IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells

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Regulatory T cells (T reg cells) play a major role in controlling the pathogenic autoimmune process in type 1 diabetes (T1D). Interleukin 2 (IL–2), a cytokine which promotes T reg cell survival and function, may thus have therapeutic efficacy in T1D. We show that 5 d of low-dose IL–2 administration starting at the time of T1D onset can reverse established disease in NOD (nonobese diabetic) mice, with long-lasting effects. Low-dose IL–2 increases the number of T reg cells in the pancreas and induces expression of T reg cell–associated proteins including Foxp3, CD25, CTLA-4, ICOS (inducible T cell costimulator), and GITR (glucocorticoid-induced TNF receptor) in these cells. Treatment also suppresses interferon γ production by pancreas-infiltrating T cells. Transcriptome analyses show that low-dose IL–2 exerts much greater influence on gene expression of T reg cells than effector T cells (T eff cells), suggesting that nonspecific activation of pathogenic T eff cells is less likely. We provide the first preclinical data showing that low-dose IL–2 can reverse established T1D, suggesting that this treatment merits evaluation in patients with T1D.
et al., 2008). We and others also showed that young prediabetic NOD mice treated with low-dose IL-2 alone, or together with rapamycin, can be protected from the development of disease (Serreze et al., 1989; Rabinovitch et al., 2002; Tang, et al., 2008). However, although >200 different treatments can prevent T1D in NOD mice, only very few are effective to cure established disease (Shoda et al., 2005). In this paper, we show that only 5-d administration of low-dose IL-2 at diabetes onset can induce long-lasting remission in the treated animals. IL-2 did not stimulate the diabetogenic effector T cells (T eff cells) but rather specifically stimulated CD4+Foxp3+ T reg cells in the pancreas to dampen the inflammatory milieu. Thus, in the presence of pathogenic T cells, IL-2 at a low dose is a selective T reg cell stimulator endowed with a great therapeutic potential.

RESULTS AND DISCUSSION
Short-term administration of low-dose IL-2 induces long-lasting diabetes remission in NOD mice but is inefficient in T reg cell–deficient mice
We assessed if 5 d of low-dose IL-2 administration could be effective to cure clinical diabetes in NOD mice. Remarkably,
this treatment induced diabetes remission in 60% of the mice within 1 wk, and most of them remained normoglycemic over the 10-wk duration of the experiment (Fig. 1, a and b). Five mice were followed up for 30–50 wk after treatment and were still diabetes free (unpublished data). Administration of an IL-2/anti–IL-2 mAb complex, which induces a more dramatic T reg cell expansion than IL-2 alone (Tang et al., 2008), provided a similar but not better curative effect. Histological examination of mice that remained diabetes free for 12–35 wk after IL-2 treatment showed that pancreatic islets were more infiltrated than age-matched nondiabetic controls, indicating that the treatment did not lead to a long-lasting clearance of insulin (Fig. 1 c). We tried to optimize treatment efficacy by prolonging IL-2 administration or by combining IL-2 with drugs that are favorable for T reg cells, such as rapamycin, glucocorticoids, and α-CD3 (Battaglia et al., 2005; Chen et al., 2006; Chatenoud and Bluestone, 2007). Prolonging IL-2 administration from 5 to 10 d induced disease remission in three out of eight mice that had not reverted after 5 d (Fig. 1 d). The other treatments did no show obvious improved outcome (unpublished data).

Our previous results indicated that disease prevention by the IL-2/anti–IL-2 mAb complex was associated with increased T reg cell survival in the pancreas (Tang et al., 2008). To address the role of T reg cells in IL-2 remission of overt diabetes, we repeated the treatment in CD28 KO NOD mice, which have a profound deficit in T reg cells (Salomon et al., 2000). IL-2 administration did not reverse hyperglycemia in CD28 KO mice, whereas diabetes remission was observed in two out of three WT littermates (Fig. 1, e and f), suggesting that low-dose IL-2 therapy is inefficient in the absence of natural T reg cells. Overall, our results show, for the first time, that low-dose IL-2 administration is one of the few treatments that can cure spontaneous overt diabetes in NOD mice (Shoda et al., 2005).

Short-term administration of low-dose IL-2 does not modify the peripheral homeostasis of hematopoietic cells

Given that T reg cells are not the only cells sensitive to IL-2 and that a systemic IL-2–mediated activation of T reg cells could lead to detrimental global immunosuppression, we performed a comprehensive analysis of the effect of IL-2 administration on the different hematopoietic subpopulations potentially sensitive to IL-2 in the spleen and pancreatic LN (PLN). Prediabetic (8–14 wk old) NOD mice were treated for 5 d with 25,000 IU (the low dose mentioned in the Introduction) or 250,000 IU IL-2. No significant change was observed at low-dose IL-2 on the total cell number, the proportion of CD4+, CD8+, CD4+Foxp3+, CD8+Foxp3+, NK, B, and CD11b+ cells (Table S1), or their activation status (unchanged CD4+ expression; unpublished data). Also, the proportions of the different dendritic cell subpopulations remained unchanged (unpublished data). Only when high-dose IL-2 was used was cell homeostasis modified, with a significant increase of the cell numbers in PLN and the proportion of CD4+Foxp3+, CD4+CD25+Foxp3−, and CD8+Foxp3− populations in the spleen. These data indicated that 5 d of low-dose IL-2 administration did not modify steady-state homeostasis of hematopoietic cells in lymphoid organs. In contrast, we have previously reported that low-dose administration of the IL-2/anti–IL-2 complex favored the expansion of T reg, T eff, NK, and CD8+ T cells in lymphoid tissues leading to a possible undesired systemic response (Tang et al., 2008). We thus focused on the mechanism of action of low-dose plain IL-2 for the rest of our study.

Low-dose IL-2 shows a profound and specific effect on pancreatic T reg cells

Data suggest that T reg cells present in the pancreas control the progression to destructive insulitis in prediabetic mice (Feurer et al., 2009). However, under highly inflammatory conditions, reduced survival or insufficient function of T reg cells may unleash T eff cell autoimmune attack (Tang et al., 2008). To establish whether the IL-2 curative effect was related to a modification of T reg cell numbers and/or characteristics in the pancreas, we quantified their proportion in the spleen, PLN, and pancreas of 8–9- and 15–16-wk-old prediabetic and new onset diabetic NOD mice. The proportion of T reg cells gradually increased with age and disease evolution in the spleen, PLN, and pancreas (Fig. 2). Unexpectedly, in prediabetic mice (8–16 wk old), significantly lower proportions of T reg cells (8% of CD4+ T cells) were found in the pancreas compared with normal ratios (10–15% of CD4+ T cells) in the spleen and PLN (Fig. 2 legend, Fig. 2).
Figure 3. IL-2 treatment increases T reg cell proportions and reinforces the T reg cell phenotype specifically in inflamed pancreas. Prediabetic (a, c, and d) or new onset diabetic (e and f) NOD mice received five daily injections of PBS (Ctrl) or 25,000 IU IL-2 and were sacrificed 2 h after the last injection. (a and e) The percentage of Foxp3+ cells among CD4+ cells was determined by FACS in the spleen, PLN, and pancreas. Symbols represent individual mice and horizontal bars are the means. (b) Percentage of Ki67+ cells among CD4+Foxp3+ cells from islets, PLN, and nondraining LN (NDLN) of prediabetic mice untreated (day 0).
(p-values). At disease onset, the percentage of pancreatic T reg cell was significantly increased to reach 14% of CD4+ T cells. A similar increase in the proportion of T reg cell in PLN with aging has been reported (Mellanby et al., 2007). However, in this same study, T reg cell proportions were unchanged between nondiabetic and recent-onset diabetic mice, which is at odds with our results. This could be a result of variations between different NOD mice colonies or of the technique used to purify islet-infiltrating T cells. Our results confirm and complete our previous and paradoxical observation that T reg cells increase with age and disease development but not sufficiently to control autoimmunity (Tang et al., 2008). Also, we show for the first time that over time, consistently lower proportions of T reg cell are found in the pancreas compared with lymphoid tissues.

We then studied the IL-2 effect on the inflammatory pancreatic infiltrate of late prediabetic mice (14–19 wk old). After 5 d of low-dose IL-2 administration, the proportion of T reg cells increased 1.7-fold in the pancreas of prediabetic mice, reaching similar proportions to those in the spleen and PLN, where T reg cell proportions were unchanged (Fig. 3 a). This increase was not associated with augmented T reg cell division, as the percentage of cycling cells, determined by the expression of Ki67 (Fig. 3 b) and EdU incorporation (not depicted), was unaffected during IL-2 treatment. T reg cell division in LN was also unaffected by IL-2 treatment (Fig. 3 b). We also verified that T eff cell cycling was not modified under IL-2 treatment (Fig. S1 a).

The increase in the numbers of pancreatic T reg cells induced by IL-2 is most probably the result of an improvement of T reg cell survival (Tang et al., 2008) or, alternatively, of the conversion of T eff cells into induced T reg cells (Zheng et al., 2007), the re-expression of Foxp3 in ex–T reg cells (Zhou et al., 2009), or the increased recruitment of T reg cells in the pancreas.

We also observed that IL-2 treatment was associated with a rise in the expression of molecules associated with T reg cell function such as CD25, Foxp3, CTLA-4 (CTL antigen 4), inducible T cell costimulator (ICOS), and glucocorticoid-induced TNF receptor (GITR; Fig. 3, c and d). Again, this effect was only noticed in pancreas-infiltrating T reg cells and not in T eff cells of the islets or PLN (Fig. S1 b).

Similar analyses were repeated in new-onset diabetic mice. In this case, in which high percentages of pancreatic T reg cells are already present before treatment, we did not observe a further increase in their numbers (Fig. 3 e). However, and as in prediabetic mice, IL-2 treatment induced a significant rise or treated with daily injections of 25,000 IU IL-2 for 3 or 5 d (days 3 and 5), n = 2–5 mice per group. The graph is representative of three independent experiments. (c and f) Mean fluorescence intensity (MFI) of CD25 and Foxp3 expression among CD4+Foxp3+ cells infiltrating the spleen, PLN, and pancreas, expressed as relative percentage from the mean fluorescence intensity value in the spleen, which has an arbitrary value of 100%. Graphs show cumulative mean data ± SEM from control mice [black] and mice treated with IL-2 for 5 d [white], from four to six independent experiments. *, P < 0.05; **, P < 0.001; ***, P < 0.0001. A representative histogram of CD25 and Foxp3 intensities on CD4+ gated islet cells is shown for new onset diabetic animals [f, right]. (d) Expression of the indicated molecules by CD4+Foxp3+ cells from mice treated with PBS [gray] or IL-2 [white] for 5 d. Graphs are representative of two to six independent experiments.
IL-2 treatment decreases IFN-γ production in the pancreas

IL-10, IL-2, and TGF-β can contribute to the control of pancreatic inflammation (Rabinovitch et al., 2002; You et al., 2007; Tang et al., 2008). Thus, we assessed the production of these cytokines by pancreas-infiltrating T cells after 5 d of IL-2 or PBS treatment in prediabetic mice. The proportions of CD4+ and CD8+ T cell subsets among total CD45+ cells were similar in PBS- versus IL-2–treated animals (33 ± 13 vs. 31 ± 15, n = 11, respectively for CD4+ T cells and 10 ± 5 vs. 9 ± 7, n = 10, respectively for CD8+ T cells). IL-10 and IL-2 production were not significantly increased in the CD4+ T cells of IL-2–treated animals and we could not detect TGF-β under the same stimulation conditions (Fig. 4 a).

We then studied the production of TNF, IL-17, and IFN-γ, which are known to be toxic for β cells. TNF production by T cells, macrophages, and dendritic cells remained unchanged under IL-2 treatment (Fig. 4 a and not depicted) and similar small amounts of IL-17 were detected in control and IL-2–treated mice (Fig. 4 a). Remarkably, IL-2 treatment induced an important reduction of IFN-γ production by both CD4+ and CD8+ pancreas-infiltrating T cells but no change in the PLN of prediabetic treated animals (Fig. 4, b and c).

Interestingly, when we repeated the experiment in new onset diabetic mice, IFN-γ production in IL-2–treated mice was also massively suppressed, specifically in pancreatic CD8+ T cells and to a lesser extent in PLN CD4+ T cells (Fig. 4 d). Consistent with this, it has been shown that T reg cells limit IFN-γ production specifically in the pancreas, precluding the progression from nondestructive insulitis to diabetes (Feuerer et al., 2009). Also, a similar reduction of intraislet IFN-γ production is associated with other treatments shown to prevent T1D development in NOD mice, such as CFA, BCG, all-trans retinoic acid, and p277 peptide treatments (Ablamunits et al., 1998; Serreze et al., 2001; Van et al., 2009). These data suggest that one of the mechanisms of action of low-dose IL-2 is the limitation of IFN-γ production by the pancreas-infiltrating T cells.

In vivo low-dose IL-2 treatment selectively modifies the T reg cell transcriptome, leaving the T eff cell gene profile practically unaltered

To have a global understanding of the mode of action of low-dose IL-2 on T cells in vivo, we performed gene array analysis.
We compared the transcriptome of highly purified T reg cells and T eff cells obtained from spleen and LNs of Foxp3-GFP NOD mice that received the same low-dose IL-2 treatment that cured new onset diabetic mice (T reg cell–IL-2 and T eff cell–IL-2) or PBS (T reg cell–PBS and T eff cell–PBS). Analysis of genes with a fold change equal to or greater than two revealed that in T reg cells, IL-2 induced the overexpression of 81 genes and down-regulation of 39 genes (Fig. 5, green). Surprisingly, only the expression of five genes (out of the ~31,500 present in the gene chip) was modified in T eff cells. Four were also up-regulated in T reg cells (Socs2, IL2Ra, Serpin3g, and S100a8; Fig. 5, blue) and one was up-regulated only in T eff cells (Hist1h1c; Fig. 5, red). This result puts forward that nonspecific activation of pathogenic T eff cells as a result of low-dose IL-2 administration is less likely.

The complete list of genes affected by IL-2 treatment in T reg cells is shown in Fig. S3. Globally, most of these 120 genes do not have a direct relationship with the biology and suppressive function of T reg cells, which is in accordance with the absence of effect of IL-2 on peripheral T reg cells that we observed in our FACS analysis (Table S1 and Fig. 3). Only a few genes involved in T reg cell function and survival, such as IL-2Ra, Foxp3, kis, and Bel-2, were up-regulated by IL-2. This increased mRNA expression was only reflected by a minor, but nonstatistically significant, augmentation of the protein expression for CD25 and Foxp3 (Fig. 3 c) and no modification for ICOS protein expression (Fig. 3 d) in T reg cells of lymphoid tissues. Interestingly, the important rise in the expression level of these three proteins in pancreatic T reg cells (Fig. 3, c and d) could reflect an enhanced sensibility to IL-2 caused by a local deficit of this cytokine in this tissue (Tang et al., 2008).

In conclusion, we show that a short course of low-dose IL-2 administration at diabetes onset can reverse established disease in NOD mice. This effect is long lasting and inefficient in the absence of naturally occurring T reg cells. The disease in NOD mice. This effect is long lasting and inefficient in the absence of naturally occurring T reg cells. The disease in NOD mice. This effect is long lasting and inefficient in the absence of naturally occurring T reg cells. The disease in NOD mice. This effect is long lasting and inefficient in the absence of naturally occurring T reg cells. 

**MATERIALS AND METHODS**

**Mice.** NOD mice were obtained from Charles River Laboratories or from our own colony. NOD Cd28−/− (Lenschow et al., 1996) and NOD mice expressing GFP under the control Foxp3 gene promoter (generated by V. Kuchroo, Brigham and Women’s Hospital, Boston, MA) were maintained in our animal facility under specific pathogen-free conditions in agreement with current European legislation on animal care, housing, and scientific experimentation. All protocols were approved by The Regional Ethics Committee in Animal Experiment No.3 of Ile-de-France region.

**IL-2 treatment.** Mice were treated with daily i.p. injections of 25,000 IU of recombinant human IL-2 (Proleukin; Novartis), 250,000 IU IL-2, or 0.5 µg of recombinant mouse IL-2 and 5 µg of anti–IL-2 mAb complex for 5 consecutive days. For Cd28−/− mice, treatment was done during 10 d. De-natured IL-2 was used as control. Glycosuria was measured using colorimetric strips (Multistix; Bayer) and hydroxyprolin was quantified by a colorimeter (Optium Xceed; Abbott Diabetes Care).

**Spleen, LN, and tissue-infiltrating lymphocyte preparation.** Spleen and LN were isolated and dissociated in PBS 3% FCS. For the other tissues and organs, mice were perfused with 0.9% NaCl, digested with collagenase/ DNase solution, and filtered, as previously described (Cassan et al., 2006). For pancreas and liver, a percoll density gradient step was performed as previously described (Bakkekeok et al., 1981).

**Antibodies and flow cytometry analysis.** Anti–CD4, anti–CD25, anti–CD314 (NK22D), anti–ICOS, anti–CTLA-4, anti–CD11b, anti–CD11c, anti–IL-2, anti–IL-10, anti–TNF, anti–IL-17, anti–IFN-γ, and anti–K67 labeled with PE, allophycocyanin, PerCP, or Alexa Fluor 700 were purchased from BD. The PE- or Pacific blue-labeled anti–Foxp3 staining was performed using a kit (eBioscience). In some cases, anti–K67 antibody (Ab) was added together with the anti–Foxp3 Ab. For intracellular cytokine staining, cells were restimulated with 1 µg/ml PMA (Sigma-Aldrich) and 0.5 µg/ml ionomycin (Sigma-Aldrich) for 4 h in the presence of 1 µl/ml GolgiPlug (BD). After cell surface staining, intracellular staining was performed using the Cytosorb/Cytoperm kit (BD). Cells were acquired on an LSR II (BD) and analyzed with Flowjo (Tree Star, Inc.) software.

**Histology.** Pancreas were fixed in formalin solution and processed for paraffin embedding. 5-µm-thick sections were stained with hematoxylin/eosin and the degree of insulin was evaluated microscopically.

**Sample generation and DNA microarray hybridization and analysis.** GFP-NOD mice were treated for 5 d with PBS or 25,000 IU IL-2 and sacrificed 2 h after the last injection. Untouched CD4+ T cells were enriched by magnetic cell separation (CD4+ T Cell Isolation Kit; Miltenyi Biotech), labeled with CD4-PE Ab, and FACs sorted as CD4+GFP+ (T reg cells) and CD4+GFP− (T eff cells). RNA was generated (RNasey Mini kit; QIAGEN) from the sorted cells. RNA quality was verified in a Bioanalyzer (Agilent Technologies) and measured with a NanoDrop (Thermo Fisher Scientific).

Microarray experiments were performed on a Mouse-WG-6 BeadChip (Illumina), a genome-wide array with 45,282 probes. Data were extracted and Quantile normalized using BeadStudio software (Illumina). The working lists were created, filtering probes with detection p-values <0.05 for at least half of the chips involved in the comparison and discarding overlapping probes. Each dataset was derived from two biologically independent replicate samples. Independent samples were compared by computing fold ratios and filtered at twofold. The Microarray data accession no. is E-MEXP-2689.
Statistical analyses. Statistical significances were calculated using the two-tailed unpaired Student *t* test with 95% confidence intervals.

Online supplemental material. Fig. S1 shows that T eff cell division and phenotype are not modified by low-dose IL-2 treatment. Fig. S2 provides information on IL-2 effect on T reg cells in different nonlymphoid tissues. Fig. S3 shows a heat map of IL-2 effect on T reg cell gene expression. Table S1 shows information on IL-2 effect on T reg cells in different nonlymphoid tissues. Fig. S3 phenotype are not modified by low-dose IL-2 treatment. Fig. S2 provides information on IL-2 effect on T reg cells in different nonlymphoid tissues. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100209/DC1.

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Figure S1. T eff cell division and phenotype are not modified by low-dose IL-2 treatment. (a) Percentage of Ki67+ cells among CD4+Foxp3− cells from islets, PLN, and nondraining LN (NDLN) of PBS or IL-2–treated prediabetic mice. Shown are mean data from three independent experiments with three to seven mice per group. (b) Expression of the indicated molecules by CD4+Foxp3− cells from mice treated with PBS (gray) or IL-2 (white) for 5 d, as in Fig. 3. Histograms are representative of two to four independent experiments.
Figure S2. IL-2 effect on T reg cells in different nonlymphoid tissues. Prediabetic NOD mice were treated for 5 d with PBS (Ctrl) or 25,000 IU IL-2. Indicated tissues were harvested and stained for CD45, CD4, CD25, and FoxP3. (a) Percentage of FoxP3+ cells among CD45+CD4+ T cells. Graph shows cumulative mean data ± SEM from control (black) and IL-2–treated mice (white) from four independent experiments with two to six mice per group. *, P < 0.05. (b) Representative images of CD25 and Foxp3 staining on CD4+CD45+ gated cells from each tissue. Quadrants delimit the nonspecific staining obtained with the isotype control antibodies. Mean fluorescence intensity values of Foxp3 and CD25 gated on Foxp3+ cells are indicated in each image.
Figure S3. Heat map of IL-2 effect on T reg cell gene expression. The list includes the genes from the comparison of the T reg cell–PBS versus the T reg cell–IL-2 lists, filtered at twofold changes. The intensity of blue and red colors indicates the degree of down or up-regulation, respectively. The fold change for each gene is indicated in the Fc column. Data are from one experiment with biological duplicates for each condition.
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<th>CD8*</th>
<th>FoxP3+/CD8*</th>
<th>CD11b+</th>
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Prediabetic (8–15 wk) NOD mice were treated during 5 d with PBS (control), 25,000 IU, or 250,000 IU IL-2 and analyzed 2 h after the last injection. Total cell numbers in the spleen and PLN were quantified and percentage of different hematopoietic populations was obtained by FACS. *, P < 0.05; **, P < 0.001. Data were obtained from two to six independent experiments.