Autoantigens Plus Interleukin-10 Suppress Diabetes Autoimmunity

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Abstract

Background: Recombinant vaccinia virus (rVV) strains expressing the immunomodulatory cholera toxin B subunit (CTB) fused to the autoantigen glutamic acid decarboxylase (GAD) or the immunosuppressive cytokine interleukin-10 (IL-10) were independently able to generate only low levels of immune suppression of type 1 diabetes mellitus (T1DM). Here we suggest that a vaccinia virus (VV)-mediated combination of CTB::GAD fusion and IL-10 proteins promises a effective and durable immunotherapeutic strategy for T1DM.

Methods: To explore this hypothesis, a CTB::GAD fusion gene was co-delivered with a gene encoding IL-10 by rVV infection (rVV-CTB::GAD + rVV-IL10) into 5–7-week-old non-obese diabetic (NOD) mice. The mice were assessed for vaccine protection against development of hyperglycemia from 12 to 64 weeks of age by assessment of pancreatic inflammation (insulinitis) and splenocyte-secreted interferon- γ and IL-10 cytokine levels.

Results: By 36 weeks of age, from 54% to 80% of the mice in the negative control animal groups (either mock-infected or inoculated with unrelated plasmid or VV) had developed hyperglycemia. Similarly, no statistically significant improvement in protection against diabetes onset was achieved by inoculation with VV expressing CTB::GAD or IL-10 independently. Surprisingly, only 20% of mice co-inoculated with rVV-CTB::GAD + rVV-IL10 developed hyperglycemia by 28 weeks of age. Other treatment groups developed hyperglycemia by 32–36 weeks. After 36 weeks, diabetes incidence no longer increased in any groups until the end of experiment at 64 weeks of age. Histological analysis of pancreatic tissues of hyperglycemic mice revealed high levels of intra-islet insulinitis. Analysis of insulinitis at termination of the experiment showed that euglycemic mice co-inoculated with VV expressing CTB::GAD and IL-10 had more effectively reduced inflammation in comparison with the other groups.

Conclusions: A combinatorial vaccination strategy based on VV co-delivery of genes encoding the immuno-enhanced autoantigen CTB::GAD and the anti-inflammatory cytokine IL-10 can maintain effective and durable euglycemia and immunological homeostasis in NOD mice with prediabetes.

Introduction

TYPE 1 DIABETES MELLITUS (T1DM) is an organ-specific autoimmune disease in which pancreatic insulin-producing islet β -cells are attacked and destroyed by the body's immune system. This perturbation of immunological homeostasis results in a progressive loss of islet β -cell function generating an insulin deficiency that elevates blood sugar levels (hyperglycemia) and increases cellular oxidative stress, which leads to chronic inflammation and associated risk for secondary neural and circulatory health problems, including amputation, blindness, heart attack, and stroke.¹ Approximately 3 million Americans, or about 15–20% of citizens afflicted with all forms of diabetes, currently suffer from T1DM,

and >13,000 children are diagnosed with T1DM in the United States annually.² Hyperglycemia (clinical disease) represents the end stage of immunological processes that develop over months in mice to years in humans.³ The diagnosis and treatment of hyperglycemia are poor, as islet β -cell destruction is completely asymptomatic until more than half of the approximately 1×10^6 pancreatic islets have been destroyed or inactivated.⁴ A variety of immune cells, including auto-reactive T helper (Th), cytotoxic T lymphocytes, B cells, dendritic cells, macrophages, and natural killer cells, have been shown to participate in diabetes pathogenesis.^{5–10}

Genetic factors play a significant role in T1D development,^{11,12} although they are insufficient to account for disease occurrence.¹³ Environmental factors, including virus infection

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and dietary components, are thought to modify diabetes susceptibility.^{14–17} More recently, an altered mucosal immune system has been associated with disease onset and is now considered to be a major contributor to the failure to develop immunological tolerance, resulting in the autoimmunity responsible for T1DM (for review, see Vaarala et al.¹⁸).

Insulin was identified as an early major diabetes autoantigen.^{19–22} Progressive islet β -cell destruction leads to the appearance of additional β -cell autoantigens that further exacerbate β -cell destruction.²³ Retardation or prevention of T1DM onset by repeated oral inoculation with small amounts of pancreatic islet autoantigens was demonstrated in animal models of autoimmune diabetes.²⁴ In addition, immunotherapy with major β -cell antigens such as insulin, glutamic acid decarboxylase (GAD), or heat shock protein 60, was shown to delay or prevent T1DM onset.^{25–27} Mucosal inoculation with small amounts of islet autoantigens was demonstrated to induce self-tolerance through interleukin (IL)-4 and signal transducer and activation of transcription 6 activation of Th2 and Th3 regulatory T cells that down-modulate autoreactive effector T cell inflammation at close proximity by “bystander suppression.”^{28,29} In addition, inoculation with autoantigens was shown to generate partial diabetes suppression in patients.^{30–32} Mucosal delivery of the cholera toxin B subunit (CTB) stimulated dendritic cell-mediated induction of IL-4 secretion by T cells and exerted a variety of distinct anti-inflammatory effects resulting in immunological suppression.^{33–35} Oral delivery of CTB conjugated with specific autoantigens demonstrated protection of mice against several Th1 cell-mediated autoimmune diseases, including autoimmune encephalomyelitis,^{36,37} autoimmune chondritis,³⁸ and uveitis.³⁹ In addition, oral delivery of CTB-insulin conjugates suppressed diabetes insulinitis and hyperglycemia in non-obese diabetic (NOD) mice and several other autoimmune diabetes animal models.^{40–45} This result was associated with reduced interferon (IFN)- γ production and Tr1 regulatory T cell migration into pancreatic islets.^{46,47} Mucosal (oral) inoculation of NOD mice with a plant-based CTB-GAD fusion protein resulted in a moderate, measurable suppression of diabetes.⁴² Low-level diabetes suppression was observed following vaccinia virus (VV)-mediated mucosal or intraperitoneal inoculation of NOD mice with CTB::GAD fusion or IL-10.^{48,49} Here we show that a systemically delivered combination of VVs expressing autoantigen CTB::GAD and the cytokine IL-10 is highly effective in long-term prevention of the onset of diabetes in NOD mice.

Materials and Methods

Viruses

The CV-1 cells were maintained and grown as previously described.⁴⁸ The Lister vaccine (LIVP) strain of VV was used as the parental virus. Construction, propagation, and purification of the recombinant viruses expressing CTB::GAD and IL-10 (recombinant VV [rVV]-CTB::GAD and rVV-IL10) and of control virus rVV-L15 were previously described.^{48–50} Virus titers were determined by plaque assay on CV-1 cells. The virus constructs used in this study are presented in Figure 1. A cDNA fragment encoding GAD55, a truncated form of human GAD65 minus the N-terminal membrane binding region (amino acids 89–585), was linked to the C-terminus of CTB gene as previously described.⁴⁹ The bacterial pWS4

FIG. 1. Physical map of recombinant vaccinia virus (rVV) strains used in the study. The TK_L and TK_R designate flanking thymidine kinase sequences from the VV genome. The *lacZ* designates the β -galactosidase gene of *Escherichia coli*; this reporter was used for selection of recombinant viruses. The autoantigen (cholera toxin B subunit [CTB]:glutamic acid decarboxylase [GAD]) and interleukin-10 (*IL-10*) genes are driven by the synthetic early/late promoter pE/L, whereas the *lacZ* is driven by the p7.5 promoter. *luc* = luciferase.

plasmid containing cytomegalovirus promoter was described previously.⁵¹

Detection of hyperglycemia in NOD mice immunized with rVVs

Four-week-old female NOD mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal care facility of the Central Veterinary Institute (Budapest, Hungary). The protocol for mouse rVV inoculation was approved by the Animal Research and Care Committees of Loma Linda University School of Medicine (Loma Linda, CA) and the Central Veterinary Institute. Prior to measurement of hyperglycemia, three groups of mice ($n = 11$) were subjected to intraperitoneal injection with rVV in 0.3 mL of phosphate-buffered saline (PBS) at 5 weeks of age. One mock-infected animal group ($n = 10$) was inoculated with PBS only. Two weeks after the first injection, the inoculations were repeated. One additional group of mice ($n = 10$) received four intradermal injections of 50 μ g of pWS4 plasmid DNA into ventral skin at weekly intervals using a tuberculin syringe fitted with a 27-gauge needle. The mice were fed complete mouse chow and water ad libitum. To detect the onset of hyperglycemia, beginning at 14 weeks of age the mice in each experimental group (Table 1) were bled from the tail vein every other week for 64 weeks, and blood sugar levels were quantified using a blood glucose analyzer (Glucometer® ESPRIT®, Bayer AG, Leverkusen, Germany). Diabetes was confirmed when blood glucose levels exceeded 14 mmol/L for two consecutive weeks.⁵² Blood glucose levels were confirmed with Keto-Diastix® urinary glucose test strips (Bayer AG). The “Test for the Equality of Two Proportions” was applied to assess the statistical significance of hyperglycemia incidence among experimental animal groups. The incidence of hyperglycemia was considered to be insignificant between groups when the calculated Z value was between -1.96 and 1.96 .

Histopathological analysis of pancreatic islets

Pancreatic tissues isolated from sacrificed mice were fixed in 8% phosphate-buffered formalin, embedded in paraffin,

TABLE 1. NOD MOUSE TREATMENT GROUPS FOR RECOMBINANT VACCINIA VIRUS-MEDIATED SUPPRESSION OF HYPERGLYCEMIA

Group	Treatment (intraperitoneal inoculation)	PFU (rVV) or mg (pWS4)	Age at injection
1 (<i>n</i> = 11)	rVV-L15 (naive)	$2 \times 5 \times 10^7$	5 and 7 weeks
2 (<i>n</i> = 11)	rVV-CTB::GAD	$2 \times 5 \times 10^7$	5 and 7 weeks
3 (<i>n</i> = 11)	rVV-CTB::GAD + rVV-IL10	$2 \times (5 \times 10^7) + (1 \times 10^7)$	5 and 7 weeks
4 (<i>n</i> = 10)	pWS4 (naive)	4 × 0.05 mg	5, 6, 7, and 8 weeks
5 (<i>n</i> = 10)	PBS (naive)	0	5 and 7 weeks

CTB, cholera toxin B subunit; GAD, glutamic acid decarboxylase; PBS, phosphate-buffered saline; PFU, plaque-forming units; rVV, recombinant vaccinia virus.

sectioned, and stained with hematoxylin and eosin prior to microscopic detection of pancreatic inflammation (insulinitis). The level of insulinitis was measured in each mouse based on the extent of lymphocyte islet infiltration. The degree of insulinitis was scored based on a five-level semiquantitative scale ranging from 0 to 4, where an insulinitis score of 0 was considered to be normal regarding islet morphology with no indication of autoreactive lymphocyte infiltration. Insulinitis scores of 1–2 indicated progressively increasing levels of peri-islet insulinitis, and scores of 3–4 indicated progressive levels of intra-islet insulinitis, with a score of 4 equivalent to total invasion of the islet by autoreactive lymphocytes.⁴⁸

Computer-assisted morphometry measurement of pancreatic islets

Pancreatic tissues isolated from sacrificed mice were fixed in 8% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to microscopic detection of pancreatic inflammation (insulinitis). The image of each pancreatic cross-section was digitized using an Axiovert 200 microscope (Carl Zeiss Inc., Jena, Germany) connected to a computer through an AxioCam video camera (Carl Zeiss Inc.). The stage of insulinitis was measured in each mouse based on the extent of lymphocyte islet infiltration. To determine the extent of islet infiltration, the percentage of the infiltrated area (infiltrated area per micrometer squared of pancreatic islet⁵²) was measured using AxioVision 4 microscope software (Carl Zeiss Inc.). The degree of insulinitis was scored based on a five-level semiquantitative scale ranging from 0 to 4, where an insulinitis score of 0 was considered to be normal islet morphology with no indication of autoreactive lymphocyte infiltration. A score of 1 was considered to be peri-islet T-lymphocyte infiltration with cytotoxic lymphocytes occupying less than 20% of the islet. A score of 2 was also considered to be peri-islet T-cell infiltration, with some intra-islet infiltration (e.g., occasional islets showing lymphocytes occupying as much as 20–40% of the islet). A score of 3 was considered to be intra-islet T-cell infiltration with lymphocytes occupying 40–70% of the islet. A score of 4 indicated massive intra-islet T-cell infiltration involving 70–100% of the islet.⁴⁸

Analyses of cytokine-secreting splenocytes

After more than 1 year (64 weeks), the surviving mice were euthanized by CO₂ asphyxiation. The spleens were immediately excised from each animal (*n* = 4–9 mice per group) and teased apart with scissors into cold RPMI 1640 medium

without fetal bovine serum. The spleen cell suspensions were passed through a fine stainless steel mesh (48 strands per inch). Individual splenocytes were harvested by centrifugation of the macerate in 15-mL polypropylene conical centrifuge tubes in a Universal 16 R centrifuge (A. Hettich GmbH, Tuttlingen, Germany) at 250 *g* for 5 min. The splenocyte pellet was washed three times by resuspension in an equal volume of ice-cold RPMI 1640 medium without fetal bovine serum followed by centrifugation. The splenocytes were suspended in complete medium (1×10^7 cells/mL) and cultured with 30 μ g/mL GAD65 autoantigen protein (Fisher Scientific, Pittsburgh, PA) in 15-mL polypropylene tubes held at an angle of 5° for 72 h at 37°C in a humidified atmosphere of 5% CO₂ in air. Following the incubation, the splenocyte preparations were centrifuged at 250 *g* for 10 min at room temperature to sediment the cells. The supernatant culture medium was collected and stored at –80°C until examined for secreted cytokine content. The concentrations of IFN- γ and IL-10 secreted into the medium were determined in triplicate wells of an enzyme-linked immunosorbent assay (ELISA) plate using mouse IL-10 and IFN- γ ELISA kits (eBioscience, Inc., San Diego, CA), according to the manufacturer's protocol. In brief, anti-mouse IFN- γ - and anti-mouse IL-10-specific antibodies were added to the wells of an ELISA 96-well microtiter plate at a dilution of 1,000 or 250 times per well, respectively, to capture IFN- γ and IL-10 from the medium generated by the cells of each mouse spleen. The ELISA plates were incubated with primary anti-IL-12 or anti-IL-10 antibodies for a period of 18 h at 4°C. The wells were washed five times with 300 μ L of PBS containing 0.05% Tween-20 (PBST), pH 7.0. Nonspecific background antibody binding was then blocked by incubating each well with 200 μ L of 1 × Assay Diluent (eBioscience, Inc.) at 37°C for 1 h. The diluent was aspirated, and the plates were washed five times with PBST. The supernatant medium from each spleen was added (100 μ L per well), and the plates were incubated for 2 h at room temperature to allow binding of the secreted cytokines. Following incubation, the plates were washed five times with PBST, a solution of either biotinylated anti-mouse IFN- γ (1,000 times dilution) or biotinylated anti-mouse IL-10 (250 times dilution) was added to each well, and the mixture was incubated for 1 h at room temperature. Following incubation, the plates were washed seven or 14 times, respectively, with PBST, then avidin-conjugated horseradish peroxidase diluted with 1 × Assay Diluent was added (100 μ L per well) to each well, and the mixture was incubated for 30 min at room temperature. The peroxidase activity was visualized by addition of 100 μ L of tetramethylbenzidine substrate solution per well. After a 15-min

incubation at room temperature, the colored reaction was stopped by addition of 50 μ L of 2 N H_2SO_4 solution per well. Spectrophotometric analysis of each sample was performed at 450 nm in a Multiscan Ms reader spectrophotometer (Labsystems Oy, Helsinki, Finland). In addition, each cytokine assay plate also contained serial dilutions of either an IFN- γ or IL-10 cytokine standard to establish a standard curve for accurate determination of the secreted cytokine levels in each sample.

Results

Expression of recombinant proteins in mammalian cell culture

The rVVs expressing the diabetes pancreatic islet autoantigen GAD fused to the C-terminus of CTB or IL-10 were used for treatment of NOD mice. All recombinant viruses expressed the CTB::GAD protein and IL-10 in rVV-infected CV-1 cells under control of the strong vaccinia E/L promoter (Fig. 1). Biosynthesis of the recombinant proteins was monitored following rVV infection as described previously.⁴⁸ Infection of CV-1 cells with rVV expressing CTB::GAD showed that the fused proteins assembled into pentameric structures with GM1 ganglioside membrane receptor binding activity.⁴⁹

Hyperglycemia detection in NOD mice

Following rVV, plasmid, and PBS (mock-infected) inoculation, the frequency of hyperglycemia increased in all experimental and control NOD mouse groups from 12 to 36 weeks of age (Table 1 and Fig. 2A). This study included three NOD mouse control groups considered to be naive regarding their therapeutic capabilities: mice inoculated with (1) PBS, (2) rVV-L15 viral vector, and (3) pWS4 plasmid vector. The incidence of diagnosed diabetes in control animal groups rose steadily throughout the duration of the immunization experiment until 32–36 weeks of age and thereafter plateaued in the range of 54.5–80% throughout the remainder of the experiment (Fig. 2A).

Prediabetic NOD mice inoculated with rVV expressing CTB::GAD or the suppressor cytokine IL-10 alone, while predicted to show a decrease in the incidence of diabetes, showed no significant effect on the prevention of hyperglycemia in comparison with control mice (Fig. 2A and B). However, mice co-inoculated with rVV-CTB::GAD and rVV-IL10 developed diabetes at the level of 20% up to 28 weeks of age. After 28 weeks, diabetes incidence and glucose levels remained unchanged until the end of experiment (64 weeks) (Fig. 2A, C). The highest blood glucose levels (averaging at approximately 28 mmol/L) were detected in naive mice inoculated with the empty plasmid pWS4. The lowest,

FIG. 2. Incidence of diabetes in non-obese diabetic (NOD) mice. (A) Comparison of immunological suppression protocols on diabetes onset in NOD mice as determined by increased incidence of hyperglycemia. Treatment with recombinant vaccinia virus (rVV)-cholera toxin B subunit (CTB)::glutamic acid decarboxylase (GAD) demonstrated little, if any, suppression of diabetes development, but co-inoculation with rVV-CTB::GAD + rVV-interleukin-10 (IL10) reduced new-onset diabetes to 20% within the multicomponent vaccine experimental group. Statistically significant differences between this and the phosphate-buffered saline (PBS) (mock-infected) control group during the experiment are designated (*). Differences between pWS4 and PBS, rVV-L15, or rVV-CTB::GAD treatment groups are statistically insignificant. (B) Comparison of rVV-IL10-inoculated mice with mock-infected naive mice shows the difference in diabetes onset (hyperglycemia) at 31 weeks of age. However, the IL10-mediated reduction from 70% to 40% was statistically insignificant. (C) Blood glucose levels in all experimental animal groups were monitored biweekly. Each data point represents the average blood glucose determination for all mice in that group. Blood glucose levels gradually increased throughout the course of the experiment until 42 weeks of age, at which time no significant increase in amount of glucose in the blood was detected throughout the remainder of the experiment. The blood glucose level varied from 3.4 to 27.5 mmol/L. Highest blood glucose levels were detected in naive mice inoculated with empty vaccinia plasmid pWS4, reaching average levels of about 28 mmol/L. Lowest and most significant levels of normal blood glucose maintenance were found in NOD mice inoculated with rVV-CTB::GAD + rVV-IL10, with almost normal levels of 8 mmol/L.

FIG. 2. (Continued).

approximately normal, levels of blood sugar (averaging at about 8 mmol/L) were identified in experimental animal groups treated with rVV-CTB::GAD + rVV-IL10 (Fig. 2C).

Development of insulinitis

Pancreatic islets of hyperglycemic and euglycemic mice were analyzed separately. Mice of all groups that developed diabetes, in which the blood glucose level reached 33 mmol/L concentration, were sacrificed, and the extent of lymphocyte islet infiltration was evaluated. As expected, all of the analyzed mice developed a high intra-islet insulinitis score of 4 (data not shown). Mice from the four experimental groups that did not develop hyperglycemia (euglycemic mice) were sacrificed at the end of experiment (64 weeks of age), and the lympho-

cyte islet infiltration of each mouse was analyzed (Fig. 3). Of 21 analyzed euglycemic mice, 10 (48%) showed no sign of insulinitis (score 0), whereas seven mice (33%) had peri-islet insulinitis of scores of 1 and 2. Four mice (19%) developed intra-islet insulinitis scores of 3 and 4. Images of representative stained pancreas sections with insulinitis scores from 0 to 3 are shown in Figure 3a. Of note is that euglycemic mice from the rVV-CTB::GAD + rVV-IL10 treatment group were found to appear healthier than those of other groups. Two-thirds of this group (six mice) were shown to be insulinitis-free (score = 0), and only one-third (three mice) had peri-insulinitis of scores of 1–2 (Fig. 3b). In contrast, within the rVV-CTB::GAD group, the mice analyzed had insulinitis scores ranging from 1 to 4. In both the PBS and rVV-L15 control groups, two of four mice were insulinitis-free (score = 0), whereas the remaining two mice in

a

FIG. 3. Histological analyses of euglycemic non-obese diabetic (NOD) mice. (a) Images of typical stained sections of pancreatic islets. (A) Islets with an insulinitis score of 0 with no effector lymphocytes in the vicinity of the islets isolated from a recombinant vaccinia virus (rVV)-cholera toxin B subunit (CTB)::glutamic acid decarboxylase (GAD) + rVV-interleukin-10 (IL10)-treated mouse. (B and C) Islets with insulinitis scores 1 (8%) and 3 (42.5%), respectively, were isolated from rVV-CTB::GAD-treated euglycemic mice. (D) Islets with an insulinitis score of 3 (52.2%) isolated from a control phosphate-buffered saline (PBS)-treated euglycemic mouse. Autoreactive lymphocytes can be seen as small dark stained cells (shown by arrows in B–D). Magnification $\times 400$. Morphometry of pancreatic islets was performed as described in Materials and Methods. (b) Histological analysis of insulinitis in mice at the end of the hyperglycemia study. Data on pancreatic tissue cross-sections of euglycemic mice of different treatment groups obtained from mice at 64 weeks of age are shown. Altogether, 21 mice (presented in the table below the graph) were analyzed and scored for insulinitis: four mice of the PBS and rVV-L15 control groups and the rVV-CTB::GAD treatment group and nine mice of the rVV-CTB::GAD + rVV-IL10 group. Blood glucose concentrations of individual (in mmol/L) or grouped (mean $\pm$ SD, in mmol/L) mice are also presented.

each group generated scores of 1 and 3 (Fig. 3b). Thus, mice from experimental groups that have similar hyperglycemia outcomes following PBS, rVV-L15, and rVV-CTB::GAD treatment (see Fig. 2), in spite of their euglycemic status, were shown to have detectable levels of pancreatic islet inflammation. The level of inflammation was shown to be significantly less pronounced in euglycemic mice from the rVV-CTB + rVV-IL10 experimental group, which was characterized with lower levels of diabetes (see Fig. 2).

Analysis of splenocyte secreted IL-10 and IFN- γ

Unexpectedly, splenocytes isolated from 64-week-old surviving PBS-treated control mice stimulated with GAD

protein secreted the highest detectable levels of inflammatory IFN- γ and immunosuppressive IL-10 cytokine secretion (Fig. 4A, C). The highest detectable level of IFN- γ secretion was found to be only about half of the level of IL-10 secreted by PBS-inoculated mice. The experimental mouse group inoculated with rVV-CTB::GAD + rVV-IL10 showed lower but measurable levels of splenocyte IFN- γ secretion. In contrast, splenocytes isolated from mice of other experimental groups, including mice inoculated with rVV-CTB::GAD, secreted virtually undetectable levels of IFN- γ . The pattern of IFN- γ secretion in mouse groups that did not receive GAD protein stimulation remained essentially the same as that in mouse groups that received GAD stimulation. However, the level of IFN- γ secretion was somewhat

FIG. 3. (Continued).

higher in GAD-stimulated experimental animal groups (Fig. 4B).

Mice co-vaccinated with rVV-CTB::GAD + rVV-IL10 were found to be the second highest IL-10 secreting group, followed by the rVV-CTB::GAD treatment group, which secreted IL-10 approaching baseline levels. Experimental animal groups inoculated with either rVV-L15 or the plasmid pWS4 secreted only baseline levels of IL-10. The same pattern of IL-10 secretion levels was observed in splenocytes isolated from experimental animal groups that were not incubated with GAD protein prior to cytokine analysis. One exception was the experimental group inoculated with plasmid pWS4: the two animals secreted approximately 1,600 pg/mL IL-10, which substantially exceeded the levels of IL-10 (500–700 pg/mL) secreted by the three mice inoculated with rVV-CTB::GAD (Fig. 4D).

Discussion

In our earlier animal immunization experiments, juvenile prediabetic NOD mice inoculated orally or systemically with

rVV-CTB::GAD showed reduced levels of pancreatic islet inflammation and hyperglycemia in comparison with virus-delivered GAD alone, which failed to provide a beneficial effect.^{48,49} However, in the present study, the incidence of diabetes in rVV-CTB::GAD-treated NOD mice was similar to that observed in the negative control experimental groups. Furthermore, intraperitoneal injection of rVV-IL10 expressing the anti-inflammatory cytokine IL-10 caused a small but statistically insignificant reduction in both pancreatic islet inflammation and hyperglycemia (Fig. 2B). However, a multifactorial approach of systemic co-inoculation of juvenile NOD mice with both rVV-CTB::GAD and rVV-IL10 demonstrated a synergistic decrease in hyperglycemia and pancreatic islet inflammation. Thus, our experimental results indicate that only combined vaccination with these two viral constructs provides a significant reduction in diabetes onset in NOD mice. It is important that no substantial increase in blood sugar levels or appearance of hyperglycemia was detected in any mouse starting from 36 weeks of age until sacrifice of the remaining surviving mice at 64 weeks. Our results suggest that immunological homeostasis was re-established in the immunized

FIG. 4. Cytokine secretion in splenocytes isolated from euglycemic non-obese diabetic (NOD) mice. Splenocytes were isolated from minced spleen tissues of 64-week-old immunized NOD mice and incubated in culture medium (A and C) containing glutamic acid decarboxylase (GAD) protein for 72 h or (B and D) in the absence of the autoantigen prior to enzyme-linked immunosorbent assay of the cell-free culture medium for the presence of secreted inflammatory or anti-inflammatory cytokines. In (A), the highest levels of interferon (IFN)- γ were detected in phosphate-buffered saline (PBS)-inoculated mice and then in mice inoculated with recombinant vaccinia virus (rVV)-cholera toxin B subunit (CTB)::GAD + rVV-interleukin-10 (IL10). Low levels of IFN- γ were detected in mice inoculated with empty plasmid pWS4, VV strain L15, or CTB::GAD. In (B), a distinct reduction in levels of IFN- γ was detected in all experimental groups in which the splenocytes were not stimulated with GAD protein. Reduced IFN- γ secretion was detected in PBS and rVV-CTB::GAD + rVV-IL10 experimental groups, but almost no increase above background levels of IFN- γ was detected in splenocytes from all other experimental groups incubated in the absence of GAD stimulation. The horizontal bar represents the mean of the sample values of each experimental group. In (C, D), splenocytes isolated from surviving 64-week-old mice were analyzed for the presence of secreted IL-10 after incubation in culture medium for 72 h in the (C) presence or (D) absence of GAD protein stimulation. In comparison with secreted IFN- γ levels, the amounts of secreted IL-10 were substantially greater in all autoantigen-stimulated experimental animal groups. In (C), maximum secretion of IL-10 was detected in mice inoculated with PBS and then in mice inoculated with rVV-CTB::GAD + rVV-IL10. Animal groups inoculated with empty plasmid pWS4 or VV strain L15 secreted almost undetectable levels of IL-10. In (D), a similar pattern but somewhat higher levels of IL-10 secretion were detected in all remaining experimental animal groups that were not stimulated with GAD autoantigen.

mice by 28–36 weeks of age. The mechanism underlying the observed synergy of two viral constructs observed following their delivery to the mice remains to be elucidated.

An interesting phenomenon was observed when the level of insulinitis was analyzed in pancreatic islets of euglycemic

mice that by the end of the experiment had not developed hyperglycemia (see Fig. 2A, B). At least 50% of euglycemic NOD mice in the PBS, rVV-L15, and rVV-CTB::GAD treatment groups developed early stages of intra-insulinitis and eventually would have developed diabetes. Analysis of eu-

glycemic mice in the rVV-CTB::GAD + rVV-IL10 treatment group showed much better prognosis for cure as only one-third of the mice in this group displayed early stages of insulinitis (scores of 1 and 2), whereas the majority (two-thirds) of the mice remained insulinitis-free (score 0). Thus, rVV-CTB::GAD + rVV-IL10 co-inoculated mice showed a significant and lasting reduction in both hyperglycemia and insulinitis.

Our results from VV-delivered IL-10 are inconsistent with earlier data that suggested delivery of the IL-10 gene and IL-10 protein or virus-mediated delivery of IL-10 may delay the appearance of diabetes symptoms and reduce diabetes incidence in NOD mice.^{53–61} These studies remain somewhat in question as alternative experiments showed that in IL-10 knockout NOD mice and in transgenic mice overproducing IL-10, the protection of exogenously added IL-10 against development of hyperglycemia has so far remained unconfirmed.⁵⁹ Moreover, there is some indication that the overproduction of IL-10 may contribute detrimental effects.^{62,63} Furthermore, the time and site of IL-10 administration in addition to the duration and level of IL-10 transgene expression may exert additional and potentially conflicting effects on IL-10-mediated diabetes suppression.⁶⁴

Pancreatic islet cells isolated from NOD mice were shown to express IL-10 at early prediabetes stages. Later, after 14 weeks of age, corresponding to the onset of hyperglycemia, IL-10 expression was down-regulated,⁶⁵ which suggests that the concentration and early delivery of exogenous IL-10 addition may be critical for modulation of diabetes development.^{58,66} Our cytokine analysis of splenocytes performed at the end of experiment (at 64 weeks of age) seems to be in agreement with this proposal (Fig. 4).

Splenocytes isolated from mice inoculated with empty virus, empty plasmid, or rVV-CTB::GAD alone secreted low to undetectable levels of the inflammatory cytokine IFN- γ , whereas splenocytes of mock-infected mice secreted high levels of IFN- γ (Fig. 4). This result is in agreement with previous findings that VV infection of dendritic cells resulted in antigen-presenting cells that did not secrete inflammatory cytokines or initiate T-cell activation.⁶⁷ However, data on diabetes incidence presented in Figure 2A indicate that all three animal groups developed diabetes similar to that of mock-infected mice, and thus virus infection (or plasmid delivery) by itself does not suppress pancreatic islet inflammation. Interestingly, splenocytes isolated from mock-infected mice stimulated with GAD protein at 64 weeks of age showed significantly higher levels of IFN- γ expression than splenocytes from unstimulated mice, suggesting that adjuvant-linked autoantigens may stimulate strong immune suppression responses during later stages of life as well. Furthermore, in spite of significant differences in diabetes levels between NOD mice co-inoculated with VV expressing CTB::GAD and IL-10 proteins and mock-infected mice, splenocytes representative of these two groups of animals secreted similar levels of IL-10 (1,500–3,500 pg/mL and 1,500–4,000 pg/mL, respectively) at 64 weeks of age. Results suggest the functional role of VV-mediated cytokines may be restricted to early stages (1–36 weeks) of NOD mouse development. Thereafter, alternative IL-10-dependent or -independent pathways may be involved in suppression of islet inflammation. A recent study confirmed that intraperitoneal inoculation of mice with recombinant virus carrying the *IL-10*

gene generated a measurable concentration of intraperitoneal IL-10 only for up to 3 weeks following inoculation.⁶⁸ Other laboratories have reported detection of measurable levels of endogenous IL-10 in NOD mouse pancreatic tissues only until 14 weeks of age.⁶⁵

Interestingly, high levels of IL-10 secretion but no suppression of diabetes onset was observed in pWS-4 plasmid-inoculated control mice (Fig. 2A), which is in agreement with our additional data that IL-10 alone is unable to suppress hyperglycemia (Fig. 2A). Thus, these data also support our observation that inoculation with both CTB::GAD and IL-10 is required for immunological suppression of type 1 diabetes. Human IL-10 is a homodimeric anti-inflammatory cytokine protein with a subunit length of 160 amino acids and 73% amino acid homology with its murine form.^{69,70} The anti-inflammatory cytokine IL-10 is produced primarily by monocytes and to a lesser extent by mast cells and lymphocytes.⁷¹ IL-10 demonstrates pleiotropic effects in both immunoregulation and inflammation. The immunosuppressive cytokine down-regulates expression of Th1 cytokines, major histocompatibility complex class II antigens, and co-stimulatory molecules on macrophages, although it also enhances B-cell survival and proliferation and antibody production. It is generally known that IL-10 can block nuclear factor κ B activity and is involved in the regulation of the Janus kinase-signal transducers and activators of transcription signaling pathway.

In earlier experiments, adenovirus and adeno-associated viruses were used to deliver islet autoantigens and the immunomodulatory cytokine IL-10, resulting in a reduction in the frequency of hyperglycemia in diabetic mice.^{55–58,60,72–74} More closely related to this work, in a transgenic, lymphocytic choriomeningitis virus-associated mouse model of diabetes, VVs expressing lymphocytic choriomeningitis virus autoantigens were able to suppress diabetes onset.^{75,76} Furthermore, VVs expressing GAD were shown to partially prevent diabetes in NOD mice.⁷⁷ However, VV-GAD inoculation experiments performed in our laboratory did not confirm this result.⁴⁹ We also previously demonstrated that systemic delivery of rVV expressing the immunomodulated autoantigen insulin (CTB::INS) can provide significant protection against development of T1D in NOD mice.⁴⁹ Mucosal immunization with rVV induced a potent immune response against viral antigens in mice, especially following co-immunization with cholera toxin as an adjuvant.⁷⁸ Recently, vaccination of BALB/c mice with a recombinant modified VV strain Ankara prevented the onset of allergen-specific sensitization to ovalbumin.⁷⁹ It is noteworthy that, in spite of induction of the antiviral humoral immune response as we have observed in mice in 2 weeks after VV infection,⁸⁰ VV infection alone has no effect on diabetes progression in NOD mice (see VV-L15 in Fig. 2A, C).

Based on their wide host range, rapid infection, efficient expression of transgenes, and production of foreign proteins that undergo mammalian posttranslational modification, vaccinia is an attractive virus vehicle for transgene delivery into a variety of eukaryotic cells for vaccination against animal and human infectious diseases.^{81–84} Cytoplasmic replicating VV leaves no residual DNA integration into the host genome and was shown to provide effective delivery of CTB-autoantigen fusion genes for expression in prediabetic NOD mice. An additional attractive feature of VV as a vaccine

delivery vehicle is that multiple antigens or autoantigen genes can be inserted into dispensable regions of the vaccinia genome without causing adverse effects on virus growth and multiplication. The relative safety of live VV vaccines for human applications was clearly demonstrated in a major smallpox eradication program.⁸³ To reduce potential side effects of live attenuated VV vaccines in immunocompromised individuals, the virus can be further attenuated by genetic manipulation.^{80,85} Thus, the data reinforce the potential for application of VV as a vehicle for autoantigen-mediated suppression of inflammatory immune responses.

Molecular mechanisms involved in immune suppression detected in NOD mice treated with rVV-CTB::GAD + rVV-IL10 remain to be elucidated. However, partial immune suppression may be due to elevated dendritic cell or lymphocyte IL-10 secretion. The elevated IL-10 levels would be expected to promote inhibition of dendritic cell maturation, enhancement of anti-inflammatory Th cell cytokine responses, and possibly the induction of regulatory T-cell populations capable of suppressing autoreactive Th1 lymphocyte activation and proliferation. The notion that exogenous IL-10 may inhibit dendritic cell maturation was further supported by additional experiments that showed dendritic cells transfected with the *IL-10* gene inhibited Th1 cell-mediated alloimmune responses and promoted immunological tolerance for diabetes inhibition.⁸⁶ Also, immature dendritic cells may secrete IL-4 and IL-10 anti-inflammatory cytokines in amounts sufficient to suppress insulinitis and hyperglycemia, thereby maintaining immunological homeostasis. Furthermore, increased levels of diabetes immunosuppression following co-delivery of CTB::GAD and IL-10 suggest that cognate naive Th0 lymphocytes that recognize CTB and bind to dendritic cells may develop into immunosuppressive Th2 cells or Tr1 regulatory T cells, which may help to suppress cytotoxic T-cell expansion and activation leading to down-regulation of the immune response.⁸⁷

Conclusions

The experimental results presented here demonstrate that systemic delivery of rVV expressing the immunostimulated autoantigen CTB::GAD in combination with virus delivery of the immunosuppressive cytokine IL-10 can provide effective and durable protection against T1DM onset in NOD mice. Further optimization of rVV dosage along with IL-10-mediated anti-inflammatory responses may provide complete protection of prediabetic NOD mice against the progression of insulinitis and hyperglycemia. An important goal of this study was to explore the duration of the therapeutic effects of vaccination. We found that the majority of CTB::GAD + IL-10-treated mice remained euglycemic at least up to 64 weeks of age, whereas most control mice developed hyperglycemia by 32 weeks of age. Insulinitis and cytokine analyses performed at the end of the experiment (64 weeks) allowed us to gain some important information concerning the state of T1DM in phenotypically healthy surviving NOD mice. Modulation of the immune response is a complex set of conditions with many players involved. Thus, further studies are required to better understand the molecular mechanisms leading to the development of T1DM. First, of perhaps greatest interest, molecular mechanisms involved in immunosuppression

at early stages (12–20 weeks of age) after vaccination will be explored. Second, the therapeutic effect of vaccination strategy will be assessed in NOD mice that have developed insulinitis and hyperglycemia by 15–20 weeks of age.

This “immunization” method could be supplemented in the future with our viral construct rVV-CTB::INS, which demonstrated a dramatic suppression of new diabetes onset in NOD mice. The effectiveness of this VV immunostimulated autoantigen/IL-10 multicomponent immunosuppression strategy for arresting or reversing the progression of diabetes symptoms of insulinitis and hyperglycemia remains to be determined. However, if the proposed immunological suppression strategy is found to be less effective in human trials, this strategy may be coupled with transplanted pancreatic islet engraftment^{87,88} and mesenchymal stem cell therapies^{87,89} as additional promising cellular alternatives for diabetes prevention or cure. Thus, it is conceivable that VV-delivered immunomodulated autoantigens and IL-10 could be combined to arrest diabetes progression coupled with pancreatic islet grafts or stromal stem cells to repopulate the pancreas with “beta-like” cells to provide an effective and durable interventional therapy for restoration of euglycemia and immunological homeostasis in the large patient population suffering from T1DM.

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Author Disclosure Statement

No competing financial interests exist.

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