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Cholera Toxin B Subunit Linked to Glutamic Acid Decarboxylase Suppresses Dendritic Cell Maturation and Function

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Abstract

Dendritic cells are the largest population of antigen presenting cells in the body. One of their main functions is to regulate the delicate balance between immunity and tolerance responsible for maintenance of immunological homeostasis. Disruption of this delicate balance often results in chronic inflammation responsible for initiation of organ specific autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and type I diabetes. The cholera toxin B subunit (CTB) is a weak mucosal adjuvant known for its ability to stimulate immunity to antigenic proteins. However, conjugation of CTB to many autoantigens can induce immunological tolerance resulting in suppression of autoimmunity. In this study, we examined whether linkage of CTB to a 5 kDa C-terminal protein fragment of the major diabetes autoantigen glutamic acid decarboxylase (GAD₃₅), can block dendritic cell (DC) functions such as biosynthesis of co-stimulatory factor proteins CD86, CD83, CD80 and CD40 and secretion of inflammatory cytokines. The results of human umbilical cord blood monocyte-derived DC - GAD₃₅ autoantigen incubation experiments showed that inoculation of immature DCs (iDCs), with CTB-GAD₃₅ protein dramatically suppressed levels of CD86, CD83, CD80 and CD40 co-stimulatory factor protein biosynthesis in comparison with GAD₃₅ alone inoculated iDCs. Surprisingly, incubation of iDCs in the presence of the CTB-autoantigen and the strong immunostimulatory molecules PMA and Ionomycin revealed that CTB-GAD₃₅ was capable of arresting PMA + Ionomycin induced DC maturation. Consistent with this finding, CTB-GAD₃₅ mediated suppression of DC maturation was accompanied by a dramatic decrease in the secretion of the pro-inflammatory cytokines IL-12/23p40 and IL-6 and a significant increase in secretion of the immunosuppressive cytokine IL-10. Taken together, our experimental data suggest that linkage of the weak adjuvant CTB to the dominant type 1 diabetes autoantigen GAD strongly inhibits DC maturation through the down regulation of major co-stimulatory factors and inflammatory cytokine biosynthesis. These results emphasize the possibility that CTB-autoantigen fusion proteins enhance DC priming of naïve Th0

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cell development in the direction of immunosuppressive T lymphocytes. The immunological phenomena observed here establish a basis for improvement of adjuvant augmented multi-component subunit vaccine strategies capable of complete suppression of organ-specific autoimmune diseases *in vivo*.

Keywords

adjuvant; autoimmunity; cholera toxin-B (CTB); dendritic cells; IDDM; juvenile diabetes

Introduction

TYPE 1 DIABETES MELLITUS (T1D) is an organ-specific autoimmune disease that results from autoreactive lymphocyte destruction of pancreatic islet insulin-producing beta cells[1, 2]. The progressive loss of islet β -cell function leads to insulin deficiency and resultant elevated blood sugar levels (hyperglycemia). The appearance of hyperglycemia increases levels of cellular oxidative stress leading to chronic inflammation throughout the body. Therefore, type 1 diabetics are not only subject to acute glucose dysregulation that can result in coma and death, but have an increased premature risk for secondary neural and circulatory pathologies, including blindness, heart attack, and stroke[3].

Dendritic cells (DCs), the most abundant subset of professional antigen presenting cells, are implicated in mediating islet β -cell destruction in T1D. Through antigen presentation, DCs play a key role in the polarization and development of naïve T helper cells (Th0) into autoreactive Th1 lymphocytes or immunosuppressive Th2 cells. This process is critical for maintenance of immunological homeostasis[4-6]. Maturation of DCs in response to “self” antigens such as pancreatic islet beta cell proteins induces the development of autoreactive CD8+ and CD4+ T helper (Th1) lymphocytes in addition to initiating B cell production of autoantibodies. Subsequently, autoreactive Th1 cells infiltrate the pancreatic islets in non-obese diabetic (NOD) mice while secreting inflammatory cytokines such as IFN- γ and IL-2. These cytokines are known to stimulate macrophage and CTL secretion of oxidative compounds NO, O₂, H₂O₂ in addition to other inflammatory cytokines (IL-1 β , TNF- α , TNF- β)[5-7]. Consequently, these immune responses induce chronic pancreatic inflammation (insulinitis), resulting in apoptosis of the islet β -cells and insufficient levels of insulin production and availability[7, 8].

In addition to induction of conventional Th1 and Th2 lymphocyte morphogenesis and proliferation, DCs can also direct the development of adaptive regulatory T cells (iTregs) required for the maintenance of immunologic tolerance[9]. Immuno-cytochemical analyses have shown that M cells in the intestinal epithelium uptake autoantigens for presentation to peripheral DCs via several routes which may aid in the establishment of immune tolerance[8, 10, 11]. Following activation, migratory DCs travel to adjacent lymph nodes where they present antigen peptides generally on MHCII receptors, synthesize costimulatory factor proteins, and secrete inflammatory or anti-inflammatory cytokines. Together, these three molecular signals guide the morphogenesis of naïve cognate Th0 cells into Ag-specific inflammatory effector Th1 lymphocytes or anti-inflammatory Th2 lymphocytes. Immature peripheral DCs (iDCs), that display high levels of costimulatory molecule expression and secrete cytokine IL-12, were found to induce Th1 lymphocyte differentiation; while those that displayed low levels of costimulatory molecule expression and secreted the anti-inflammatory cytokine IL-10 induced the development of Th2 lymphocytes[12-15].

Oral delivery of autoantigens such as insulin and glutamic acid decarboxylase (GAD) resulted in partial suppression of diabetes symptoms in diabetic animals[16, 17]. However,

this form of anti-inflammatory molecular therapy requires repeated autoantigen administration over an extended period of time. Therefore, adjuvants were considered for further enhancement of autoantigen therapy for prevention of T1D onset[16, 18]. The cholera enterotoxin B subunit (CTB) mucosal adjuvant from *Vibrio cholerae*, was shown to significantly stimulate induction of oral tolerance in T1D when physically conjugated with islet autoantigens[19-22]. In terms of the mechanism of adjuvant function, the CTB subunit was shown to bind specifically to GM1-ganglioside (GM1), a receptor molecule found commonly embedded in the plasma membrane of most types of epidermal cells. The interaction of CTB with GM1 was shown to facilitate entry of the holotoxin into the cell[23]. Thus, CTB was considered by many investigators to be an efficient trans-mucosal carrier molecule for autoantigen induction of peripheral tolerance[19, 24]. Further experiments in our laboratory compared the efficacy of CTB to several additional enterotoxin B subunits[25]. The results of these experiments revealed that enterotoxin B subunits from different bacterial as well as plant sources were approximately equal in their efficacy for stimulating autoantigen mediated partial suppression of diabetes onset.

To identify mechanisms underlying early CTB-autoantigen interactions with cells of the immune system responsible for initiation or suppression of T1D onset, we investigated DC interactions with CTB - autoantigen fusion proteins[26]. While it is generally accepted that fusion of CTB with autoantigenic proteins results in enhanced levels of immune suppression, several exceptions have been shown to occur in which specific CTB-autoantigen fusion proteins appear to stimulate rather than suppress autoimmunity^{27,28}. Thus, in addition to establishment of mechanisms underlying CTB-GAD autoantigen fusion protein - DC interactions, these experiments will provide a basis for development of combinatorial CTB-GAD/Insulin fusion protein therapies for additive or synergistic enhancement of diabetes suppression.

Materials and Methods

Expression and purification of CTB-GAD₃₅ Fusion Proteins in *E. coli*

A cDNA fragment encoding a 5-kDa peptide (amino acid 509-543) proximal to the carboxyl-terminus of GAD₆₅—designated GAD₃₅ was genetically linked to the carboxyl-terminus (309bp) of CTB to generate the fusion gene CTB-GAD₃₅ (Figure 1a). The cholera toxin B subunit–autoantigen fusion gene CTB-GAD₃₅ was cloned into the (A) configuration of the *E. coli* expression vector pRSET (Invitrogen™, Carlsbad, CA), under control of the bacteriophage T7 promoter in order to achieve high levels of transgene expression. The pRSET vector also contains an oligonucleotide encoding 6 histidines immediately upstream of the CTB permitting nickel affinity isolation of the transgene product. Selective clones were assessed by DNA sequence analysis to confirm the in-frame linkage of CTB and CTB-GAD₃₅ DNA fragments. The recombinant vectors were introduced into *E. coli* producer strain BL21 (DE3)pLysS (Invitrogen, Carlsbad, CA) by electroporation for nickel affinity column isolation recombinant protein from the lysed cells [25].

The recombinant CTB-GAD₃₅ fusion protein expressed in *E. coli* was purified from lysed cells using metal chelation chromatography. The transformed *E. coli* BL-21 strain, was grown in 250 ml Luria Broth (LB) medium containing ampicillin (100mg/ml) with shaking at 37°C. While still in log phase of growth, CTB-GAD₃₅ protein synthesis was stimulated by addition of 90 mg isopropyl β-D-1thiogalacto-pyranoside (IPTG), (Sigma Chemical Co. St. Louis, MO), to the bacterial culture. After 6 hr continued growth at 37°C, the bacterial culture was pelleted by centrifugation in a SA-600 rotor for 10 min, at 5,000 rpm and 4°C, in a Sorvall RC5B centrifuge. The cell pellet was resuspended in 1.0 ml / tube of 10 mM HEPES buffer (pH 7.5), containing 100 mM imidazole. The cells were disrupted by sonication at 3 × 10 sec bursts at 10 W, with a Sonic 60 Dismembrator, (Fisher Sci.

Sunnyvale, CA). The CTB-GAD₃₅ protein was isolated and purified from the bacterial homogenate using a Maxwell Model 16 robotic protein purification system (Promega Inc. TM), according to the protein isolation protocol provided by the manufacturer (Promega Inc., Madison, WI). In order to obtain a pure protein product, the robot employs electromagnetically charged Magne-His Nickel-Iron alloy particles with an affinity for the 6-HIS tag linked to the N terminus of the recombinant CTB-GAD₃₅ fusion protein. Imidazole was removed from the protein mixture by dialysis of the preparation against 2 × 1.0 Liter, 10 mM HEPES buffer (pH 7.5), for 4 hr at 4°C. The purity of the isolated CTB-GAD₃₅ protein (~22kDa) was determined by electrophoretic mobility analysis in a 12% polyacrylamide gel in comparison with protein molecular weight standards (Figure 1b). The purified CTB-GAD₃₅ protein was confirmed by immunoblot analysis using an anti-polyhistidine primary antibody and stored at -20°C until further use.

Isolation and Culture of Monocyte-derived DC from Human Cord Blood

Monocyte-derived dendritic cells (MDDC) were prepared from freshly collected human umbilical cord blood. The leukocyte fraction of cord blood obtained from normal healthy placenta donors (by LLU IRB approved protocols), was separated from red blood cell and platelet fractions by Ficoll-paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation for 30 minutes at 2,000 RPM @ 4°C, in a Beckman Coulter Allegra X-15R centrifuge, equipped with a SX4750 rotor. The CD14⁺ monocytes were obtained from the total lymphocyte fraction by incubation with anti-CD14 PE (Phycoerythrin) (Miltenyi Biotech, Auburn, CA) for 10 minutes @ 4°C followed by incubation with anti-PE magnetic microbeads for 15 minutes at 4°C. The cells were separated magnetically by passing them through the MACS column as described by the manufacturer (Miltenyi Biotech, Auburn, CA) [29, 30]. The purity of the monocyte fraction was determined by flow cytometry in a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

The isolated monocytes were cultured in 6 cm non-pyrogenic polystyrene culture plates in RPMI 1640 culture medium (Mediatech Inc. Manassas, VA, USA) in a humidified atmosphere of 5% CO₂ at 37 °C (Preprotech, Rocky Hill, NJ). The medium was supplemented with 10% FBS, 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml human recombinant GM-CSF, and 10 ng/ml human recombinant IL-4 (ProSpec-Tany TechnoGene, Rehovot Science Park, Israel). The monocyte cell culture was fed every 2 days by gentle replacement of 50% of the medium with fresh medium. The cell cultures were kept in their original plates until harvested to avoid induction of DC maturation caused by mechanical stress associated with replating the cells. The percentage of monocyte-derived immature dendritic cells (M-iDC) was determined directly by flow cytometry based on expression of DC specific cell surface markers (CD14⁺HLA-DR⁺CD11c⁺) after 6 days further incubation.

DC Maturation Assay and Phenotyping

Immature dendritic cells (iDCs) were stimulated by the addition of CTB (10ug/ml), GAD₃₅ (10ug/ml), CTB-GAD₃₅ fusion protein (20ug/ml) and Phorbol myristate acetate (PMA) and Ionomycin at 10ng/ml (PMA+Ionomycin) for 48hrs at 37 degrees, 5% CO₂. After incubation, the expression of DC surface markers indicating their state of activation and maturation (CD14, CD11c, HLA-DR), was determined by flow cytometry. The gated CD14⁺ HLA-DR⁺ CD11c⁺ cell population was analyzed for the expression of CD86, CD83, CD80, and CD40 activation markers (BD Pharmingen, San Jose, CA USA).

Flow Cytometry Analysis

For flow cytometry examination of DC surface markers, the DCs were surface stained with antibodies conjugated to Phycoerythrin (PE), Fluorescein isothiocyanate (FITC),

Allophycocyanin (APC), and Peridinin Chlorophyll Protein Complex (PerCP) [31] and resuspended in 1.0% of paraformaldehyde for analysis using the FACSCalibur flow cytometer. The following antibodies were used to identify the different lymphocyte types: anti- CD86-FITC, anti- CD83-PE, anti- CD40-FITC, anti- CD80-PE, anti- HLA-DR PerCP anti- CD14-APC anti- Mouse IgG1 FITC, anti- Mouse IgG1 PE, anti- Mouse IgG1 HLA-DR, anti- Mouse IgG1 APC (BD Pharmingen); 7-Amino-actinomycin D (7-AAD) was used to assess viability of the collected cells. Briefly, a pooled sample of harvested cells was incubated with 7-Amino-actinomycin D (7-AAD) and prepared for flow cytometry according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Fluorescence intensity on all flow histograms is shown on a log scale.

Secreted Cytokine Analysis by Cytometric Bead Assay

Immature DCs were stimulated with PMA and Ionomycin and the recombinant proteins for 48 hours as described above. The supernatant was collected and stored at -20°C until analyzed for concentrations of IL-6, IL-10 and IL-12/23p40 using the cytometric Bead Array (CBA) kit (Becton Dickinson Biosciences, San Jose, CA, USA). Premixed beads (50 μl), coated with capture antibodies were incubated with 50 μl of cytokine standards or test samples in the dark at room temperature for 1 hour. Following the incubation, 50 μl of a mixture of Phycoerythrin-conjugated antibodies prepared against the specific cytokines was added to each preparation, and the samples incubated for 2 hours in the dark at room temperature. The beads were washed once with the wash buffer supplied with the CBA kit and analyzed immediately in a BD FACSCalibur flow cytometer (Becton, Dickinson Inc.). Data analysis was performed using the FCAP Array software packages supplied by BD Biosciences. Three thousand counting events were acquired from each sample. Cytokine calibration curves were generated using cytokine standards supplied by BD Biosciences [32, 33].

Results

CTB-GAD₃₅ Fusion Protein Expression in E.coli

Recombinant GAD₃₅ and CTB-GAD₃₅ were identified by immunoblot with a monoclonal antibody made against the carboxyl terminal epitope of GAD₆₅. The CTB-GAD₃₅ protein was identified in both monomeric (~22kDa) and multimeric form (Figure 1b). Identification of GAD₃₅ and CTB-GAD₃₅ was confirmed by immunoblot analysis using an anti-polyhistidine primary antibody.

CTB-GAD₃₅ Suppresses Monocyte-derived Dendritic cell Activation and Maturation

We examined the influence of CTB, GAD₃₅, CTB-GAD₃₅ and LPS on immature DC maturation by measuring the surface expression of costimulatory molecules CD86, CD83, CD80 and CD40. Immature dendritic cells (iDC) were differentiated from monocytes and assessed for the expression of CD14-HLA-DR+CD11C+ surface markers indicating DC differentiation from monocytes. Culture-generated iDCs (CD14-HLA-DR+CD11C+) were stimulated with CTB, GAD₃₅ and CTB-GAD₃₅ fusion proteins and assessed for surface expression of CD86, CD83, CD80 and CD40. Flow cytometric data revealed that iDC exposure to CTB-GAD₃₅ resulted in a significantly lower expression of CD86, CD83, CD80 and CD40 costimulatory factors as compared with CTB, GAD₃₅, and LPS. These results suggest that the physical linkage of CTB to the GAD₃₅ autoantigen is responsible for suppression of dendritic cell activation and maturation by CTB-GAD₃₅.

CTB-GAD₃₅ downregulates PMA and Ionomycin induced DC surface co-stimulatory molecule expression

Based on the data obtained showing that CTB-GAD₃₅ suppresses DC maturation, we further assessed the capacity of CTB-GAD₃₅ to prevent activation of DCs in the presence of PMA and Ionomycin stimulation. Separate cultures of DCs were treated with PMA and Ionomycin alone and a combination of PMA and Ionomycin plus CTB-GAD₃₅. In preliminary experiments, 24-h and 48-h treatments were tested. The experimental results showed that the inhibitory effects of CTB-GAD₃₅ on PMA and Ionomycin DCs were more evident during the first 24 hours (data not shown). Therefore, in subsequent experiments, we used the 24-hr time points. As shown in figure 3, CTB-GAD₃₅ induces a statistically significant reduction of PMA and Ionomycin induced up-regulation of CD86, CD83 and CD80 assessed by mean fluorescence intensity measurements ($p=0.05$).

CTB-GAD₃₅ Suppresses DC pro-inflammatory Cytokine secretion

In these experiments, we determined whether CTB-GAD₃₅ suppression of DC maturation (suppression of costimulatory factor surface expression) resulted in a decreased synthesis of IL-12/23p40 and IL-6, along with an increased secretion of immunosuppressive cytokine IL-10. The culture medium was collected from DCs exposed to 48hrs incubation with CTB-GAD₃₅ fusion protein and analyzed for the presence of secreted cytokines by Cytometric bead array based flow cytometry. The results of this experiment showed that there was a significant decrease in pro-inflammatory cytokines IL-12/23p40 and IL-6 with a concomitant increase in IL-10 production by DCs incubated with CTB-GAD₃₅ (Figure 4). Taken together, the observed down regulation of co-stimulatory surface marker expression, the decrease in secreted IL-12/23p40 and IL-6 inflammatory cytokines, and the increased secretion of the anti-inflammatory cytokine IL-10, demonstrate that CTB-GAD₃₅ can suppress DC maturation and activation.

Discussion

Using a CTB-insulin fusion protein, Bergerot et. al., demonstrated sustained prevention of diabetes onset in NOD mice as late as 15 weeks after birth. Further, the protective effect against development of insulinitis was shown to be transferable to un-inoculated NOD mice through adoptive transfer of CD4⁺ T cells from CTB-INS inoculated animals[34]. Experiments in our laboratory have shown that inoculation with CTB-autoantigen fusion proteins suppresses insulinitis and autoimmune diabetes in NOD mice[34-36]. Specifically, our animal immunization experiments revealed that CTB-GAD₃₅ inoculated animals showed reduced levels of hyperglycemia and pancreatic islet inflammation in comparison with control animals [37, 38]. In a separate *in vivo* study, our laboratory recently demonstrated that a combinatorial DNA vaccine therapy consisting of genes encoding CTB-GAD₃₅ and IL-10 can completely suppress early onset of diabetes in NOD mice[39]. However, molecular and cellular mechanisms underlying vaccine suppression of T1D remain to be elucidated. The dominant role of DCs in the induction of inflammation and tolerance implicates them at an early stage of CTB-GAD₃₅ mediated suppression of diabetes onset.

Dendritic cell synthesis of IL-12 and expression of surface co-stimulatory molecules are responsible for induction of T cell development into pro-inflammatory autoreactive Th1 effector cells which secrete inflammatory cytokines IFN- γ and IL-2 (reference?). This T cell response is responsible to a large extent for the onset and progression of diabetes insulinitis and beta cell death [4, 6, 40, 41]. Conversely, production of IL-10 by iDCs was shown to suppress biosynthesis and secretion of pro-inflammatory cytokines and other immunostimulatory molecules (IL-12, TNF- α , IL-6, LTB4, NO, PGE2). Further, synthesis

of IL-10 was also shown to be essential for the inhibition of DC maturation by suppressing IL-12 synthesis. In addition, increased DC IL-10 production has been shown to inhibit autoreactive Th1 cell proliferation and the secretion of downstream inflammatory cytokines IFN- γ and IL-2 (reference). Here we report that the CTB-GAD₃₅adjuvant – autoantigen fusion protein stimulated iDCs to synthesize significantly higher levels of IL-10 than was observed for iDCs stimulated with CTB alone or PMA + Ionomycin.

More recently, inflammatory TH17 lymphocytes have become prominent targets in the treatment of organ specific autoimmune diseases since they were identified as a new and significant class of effector Th cells found to be involved in both human and mouse autoimmune disease models[42-44]. A major cytokine secreted by TH17 cells, interleukin-17 (IL-17), was detected in the serum and tissues of patients with autoimmune diseases such as multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)[45, 46]. In addition, IL-17 blockade was shown to prevent development of encephalomyelitis (EAE) while IL-17 knock out mice showed resistance to the development of EAE and collagen-induced arthritis (CIA)[43, 47]. Together, these findings suggest that down regulation of TH17 cell differentiation and function may prove to be essential for effective treatment for autoimmune diabetes. The inflammatory cytokine interleukin - 6 (IL-6), was shown to be an effective target for down-regulating the differentiation of TH17 cells as a treatment for autoimmune diseases[48, 49]. This inflammatory cytokine was shown to have evolved as a pleiotropic cytokine involved in regulating the balance of IL-17 producing Th17 cells and Treg cells[50, 51]. Our data show that CTB-GAD₃₅ suppresses DC synthesis of IL-6. The reduction in IL-6 biosynthesis in combination with CTB-GAD₃₅ inhibition of DC co-stimulatory factor up-regulation may be critical elements in the elucidation of cellular mechanisms responsible for CTB-GAD₃₅ mediated immune suppression of diabetes onset.

Based on our cumulative experimental data, the efficacy of autoantigens for suppression of diabetes autoimmunity appears to be significantly increased when physically conjugated to bacterial or plant enterotoxin B subunit adjuvants such as CTB. The conjugation of CTB to antigens was shown to significantly enhance the immunogenicity of linked antigens[52, 53] through induction of DC upregulation of CD86 and CD83[52, 54]. D'Ambrosio et al. demonstrated that CTB could partially prevent LPS-induced maturation of monocyte derived DCs[53]. Recently, CTB conjugated to the autoantigen proinsulin (CTB-INS) was shown to be effective in the suppression of DC surface costimulatory molecules CD86 and CD83 and stimulated a significant increase in iDC biosynthesis of IL-10 in addition to suppression of IL-12/23p40 biosynthesis[26]. Similar to D'Ambrosio's findings, our data suggest that CTB can alter iDC phenotype and function. Furthermore, CTB may possess an intrinsic capacity to modulate either inflammation or tolerance depending on whether it is conjugated to an antigen or an autoantigen. Further experiments with specific CTB-autoantigens will help to resolve the mechanism(s) underlying the duality of CTB's function as an adjuvant for enhancement of inflammatory or anti-inflammatory antigen mediated immune responses.

In this study, we found that CTB-GAD₃₅ fusion protein suppressed human iDC capacity to undergo maturation and become activated *ex vivo*. Furthermore, our data suggests that the CTB-GAD₃₅ fusion protein inhibits PMA and Ionomycin induction of DC maturation as determined by failure of the two immunostimulatory molecules to up-regulate iDC synthesis of CD86, CD83 and CD80 co-stimulatory molecules after pre-incubation with CTB-autoantigen fusion protein. This experimental result suggests that inoculation of diabetic patients with the adjuvant-autoantigen fusion protein may inhibit further progression of previously established autoimmunity. In the case of autoimmune diabetes, inhibition of DC maturation could arrest continued development of diabetes insulinitis and hyperglycemia, a

result already partially demonstrated in earlier studies from our laboratory[55, 56]. Further, the experiments presented here show that CTB-GAD₃₅ inoculated DCs are inhibited in their ability to synthesize the major pro-inflammatory cytokines IL-12/23p40 and IL-6, known to be involved in diabetes onset. This loss of inflammatory cytokine biosynthesis was further enhanced by a concomitant increase in DC synthesis of the anti-inflammatory cytokine IL-10. The results generated by previous CTB-INS studies helps to establish a molecular basis for CTB-GAD₃₅ suppression of diabetes onset in NOD mice[37, 57, 58]. In earlier studies, our laboratory demonstrated that fusion of CTB to diabetes autoantigens was essential for enhancement of immune suppression, as co-delivery of CTB and the insulin autoantigen did not result in a significant inhibition of DC activation and maturation[26]. Taken together, our experimental findings suggest linkage of CTB to diabetes autoantigens provides an effective tool for analysis of mechanisms responsible for adjuvant-enhanced immunological tolerance. Determination whether CTB-GAD₃₅ mediated immunological suppression of DCs can halt or reverse diabetes progression will establish a basis for improvement of combinatorial vaccine strategies that can prevent, interrupt or reverse autoimmunity in diabetes patients.

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List of Abbreviations

DC	dendritic cell
TLR	Toll Like receptor
T1D	type 1 diabetes
PMA	Phorbol myristate acetate
LPS	lipopolysaccharide

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Figure 1.

A. Diagram of the recombinant *E. coli* pRSET(A) expression vector. A 5kDa C-terminal cDNA fragment encoding the pancreatic islet beta cell autoantigen glutamic acid decarboxylase (GAD) was linked to the C-terminus of a cDNA fragment encoding the cholera toxin B subunit (CTB). The vector includes a bacteriophage T7 promoter to

generate high expression levels of inserted genes and a 6-histidine amino acid sequence tag for nickel affinity column isolation of the recombinant protein. (B) Immunoblot identification of renatured GAD₃₅ and CTB-GAD₃₅ subunit fusion proteins synthesized in *E. coli*. Recombinant proteins were identified with anti-His primary antibody.

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Figure 2.

The CTB-GAD₃₅ fusion protein suppresses dendritic cell maturation.. Umbilical cord derived dendritic cells were stimulated for 24hrs with the following proteins CTB, GAD₃₅, CTB-GAD₃₅ or LPS. Surface markers of DC maturation were identified by flow cytometry. The histograms depict expression of CD86, CD83, CD80 and CD40 after exogenous protein stimulation. The shaded histogram peak represents the untreated dendritic cell sample (negative control).

Figure 3.

The CTB-GAD₃₅ fusion protein suppresses PMA and Ionomycin induced dendritic cell maturation. Immature cord derived dendritic cells were stimulated with PMA and Ionomycin or a combination of PMA and Ionomycin + CTB-GAD₃₅ for 24 hrs. Dendritic cell maturation was determined based on flow cytometric analysis of CD86, CD83 and CD80 co-stimulatory factor biosynthesis. The histograms depict the mean fluorescence intensity of dendritic cell CD86, CD83, CD80 following CTB-GAD₃₅ fusion protein and PMA + Ionomycin stimulation.

Figure 4.

The CTB-GAD₃₅ fusion protein suppresses dendritic cell secretion of pro-inflammatory cytokines IL-12/23p40 and IL-6 while increasing synthesis of the anti-inflammatory cytokine IL-10. Premixed plastic beads coated with capture antibodies (BD Biosciences, San Jose, CA, USA) and a mixture of Phycoerythrin-conjugated antibodies against IL-12/23p40, IL-6 and IL-10 were incubated for 2 h with culture medium removed from DC cultures previously incubated with CTB and GAD autoantigen treatment conditions as described in the section on materials and methods. The beads were washed and analyzed by flow cytometry to determine the concentrations of IL-10, IL-6 and the IL-12/23p40 subunit present in the culture media. The cytokine concentrations in the samples were normalized to standard IL-10, IL-6 and IL-12/23p40 cytokine curves and the respective cytokine concentrations were determined in pg/ml. The data represent the Mean and SE (*P < 0.01) of repeated independent experiments in comparison with the un-inoculated cell medium control sample.