Interleukin-7 Is a Survival Factor for CD4<sup>+</sup> CD25<sup>+</sup> T-Cells and Is Expressed by Diabetes-Suppressive Dendritic Cells

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Dendritic cells can facilitate allograft survival and prevent autoimmunity via direct and indirect cell-mediated mechanisms. Recent studies demonstrate that immunoregulatory dendritic cells (iDCs) confer immune hyporesponsiveness in part through CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells (Tregs). Herein, we provide evidence to support the hypothesis that dendritic cells derived from NOD mice and engineered ex vivo to exhibit suppressed expression of the CD40, CD80, and CD86 costimulatory molecules mediate an increase in the prevalence of regulatory CD4<sup>+</sup> CD25<sup>+</sup> T-cells via interleukin (IL)-7. Unlike control dendritic cells, these dendritic cells expressed significant levels of IL-7. Exogenous addition of IL-7 to NOD T-cells did not promote expansion or proliferation, but instead selectively maintained the number of CD4<sup>+</sup> CD25<sup>+</sup> T-cells by inhibiting activation of apoptosis in these cells. In vitro, IL-7 receptor α-chain (IL-7Rα) was expressed at significantly higher levels on CD4<sup>+</sup> CD25<sup>+</sup> T-cells compared with CD4<sup>+</sup> CD25<sup>−</sup> T-cells irrespective of resting or stimulated state. In vivo, CD4<sup>+</sup> CD25<sup>+</sup> T-cells obtained from NOD-scid mice reconstituted with ex vivo engineered iDCs and NOD splenocytes expressed significantly higher levels of IL-7Rα compared with levels in the CD4<sup>+</sup> CD25<sup>−</sup> subset, especially in diabetes-suppressive dendritic cell–administered NOD-scid recipients. Taken together, our data suggest a novel mechanism by which iDCs delay autoimmunity through the CD4<sup>+</sup> CD25<sup>+</sup> Treg pathway and suggest IL-7 as a survival factor for these putative Tregs, which express the α-chain of its receptor at considerably higher levels than CD4<sup>+</sup> CD25<sup>−</sup> T-cells. Diabetes 55:158–170, 2006
production and acts in synergy with IL-12 on T-cells (31,34,35). However, the critical function served by IL-7 as a homeostatic regulator is in inhibiting cell death through apoptosis largely through the bcl family of antiapoptotic molecules (36–41).

Recent data suggest that Tregs can be generated in the absence of IL-2 and that a complementary regulator could exist to maintain their number (42,43). IL-2 signaling passes largely through two main pathways. One leads to the activation of the Akt kinase cascade and the upregulation of antiapoptotic proteins and activity (44,45). The other passes through the activation of the signal transducer and activator of transcription (STAT)5 transcription factor and results in T-cell proliferation and differentiation (44–47). A subset of mice deficient in STAT5A/5B showed autoimmune phenomena similar to those lacking IL-2 or its receptor and correlated with decreased numbers of CD4+ CD25+ Tregs (48). Tregs in these doubly deficient mice underwent apoptosis at an accelerated rate (48). Farrar and colleagues (49,50) have since proposed that the activation of an IL-7–sensitive transcription factor (STAT5) is important in the development or survival of CD4+ CD25+ Tregs. Using a transgenic mouse expressing a constitutively activated STAT5 in B- and T-cell compartments, these studies showed that CD8+ T-cell numbers were dramatically increased with a cell surface phenotype identical to that of cells undergoing homeostatic proliferation or with a memory-like state (49). Additionally, they showed that these transgenic mice were no longer dependent on IL-15 or IL-7 to undergo homeostatic proliferation. Moreover, they demonstrated an increase in prevalence of CD4+ CD25+ Tregs expressing the Foxp3 transcription factor. Finally, these Tregs were capable of suppressing the proliferation of CD4+ CD25− wild-type T-cells prestimulated by antigen-presenting cells (CD8−). It is noteworthy that STAT5 is one of the transcription factors responsible to IL-7 (51–57).

In this report, in addition to confirming the diabetes-suppressive properties of iDCs as we have previously published (25), we demonstrate that, at least in the NOD mouse, treatment of NOD-derived T-cells with IL-7 in vitro results in an increase in the steady-state numbers of CD4+ CD25+ T-cells in vitro. This increase is not due to proliferation but rather to the suppression of apoptosis of the CD4+ CD25+ T-cells. Furthermore, we show that IL-7 receptor α-chain (IL-7Rα) is expressed at significantly higher levels on CD4+ CD25+ T-cells compared with CD4+ CD25− cells in culture and in vivo. We also demonstrate a dramatic induction of IL-7 in diabetes-delaying iDCs. These iDCs, when injected in vivo in immunocompromised hosts reconstituted with NOD-derived T-cells, promote an increased prevalence of CD4+ CD25+ T-cells that selectively express IL-7Rα. Taken together, we propose a novel mechanism of iDC action in the suppression of type 1 diabetes onset, in which iDC-derived IL-7 prevents apoptosis of Treg populations that selectively express the α-chain of the IL-7 receptor.

**RESEARCH DESIGN AND METHODS**

Female NOD and NOD-scid mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and were used between the ages of 5 and 10 weeks. They were maintained in a specific pathogen-free environment in the Animal Facility of the Rogosn Research Center and used under the approval of the Animal Research Care Committee.

**Generation of iDCs.** iDCs were engineered using previously published protocols (25,58). Basically, bone marrow was obtained from female NOD mice, and immature dendritic cell progenitors were propagated in vitro in granulocyte macrophage colony-stimulating factor and IL-4. At the end of the propagation, the dendritic cells were treated with a mixture of antisense oligonucleotides targeting the primary transcripts of CD40, CD80, and CD86 in which each of the antisense oligonucleotide mixtures was at a final culture concentration of 50 μg/ml. The term “iDC” to describe this engineered embodiment of NOD bone marrow–derived dendritic cells, and its immunoregulatory capability in delaying the onset of type 1 diabetes has been described previously (25).

**iDC transfer to NOD mice and diabetes incidence.** Prepared as described previously (25), 2 × 10^6 NOD iDCs in PBS were injected by intraperitoneal route into 5–8-week-old female NOD mice at weekly intervals for a period of 1 month. Diabetes incidence was ascertained twice weekly in tail vein blood by electronic sampling (One-Touch; LifeScan Technologies, Milpitas, CA). Confirmation of diabetes was noted on two consecutive readings of blood glucose >280 mg/dl.

**iDC-stimulated generation of Tregs in vivo.** Control dendritic cells or iDCs (1 × 10^6–1 × 10^7) from 5–8-week-old female NOD mice were transferred intraperitoneally into female NOD-scid mice between the ages of 5–10 weeks. Three to 5 days later, 1 × 10^7 column-enriched splenic T-cells (CD3+ T-cell enrichment columns; R&D Systems, Minneapolis, IN) with or without prelabelling with carboxyfluorescein ester (CFSE) (see below) were transferred through the tail vein in the same mice. One week later, splenic T-cells from the reconstituted NOD-scid mice were enriched over the T-cell column. Cell surface phenotype of the T-cells was assessed by fluorescence-activated cell sorter (FACS) analysis in a FACSCalibur (BD Biosciences/Pharmingen) and CellQuest modules (BD Biosciences, San Jose, CA).

**Determination of cell proliferation and apoptosis activation.** To determine proliferation in vivo, NOD-derived enriched T-cells (female NOD mice were used at 5–8 weeks of age) were labeled in vitro with CFSE (Molecular Probes, Eugene, OR) before injection into the NOD-scid mice described immediately above. At specific times, NOD-scid mice reconstituted with the CFSE-labeled cells were killed for splenic T-cell enrichment. Proliferation was assessed by FACS analysis, measuring CFSE dilution in specific T-cell subpopulations marked by fluorescent antibodies or fluorescent reagents (like annexin V, described below). In parallel, selected T-cell populations were cocultured in vitro in the presence of control dendritic cells or iDCs followed by proliferation assessment using the CyQuant reagent (Molecular Probes) at 5 days after the initiation of coculture. Apoptosis of selected T-cell subpopulations was determined by annexin V staining/propidium iodide uptake of cells positive for defined cell surface antigens, as assessed by FACS analysis.

**FACS analysis.** The following antibodies were direct conjugates of either fluorescein isothiocyanate, phycoerythrin, Cy5, CyChrome, or allophycocyanin and purchased from BD Biosciences/Pharmingen (San Diego, CA): CD4 clone, RM4–5; CD25 clone, 7D4; CD62 ligand clone, MEL-14; CD3 clone, Leu-4; and IL-7Rα (CD127 clone) and IL-7Rβ (CD100 clone, SR/B9). Relevant isotype-specific, fluorescently conjugated antibodies were used throughout as controls for non-specific cell surface binding. Cells were incubated with mixtures of specific antibodies and, in parallel, with the isotype controls at titers between 1:100 and 1:500 per the manufacturer’s suggestions. They were also labeled with propidium iodide, 7-AAD, and/or the annexin V-staining reagent and then used for FACS analysis. Mean fluorescence intensity was measured for gated cells gated on forward and side scatter properties representing T-cells. Dead cells as well as cell clumps were excluded from the analyses, which were performed using the CellQuest software package.

**Effects of IL-7 on NOD-derived T-cells in vitro.** Column-enriched splenic T-cells were treated with 50 ng/ml murine recombinant IL-7 (R&D Systems) with or without 5 μg/ml concanavalin A (Sigma, St. Louis, MO) for periods between 18 and 96 h in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine. At the end of the specific treatment period, annexin V positivity in these T-cells was used to assess apoptosis. In parallel, the degree of proliferation in these T-cells was measured using the CyQuant fluorescence reagent (Molecular Probes). To determine whether IL-7 was directly responsible for the augmentation of CD4+ CD25+ putative Tregs in culture, we added 5 μg/ml blocking IL-7 (Peprotech, Rocky Hill, NJ) or IL-7Rα (clone A7R34) antibody to the culture well. We used a combination of iDCs (clone A7R34) and IL-7Rα (clone A7R34). IL-7Rα (clone A7R34) and IL-7Rα (clone A7R34) were transferred through the tail vein in the same mice. One week later, splenic T-cells from the reconstituted NOD-scid mice were enriched over the T-cell column. Cell surface phenotype of the T-cells was assessed by fluorescence-activated cell sorter (FACS) analysis in a FACSCalibur (BD Biosciences/Pharmingen) and CellQuest modules (BD Biosciences, San Jose, CA). Reverse transcription was performed with 5 μg total RNA by real-time PCR using the specific primers listed further below. The mRNA levels for each
FIG. 1. Differentiation between pro-proliferative or antiapoptotic effects of iDCs on T-cell subsets. A: Frequent iDC administration to pre-diabetic NOD mice completely prevents diabetes. Weekly injections of $2 \times 10^6$ iDCs into NOD female recipients beginning at 8 weeks of age for a period of 1 month resulted in all recipients being diabetes free at >32 weeks of age. $P < 0.0001$ by Kaplan-Meier log-rank analysis. B: Reconstitution of NOD-scid mice with immune cell populations. To determine the effects of the iDCs in vivo, we first injected $1 \times 10^5–2 \times 10^6$ dendritic cell embodiments (control dendritic cells or iDCs) into 5- to 8-week-old NOD-scid female mice. In parallel, column-enriched splenic T-cells from age-matched female NOD mice were labeled in culture with CFSE. Three days later, $1 \times 10^7$ of the CFSE-labeled column-enriched NOD splenic T-cells were injected into the NOD-scid recipients by tail vein injection. These “incubator mice” were killed 1 week later, and the spleens were collected for T-cell enrichment, processing, and downstream FACS analysis. C: General schema of FACS analysis. This panel describes the strategy for differentiating between proliferation and apoptosis activation in selected T-cell subsets. After staining with fluorescent label–coupled antibodies to CD4 and CD25, cells representing T-cells were first gated (top left panel; P1) on forward and side scatter. CD4$^+$ CD25$^-$ double-positive cells were then isolated inside a new gate (middle left panel; P2). Within this gate, the dilution of CFSE (top right panel) or the level of annexin V (bottom right panel) was ascertained. D: Freshly isolated splenic T-cells from NOD-scid mice reconstituted with dendritic cells and NOD T-cells; gating into proliferative and apoptotic double-positive populations. Freshly isolated splenic T-cells from NOD-scid mice reconstituted with dendritic cells and NOD T-cells as described (top panel; cells selected for subsequent FACS analyses) were assigned into one of four FACS gates based on CFSE dilution and annexin V positivity: P2, proliferating and apoptotic; P3, nonproliferating and apoptotic; P4, proliferating and low apoptotic; and P5, nonproliferating and low apoptotic. Each of these gates was then probed for the analysis presented in E. E: iDCs do not promote discernible proliferation of CD4$^+$ CD25$^-$ T-cells compared with control dendritic cells in vivo. Comparing the CFSE dilution among CD4$^+$ CD25$^-$ T-cells from iDCs and control dendritic cell–preconditioned NOD-scid incubator mice, it is evident that negligible proliferation occurred in vivo. The CFSE dilution reflects the proliferation of the CD4$^+$ CD25$^-$ double-positive cells shown herein. These data are representative of the prevalence of each cell population as observed in freshly isolated T-cells from three different NOD-scid mice (iDC-preconstituted mice $n = 3$; control dendritic cell–preconstituted mice $n = 3$).
of the cassettes were analyzed using the iCycler system (Bio-Rad, Hercules, CA). The mRNAs were reverse transcribed into cDNAs by using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed by using a SYBR supermix kit (Bio-Rad) and running for 45 cycles (94°C for 10 s, 55°C for 10 s, and 72°C for 10 s, followed by a 5-min extension at 72°C). The mRNA level of each sample for each cassette was normalized to that of 18S RNA control PCR product (18S Housekeeping Gene; Ambion Classic II Internal Standards; Ambion, Austin, TX). Relative mRNA levels are presented as $2^{(Ct_H252-Ct_H11002)}$. The primers used to amplify the mouse IL-7 cDNA product were sense, 5'-ATT ATG GGT GGT GAG AGC CG-3', and antisense, 5'-GTT CCT GTC ATT TTG TCC AAT TCA-3'. The IL-7 and 18S RNA PCR products were also resolved in parallel in a 2% agarose gel and visualized after ethidium bromide staining.

**Statistical analysis.** Student’s t test, Kaplan-Meier log-rank analysis, and ANOVA, where described, were facilitated using Prism software (version 4; GraphPad, San Diego, CA).

**RESULTS**

Administration of iDCs to NOD mice in vivo promotes the preferential survival but not the considerable proliferation of CD4$^+$ CD25$^+$ T-cells. In a recent study, we showed that administration of iDCs to pre-diabetic NOD mice significantly delayed the onset of diabetes with a concomitant increase in the prevalence of CD4$^+$ CD25$^+$ T-cells (25). We now demonstrate that frequent administration of these dendritic cells completely prevents the onset of diabetes in NOD mice (Fig. 1A). Toward a mechanistic understanding of how the iDC administration was associated with the increased prevalence of these putative regulatory T-cells, we analyzed proliferation of CD4$^+$ CD25$^+$ T-cells in apoptotic and nonapoptotic states in vivo. CFSE-labeled NOD T-cells from pre-diabetic NOD female mice were injected into age- and sex-matched NOD-scid recipients that had been reconstituted with either untreated bone marrow–derived NOD dendritic cells (from 5- to 8-week-old NOD females) or iDCs 3 days before T-cell addition, as illustrated in Fig. 1B. One week later, the spleens were harvested and CD3$^+$ T-cells were then enriched over columns. The T-cells were then stained with fluorescent antibodies to CD4 and CD25 as well as annexin V and propidium iodide. The cells were then subjected to FACS analysis as follows. First, annexin V and propidium iodide uptake levels were ascertained inside the CD4$^+$ CD25$^+$ gate. Each of the resulting quad-
FIG. 2. A: IL-7 promotes survival of CD4⁺ CD25⁺ NOD splenic T-cells in vitro. Increased number of CD4⁺ CD25⁺ T-cells is observed after a 24-h exposure to 50 ng/ml IL-7 in culture. Freshly isolated splenocytes from 5- to 8-week-old NOD mice were column enriched and cultured in the presence or absence of IL-7, with or without concanavalin A stimulation (5 μg/ml). Twenty-four hours later, the cells were stained with fluorescence-coupled antibodies to CD4 and CD25 and processed for FACS analysis. At the top of the panel, P1 shows the cells selected for FACS analysis based on forward and side scatter properties associated with T-cells. Each of the FACS plots represents CD4 and CD25 cells. These data are representative of four independently performed experiments.

B: IL-7 maintains CD4⁺ CD25⁺ T-cell numbers at 96 h after exposure in vitro. CD4⁺ CD25⁺ cell numbers observed at 24 h after IL-7 exposure were maintained at 96 h in vitro. Freshly isolated splenocytes from 5- to 8-week-old NOD mice were column enriched and cultured in the presence or absence of IL-7, with or without concanavalin A stimulation (5 μg/ml) for 5 days. FACS analysis for cell prevalence was performed identically as above. These data are representative of four independently performed experiments.
rants reflecting propidium iodide/annexin V was further analyzed for CFSE dilution. The degree of CFSE peak shift and the number of peaks within each of the propidium iodide/annexin V quadrants represents the degree of proliferation of CD4+ CD25+ T-cells in the different apoptotic states.

As a prelude to the experiments of which the data are shown in Fig. 1D and E, the general procedure followed to determine proliferation/expansion of putative Tregs or the survival of already-existing populations through ascertainment of apoptosis activation is illustrated in Fig. 1C. Starting from freshly isolated spleen, we first gated CD4+ CD25- T-cells, and we then probed the gate for either CFSE dilution and/or annexin V positivity. Figure 1C, as a general schema, depicts the CFSE content and the annexin V positivity of CD4+ CD25+ T-cells that were cultured in the absence of IL-7 or IL-2 for a 72-h period.

To establish potential mechanisms by which iDCs lead to an augmentation of CD4+ CD25+ putative Tregs, we concurrently determined the proliferation and the incidence of apoptosis of CD4+ CD25+ T-cells obtained from NOD-scid mice previously reconstituted with iDCs and NOD splenic T-cells, the latter cells prelabeled with CFSE. Specifically, we first labeled freshly isolated NOD splenic T-cells with CFSE and subsequently injected them into NOD-scid mice that had been administered either control syngeneic dendritic cells or iDCs. Five days later the spleens of the NOD-scid recipients were excised, and enriched T-cells were subjected to FACS analysis for CFSE dilution and annexin V positivity (Fig. 1D). We gated cells around four quadrants (P2–P5), each of which represents variable CFSE dilution and annexin V positivity (and therefore variable proliferation and apoptosis; Fig. 1D). In each of these quadrants, we then ascertained the prevalence of CD4 CD25 double-positive cells (Fig. 1E). Inside the double-positive cell populations, we ultimately determined CFSE dilution (Fig. 1E).

By comparing the CFSE dilution profiles in CD4+ CD25+ cells (Fig. 1E) gated inside annexin V+ CFSE+ double-positive populations in the annexin V/CFSE dot plot (Fig. 1D), we propose that the degree of CFSE dilution of CD4+ CD25+ cells from control and iDC NOD-scid recipients is essentially identical. These data suggest that the augmentation of CD4+ CD25+ T-cell numbers in the iDC NOD-scid recipients was not due to expansion/proliferation of existing or progenitor cells (Fig. 1E; compare the CFSE dilution histograms in the CD4+ CD25+ cells from each of the annexin V/CFSE quadrants (P2–P5; Fig. 1D).

**IL-7 increases the steady-state percentage of CD4+ CD25+ T-cells in vitro from the spleens of NOD mice.**

Given the importance of IL-7 in homeostasis of T-cells, specifically with respect to survival without the need for proliferation, we speculated that our findings shown in Fig. 1E might be attributable to iDC-induced homeostatic maintenance of existing CD4+ CD25+ putative Tregs, perhaps through IL-7. We initially postulated that CD4+ CD25+ T-cells would respond identically to the effect of IL-7 as unenriched T-cell populations as previously reported (28–30,33,37,40,59–61). Reombinant IL-7 (50 ng/ml) exogenously added to freshly isolated NOD splenic T-cells (derived from the spleens of female, pre-diabetic NOD mice at 5–8 weeks of age) resulted in a greater prevalence of CD4+ CD25+ T-cells at 24 h after IL-7 addition. (Fig. 2A). The addition of concanavalin A led to an even greater increase in the prevalence of CD4+ CD25+ T-cells in IL-7–pretreated cells at this time over cells not stimulated with concanavalin A. The increased prevalence of CD4+ CD25+ T-cells was maintained at even 96 h after IL-7 addition with or without concanavalin A stimulation at a time when primary T-cells are expected to poorly survive in culture without the addition of IL-2 (Fig. 2B) (31,37,41,60,62).

**Increased prevalence of IL-7–induced NOD CD4+ CD25+ T-cells in vitro is due to apoptosis suppression.**

The observation of a considerable number of CD4+ CD25+ T-cells at 96 h after culture in the presence of IL-7 with or without concanavalin A stimulation strongly suggested a selective effect of IL-7 on survival over proliferation. We chose to examine the state of apoptosis activation by ascertaining annexin V levels in vitro. Figure 3A confirms the well-known effect of IL-7 on the survival of freshly isolated, enriched yet unfractionated T-cells (31,37,41,60,62), this time derived from the NOD strain. Figure 3B shows dramatically decreased annexin V staining of CD4+ CD25+ T-cells in IL-7–treated, freshly isolated NOD T-cell cultures with or without concanavalin A stimulation. This degree of survival was maintained at 96 h after the initiation of T-cell culture.

**IL-7Rxα is expressed at higher levels on NOD CD4+ CD25+ T-cells than CD4+ CD25− T-cells in vitro.**

Because CD4+ CD25+ T-cells from NOD mice demonstrated significantly lower apoptosis activation as measured by annexin V staining in response to exogenous IL-7 addition, we proposed that these low-apoptotic cells would express more IL-7Rxα. In Fig. 4, by FACS analysis, we show an increased level of IL-7Rxα in the CD4+ CD25+ T-cell population in freshly isolated NOD splenic T-cells compared with that on CD4+ CD25− T-cells. Although concanavalin A treatment resulted in a decrease in the overall numbers of cells in both populations, the difference in the magnitude of IL-7Rxα expression between CD4+ CD25+ and CD4+ CD25− T-cells remained similar.

**IL-7Rxα is expressed at significantly higher levels on CD4+ CD25+ T-cells compared with CD4+ CD25− T-cells in vivo.**

To examine whether our in vitro observations on preferential expression of IL-7Rxα on CD4+ CD25+ T-cell populations compared with levels on CD4+ CD25− held true in vivo, we reconstituted female NOD-scid mice with bone marrow–derived NOD dendritic cells and purified T-cells by sequential transfer of the dendritic cells followed 3 days after by enriched T-cells freshly isolated from Thy1.1–NOD syngeneic mice. We chose to use the Thy1.1 marker to ensure that the T-cells phenotyped in downstream analyses would, in fact, derive from donors, even in T-cell–deficient NOD-scid recipients. Furthermore, the Thy1.1 marker would also aid in normalizing the data given the predicted homeostatic expansion expected in the NOD-scid lymphopenic host. Five days after T-cell transfer, the spleens of the NOD-scid recipients were processed to obtain T-cells. In Table 1, we show increased prevalence of IL-7Rxα Thy1.1+ cells in the CD4+ CD25+ population in NOD-scid hosts preconditioned with iDCs than in recipients pretreated with control dendritic cells. The prevalence of IL-7Rxα Thy1.1+ cells in the CD4+ CD25+ populations was very low or undetectable in iDC-preconditioned NOD-scid mice and control dendritic cell recipients, respectively (Table 1).

**NOD bone marrow–derived iDCs express IL-7 in vitro.**

Up to this point, our studies began suggesting to us a model whereby the levels of IL-7Ra, expressed at significantly higher levels on CD4+ CD25+ compared with
FIG. 3. A: Exogenous IL-7 addition to NOD T-cells reduces apoptosis activation in vitro. Significant decrease in the number of annexin V⁺ CD4⁺ CD25⁺ T-cells after IL-7 treatment of NOD T-cells reflects an effect of IL-7 on apoptosis suppression. Freshly isolated splenocytes from 5- to 8-week-old NOD mice were column enriched and cultured in the presence or absence of IL-7, with or without concanavalin A stimulation (5 μg/ml). Twenty-four hours later, the cells were stained with fluorescence-coupled antibodies to CD4 and CD25, stained for annexin V, and processed for FACS analysis. CD4⁺ CD25⁺ cells were gated and then examined for annexin V positivity. Each of the histograms indicates the level of apoptosis activation. These data are representative of four independently performed experiments. B: IL-7 maintains suppressed apoptosis activation of CD4⁺ CD25⁺ T-cell numbers at 96 h after exposure in vitro. The levels of annexin V in CD4⁺ CD25⁺ cells observed at 24 h after IL-7
CD4^+ CD25^- T-cells, were partly responsible for conferring to them a diabetes-delaying immunoregulatory phenotype in the NOD mouse. We further proposed that iDCs promoted the diabetes-delaying effects (25) by producing IL-7 in close proximity to, and affecting the activity of putative Tregs within, the IL-7R+ CD4^+ CD25^+ subpopulation. To address this possibility, we first looked at IL-7 production in NOD bone marrow-derived control, untreated dendritic cells and then compared it with production in iDCs. Consistent with our observations, others have previously detected IL-7 production in dendritic cells (Fig. 5A) (63–66). By real-time PCR, iDCs expressed significantly higher IL-7 steady-state mRNA than did control, untreated dendritic cells (1,800 ± 265 pmol IL-7 in iDCs vs. undetectable in control dendritic cells; n = 3, data extrapolated from C (cycle number where fluorescence crosses the threshold) values as described in RESEARCH DESIGN AND METHODS).

To verify that it is iDC-derived IL-7 that directly promotes augmented CD4^+ CD25^+ putative Treg numbers and is directly involved in suppressing apoptosis activation in these cells, we repeated the coculture experiments with iDCs and freshly isolated NOD splenic T-cells in the presence of blocking IL-7 or IL-7R antibodies. As an additional demonstration that the cells are part of the Treg family, after the coculture, we determined the percentage of CD25^+ CD62L^- cells inside a CD4^- gate (Fig. 5B) and the percentage of annexin V^+ cells inside a CD4^- CD25^- CD62L^+ population (Fig. 5C). The addition of blocking antibodies significantly decreased the prevalence of CD25^+ CD62L^- cells inside a CD4^- gate (Fig. 5B) and, moreover, blocking antibodies significantly increased apoptotic putative Tregs (Fig. 5C). Over a 96-h period, we observed a greater prevalence of dead or apoptotic cells in iDC:T-cell cocultures treated with the blocking IL-7 antibody (Fig. 5D).

These data support a causal role for iDC-elaborated IL-7 in exposure were maintained or significantly improved at 96 h in vitro. Freshly isolated splenocytes from 5- to 8-week-old NOD mice were column enriched and cultured in the presence or absence of IL-7, with or without concanavalin A stimulation (5 μg/ml) for 5 days. FACS analysis for annexin V prevalence in the CD4^- CD25^- cell population was performed identically as above. These data are representative of four independently performed experiments.
augmenting the prevalence of putative Tregs, at least in part through activation of antiapoptotic mechanisms in Tregs that express IL-7R. We also ascertained the cytokine production in these cocultures in the presence or absence of the blocking antibodies. With the exception of tumor necrosis factor-α and IL-6, whose levels were significantly increased in the cocultures with the blocking antibodies (Fig. 5E), the levels of all other cytokines detectable (interferon-γ, IL-2, IL-4, IL-10, transforming growth factor-β, and IL-12p70) were almost identical between control and iDC-seeded cocultures (data not shown).

**IL-7Rα is constitutively expressed on the surface of NOD dendritic cells and induced at higher levels on iDCs.** We have concurrently speculated that in addition to its effects on T-cell survival, IL-7 could also promote the survival of dendritic cells in an autocrine manner. In such a putative autocrine system, dendritic cells should express IL-7Rα. As shown in Fig. 6, not only was IL-7Rα expressed on control, untreated NOD dendritic cells, but it was expressed at higher levels on iDCs.

**DISCUSSION**

Our previous data indicated that iDC treatment of prediabetic NOD mice significantly delayed diabetes onset with a single injection (25). We now demonstrate that weekly injection of iDCs for a period of 1 month commencing on the 8th week of life completely prevents diabetes in the NOD mouse. The simplistic reason could be that increased injection frequency replenished the iDCs whose lifespan is expected to be limited in vivo after exogenous administration. We had also shown that iDCs promoted an increased prevalence of CD4+ CD25+ T-cells (25). In parallel, we have observed that adoptive cotransfer of T-cells from iDC-treated, diabetes-free NOD mice along with T-cells from diabetic NOD mice into NOD-scid recipients did not result in diabetes (J.H., N.G., unpublished observations). This strengthens the argument that iDCs induce a T-cell population, possibly the same CD4+ CD25+ T-cells whose prevalence was increased, which somehow mediates the diabetes-suppressive effect. The maintenance of Treg numbers, in our model, would be dependent on the presence and activity of iDCs. We initially envisioned two possible mechanisms that could result in increased prevalence of the CD4+ CD25+ T-cell population: proliferation of Tregs within the CD4+ CD25+ T-cell population and/or preferential survival of this cell subpopulation in vivo. From these studies, we suggest that iDCs may not affect proliferation of CD4+ CD25+ T-cells in vivo but are responsible for maintaining their numbers, which is very likely through apoptosis suppression as evidenced by a dramatic decrease in the levels of apoptosis activation measured in annexin V/propidium iodide positivity.

Exogenous IL-7 addition to NOD T-cells resulted in preferential suppression of apoptosis activation of CD4+ CD25+ cells over CD4+ CD25- T-cells. Given the potential for regulatory activity of the former population, we propose IL-7 as an additional "costimulatory" signal promoting the preferential survival especially of putative regulatory T-cells and envisage a "coregulatory" mechanism of iDC action on these specific cell subtypes. Because our iDCs express low levels of CD40, CD80, and CD86, T-cells with TCR:antigen specific for the MHC on the iDCs would have a higher activation threshold and would be susceptible to apoptosis. An additional signal would therefore have to be supplied to suppress apoptotic pathways and to permit TCR:MHC interaction time to be prolonged enough to permit adequate activation. We propose this signal to be IL-7, which would be expected to act on all T-cells with a functional IL-7 receptor coming into contact with the iDCs. Our data showing that blocking antibodies to IL-7 and IL-7Rα in iDC:T-cell cocultures increase the incidence of apoptotic CD4+ CD25+ cells and decrease their number are consistent with such a potential mechanism of action, although we do not exclude the existence of additional, complementary mechanisms that can underlie the iDC-dependent increase in Treg prevalence.

Although these data show that IL-7 is of relevance in an
C: Blocking antibodies to IL-7 or IL-7Ra increase the prevalence of apoptotic CD4⁺ CD25⁺ T-cells in iDC:T-cell co-cultures. Shown are the FACS data of annexin V⁺ cells inside a CD4⁺ CD25⁺ CD62L⁺ gate, where the cells derive from dendritic cell:T-cell co-cultures at the end of 5 days. These data are representative of three independent experiments, each performed in triplicate. The error bars represent the SE. *P < 0.05 between control co-cultures and those in which blocking antibodies were added (ANOVA).

D: Prevalence of apoptotic and dead CD4⁺ CD25⁺ CD62L⁺ T-cells derived from iDC:T-cell co-cultures increases over time in the presence of blocking IL-7 antibodies. T-cells from the co-cultures were first gated into CD4⁺ cells, and within this gate, CD25⁺ CD62L⁺ cells were then gated. Propidium iodide⁺ annexin V⁺ double-positive cells were determined inside this putative Treg population. Cells were removed from co-cultures at the end of 24, 72, and 96 h incubation. These data are representative of two independent experiments in triplicate, and the error bars represent the SE. *P < 0.001 between control co-cultures and those treated with the blocking IL-7 antibody, at all time points (ANOVA).

E: Blocking antibodies to IL-7 or IL-7Ra increases the production of tumor necrosis factor-α and IL-6 in iDC:T-cell co-cultures. Cytokine production was assessed in co-culture supernatant at the end of a 5-day incubation. These data are representative of three independent experiments, each performed in triplicate. The error bars represent the SE. *P < 0.05 between control co-cultures and those in which blocking antibodies were added (ANOVA).
NOD system, we have nevertheless confirmed that IL-7 acts identically on CD4⁺ CD25⁺ putative Tregs in the C57BL/6 and Balb/c mouse (J.H., J.M., N.G., unpublished observations). Therefore, we consider it unlikely that the effects of IL-7, exogenously supplied or produced by iDCs, in promoting the augmentation of CD4⁺ CD25⁺ Tregs are NOD specific. The action of IL-7 in regulating diabetes in the NOD system via Tregs has been suggested in previous studies (67,68). Herbelin et al. (68) identified a subset of CD4⁺ CD62L⁺ T-cells able to inhibit diabetes transfer into immunodeficient NOD-scid recipients. IL-7 exhibited potentiating activity on diabetes protection via these cells that were also generated among thymocytes in vitro with exogenous IL-7 addition (68). In a subsequent study, Gombert et al. (67) reported that exogenous addition of IL-7 restored immunosuppressive IL-4 production in NOD mature thymocytes and, furthermore, that IL-7 additionally increased IL-4 production by NK1⁺ NOD spleen T-cells stimulated by an antibody to the CD3 complex.

Our data also suggest a method that could be useful alone or in conjunction with other published methods by which CD4⁺ CD25⁺ T-cells with regulatory activity can be expanded in vitro (22,69–73). IL-7 could, therefore, be of practical utility in improving the yield, persistence in vivo, and activity of Tregs in suppressing autoimmune or in facilitating allograft survival. Although we are excited by these novel observations suggesting IL-7 as a potential regulator/facilitator of costimulation of Tregs through suppression of apoptosis, much more work needs to be done to understand the mechanism and to translate a method into a relevant therapeutic.

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Figure 6. Detectable cell surface IL-7Rα on NOD dendritic cells and increased level on iDCs. FACS analysis demonstrates detectable levels of IL-7Rα chain on the surface of bone marrow–derived dendritic cells from NOD mice (5- to 8-week-old females) and increased in iDCs. The cells were produced in vitro as described in RESEARCH DESIGN AND METHODS. They were then stained with a fluorescence-coupled antibody to the α-chain of IL-7R and processed for FACS. These data are representative of measurements made in three independently propagated dendritic cell cultures.


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