Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients

Michelle Rosenzwajg a, b, c, Guillaume Churlaud a, b, c, Roberto Mallone d, c, f, g, Adrien Six a, b, c, Nicolas Dérian a, b, c, Wahiba Chaara a, b, c, Roberta Lorenzon a, b, c, S. Alice Long h, Jane H. Buckner h, Georgia Afonso d, e, f, g, Hang-Phuong Pham l, m, Aines Hartemann l, Aixin Yu k, m, Alberto Pugliese k, l, m, Thomas R. Malek k, m, David Klatzmann a, b, c, *

* Corresponding author. Hôpital Pitié-Salpêtrière, Clinical Investigation Center for Biotherapies and Inflammation-Immunopathology-Biotherapy Department, 83 Bd de l'Hôpital, F-75013, Paris cedex 13, France. Tel: +33 1 42 17 74 61; fax: +33 1 42 17 74 62.
E-mail address: david.klatzmann@upmc.fr (D. Klatzmann).

Most autoimmune diseases (AID) are linked to an imbalance between autoreactive effector T cells (Teffs) and regulatory T cells (Tregs). While blocking Teffs with immunosuppression has long been the only therapeutic option, activating/expanding Tregs may achieve the same objective without the toxicity of immunosuppression. We showed that low-dose interleukin-2 (ld-IL-2) safely expands/activates Tregs in patients with AID, such HCV-induced vasculitis and Type 1 Diabetes (T1D). Here we analyzed the kinetics and dose-relationship of IL-2 effects on immune responses in T1D patients. Ld-IL-2 therapy induced a dose-dependent increase in CD4+ Foxp3+ and CD8+ Foxp3+ Treg numbers and proportions, the duration of which was markedly dose-dependent. Tregs expressed enhanced levels of activation markers, including CD25, GITR, CTLA-4 and basal pSTAT5, and retained a 20-fold higher sensitivity to IL-2 than Teff and NK cells. Plasma levels of regulatory cytokines were increased in a dose-dependent manner, while cytokines linked to Teff and Th17 inflammatory cells were mostly unchanged. Global transcriptome analyses showed a dose-dependent decrease in immune response signatures. At the highest dose, Teff responses against beta-cell antigens were suppressed in all 4 patients tested. These results inform of broader changes induced by ld-IL-2 beyond direct effects on Tregs, and relevant for further development of Il-Il-2 for therapy and prevention of T1D, and other autoimmune and inflammatory diseases.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Most self-reactive T lymphocytes are physically eliminated during thymic selection to avoid immune responses against self-antigens. Yet this process is imperfect, and potentially harmful self-specific effector T cells (Teffs) survive thymic deletion and...
populate the periphery. In the normal immune system, these autoreactive T effs do not mediate autoimmunity as their expansion and function are inhibited by regulatory T cells (Tregs) [1]. In most autoimmune diseases (AID), there is an imbalance between T effs that attack normal tissues and Tregs that normally control them. Tregs are essential players in the control of all immune responses, including responses to self, tumors, and infectious agents [1], and in the control of inflammatory disorders [2]. The discovery of Tregs revolutionized our understanding of AID pathophysiology and opened new avenues for the treatment of autoimmunity. Human beings and mice presenting a FOXP3 genetically-determined Treg and their mouse models [9–13]. Moreover, administration/restoration of Tregs improves symptoms in experimental animals and patients with AID [14–19]. Thus, while blocking T effs with immunosuppressive drugs has long been the only therapeutic option for AID, activating/expanding Tregs is becoming a novel approach that may lead to improved outcomes with better safety [14–19].

Until recently, most Treg-based therapies have been based on the infusion of ex vivo expanded Tregs, as there were no drugs or molecules that could specifically expand/activate Tregs in vivo. We and immunorecently showed that IL-2, a molecule identified almost 30 years ago for its capacity to stimulate T cells in vitro, could be such a molecule [14–17]. We performed the first clinical trial aimed at inducing Tregs with IL-2 in patients with an AID [17], namely hepatitis C virus (HCV)-related vasculitis (NCT00574652) [20]. We treated these patients with low-dose (ld) IL-2 (1.5–3 million international units MIU/day; 52.5 MIU cumulative dose; Proleukin®). The treatment was very well tolerated. Importantly, we observed a major expansion of Tregs with subsequent clinical improvement in 8/10 patients [17]. Our results showed for the first time that ld-IL-2 could be exploited as an immunoregulatory drug for the treatment of AID, acting via Treg expansion/activation. Others have reported that low dose IL-2 is safe and beneficial for the treatment of aloimmunity, i.e. chronic graft versus host disease (GVHD) [15,16].

Based on these results, we evaluated ld-IL-2 in patients with type 1 diabetes (T1D), a condition resulting from the chronic autoimmune destruction of pancreatic beta-cells, eventually leading to insulin deficiency. There are several reasons for using IL-2 in T1D patients: (i) several susceptibility genes are directly connected to the IL-2 pathway: IL-2, CD25 (IL-2RA) [21–23], IL-2RB [24], and PTPN2 [25,26]; (ii) some studies reported Treg deficiency in peripheral blood [9,10], and in pancreatic lymph nodes [27,28]; (iii) at the time of clinical diagnosis, there is significant residual beta-cell function in most patients, so that immunotherapy could curtail inflammation, promote immune tolerance, and in turn preserve beta-cell mass and function [29]. In Non Obese Diabetic (NOD) mice, a model of spontaneous autoimmune diabetes with remarkable similarities to the human disease, IL-2 prevents T1D and we showed that a short course of IL-2 at diabetes onset led to disease reversal in one third of the mice [14,30]. Finally, the use of immunosuppressants such as cyclosporine A (CsA), a calcineurin inhibitor that reduces T cell activation and expansion, provided proof of principle that newly-diagnosed T1D could be treated with immunotherapy [31–33]. CsA demonstrated clinical efficacy in prolonging endogenous insulin production, but remission from autoimmunity was limited to the duration of the treatment and CsA toxicity precluded its clinical use. Other immunosuppressive drugs or immunomodulators have been tested in clinical trials in T1D, both as short therapy courses or chronic regimens, only in some cases resulting in temporary preservation of insulin secretion with better results in subsets of responder patients, as reported recently for therapies with anti-CD3, anti-CD20 and CTLA4-ig [34–38].

The identification of a dose of IL-2 capable of safely tipping the Treg/Teff balance towards Tregs is of major importance. In our vasculitis trial, we showed that IL-2 at the dose of 1.5 MIU induced Tregs in all 10 patients and was well tolerated. However, the dose to be used in T1D was not predictable as (i) some T1D patients may have defects in the IL-2/IL-2R activation pathway [39] (ii) HCV vasculitis is an antibody-mediated disease, while in T1D beta-cell destruction is mediated by pathogenic T effs that could respond to IL-2 and thus exacerbate disease. Hence, we designed a dose finding trial to define safety and immunological responses; we previously reported that a 5-day course of single IL-2 injections, given at 0.33, 1 or 3 MIU, were well tolerated and stimulated Tregs [40].

Here, we report the results of detailed immunomonitoring of these patients, showing that ld-IL-2 induces a dose-dependent, regulatory milieu characterized by broad changes extending beyond the primary effect on Tregs. Thus, our study provides important information for further developing ld-IL2 therapies.

2. Materials and methods

2.1. Patient characteristics

Male and female patients aged from 18 to 55 years, with confirmed T1D were recruited at the Diabetology Department of the Pitié-Salpetrière Hospital (Paris, France) and thereafter followed at the Clinical Investigation Centre-Paris Est. Written informed consent was obtained from all subjects before they were enrolled in the DF-IL2 trial (NCT01353833). Detailed patients description, safety analyses and peak effects of the treatment on Tregs have been reported [40]. This clinical trial was conducted according to Declaration of Helsinki principles. All human studies were approved by the appropriate institutional review boards.

2.2. Immunomonitoring

Blood samples were collected according to the planned protocol: absolute numbers of lymphocyte subsets and Tregs were monitored at each patient visit: day-1, before treatment; day 2–5, during IL-2 administration; day-6; 15, 22, and 60 end of the follow up.

Tregs phenotyping and cytokines assay were performed at baseline, day 6, 15, 22 and 60. Because of the limited amount of blood collected, other phenotypic markers where only investigated at baseline and day 15. When possible, cells were frozen for other investigations. Stat5 phosphorylation (pStat5) was examined on frozen cells from 2 controls and 3 patients per treated group. Beta-cell-specific Teff responses were studied in 1 control, 3 patients for the dose 0.33, 2 for the dose 1 and 4 for the dose 3.

2.3. Flow cytometry and pStat5 analysis

Flow cytometry and basal pStat5 experiments were performed according to previously published methods [17,41]. Monoclonal antibodies (mAbs) used were CD3-APC750, CD4-ECD, CD8-APCα700, CD16-FITC, CD19-ECD, CD45RO-FITC, CD56-PCy7 and CD152-PE from Beckman Coulter; CD25-PE, CD45RA-APC and CD45RA-PCy7 from BD Biosciences; CD127-FITC from eBioscience; CD25-APC and IAP-PE from R&D Systems and GITR-PE from Milltenyi.

For pStat5 activation experiments, peripheral blood mononuclear cells (PBMCs) were cultured (500,000/well) for 30 min at 37 °C and IL-2 was added for 15 min. Cells were then fixed with 1% paraformaldehyde, permeabilized with 100% methanol, stained for pSTAT5 and appropriate surface markers. Events were collected.
using a LSRFortessa flow cytometer (BD Biosciences) and data were analyzed using Diva software (BD Biosciences). To calculate EC50, after subtracting the value from the media control, binding data for each sample was normalized to 100% based on the maximal response and non-linear regression of these data was performed assuming a variable slope using Graph-Pad Prism 6.0.

2.4. Plasma cytokine measurement

Plasma samples were collected, aliquoted and stored at –80 °C until analyzed. Quantitative determination of 26 cytokines/chemokines (GM-CSF, IFN-α2, IFN-γ, IL-1RA, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17a, CXCL-10, CCL-2, CCL-3, CCL-4, CCL-2, TNF-α and IL-2RA) was performed using Human Milliplex HCYTOMAG-60 kits (Millipore) and TGF-beta using Human TGF-beta1 Platinium ELISA (eBioscience) in accordance with the manufacturer protocols.

2.5. Analysis of beta-cell antigen-specific T-cell responses

CD4+ and CD8+ T-cell responses were evaluated according to previously published assay [42].

2.6. Anti-IL-2 antibodies assay

The presence of plasma IL-2-binding antibodies was studied by ELISA, as previously described [43]. Data are the average of two replicates. Positive controls are from ApoE−/− mice that were injected three times a week with 25,000 IU of Proleukin® for 4 months. Mouse studies were approved by the appropriate institutional review boards.

IL-2 neutralizing activity of plasma was assayed in a proliferation assay with Kit 225 cells [44]. 10,000 cells/well were cultured (37 °C in 5% CO2) in 100 μL of RPMI (Gibco) and 10% of BSA. Plasma were preincubated for 1 h with IL-2 10 IU/ml at 37 °C and then added at the start of the cultures at a concentration of 20%. Cell proliferation was assayed by counting cells at day 3.

2.7. Transcriptomic analysis

Transcriptomic analysis was performed using the SurePrint G3 Human GE v2 8 × 60K Microarray (Agilent Technologies, ID: 039494) according to the manufacturer’s protocol and to previously published [17]. Cyanine-3 labeled cRNAs were prepared using the One-Color Low Input Quick Amp Labeling kit (Agilent Technologies).

The slides were scanned using a G2565CA Scanner System (Agilent Technologies), and using a scan protocol with a resolution of 3 μm and a dynamic range of 20 bit. The resulting tiff images were analyzed with the Feature Extraction Software v10.7.3.1 (Agilent Technologies) and using the GE2_107_Sep09 protocol. Original dataset was normalized using quantile method implemented in Limma’s package. Probes with bad detection p-values (<0.01) in half of the samples of a biological group were discarded. Ranked gene lists were obtained using statistical score of paired ebayes implemented in Limma’s package. Gene set enrichment analyses were performed using the Blood Transcription Modules [45] added with a selection of molecular signatures from C2 database provided by GSEA software [46,47]. Network analyses were performed using Cytoscape 2.8.3 and the plugin Enrichment Map dedicated to gene set enrichment analyses.

All datasets can be accessed in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2858.

2.8. Statistical analysis

Data were compared between patients groups using a two-way analysis of variance (ANOVA) for repeated measures using Graph-Pad Prism version 5.0. When the p value of the time by treatment interaction was significant (p < 0.05), each IL-2 group of patients was compared to placebo group.

Detailed statistical results of two-way ANOVA for repeated measures are shown in the Supplementary Table 1.

Principal Component Analyses were then performed on log10-transformed data expressed as fold change compared to day 1. Multivariate analyses were performed using R platform (www.r-project.org).

3. Results

3.1. Clinical trial design, patients and safety

Results presented here are from a randomized, double-blind, placebo-controlled, dose-finding trial. Patients received a daily sub-cutaneous injection of placebo or 0.33, 1 or 3 MIU of IL-2 for 5 consecutive days. They were sampled every day just before the IL-2 injection for the first 5 days, then at day 6, 15, 22 and 60 (Supplementary Fig. 1). Twenty-four patients (6 per dose) were included in the per-protocol analysis. No serious adverse events were reported during the trial [40].

3.2. Dose dependent effects on peripheral blood mononuclear cells

3.2.1. T, B and NK cells

There were no significant changes in CD4+ and CD8+ T cell, B cell and NK cell numbers in the two lower dose groups compared to placebo (Supplementary Fig. 2). At the dose of 3 MIU/day, T and NK cell numbers were decreased approximately 2 fold during treatment, followed by an immediate return to baseline thereafter (Supplementary Fig. 2). Regarding cell percentages (Supplementary Fig. 3), a B cell reduction was observed at the two highest doses (p = 0.03 at 1 MIU/day and 0.003 at 3 MIU/day) during treatment, followed by a return to baseline levels thereafter. No statistically significant variations of NK cell percentages were observed at the doses of 0.33 and 1 MIU/day while increase NK proportions were observed at the dose of 3 MIU/day (p = 0.003). However, at this dose, individual variations of NK cell percentages remained in the range observed in the placebo group (see below). Finally, there was an increase in CD56bright regulatory NK cells [48] at the dose of 1 MIU/day (p = 0.015) (Supplementary Fig. 4).

3.2.2. CD4 and CD8 Tregs

Effects of treatment on the proportions of CD4+ Tregs are shown in Fig. 1A. Comparisons between placebo and each of the three doses of IL-2 by repeated measures ANOVA showed that the day/ treatment interaction was highly significant (p < 0.0001). Tregs were expanded at all doses, and the duration of increase was dose-dependent. By day 15, Tregs returned to baseline at the dose of 0.33 MIU/day, but persisted elevated at the dose of 1 MIU/day; increased Treg levels were still detected at day 60 at the dose of 3 MIU/day. The increased percentage of CD4+ Tregs was associated with an increase in their absolute numbers (p < 0.0001) (Fig. 1B), leading to an increase in the Tregs/Teff ratio (p < 0.0001) (Fig. 1C).

We previously reported that the very small population of CD8+CD25+Foxp3+ T cells, which are suppressive and expanded in colorectal cancer [49], was expanded in the VASCIL-IL-2 trial [17]. Here, we show a dose-dependent expansion of these CD8+ Tregs (p = 0.0002), remarkably high at the highest IL-2 dose (p < 0.0001) (Fig. 1D).
3.3. Treg immunophenotyping

Expanded Tregs displayed a memory phenotype \((p = 0.0051)\) and phenotypic features characteristic of enhanced activation (Fig. 2A). The expression of IL-2 receptor-alpha (CD25) was increased in Tregs (Fig. 2B), but not in CD4\(^+\)CD45RA\(^-\)/C0 T effector/memory (TEM) cells (not shown), during IL-2 therapy, from day 2 or 3, for all doses \((p < 0.0001)\), and rapidly returned to baseline values thereafter. This was associated with increased expression of GITR \((p = 0.0058)\) and CTLA-4 \((p < 0.0001)\) (Fig. 2C, D), whereas TGF-\(\beta\) latency-associated peptide (LAP) remained unchanged (data not shown).

3.4. Tregs show stable, selective, and highly sensitive responses to IL-2

An immediate consequence of IL-2 binding to the IL-2R is tyrosine phosphorylation of STAT5 (pSTAT5). Basal pSTAT5 levels in Tregs were transiently increased at the 3 MIU/day dose \((p = 0.0308)\) (Fig. 2E), which may be related to the increased proportions of CD45RA\(^-\)/C0 Tregs (Fig. 2A). We also assessed the IL-2-dependent activation of pSTAT5 by CD4\(^+\) Treg and TEM cells by various amounts of IL-2, for patients’ samples that were obtained before and 4–6 days after the initiation of IL-2 therapy. For each patient’s sample examined, Tregs showed increased sensitivity to IL-2 when compared to TEM cells. These responses were largely comparable to cells from normal controls that did not receive IL-2 therapy (Fig. 3A). This trend was similar regardless of the dose of IL-2 used to treat the patients. In addition, the responses during treatment were essentially identical to those detected before il-IL-2 therapy. Thus, a short course of IL-2 therapy did not rapidly alter IL-2-dependent pSTAT5 activation. By calculating the EC50s of these responses, Tregs from T1D patients were approximately 15–20-fold more sensitive to IL-2 than TEM cells (Fig. 3B). These analyses of frozen PBMCs resulted in IC50s in a similar range detected for fresh PBMCs, i.e. 3–7 pM for Tregs and 30–35 pM for CD4\(^+\) TEM cells (A.Y, Fig. 1.

Dose-dependent increase of Treg in T1D patients treated by id-IL-2. Each curve represents changes in CD25\(^+\)/CD127\(^-\) Foxp3\(^+\) among CD4\(^+\) T cells (percentages in A and absolute numbers in B), in Treg/Teff ratio (C) and in percentages of CD8\(^+\)CD25\(^-\) Foxp3\(^+\) cells (D). Data are shown as mean ± SEM of individual results normalized by baseline values for each patient at different time points (the grey zone represents the 5 days IL-2 treatment).
A.P. and T.R.M, unpublished data). Overall, these data are consistent with stable, highly sensitive, and selective responses by Tregs to IL-2 in these T1D patients.

3.5. Dose dependent modulation of plasma cytokine profiles

A panel of 26 cytokines and chemokines were assayed to investigate whether IL-2 could influence the balance of Th1/Th2/Th17 cytokines during treatment. The entire cytokine profile was tested at day 6, according to dose and to the functional classification of cytokines. Dose dependent IL-2 levels could be detected in the plasma. A radar chart (Fig. 4A) illustrates a clear trend for a dose-dependent increase of Treg-related cytokines, while the other cytokines are modestly affected at the 0.33 and 1 MIU/day doses. At 3 MIU/day, there are discrete and more pronounced cytokine increases, notably for CXCL10 and IL-5 whereas other cytokines tested remained unchanged (Fig. 4A). Principal Component Analyses of these results illustrate this dose-dependent trend: the 0.33 MIU/day patients largely overlap with placebo-treated patients, while the 1 and 3 MIU/day patients show more distinct behaviors (Fig. 4B).

Repeated measures ANOVA for the day/treatment interaction were significant for IL-2, IL-2RA, IL-5, IL-10, IL-17, TNF-alpha, TGF-beta1, CCL22, and CXCL10 (p < 0.0001 for IL-2, IL-2RA, IL-5, IL-10, CXCL10 and TNF-alpha, p = 0.0006 for TGF-beta1, p = 0.0059 for IL-17 and p = 0.0096 for CCL22) (Fig. 4A and Supplementary Table 1).
The time-course changes for cytokines show a dose-dependent and rapid increase, with values already elevated at day 2 (Fig. 4C). The increase of IL-17 was only observed at the 3 MIU/day dose (Fig. 4C), but this was paralleled by an increase in the regulatory cytokine TGF-beta.

3.6. Transcriptomic analysis

We compared the global transcriptome of PBMCs at baseline and day-6. For each IL-2 treatment dose (0.33, 1 and 3 MIU), Enrichment Map analysis was performed on significantly up- and down-regulated molecular signatures ($q$-value < 0.05). Network analyses of significantly enriched molecular signatures show a good consistency for all IL-2 doses (Fig. 5). Whatever the IL-2 dose, there is a striking down-regulation of B-cells related signatures, and an up-regulation of molecular signatures related to cell cycle and transcription. Chemokines-associated signatures are found down-regulated with the 2 lowest IL-2 doses, but up-regulated at the dose of 3 MIU/day, a dose at which NK cells signatures also became up-regulated.

The molecular signature related to FOXP3 target genes [51] is non-significantly up-regulated at the lowest dose ($q$-val = 0.2), and significantly enriched and up-regulated at the dose of 1 and 3 MIU/day.

3.7. Modulation of beta-cell-specific Teff responses

Preproinsulin (PPI) and glutamic acid decarboxylase (GAD) are beta-cell autoantigens commonly targeted by pathogenic Teffs in T1D patients. We therefore investigated T-cell responses directed to GAD, proinsulin (PI), C-peptide and the PPI signal peptide during ld-IL-2 treatment in 10 patients for whom samples from different time points (day 1, 6, 15 and 22) were available. Compared to pretreatment values, many treated patients showed a reduction or stabilization of IFN-gamma responses to most antigens between day 6 and 22 (Fig. 6). Specifically, we observed consistent suppression of all Teff responses tested in 1/3, 1/2 and 4/4 patients treated with 0.33, 1 and 3 MIU of IL-2, respectively. The suppressive effect on these responses was maintained after cessation of IL-2 therapy at the higher 3 MIU/day dose (Fig. 6), up to day 22.

3.8. Anti-IL-2 antibodies are not induced by ld-IL-2

We did not observe induction of anti-IL-2 antibodies and neutralizing antibodies in the plasma, at any time during the follow-up (Fig. 7). While a very small reactivity against IL-2 could be detected in some patients, (i) it was not increased by IL-2 administration (Fig. 7A and B) and (ii) it did not neutralize the effect of IL-2 on the proliferation of an IL-2-dependent cell line (Fig. 7C).

4. Discussion

4.1. On finding an optimal IL-2 dose for Treg specific expansion/activation

IL-2 has been initially described as a T cell growth factor [52] and developed for the treatment of conditions calling for the boosting of Teffs, i.e. cancer and infections [53,54]. However, IL-2 is dispensable for the differentiation, survival and function of Teffs, as IL-2−/− mice develop T-cell-mediated autoimmunity [55]. In contrast, IL-2 is essential for the differentiation, survival and function of Tregs [56,57]. IL-2 also has pleiotropic functions and can activate Teffs and notably memory CD8+ Teffs, and using IL-2 for treating T-cell mediated AID by promoting Treg function carries the risk of stimulating Teffs and could potentially worsen disease. Thus, therapeutic use of IL-2 to control autoimmunity requires identifying doses that selectively promote Treg function without activating effector populations.

The present study shows that the 0.3–3 MIU/day dose range is safe and effectively expanded/activated Tregs in T1D patients. The IL-2 dose-range of 0.3–1 MIU/day, given for 5 days effectively expands and activates Tregs without untoward effects on Teffs or NK
cells. These results are in line with a recent study of low dose IL-2 (< 0.1–0.4 MIU/day for five days) in healthy adults [58]. At the dose of 3 MIU/day, the effect on Tregs is even more pronounced and importantly more durable; yet at this dose, we observed a trend toward NK cell expansion (Supplementary Fig. 2), a trend for chemokine/cytokine increase (Fig. 4), and more frequent mild to moderate side effects [40].

Under the assumption that chronic diseases will require maintaining increased proportion of Tregs over time, repeated treatment will likely be necessary. The appropriate dosing should consider the patient population, the duration and frequency of the treatment, and the cumulative dosing effects in relation to time. The duration of Tregs expansion, which is clearly dose-dependent, is a major parameter to take in consideration for designing regimen of administration. Using results from this trial, we modeled that at a dose of 1 MIU/day, a 5-day induction course followed by one injection every two weeks would allow to maintain a modest increase (<30%) of Tregs throughout the treatment period, a dosing that we currently use in a trial investigating ld-IL-2 in 11 different AIDs (NCT01988506).

4.2. On the effect of IL-2 on the immune environment

At the dose of 1 and 3 MIU/day, we observed a significant and unexpected decrease in B cell percentages, correlating with increases in Treg but not NK cells (Supplementary Fig. 2). Interpretation of this finding is not straightforward. A direct effect of IL-2 on mature B cells is unlikely because they do not express CD25 nor the signal transducing beta and gamma chains of the IL2 receptor [59]. The clinical significance of these findings

---

**Fig. 4.** Dose-dependent changes in cytokines and chemokines in the plasma of T1D patients treated by ld-IL-2. Plasma cytokines and chemokines were measured by luminex assay, except for TGF-beta measured by ELISA. Radar chart representation of Treg/anti-inflammatory, Th1/Th17/inflammatory and Th2 cytokines at day 6 of follow-up (A). For each cluster of cytokines, patient projections according to the first two PCA components (capturing more than 70% of the overall variability) (B). Time course changes of representative cytokines (C). Data are shown as mean ± SEM of patients’ values normalized by individual baseline values at different time points.
remains to be clarified in future studies, particularly because a decrease in B cells could be advantageous for the treatment of B cell dependent AIDs.

IL-2 induces a clear shift of the peripheral blood immune environment towards a regulatory milieu, as reflected not only by changes in cell proportions, but also by plasma proteomics. This shift is dose-dependent, as well illustrated by the unsupervised statistical analyses (Fig. 4B). These results suggest a broad anti-inflammatory effect of IL-2 and thus a broader therapeutic scope.

IL-10 and TGF-beta, two cytokines involved in suppressive functions, are also increased by IL-2 therapy, more pronouncedly at 3 MIU/day. At the same dose, there is an increase in IFN-gamma, which is usually classified as a Th1 cytokine. However, this is the only Th1 cytokine impacted and it was only modestly elevated, possibly coming from IFN-gamma secreting Tregs [60]. IL-17 was moderately increased only at the 3 MIU/day dose, a dose at which IL-2, IL-10 and TGF-beta were markedly increased; thus, the overall balance still appears tipped towards a regulatory milieu at this dose. Consistent with this interpretation, beta-cell-specific Teff responses were reduced in 6/9 patients tested during treatment, importantly in 4/4 treated with the 3 MIU/day dose (Fig. 6), with no reductions in the placebo-treated patient tested. Thus, altogether, there is a clear IL-2 dose-dependent tuning of the immune response towards regulation of effector responses, including disease-specific, autoreactive Teff responses.

At the dose of 3 MIU/day, there was a marked increase of IL-5 levels (Fig. 4A), a cytokine involved in eosinophil homeostasis. However, at the single and cumulative doses used, our available data show only a modest increase in eosinophil counts. This contrasts with the marked increase reported by others at doses of IL-2 that were higher and/or given over a longer time course [15,61]. These results are compatible with IL-2 triggering IL-5 production by stimulating ILC2 cells, which are CD25⁺ [62]. However, ILC2 also produce IL-13, IL-9 and IL-4 [62], which were not increased in patients’ plasma. Of note, it has been reported that IL-5 promotes induction of antigen-specific Treg that suppress autoimmunity [63]. Importantly, at the 1 MIU/day dose, IL-5 was not induced, further pointing at this dose as safer choice for chronic administration.

**Fig. 5.** Clustering of significantly regulated molecular signatures from PBMCs. For each IL-2 treatment dose (0.33, 1 and 3 MIU), Enrichment Map analysis was performed on significantly up- and down-regulated molecular signatures, in red and green, respectively (q-value < 0.05). Clusters have been labeled according to their main biological feature. Size of nodes (circles) is proportional to the number of genes in the signature; width of bars linking nodes is related to the Jaccard coefficient between two signatures. Signatures without connection are not shown.
4.3. On IL-2 and therapy and prevention of AIDs

AIDs often develop over a long period of time and are chronic, with a progressive aggravation. Early intervention with ld-IL-2 could help reestablish a proper immune milieu and slow-down or even reverse the process. This is exemplified in T1D, for which at diagnosis most patients still maintain a functional mass of pancreatic ß-cells and produce insulin upon stimulation. Halting beta-cell autoimmune destruction and preserving residual beta-cells soon after diagnosis could prevent further disease progression, thus improving clinical management and long-term outcomes for patients with T1D. In this perspective, our initial results showing the blunting of beta-cell-specific Teffs during ld-IL-2 are very encouraging. We caution that such analyses could be performed only in a subset of patients and thus further study and validation are needed, which we will pursue in our efficacy trial of ld-IL-2 in patients with T1D (NCT01862120).

We believe that there may be even greater potential for using ld-IL-2 to prevent AIDs. Providing a boost to Tregs in patients at risk for AIDs, such as autoantibody-positive relatives of T1D patients, could help delay or prevent the onset of the full-blown disease. Moreover, boosting Tregs with ld-IL-2 could also prevent or decrease the frequency and severity of relapses in relapsing-remitting diseases like multiple sclerosis (MS).

Finally, ld-IL-2 has now been used successfully in the setting of chronic and acute GVHD [15,64] and several AIDs, such as HCV-vasculitis [17], T1D [40], alopecia areata [65], and SLE [66--68]. A therapeutic benefit was observed in most patients treated in these trials. This changes the paradigm of a highly focused effect of ld-IL-2 on Tregs to one where IL-2 has a broader regulatory influence on the homeostasis of the immune system, and notably inflammation. As such, future studies should explore further the effects of IL-2 and define efficacious ld-IL-2 regimens that can benefit patients currently suffering from several forms of AID and inflammatory disorders.

**The DF-IL2 study group**

*Principal Investigator:* Prof D Klatzmann MD, PhD  
*Principal Clinical Investigator:* Prof A Hartemann MD
Methodologist: G. Bensimon MD

Data management & Statistical Analysis: C. A. Payan MD

Clinical Investigations

Centre-Centre d’Investigation Clinique Paris-Est Hôpital de La Pitié-Salpêtrière Paris France

Prof. C. Funck-Brentano MD, B. Charbit MD, N. Nicolas MD, S. Delroise MD

Biological Investigation

M. Rosenzwajg MD, M. Fontfrede PharmD

Safety committee

Pr. P. Bougnères MD, Pr. C. Boitard MD

Scientific watch

E. Piaggio PhD

Modeling and Interim Analysis

Clinical Pharmacology, Angers Hospital, Angers, France (Prof. B. Diquet PharmD, C. Abbara PharmD)

Biometry, Curie Institute, Paris, France (Prof. B. Asselain MD, Dr. Y. de Rycke PhD)

Regulatory affairs and Monitoring

Clinical Research and Development Department, APHP, Paris, France (V. Millul PhD)

Clinical Research Unit Pitié-Salpêtrière, APHP, Paris, France (N. Dedisse CRA, D. Wenjie CRT)

Randomization

Clinical Research Unit- EST GH St Antoine, APHP, Paris, France (A. Rousseau PhD)

Treatment Management

Pharmacy, Pitié-Salpêtrière Hospital, APHP, Paris, France (MH. Fievet PharmD, G. Churlaud PharmD, PhD)

Clinicians at recruiting centres

- Department of endocrinology-diabetology, AP-HP, Ambroise Paré Hospital, Boulogne, France
- Univ Versailles SQY, Versailles, France (M-L. Raffin-Sanson, MD)
- Department of diabetology, AP-HP, Hôtel-Dieu Hospital, Paris, France (D. Dubois-Laforgue, MD)
- Department of endocrinology-diabetology-nutrition, AP-HP, La Pitié-Salpêtrière Hospital, Paris, France (Prof. A. Hartemann, O. Bourron MD, S. Jacqueminet MD)
- Department of Internal Medicine, AP-HP, Henri Mondor Hospital, Creteil, France (G. Lagadec, MD)
- Department of internal medicine, Institut Mutualiste Montsouris, Paris, France (C. Deybach, MD)
- Centre Hospitalier de Rambouillet, Department of internal medicine, France, (M. Popelier MD)
- Department of endocrinology-diabetology, AP-HP, Saint-Antoine Hospital, Paris, F-75012, France (N. Bourcigaux, MD, E. Laroche MD, S. Christin-Maitre MD)
- Department of endocrinology-diabetology-nutrition, AP-HP, Jean Verdier Hospital, Bondy, France (E. Cosson MD, B. Donadille, MD, B. Lormeau, MD)

Acknowledgments

We are indebted to Christophe Misse, director of the Département de la Recherche Clinique et du Développement (DRCD) of the AP-HP, for initiating the support for this study. We thank the patients and the members of the DF-IL2-study group (listed above), and more specially Gilbert Bensimon for the study design and Christine Payan for data management and statistical analyses. We also thank all the personnel of the Endocrinology department, the Clinical Investigation Center Paris-est (CIC-PE) and the Clinical Investigation Center for Biotherapies (CIC-BTI) for their participation and more especially Nathalie Fery and Cornélia Degbe. DK, MR and TRM are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

MR and DK are inventors on a patent application related to the therapeutic use of Id-IL-2, which belongs to their academic institutions and has been licensed to ILTOO Pharma. MR, GC and DK hold shares in ILTOO Pharma. HPP is an employee of ILTOO Pharma. No other potential conflicts of interest relevant to this article were reported.

This work was funded by the DRCD, the CIC-PE and CIC-BTI, the Inserm and French state funds managed by the ANR within the Investissements d'Avenir programme (ANR-11-IDEX-0004-02). RM was supported by the Aviesan/AstraZeneca program. JB and AL were supported by the Immune Tolerance Network (NIH Contract #N01 AI15416), an international clinical research consortium headquartered at the Benaroya Research Institute and supported by the National Institute of Allergy and Infectious Diseases and the Juvenile Diabetes Research Foundation. This work was also supported by the Diabetes Research Institute Foundation, Hollywood, FL, the Peacock Foundation, Inc., Miami, FL, and the Anton E. B. Schefler Foundation, which supported AV, AP and TRM.

MR supervised immunomonitoring, participated to the study design and analyzed results; GC, RM, AS, ND, WC, RL, AL, JB, GA, HPP, AV, AP and TRM performed some experiments and analyzed results; AH was the principal clinical investigator of the study, participated to the study design and analyzed results; DK was the principal investigator of the study, conceived the study, participated to the study design, analyzed results and wrote the manuscript. All authors edited the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2015.01.001.

References


Klotzmann D. Immune regulation without immunosuppression: the promise of low dose IL-2. In: FOCIS meeting Chicago; 2014.