Journal of Translational Autoimmunity 3 (2020) 100065

Contents lists available at ScienceDirect

Journal of Translational Autoimmunity

journal homepage: www.journals.elsevier.com/journal-of-translational-autoimmunity/

Axenic *Caenorhabditis elegans* antigen protects against development of type-1 diabetes in NOD mice

Belinda M. Jackson-Thompson [a,*], Marina Torrero [a,1], Blima K. Mitre [b], James Long [c], Mathanraj Packiam [a], Edward Mitre [a]

[a] Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD, 20814, USA
[b] UPMC Passavant Hospital, Pittsburgh, PA, USA
[c] Walter Reed National Military Medical Center, 8901 Wisconsin Ave, Bethesda, MD, 20889, USA

ARTICLE INFO

Keywords:
Autoimmune diabetes
Immunotherapy
Caenorhabditis elegans
Non-obese diabetic (NOD) mice
Helminth therapy

ABSTRACT

Studies in humans and animals have demonstrated that infection with helminths (parasitic worms) is protective against a range of hyperinflammatory diseases. A number of factors limit translation into clinical use, including: potential contamination of helminths obtained from infected humans or animals, lack of batch to batch stability, and potential pathological risks derived from live worm infections. To overcome these limitations we tested whether an antigen homogenate of the non-pathogenic nematode *Caenorhabditis elegans* confers protection against type 1 diabetes mellitus (T1D) using the Non Obese Diabetic (NOD) mouse model.

Our study demonstrates that twice weekly intraperitoneal injections of axenically cultured *C. elegans* antigen (aCeAg) confers substantial protection against type 1 diabetes in NOD mice. Whereas 80% of control mice (PBS-injected) developed clinical disease, only 10% of aCeAg-treated mice became diabetic. Additionally, aCeAg treated mice had significantly greater numbers of insulin-producing pancreatic islets and greater numbers of islets negative for lymphocyte infiltration. Immunological changes observed in aCeAg treated mice included increases in total IgE and total IgG1, consistent with induction of a type 2 immune response similar to that typically seen in parasitic worm infection. Although evidence suggests that helminth infections induce strong immunoregulatory signals, we did not observe significant changes in regulatory T cell numbers or in production of the regulatory cytokines TGFβ and IL-10. The lack of a regulatory response may be due to our time point of observation, or perhaps the mechanism of aCeAg efficacy may differ from that of helminth infection.

Discovery that antigens obtained from a non-parasitic environmental nematode replicate the protective phenotype induced by parasitic worm infections may accelerate our ability to develop nematode-derived therapies for allergy and autoimmune diseases.

1. Introduction

The 'Old Friends Hypothesis' (a modification of the 'Hygiene Hypothesis') suggests early exposure to microbes may be required for proper development of the human immune system [1–3]. Exposure to a variety of microorganisms is believed to be particularly relevant in the establishment of homeostasis between T effector and T regulatory immune responses, as well as development of immune-tolerance against autoantigens and innocuous environmental antigens. Epidemiologic evidence indicates a reverse correlation exists between geographic regions with high levels of helminth infections (parasitic flukes, tapeworms, and nematodes) and areas with increasing rates of hyper-inflammatory disease [4–6].

We and others have shown in animal models that chronic helminth infections can be protective against inflammatory diseases [7–9]. Given the inherent risks involved in administering live parasites, we have focused on developing helminth antigens as potential therapeutics for inflammatory diseases. Our group and others have demonstrated that administration of soluble homogenates prepared from parasitic worms results in immune regulation and protection against type 1 diabetes and

* Corresponding author.
E-mail addresses: Belinda.Jackson.ctr@usuhs.edu (B.M. Jackson-Thompson), bmitre@comcast.net (B.K. Mitre), james.o.long6.mil@mail.mil (J. Long), Mathanraj.packiam.mil@mail.mil (M. Packiam), Edward.Mitre@usuhs.edu (E. Mitre).
[1] Deceased June 16, 2015.

https://doi.org/10.1016/j.jtauto.2020.100065
Received 22 April 2020; Received in revised form 23 July 2020; Accepted 11 August 2020

other autoimmune diseases [8,10].

To date, however, development of helminth antigens as novel immunotherapeutics has been slow. Helminths are parasitic worms, and as yet none can be maintained completely in vitro. Thus, obtaining these worms requires maintenance of complex lifecycles and extraction from animal feces or by animal dissection after euthanasia. These processes are laborious and expensive, exhibit batch to batch variability, and often result in contamination of parasitic worms with host antigens or intestinal microbes [7,11].

In this study we investigated whether soluble antigens obtained from *Caenorhabditis elegans*, a non-parasitic environmental nematode that can be readily maintained in laboratory settings, can confer protection against inflammatory disease. Our results demonstrate that repeated treatment of non-obese diabetic (NOD) mice with an antigen homogenate derived from axenically cultured *C. elegans* (aCeAg) reduces destruction of pancreatic islets and confers clinical protection against the onset of type 1 diabetes.

2. Materials and methods

2.1. Axenic growth of C. elegans

Wild-type *C. elegans* colonies (Bristol N2) were established on Nematode Growth Medium (NGM) agar and fed OP50 *Escherichiae coli* as per Stiernagle [12]. Briefly, ~1000 worms were transferred to a 100 mm Petri dish containing NGM media (1.7% Agar w/v, 50 mM NaCl, 0.25% Peptone w/v, 1 mM $CaCl_2$, 5 μg/mL cholesterol, 25 mM KH_2PO_4, and 1 mM $MgSO_4$) and seeded with an OP50 lawn. Worms were cultured at ambient temperature until ~85% became gravid (3–5 days). They were rinsed from the plate in sterile dH_2O, and pelleted at 10,000×*g* for 30 s; the supernatant was discarded. The worms were repeatedly rinsed and pelleted as above until cleared of visible bacteria. *C. elegans* eggs were isolated from the gravid adults by incubating the worm pellet in freshly prepared 20% alkaline hypochlorite solution (0.25 M NaOH, 20% bleach) for 3–5 min with occasional vortexing. The suspension was monitored for dissociation of adult worms and egg release. The eggs were pelleted (10,000×*g* for 30 s), rinsed 3x with sterile dH_2O, and resuspended in 3 mL of fresh sterile dH_2O. The egg suspension was transferred to a clean 15 mL conical tube and incubated overnight at ambient temperature with gentle shaking, allowing for egg hatch and developmental arrest of larval stage 1 (L1) worms.

To establish axenic *C. elegans* cultures the newly hatched L1 larvae were pelleted (800×*g* for 3 min), the supernatant discarded, and the worms resuspended in 10 mL of axenic media. The suspension was transferred to a T25 polystyrene culture flask and incubated at ambient temperature with gentle shaking. Cultures were expanded to 50 mL of media in T150 flasks and sub-cultured weekly to maintain a viable worm concentration of ~ 50,000 worms/ml. The axenic media used in this study is a modified version of that previously described by R. Nass and I. Hamza [13], and N.J. Szewczyk et al. [14]. A 2X stock of custom ordered *C. elegans* Maintenance Medium (CeMM, MediaTech Inc.) was diluted to a 1x working stock and supplemented with 20 μM Hemin Chloride, 10% Ultra-pasteurized (UHT) skim milk, 5 μg/ml cholesterol, 100 μg/ml tetracycline, and 100 Units/ml of Penicillin-Streptomycin (Thermo Fisher, Product no. 15140163).

2.2. Preparation of C. elegans antigen

C. elegans were collected from OP50 seeded agar plates (via rinsing with sterile dH_2O), or from axenic liquid media, for preparation of *C. elegans* antigen (CeAg) and axenic *C. elegans* antigen (aCeAg) respectively. Worms were pelleted (800×*g* for 5 min), rinsed two times with PBS, and resuspended in ~20 ml PBS. Worm suspensions were transferred to "D" lysing tubes (MP Biomedicals), and mechanically homogenized using the MP Biomedical FastPrep-24 system. Three consecutive 20 s cycles were run on setting 4.0 M/s^2, following which samples were centrifuged at 16,000×*g* for 1 min and supernatants were collected. Supernatant samples were combined as appropriate and passed through a 0.45-μm filter. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher, catalog no. 23225).

2.3. NF-κB reporter assay of lipopolysaccharide (LPS) activity

Human embryonic kidney (HEK) cells expressing the TLR4-MD2-CD14 receptor complex and carrying the NF-κB induced Secreted Embryonic Alkaline Phosphatase (SEAP) reporter gene were used to assess the LPS activity of the various antigen preparations. The HEK TLR4 cells were incubated in 96-well plates at 0.25×10^6 cells/ml in the presence of purified LPS (10 ng/ml; Invivogen, Product code: tlrl-smlps), CeAg (10 μg/ml), or aCeAg (10 μg/ml) for 20 h at 37 °C. To detect NF-κB induced expression of SEAP, using the QUANTI-blue colorimetric assay (InvivoGen, Product code: rep-qbs), 20 μl of cell culture supernatant was incubated with 180 μl of substrate at 37 °C for 30 min. Sample activity was assessed by reading the optical density at 650 nm. Supernatants from untreated cells were used as negative controls.

2.4. Limulus amoebocyte lysate (LAL) assay of endotoxin content

The amount of LPS (endotoxin) present in each antigen preparation was determined using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher, catalog no. 88282). Briefly, 50 μl of each antigen sample was dispensed into a preheated (37 °C) 96-well plate, followed by 50 μl of LAL reagent. The plate was incubated at 37 °C for 10 min. One hundred microliters of chromogenic substrate were added and incubation was continued for 10 min. One hundred microliters of stop solution was added and sample absorbance was read at 405 nm. The concentration of endotoxin was extrapolated using a standard curve generated by purified *E. coli* (serotype 011: B4) LPS. The range of detection was 0.1–1 EU/ml (EU = endotoxin units).

2.5. In vivo analysis of aCeAg protective therapy in NOD mice

Female NOD/LtJ mice (The Jackson Laboratory) were maintained at the Uniformed Services University (USU) animal facility with free access to food and water. All experiments were performed under protocols approved by the USU Institutional Animal Care and Use Committee. One hundred micrograms of aCeAg was administered in 100 μl of PBS by intraperitoneal (I.P.) injection starting at 8 weeks of age and continued twice weekly for 14 weeks.

For analysis of NOD mice with depleted circulating TGFβ, in addition to receiving bi-weekly aCeAg injections, mice received 100 μg anti–TGFβ (clone 1D11.16.8; Bio X Cell, catalog no. BE0087) or 100 μg mouse IgG1 isotype (clone MOPC-21; Bio X Cell, catalog no. BE0083) via I.P. injection 3x per week through studies end.

2.6. Assessment of diabetes in NOD mice

Glucose levels of mice were monitored by analysis of blood taken via tail vein bleeds every week using a standard blood glucose meter (Accu-Check Advantage, Roche Diagnostics, Mannheim, Germany). NOD mice with glucose levels >230 mg/dl on two consecutive weeks were considered diabetic. Mice were euthanized after diabetes onset or at study endpoint at 20–24 weeks of age.

2.7. Assessment of pancreas inflammation

Pancreases were isolated and fixed in 10% formalin. H&E-stained slices were assessed for inflammation by pathologists (B.K.M. and J.L) blinded to the intervention group. For each pancreas, total numbers of islets were counted in two longitudinal sections separated by 400 μm. The severity of insulitis was scored as non-infiltrated, peri-insulitis (lymphocytes at the periphery of islets), or intra-insulitis (lymphocyte

infiltration into the interior of the islets of less than or greater than 50%).

2.8. Measurement of IgE, IgG1, IgG2c, and circulating TGFβ concentrations by ELISA

At study endpoint, blood was drawn from CO2 euthanized mice by cardiac bleed, and plasma was isolated using heparinized microtubes (BD Microtainer, Product no. 365985). Plasma samples were kept at −20 °C until assays were performed.

2.8.1. IgE analyses - total, insulin-specific, and aCeAg-specific

Prior to running IgE ELISAs, IgG antibodies were adsorbed from the plasma by overnight incubation with GammaBind Plus Sepharose beads (GE Healthcare, catalog no. 17088601). Total IgE concentrations were measured using eBIoscience ELISA kit (Product no. 88-50460-86) according to the manufacturer's instructions.

For insulin-specific and aCeAg-specific analysis, Corning™ 96-Well Half-Area EIA/RIA plates (Fisher Scientific, catalog no. 10629151) were coated overnight at 4 °C with 20 μg/ml aCeAg or recombinant human insulin (Sigma-Aldrich, catalog no. 91077C) in PBS. Wells were washed 3x with PBS plus 0.025% Tween-20 using a microplate washer (BioTek Instruments, Model ELX 405) and blocked with PBS plus 5% bovine serum albumin (BSA) and 0.05% Tween-20 at room temperature for 2 h. Plates were washed 3x as above, and plasma samples were added and incubated overnight at 4 °C. For detection of captured antibody, plates were washed 3x and incubated for 1 h with biotinylated anti-mouse IgE antibody (Clone RME-1; BioLegend, catalog no. 406903), followed by 6x plate washes and a 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson Immuno-Research Laboratories, catalog no. 016-050-084). The substrate 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) was added for 15 min and absorbance was measured at 405 nm using the BioTek Synergy/HTX plate reader. All the insulin-specific samples from each experiment were assayed on a single plate, as were all the aCeAg samples, allowing for accurate comparison between groups. Samples were analyzed in duplicate.

2.8.2. IgG1 and IgG2c analysis - total and insulin-specific

Total IgG1 and IgG2c concentrations were measured by ELISA (eBioscience, catalog no. 88-50410-22 and Invitrogen, catalog no. 88-50670-22) following the manufacturer's instructions.

For insulin-specific analysis, Corning™ 96-Well Half-Area EIA/RIA plates (Fisher Scientific, catalog no. 10629151) were coated overnight at 4 °C with 20 μg/ml recombinant human insulin (Sigma-Aldrich, catalog no. 91077C) in PBS. Wells were washed 3x with PBS plus 0.025% Tween-20 using a microplate washer (BioTek Instruments, Model ELX 405), and blocked with PBS plus 5% BSA and 0.05% Tween-20 at room temperature for 2 h. Plates were washed 3x as above, and plasma samples were added and incubated overnight at 4 °C. For detection of captured antibody, plates were washed 3x and incubated for 1 h with either biotinylated anti-mouse IgG1 antibody (clone RMG1-1) or biotinylated anti-mouse IgG2c antibody (Clone RM223). Plates were washed 6x followed by a 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson Immuno-Research Laboratories, catalog no. 016-050-084). The substrate 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) was added for 15 min and absorbance was measured at 405 nm using the BioTek Synergy/HTX plate reader. All the insulin-specific IgG1 samples for each experiment were assayed on a single plate, as were the insulin-specific IgG2c samples, allowing for accurate comparison between groups. Samples were analyzed in duplicate.

2.8.3. Circulating TGFβ analyses - total and bio-active

TGFβ levels were analyzed using the Human/Mouse TGFβ1 ELISA Kit (eBioscience) per the manufacturer's instructions. For analysis of total TGFβ levels the plasma samples were acid activated for 10 min with 10 mM HCl, followed by a 10-min neutralization step with 10 mM NaOH, prior to ELISA analysis.

2.9. Ex vivo splenocyte stimulation and cytokine quantification

Spleens were collected from NOD mice following euthanasia and single cell suspensions prepared by pressing tissue through a 70-μm cell strainer. Cells were pelleted, resuspended in ACK Lysing buffer (Thermo Fisher), and incubated at room temperature for 5 min to lyse red blood cells. Cells were washed twice with PBS and then resuspended in RPMI media supplemented with 10% fetal calf serum (Valley Biomedical, Winchester, VA), 1% L-glutamine (Thermo Fisher), and 100 Units/ml of Penicillin-Streptomycin (Thermo Fisher, Product no. 15140163). Cells were cultured (1×10^6 cells in 1 ml of media) in the presence of aCeAg (20 μg/ml), plate-bound anti-mouse CD3e (5 μg/ml; Invitrogen) and anti-mouse CD28 (2 μg/ml; Invitrogen) antibodies, or media alone for 72 h. Cell culture supernatants were collected and kept at −20 °C until assays were performed. IFN-gamma, IL-4, IL-10, and bio-active TGFβ were quantified by enzyme linked immunosorbent assays (ELISA) according to manufacturer's instructions (Thermo Fisher: Product no. 88-7314-88 [IFN-γ], Product no. 88-7044-88 [IL-4], Product no. 88-7105-88 [IL-10], and Product no. [TGFβ]). For analysis of total TGFβ production supernatant samples were acid activated for 10 min with 10 mM HCl, followed by a 10-min neutralization step with 10 mM NaOH, prior to ELISA analysis.

2.10. Flow cytometric detection of regulatory T cells

Splenocytes were cultured and stimulated as described above, at a total of 4×10^6 cells in 4 ml RPMI supplemented media (10% fetal calf serum, 1% L-glutamine, 100 Units/ml of Penicillin-Streptomycin). After 2 h of incubation at 37 °C, GolgiStop was added (BD Biosciences) and cells were incubated for an additional 4 h. Cells were collected, washed with PBS plus 1% BSA, and stained with fixable eFluor™ 450 viability dye (Thermo Fisher). Cells were washed again and incubated overnight at 4 °C in Fixation/Permeabilization Solution (Thermo Fisher). Cells were washed in PBS plus 1% BSA and cryopreserved in PBS/10% dimethyl sulfoxide (Sigma-Aldrich) until study end. For analysis cells were washed once with PBS/1% BSA, followed by a blocking step with PBS plus 5% BSA. Cells were surface stained for flow cytometry with mouse anti-CD4 (clone RM4-5) conjugated with FITC (Thermo Fisher), mouse anti-CD8a (clone 53–6.7) conjugated with PE-Cyanine7 (Thermo Fisher), and mouse anti-CD25 (clone eBio7D4) conjugated with eFluor 660 (Invitrogen); prior to use all surface antibodies were individually titrated allowing for stain optimization. Cells were then washed 2x with Fixation/Permeabilization buffer, blocked with anti-mouse CD16/ant-CD32 (eBioscience, catalog no. 14-0161-82), and internally stained with mouse anti-Foxp3 (clone FJK-16s) conjugated with PE- Cyanine 5 (Thermo Fisher). Stained cells and compensation controls (Thermo Fisher OneComp eBeads) were detected with the BD LSRII Flow cytometry system. Subsequent analysis was performed using the Flow Jo software platform. The fluorescence minus one approach was used to determine background thresholds for each of the conjugated cytokines.

2.11. Statistical analysis

Statistical analyses were performed with GRAPHPAD PRISM software (GraphPad Software, San Diego, CA). Differences between multiple groups were tested for significance using the Kruskal–Wallis test, followed by Dunn's post-hoc multiple comparisons. Comparisons between two groups were performed by Mann–Whitney *U* test. P-values < 0.05 were considered significant. Data are shown as median values. Glucose levels are shown as means ± SEM.

3. Results

3.1. Nematode antigen derived from axenically cultured C. elegans does not induce TLR4 activity

Traditionally *C. elegans,* a soil dwelling nematode, is cultured in laboratory settings on a lawn of *E. coli*. To ensure that our *C. elegans* derived antigen was free of confounding metabolites, we cultured these non-parasitic worms in axenic media (media absent of living organisms). We transitioned the wild-type N2 *C. elegans* strain from an OP50 *E. coli* lawn to axenic media following a published protocol [15]. Once a thriving culture was obtained, 50 mL aliquots of viable worms (~5000 worms per ml) were collected every two weeks and homogenized. Soluble antigen was collected from the homogenate and the protein concentration determined via Bicinchoninic Acid protein assay. To confirm the exclusion of *E. coli* based LPS contamination, we analyzed axenic *C. elegans* antigen (aCeAg) for Toll-like receptor 4 (TLR4) activity using an NF-κB-inducible reporter assay. As shown in Fig. 1, 10 μg/ml of soluble antigen prepared from *C. elegans* grown with *E. coli* as a food source had TLR4 activity similar to that of a 10 ng/ml preparation of purified bacterial LPS. In contrast, antigen prepared from axenic *C. elegans* (aCeAg) had negligible TLR4 activity. Consistent with these results, the endotoxin concentration found in 1 mg of aCeAg was only 2.56 EU/mg vs 10 million EU/mg in purified LPS.

3.2. aCeAg injections delay the onset of type-1 diabetes in NOD mice

We previously reported that helminth therapy protects against disease in NOD mice - a model for autoimmune type 1 diabetes [8]. To determine whether similar protective efficacy would be conferred by aCeAg, NOD mice were given PBS or aCeAg injections twice weekly from 8 to 22 weeks of age (Fig. 2A). Significantly fewer aCeAg treated mice developed diabetes compared to PBS-injected control mice (Fig. 2B, PBS controls: 80%, aCeAg: 10%, $p = 0.037$). Mean glucose values for NOD mice injected with aCeAg were 174.5 mg/dl versus 387.9 mg/dl for the control group, with mean area under the curve values of 1342 and 2342 respectively ($p < 0.001$, Fig. 2C).

Individual pancreases were examined to determine whether aCeAg therapy protected against organ inflammation. Following euthanasia pancreatic samples from each animal were prepped for histological assessment, and were analyzed by a pathologist blinded to the treatment groups. For each pancreatic sample, the total number of islets found within two longitudinal sections (separated by 400 μm) were counted. Additionally, the islets were assessed for inflammation and classified as normal, having peri-insulitis, or having intra-insulitis with less than or greater than 50% lymphocyte infiltration. As seen in Fig. 2D, mice treated with aCeAg had a greater mean number of total islets than the untreated control mice, although the difference was not statistically significant (mean # islets - PBS controls: 4.4, aCeAg: 10.8, $p = 0.064$). Similarly, aCeAg treated mice had greater numbers of normal β-islets than did the controls (Fig. 2E, per two longitudinal sections - PBS controls: 0.7, aCeAg: 2.4).

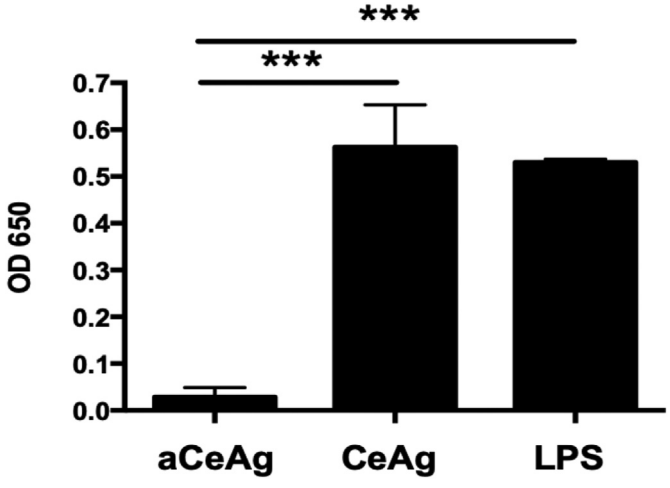

Fig. 1. TLR4 activity of soluble antigen homogenates produced from *C. elegans* cultured axenically (aCeAg) or with OP50 *E. coli* (CeAg). HEK cells expressing TLR4 receptor complex and carrying an NF-κB SEAP reporter gene were incubated in the presence of soluble antigen extracts prepared from *C. elegans* cultured axenically (aCeAg: 10 μg/ml) or with *E. coli* (CeAg: 10 μg/ml). Purified LPS (10 ng/ml) was used as a positive control. SEAP activity was measured after 20 h of incubation and plotted as an OD 650 value. ***$p < 0.001$; Kruskal–Wallis test, followed by Dunn's post-hoc multiple comparisons.

3.3. Assessment of aCeAg induced immune responses

To gain insight on the potential mechanisms by which aCeAg confers protection in NOD mice, we conducted a number of immunological investigations. Results from ELISA analyses of circulating antibody levels demonstrated that aCeAg significantly increases circulating concentrations of both total IgE (Fig. 3A, PBS controls: 14.3 μg/ml, aCeAg: 32.8 μg/ml; $p = 0.0437$) and total IgG1 (Fig. 3B, PBS controls: 16.9 μg/ml, aCeAg: 37.5 μg/ml; $p = 0.0001$). Total IgG2c antibody levels (Fig. 3C), as well as insulin-specific levels of IgE, IgG1, and IgG2c (Fig. 3D, E, and 3F) were not altered by aCeAg. Finally, repeated administration of aCeAg did not result in allergic sensitization as aCeAg-specific IgE levels were not elevated in aCeAg-treated mice (Fig. 3G).

To assess for changes in cellular responses, we cultured splenocytes from each animal with aCeAg, anti-CD3/anti-CD28, or media alone. We analyzed the culture supernatants for secreted cytokines, measured by ELISA, indicative of a TH1 (IFNγ, Fig. 4A and B) and/or a TH2 (IL-4, Fig. 4D and E) shift. Cells from aCeAg-treated mice demonstrated an insignificant increase in IL-4 production in response to T-cell receptor (TCR) stimulation (Fig. 4B, anti-CD3/CD28 - PBS controls: 0.0 pg/ml, aCeAg: 37.0 pg/ml; $p > 0.999$) and no change in IFNγ levels. Unlike the antigen-specific response of cultured splenocytes from NOD mice treated with helminth derived antigen (*L. sigmodontis* antigen - LsAg) [8], aCeAg does not induce an antigen-specific increase in IL-4 levels (Fig. 4C). A modest, but statistically insignificant antigen-specific increase in IFNγ levels was detected from these cells with aCeAg stimulation (Fig. 4F, aCeAg - PBS controls: 0.0 pg/ml, aCeAg: 20.6 pg/ml; $p > 0.999$).

To determine if aCeAg-induced production of regulatory cytokines we measured TGFβ and IL-10 production from cultured splenocytes as well as plasma levels of TGFβ. The levels of circulating TGFβ, both total and bio-active, were unaltered in mice receiving aCeAg treatments (Fig. 5A and B). Total TGFβ produced spontaneously and in response to anti-CD3/anti-CD28 was lower, though not to a statistically significant degree, from splenocytes of aCeAg-treated mice (Fig. 5C, media alone - PBS controls: 639.3 pg/ml, aCeAg: 128.0 pg/ml; $p = 0.1799$ and Fig. 5D, antiCD3/CD28 - PBS controls: 604.3 pg/ml, aCeAg: 204.6 pg/ml; $p > 0.999$). When splenocytes from these mice were co-cultured with aCeAg, production of TGFβ increased to levels equivalent to those seen in the controls (Fig. 5E, aCeAg - PBS controls: 464.0 pg/ml, aCeAg: 499.7 pg/ml; $p > 0.9999$). Production of bio-active TGFβ was no different for any of the culture conditions. A modest, but non-significant, increase in IL-10 production was observed for all the in vitro culture conditions (Fig. 5I, media alone - PBS controls: 0.0 pg/ml, aCeAg: 200.2 pg/ml, $p > 0.999$; 5 J, anti-CD3/CD28 - PBS controls: 0.0 pg/ml, aCeAg: 1149.9 pg/ml, $p > 0.999$; 5 K, aCeAg - NOD controls: 0.0 pg/ml, NOD aCeAg: 166.6 pg/ml, $p > 0.999$).

3.4. aCeAg induced protection is independent of TGFβ

As results from a prior study we conducted indicated that NOD mice infected with the parasitic nematode *L. sigmodontis* were protected against diabetes through the actions of host-derived TGFβ [9], we tested

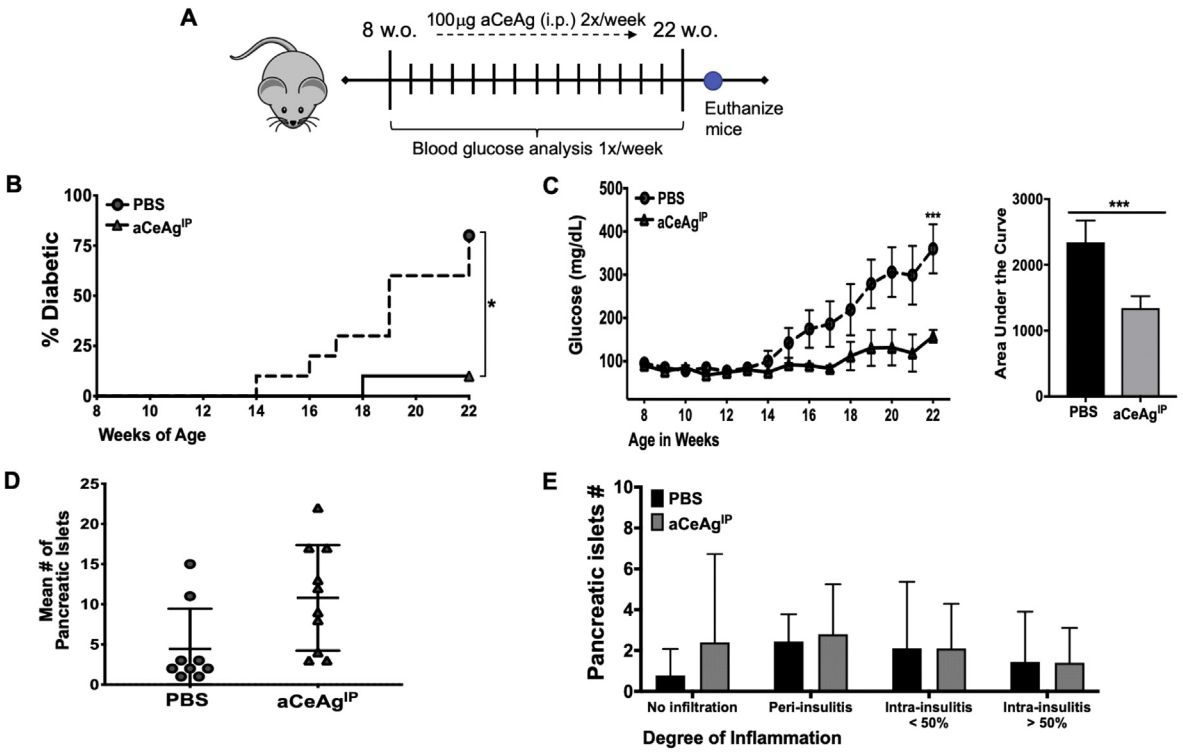

Fig. 2. aCeAg delays the onset of Type 1 Diabetes pathology in NOD mice. A) aCeAg (~5 mg/kg) was injected i.p. twice a week, starting at 8 weeks of age, for 14 weeks. Control mice received PBS (100 μl) injections at identical intervals (n = 10). B) The percent of mice developing diabetes (defined as a blood glucose level > 230 mg/dl) is significantly greater in the untreated control NOD group (80%) versus those treated with aCeAg (10%) (*p = 0.0366, Gehan-Breslow-Wilcoxon test). C) Glucose levels were measured weekly from blood obtained by tail vein puncture; the mean Area Under the Curve values from week 22 demonstrate a significant decrease in hyperglycemia with aCeAg treatment (***p < 0.001, Mann–Whitney *U* test). D) The mean number per mouse of pancreatic islets identified in two longitudinal sections separated by 400 μm. E) Pancreatic islets categorized by degree of inflammation.

whether TGFβ was required for aCeAg protection. For depletion of the cytokine mice were treated 3 times a week with mouse anti–TGFβ or the IgG1 isotype control, in addition to twice weekly injections of aCeAg or PBS. Reduction of circulating TGFβ levels was confirmed by ELISA analysis (Fig. 6). Results show that aCeAg-mediated protection against diabetes is not dependent on TGFβ. Ninety percent of mice treated with aCeAg plus TGFβ antibody, and 100% of those treated with aCeAg plus the IgG isotype control, maintained a non-diabetic status compared to only 30% of the PBS controls (Fig. 7A). Glucose levels remained in the normal range in the aCeAg treated mice (with or without TGFβ depletion), and area under the curve calculations of the treated mice were significantly lower than that of PBS control animals (Fig. 7B; AUC PBS controls: 2,731, aCeAg/IgG1: 1,351, aCeAg/anti-TGFβ: 1,491, $p < 0.001$). The total number of islets identified in both groups of aCeAg treated mice were higher than the number identified in the non-treated control group (Fig. 7C). No significant differences were found when comparing degree of islet inflammation (Fig. 7D).

As seen in Supplemental Figs. 1 and 2, changes in antibody and cytokine production induced by aCeAg administration are not substantially altered by TGFβ depletion. aCeAg increases total and insulin-specific IgE levels as well as insulin-specific IgG1 levels independent of TGFβ depletion (Supplemental Fig. 1). Additionally, TGFβ depletion caused no significant changes in cytokine production by ex vivo splenocytes in response to aCeAg or T-cell receptor stimulation (Supplemental Fig. 2).

3.5. Regulatory T cells are not increased by aCeAg administration

To further explore the impact of aCeAg treatment on the host immune response, we looked for possible alterations in regulatory $CD4^+$ T cell (Treg) differentiation. T cells were identified as $CD4^+$CD25+Foxp3+, by flow cytometry. Analysis was performed on splenocytes collected from NOD mice between 21 and 24 weeks of age. No changes were observed in either $CD4^+$ Treg numbers or percentages as a result of aCeAg treatments or TGFβ depletion (Fig. 8).

4. Discussion

Numerous studies have demonstrated that helminths and helminth antigens are protective against autoimmune diseases such as T1DM [16–25]. Given the risks associated with administration of live parasites, we and others have focused on developing helminth antigens as potential therapeutics for inflammatory diseases. A major limitation to progress in this field has been the technical difficulty inherent in obtaining and purifying large quantities of parasitic worm antigens. In this study, we have overcome this obstacle by demonstrating that a homogenate of soluble antigens derived from the non-parasitic, axenically grown *C. elegans* (aCeAg) is protective against type 1 diabetes in the NOD mouse model. Weekly injections of aCeAg conferred marked protection against onset of diabetes and a dramatic reduction in hyperglycemia (from a mean glucose level at 22 weeks of 388 mg/dL in untreated controls to 175 mg/dL in aCeAg-treated mice).

The degree of protection conferred by aCeAg against T1DM in this study is similar to that obtained by our group and others when administering live helminth infection or helminth-derived antigens to NOD mice [8,9,16,17]. As with live nematodes [8,9], aCeAg treatment increased levels of total IgE and total IgG1, consistent with induction of a type 2 immune response. Interestingly, we did not observe detectable aCeAg-specific IgE at study endpoint. In vitro T-cell receptor stimulation revealed increased IL-4 and decreased IFNγ production by splenocytes of aCeAg-treated mice, consistent with a type 2 immune shift, but these differences were not statistically significant.

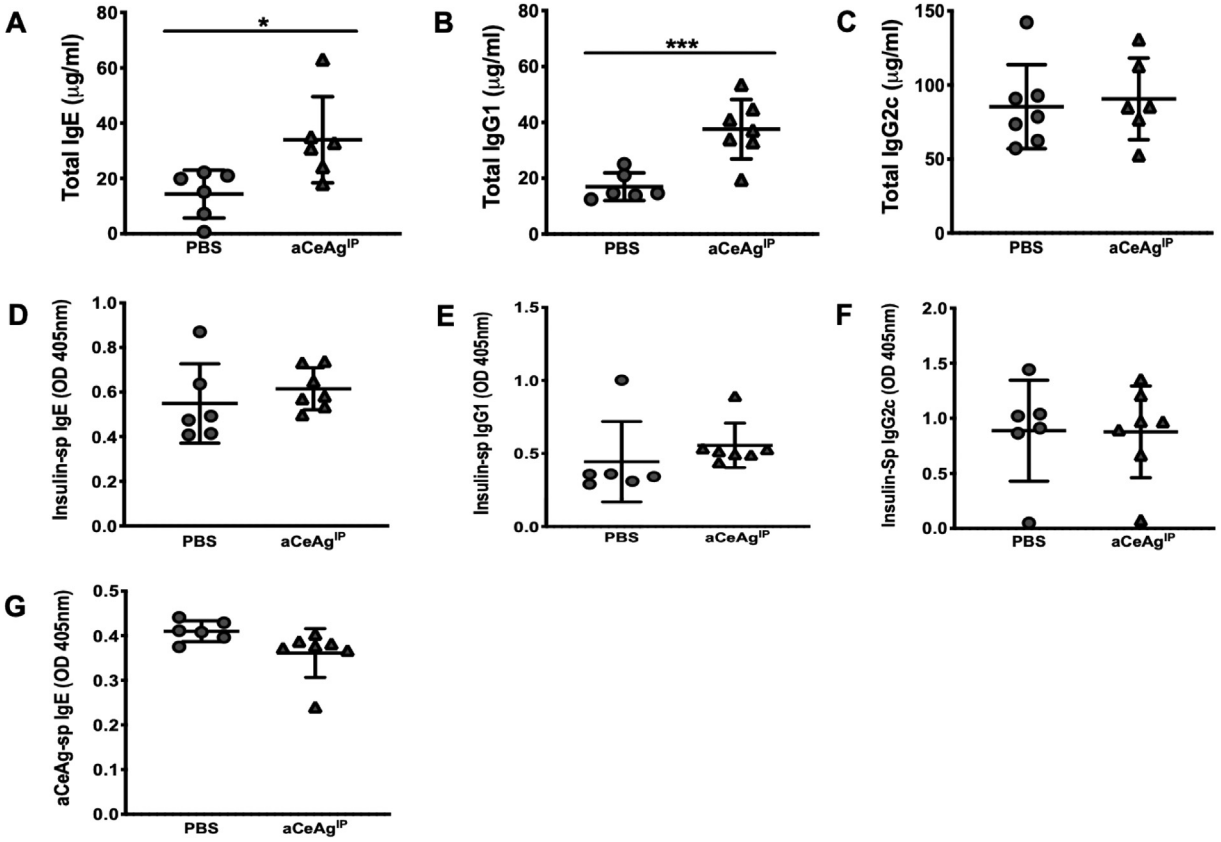

Fig. 3. Effects of aCeAg on polyclonal and insulin-specific antibody levels. ELISA analysis of plasma antibody levels in control and aCeAg treated NOD mice: Total IgE (A), IgG1 (B), and IgG2c; insulin-specific IgE (D), IgG1 (E), and IgG2c (F); and aCeAg-specific IgE (G). *$p < 0.05$, ***$p < 0.001$; Kruskal–Wallis test, followed by Dunn post hoc multiple comparisons.

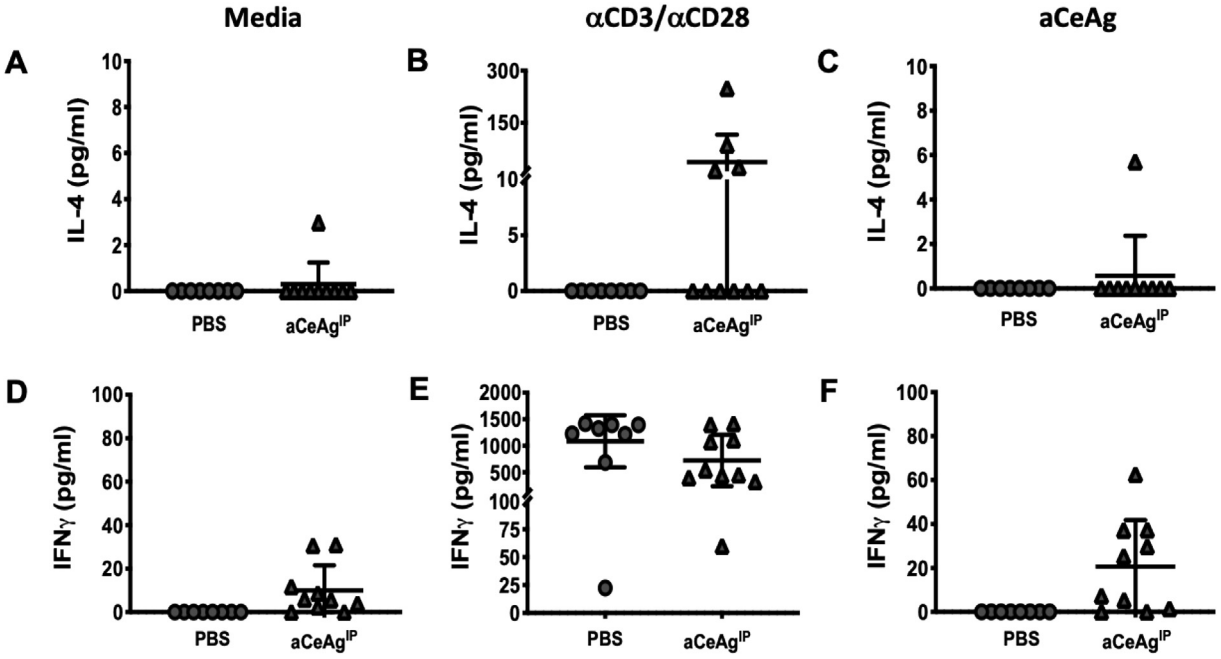

Fig. 4. IL-4 and IFNγ production from splenocytes of aCeAg-treated and control NOD mice. IL-4 concentrations in supernatants of (A) unstimulated cells, (B) anti-CD3/CD28 stimulated cells, and, (C) aCeAg stimulated cells. IFNγ concentrations in supernatants of (D) unstimulated cells, (E) anti-CD3/CD28 stimulated cells, and (F) aCeAg stimulated cells.

Our group and others have demonstrated that helminth-mediated protection against autoimmunity predominantly occurs through induction of immunoregulatory pathways. In this study, circulating levels of total and bioactive TGFβ were slightly higher in aCeAg-treated mice

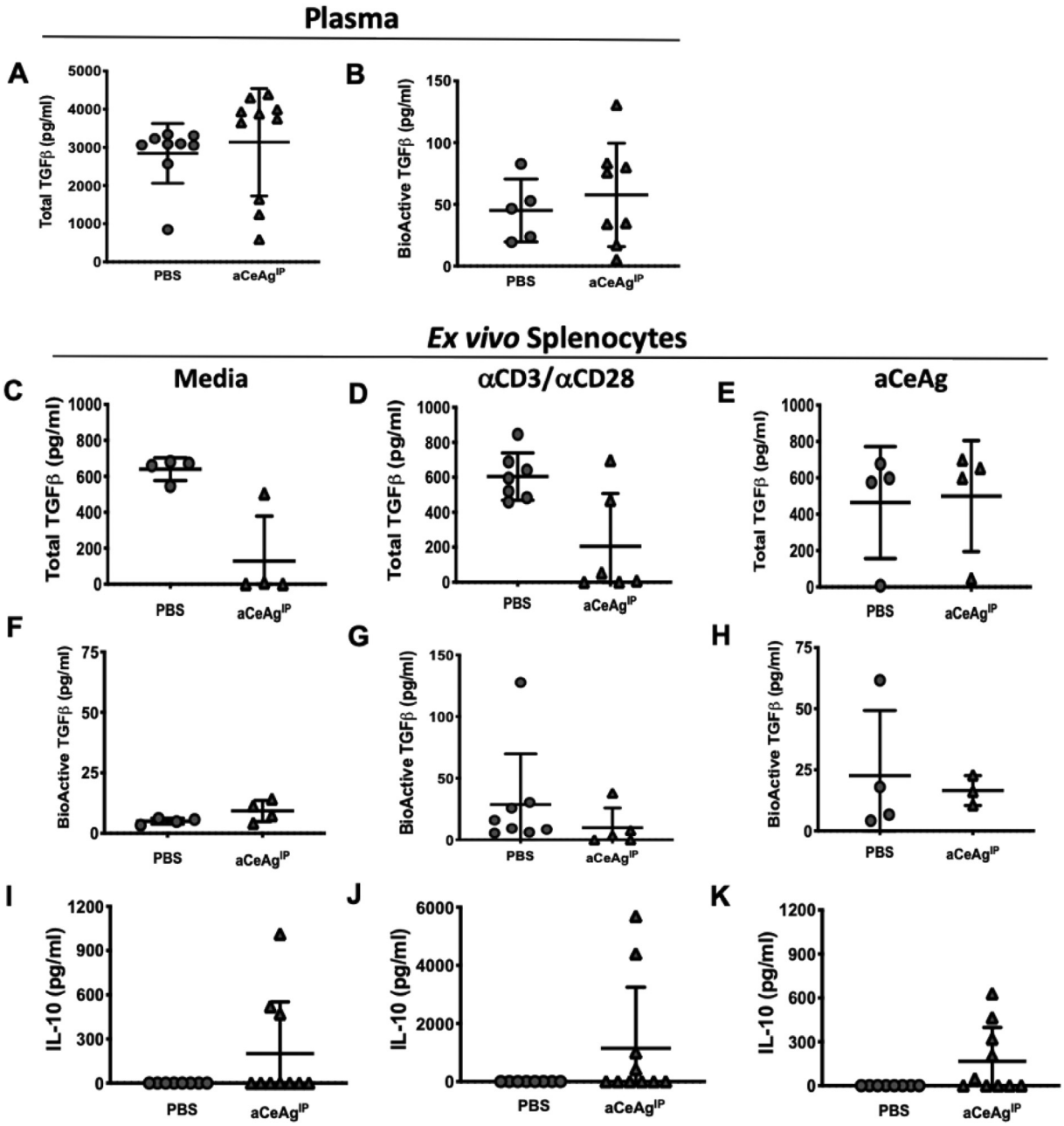

Fig. 5. Regulatory cytokine production in aCeAg-treated and control NOD mice. Concentrations of circulating bio-active (A) and total (B) TGFβ in plasma samples of control and aCeAg treated NOD mice. Comparison of ex vivo cultured splenocytes isolated from control and aCeAg treated NOD mice. Total TGFβ: unstimulated cells (C), anti-CD3/CD28 stimulated cells (D), and aCeAg stimulated cells (E). Bio-active TGFβ: unstimulated cells (F), anti-mouse CD3/CD28 stimulated cells (G), and aCeAg stimulated cells (H). IL-10: unstimulated cells (I), anti-mouse CD3/CD28 stimulated cells (J), and aCeAg stimulated cells (K).

than controls, but these differences were not statistically significant. In vitro anti-CD3/anti-CD28 and aCeAg stimulation of splenocytes also did not reveal increased production of total TGFβ, bioactive TGFβ, or IL-10 in aCeAg-treated mice. In contrast to studies using helminths and helminth antigens, aCeAg-treatment did not alter percentages or total numbers of $CD4^{+}CD25+FoxP3+$ T-regulatory cells in spleens of NOD mice.

As we have previously demonstrated that protection against T1DM by the parasitic nematode *Litomosoides sigmodontis* is dependent on TGF-beta [9], we tested whether TGFβ depletion would negate the beneficial effects of aCeAg. While TGFβ-neutralizing antibody significantly reduced circulating levels of TGFβ, depletion of TGFβ did not reverse the protective effects of aCeAg. It is possible that aCeAg protects against autoimmunity by inducing other immunoregulatory pathways. One possibility is enhancement of host IL-10 production, which was shown to be essential for protection against T1DM conferred by the intestinal nematode *Heligmosoides polygyrus* [20]. Alternatively, it may be that aCeAg protects against T1DM either through direct immunoregulatory activity of one (or a combination) of its constituent antigens, or perhaps by altering the host microbiome, which is increasingly recognized as playing a key role in the expression of inflammatory diseases [26–28]. Elucidating the mechanisms by which aCeAg protects will be the focus of future studies.

We found increased levels of the murine immunoglobin isotype IgG1 in association with suppression of T1D onset in the NOD mouse model. This finding may have translational relevance, as the mechanisms associated with murine IgG1 anti-inflammatory properties are reportedly similar to those of the human isotype IgG4 [29,30]. It has been shown that like human IgG4, murine IgG1 preferentially interacts with

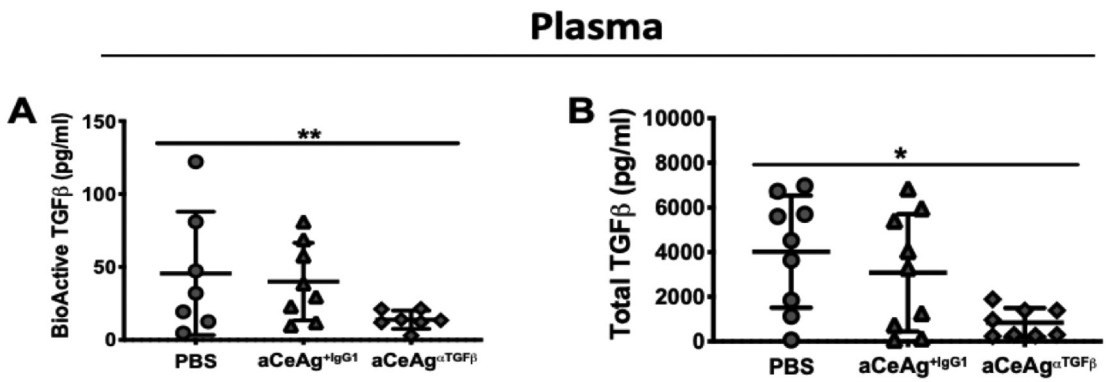

Fig. 6. TGFβ depletion using anti-mouse TGFβ, or IgG1 isotype control. NOD mice were given three i.p. injections per week of PBS, anti-mouse TGFβ, or IgG1 isotype control. Plasma concentrations of TGFβ were determined by ELISA following 18-weeks of treatment. *p < 0.05, **p < 0.01; Kruskal–Wallis test, followed by Dunn's post-hoc multiple comparisons.

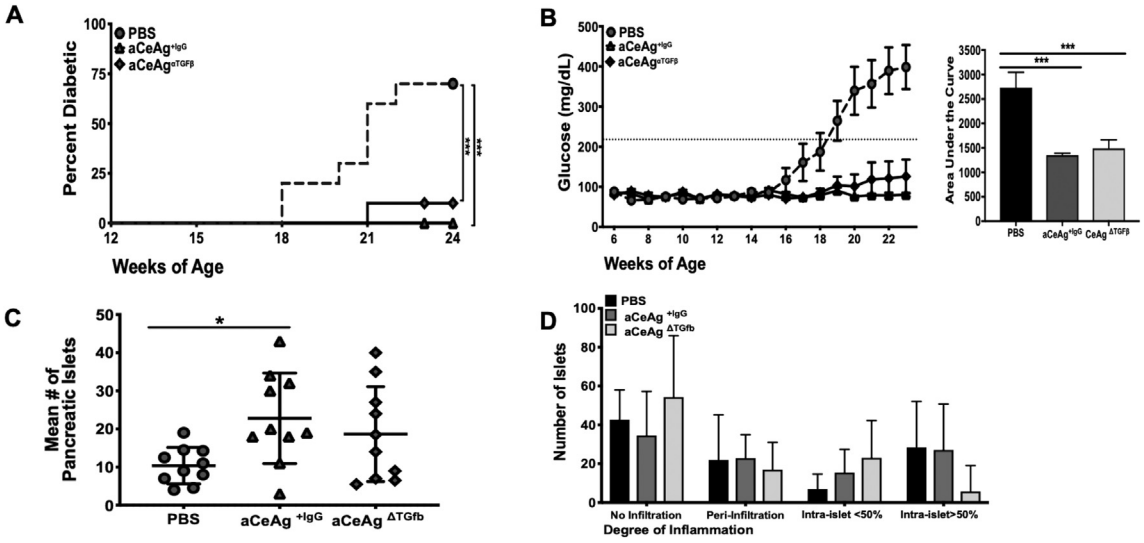

Fig. 7. TGFβ is not required for aCeAg protection against Type 1 Diabetes. aCeAg (~5 mg/kg) was injected i.p. twice weekly, starting at 6 weeks of age for 18 weeks. Antigen treated mice also received i.p. injections of anti-TGFβ or an IgG1 isotype antibody 3 times per week throughout the study. Control mice received injections of PBS alone (n = 10). A) The percent of mice developing diabetes (defined as a blood glucose level > 230 mg/dl) is significantly greater in the PBS control group (70%) versus those treated with aCeAg plus IgG1 (0%), or aCeAg plus anti-TGFβ (10%). B) Glucose levels and the associated area under the curve values demonstrate a significant decrease in hyperglycemia with aCeAg treatment; an effect unaltered by depletion of TGFβ. C) The mean number of pancreatic islets per mouse identified in two longitudinal sections separated by 400 μm. D) Pancreatic islets categorized by degree of inflammation. *p < 0.05, ***p ≤ 0.001 using the Kruskal–Wallis test, followed by Dunn's post-hoc multiple comparisons.

Fc-gamma RIIB, a low affinity inhibitory receptor involved in the down regulation of B cell antibody production. Further, murine IgG1 inhibits activation of the classical complement pathway, as it does not interact with C1q and inhibits C1q binding of murine IgG2a, IgG2b, and IgG3.

A key limitation to our study was that we conducted immunological assays at study endpoint after 14 weeks of aCeAg administration. Consequently, we may have missed type 2 cytokine changes, as well as innate and/or immunoregulatory responses that occur earlier in the course of aCeAg treatment. Additionally, the long-term efficacy of aCeAg was not addressed in this study. Thus, another goal of future analysis will be to characterize the immunological changes induced by aCeAg treatment at multiple timepoints, and to evaluate if aCeAg can induce non-TLR4 innate activation pathways.

Kim and colleagues previously reported that a homogenate of *C. elegans* antigens is protective in a murine model of airway inflammation [31]. In contrast to our study, the *C. elegans* they used for antigen production was grown on lawns of *Escherichiae coli*. A major drawback of this approach is the risk of contaminating the nematode extract with lipopolysaccharide (LPS). LPS is a pathogen-associated molecular pattern in gram negative bacteria that induces substantial inflammation by activation of toll-like receptor 4 (TLR-4). As we show in our study, antigen homogenate obtained from *E. coli*-grown *C. elegans* can have extremely high TLR-4 activating properties. By growing *C. elegans* in axenic media, we avoid the risk of LPS contamination.

One of the advantages of identifying aCeAg as a protective nematode homogenate for T1DM is that we are no longer constrained by production limits. A major obstacle to working with homogenates of parasitic nematodes is that, by definition, parasitic nematodes require animal hosts. To date, no parasitic nematode can be maintained entirely in vitro. Thus, production of homogenates of parasitic nematode antigens is time-consuming, costly, laborious, and carries a risk of contamination with host antigens. Because *C. elegans* is an environmental nematode whose lifecycle can be maintained in axenic media in vitro, we can readily produce large quantities of aCeAg rapidly and inexpensively. As such, we can now conduct large scale fractionation studies to determine which nematode antigens within aCeAg are required for protection against autoimmunity.

Further, because we can produce aCeAg under laboratory conditions

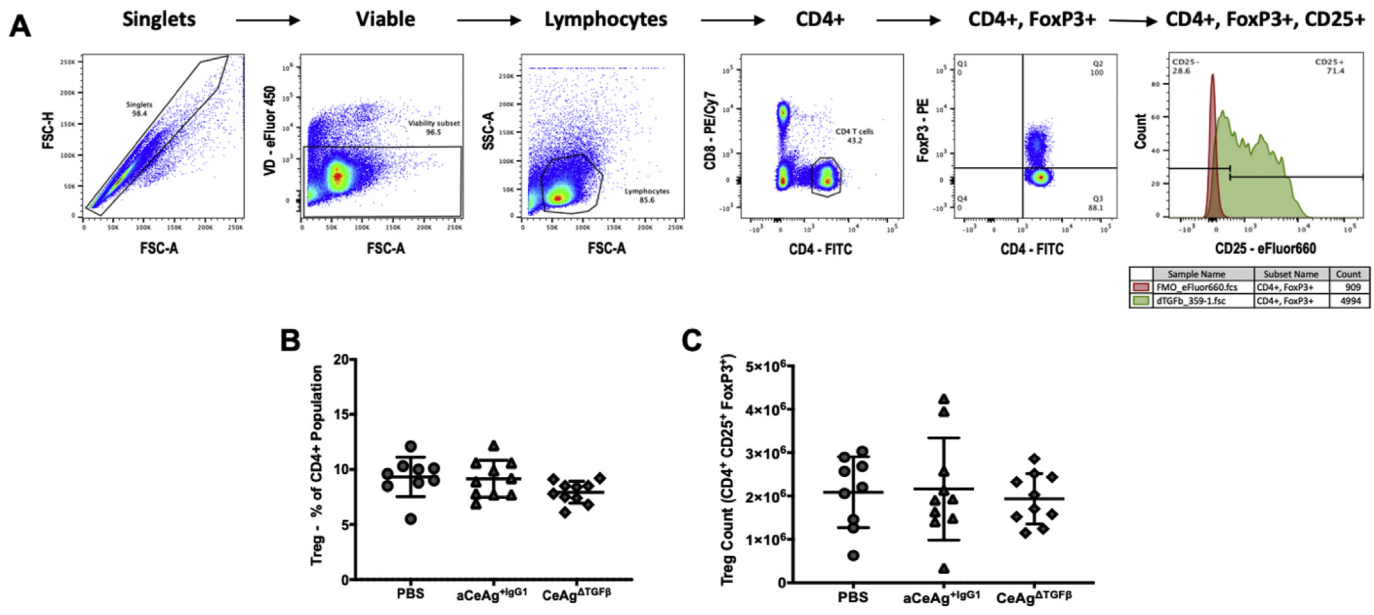

Fig. 8. aCeAg therapy does not alter CD4$^+$ Treg differentiation. (A) Gating strategy for flow cytometric identification of regulatory T cells. Viable, singlet cells were gated by forward scatter (FSC) and side scatter (SSC) for lymphocyte identification. CD4$^+$ lymphocytes were gated and analyzed for CD25 and Foxp3 expression. Frequency (B) and total number (C) of splenic CD4$^+$CD25+Foxp3+ T cells obtained from PBS control versus aCeAg-treated NOD mice, with and without TFGβ depletion.

that enable homogeneity between batches without risk of contaminating antigens from other organisms, we also plan to explore the feasibility of developing the entire aCeAg extract as a treatment for inflammatory diseases. This would require testing aCeAg on a number of allergy and autoimmunity models, and exploring whether oral administration of aCeAg can also exert protective efficacy.

Key to our future studies is the identification of components essential for aCeAg efficacy and the mechanism(s) of protection. Type 1 diabetes is a chronic autoimmune disease in which the critical loss of pancreatic beta-cells is associated with auto-reactive T cell infiltration. We have preliminary data demonstrating aCeAg efficacy in suppressing activation of a human T cell line in vitro. Our future research will use this model to tease out the essential components of the aCeAg homogenate required for suppression of T cell activation. Additionally, this in vitro analysis will allow us to screen fractions of the aCeAg homogenate for efficacy, thereby reducing the pool of non-essential components. Ultimately we hope to identify the specific antigens responsible for protection by using mass spectrophotometry on the effective sub-fractions.

Parasitic worms are increasingly recognized as having beneficial effects against inflammatory diseases. Even though numerous individuals purposefully infect themselves with nematodes to ameliorate symptoms of allergic and autoimmune diseases [32,33], to date no helminth or helminth-derived therapies have been approved for clinical use. The discovery that antigens obtained from an axenically cultured non-parasitic environmental nematode are protective in an inflammatory disease model should accelerate our ability to develop nematode-derived therapies for allergy and autoimmune diseases.

Author contributions

Belinda M. Jackson-Thompson: Study design, Data acquisition, Formal analysis and interpretation, Writing - original draft, Writing - review & editing. Marina Torrero: Study design, Formal analysis. Blima K. Mitre: Data acquisition, Formal analysis. James Long: Data acquisition, Formal analysis. Mathanraj Packiam: Data acquisition, Formal analysis. Edward Mitre: Conceptualization, Funding acquisition, Study design, Formal analysis and interpretation, Manuscript - Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the Drs. Kateryna Lund and Kheem Bisht in the Biomedical Instrumentation Center of USUHS for their expertise and assistance in Flow Cytometry acquisitions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2020.100065.

Funding

This work was supported by the National Institutes of Health [Grant: 1 DP2 DK083131], the Uniformed Services University [Grant: F173424117], and the Uniformed Services University/Henry Jackson Foundation Joint Office of Technology Transfer [Grant: Q573427815].

References

[1] B.-S.P. Alexandre-Silva GM, A.C.S. Oliveira, F.A. Cerni, U. Zottich, M.B. Pucca, The hygiene hypothesis at a glance: early exposures, immune mechanismand novel therapies, Acta Trop. 188 (2018) 16–26.
[2] H. Okada, C. Kuhn, H. Feillet, J.F. Bach, The 'hygiene hypothesis' for autoimmune and allergic diseases: an update, Clin. Exp. Immunol. 160 (1) (2010) 1–9.
[3] G.A. Rook, L.R. Brunet, Old friends for breakfast, Clin. Exp. Allergy 35 (7) (2005) 841–842.
[4] S. Scrivener, H. Yemaneberhan, M. Zebenigus, D. Tilahun, S. Girma, S. Ali, P. McElroy, A. Custovic, A. Woodcock, D. Pritchard, A. Venn, J. Britton, Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study, Lancet 358 (9292) (2001) 1493–1499.
[5] M. Yazdanbakhsh, A. van den Biggelaar, R.M. Maizels, Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease, Trends Immunol. 22 (7) (2001) 372–377.

[6] A.H. van den Biggelaar, R. van Ree, L.C. Rodrigues, B. Lell, A.M. Deelder, P.G. Kremsner, M. Yazdanbakhsh, Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10, Lancet 356 (9243) (2000) 1723–1727.
[7] T.B. Smallwood, P.R. Giacomin, A. Loukas, J.P. Mulvenna, R.J. Clark, J.J. Miles, Helminth immunomodulation in autoimmune disease, Front. Immunol. 8 (2017) 453.
[8] M.P. Hubner, J.T. Stocker, E. Mitre, Inhibition of type 1 diabetes in filaria-infected non-obese diabetic mice is associated with a T helper type 2 shift and induction of FoxP3+ regulatory T cells, Immunology 127 (4) (2009) 512–522.
[9] M.P. Hubner, Y. Shi, M.N. Torrero, E. Mueller, D. Larson, K. Soloviova, F. Gondorf, A. Hoerauf, K.E. Killoran, J.T. Stocker, S.J. Davies, K.V. Tarbell, E. Mitre, Helminth protection against autoimmune diabetes in nonobese diabetic mice is independent of a type 2 immune shift and requires TGF-beta, J. Immunol. 188 (2) (2012) 559–568.
[10] C. Shepherd, S. Navarro, P. Wangchuk, D. Wilson, N.L. Daly, A. Loukas, Identifying the immunomodulatory components of helminths, Parasite Immunol. 37 (6) (2015) 293–303.
[11] K. Sobotkova, W. Parker, J. Leva, J. Ruzkova, J. Lukes, K. Jirku Pomajbikova, Helminth therapy - from the parasite perspective, Trends Parasitol. 35 (7) (2019) 501–515.
[12] T. Stiernagle, Maintenance of C. elegans, in: T.C.e.R. WormBook, Community (Eds.), February 11 (2006).
[13] R. Nass, I. Hamza, The nematode C. elegans as an animal model to explore toxicology in vivo: solid and axenic growth culture conditions and compound exposure parameters, Curr Protoc Toxicol 31 (1) (2007) 1.9.1–1.9.18.
[14] N.J. Szewczyk, E. Kozak, C.A. Conley, Chemically defined medium and Caenorhabditis elegans, BMC Biotechnol. 3 (2003) 19.
[15] T.K. Samuel, J.W. Sinclair, K.L. Pinter, I. Hamza, Culturing Caenorhabditis elegans in axenic liquid media and creation of transgenic worms by microparticle bombardment, JoVE 90 (2014), e51796.
[16] A. Cooke, P. Tonks, F.M. Jones, H. O'Shea, P. Hutchings, A.J. Fulford, D.W. Dunne, Infection with Schistosoma mansoni prevents insulin dependent diabetes mellitus in non-obese diabetic mice, Parasite Immunol. 21 (4) (1999) 169–176.
[17] K.A. Saunders, T. Raine, A. Cooke, C.E. Lawrence, Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection, Infect. Immun. 75 (1) (2007) 397–407.
[18] A.C. La Flamme, K. Ruddenklau, B.T. Backstrom, Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis, Infect. Immun. 71 (9) (2003) 4996–5004.
[19] R.W. Summers, D.E. Elliott, J.F. Urban Jr., R.A. Thompson, J.V. Weinstock, Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial, Gastroenterology 128 (4) (2005) 825–832.
[20] P.K. Mishra, N. Patel, W. Wu, D. Bleich, W.C. Gause, Prevention of type 1 diabetes through infection with an intestinal nematode parasite requires IL-10 in the absence of a Th2-type response, Mucosal Immunol. 6 (2) (2013) 297–308.
[21] C.L. Tang, J.N. Zou, R.H. Zhang, Z.M. Liu, C.L. Mao, Helminths protect against type 1 diabetes: effects and mechanisms, Parasitol. Res. 118 (4) (2019) 1087–1094.
[22] S.M. Quinn, M. Raverdeau, A.M. McGinley, K.H.G. Mills, Helminths products directly modulate T cells that mediate experimental autoimmune encephalomyelitis, Eur. J. Immunol. 49 (8) (2019) 1291–1294.
[23] R.M. Maizels, Regulation of immunity and allergy by helminth parasites, Allergy 75 (3) (2020) 524–534.
[24] P.H. Gazzinelli-Guimaraes, T.B. Nutman, Helminth parasites and immune regulation, F1000Res 7 (2018).
[25] R.M. Maizels, H.H. Smits, H.J. McSorley, Modulation of host immunity by helminths: the expanding repertoire of parasite effector molecules, Immunity 49 (5) (2018) 801–818.
[26] M.M. Zaiss, N.L. Harris, Interactions between the intestinal microbiome and helminth parasites, Parasite Immunol. 38 (1) (2016) 5–11.
[27] A. Paun, J.S. Danska, Immuno-ecology: how the microbiome regulates tolerance and autoimmunity, Curr. Opin. Immunol. 37 (2015) 34–39.
[28] T.P. Brosschot, L.A. Reynolds, The impact of a helminth-modified microbiome on host immunity, Mucosal Immunol. 11 (4) (2018) 1039–1046.
[29] R.J. Lilienthal GM, J. Petry, Y.C. Bartsch, A. Leliavski, M. Ehlers, Potential of murine IgG1 and human IgG4 to inhibit the classical complement and Fcγ receptor activation pathways, Front. Immunol. 9 (2018) 958.
[30] F. Nimmerjahn, J.V. Ravetch, Divergent immunoglobulin g subclass activity through selective Fc receptor binding, Science 310 (5753) (2005) 1510–1512.
[31] S.E. Kim, J.H. Kim, B.H. Min, Y.M. Bae, S.T. Hong, M.H. Choi, Crude extracts of Caenorhabditis elegans suppress airway inflammation in a murine model of allergic asthma, PloS One 7 (4) (2012), e35447.
[32] M. Velasquez-Manoff, The parasite underground, in: The New York Times Magazine, 2016.
[33] S.J. Donkers, M.C. Kirkland, M. Charabati, L.C. Osborne, Perspectives of people with multiple sclerosis about helminth immunotherapy, Int J MS Care 22 (1) (2020) 43–51.