Original Article

CCL4 Protects From Type 1 Diabetes by Altering Islet β-Cell–Targeted Inflammatory Responses

Craig Meagher,[1,2] Guillermo Arreaza,[2] Andrew Peters,[3] Craig A. Strathdee,[4] Philippe A. Gilbert,[3] Qing-Sheng Mi,[3] Pere Santamaria,[5] Gregory A. Dekaban,[1,3] and Terry L. Delovitch[1,2]

We previously reported that interleukin (IL)-4 treatment of nonobese diabetic (NOD) mice elevates intrapancreatic CCL4 expression and protects from type 1 diabetes. Here, we show that antibody neutralization of CCL4 abrogates the ability of T-cells from IL-4–treated NOD mice to transfer protection against type 1 diabetes. Intradermal delivery of CCL4 via a plasmid vector stabilized by incorporation of the Epstein-Barr virus EBNA1/*oriP* episomal maintenance replicon (pHERO8100-CCL4) to NOD mice beginning at later stages of disease progression protects against type 1 diabetes. This protection was associated with a Th2-like response in the spleen and pancreas; decreased recruitment of activated $CD8^+$ T-cells to islets, accompanied by diminished CCR5 expression on $CD8^+$ T-cells; and regulatory T-cell activity in the draining pancreatic lymph nodes. Thus, inflammatory responses that target islet β-cells are suppressed by CCL4, which implicates the use of CCL4 therapeutically to prevent type 1 diabetes. *Diabetes* 56: 809–817, 2007

Development of type 1 diabetes depends upon the selective recruitment of pathogenic leukocytes to pancreatic islets by chemokines and their receptors (1–5). Previously, we demonstrated in nonobese diabetic (NOD) mice that treatment with interleukin (IL)-4 elevates intrapancreatic CCL4 levels that correlate with decreased intraislet CCR5 expression and protection from type 1 diabetes (4). Increased intrapancreatic CCL4 expression is associated with enhanced recruitment of insulin B-chain reactive regulatory $CD4^+$ T-cells to the pancreas and prevention of destructive insulitis upon oral insulin administration (6), and protection from type 1 diabetes by a neutralizing anti–IL-16 monoclonal antibody depends on CCL4 activity. These results suggest that enhanced CCL4 expression in secondary lymphoid organs and the pancreas blocks the development of type 1 diabetes.

In this study, we tested whether CCL4 protects against type 1 diabetes using a gene transfer approach. A plasmid vector stabilized by the Epstein-Barr virus EBNA1/*oriP* episomal maintenance replicon was utilized to sustain CCL4 expression in vivo. We demonstrate that intradermal gene transfer of CCL4 prevents type 1 diabetes and that antibody-mediated CCL4 blockade reverses the ability of T-cells from IL-4–treated NOD mice to transfer protection against type 1 diabetes. This is the first demonstration that IL-4–mediated protection against type 1 diabetes relies upon CCL4 activity in vivo and that CCL4 inhibits progression to type 1 diabetes.

RESEARCH DESIGN AND METHODS

NOD/Del, NOD.*Scid* (from Dr. Len Schultz; The Jackson Laboratories, Bar Harbor, ME) and NOD8.3 TCR transgenic mice were bred in a specific pathogen-free barrier facility at the Robarts Research Institute (7,8). Mice were maintained in a specific pathogen-free facility at the University of Western Ontario according to institutional guidelines.

IL-4 treatment, cell transfer, and CCL4 neutralization. One hundred nanograms (1,000 units) of recombinant mIL-4 (Immunex, Seattle, WA) in 0.1 ml of vehicle (PBS plus 1% serum from NOD.*Scid* mice) or vehicle was administered intraperitoneally three times per week to NOD mice from 3 to 12 weeks of age (9). Spleen T-cells (5×10^6) were purified on T-cell columns (R&D Systems, Minneapolis, MN) and transferred intraperitoneally into NOD.*Scid* recipients. NOD.*Scid* mice (n = 9–10/group) receiving spleen T-cells from IL-4–treated NOD mice were treated intraperitoneally three times per week with 100 μg of either a neutralizing polyclonal anti-CCL4 antibody (R&D Systems) or a control goat IgG (Jackson ImmunoResearch, West Grove, PA). Recipients of spleen T-cells from vehicle-treated control mice were treated with either anti-CCL4 or control goat IgG. Blood glucose levels were measured biweekly, and two consecutive blood glucose level readings of >11.1 mmol/l were indicative of diabetes onset.

Expression constructs. Trizol (Invitrogen, Burlington, ON, Canada) was used to isolate spleen RNA from 10- to 12-week-old NOD mice. After treatment with RNase-free DNase (Invitrogen), full-length mCCL4 cDNA was generated using Superscript II and oligo(dT) primers (Invitrogen). cDNA encoding murine CCL4 was amplified using Expand High Fidelity *Taq* Polymerase (Roche, Laval, QC, Canada) in combination with the sense (5′-TGAAGCTTCT GGGCCCTGCA-3′) and antisense (5′-TCAGTGAGAAGCATCAGGGCT-3′) primers. CCL4 cDNA was subcloned into the *Eco*RI site of pHERO8100 to generate pHERO8100-CCL4. Plasmids were propagated in *Escherichia coli* DH5α (Invitrogen) and purified free of contaminating bacterial endotoxin using the Endo-Free Maxi kit (Qiagen, Mississauga, ON, Canada).

CCL4 expression. To assay in vitro expression, COS-7 cells (10^6) were transfected in triplicate with pHERO8100 or pHERO8100-CCL4 prepared in Lipofectamine Plus (Invitrogen). At 48 h posttransfection, CCL4 levels in culture supernatants were detected by enzyme-linked immunosorbent assay

From the [1]Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada; the [2]Laboratory of Autoimmune Diabetes, Robarts Research Institute, London, Ontario, Canada; the [3]Bio-Therapeutics Research Group, Robarts Research Institute, London, Ontario, Canada; the [4]Amgen, Seattle, Washington; and the [5]Julia McFarlane Diabetes Research Centre, University of Calgary, Calgary, Alberta, Canada.

Address correspondence and reprint requests to Dr. Terry L. Delovitch, Laboratory of Autoimmune Diabetes, Robarts Research Institute, 100 Perth Dr., London, Ontario, N6A 5K8, Canada. E-mail: del@robarts.ca.

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C.M. is currently affiliated with the Diabetes Research Center, University of California San Francisco, San Francisco, California.. Q.-S.M. is currently affiliated with the Center for Biotechnology and Genomic Medicine, Departments of Pathology and Medicine, Medical College of Georgia, Augusta, Georgia.

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ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IGLN, inguinal lymph node; IL, interleukin; IFN, interferon; PLN, pancreatic draining lymph node; Treg, regulatory T.

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(ELISA) using a mouse DuoSet kit (R&D Systems). The internal luciferase expression vector pGL3-control (Promega, Madison, WI) was used to correct for differences in transfection efficiency between samples. To assay in vivo expression, CCL4 in serum samples was quantitated using the CCL4 Quantikine ELISA kit (R&D Systems). For each treatment group, six or more serum samples were collected per time point.

Intradermal gene delivery. Plasmid DNA (50 μg) was precipitated onto 25 mg of 1.6 μm gold particles (Bio-Rad, Mississauga, ON, Canada) to generate plasmid DNA/gold-coated cartridges (1 μg of DNA/cartridge). Intradermal gene inoculations to the abdomen were performed at 400 psi of helium (10). NOD mice were inoculated weekly with 1 μg of either pHERO8100 or pHERO8100-CCL4 from 3 to 14, 3 to 7, or 9 to 14 weeks of age. NOD8.3 TCR transgenic mice were similarly treated from 3 weeks of age until either diabetes onset or termination of the experiment. Each treatment protocol was repeated at least twice.

Histopathology. Formalin-fixed and paraffin-embedded pancreata were sectioned at 6-μm intervals. For each mouse, ≥10 nonadjacent sections and islets were scored for the incidence and severity of insulitis. Mononuclear cell infiltration was scored blindly by two observers, and the insulitis index was calculated (11). Insulin immunostaining was performed with a polyclonal rabbit anti-insulin (Santa Cruz Biotechnologies, Santa Cruz, CA) antibody and detected with a horseradish peroxidase goat anti-rabbit antibody conjugate (Santa Cruz Biotechnologies).

T-cell activation and cytokine/chemokine secretion. Spleen- and draining pancreatic lymph node (PLN)-derived T-cells were isolated on T-cell columns (R&D Systems) to a purity of >93%, as assessed by fluorescence-activated cellk sorter analysis of CD3 cell surface expression. Cells were cultured (10^6/ml) in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 10 mmol/l HEPES buffer, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 mmol/l 2-mercaptoethanol (all from Invitrogen) at 37°C and stimulated with plate-bound 145-2C11 anti-CD3ε (2 μg/ml; Cedarlane Laboratories, Hornby, ON, Canada). Culture supernatants collected after 48 h were assayed for T-cell cytokine and chemokine production by ELISA (4,9).

Intrapancreatic chemokine analysis. Intrapancreatic levels of CCL3 and CCL4 were quantitated as described (4).

Isolation of islets and islet-infiltrating lymphocytes. Islet-infiltrating cells were isolated from six 14- to 15-week-old NOD mice treated with 1 μg/week of pHERO8100 or pHERO8100-CCL4 from 3 to 14 weeks of age, as described (12). After characterization flow cytometrically by gating on forward and side scatter, the lymphocytes were cultured (10^6/ml) for 48 h in complete RPMI-1640 medium (Invitrogen) during activation by plate-bound anti-CD3ε. The concentrations of secreted cytokines and chemokines were assayed by ELISA (4,9).

Adoptive cell transfer and cotransfer. Spleen T-cells from NOD mice treated once weekly from 3 to 14 weeks of age with 1 μg of pHERO8100 or pHERO8100-CCL4 were transferred intraperitoneally (4×10^6/mouse) into NOD.*Scid* mice (5–7 week old). For cotransfers, NOD.*Scid* mice were transferred intraperitoneally with diabetogenic spleen T-cells (4×10^6/mouse) from recent-onset diabetic NOD mice and either spleen- (2×10^6/mouse) or PLN-derived T-cells (1.8×10^6/mouse) from 20-week-old NOD mice. The latter mice were previously treated once weekly with 1 μg of pHERO8100 or pHERO8100-CCL4 from 3 to 14 weeks of age. Mice were monitored twice weekly for the onset of type 1 diabetes.

Cell purification and fluorescence-activated cell sorter analysis. Spleen-, PLN-, and inguinal lymph node (IGLN)-derived T-cells were enriched (>94%) on T-cell columns (R&D Systems). Islet-infiltrating lymphocytes were purified as above. Cells were incubated (4°C, 20 min) with 2.4G2 anti-FcγII/IIIR monoclonal antibody and stained (4°C, 40 min) with fluorescein isothiocyanate (FITC)-anti-mouse CD3ε (145–2C11), FITC–anti-mouse CD8α (53–7.3), allophycocyanin–anti-mouse CD8α (53–7.3), PerCP–anti-mouse CD8α (53–7.3), FITC–anti-mouse CD4 (GK1.5), PerCP–anti-mouse CD4 (GK1.5), FITC–anti-mouse B220 (RA3–6B2), FITC–anti-mouse CD25 (7D4), phycoerythrin–anti-mouse CCR5 (C34–3448), and phycoerythrin–anti-CD69 (H1.2F3). All reagents were purchased from BD Biosciences and eBioscience. Analyses were performed using a FACSCalibur, and results were analyzed with CellQuest (BD Biosciences) and FlowJo (Treestar) software.

Statistical analysis. Data from one of three representative experiments are shown in each figure. The Mantel-Haenszel log-rank test was used to compare the development of type 1 diabetes between treatment groups, the Wilcoxon's signed-rank test to compare times of disease onset, the unpaired Student's *t* test to compare frequencies of cell populations determined by flow cytometry, and the Mann-Whitney nonparametric test for all additional statistical comparisons unless noted. $P \leq 0.05$ was considered significant.

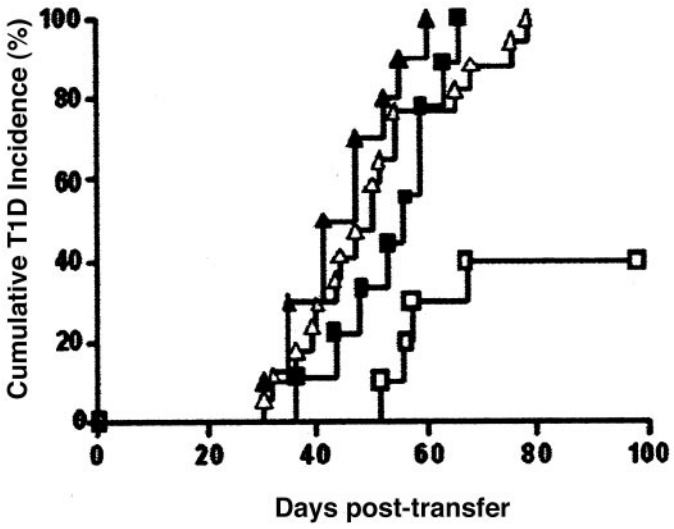

FIG. 1. CCL4 mediates IL-4–induced protection from type 1 diabetes (T1D). Spleen T-cells (5×10^6) from female NOD mice treated three times per week from 3 to 12 weeks of age with IL-4 or vehicle were transferred intraperitoneally into NOD.*Scid* recipients. Recipients of T-cells from IL-4–treated donor mice were divided into two groups. Group 1 ($n = 10$) was administered 100 μg of control goat IgG three times per week and group 2 ($n = 9$) was treated with 100 μg of goat anti-mouse CCL4 polyclonal antibody three times per week until the experiment was terminated. Recipients of T-cells from vehicle-treated donor were also divided into goat IgG ($n = 17$) and goat anti-mouse CCL4 ($n = 10$) treatment groups. □, IL-4/IgG ($n = 10$); ■, IL-4/anti-CCL4 ($n = 9$); △, vehicle/IgG ($n = 17$); ▲, vehicle/anti-CCL4 ($n = 10$).

RESULTS

CCL4 mediates IL-4–induced protection from type 1 diabetes. IL-4–induced protection of NOD mice from type 1 diabetes is associated with increased CCL4 production by spleen and islet-infiltrating T-cells (4,13). To determine the significance of CCL4 in this protection, we analyzed whether CCL4 diminishes the capacity of spleen T-cells from IL-4–treated NOD mice to transfer type 1 diabetes into NOD.*Scid* mice. Compared with spleen T-cells from vehicle-treated control mice, transferred spleen T-cells from IL-4–treated NOD mice reduced the incidence ($P = 0.0003$) and delayed the time of onset of type 1 diabetes (Fig. 1). All recipients of T-cells from vehicle-treated mice administered goat IgG (vehicle/IgG) developed type 1 diabetes by 78 days posttransfer, while only 4 of 10 recipients of T-cells from IL-4–treated donors subsequently treated with goat IgG (IL-4/IgG) developed type 1 diabetes at 98 days posttransfer. The ability of T-cells from IL-4–treated donors to delay and reduce the incidence of type 1 diabetes was eliminated ($P = 0.004$) upon neutralization of CCL4 activity by an anti-CCL4 antibody from the time of transfer (IL-4/anti-CCL4), as all nine recipient mice developed type 1 diabetes by 66 days posttransfer. Thus, while spleen T-cells from IL-4–treated mice are less pathogenic than those from vehicle-treated mice, CCL4 blockade enabled the T-cells from IL-4–treated donors to regain their pathogenic competence. Moreover, although CCL4 neutralization in recipient mice did not affect the ability of spleen T-cells from vehicle-treated donor mice to transfer type 1 diabetes (vehicle/anti-CCL4), CCL4 neutralization accelerated type 1 diabetes development noted by the onset at day 60 posttransfer in vehicle/anti-CCL4–treated mice and at day 78 posttransfer in vehicle/IgG-treated mice. Therefore, CCL4 may be an important mediator of IL-4–induced protection from type 1 diabetes.

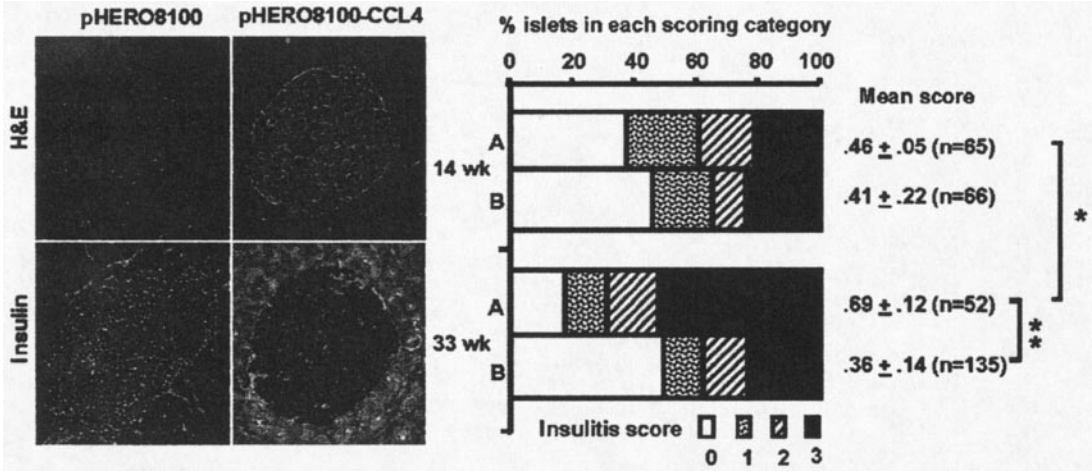

FIG. 2. Gene transfer of pHERO8100-CCL4 prevents destructive insulitis. Mice were treated weekly from 3 to 14 weeks of age with 1 μg of pHERO8100 (A) or pHERO8100-CCL4 (B) DNA before harvesting pancreata at the indicated ages. Numbers of islets analyzed are in brackets, bar graphs represent the percent of islets in each scoring category, and the mean insulitis index ± SD is shown for each treatment group (*$P < 0.03$; **$P < 0.03$). A representative islet analyzed at 33 weeks of age for each treatment group is presented in the *left panels*.

Intradermal CCL4 gene transfer sustains CCL4 expression in vivo. To investigate the protective capacity of CCL4 against type 1 diabetes, we developed a protocol similar to that used for the intradermal gene transfer of IL-4 to NOD mice (10). The pHERO8100 vector constructed was modeled after our previously described pHERO EBNA1/*oriP*-based expression vector, except that a ubiquitin C promoter that drives expression of a green fluorescent protein–EBNA fusion protein (NH_2-terminus is green fluorescent protein, C-terminus is EBNA1) in the nucleus was used (10,14). Transfection of COS-7 cells with pHERO8100-CCL4 yielded a CCL4 concentration of 11 ± 0.6 ng/ml in culture supernatants, whereas only background CCL4 expression was obtained upon transfection with the pHERO8100 control vector (online appendix Fig. 1*A* [available at http://dx.doi.org/10.2337/db06-0619). To analyze CCL4 expression in vivo, pHERO8100-CCL4 or pHERO8100 (1 μg) was administered intradermally to 3-week-old NOD mice to avoid the elevated serum CCL4 levels that may be associated with early insulitis at 4–6 weeks of age (15). Significantly elevated CCL4 levels persisted in the serum of pHERO8100-CCL4–treated mice until 7 days posttreatment (online appendix Fig. 1*B*), indicating that intradermal administration of pHERO8100-CCL4 sustains the expression of CCL4 in vivo.

Intradermal CCL4 gene transfer prevents destructive insulitis and type 1 diabetes. We investigated whether pHERO8100-CCL4 prevents destructive insulitis and type 1 diabetes. NOD mice were treated weekly with 1 μg of pHERO8100 or pHERO8100-CCL4 from 3 to 14 weeks of age (i.e., until the onset of destructive insulitis [15]). In 14-week-old mice, a similar proportion of islets exhibited noninvasive insulitis (scores of 0 or 1) after pHERO8100-CCL4 or pHERO8100 treatment (Fig. 2). From 14 to 33 weeks of age, insulitis was more severe in pHERO8100-treated but not pHERO8100-CCL4–treated mice ($P < 0.03$). At 33 weeks of age, insulitis was less severe in pHERO8100-CCL4–treated mice ($P < 0.03$), consistent with the observed decrease in destructive insulitis and preservation of insulin expression in the majority of islets in these mice. Furthermore, pHERO8100-CCL4 reduced the incidence of type 1 diabetes ($P = 0.012$) from 72% in pHERO8100-treated to 27% in pHERO8100-CCL4–treated mice at 35 weeks of age (Fig. 3*A*). Thus, administration of CCL4 to NOD mice via our gene transfer approach prevents type 1 diabetes.

Since invasive insulitis and type 1 diabetes in NOD mice are associated with diminished intrapancreatic CCL4 levels at 10 weeks of age (4), we examined whether CCL4 administration at an earlier (3–7 weeks) or later (9–14 weeks) stage of insulitis protects against type 1 diabetes. Relative to pHERO8100 control-treated mice, treatment with pHERO8100-CCL4 from 9 to 14 weeks of age ($P < 0.05$; Fig. 3*B*), but not from 3 to 7 weeks of age ($P = 0.662$; Fig. 3*C*), effectively protected against type 1 diabetes. Note that pHERO8100-CCL4 treatment from 3 to 14 (Fig. 3*A*) or 9 to 14 (Fig. 3*B*) weeks of age was equally effective in preventing type 1 diabetes. Hence, intradermal delivery of CCL4 to NOD mice at later stages of disease progression affords optimal protection against type 1 diabetes.

Transfer of diabetes by spleen T-cells from pHERO8100-CCL4–treated mice. Cell transfers were performed to determine whether CCL4-induced protection against type 1 diabetes results from a decreased diabetogenic potential of T-cells in pHERO8100-CCL4–treated mice. Transfer of type 1 diabetes was significantly prevented ($P = 0.002$) in NOD.*Scid* recipients of spleen T-cells from 14-week-old pHERO8100-CCL4–treated NOD mice (Fig. 4). Whereas 100% of NOD.*Scid* recipients of spleen T-cells from control-treated mice developed type 1 diabetes by day 77 posttransfer, only 36.4% (4 of 11) of recipients of spleen T-cells from pHERO8100-CCL4–treated mice developed type 1 diabetes by day 90 posttransfer. Thus, the diabetogenic potential of spleen T-cells is significantly diminished in pHERO8100-CCL4–treated mice.

CCL4 gene transfer elicits Th2-type immune responses. As IL-4–induced protection from type 1 diabetes in NOD mice is associated with elevated T-cell production of CCL4 and a Th2-type response (9), we examined if such a response is generated in pHERO8100-CCL4–treated mice. NOD mice were administered pHERO8100 or pHERO8100-CCL4 from 3 to 14 weeks of age, and 1 week later levels of spleen- and islet-derived T-cell cytokine secretion were assayed. Both spleen- (Fig. 5*A*) and islet- (Fig. 5*B*) derived T-cells from pHERO8100-CCL4–treated mice produced less interferon (IFN)-γ and more IL-4 and IL-10 than T-cells

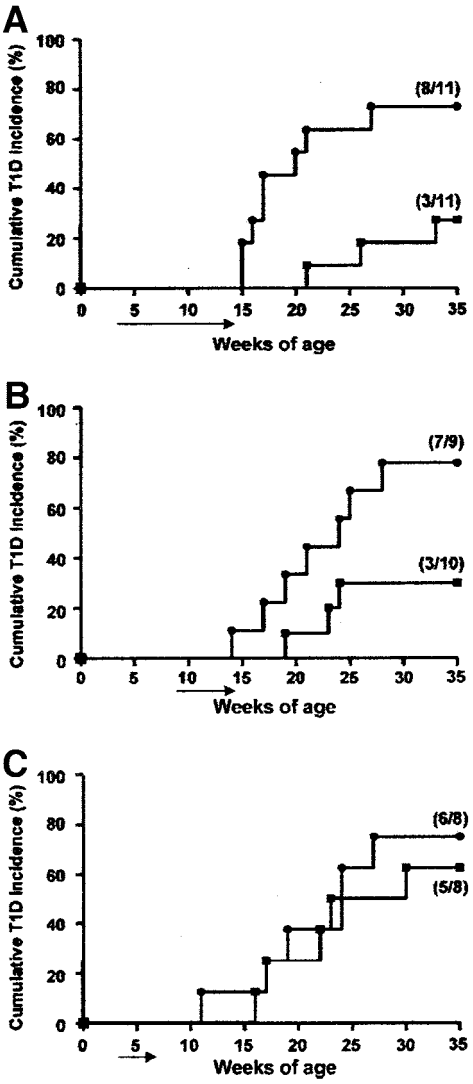

FIG. 3. Protection against type 1 diabetes is conferred by pHERO8100-CCL4 treatment in a time-dependent manner. NOD mice treated weekly with pHERO8100-CCL4 from either 3–14 (*A*) ($P = 0.012$) or 9–14 (*B*) ($P = 0.042$) weeks of age, but not from 3 to 7 weeks of age (*C*) ($P = 0.66$), were protected against type 1 diabetes compared with pHERO8100 control-treated mice. Arrows indicate the time of treatment, and the fraction of mice protected in each treatment group is shown in brackets. ●, pHERO8100; ■, pHERO8100-CCL4.

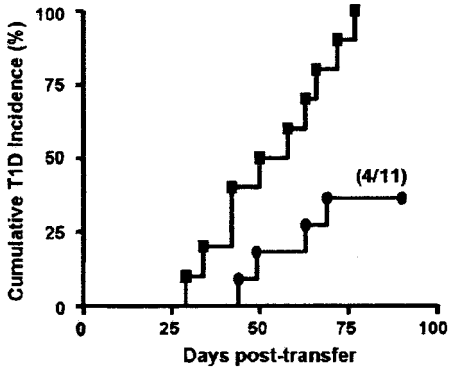

FIG. 4. Diabetogenic potential of spleen T-cells is decreased in pHERO8100-CCL4–treated mice. NOD mice were treated with pHERO8100-CCL4 or pHERO8100 from 3 to 14 weeks of age, after which their spleen T-cells were pooled and transferred intraperitoneally to NOD.*Scid* recipients (4×10^6 cells/mouse). Recipient mice were monitored for their incidence of type 1 diabetes. ■, pHERO8100 ($n = 10$); ○, pHERO8100-CCL4 ($n = 11$).

from control-treated mice. The cytokine ratios in Fig. 5*B* were calculated to account for the likelihood that different numbers of T-cells were stimulated for each treatment group, as islet-infiltrating cells represented a mixed cell population. Interestingly, production of CCL4 but not CCL3 by activated PLN-derived T-cells was elevated in pHERO8100-CCL4–treated mice (Fig. 5*C*). Thus, intradermal delivery of pHERO8100-CCL4 diminishes Th1-type and elevates Th2-type cytokine responses in the spleen and pancreas.

pHERO8100-CCL4 gene delivery is associated with decreased CCR5 expression on $CD8^+$ T-cells and reduced $CD8^+$ T-cell recruitment to islets. Expression of CCR5 (CCL4 receptor) is associated with Th1-type immune responses and the development of destructive insulitis (16,17). Since CCR5 internalization is enhanced upon interaction with its ligands (18), we investigated whether CCL4 influences CCR5 expression on peripheral and islet-infiltrated T-cells after treatment with pHERO8100-CCL4. Compared with control-treated mice, the proportion of $CD8^+CCR5^+$ T-cells in the spleen, PLN, and islets was significantly decreased in pHERO8100-CCL4–treated mice at 14 weeks of age (Fig. 6*A* and *B*). In contrast, the proportion of $CD4^+CCR5^+$ T-cells was similar in these tissues and the IGLN of both treatment groups (Fig. 6*B*). As CCL4 stimulates migration of $CCR5^+$ T-cells, this result may reflect increased $CD8^+CCR5^+$ T-cell migration away from the spleen, PLN, and islets toward the site of gene delivery. However, since similar numbers of IGLN-derived T-cells were isolated from both treatment groups at 14 weeks of age (C.M., unpublished observations), and the proportion of $CD8^+CCR5^+$ T-cells was similar in the IGLN of both treatment groups (Fig. 6*A* and *B*), this possibility is unlikely.

Alternatively, given that pHERO8100-CCL4 treatment enhances CCL4 levels in vivo, this treatment may selectively downregulate CCR5 expression on $CD8^+$ T-cells in the spleen, PLN, and islets. As CCR5 is a marker of activated $CD8^+$ T-cells and may control development of invasive insulitis in NOD mice (17), our results predict that pHERO8100-CCL4 inhibits the recruitment of activated $CD8^+$ T-cells to the pancreas by downregulation of CCR5 expression. In support of this idea, the frequency of $CD8^+CD69^+$, but not $CD4^+CD69^+$, T-cells in the PLN and islets was significantly decreased at 14 weeks of age in pHERO8100-CCL4–treated mice (Fig. 6*C*). To further examine if CCL4 inhibits $CD8^+$ T-cell recruitment to the pancreas and/or the activation of $CD8^+$ T-cells in the pancreas, we treated NOD8.3 transgenic mice weekly with pHERO8100 or pHERO8100-CCL4 beginning at 3 weeks of age (Fig. 6*D*). Although pHERO8100-CCL4 treatment significantly delayed ($P = 0.031$) onset of type 1 diabetes in these mice, protection from type 1 diabetes was not observed. Thus, pHERO8100-CCL4 treatment delays $CD8^+$ T-cell migration to islets but does not block $CD8^+$ T-cell effector functions that mediate islet β-cell destruction.

Regulatory T activity is enhanced in the PLN of pHERO8100-CCL4–treated mice. As pHERO8100-CCL4–treated mice remain protected from type 1 diabetes for 21 weeks after treatment ceases, we analyzed whether T-cells from such mice function as regulatory T (Treg) cells to maintain protection. NOD mice treated from 3 to 14 weeks of age with pHERO8100 or pHERO8100-CCL4

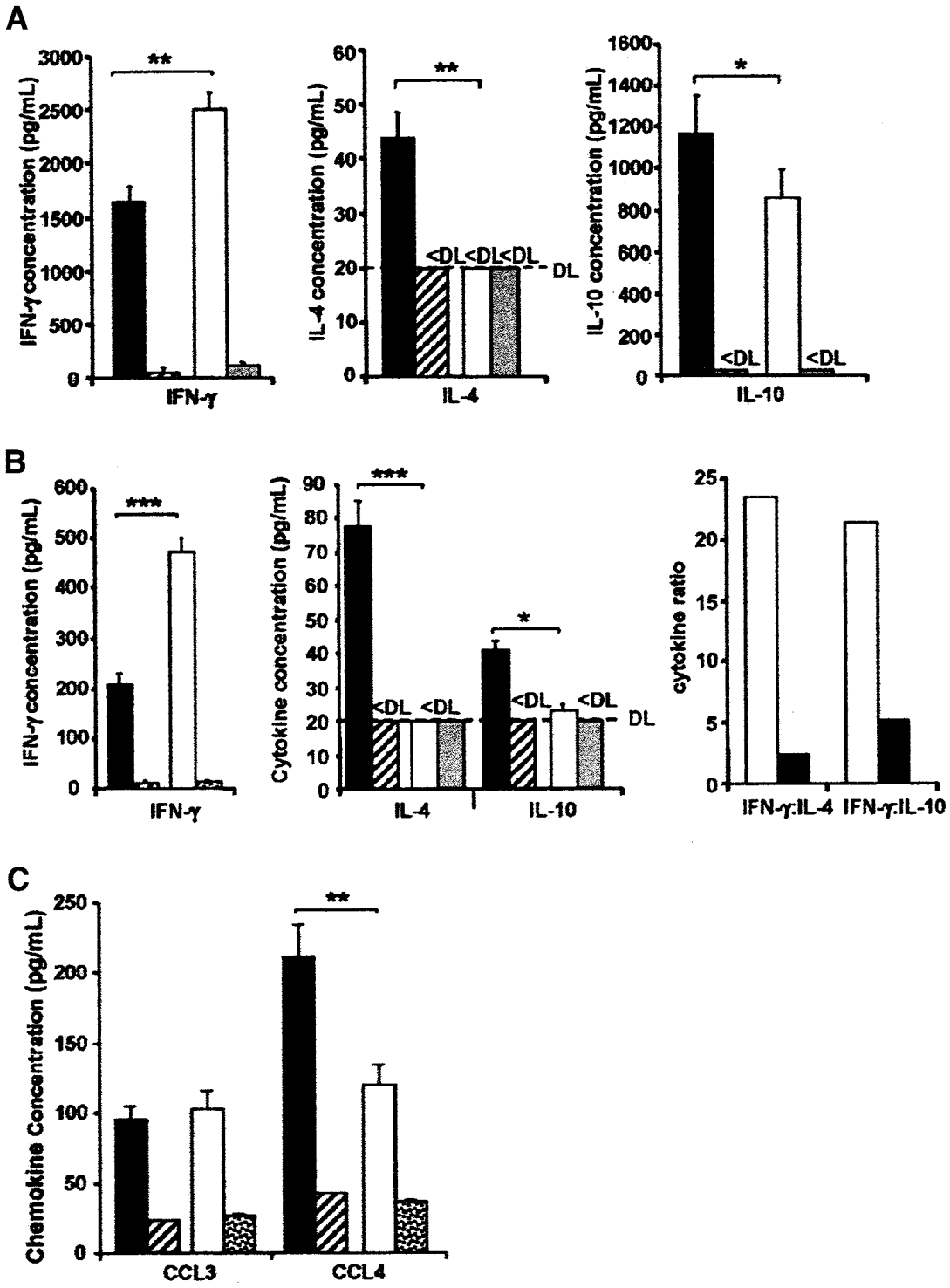

FIG. 5. Intradermal CCL4 gene delivery enhances a Th2-type cytokine response. Spleen T-cells (*A*), islet-derived lymphocytes (*B*), and PLN-derived T-cells (*C*) pooled from 4 to 5 mice treated from 3 to 14 weeks of age with pHERO8100-CCL4 or pHERO-8100 were or were not activated with plate-bound anti-CD3ε for 48 h. Cytokine (*A* and *B*) and chemokine (*C*) accumulation in supernatants were determined by ELISA. Data shown are mean values ± SD of quadruplicate determinations from three experiments (*$P < 0.05$; **$P < 0.03$; ***$P < 0.001$). DL, detection limit. ■, pHERO8100-CCL4/α-CD3; ▨, pHERO8100-CCL4/unstimulated; □, pHERO8100/α-CD3; ▩, pHERO8100/unstimulated.

were aged until 20 weeks of age, at which time NOD.*Scid* mice were injected with spleen T-cells from newly diabetic NOD mice alone or together with spleen- or PLN-derived T-cells from pHERO8100-CCL4– or control-treated mice. All recipient mice, having received diabetogenic T-cells, developed type 1 diabetes by 10 weeks posttransfer, and cotransfer with spleen-derived T-cells from either treatment group did not prevent type 1 diabetes (Fig. 7*A*). In contrast, co-transfer with PLN-derived T-cells from pHERO8100-CCL4– but not pHERO8100-treated mice significantly protected ($P = 0.013$) recipient mice against type 1 diabetes (Fig. 7*B*). Hence, Treg cells are localized to the PLN in pHERO8100-CCL4–treated mice and retain the ability to transfer protection from type 1 diabetes for at

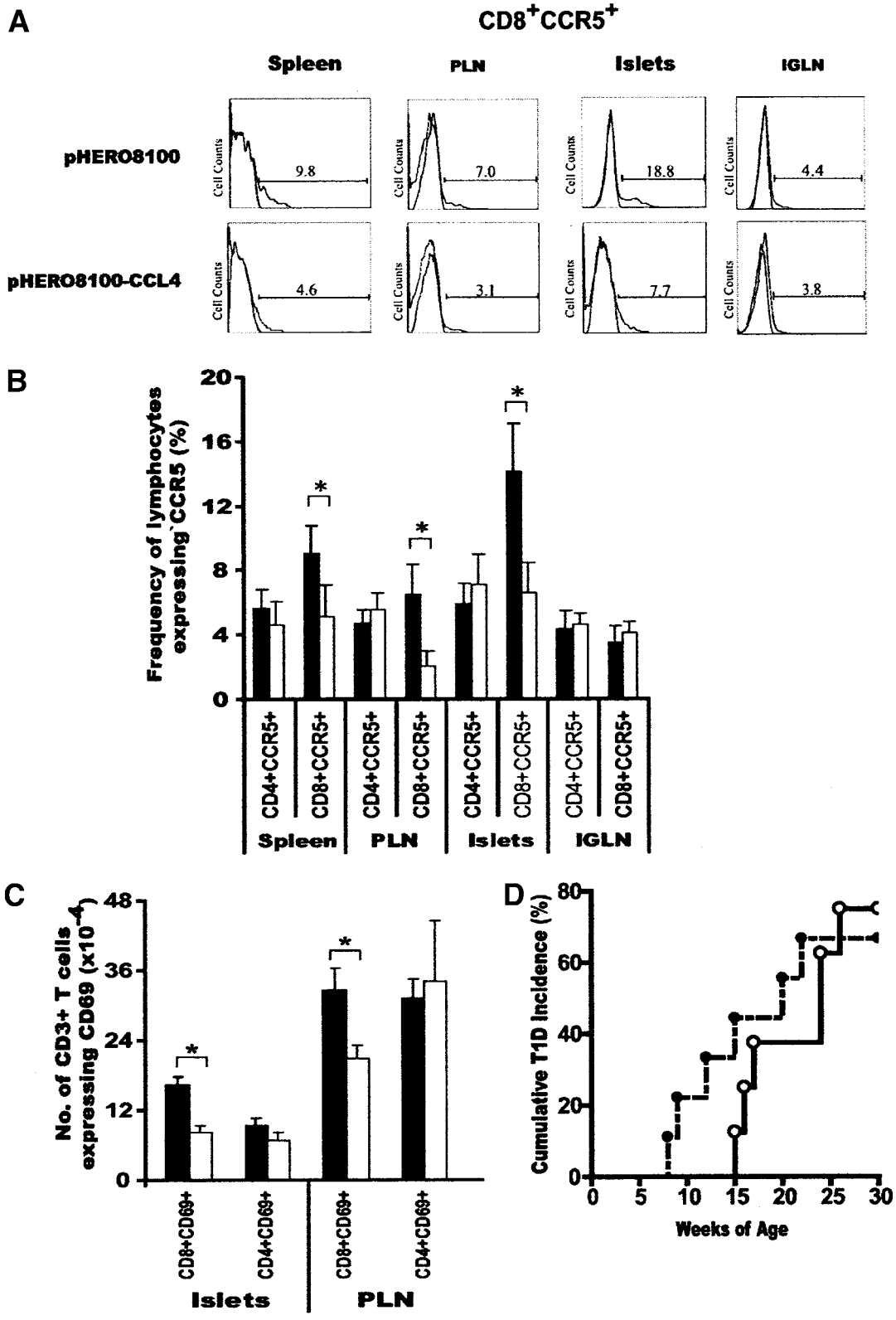

FIG. 6. Treatment with pHERO8100-CCL4 decreases CCR5 expression on CD8$^+$ T-cells and their activation in the pancreas. Spleen-, PLN-, and IGLN-derived T-cells were isolated from four mice per group per experiment. Islet-infiltrating lymphocytes were isolated from four groups of six mice per treatment group. The frequency of T-cell subpopulations expressing CCR5 and CD69 was determined by flow cytometry. *A*: Representative CCR5 profile for CD8$^+$ T-cells. *B*: Mean frequency of CCR5$^+$ lymphocytes ± SD (*$P < 0.05$) in each tissue. ■, pHERO8100; □, pHERO8100-CCl4. *C*: Percent of activated CD69$^+$ T-cells in the PLN and islets (*$P < 0.03$). ■, pHERO8100; □, pHERO8100-CCL4. *D*: Treatment of female NOD8.3 transgenic mice with pHERO8100-CCL4 delays ($P = 0.03$) the onset but does reduce the incidence of type 1 diabetes versus control-treated mice. ○, pHERO8100-CCL4 ($n = 8$); ○, pHERO8100 ($n = 9$).

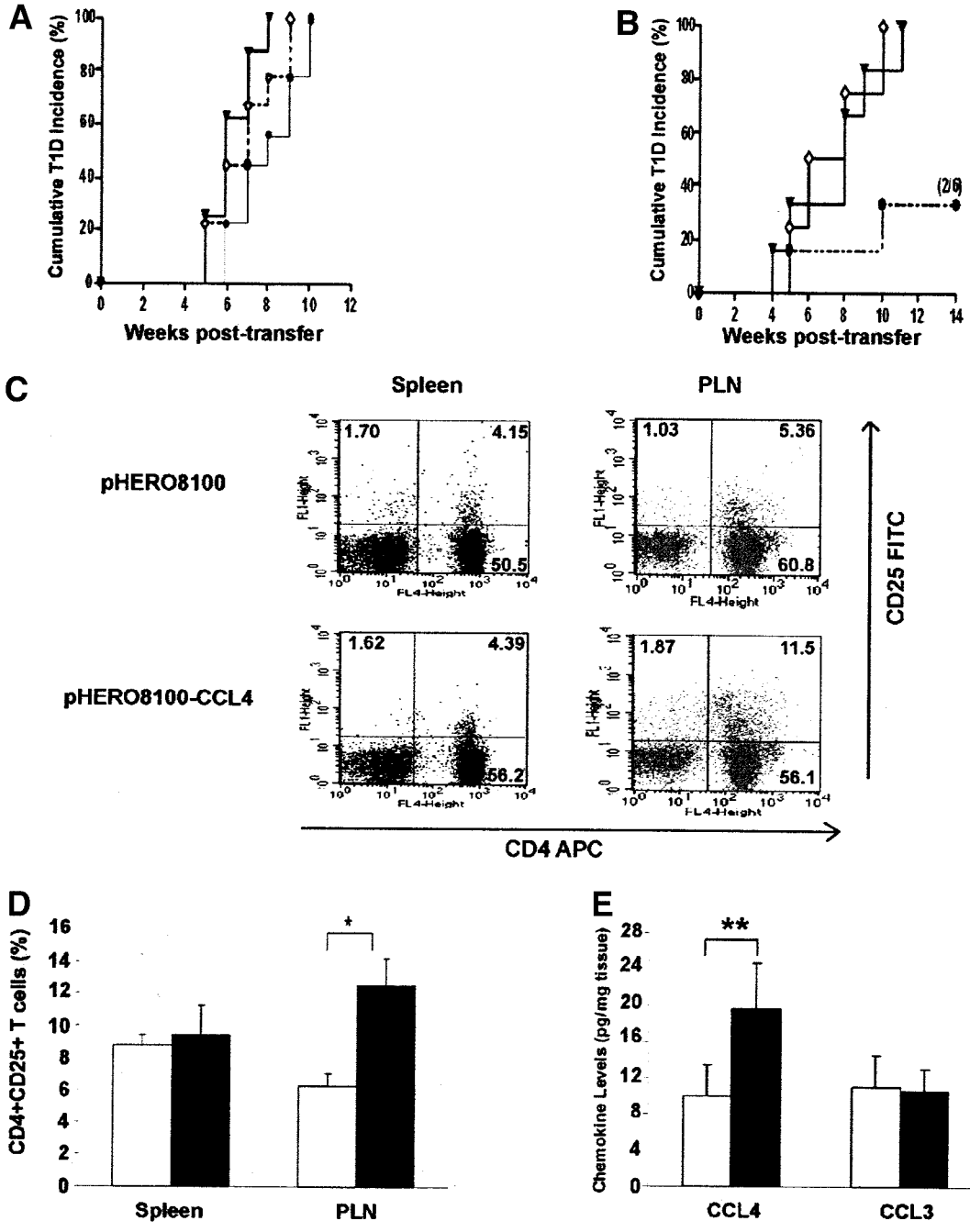

FIG. 7. Treg activity is increased in the PLN of pHERO8100-CCL4–treated mice. At 20 weeks of age, spleen-derived (4×10^6) (*A*) and PLN-derived (1.8×10^6) (*B*) T-cells from pHERO8100-CCL4– or pHERO8100-treated mice (3–14 weeks of age) were cotransferred intraperitoneally with spleen T-cells (4×10^6) from diabetic (Diab) NOD mice into NOD.*Scid* recipients. *A*: ◇, Diab T-cell ($n = 8$); ▲, Diab T-cell plus pHERO8100 ($n = 8$); ■, Diab T-cell plus pHERO8100-CCL4 ($n = 9$). *B*: ◇, Diab T-cell ($n = 8$); ▲, Diab T-cell plus pHERO8100 ($n = 6$); ■, Diab T-cell plus pHERO8100-CCL4 ($n = 6$). *C and D*: An increased frequency of $CD4^+CD25^+$ T-cells is present in PLN-derived cells from 20-week-old pHERO8100-CCL4–treated mice as determined by flow cytometry. Results from one of three reproducible experiments are shown in (*C*), and results from these three experiments are compared in (*D*) (*$P < 0.03$). □, pHERO8100; ■, pHERO8100-CCL4. *E*: Intrapancreatic levels of CCL4, but not CCL3, are elevated in pHERO8100-CCL4–treated mice (four pancreata per treatment group) at 20 weeks of age as determined by ELISA. Data represent the means ± SD ($P < 0.02$) from three experiments. □, pHERO8100; ■, pHERO8100-CCL4.**

least 6 weeks posttreatment. In addition, at 20 weeks of age, the frequency of $CD4^+CD25^+$ T-cells was selectively increased more than twofold ($P < 0.03$) in the PLN but neither in the spleen nor IGLN (data not shown) of pHERO8100-CCL4–treated NOD mice (Fig. 7*C* and *D*).

Treg activity in the PLN is associated with elevated intrapancreatic levels of CCL4. As Tregs migrate in response to CCL4 (19) and intraislet localization of antigen-specific Tregs is associated with elevated CCL4 levels (6), we quantitated intrapancreatic CCL4 levels at 20 weeks of age when Treg activity was detected. Intrapancreatic CCL4 levels were elevated ($P < 0.02$) in pHERO8100-CCL4–treated mice relative to control-treated mice (Fig. 7*E*). In contrast, intrapancreatic levels of CCL3 were similar in both treatment groups. Thus, CCL4 levels remain high in the pancreas of pHERO8100-CCL4–treated mice well after treatment is stopped and at a time when Treg activity is detectable in the draining PLN.

DISCUSSION

Elevated CCL4 expression demarcates inflammatory Th1-type immune responses (20), and effector Th1-cells are diabetogenic (15). However, we reported that enhanced CCL4 expression in the spleen and pancreas is associated with a Th2-type response that protects NOD mice from destructive insulitis and type 1 diabetes (4,6,11). To explain these different findings, we evaluated whether CCL4 prevents type 1 diabetes.. We found that neutralization of CCL4 abrogates the ability of IL-4 to protect NOD mice from type 1 diabetes and that intradermal CCL4 gene transfer prevents destructive insulitis and type 1 diabetes. This study clearly demonstrates that direct administration of CCL4 to NOD mice blocks the pathogenesis and onset of type 1 diabetes.

Gene transfer of a plasmid stabilized by the Epstein-Barr virus–derived EBNA1/*oriP* episomal maintenance replicon was used here to express CCL4 in NOD mice, since we had determined that the EBNA1/*oriP* system sustains gene expression in vivo (10). Previous DNA vaccination studies reported that anti-chemokine antibodies naturally exist in rodents and that these humoral responses are enhanced by overexpression of chemokines after vaccination (21–23). We therefore used pHERO8100-CCL4 to sustain a moderate level of CCL4 expression and minimize such an antibody response. Indeed, no difference was detected in the anti-CCL4 antibody serum titer (1/100) in 15-week-old mice previously treated from 3 to 14 weeks of age with either pHERO8100 or pHERO8100-CCL4. Consequently, anti-CCL4 antibody responses do not seem to protect pHERO8100-CCL4–treated mice against type 1 diabetes.

pHERO8100-CCL4 effectively protected NOD mice from type 1 diabetes even when administered at a later stage of disease progression (10 weeks of age). This result supports previous observations that onset of destructive insulitis is associated with reduced intrapancreatic CCL4 levels in 10-week-old NOD mice and that an increase in this level before this age leads to protection from type 1 diabetes (4,11). Our finding may also explain why enhanced T-cell production of CCL4 in the PLN induced by pHERO8100-CCL4 (Fig. 5*C*) protects from invasive insulitis and type 1 diabetes. Interestingly, this protection was mediated by reduced IFN-γ and increased IL-4 and IL-10 production by T-cells in the spleen and islets. Furthermore, pHERO8100-CCL4 treatment increased the production of IgG1 but not IgG2c anti-GAD65 Abs (C.M., unpublished data). These results link elevated CCL4 expression with a diminished Th1-type response and are consistent with reports that elevated CCL4 levels promote recruitment of insulin-reactive $CD4^+$ regulatory Th2 cells to the pancreas (6), islet-specific Th2 cells express CCL4 (2), and CCL4 promotes adaptive and mucosal immunity by inducing antigen-specific Th2-like responses (24,25). Given that a deficiency in Th2-type immunity occurs in NOD mice (15,26), our results suggest that CCL4 treatment may prevent type 1 diabetes by correcting this deficiency in islets, as Treg cells exert more regulatory function in islets than in PLN (27).

Whereas CCL4 can prevent type 1 diabetes, its CCR5 receptor can promote invasive insulitis, as CCR5-deficient islets are protected against islet allograft rejection and neutralization of CCR5 prevents type 1 diabetes (17,28). It is possible that CCR5 ligands have distinct roles during the development of insulitis (2,4,17) and that CCR5 antagonists may provide a promising therapy to prevent type 1 diabetes. In this regard, elevated circulating levels of CCL4 may attenuate CCR5 function by interfering with inflammatory chemokine gradients that recruit $CCR5^+$ autoreactive T-cells to islets. Alternatively, CCL4-CCR5 interactions may induce CCR5 internalization and account for our finding of a decreased amount of CCR5-dependent effector cell recruitment to islet lesions (18). As a lower percentage of $CD8^+CCR5^+$ T-cells were localized to the PLN and islets following pHERO8100-CCL4 treatment, and CCR5 is an activation marker of $CD8^+$ T-cells in NOD mice (17), our results suggest that pHERO8100-CCL4 treatment inhibits recruitment of activated $CD8^+$ T-cells to the pancreas. In support of this notion, the frequency of $CD8^+CD69^+$ T-cells was significantly decreased in the islets of pHERO8100-CCL4–treated mice and pHERO8100-CCL4 treatment significantly delayed the onset of type 1 diabetes in NOD8.3 transgenic mice.

Although often categorized as a proinflammatory receptor, CCR5 can also suppress immune responses. Blockade of CCR5 activation exacerbates tissue destruction in glomerulonephritis (29), CCR5-deficient effector cells are less susceptible to Treg suppression of graft versus host disease (30), defects in IFN-γ production are overcome to control *Leishmania* infection in CCR5-deficient mice (31), and IFN-γ and tumor necrosis factor-α production by splenocytes as well as T-cell engraftment in the spleen are increased in recipients of CCR5-deficient donor cells during acute GVHD (32). Thus, CCR5 may suppress Th1-cytokine production in certain environments, which may explain how pHERO8100-CCL4 attenuates Th1 cytokine production and protects from type 1 diabetes.

Finally, our cell transfer studies raise the possibility that Tregs may play a role in CCL4-induced protection from type 1 diabetes. The diabetogenic potential of spleen T-cells was diminished in pHERO8100-CCL4–treated mice, and PLN T-cells from such mice suppressed the ability of diabetogenic T-cells to transfer type 1 diabetes. Note that these PLN T-cells consist of an increased proportion of $CD4^+CD25^+$ T-cells and that their suppressive activity correlated with an elevated level of CCL4 in the pancreas. Although it is curious that Tregs possess a $CD4^+CD25^+$ phenotype, Treg function correlates with an increased expression of CCL4 (4,6,19), and CCR5 expression by Tregs is required for their suppressive function (30), further studies are required to determine whether $CD4^+CD25^+$ T-cells purified from the PLN of pHERO8100-CCL4–treated mice indeed function as Tregs. CD25 is also a marker of activated T-cells, and the proportion of activated T-cells rather than Tregs may be elevated in the PLN of these mice. Nonetheless, enhanced CD69 expression was not observed on PLN T-cells from these CCL4-treated mice. The possibility that IL-10–producing $CD4^+$ Tr1 cells (33,34) and/or other Treg subsets are localized to the pancreas and mediate protection from type 1 diabetes in these mice must also be considered.

We conclude that CCL4 protects against the development of destructive insulitis and type 1 diabetes.. This study adds to the mounting evidence that CCL4 can protect from autoimmune disorders (19,21). Our finding that CCL4 treatment may be beneficial even at a later stage(s) of autoimmune disease pathology is of particular significance, as it suggests that the therapeutic value of CCL4 in the prevention type 1 diabetes may be to arrest the transition from peri-insulitis to invasive/destructive insulitis and subsequent islet β-cell destruction.

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