUC) (Table 1), nor for biological/immunological parameters including Tregs (Fig.
8 2a). According to the inclusion criteria, all patients had at least one positive type 1 diabetes-
9 associated autoantibody. Anti-GAD and anti-IA2 antibodies were the most frequent such
10 autoantibodies, in accordance with the literature.

11 *Safety*

12 Clinical safety was satisfactory at all doses; no serious adverse events occurred during the
13 treatment and off-treatment follow-up periods (Table 2). Over the entire observation period,
14 non-serious adverse events (NSAEs) were all transient and mild to moderate. During the
15 treatment period, there was a dose-effect relationship for all NSAEs taken together. Local
16 reactions at the injection site accounted for most of the common NSAEs, with a dose-effect
17 relationship from 3.4% of administration for placebo-treated patients to 26.2%, 36.9% and
18 47.7% at the 0.125, 0.25 and 0.5MIU/m²/day doses, respectively. The other non-serious
19 adverse events (headache, gastrointestinal symptoms, transient asthenia and fever) had the
20 same frequency in the different therapy groups, including placebo. Importantly, the one-year
21 treatment period covered the seasons with a high rate of infections. Four upper respiratory
22 tract infections were noted and all resolved rapidly without complications (Table 2).

23 Two patients had hypereosinophilia during the maintenance period, but no concomitant
24 allergic disease or other symptoms related to hypereosinophilia were observed (ESM Fig. 3).

25 One patient had anti-TPO antibodies at baseline that doubled at month 6 with normal thyroid

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

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

4 The mean (95% CI) baseline percentage of Tregs in patients was 5.5% (5.0; 6.1) of CD4⁺ T
5 cells (Table 1 and Fig. 2a). At the end of the induction period, a significant dose-response
6 relationship between Treg increase and IL-2 dose (p=0.0002) was observed as the primary
7 efficacy endpoint. The mean relative change in Tregs was -0.2% (-30.4; 30.0) in the placebo
8 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)
9 (p=0.0002) for the 0.125, 0.25 and 0.5 MIU/m²/day doses, respectively (Fig. 2b, 2c and ESM
10 Table 4). Although mean Treg values were significantly different from those of placebo at all
11 IL-2 doses, the individual Treg response to IL-2 appeared variable.

12 As pre-specified in the protocol, an individual was defined as a Treg high-responder (H-Treg
13 patients) if his/her Treg response showed a $\geq 60\%$ increase over baseline at day 5. According
14 to this criterion, 7 patients were H-Treg, 3 and 4 of whom received the 0.250 and 0.500
15 MIU/m²/day doses, respectively (Fig. 2b). The other patients had a low Treg response (L-
16 Treg patients) (Fig. 2b). This heterogeneity of the Treg response to IL-2 was reminiscent of
17 what we observed in our previous trial in adults with established type 1 diabetes (Fig. 3) [12].

18 ***Immunological secondary efficacy criteria***

19 Time-dependent changes in Tregs during the maintenance course differed between groups.
20 While the mean Treg values rapidly returned to baseline after the induction course for patients
21 receiving the lowest dose, they remained elevated over the baseline during the entire
22 maintenance course for the two highest doses, with a significant effect only for the highest
23 (p=0.02 for 0.5 MIU/m²/day) (Fig. 2c and ESM Table 4 & 5). The increased percentage of
24 CD4⁺ Tregs was associated with an increase in the Treg/Teff ratio (Fig. 2d). There were no

1 statistically significant changes during induction and maintenance periods in activated CD25⁺
2 Tregs (ESM Fig 4), B cells or natural killer (NK) cells (ESM Fig 5) in any of the dose groups.
3 As a mean, the H-Treg patients maintained a 50% increase of Tregs throughout the treatment
4 period (Fig. 4b). However, there were individual variations (Fig. 4c) that we did not see in
5 other clinical trials of ld-IL2[12, 27]. In contrast, the L-Treg patients had Treg values that
6 never exceeded the threshold of a 60% increase. As a mean, L-Treg patients (treated with IL-
7 2 or placebo) maintained Treg levels around baseline values (Fig. 4d).

8 ***Metabolic secondary efficacy criteria***

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

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

19 ***Identification of potential biomarkers of patients' responses***

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

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

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

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

15

16 **Conclusion**

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

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

4 As this trial was a dose-finding one, the main primary outcome was the Treg response after
5 the 5 daily consecutive IL-2 injections. In our previous trial in adults with established type 1
6 diabetes, we reported at the same time point a dose-dependent and significant increase in
7 Tregs at all doses (3, 1 and 0.33 MIU/injection). Due to the large variation in body surface
8 area in children, we adapted our injected dose of IL-2 according to this parameter,
9 approximating the body surface area of adults to around 1.8 m². In line with our previous
10 results, we observed a dose-dependent increase of Tregs that was significant at all doses,
11 including the lowest dose of 0.125 MIU/m². We had noticed some variability in the Treg
12 response in our trial in adults, with some patients receiving the highest dose who responded
13 less than others receiving the lowest dose. We had thus pre-specified the threshold of a $\geq 60\%$
14 increase in Tregs to define a robust response to IL-2. According to this criterion, no patient
15 had such a response at the lowest dose, while 3/6 and 4/6 had it in the two highest dose
16 groups; thus, 58% of the type 1 diabetes children treated with the two highest doses had a
17 high Treg response. For reference, we had 35/46 (76%) high responders in our TRANSREG
18 trial of adult patients with one of 11 autoimmune diseases receiving 1 MIU/injection[27]. It is
19 noteworthy that, at baseline, the H-Treg responders had a lower proportion of peripheral
20 blood Tregs than L-Treg responders, and also had higher plasma sIL2RA levels, which are
21 known to reflect Treg activation[16, 36, 37]. These observations suggest that H-Treg
22 responders have actively engaged Tregs that may not be receiving the amount of IL-2 they
23 need for optimal efficacy. This is further supported by the capacity to predict the Treg
24 response based on plasma sIL2RA levels at baseline. Altogether, the dose of 1 MIU/injection,
25 adjusted to body surface area in the case of children, appears to be optimal regarding our

1 administration scheme. Indeed, it is safe and the only one that maintained a significant
2 increase of Tregs throughout the maintenance period. This dose is close to the 260,000 IU/m²
3 every 3 days proposed by others [17, 38, 39]

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

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

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

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

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

22 We envision that ld-IL2 could be beneficial not just at onset, but even later in patients with
23 more established type 1 diabetes, a notion that will be tested in a planned trial
24 (NCT03243058). Finally, the recent milestone results showing that it is possible to delay type
25 1 diabetes onset by a single injection of teplizumab[44] should also prompt the use of ld-IL2

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

4

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

8 **Data availability**

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

13

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

17 **Contribution Statement:**

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

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

22 RL, CA and AH contributed to data analyses.

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

24 MR supervised immunomonitoring and analysed results.

25 AR and PC performed the immunomonitoring and analysed results.

26 CBe participated in the study design.

27 EV performed the statistical analyses and interpretation of the data.

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

29 All authors edited the article and approved the final version.

30 DK is the guarantor of this work.

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22

23 **FIGURE LEGENDS**

24 **Figure 1:** Trial profile

25 Twenty-four patients were assessed for eligibility and randomised. Seven were assigned to
26 placebo, five to 0.125 MIU/m²/day, six to 0.25 MIU/m²/day and six to 0.5 MIU/m²/day. In the
27 initial version of the protocol, the first five patients (2 placebo and 1 for each dose) received a
28 prior single administration of IL-2 one week before the induction course and their Treg
29 response was measured at day 8. In a modified version of the protocol aimed at facilitating
30 recruitment, this single injection was eliminated and Tregs were determined at day 5 just prior
31 to the IL-2 injection. One patient dropped out of the study at day 270 because of grade 2
32 abdominal pain. All patients were analysed for primary and secondary endpoints

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

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

1 or day 8 (triangle) compared to baseline per IL-2 dose; (c) Secondary outcome: mean \pm sd
2 changes in Tregs over the whole treatment period and follow-up per IL-2 dose. (d) Data
3 represent changes in Treg/Teff ratio defined as the percentage of Tregs divided by the
4 percentage of CD4⁺ CD25^{lo/+} Foxp3⁻ T cells; mean \pm sd changes in Treg/Teff ratio over the
5 whole treatment period and follow-up per IL-2 dose. (e) Changes in C-peptide AUC from
6 baseline to day 436 per IL-2 dose. Each colour corresponds to an IL-2-dose: grey for placebo,
7 blue for 0.125 MIU/m²/day, red for 0.25 MIU/m²/day and black for 0.5 MIU/m²/day. Data
8 were normalised by baseline values for each patient at the different time points and are
9 represented as fold change, but all statistics were calculated using the raw data.

10

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

17

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

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

25

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

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

1 expression levels for cytokines found to be statistically different between the groups of low-
2 responders (grey dots) and high-responders (black dots) to ld-IL2 treatment. The p-values
3 obtained by the Wilcoxon rank-sum test are indicated for each cytokine (h,i) Barplot and
4 dotplot representations showing the estimated variable importance, quantified using the
5 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 \pm SD or Number of patient (n).

	Placebo (n = 7)	0.125 MUI/m ² (n=5)	0.25 MUI/m ² (n=6)	0.5 MUI/m ² (n=6)	<i>p-value</i>	L-Treg (n=17)	H-Treg (n=7)	<i>p-value</i>
Demographics								
Sex (Male/Female)	5/2	1/4	2/4	4/2	0.2748 [‡]	8/9	4/3	1.000 [‡]
Age (years)	9.3 \pm 1.4	10.6 \pm 1.1	9.7 \pm 1.6	10.2 \pm 2	0.4985 ^{††}	10 \pm 1.6	9.6 \pm 1.6	0.5586 [†]
BMI (kg/m ²)	16.4 \pm 2	19.4 \pm 2.9	16.5 \pm 1.8	18.2 \pm 2.3	0.1100 ^{**}	17.7 \pm 2.4	17.1 \pm 2.6	0.6176 [*]
Body surface area (BSA) (m ²)	1.2 \pm 0.2	1.3 \pm 0.2	1.2 \pm 0.3	1.2 \pm 0.3	0.8574 ^{**}	1.2 \pm 0.2	1.1 \pm 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 \leq 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 \pm 0.3	0.5 \pm 0.1	0.3 \pm 0.2	0.6 \pm 0.5	0.5267 ^{**}	0.5 \pm 0.2	0.4 \pm 0.4	0.7669 [*]
Fasting glycemia (mmol/L)	5.6 \pm 1.6	5.7 \pm 0.7	5.5 \pm 1.2	5.8 \pm 1.8	0.9796 ^{**}	5.7 \pm 1.4	5.5 \pm 1.1	0.6498 [*]
Fasting C-peptide (nmol/L)	0.23 \pm 0.10	0.33 \pm 0.07	0.30 \pm 0.10	0.33 \pm 0.13	0.2668 ^{**}	0.30 \pm 0.13	0.30 \pm 0.10	0.9986 [*]
C- peptide AUC (nmol h/L)	0.96 \pm 0.49	1.23 \pm 0.13	1.13 \pm 0.49	1.19 \pm 0.56	0.7810 ^{**}	1.13 \pm 0.46	1.13 \pm 0.43	0.9332 [*]
HbA _{1C} (mmol/mol) (%)	61.6 \pm 14.6 (7.8 \pm 1.3)	50.6 \pm 3.5 (6.8 \pm 0.3)	64.7 \pm 21.3 (8.1 \pm 1.9)	57.4 \pm 9.2 (7.4 \pm 0.8)	0.1794 ^{††}	58.0 \pm 10.8 (7.5 \pm 1.0)	61.4 \pm 21.2 (7.8 \pm 1.9)	0.6558 [†]
IDAA1C	9.8 \pm 1.2	8.9 \pm 0.7	9.3 \pm 2.5	9.5 \pm 2.5	0.5934 ^{††}	9.4 \pm 1.1	9.6 \pm 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 \pm 1.1	5.8 \pm 0.9	4.9 \pm 1.9	4.9 \pm 0.7	0.1232 ^{**}	6.1 \pm 1.1	4.3 \pm 1.0	0.0018
CD4+ T cells (cells per mm ³)	778 \pm 146	931 \pm 271	1133 \pm 427	731 \pm 190	0.0977 ^{**}	884 \pm 309	877 \pm 299	0.9607
CD8+ T cells (cells per mm ³)	500 \pm 194	522 \pm 123	738 \pm 339	542 \pm 296	0.3610 ^{**}	556 \pm 256	620 \pm 274	0.5894
CD19+ B cells (cells per mm ³)	357 \pm 116	320 \pm 131	344 \pm 174	268 \pm 151	0.7132 ^{**}	328 \pm 138	314 \pm 151	0.8244
CD56+CD3- NK cells(cells per mm ³)	149 \pm 146	99 \pm 59	127 \pm 86	99 \pm 68	0.8141 ^{††}	116 \pm 106	131 \pm 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