Administration of low-dose interleukin-2 (IL-2) alone or combined with rapamycin (RAPA) prevents hyperglycemia in NOD mice. Also, low-dose IL-2 cures recent-onset type 1 diabetes (T1D) in NOD mice, partially by boosting pancreatic regulatory T cells (Treg cells). These approaches are currently being evaluated in humans. Our objective was to study the effect of higher IL-2 doses (250 000–500 000 IU daily) as well as low-dose IL-2 (25 000 IU daily) and RAPA (1 mg/kg daily) (RAPA/IL-2) combination. We show that, despite further boosting of Treg cells, high doses of IL-2 rapidly precipitated T1D in prediabetic female and male mice and increased myeloid cells in the pancreas. Also, we observed that RAPA counteracted IL-2 effects on Treg cells, failed to control IL-2-induced tolerance in a reversible way. Notably, the RAPA/IL-2 combination failure to cure T1D was associated with an unexpected deleterious effect on glucose homeostasis at multiple levels, including β-cell division, glucose tolerance, and liver glucose metabolism. Our data help to understand the therapeutic limitations of IL-2 alone or RAPA/IL-2 combination and could lead to the design of improved therapies for T1D. Diabetes 62:3120–3131, 2013

In type 1 diabetes (T1D), the immune system destroys the pancreatic β-cells (1). At clinical onset, ~30% of β-cells are still able to produce insulin (2), thus stopping autoimmune destruction, which at this stage is a promising approach (3). Along the same lines, there is a growing list of phase II/III clinical trials based on immunomodulation that are currently being conducted in T1D patients (4).

NOD mice, which develop spontaneous T1D, represent an accepted model for testing new therapies (5), the gold standard being that treatments that cure overt hyperglycemia in these mice may be most appropriate for translation into the clinic, as was the case for anti-CD3 antibodies (Abs) (6), which have been tested in patients with promising results (7). In addition, results from our own group showing that low-dose interleukin-2 (IL-2) can prevent (8) and revert disease in NOD mice (9) have led to the translation of this strategy into clinical trials in T1D patients (clinical trial reg. no. NCT01353833, clinicaltrials.gov).

We have shown that in NOD mice, administration of low-dose IL-2 for 5 days induced the remission of new-onset T1D by specifically boosting regulatory T cells (Treg cells) in the pancreas without activating pathogenic effector T cells (Teff cells). However, remission was obtained in only 60% of treated mice, and half of them became diabetic again during the following months (9). Consequently, improving IL-2 therapy by optimizing dosing or combining IL-2 with other immunomodulatory drugs, such as rapamycin (RAPA), could be of great importance for the goal of translating this therapy to humans.

RAPA has been used in clinical transplantation for many years (10), and it has been safely administered to T1D patients during islet transplantation (11,12). In mice, RAPA monotherapy can prevent T1D development (13); however, it is unable to induce disease reversal (14). Moreover, RAPA and IL-2 were found to be synergistic for the prevention of diabetes in NOD mice (13). Consequently, we decided to test whether RAPA could synergize with short-term IL-2 therapy to reverse T1D and reinforce the development of long-term tolerance.

In this work, we have further studied the mechanisms of action of IL-2 and RAPA alone or in combination in the NOD model of T1D.

RESEARCH DESIGN AND METHODS

Mice. NOD mice were bred in our animal facility under specific pathogen-free conditions in agreement with current European legislation. Protocols were approved by The Ethics Committee in Animal Experiment Charles Darwin, France (no. Ce5/2012/21).

IL-2 and RAPA treatment. Mice were treated with daily intraperitoneal injections of 25 000, 250 000, or 500 000 IU of recombinant human IL-2 (Proleukin; Novartis France) for the indicated time. RAPA (Rapamune; Wyeth-Lederle) was administered at 1 mg/kg per os, a dose that has been previously reported not to be toxic to pancreatic islets (13,14) and to prevent T1D onset in NOD mice (13). Glicosuria was measured using colorimetric strips (Multistix; Bayer), and blood glucose levels were quantified by a glucometer (Optium Xceed; Abbott).

Spleen-, lymph node-, and tissue-infiltrating lymphocytes preparation. Spleen and lymph nodes (LNs; axillary and brachial) and pancreatic draining LNs (DLNs) were isolated and dissociated in PBS-3% FCS. For panescreatic infiltrating lymphocyte preparation, the whole pancreas was digested with collagenase/DNase solution and submitted to Percoll density gradient as described (15,16).

Abs and flow cytometry analysis. Anti-CD3, anti-CD4, anti-CD8, anti-CD45.1, anti-inducible T-cell costimulatory (ICOS), anti-B220, anti-glucocorticoid-induced tumor necrosis factor receptor (GITR), anti-Ly6C, anti-Ly6G, anti-CD11b, anti-CD11c, anti-CD19, anti-Gr1, anti–IFN-γ, anti-Ki67, anti–pSTAT5 (pY694), and streptavidin labeled with phycoerythrin (PE), allophycocyanin, PerCP, PerCP-Cy5.5, V500, allophycocyanin (APC)-H7, PE-Cy7, Alexa Fluor-700, Alexa Fluor-647, or biotin, were from BD Biosciences. Anti-CD25, anti-cytotoxic...
T-lymphocyte antigen 4 (CTLA-4), anti-NKp46, and anti-F4/80 labeled with fluorescein isothiocyanate (FITC), PE-Cy7, APC, or eFluor 450 were from eBioscience. The eFluor 450-anti-Foxp3 staining was performed using the eBioscience kit. For intracellular cytokine staining, cells were restimulated with 1 µg/mL anti-CD3ε (clone 145-2C11) and anti-CD28 (clone 37.5.1) (BD Biosciences). The eFluor 450-anti-Foxp3 staining was performed using the eFluor 450-anti-Foxp3 staining kit (eBioscience). For phospho-specific-staining, cells were incubated in RPMI 2% medium containing NaF (10 mM), sodium orthovanadate (10 mM), and protease inhibitors (Roche). CD45.1+ cells were isolated by MACS separation using anti-CD45.1 microbeads (Miltenyi Biotec). Cell counting and analysis were performed on a LSR II (BD Biosciences) and analyzed with Flowjo (Tree Star) software.

**RESULTS**

High doses of IL-2 are toxic and can precipitate T1D development. We have previously shown that five doses of 25,000 IU IL-2 could revert new-onset T1D in NOD mice in part by specifically boosting pancreatic T_{reg} cells (9). However, not all treated mice were cured, and in some of them the beneficial effects were transient. We reasoned that increasing the dose of IL-2 administered may further increase the frequency of T_{reg} cells and thus improve the treatment efficacy.

We first tested the capacity of higher IL-2 doses to prevent T1D development. We found that daily treatment of NOD mice with 250,000 or 500,000 IU IL-2 (i.e., doses 10- or 20-fold higher than the dose shown to prevent T1D) (8) could be lethally toxic in a dose-dependent manner in 5-week-old mice (Fig. 1A, top). In prediabetic mice at 12-14 weeks of age, this treatment was less toxic, but it dramatically precipitated the onset of diabetes after only a few daily injections and in a dose-dependent manner (Fig. 1A, middle and bottom). At the 250,000 IU IL-2 dose, females were significantly more sensitive than males to IL-2-induced acceleration of diabetes (Fig. 1A, bottom).

IL-2 toxicity is mainly associated with vascular leak syndrome, which can lead to hypotension, pulmonary edema, liver cell damage, and even death (17). We thus measured organ edema after 5 days of high-dose IL-2 administration (Fig. 1B). Unlike C57BL/6 mice (17), higher cumulative doses of IL-2 were necessary before vascular leak syndrome became evident in NOD mice. Moreover, we observed sex-dependent differences, with lung edema being most prominent in males and liver edema in females. Brain edema did not develop in either group. Additionally, we measured islet infiltration in these mice and observed that 5 days of high-dose IL-2 administration induced a mild increase of invasive insulitis in males (Fig. 1C).

The rapid onset of T1D, observed as early as after only 3 days of treatment, was not due to an immediate detrimental effect of IL-2 on glucose homeostasis, as administration of 250,000 IU IL-2 did not induce any apparent alteration in glucose metabolism after a glucose bolus administration 2 h after the IL-2 injection (Fig. 1D).

Low-dose IL-2-induced T1D remission was associated with T_{reg}-cell activation only in the pancreases, whereas T_{eff}, CD8^+, and NK cells were not noticeably affected by this treatment (9). On the contrary, high-dose IL-2 induced systemic effects, including increased cell numbers in secondary lymphoid organs, most significantly in the pancreas DLN (Fig. 2A). In the DLN (Fig. 2B), nonadherent LN cells, and spleen (not shown), significantly higher proportions of NK cells along with lower frequencies of total CD4^+ T cells, but with increased T_{reg}-cell proportions, were observed.

In the islets, high-dose IL-2 effects were more pronounced (Fig. 2B). Notably, total CD4^+ T cells were unchanged, but an almost double frequency of T_{reg} cells was seen after IL-2 treatment. Also, NK, CD11c^+, and CD11b^+ cells increased after IL-2 administration. Interestingly, in almost all analyzed organs, T_{reg}, T_{eff}, CD8^+, NK, B, and CD11b^+ cells increased their division after high-dose IL-2 administration, as assessed by quantification of Ki67 expression (Fig. 2C). In particular, >80% of NK and CD11b^+ cells had cycled in the pancreas. Indeed, by immunohistology analysis these highly proliferative CD11b^+ cells were found interspersed around the islets and surrounding blood vessels (Fig. 3A). Further phenotypic analysis indicated that two subpopulations among CD11b^+ cells increased during IL-2 treatment: CD11b^+ Ly6G^-Ly6C^+ cells, likely representing tissue macrophages; and CD11b^+ Ly6G^+ cells, likely representing neutrophils (Fig. 3B).

The detailed analysis of the effects of IL-2 on T_{reg} cells indicated that the cytokine increased the expression of Foxp3 and CD25 in a dose-dependent way, suggesting an enhancement of T_{reg} cell fitness (Fig. 4A). Similarly, even though the frequency of T_{eff} cells was diminished, their activation was potentiated by IL-2 administration, as...
indicated by the dose-dependent increase in the fraction of CD25+ Teff and CD8+ T cells, mainly observed in the islets (Fig. 4B). Moreover, Teff, CD8+, and NK cells showed increased IFN-γ production (Fig. 4C) during treatment with high-dose IL-2. Additionally, among the expanded CD8+ T-cell population, we observed a significant increase in the frequencies of NRPV7+ islet-specific glucose-6-phosphatase catalytic subunit-related protein-specific autoreactive CD8 T cells (19) in the blood and the islets of the treated mice (Fig. 4D).

RAPA partially counteracts the activation of pancreatic Treg cells induced by low-dose IL-2. The immunomodulatory effects of RAPA have been attributed to its capacity to preferentially affect activated Teff cells, while Treg cells are less susceptible to its action (20). Consequently, we hypothesized that the beneficial effect of low-dose IL-2 on Treg cells could synergize with the concomitant elimination of pathogenic Teff cells by RAPA after administration of a RAPA/low-dose IL-2 combination.

We analyzed the effects of combined treatment on lymphoid cells in prediabetic NOD females, in which insulitis is already important. Administration for 5 days of RAPA alone, low-dose IL-2 alone, or both drugs combined did not induce major changes in absolute numbers and frequencies of T cells in the spleen, LNs, and DLN (data not shown). In the pancreas, low-dose IL-2 alone did not modify the frequency of total CD8+ or CD4+ T cells (data not shown). However, it modified the Treg/Teff balance by increasing the
FIG. 2. Administration of high doses of IL-2 to NOD mice: effects on immune cells. Prediabetic female NOD mice 12–14 weeks of age were treated daily with PBS or 250,000 or 500,000 IU IL-2 over 5 days and analyzed 2 h after the last injection. A: Absolute cell numbers in DLN, nondraining LNs, and spleen. B: Percentage of total CD4+, CD8+, NKp46+ CD3^+ (NK), B, CD11c^+, and myeloid CD11b+ cells in DLN (top) or in pancreas (bottom). Right panels indicate the percentage of T_{reg} cells among total CD4^+ T cells. C: Representative histograms of Ki67 expression (left) and percentages of Ki67^+ cells among indicated populations (right) in the DLN and pancreas. Similar results were obtained in male NOD mice (data not shown). Data are cumulative of three to five independent experiments with 4 to 14 mice per group. Symbols represent individual mice, and horizontal lines represent the median. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired, two-tailed Student t test).
percentage of T_{reg} cells (Fig. 5A), which was associated with increased cell division (Fig. 5B) and increased expression of Foxp3, CD25, GITR, ICOS, and CTLA-4 (Fig. 5C). A similar tendency was observed when low-dose IL-2 was combined with RAPA. Notably, the effect of IL-2 on T_{reg} cell numbers or activation was significantly less pronounced in the presence of RAPA (Fig. 5A–C).

Finally, we examined the effects of treatment on NK cells in the pancreas and observed that their proportion doubled after 5 days of treatment with low-dose IL-2 alone, with the percentage of proliferating cells increasing from low basal levels up to ~60% after treatment (Fig. 5D–E). Addition of RAPA to IL-2 treatment did not modify the effect of IL-2 on NK cells.

We discarded that the partial counteraction of IL-2 effects on T_{reg} cells by RAPA was due to interference of the JAK/STAT pathway, as IL-2 phosphorylation was not modified by the administration of RAPA in vivo (Fig. 6).

**RAPA inhibits the ability of low-dose IL-2 to revert T1D.** To test whether RAPA could reinforce the development of long-term tolerance when combined with IL-2, we treated new-onset T1D NOD mice with 25,000 IU (low-dose) IL-2 with or without RAPA. In agreement with our previously reported results (9), low-dose IL-2 treatment induced diabetes remission in 57% of the mice. However, none of the 12 mice that received the combined treatment were cured (Fig. 7A). We assessed the effects of treatment on pancreatic T cells from these mice: RAPA/IL-2 combination did not modify the percentage of total CD8+ or CD4+ T cells (not shown), but it significantly increased the frequency of T_{reg} cells (Fig. 7B). Interestingly, RAPA hampered the IL-2–induced reduction in IFN-γ production by CD8+ T cells infiltrating the pancreas (Fig. 7C), which we had previously shown to be associated with T1D reversal (9). These results may partially explain why RAPA inhibits the ability of IL-2 to revert disease.

To determine whether RAPA had any effect in mice that had reverted from new-onset T1D after low-dose IL-2 therapy, we administered RAPA to NOD mice 10 days after IL-2–induced disease remission. Surprisingly, RAPA precipitated hyperglycemia in all previously cured mice (Fig. 7D). Of note, in two of eight treated mice, RAPA induced irreversible hyperglycemia, but in the other six IL-2–treated mice, the hyperglycemia triggered by RAPA was transient. Indeed, after RAPA withdrawal and without further addition of IL-2, mice spontaneously became normoglycemic again until RAPA treatment was resumed, at which point mice reverted to diabetes. In some of these mice, the transient occurrence of diabetes upon adding and removing RAPA could be repeated at least three times, indicating that RAPA can reversibly inhibit the tolerogenic effect of IL-2. We analyzed the temporal effect of RAPA on the pancreatic infiltrate. Intriguingly, T_{reg} cell levels in the pancreas were significantly lower in mice cured of diabetes with IL-2, but which had become diabetic again after RAPA treatment, compared with mice that did not receive RAPA, and a significant parallel increase in these cells was observed in the DLN (Fig. 7E and F). T_{reg} cells returned to initial levels after RAPA withdrawal and restoration of euglycemia, suggesting that under RAPA treatment the migration pattern of CD4+ T cells may be altered.

**Combination IL-2 plus RAPA impairs glucose tolerance.** The rapid reversibility of the effect of RAPA on diabetes led us to evaluate whether RAPA was affecting glucose homeostasis. We measured fasting blood glucose levels and performed glucose tolerance tests in prediabetic NOD mice previously treated with IL-2, RAPA, or both...
FIG. 4. Administration of high doses of IL-2 to NOD mice: effects on cell activation and cytokine production. Mice were treated as in Figure 2, and DLN and pancreas-infiltrating cells were analyzed by flow cytometry. A. Left: Representative contour plots of Foxp3 and CD25 expression in CD4+ T cells in indicated groups. Right: Relative mean fluorescence intensity (MFI) of Foxp3 and CD25 in Treg cells expressed as the relative percentage of the MFI value in nondraining LNs of PBS-treated mice, which was assigned an arbitrary value of 100%. B: Percentages of CD25+ cells among CD4+ Foxp3+ (Teff cells) (top) and CD8+ T cells (bottom). C: Representative contour plots of IFN-γ staining (left) and the percentage of IFN-γ–secreting cells (right) among islet-infiltrating CD4+ Foxp3+ (top), CD8+ (middle), and NK cells (bottom) quantified after ex vivo stimulation with PMA-ionomycin. Data are cumulative of two to three independent experiments with four to nine mice per group. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired, two-tailed Student t test). D. Top: Representative histograms of control TUM and NRP-V7 tetramer expression on CD8+ T cells from blood and islets. Bottom: Percentage of NRP-V7+ CD8+ T cells among total CD8+ T cells in blood (left) and in the pancreatic islets (right). Data are from one experiment, symbols represent individual mice, and horizontal lines represent the median. *P < 0.05 (unpaired, two-tailed Mann-Whitney test).
combined. Low-dose IL-2 treatment did not modify glucose homeostasis (Fig. 8A and B), in agreement with results obtained with high doses of IL-2 (Fig. 1D). However, RAPA/IL-2 combination induced elevated fasting blood glucose levels (Fig. 8A), and also RAPA- and RAPA/IL-2–treated mice displayed highly impaired glucose tolerance (Fig. 8B). Mechanistically, RAPA-induced glucose intolerance could be due to direct β-cell toxicity or to peripheral insulin resistance; we thus monitored β-cell division and performed insulin tolerance tests (Fig. 8C and D). Even if neither RAPA nor RAPA/IL-2 treatments visibly modified the response to an exogenous insulin boost, RAPA/IL-2 administration significantly reduced basal β-cell proliferation in vivo.

Finally, to better understand how RAPA alone or combined with IL-2 interfered with glucose homeostasis, we studied by microarray analysis the liver response to a glucose challenge. As depicted (Fig. 8E and Supplementary Fig. 1), the liver transcriptome signature was highly modified by IL-2 alone (81 genes) or combined with RAPA (40 genes), whereas fewer genes were affected by RAPA alone (16 genes). To retrieve relevant biological processes associated to the different treatments, we analyzed the canonical
pathways that were most significant to our datasets (Supplementary Table 1). RAPA- and IL-2-modified transcripts were associated mainly with metabolic and immune pathways, respectively. Interestingly, RAPA/IL-2 combination modified other pathways than each drug alone, most of them involving metabolic functions. These data further document the complex effects of these drugs beyond immune modulation and may partially explain the associated detrimental effects on glucose homeostasis.

DISCUSSION
Low-dose IL-2 administration represents one promising approach (9) (clinical trial reg. no. NCT01353833, clinicaltrials.gov) among the novel immunotherapies being evaluated in T1D patients (4). We reasoned that we could enhance the efficiency with which IL-2 induces a tolerogenic state in NOD mice (9) by increasing its dose. However, higher IL-2 doses dramatically accelerated disease onset and demonstrated a toxicity that could even be lethal. Interestingly, female NOD mice were significantly more susceptible than males to diabetes induction, correlating with the higher incidence of spontaneous T1D in female mice (70%) compared with male mice (30%) (21). Additionally, high-dose IL-2–associated organ edema and insulitis appeared dissimilar in males and females, suggesting that IL-2–related side effects may be sex-dependent in the NOD mice.

T1D appeared in some of the mice treated with high-dose IL-2 despite substantial local and systemic increase in the frequency and activation of Treg cells. Disease occurrence could be explained by the concurrent activation of Teff, NK, B, and CD8+ T cells, all of which have been implicated in T1D development (3,22). Of note, among CD8+ T cells, islet-specific ones were enriched in the pancreas, potentially contributing to the destruction of the β-cells. Remarkably, high doses of IL-2 induced a previously unreported yet striking increase in CD11b+ myeloid cells in the pancreas. Historically, macrophages have been regarded as mediators of insulitis (23). However, recently myeloid cells have been associated with T1D resistance and prevention in NOD mice (19,24,25). The role of different myeloid subpopulations in disease pathogenesis is nevertheless still largely unknown.
Overall, higher doses of IL-2 resulted in a shift from immune tolerance to overt destructive autoimmunity. In the context of human therapy, these results highlight the need to perform thorough immunomonitoring of the broad effects of IL-2 so as to determine the dose that would uniquely act on T\(_{\text{reg}}\) cells or other regulatory populations.

The potent immunosuppressive properties of RAPA are associated with its capacity to block cell cycle progression and induce T-cell anergy and depletion (26), thus impacting on T-cell differentiation and function. In our model, RAPA specifically dampened the IL-2 effect on pancreatic T\(_{\text{reg}}\) cells. Notably, diabetic animals receiving IL-2/RAPA combination showed higher frequencies of pancreatic T\(_{\text{reg}}\) cells compared with treatment with IL-2 alone, which nevertheless were associated with inefficient control of IFN-\(\gamma\) production by infiltrating T cells. These T\(_{\text{reg}}\) cells could originate from the expansion of preexisting natural T\(_{\text{reg}}\) cells or from the generation of induced T\(_{\text{reg}}\) cells from T\(_{\text{eff}}\).
D) probes from reference PBS. Each dataset was derived from three biologically independent replicate samples.

number represents the number of genes in each subgroup: total number of genes (Total), and upregulated (Up, or U) and downregulated (Down, or D) probes from reference PBS. Each dataset was derived from three biologically independent replicate samples.

cells, probably favored by the proinflammatory environment in the islets (27).

We could not attribute the deleterious impact of IL-2/RAPA combination on Treg-cell function to interference of RAPA on IL-2–mediated activation of STAT-5. However, although Treg cells are less dependent for survival on the AKT/mammalian target of RAPA pathway than are Teff cells (28), it is possible that RAPA inhibition of this pathway may still affect IL-2 action on Treg cells (29,30).

Although our data suggest that the deleterious effects of RAPA in IL-2–treated NOD mice may be related to its action on Treg-cell function or trafficking, we also found that a short course of RAPA and IL-2 at low doses significantly impaired glucose homeostasis. There have been some reports of renal transplant patients who received long-term treatment with RAPA becoming at risk for developing new-onset diabetes, associated with abnormal glucose and lipid homeostasis and with reduced insulin sensitivity (40). Furthermore, in rodents, long-term RAPA treatment severely impairs glucose tolerance, affecting hepatic gluconeogenesis (41,42), adipocyte lipid uptake (41), skeletal muscle insulin sensitivity (43), and β-cell homeostasis (41). However, RAPA alone prevents TID development in NOD mice (13,44) and has been reported to improve Treg-cell suppressive function in TID patients (12). Here, we demonstrate that RAPA administration even for a short period and at doses two to five times lower than those reported in the literature (41,42); impaired glucose tolerance, as previously suggested (14); and modified liver glucose metabolism in the NOD model. Moreover, when combined with IL-2, the negative effects on glucose metabolism were broadened, also inducing elevated basal blood glucose levels and

![Image](diabetes.diabetesjournals.org)

FIG. 8. A short course of RAPA alone or combined with IL-2 induces glucose intolerance. Prediabetic female NOD mice were treated for 5 days with PBS, 25,000 IU IL-2, 1 mg/kg RAPA, or IL-2 and RAPA combined. A, B, and E: On day 5, an IPGTT was performed after an overnight fast or mice were killed 4 h after the glucose bolus for transcriptome analysis of the liver. A: Fasting blood glucose levels were determined before glucose injection. B: Blood glucose levels and area under the blood glucose curve (AUC) of the treated mice of the different groups (n = 7–8 per group). Data are cumulative of two independent experiments with seven mice per group. Symbols represent individual mice, and horizontal lines represent the median. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired, two-tailed Student t test). C: Percentage of Ki67+ cells among total β-cells (insulin+CD45.1+) analyzed at day 5. Each column represents the mean ± SEM of all islets counted for all mice of the same group (n = 3–4 per group). *P < 0.05 (Mann-Whitney test). D: On day 5, an ITT was performed after a 4 h fast. Shown are blood glucose levels and area under the blood glucose curve of the treated mice of the different groups (n = 7 per group). Symbols represent individual mice, and horizontal lines represent the median. E: Venn diagram comparing differentially expressed genes in the liver after glucose challenge of mice in IL-2 vs. PBS, IL-2/RAPA vs. PBS, and RAPA vs. PBS groups. The threshold for differential expression was defined as 1.5-fold changes in expression with overlapping probes discarded. Each number represents the number of genes in each subgroup: total number of genes (Total), and upregulated (Up, or U) and downregulated (Down, or D) probes from reference PBS. Each dataset was derived from three biologically independent replicate samples.
imparing \( \beta \)-cell proliferation. Interestingly, our results showing that RAPA restored diabetes in NOD mice, which had been previously cured of new-onset disease by IL-2 treatment, evoke RAPA effects counteracting anti-CD3 treatment in NOD mice (14).

Recently, a clinical trial testing RAPA/IL-2 combined therapy in new onset T1D patients was halted due to a transient drop in C-peptide levels in all patients, despite effective T\( _{reg} \)-cell induction (30). Our results showing that RAPA breaks IL-2–induced tolerance and that, in combination with IL-2, it induces glucose intolerance, help to explain the inefficacy and deleterious consequences of the combined treatment in T1D. However, the RAPA/IL-2 combination can be efficient at boosting T\( _{reg} \) cells and inducing tolerance in graft-versus-host disease (37,45). The latter results, which were observed in hosts devoid of the metabolic alterations associated with T1D, demonstrate the different potential outcomes of the combined treatment depending on the underlying pathology. And even when referring to T1D, it is surprising to observe that the RAPA and IL-2 combination can have a completely different outcome in preventive or curative schedules in the NOD mice (13). Probably, in prediabetic mice, the effects of RAPA are not strong enough to cause hyperglycemia because, at odds with already diabetic mice, there are enough healthy islets to compensate for the negative effects of RAPA, at least for a short-term treatment.

Our results, together with the accumulated experience in the use of IL-2 and RAPA in the context of T1D prevention or reversal (see recapitulation in Supplementary Table 2), help define the limitations of the application of these drugs in T1D and may contribute to the design of improved IL-2–based therapy.

ACKNOWLEDGMENTS

E.P. has received Agence Nationale de la Recherche grant ANR-09-GENO-006-01 and an INSERM/Direction Générale de l’Offre de Soins 2011 grant. O.B. has received a University of Zurich Fonds zur Förderung des Akademischen Nachwuchses grant. A.B. and L.P. were supported by the Ministère de la Recherche.

E.P. has received a European Foundation for the Study of Diabetes/Juvenile Diabetes Research Foundation International/Novo Nordisk 2011 grant. E.P. is the inventor of a patent application related to the use of low-dose IL-2 owned by her public institutions. No other potential conflicts of interest relevant to this article were reported.

A.B., L.P., G.F., J.W., O.B., and E.P. designed and performed the experiments and analyzed data. N.C and W.C. performed and analyzed microarray experiments. E.P. conceived the project. A.B., L.P., and E.P. wrote the manuscript. A.H. and all the authors discussed the results and commented on the manuscript. E.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Pere Santamaria, University of Calgary, Canada, for kindly providing the NRP-V7 and TUM tetramer, and Benoit Salomon, INSERM U959, Paris; Olivier Boyer, INSERM U905, Rouen; and José Cohen, INSERM U855, Créteil, for constructive and critical reading of the manuscript. The authors especially thank Bertrand Blondeau, UMRS 872, Paris, for his valuable advice and technical help and Pedro Carranza (supported by Cardiovascular Diseases, Diabetes and Obesity) Ile-de-France, INSERM U959, Paris, and Hanem Sadek, INSERM U950, Paris, for technical help. The authors thank Christelle Enond, Flora Issert, François Bodin, and Serban Morosan (all from the Centre d’Exploration Fonctionnelle, Université Pierre et Marie Curie) for taking good care of the mice.

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