

Early Treatment of NOD Mice With B7-H4 Reduces the Incidence of Autoimmune Diabetes

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OBJECTIVE—Autoimmune diabetes is a T cell–mediated disease in which insulin-producing β -cells are destroyed. Autoreactive T cells play a central role in mediating β -cell destruction. B7-H4 is a negative cosignaling molecule that downregulates T-cell responses. In this study, we aim to determine the role of B7-H4 on regulation of β -cell–specific autoimmune responses.

RESEARCH DESIGN AND METHODS—Prediabetic (aged 3 weeks) female NOD mice (group 1, $n = 21$) were treated with intraperitoneal injections of B7-H4.Ig at 7.5 mg/kg, with the same amount of mouse IgG (group 2, $n = 24$), or with no protein injections (group 3, $n = 24$), every 3 days for 12 weeks.

RESULTS—B7-H4.Ig reduced the incidence of autoimmune diabetes, compared with the control groups (diabetic mice 28.6% of group 1, 66.7% of group 2 [$P = 0.0081$], and 70.8% of group 3 [group 1 vs. 3, $P = 0.0035$]). Histological analysis revealed that B7-H4 treatment did not block islet infiltration but rather suppressed further infiltrates after 9 weeks of treatment (group 1 vs. 2, $P = 0.0003$). B7-H4 treatment also reduced T-cell proliferation in response to GAD65 stimulation *ex vivo*. The reduction of diabetes is not due to inhibition of activated T cells in the periphery but rather to a transient increase of Foxp3⁺ CD4⁺ T-cell population at one week posttreatment (12.88 ± 1.29 vs. $11.58 \pm 1.46\%$; $n = 8$; $P = 0.03$).

CONCLUSIONS—Our data demonstrate the protective role of B7-H4 in the development of autoimmune diabetes, suggesting a potential means of preventing type 1 diabetes by targeting the B7-H4 pathway. *Diabetes* 60:3246–3255, 2011

Autoimmune diabetes is a T cell–mediated chronic disease (1–4). Insulin-producing β -cells are destroyed by inflammatory autoreactive T cells that recognize islet autoantigens (1). The non-obese diabetic (NOD) mouse is the best available animal model for human type 1 diabetes (5). NOD mice have many key features of human type 1 diabetes. Similar to human type 1 diabetes, most leukocytes in the islet infiltrates are T cells in NOD mice (3,4). Accordingly, disease can be prevented in NOD mice by anti-T-cell antibodies, such as anti-CD3, anti-CD4, or anti-CD8 monoclonal antibodies (mAbs)

(6–8). A similar result is confirmed in humans. Loss of insulin is prevented by anti-CD3 treatment in new-onset type 1 diabetic patients (9–11). The central role of T cells in the development of autoimmune diabetes is further confirmed in adoptive transfer experiments (12). Diabetes can be transferred by injection of T cells from diabetic donors to healthy recipients (12). These promising data from both NOD mice and humans suggest that autoimmune diabetes can be reduced by controlling the autoreactive T-cell population. However, global deletion of T cells results in severe side effects caused by a nonspecific reduction of immunity. In fact, some patients given anti-CD3 treatment experienced “flu-like” symptoms due to significant cytokine release by antibody-bound T cells, recurrence of Epstein-Barr viral infections, and only transient protection (11). Therefore, reduced autoreactive T-cell proliferation by removal of T cells is not a cure.

T-cell activation requires two signals. Signal 1 refers to the interaction of peptides presented on the major histocompatibility complex and T-cell receptor. Signal 2 refers to a positive or negative signal. In the absence of signal 2, no response develops (13). Signal 1 plus signal 2 leads to activation or termination of T-cell responses, depending on which cosignaling pathway dominates. The “on” and “off” autoreactive T-cell responses controlled by positive and negative cosignaling molecules demonstrate the rationale for using costimulation blockade as a therapeutic target. The classic CD28/CTLA-4/B7 pathway plays an important role in maintaining T-cell homeostasis (14–16). B7.1 and B7.2 can up- and downregulate T-cell responses by engaging two opposing receptors, the activating receptor CD28 and the inhibitory receptor CTLA-4 (17), respectively. The interaction between CD28 and B7.1/B7.2 promotes T-cell proliferation, whereas engagement of CTLA-4 with B7 terminates activated T-cell response. A profound role of cosignaling molecules in the autoreactive T-cell response is well established in CTLA-4 knockout mice (18,19). Loss of function of CTLA-4 results in massive lymphoproliferation in CTLA-4–deficient mice, which die 3–4 weeks after birth. In parallel, blocking of endogenous CTLA-4 by injection of anti-CTLA-4 mAbs results in rapid β -cell destruction, indicating that the autoimmune response can be augmented in the absence of coinhibitory molecules (20,21). Consistent with this notion, systemic administration of CTLA-4.Ig prevents autoimmune diabetes by competing with CD28 for binding to CD80/CD86, confirming that coinhibitory molecules can turn off autoreactive T-cell proliferation through blocking the action of positive cosignaling molecules (15). This observation suggests potential effectiveness of using coinhibitory molecules to block the autoimmune response. In fact, many experimental data reveal that costimulation blockade using CTLA-4, B7-1H (PD-L1), or anti-ICOS is able to control autoimmune diseases (15,22–25).

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B7-H4 is a novel coinhibitory B7 homology molecule and functions to suppress T-cell proliferation (26–28). Blocking endogenous B7-H4 by injection of anti-B7-H4 mAb accelerates experimental autoimmune encephalomyelitis (EAE) disease, suggesting its negative role in regulating autoimmune response in vivo (27). We have shown previously that B7-H4 inhibits the proliferation of activated CD4⁺ and CD8⁺ T cells from type 1 diabetic patients induced by anti-CD3 antibody (29). Furthermore, cell-associated B7-H4.Ig expressed on human β -cells inhibits the cytotoxicity of the T-cell clones to targeted human β -cells (29). Collectively, these data signify that B7-H4 is a negative cosignaling molecule. However, its role in tissue-specific autoimmune diabetes has never been reported. On the basis of previous data, we hypothesize that it may be able to control the development of diabetes in NOD mice by downregulating autoreactive T-cell responses. We tested our hypothesis using systemic administration of B7-H4.Ig in standard NOD mice.

RESEARCH DESIGN AND METHODS

Mice. NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Jack Bell Research Centre at the University of British Columbia. All mice were cared for according to the guidelines of the Canadian Council on Animal Care.

In vitro T-cell studies. CD3⁺ T cells that had been purified using magnetic negative selection (StemCell Inc., Vancouver, BC, Canada) were cultured in plates that had been coated with various concentrations of anti-CD3 mAb. B7-H4.Ig or mouse Ig (10 μ g/mL or indicated concentration) was cultured for 2–4 h before adding T cells.

Treatment protocol. Groups of female NOD mice were treated with B7-H4.Ig (group 1, provided by Amplimmune, Inc.) or mouse Ig (group 2) starting at the age of 3 weeks. Animals received treatment or control Ig at a dosage of 7.5 mg/kg body weight every 3 days for 12 weeks. Animals were considered diabetic after two consecutive readings with blood glucose levels of >250 mg/dL. Onset of diabetes was dated from the first sequential diabetes measurement.

Histological analysis. The pancreata from various treatment groups were snap frozen. The cryostat sections were stained with hematoxylin and eosin. At least 100 islets were counted and blindly scored. Double staining of insulin and CD45 was developed using Texas red or FITC-conjugated secondary antibody. CD4, CD8 (BD Bioscience, Mississauga, ON, Canada), and Foxp3 (eBioscience, San Diego, CA) were stained according to the manufacturer's instructions.

Autoreactive T-cell responses in the periphery. Purified CD3⁺ T cells from different treatment groups were cultured with irradiated (3,000 rads) splenocytes from NOD mice and GAD p35 (20 μ g/mL, amino acids 524–543) for 4 days for [³H] incorporation assay or cytokine measurement. In some experiments, CD25⁺ cells were depleted from purified CD3⁺ T cells by microbeads (Miltenyi Biotec Inc., Auburn, CA).

Flow cytometric analysis. Single-cell suspensions were stained with fluorochrome-conjugated mAbs CD4, CD25, Foxp3, CD69, and CD44 (eBioscience). Intracellular staining of cytokine γ -interferon (IFN- γ) was performed after 4–5 h phorbol myristic acid plus ionomycin stimulation.

Isolation of islet-infiltrating cells. Islets were isolated with collagenase (Sigma, Oakville, ON, Canada) digestion of pancreata, as described previously (30). The single-cell suspension was dissociated by a nonenzymatic digestion solution (Invitrogen, Burlington, ON, Canada). These islet-infiltrating cells (IICs) were then subjected to fluorescence-activated cell sorter (FACS) analysis and real-time PCR. Real-time PCR was performed as previously described (30). The primer pairs are listed in Table 1.

Statistical analysis. The significance of the Kaplan-Meier survival curve in Fig. 2 was determined by a log-rank test. The significance of T-cell proliferation, expression of T-cell subsets, and cytokine production in Figs. 1, 3, and 4–6 was calculated by two-tailed Student *t* test or ANOVA. Values are expressed as means \pm SEM, and differences are considered significant when *P* < 0.05.

RESULTS

B7-H4.Ig inhibits NOD mouse T-cell proliferation in vitro. B7-H4.Ig inhibits proliferation of T cells from non-diabetes-prone strains of mice such as C57BL/6 and BALB/c (26–28). To assess whether or not B7-H4 could affect the

TABLE 1
PCR primer pairs

Gene	Sense	Antisense
Foxp3	TTC ATG CAT CAG CTC TCC AC	CTG GAC ACC CAT TCC AGA CT
GAPDH	AAC TTT GGC ATT GTG GAA GG	ACA CAT TGG GGG TAG GAA CA
Gzmb	TCG ACC CTA CAT GGC CTT AC	GAG CAG CAG TCG GCA CAA AG
IFN- γ	ACT GGC AAA AGG ATG GTG AC	TGA GCT CAT TGA ATG CTT GG
IL-2	CCC ACT TCA AGC TCC ACT TC	ATC CTG GGG AGT TTC AGG TT
IL-10	CCA AGC CTT ATC GGA AAT GA	TTT TCA CAG GGG AGA AAT CG
Th17	TCC AGA AGG CCC TCA GAC TA	AGC ATC TTC TCG ACC CTG AA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gzmb, granzyme B.

autoreactive T-cell response, we detected the effects of B7-H4.Ig on activated CD3⁺ T cells from NOD mice. Figure 1A shows that B7-H4.Ig significantly inhibited T-cell proliferation in a dose-dependent manner. T-cell proliferation was reduced by 62.2% in the presence of 10 μ g/mL B7-H4.Ig (Fig. 1B). Interleukin-2 (IL-2), a hallmark of T-cell proliferation, was reduced by 60.0% (*P* = 0.002) (Fig. 1C) in the presence of 10 μ g/mL B7-H4.Ig. The expression of early T-cell activation marker CD69 in CD4⁺ and CD8⁺ subsets was also reduced (Fig. 1D, E, F, and G). In addition, we used NRP-V7 tetramer to detect the effects of B7-H4 on the autoreactive CD8⁺ population from NOD mice. B7-H4 significantly inhibited NRP-V7 CD8⁺ cells from the NOD mice (data not shown). Collectively, these data demonstrate that B7-H4 inhibits activation of T cells from diabetes-prone NOD mice in vitro.

Early treatment of NOD mice with B7-H4.Ig reduces the development of diabetes. In order to test the effects of B7-H4 on autoimmune responses in vivo, we treated NOD mice at 3 weeks of age, presumably before or just at the onset of insulinitis. Consistent with previous reports, 66.7% of the animals treated with mouse Ig (group 2, *n* = 24) developed hyperglycemia at between 10 and 33 weeks of age, similar to untreated animal colonies in our facility (in group 3, *n* = 24, the incidence of diabetes is 70.8%) (Fig. 2A). In contrast, only 28.6% of mice treated with B7-H4.Ig (group 1, *n* = 21) developed diabetes (Fig. 2A), significantly lower than the control groups (group 1 vs. 2, *P* = 0.0081; group 1 vs. 3, *P* = 0.0035).

To assess the kinetics of the development of diabetes in B7-H4.Ig-treated and control groups, the distribution of the onset of diabetes was compared among three groups. Diabetes developed at between 10 and 33 weeks of age in the mouse Ig-treated group, similar to the untreated group in which hyperglycemia developed at between 11 and 30 weeks of age (Fig. 2B). The onset of diabetes peaked at between 12 and 24 weeks of age in the two control groups (groups 2 and 3). In contrast, in the B7-H4-treated group, no mice developed diabetes until 13 weeks of age, 2–3 weeks later than the other two control groups, demonstrating a lower and later pattern of development of hyperglycemia with B7-H4 treatment (Fig. 2B).

Early treatment of NOD with B7-H4.Ig does not prevent insulinitis. Hyperglycemia in NOD mice results from destruction of insulin-producing β -cells by leukocytes that

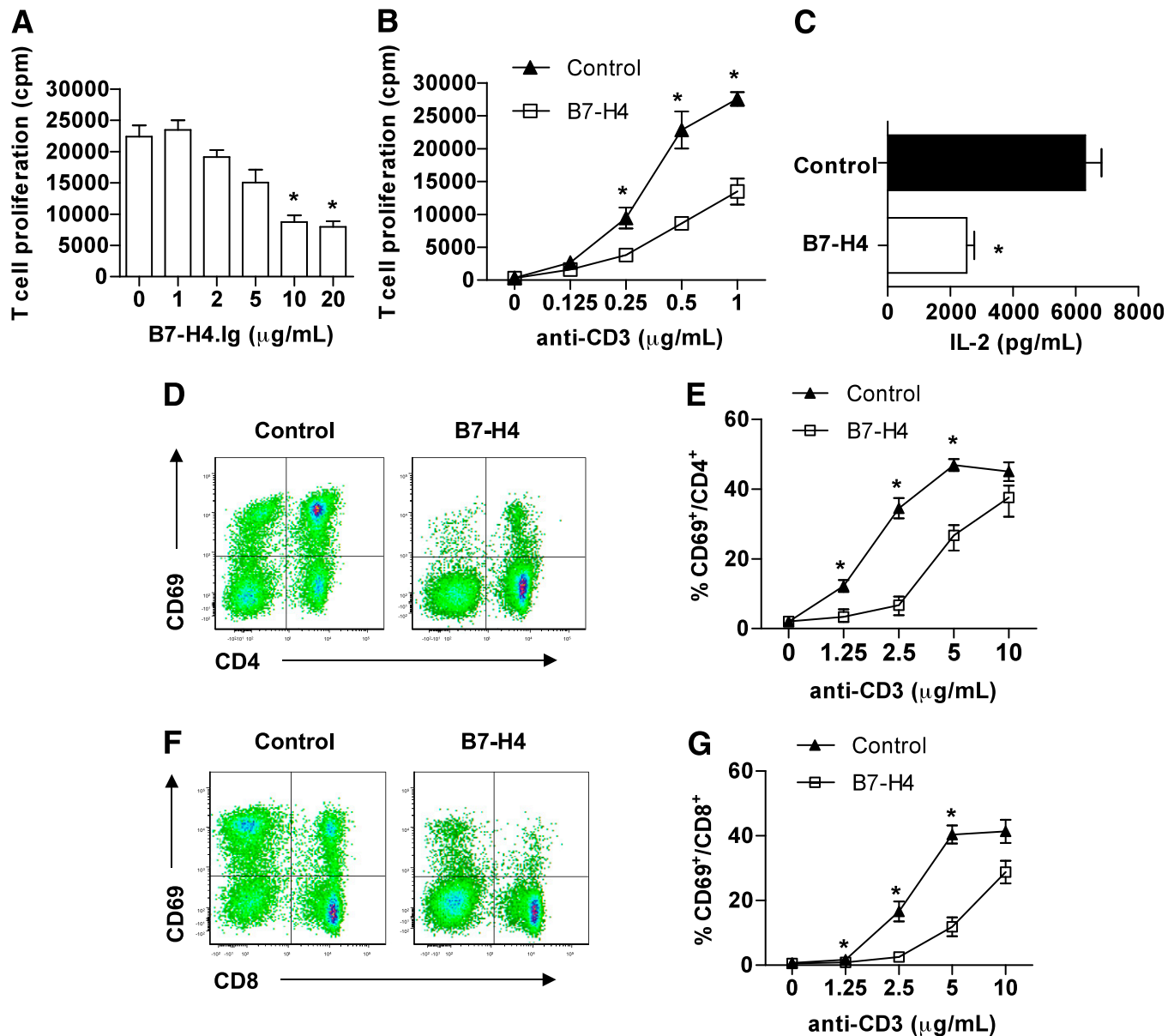


FIG. 1. B7-H4.Ig inhibits NOD T-cell proliferation and cytokine production. Purified CD3⁺ T cells were stimulated with various concentrations of plate-bound anti-CD3 for 18 h (for early T cell marker CD69 detection) (D–G), 48 h (for cytokine detection) (C), or 72 h (for T-cell proliferation) (A and B). B7-H4.Ig or mouse Ig was added at indicated concentrations. For the T-cell proliferation assay, cultures were pulsed with 1 μCi of [³H]-thymidine in the last 18 h. B7-H4.Ig inhibited T-cell proliferation in a dose-dependent manner ($P = 0.61, 0.04, 0.02, 0.01, 0.005$) (A) ($P = 0.01, 0.02, 0.05, 0.03$) (B). B7-H4.Ig inhibited IL-2 secretion ($P = 0.002$) (C) and CD69 expression on CD4⁺/CD8⁺ T-cell subsets ($P = 0.04, 0.003, 0.03, 0.08$) (E) ($P = 0.03, 0.003, 0.02$) (G). The production of IL-2 was measured by sandwich ELISA using pair antibodies from eBioscience (clone numbers of capture and detection antibodies for IL-2: JES6-1A12 and JES6-5H4). Triplicate wells were harvested and counted. Data represent three independent experiments and are expressed as means ± SEM. * Indicates significant value of $P < 0.05$. (A high-quality color representation of this figure is available in the online issue.)

infiltrate into the islets. The reduction of diabetes by B7-H4 treatment may account for the decline of infiltrates into the pancreas. Therefore, the development of insulinitis and β -cell function were examined in B7-H4– (group 1) and mouse Ig–treated (group 2) animals (Fig. 3A). Insulinitis was detected in groups 1 and 2 at 4 weeks of age. However, no destructive insulinitis was observed in either group (Fig. 3B). At 8 weeks of age, total infiltrated islets were accumulated to a similar degree in two groups. By 12 weeks of age, destructive insulinitis was detected similarly in two groups (24 and 27% for groups 1 and 2, respectively). Notably, by 16 weeks of age, the invading intrainsulinitis increased sharply to 54% in group 2, whereas it remained at 27%, a similar percentage (24%) to the 12-week results in group 1.

It thus appears that severe destructive insulinitis was arrested at 12 weeks of age in B7-H4–treated NOD mice.

At a late stage (16 weeks of age), we observed a distinct pattern of insulinitis in two groups. Inflammation and loss of islets in the B7-H4.Ig treatment group were significantly lower compared with group 2 (inflammation, $P = 0.0003$; loss of islets, $P = 0.0006$) (Fig. 3C). There was no difference in total pancreatic insulin content and insulin secretion during an intraperitoneal glucose tolerance test (IPGTT) at 12 weeks (Fig. 3D and E). By contrast, the amount of insulin contained in the pancreata of the B7-H4–treated group at 16 weeks was significantly higher than that in controls ($P = 0.003$) (Fig. 3D), demonstrating a greater β -cell mass after B7-H4 treatment. Furthermore, mice in the control

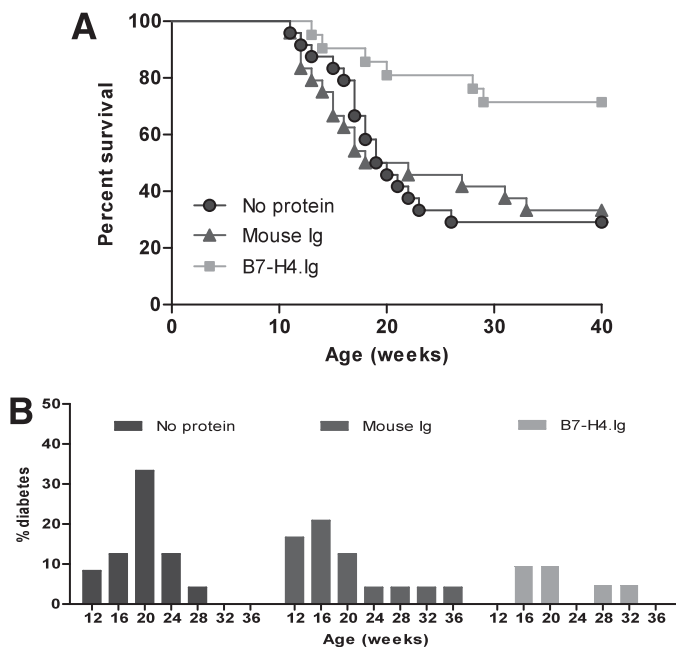


FIG. 2. B7-H4.Ig treatment reduces the incidence of autoimmune diabetes. **A:** Survival curve for mice untreated or treated with B7-H4.Ig or control mouse Ig. Female NOD mice were injected intraperitoneally with B7-H4.Ig (group 1, \square , $n = 21$) or mouse Ig (group 2, \blacktriangle , $n = 24$) at 7.5 mg/kg every 3 days for 12 weeks. Untreated female NOD mice (group 3, \bullet , $n = 24$) were also included. Systemic administration of B7-H4.Ig significantly suppressed diabetes (group 1 vs. 2, $P = 0.0081$; group 1 vs. 3, $P = 0.0035$, by log-rank test). **B:** Distribution of the incidence of diabetes among three groups. The percentage of onset of diabetes is plotted at 12, 16, 20, 24, 28, 32, and 36 weeks of age. The percentage of diabetes at various time points in groups 1, 2, and 3 is shown in dark gray, gray, and light gray, respectively.

group demonstrated deterioration in glucose tolerance and an impaired insulin secretion during IPGTT (236 ± 56 vs. 483 ± 84 ng) at 16 weeks compared with the B7-H4-treated group (Fig. 3F). Thus, B7-H4 treatment resulted in a reduced amount of aggressive islet destruction and improved β -cell function and mass at 16 weeks. These data indicate that protection against diabetes in B7-H4-treated NOD mice was not related to blockade of insulinitis. Instead, it might be associated with modulation of infiltrates at a later stage.

B7-H4.Ig suppresses activated IICs and increases Foxp3 expression in the pancreas. The distinct patterns of insulinitis in NOD mice between 12 and 16 weeks of age in B7-H4 (group 1) and control (group 2) mice prompted us to investigate the phenotype of IICs. Immunohistochemistry staining showed a similar number of CD4⁺ IICs in the two groups (74 ± 20 vs. 75 ± 18 per field; $P = 0.92$; $n = 5$) (Fig. 4A). In contrast, an increased number of Foxp3⁺ (14 ± 4 vs. 22 ± 6 per field; $P = 0.04$; $n = 5$) (Fig. 4A) were stained in group 1 compared with group 2, suggesting a B7-H4-associated regulatory T-cell (Treg) phenotype at the site of inflammation. Consistent with this immunohistochemistry result on Foxp3 expression, an increased percentage of Foxp3 expression among the CD4⁺ population was found using FACS analysis ($P = 0.008$; $n = 5$) (Fig. 4B and C). A decreased number of CD8⁺ cells were stained in the pancreas (22 ± 3 vs. 13 ± 5 per field; $P = 0.05$; $n = 5$) (Fig. 4A). However, no significant alteration was found using FACS (data not shown), reflecting a fluctuation expression of CD8⁺ T-cell subsets among infiltrates.

We next examined the activation status of CD4 and CD8 subsets by measuring the early T-cell activation marker CD69. We found that B7-H4 treatment reduced CD69 expression in both CD4⁺ and CD8⁺ subsets ($P = 0.04$ and $P = 0.02$, respectively; $n = 5$) (Fig. 4D and E), indicating that B7-H4 inhibited activation of both the CD4⁺ and CD8⁺ population.

We next investigated the effect of B7-H4 on the function of effector cells in the pancreas. Figure 4F showed relative mRNA expression levels in IICs. A lower-level expression of IL-2, IFN- γ , granzyme B, and IL-17, and a higher-level expression of Foxp3 and IL-10 was detected in group 1 compared with group 2, suggesting that B7-H4 efficiently inhibited proinflammatory cytokines by promoting transcription of Tregs and IL-10. To test this regulation at protein levels, we performed intracellular staining of IFN- γ , which showed that the production of IFN- γ is reduced in both CD4⁺ and CD8⁺ subsets ($P = 0.001$ and $P = 0.0005$, respectively; $n = 5$) (Fig. 4G and H), suggesting an inhibition of cytotoxic CD8 function and a shift from T helper 1 (Th1) toward Th2 upon local B7-H4 treatment. This finding is consistent with previous evidence of a preferential inhibition of the Th1 response in vivo by B7-H4 (31).

Collectively, B7-H4 treatment resulted in a decreased Th1 response and reduced proinflammatory cytokine expression mediated by local upregulation of Tregs. This is likely to have a profound inhibitory effect on the progression from insulinitis to overt diabetes.

B7-H4.Ig does not alter the peripheral homeostasis of T cells but transiently upregulates Foxp3⁺ T cells in the draining lymph nodes. To assess whether or not the reduction of the development of diabetes by B7-H4 occurs through the deletion of T cells or the inhibition of activated autoreactive T cells, we monitored the kinetics of CD4⁺ and CD8⁺ counts, and T-cell activation markers CD69, CD25, and CD44 at 4, 8, 12, and 16 weeks of age. No significant differences in the percentage of CD4⁺, CD8⁺, total number of T-cell subsets, CD4⁺CD69⁺, CD8⁺CD69⁺, CD4⁺CD25⁺, CD8⁺CD25⁺, CD4⁺CD44⁺, or CD8⁺CD44⁺ (data not shown) were found at any time point between the two groups, suggesting that B7-H4 neither deleted CD4⁺ and CD8⁺ T cells nor inhibited activation status in vivo in the periphery. These contradictory data may reflect the complicated micro-environment in vivo, such as the interaction between B7-H4 and other cosignaling pathways, and/or the alteration of T-cell subsets such as Th1 versus Th2 or Treg versus effector T cell (Teff).

The development of diabetes in NOD mice is the result of an imbalance of regulatory/suppressive and pathogenic/activated T cells. We showed previously that local expression of B7-H4 by a recombinant adenovirus prolongs islet allograft survival, and that this survival was associated with upregulation of Foxp3⁺ cells in the allograft (30). However, the relationship between Tregs and B7-H4 in the autoimmune response has not been elucidated. Therefore, we compared the expression of Tregs (CD4⁺CD25⁺Foxp3⁺), Teffs (CD4⁺CD25⁻Foxp3⁻), and Foxp3⁺ in the CD4⁺ population (Foxp3⁺CD4⁺) of pancreatic lymph nodes (PLNs) and spleens of NOD mice in groups 1 and 2. There was no significant difference in Tregs or Teffs (data not shown). However, there was a significant increase in the percentage of Foxp3⁺CD4⁺ cells in the PLNs of 4-week-old mice treated with B7-H4.Ig compared with group 2 ($P = 0.03$; $n = 12$) (Fig. 5A and B), but not at other time points, indicating a transient regulation of suppressive Tregs in the local draining lymph nodes.

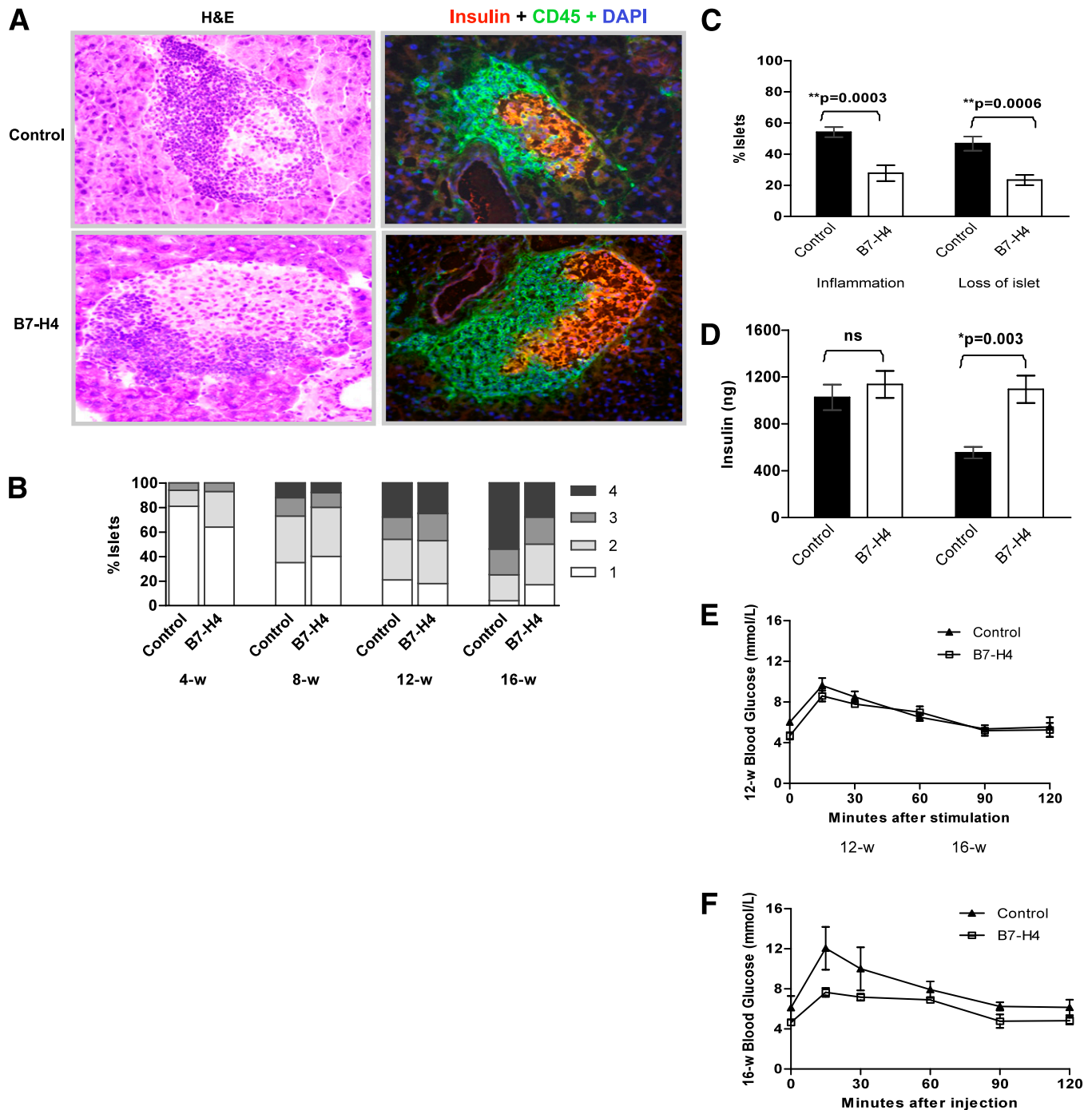


FIG. 3. B7-H4.Ig treatment does not block insulinitis but alters the aggressiveness of insulinitis at later stages. **A** and **B**: Representative sections of pancreata from 12-week-old NOD mice treated with B7-H4.Ig (group 1) or mouse Ig (group 2) were stained with hematoxylin and eosin (H&E) and insulin plus CD45 to determine the infiltrate scores and loss of islet cells. Insulinitis scores were calculated according to hematoxylin and eosin staining of the pancreata. Scores were given as follows: free of infiltrates, score 1; <25, 25–50, and >50% infiltrates per islet (scores 2, 3, and 4, respectively). **C**: Inflammation and loss of islets in the pancreata from mice at the age of 16 weeks were compared in groups 1 and 2. Percentages of inflammation and loss of islets were calculated based on the severe intraislet infiltrates and insulin staining, respectively. Both inflammation and loss of islets were significantly lower in B7-H4.Ig treatment group 1 compared with group 2 (inflammation, $P = 0.0003$; loss of islets, $P = 0.0006$). **D**: Total insulin content extracted from each pancreas was detected using a sensitive mouse insulin kit. The pancreas was homogenized in acid-ethanol (0.1 N HCl in 100% ethanol), and the supernatant was diluted in 10 mM Tris-HCl, PH7.5, 1 mM EDTA. The amount of insulin was measured by ELISA kit (Crystal Chem Inc., Chicago, IL) according to the manufacturer's instructions. There was no significant difference in the amount of insulin from group 1 or 2 at 12 weeks of age. However, there was a significant loss in the control group at 16 weeks ($P = 0.003$). An IPGTT was performed at 12 weeks (**E**) or 16 weeks (**F**). IPGTT was performed by intraperitoneal injection of 2 g/kg glucose after 5 h fasting. Blood samples were collected for examining insulin secretion. Blood glucose was tested at the indicated times. A similar blood glucose level was detected in both groups at 12 weeks. By contrast, glucose intolerance was detected in the control group compared with the B7-H4–treated group at 16 weeks. Six to eight animals are included in each group. W, weeks. (A high-quality digital representation of this figure is available in the online issue.)

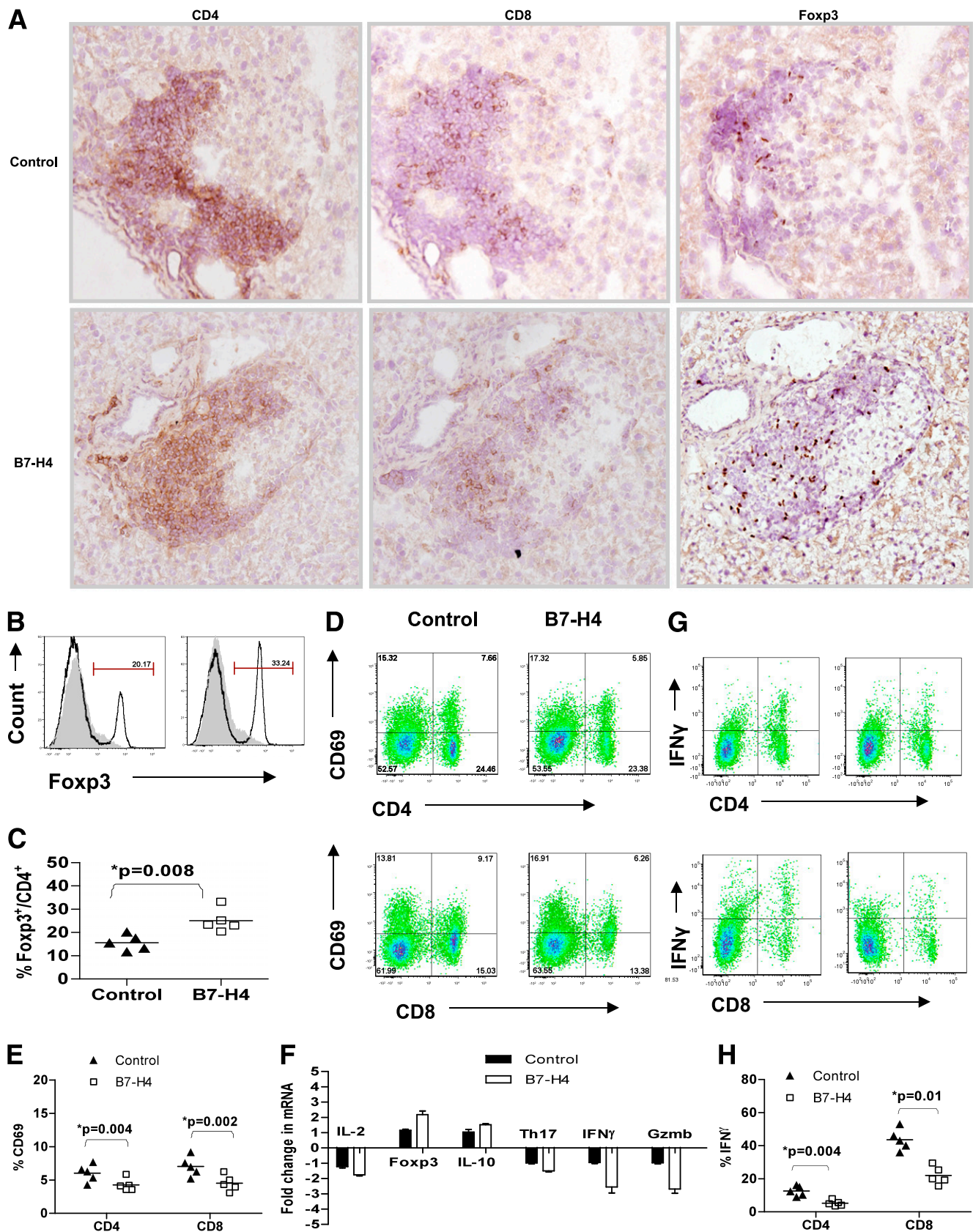


FIG. 4. Phenotype of IICs. **A:** Histology of pancreas from B7-H4.Ig⁻ (group 1) and mouse Ig-treated (group 2) NOD mice at 12–16 weeks of age. Frozen sections were stained with anti-CD4, CD8, or Foxp3 (shown in brown). The number of CD4-, CD8-, or Foxp3-positive cells was blindly counted by microscopy and described as means/field. FACS analysis of IICs (**B–E**). IICs were isolated and stained with anti-CD4, CD8, and CD69, or anti-CD4, CD25, and Foxp3. Representative plots of CD4 vs. CD69, CD8 vs. CD69, and histogram of Foxp3⁺ gated on CD4⁺ T-cell subset (**B** and **D**). Quantitative data (**C** and **E**). **F:** mRNA expression in IICs. mRNA was isolated from IICs and subjected to real-time PCR analysis. Expression level of mRNA in control group was considered as 1. Total RNA was extracted using RNeasy Mini kits (QIAGEN, Mississauga, ON, Canada). All RNA samples

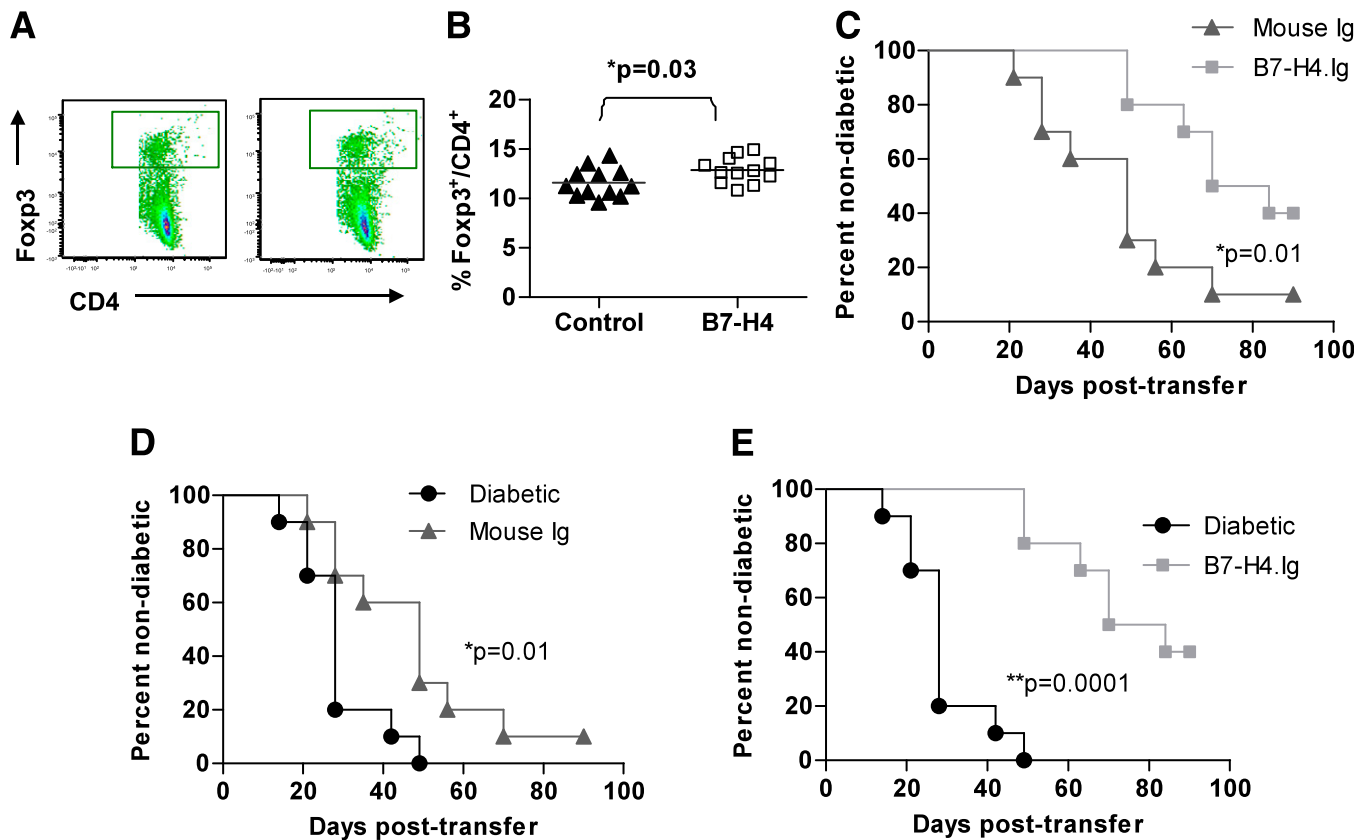


FIG. 5. B7-H4.Ig transiently upregulates Foxp3⁺ T cells in the draining lymph nodes. **A:** Representative plots of Foxp3⁺CD4⁺ subsets and pool percentage (**B**) in two treatment groups at 4 weeks of age in the PLN are shown. Control mouse Ig- and B7-H4.Ig-treated groups are shown in ▲ and □, respectively. Each dot represents one individual mouse; *n* = 8–11 per group. **C:** Lymphocytes from PLNs of B7-H4.Ig-treated NOD mice protected against the development of diabetes in NOD SCID mice. A mixture of single cells from PLNs of B7-H4.Ig- or control Ig-treated, 4-week-old NOD mice (10×10^6 cells) and splenic leukocytes from diabetic NOD mice (15×10^6 cells) was adoptively transferred intravenously into NOD SCID recipients. B7-H4.Ig treatment significantly delayed the incidence of diabetes in NOD SCID mice induced by diabetic splenic leukocytes, compared with control Ig treatment (**P* = 0.01, B7-H4.Ig vs. control Ig). Lymphocytes from a mixture of 4-week-old NOD mice of either control Ig- (**D**) or B7-H4.Ig-treated (**E**) or diabetic NOD mice showed protection against development of hyperglycemia, compared with lymphocytes from diabetic NOD mice alone in the NOD SCID mice (**P* = 0.01, mouse Ig vs. diabetic NOD alone; ***P* = 0.0001, B7-H4.Ig vs. diabetic NOD alone). Data are pooled from two independent experiments, with *n* = 10 per group. (A high-quality color representation of this figure is available in the online issue.)

In order to assess whether or not this transient increase in the percentage of Foxp3⁺CD4⁺ cells in the PLNs is necessary for suppression of autoimmune diabetes, mixed leukocytes from diabetic NOD and 4-week-old mice treated either with B7-H4.Ig or control were adoptively transferred into NOD SCID mice. Control recipients developed hyperglycemia at an average of 7–8 weeks, whereas B7-H4-treated mice survived longer (>11 weeks; *P* = 0.01, by log-rank test) (Fig. 5C). This suggests that PLNs of 4-week-old NOD SCID mice treated with B7-H4.Ig displayed a greater suppressive phenotype for the development of diabetes. Although PLNs of 4-week-old mice treated with both B7-H4.Ig and control inhibited the development of hyperglycemia induced by diabetic NOD splenocytes, they showed different degrees of suppression (Fig. 5D and E). The above finding of a transient increase in the percentage

of Foxp3⁺CD4⁺ cells in the PLNs may account for these differential phenotypes.

B7-H4 reduces the autoimmune response in the periphery. The above data, on one hand, demonstrated that inhibition of diabetes by B7-H4.Ig was not caused by a quantitative difference in the T-cell counts or activation status in the periphery. On the other hand, a transient upregulation of Foxp3⁺CD4⁺ cells in the PLNs suggested a possible alteration of T-cell function. To assess the effects of B7-H4 on the autoreactive T-cell response, we tested the proliferation of T cells in the presence of islet autoantigen GAD (Fig. 6A). T-cell proliferation was reduced by 35.8% (*P* = 0.02; *n* = 6), indicating that the ability of T cells to prime autoantigen was impaired with B7-H4 treatment.

The reduced ability of T cells to induce autoreactive T-cell proliferation in group 1 might result from a Th1/Th2

were digested with RNase-free DNase I (QIAGEN). First-strand cDNA was then synthesized from this RNA by Superscript II reverse transcriptase (GIBCO, Burlington, ON, Canada) using oligo(dT). Mouse glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal control to confirm equivalent loading of the total RNA. Quantitative real-time PCR was done in duplicate using 25 ng cDNA with 0.4 μmol/L of each primer in a final reaction volume of 20 μL containing 10 μL of 2 SYBR PCR Master Mix (QIAGEN). PCR parameters were as follows: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 30 s. Relative expression level was expressed as $2^{-(CT_{\text{ubiquitin}} - CT_{\text{gene}})}$ (where CT is cycling threshold) with ubiquitin RNA as the endogenous control for normalization. Each group represents four to six mice. Gzmb, granzyme B. **G:** Representative plots of intracellular staining of IFN-γ among CD4⁺ or CD8⁺. **H:** Expression levels of IFN-γ were generated from (**G**). Each dot represents one mouse (controls, ▲; B7-H4 treated, □). (A high-quality digital representation of this figure is available in the online issue.)

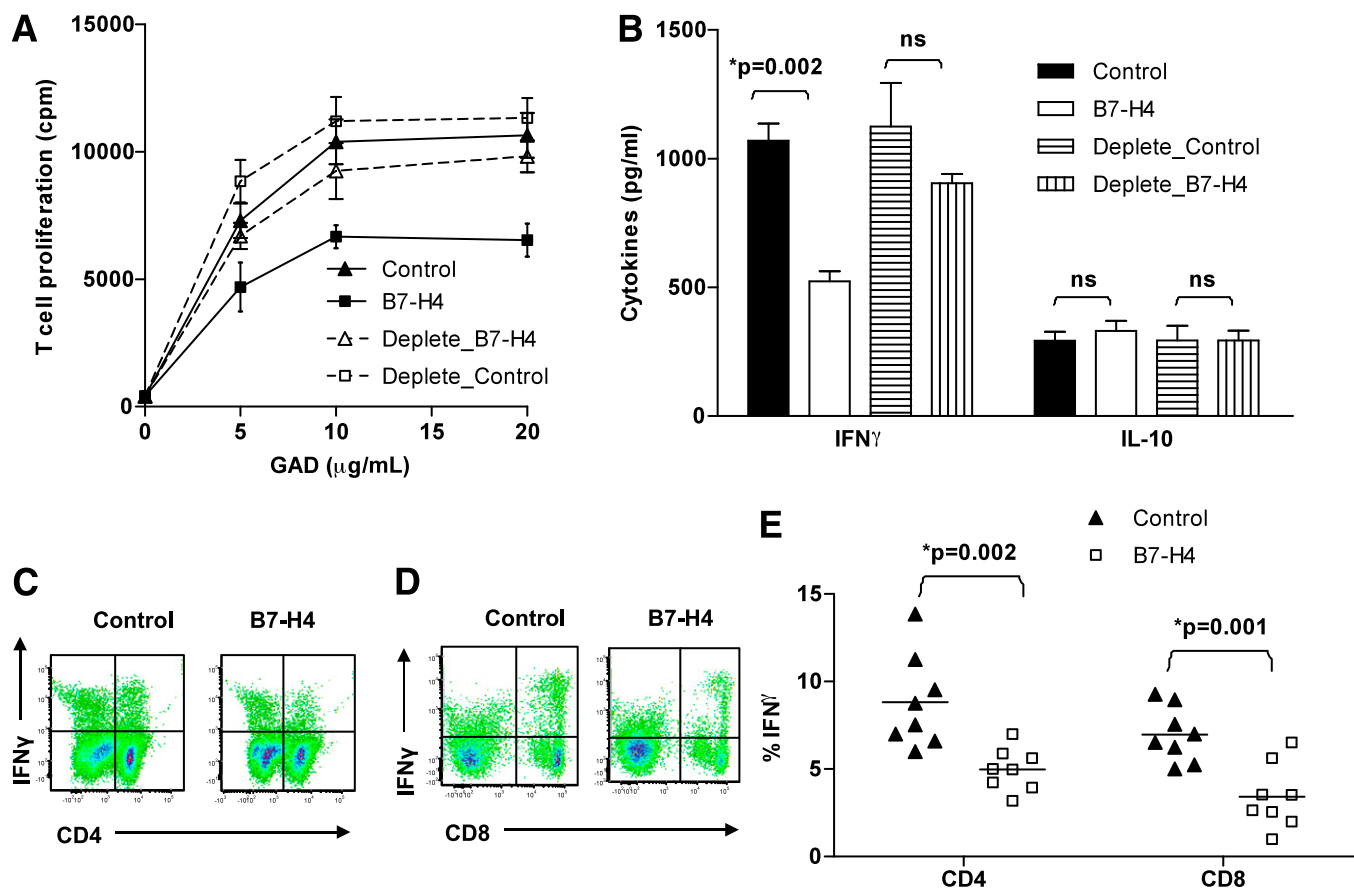


FIG. 6. A reduced autoimmune response in the periphery occurs after B7-H4 treatment. **A:** Purified CD3⁺ T cells from B7-H4- or mouse Ig-treated (group 1 and group 2, respectively) NOD mice were cultured with irradiated splenocytes and GAD p35 at indicated concentrations for 4 days and pulsed with 1 μ Ci of [³H]-thymidine in the last 18 h. [³H] incorporation is shown on the y-axis. (▲, group 1; ■, group 2; △, group 1 depleted with anti-CD25; □, group 2 depleted with anti-CD25). **B:** Purified CD3⁺ T cells from groups 1 and 2 were cultured with irradiated splenocytes, GAD p35 (20 μ g/mL), and 20 u/mL rIL-2 for 4 days and restimulated with phorbol myristic acid and ionomycin for 4 h for intracellular staining of IFN- γ or replated on a 24-well plate precoated with anti-CD3 (1 μ g/mL) for cytokine detection. Supernatants were collected at 48 h and subjected to analysis for cytokine production. The production of IFN- γ and IL-10 protein was measured by sandwich ELISA using pair antibodies from eBioscience (clone numbers of capture and detection antibodies for IFN- γ : XMG1.2 and R4-6A2; for IL-10: JES5-16E3 and JES5-2A5). Representative plots and quantitative analysis of IFN- γ expression (**C–E**). Each dot represents one individual mouse at the age of 8–12 weeks; $n = 5–9$ per group. (A high-quality color representation of this figure is available in the online issue.)

shift. We therefore examined the production of cytokines in response to autoantigens (Fig. 6B). The production of IFN- γ was reduced by 51.1% ($P = 0.002$; $n = 6$), but there was no change for IL-10 production (331 ± 96.17 vs. 292.33 ± 86.42 pg/mL; $P = 0.49$), indicating that the reduction of the autoreactive T-cell response might be associated with a decreased production of the Th1-related cytokine IFN- γ without affecting Th2-associated IL-10.

To assess the possibility of Treg involvement in B7-H4-mediated reduction in the autoimmune response, T-cell proliferation and cytokine production were also measured after CD25⁺ cells were depleted (Fig. 6A and B). T-cell proliferation was significantly higher after CD25⁺ depletion, reaching a similar level as the control group ($P = 0.46$; $n = 6$), suggesting involvement of Tregs in B7-H4-mediated reduced autoimmune response. Moreover, T-cell proliferation reached a similar level in both groups after CD25⁺ depletion, demonstrating a direct effect of B7-H4 on GAD p35-reactive T cells. The involvement of Tregs was further confirmed in the production of IFN- γ after depletion of CD25 (Fig. 6B).

The impaired ability for T-cell proliferation and IFN- γ production in response to GAD in group 1 may be due to

either a decline in the number of cells able to respond or impaired function from each cell. To test these two possibilities, intracellular staining of IFN- γ was performed at a single-cell level. A decreased percentage of IFN- γ in both CD4⁺ ($P = 0.002$; $n = 8$) (Fig. 6C and E) and CD8⁺ ($P = 0.001$; $n = 8$) (Fig. 6D and E) was detected, suggesting a decline in the number of cells to respond to GAD.

DISCUSSION

Regulation of autoreactive T-cell responses can be achieved by several mechanisms, including costimulation blockade (32,33). The concept of controlling autoreactive T-cell responses by targeting signal 2 has been investigated in several experimental models and human studies (14,15,34). B7-H4 is a new B7 homolog molecule that interacts with an unknown receptor on activated T cells to inhibit mitogenic T-cell activation of non-diabetes-prone mouse strains. In this study, we showed that it also inhibited proliferation of T cells from the diabetes-prone NOD mouse strain. Autoreactive T-cell proliferation is thought to determine the fate of autoimmune diabetes. Blockade of endogenous B7-H4 by anti-B7-H4 mAb exacerbates EAE, an animal model of the

autoimmune disease multiple sclerosis (27). Unlike EAE, which is induced by immunization with an antigen, autoimmune diabetes is a spontaneous disease caused by T cell-driven autoimmune destruction of insulin-producing β -cells. Therefore, the effect of B7-H4 in these two different autoimmune diseases and the mechanisms involved may not be the same. In this study, we found that B7-H4.Ig treatment of prediabetic mice reduced the incidence of diabetes to 28.6% compared with the control Ig group incidence of 66.7%. Our study is the first to report that B7-H4 indeed suppresses autoimmune diabetes in NOD mice.

The development of diabetes is the consequence of progressive loss of insulin-producing β -cells. Leukocytes are recruited to the pancreas and reside on the islet cells. After a long period of time, infiltrates replace the β -cells, resulting in hyperglycemia. There are two major checkpoints during the development of diabetes in NOD mice. Checkpoint 1 features the recruitment of infiltrates but free of diabetes. Leukocyte infiltrates can be stained surrounding the islets at as early as 3–4 weeks of age and accumulated further by 8–10 weeks of age (35). The second checkpoint begins at 10–12 weeks of age. At this time, nondestructive peri-insulinitis is transformed into destructive insulinitis. β -cells are killed by dense infiltrates, which results in dysfunction of secretion of insulin from the islets. Studies into the mechanisms of the reduction of diabetes reveal that B7-H4 does not affect checkpoint 1. We observed a similar degree of insulinitis in B7-H4.Ig- and control Ig-treated groups by 12 weeks of age (Fig. 3). Similar to this result, massive infiltrates are detected in allografts after islet transplantation (30). The presence of infiltrates in two different models suggests inefficient inhibition of T-cell priming by B7-H4 engagement at an early stage.

At a later stage, there is a distinct pattern of infiltration in the two groups. In controls, the severity of insulinitis progresses aggressively, whereas it remains at a similar level in terms of severity of inflammation and β -cell function in the B7-H4-treated group at between 12 and 16 weeks of age, suggesting potent regulation of diabetes development by B7-H4. In NOD mice, progression from checkpoint 1 to 2 is not random or immediate. Instead, experimental data show that the transition of insulinitis to overt diabetes is highly regulated over a long period of time (36–39). The autoimmune T-cell response is triggered by β -cell-specific autoantigens and may start within islets, where autoantigens originate and are presented by either β -cells or by antigen-presenting cells. However, naïve T cells cannot access peripheral nonlymphoid tissues (40). Instead, they only circulate through peripheral lymphoid organs. Early treatment of NOD females with anti-CD40 L mAb prevents insulinitis and diabetes. However, it does not prevent the appearance of islet-reactive T cells, indicating that priming does not occur within the islets (41). In fact, experimental data support the idea of β -cell-specific T cells being primed in the PLNs. Several lines of evidence support this notion. First, the activation status of T cells in the PLNs is correlated with islet infiltration (42). Secondly, proliferating cells appeared in the PLNs before the onset of insulinitis (42). This notion is confirmed by direct evidence of the removal of the PLNs (43). B7-H4 transiently upregulates Foxp3⁺ Tregs in the PLNs after 1 week of treatment, suggesting that the alteration of aggressiveness of insulinitis in the pancreas at the late stage may be correlated with modulation of Tregs in the PLNs at a very early time point.

Autoimmune diabetes is a chronic disease. Progression of insulinitis to overt diabetes is a long process. The number

and/or function of Tregs in NOD mice decrease over time and are correlated with disease onset, suggesting that Tregs may be defective in NOD mice (36,44). Depletion of Tregs or loss of regulatory pathways such as CTLA-4, TGF- β , and Foxp3 promotes a more aggressive disease (19,45–47). Conversely, treatment with Tregs can delay and/or cure mice of a variety of immunological diseases (16,48,49). The site of Treg action can be peripheral or local. In the current study, our data show that B7-H4-mediated protection is associated with early upregulation of Foxp3 in the PLNs and late modulation of Tregs at the site of inflammation. At an early stage, it may correlate with a reduced autoimmune response and IFN- γ production to autoantigen GAD stimulation. At a late stage, Tregs preferentially function at the site of inflammation. Our data show that the local effect of Treg in the islets appears to limit the Th1 response, proliferation and/or cytokine production by the pathogenic T cells. Tregs in the pancreas are likely to mediate this modulation and therefore control the progression from insulinitis to overt diabetes in prediabetic mice. The source of increased Tregs in the pancreas could be the result of improvement of Treg survival, or conversion of Teff into Treg, or increased recruitment of Tregs in the pancreas (37).

In the current study, we show novel data that B7-H4 inhibits T cell-mediated, spontaneously developing autoimmune diabetes in NOD mice. This result provides an additional piece of evidence to support the function of B7-H4 as a negative regulator of T-cell immunity *in vivo*. It has been reported that overexpression of B7-H4 prolongs graft survival and that recipients treated with B7-H4-transfected NIT-1 (an insulinoma cell line) were more resistant to STZ-induced diabetes. Survival was associated with augmented Tregs, and the production of IFN- γ and IL-4 by splenocytes was decreased and increased, respectively (50). It will be interesting to investigate the effect of B7-H4 on the modulation of antigen-presenting cells and the relationship between Treg and Th17. In conclusion, the ability of B7-H4 to negatively regulate autoimmune diabetes in NOD mice provides proof of principle for further investigation, such as whether or not early treatment with B7-H4 is necessary for prevention of disease, to what degree B7-H4 can reverse new-onset diabetes, and what the basic underlying mechanism of B7-H4 action on autoimmunity is.

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