

Haptoglobin as an early serum biomarker of virus-induced autoimmune type 1 diabetes in biobreeding diabetes resistant and LEW1.WR1 rats

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Abstract

Proteomic profiling of serum is a powerful technique to identify differentially expressed proteins that can serve as biomarkers predictive of disease onset. In this study, we utilized two-dimensional (2D) gel analysis followed by matrix-assisted-laser desorption/ionization time-of-flight mass spectrometry analysis to identify putative serum biomarkers for autoimmune type 1 diabetes (T1D) in biobreeding diabetes resistant (BBDR) rats induced to express the disease. Treatment with toll-like receptor 3 ligand, polyinosinic:polycytidilic acid (pIC), plus infection with Kilham rat virus (KRV), a rat parvovirus, results in nearly 100% of young BBDR rats becoming diabetic within 11–21 d. Sera collected from prediabetic rats at early time points following treatment with pIC + KRV were analyzed by 2D gel electrophoresis and compared with sera from control rats treated with phosphate-buffered saline, pIC alone or pIC + H1, a non-diabetogenic parvovirus. None of the latter three control treatments precipitates T1D. 2D gel analysis revealed that haptoglobin, an acute phase and hemoglobin scavenger protein, was differentially expressed in the sera of rats treated with pIC + KRV relative to control groups. These results were confirmed by Western blot and enzyme-linked immunosorbent assay studies, which further validated haptoglobin levels as being differentially increased in the sera of pIC + KRV-treated rats relative to controls during the first week following infection. Early elevations in serum haptoglobin were also observed in LEW1.WR1 rats that became diabetic following infection with rat cytomegalovirus. The identification and validation of haptoglobin as a putative serum biomarker for autoimmune T1D in rats now affords us the opportunity to test the validity of this protein as a biomarker for human T1D, particularly in those situations where viral infection is believed to precede the onset of disease.

Keywords: BBDR rat, biomarker, haptoglobin, KRV, type 1 diabetes

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Introduction

Type 1 diabetes (T1D) is characterized by near total insulin deficiency resulting from the autoimmune destruction of insulin-producing beta-cells in the pancreatic islets by auto-reactive T lymphocytes recognizing beta cell-specific antigens.^{1,2} The disease predominantly affects young children, and imposes a significant burden on their lives stemming from its chronicity and its potentially life-threatening complications. A strong association between disease susceptibility and certain human leukocyte antigen (HLA) genes has been shown.^{3,4} Genetic susceptibility alone, however,

is insufficient to explain disease penetrance since concordance in monozygotic twins ranges only between 30% and 50%.⁵ Therefore, genetic susceptibility in conjunction with environmental perturbants may be necessary to precipitate disease. The most important of these environmental perturbants may be viruses.^{6,7} T1D predominates as a disease of children who tend to be exposed to a variety of infectious agents that may influence predisposition to autoimmunity during a critical period during maturation of their immune system. Indeed, T-cell tolerance (and prevention of autoimmunity) is in part mediated by interactions of

maturing thymocytes with antigens expressed in the thymic microenvironment.^{1,2}

Definitive proof that any given virus directly precipitates diabetes in humans is still lacking. The paucity of data supporting causal association with viral infections may be attributable to the fact that the autoimmune reaction is not clinically apparent immediately following infection. Rather, the destruction of pancreatic islets can take an indolent course, progressing for years before clinical diagnosis is established. The recent availability of novel immunomodulatory therapies such as anti-CD3, which may preserve residual beta-cell mass in new onset diabetics,⁸ has generated a demand for non-invasive testable biomarkers that can identify the development of the autoreactive process before it becomes clinically apparent. Reliably identifying patients in the early phases of disease would provide clinicians with a window of opportunity when pharmacotherapy could be more effective in halting disease.

Currently, commonly used predictive markers for T1D include human leukocyte antigen (HLA, the name of the major histocompatibility complex in humans) typing and serum autoantibodies to pancreatic islet antigens. However, these markers are most predictive in detecting familial cases of T1D, where a proband with positive antibody titers already exists, and are less predictive of disease in the general population.^{9,10} Their lack of predictive power in the general population stems from the fact that high-risk HLA-DQ and DR genotypes occur in only a small percentage (2%) of the population, of which only 30–40% develop T1D.¹¹ Alternatively, autoantibodies against protein tyrosine phosphatase IA2, islet cell antigen 512 (together called ICA), insulin (also known as IAA), glutamic acid decarboxylase and zinc transporter ZnT8, have been shown to appear in the blood of diabetics many years prior to disease onset and are better predictors of disease in relatives of probands.^{12–14} Ideally, the most important biomarkers would identify people at risk soon after beta-cell destruction is initiated, for example, as a sequelae of a viral infection prior to the appearance of autoantibodies.

Viral infections such as cytomegalovirus, mumps, rubella, enteroviruses and parvovirus, have all been associated with human T1D.¹⁵ Indeed, the effects of diverse viruses in triggering T1D may explain the heterogeneous nature of disease onset and kinetics in the general population. In animal models of T1D, unequivocal evidence of virally-induced T1D comes from studies of biobreeding diabetes resistant (BBDR) rats infected with the UMass strain of rat virus (Kilham rat virus [KRV]-UMass), a ssDNA parvovirus.^{16,17} When maintained in a viral antibody-free (VAF) facility, BBDR rats never develop T1D, despite having the susceptible RT1^u major histocompatibility complex (MHC) haplotype.¹⁸ However, when KRV-infected BBDR rats are pretreated for three consecutive days with a low dose (1 µg/g) of polyinosinic:polycytidilic acid (polyinosinic:polycytidilic acid [pIC], a synthetic dsRNA moiety that serves as a ligand for toll-like receptor 3 [TLR3] and MDA5), the conversion to diabetes among these rats is nearly 100%.¹⁸

The exact mechanism by which treatment with KRV synergizes with pIC to induce diabetes in the BBDR rat is still unclear. Although TLR-induced activation of innate

immunity is a critical component for diabetes expression, KRV does not appear to directly infect the pancreatic islets.^{12,19} However, the highly predictable time course with which the pIC + KRV treatment in BBDR rats leads to nearly 100% conversion to diabetes 11–21 d following treatment onset allows us to identify putative serum biomarkers in the earliest phase of the autoimmune process among rats that we know will develop diabetes. In this study, we used a global proteomics technique to analyze the sera of prediabetic rats treated with pIC plus KRV infection to identify proteins that may serve as biomarkers for T1D. Our screen of the BBDR rat serum proteome has revealed a potential biomarker, haptoglobin, an acute phase and hemoglobin scavenger protein that has a differential expression pattern in the sera of pIC + KRV-treated rats as compared with control groups of BBDR rats.

Materials and methods

Animals and cell lines

VAF BBDR/Wor and LEW1.WR1 rats were obtained from Biomedical Research Models, Inc (Worcester, MA, USA). Animals were certified to be free of a panel of viruses, including KRV and rat cytomegalovirus (RCMV). All animals were housed in a VAF facility in microisolator cages and provided with water and commercial chow *ad libitum*. BBDR and LEW1.WR1 rats 21–25 d of age of either sex were used and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. KRV-UMass and NRK cells were obtained from stocks maintained in our laboratories. KRV (UMass isolate) was propagated in NRK cells grown in Dulbecco's minimal essential medium.²⁰ Toolan's H-1 virus was obtained from ATCC (Manassas, VA, USA) and was propagated in Chang liver cells.²⁰ RCMV was prepared as described.²¹

Virus infection protocols

pIC was purchased from Sigma (St Louis, MO, USA), dissolved in Dulbecco's phosphate-buffered saline (PBS) (1 mg/mL), sterile-filtered and stored at –20°C until used. The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC, USA) and was uniformly <10 units/mg. Rats were injected intraperitoneally with pIC (1 µg/g body weight on days –3, –2 and –1) starting at 21–25 d of age; pIC-treated rats were either given no further treatment or infected intraperitoneally with 1×10^7 PFU KRV or H-1 in a volume of 1 mL on treatment day 0. In designated experiments, LEW1.WR1 rats were treated with 5×10^6 PFU of RCMV alone. In experiments designed to measure the frequency of diabetes induction, all treated rats were screened for glycosuria twice weekly. Diabetes was diagnosed on the basis of a plasma glucose concentration >250 mg/dL (11.1 mmol/L).

Blood collection/serum harvesting

Blood (200–500 μ L) was collected on indicated days via tail vein nicking into unheparinized tubes. On experiment termination days, rats were first euthanized in a CO₂ chamber and blood was subsequently harvested by cardiac puncture. All blood samples were allowed to clot at room temperature for 1 h, centrifuged at 4°C at 13,000 *g* for 10 min and sera harvested. Sera used for proteomics experiments were additionally re-centrifuged at 4°C at 13,000 *g* for 10 min to remove any residual cell debris. All sera were aliquoted and frozen at –80°C until use.

Immunoaffinity removal of rat serum abundant proteins

Rat serum samples were depleted of albumin, IgG and transferrin using the Multi Affinity Removal Column (Ms-3, 4.6 \times 100 mm, Agilent Technologies, Santa Clara, CA, USA). Sera were first diluted with four volumes of Agilent solvent A and centrifuged at 16,000 *g* for 1 min through a 0.22 μ m spin filter to remove particulates. Five hundred microliters of the diluted serum (1:10) were then injected onto an Applied Biosystems Vision™ Workstation liquid chromatography system with the attached Agilent Multi Affinity Removal Column at a flow rate of 0.5 mL/min. Flow through was collected in a volume of 1.5 mL, and an aliquot was analyzed by one-dimensional (1D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins captured by the column were removed with Agilent solvent B at a flow rate of 1 mL/min and analyzed in parallel with the flow-through samples. The column was regenerated by equilibrating with solvent A.

Native and non-native 1D electrophoresis

Non-depleted serum samples were quantified using the Bradford dye-binding assay, and 3 μ g of total protein from each sample was loaded onto 8–16% Tris-HCl precast gels (Bio-Rad, Hercules, CA, USA). Gels were run in Tris glycine buffer without sodium dodecyl sulfate or any denaturing agents at a constant voltage of 200 V for 50 min. Depleted samples were concentrated on Amicon Ultra 4 10 kDa cut-off ultracentrifugation columns (Millipore, Billerica, MA, USA). Protein quantitation of the concentrated sample was achieved using the Bradford dye-binding assay. Four micrograms of the concentrated samples were reduced with 50 mmol/L dithiothreitol and subjected to electrophoresis on 4–12% Bis-Tris SDS NuPage gels (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Gels were run with the MOPS SDS running buffer (Invitrogen) at a constant voltage of 200 V for 50 min. Both native and non-native 1D gels were stained overnight with Coomassie blue and destained with milliQ water prior to imaging.

Two-dimensional electrophoresis

Prior to isoelectric focusing (IEF), samples were solubilized in 40 mmol/L Tris, 7 mol/L urea, 2 mol/L thiourea and 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), reduced with tri-butylphosphine, and alkylated with 10 mmol/L acrylamide for 90 min at room

temperature. Protein was precipitated from each sample by mixing it with nine times the sample volume of acetone for 30 min at room temperature. Following precipitation, the pellet was again solubilized and buffer exchanged in 7 mol/L urea, 2 mol/L thiourea, and 2% CHAPS until conductivity was <150 μ S/cm, and 100 μ g protein was subjected to IEF on 11 cm pH 4–7 and pH 6–11 immobilized pH gradient (IPG) strips (Amersham, Piscataway, NJ, USA). Following IEF, IPG strips were equilibrated in 6 mol/L urea, 2% SDS, 50 mmol/L Tris-acetate buffer (pH 7.0) and 0.01% bromophenol blue, and subjected to SDS-PAGE on 8–16% Tris-HCl Gel (Bio-Rad). All gels were stained in Sypro® Ruby (Molecular Probes, Eugene, OR, USA) and imaged by a charge-coupled device camera on a fluorescent imager (Bio-Rad Gel-Doc).

Axima QIT MALDI mass spectroscopy analysis

Gel spots excised with an automatic gel cutter (Bio-Rad) were washed twice for 30 min with 200 μ L of 25 mmol/L ammonium bicarbonate in 50% acetonitrile (CH₃CN). CH₃CN (50 μ L) was added to shrink the gel, the excess removed and the gel dried using a SpeedVac. Digestion was performed by the addition of 30 μ L of 2 ng/ μ L trypsin (Sigma Proteomics Grade) in 25 mmol/L ammonium bicarbonate with incubation at 37°C overnight. This solution was transferred to a clean tube and the gel was further extracted with 30 μ L of 80% CH₃CN:0.1% formic acid. The combined extracts were dried using a SpeedVac to a 10 μ L volume and acidified with 1–2 μ L of 1% trifluoroacetic acid (TFA). Samples were loaded on a μ C18 Zip Tip (Millipore) pre-equilibrated with 0.1% TFA. After washing with 2 \times 10 μ L aliquots of 0.1% TFA, samples were deposited directly onto the MALDI sample target using 1 μ L of matrix solution (15 mg/mL of 2,5-dihydroxybenzoic acid; MassPrep DHB, Waters Corp., Milford, MA, USA) in 50:50 CH₃CN:0.1% TFA. Sample plates were allowed to air dry prior to insertion into the mass spectrometer. Analysis was performed on a Kratos Axima QIT (Shimadzu Instruments, Columbia, MD, USA) matrix-assisted-laser desorption/ionization (MALDI-TOF) mass spectrometer. Peptides were analyzed in positive ion, mid-mass range mode (*m/z* 700–3000). The instrument was externally calibrated with Angiotensin II (MH⁺ at *m/z* 1046.54), P14R (MH⁺ at *m/z* 1533.86) and ACTH 18–39 (MH⁺ at *m/z* 2465.20). Precursors were selected based on signal intensity at a mass resolution width of 250 for collision-induced dissociation (CID) fragmentation using argon as the collision gas. Database searches were performed with Mascot version 2.1 (Matrix Science, Ltd, London, UK) using the mammalian index of the National Center for Biotechnology Information non-redundant (NCBI nr) protein database (04/02/06) and Peptide Mass Fingerprint program for mass spectroscopy (MS) data and the MS/MS Ion Search program for CID data. All identifications were confirmed or established with CID (MS/MS) data.

Western blot analysis

The Bradford assay was used to quantify protein levels from rat sera. Three micrograms of each serum sample were

reduced by 2-mercaptoethanol, loaded onto 4–20% precast Tris-glycine gradient gels (Invitrogen) along with purified haptoglobin (Sigma-Aldrich, St Louis, MO, USA) and molecular weight marker (Invitrogen), and run at 150 V for one hour. Gels were transferred onto polyvinylidene difluoride membranes, blocked with 5% milk for at least one hour, and incubated with primary antibody (chicken anti-rat haptoglobin, Abcam, Cambridge, MA, USA) for two hours. Membranes were washed three times with tris-buffered saline (TBS) + tween, and incubated with secondary antibody (rabbit anti-chicken HRP, Abcam) for one hour. Membranes were washed two times with TBS + Tween, once with TBS alone, and developed with ECL (Gibco). Membranes were imaged using Kodak chemiluminescent film.

Enzyme-linked immunosorbent assay

An haptoglobin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Immunology Consultants Lab (Newberg, OR, USA) and used according to the manufacturer’s instructions. Standard curves were generated utilizing haptoglobin standards supplied in the kit. All test samples were quantified in duplicate, and any values out of range were appropriately diluted and re-analyzed.

Statistical analysis

Two-tailed Student’s *t*-tests were performed to detect differences in spot values of two-dimensional (2D) gels for serum collected on the first day following KRV treatment (day +1) for each combination of the three treatment groups, pIC + KRV, pIC alone and PBS alone. Analysis of variance with

the Bonferroni test for *a posteriori* contrasts (GraphPad Prism 4.0, San Diego, CA, USA) was used to compare ELISA data on serum haptoglobin levels. *P* values of less than 0.05 were considered to be significant.

Results

2D gel and MALDI-TOF-MS/MS analysis identifies haptoglobin as an early putative biomarker for virus-induced autoimmune diabetes

To screen for early biomarkers of virus-induced autoimmune diabetes, BBDR rats were pretreated with pIC on days -3, -2 and -1 followed by KRV infection on day 0. Control rats (which will not become diabetic) were injected with PBS or pIC alone on the same days, but not infected with KRV. Blood was collected from all rats for analysis on day +1. Serum was obtained from each sample, depleted of the three most highly abundant proteins (albumin, immunoglobulin and transferrin), and subjected to 2D gel electrophoresis followed by image analysis of differentially expressed proteins. Proteins whose levels were significantly different on day +1 between the rats in each of the treatment groups were selected for identification by MALDI-TOF mass spectrometry. Table 1 contains proteins that are differentially expressed in sera from rats treated with pIC + KRV or pIC alone versus PBS, and reflect the effects of pIC treatment ± KRV. In contrast, the proteins in Table 2 show proteins that are differentially expressed in sera from rats treated with pIC + KRV versus those treated with pIC alone. Of the proteins identified, most were either acute phase proteins such as α_2

Table 1 Differentially expressed proteins in sera of rats treated with pIC + KRV or pIC alone versus PBS

Protein ID	Function	Measured pI	Measured MW (kDa)	Fold difference*	P value
Haptoglobin	Acute phase	5.694	41.240	9.642	0.0003603
	Heme scavenger	5.230	43.719	3.212	0.0009308
	Hemolysis	5.363	42.562	2.488	0.0007297
		5.114	44.196	2.049	0.002
Alpha and beta-globin	Hemolysis	7.700	12.491	6.179	0.007
Alpha 2 macroglobulin	Acute phase	6.309	32.089	4.336	0.003
	Hemolysis	6.414	42.516	4.059	0.017
	Serpin	6.319	30.937	3.258	0.007
		6.723	30.564	2.693	2.59E-05
		5.420	132.628	2.480	3.04E-05
		5.457	129.799	2.206	0.0002132
		5.386	132.628	2.032	0.023
Biliverdin reductase B	Acute phase	6.553	23.304	2.970	0.008
	Heme degradation				
Major beta-hemoglobin	Hemolysis	7.312	13.037	2.904	4.92E-07
		7.004	12.199	2.822	0.011
Complement component 3	Acute phase	8.635	61.733	2.841	0.000105
		5.834	17.071	-2.375	0.046
Plasminogen protein	Plasmin precursor	7.095	166.928	2.399	0.001
Hemopexin	Acute phase	6.685	50.229	2.112	6.86E-05
	Heme degradation	6.685	46.493	2.016	0.0001485
Alpha 1 inhibitor III	Serpin	5.156	38.697	-2.324	8.64E-08
		4.497	19.921	-2.340	0.008
		4.916	23.870	-2.674	0.001
Spin 2a	Serpin	4.623	17.290	-2.437	0.0001016

pIC, polyinosinic:polycytidilic acid; KRV, Kilham rat virus; PBS, phosphate-buffered saline

*Positive values indicate the fold increase in serum protein levels while negative values indicate the fold decrease in pIC + KRV- and pIC-alone-treated rats compared with PBS-treated controls

Table 2 Differentially expressed proteins in sera of rats treated with pIC + KRV versus pIC alone

Protein ID	Measured pI	Measured MW (kDa)	Fold difference*	P value
Preprohaptoglobin	5.902	29.093	-3.579	0.008
	5.174	32.225	-3.156	0.00001633
Haptoglobin Precursor	4.994	12.967	-2.854	0.014
	5.360	12.695	-2.624	0.007
Haptoglobin precursor or haptoglobin alpha 1 s bal-647	5.871	30.625	-2.501	0.001
	5.202	30.411	-3.060	0.039
	5.388	29.891	-2.245	0.022
Complement component 3	7.921	231.297	-2.115	4.81E-06

pIC, polyinosinic:polycytidilic acid; KRV, Kilham rat virus

*Negative values indicate the fold decrease in serum protein levels in pIC + KRV-treated rats relative to those treated with pIC alone

macroglobulin or complement component 3, or proteins involved in hemolysis and the heme scavenging pathway such as hemoglobin, biliverdin reductase B and hemopexin. However, the serum protein whose levels were most affected by treatment was haptoglobin (~9-fold increase in pIC + KRV or pIC-alone-treated rats compared with PBS controls).

We observed that several modified variants of the activated haptoglobin protein were identified by MALDI-TOF mass spectrometry (Table 1). These isoforms ranged in pI from 5.1 to 5.6 and in molecular weight from 41 to 44 kDa, and all isoforms appeared to be significantly elevated (between 2- and 9-fold) in the sera of rats treated with pIC + KRV or pIC alone, when compared with rats treated with PBS alone. Interestingly, the other significantly elevated protein (~6-fold) in the sera of pIC + KRV or pIC-alone-treated rats compared with rats treated with PBS alone included the alpha- and beta-globin chains of hemoglobin, targets of haptoglobin binding. In addition, it is intriguing that the preprohaptoglobin was *lower* in pIC + KRV-treated rats than those receiving pIC alone (Table 2), consistent with an *increased* conversion of precursor haptoglobin to its active form in the pIC + KRV-treated rats. Thus, as early as one day after virus infection, significant differences were found in the sera of rats that did, or did not, progress to diabetes.

Both precursor and activated forms of haptoglobin remain elevated in pIC + KRV-treated rats compared with those treated with pIC +/- non-diabetogenic H1 virus

To next validate the results obtained in the 2D gel experiments using a different assay, Western blot analysis of undepleted serum samples was performed. BBDR rats were randomized to one of four treatment groups: PBS alone, pIC alone, pIC + KRV or pIC + H-1 virus. KRV and H-1, both parvoviruses, share >80% sequence homology within their structural proteins, and 100% homology within their non-structural proteins. H-1, however, when injected alone or with pIC, does not cause diabetes in BBDR rats.²² In one study, blood was collected by tail bleed. Because we were concerned about the extent to which the method of bleeding rats might have promoted hemolysis in our samples, thereby contributing to the selective identification of heme-associated proteins, we also performed a second study using cardiac puncture. In our

experience, this method is least likely to rupture red blood cells and promote hemolysis.

Blood was collected from BBDR rats over an approximately two-week time course (where day -4 is pretreatment). As above, rats were treated with pIC (or PBS) on days -3, -2 and -1 followed by KRV (or H1) infection on day 0. Representative Western blot data from the tail bleed and cardiac puncture studies are shown in Figures 1a and b, respectively. Three haptoglobin-specific bands were typically found in each sample: (1) prohaptoglobin (47 kDa), the uncleaved protein that normally circulates in the body; (2) beta (37 kDa); and (3) alpha (10 kDa) chain of the cleaved, activated haptoglobin molecule. On day 0 and day +1, in both the tail bleed and cardiac puncture experiments, Western blot analysis demonstrated an increase in all three bands in the pIC-treated group with or without concomitant virus infection of rats compared with rats treated with PBS alone. The intensity of all three bands decreased subsequent to day +1, although levels remained highest in the pIC + KRV group. This decrease in prohaptoglobin and activated haptoglobin in the serum appears to correspond with the serum clearance of pIC, which is undetectable 24-48 h following intraperitoneal injection (Dr Danny Zipris, personal communication).

ELISA quantitation of serum haptoglobin levels distinguish pIC + KRV-treated rats from those treated with pIC alone or with concomitant infection with the non-diabetogenic H1 virus

The above studies show that serum haptoglobin is increased in virus-induced diabetes, but to have utility as a biomarker it must also be quantifiable. Therefore, we next used ELISA analysis to quantify serum haptoglobin and correlate the levels with diabetes outcome. In sera from tail bleeds or cardiac punctures of rats treated with PBS alone, pIC alone, pIC + KRV or pIC + H-1, we observed similar trends in serum haptoglobin levels quantified by ELISA (Figure 2) as observed by Western blot (Figure 1). Although the total amount of haptoglobin was lower in the sera harvested from tail blood than cardiac blood, perhaps due to increased clotting and consumption of free serum haptoglobin, the overall changes in haptoglobin levels over time in each of the treatment groups appeared remarkably similar.

In both tail and cardiac bled rats, haptoglobin levels were dramatically elevated on days 0 (after pIC treatment but

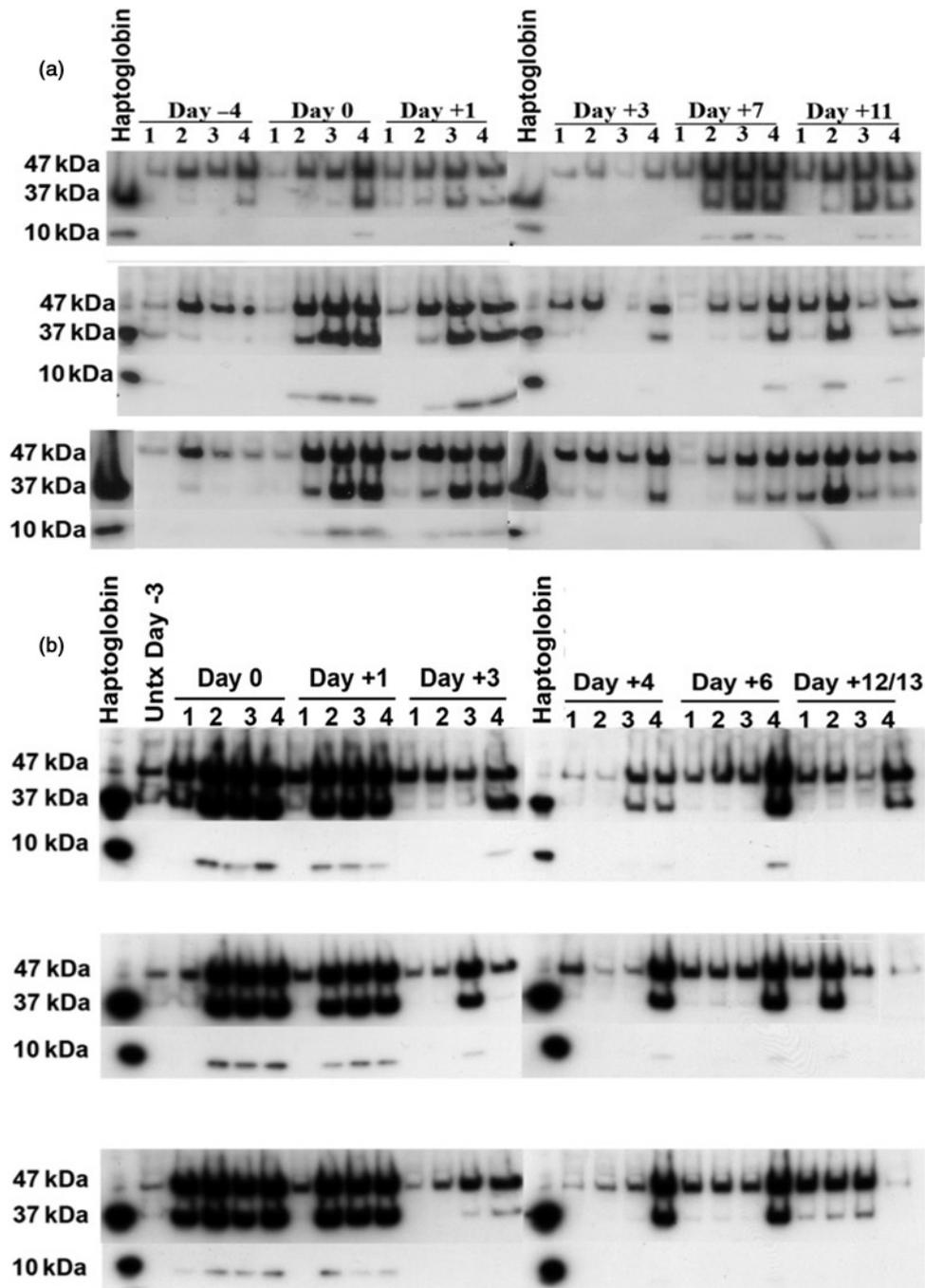


Figure 1 Western blot analysis of undepleted sera from rats treated with PBS alone (1), pIC alone (2), pIC + H-1 (3) or pIC + KRV (4). Protein concentration was assessed by the Bradford assay and equal protein was loaded in each well. Rats were tail bled (a) or cardiac bled (b) at indicated time points. Purified rat haptoglobin was used as a positive control; molecular weight of precursor prohaptoglobin is 47 kDa and cleaved, activated haptoglobin is 37 kDa (β chain) and 10 kD (α chain). In (a) serum was collected from a single rat in each group for the time points indicated; shown are data from three rats per group. All pIC + KRV-treated rats developed diabetes by 21 d; the rats in all other treatment groups remained diabetes-free. In (b) each lane represents serum from a single rat. pIC, polyinosinic:polycytidilic acid; KRV, Kilham rat virus

prior to virus infection) and +1 (1 day after virus infection) in all groups that received pIC relative to rats treated with PBS alone. Haptoglobin levels declined thereafter, perhaps as a consequence of clearance of pIC from the bloodstream. In contrast, serum haptoglobin levels remained significantly elevated in the pIC + KRV-treated rats when compared with the pIC + H-1-treated rats on days +3 and day +7 in tail bled rats (Figure 2a, $P < 0.05$), and on day +3 and

day +6 in rats that were bled via the cardiac route (Figure 2b, $P < 0.05$). Both tail and cardiac bled rats also had higher haptoglobin levels on day +4 in the pIC + KRV group, although this elevation was not statistically significant. This rapid increase and decline in total haptoglobin levels, with a more sustained elevation only in the pIC + KRV-treated animals, was also observed in the Western blot analysis (Figure 1).

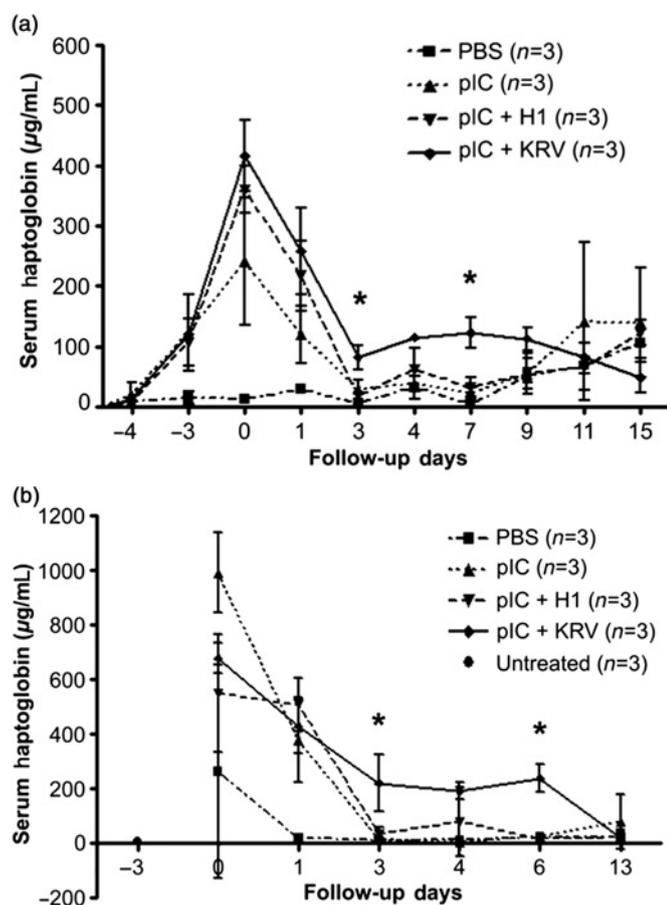


Figure 2 Serum haptoglobin levels determined by ELISA in BBDR rats treated with PBS alone, pIC alone, pIC + H-1 and pIC + KRV. Serum samples were obtained from rats that were (a) tail bled or (b) cardiac bled at indicated time points, and were run in duplicate against a standard curve of purified rat haptoglobin. * $P < 0.05$ between pIC + KRV and pIC + H-1 groups. In (a) all pIC + KRV-treated rats developed diabetes by 21 d; the rats in all other treatment groups remained diabetes free. pIC, polyinosinic:polycytidilic acid; KRV, Kilham rat virus; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BBDR, biobreeding diabetes resistant

Identification of hemoglobin:haptoglobin complexes in sera of BBDR rats treated with pIC ± KRV

Our 2D and MALDI-TOF mass spectrometry analysis identified a significant increase in both haptoglobin and hemoglobin alpha and beta chains in pIC alone and pIC + KRV-treated rats (Table 1). Since haptoglobin is known to complex with hemoglobin, we investigated the potential formation of haptoglobin:hemoglobin or other protein complexes by native gel. We subjected day +1 sera from rats treated with PBS alone, pIC alone or pIC + KRV, to native gel analysis (without denaturation). Bands that appeared to be different between treatment groups by gross visualization were excised, tryptically digested and peptides identified by MALDI-TOF mass spectrometry. Interestingly, the most prominent band that appeared in the native gel of serum samples from day +1 of pIC alone and pIC + KRV-treated rats was identified as a complex of hemoglobin alpha chain and haptoglobin (Figure 3). This band disappeared in the sera from rats treated with pIC alone on days +3 and +6, but persisted in the sera from rats treated with pIC + KRV on those same days.

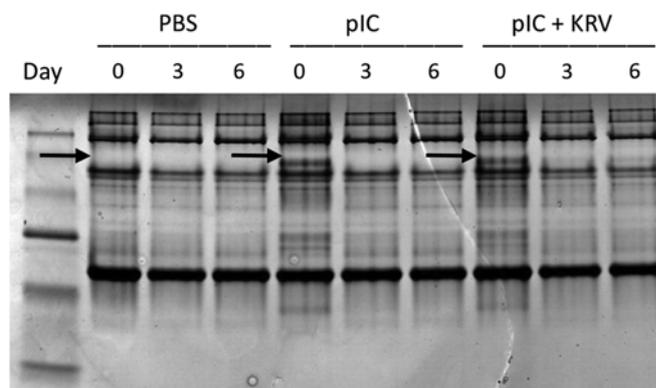


Figure 3 Native gel of sera from BBDR rats treated with PBS alone, pIC alone or pIC + KRV on indicated days. Protein concentration was quantified using the Bradford assay and equivalent concentrations of proteins were loaded into each well. Protein bands (indicated by arrow) that differed between groups were excised from individual lanes using a spot cutter; a molecular weight marker is shown in lane 1. Gel plugs from each lane were tryptically digested and peptides identified by MALDI-TOF mass spectrometry. pIC, polyinosinic:polycytidilic acid; KRV, Kilham rat virus; PBS, phosphate-buffered saline; BBDR, biobreeding diabetes resistant; MALDI-TOF, matrix-assisted-laser desorption/ionization time-of-flight

Serum haptoglobin levels do not distinguish diabetes outcome in BBDR rats treated with KRV alone

Validation of haptoglobin as a biomarker of T1D requires testing the predictive value of the marker in a variety of experimental settings where diabetes penetrance is not complete. For the next study, we again employed BBDR rats infected with KRV, but without pIC pretreatment. BBDR rats treated with KRV alone develop T1D with a cumulative frequency of ~30% within 21 d.²⁰ Because haptoglobin levels by Western blot and ELISA analyses gave concordant results, these subsequent studies utilized ELISA alone.

Following treatment with a single intraperitoneal dose of KRV alone, 42% (10 of 24) of BBDR rats became diabetic within 17 d (Figure 4a). Haptoglobin levels of KRV-treated rats, regardless of diabetes status, were always higher than untreated controls for all time points following infection, indicating that KRV alone promotes an increase in serum haptoglobin levels (Figure 4b). However, at any given time point, there was no statistically significant difference in serum haptoglobin levels between rats that became diabetic compared with those that remained diabetes-free.

Serum haptoglobin levels trend higher in prediabetic compared with diabetes-free LEW1.WR1 rats treated with RCMV alone

Because multiple viruses have been implicated in the initiation of human T1D, we next wished to investigate whether serum haptoglobin levels associate with diabetes outcome in a different rodent model of virally-induced diabetes. For these studies we used LEW1.WR1 rats, which develop T1D in response to RCMV infection with the same frequency and time course as BBDR rats in response to KRV.²³ In our studies, we found that 22% (3 of 11) of LEW1.WR1 rats treated with a single dose of RCMV developed diabetes within 16 d (Figure 5a). RCMV-treated rats that went on to become diabetic (but not those that

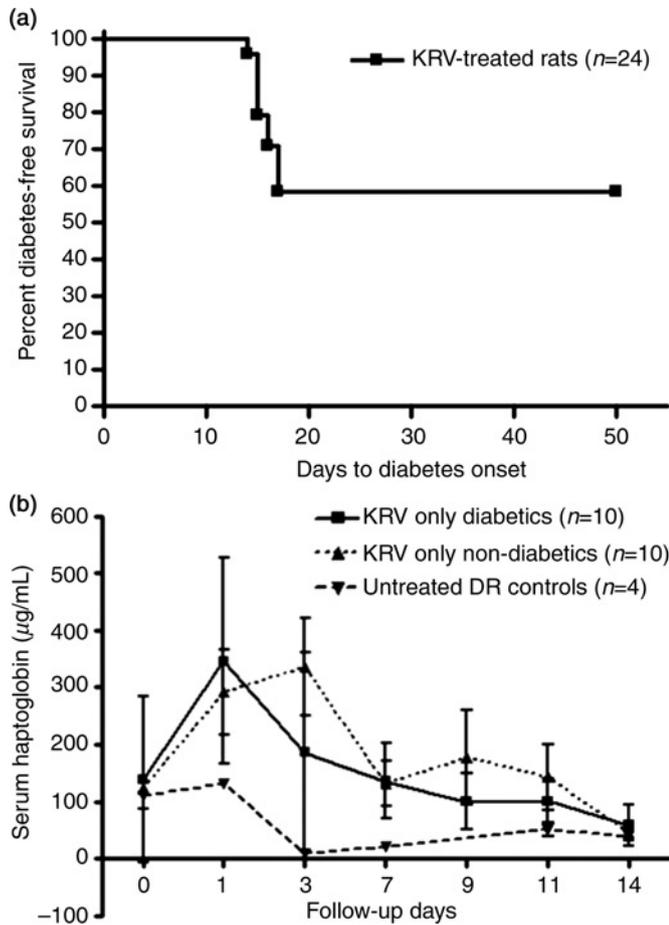


Figure 4 Diabetes incidence and serum haptoglobin levels in BBDR rats treated with KRV alone. (a) Kaplan-Meier survival curve depicts the percent of rats exhibiting diabetes-free survival; $n = 24$ KRV-treated rats, 10 developed diabetes. (b) Rats were tail bled on the indicated days and serum haptoglobin levels determined by ELISA. For any given time point, there were no significant differences in serum haptoglobin levels between KRV-treated rats that became diabetic and those that remained diabetes-free. KRV, Kilham rat virus; BBDR, biobreeding diabetes resistant; ELISA, enzyme-linked immunosorbent assay

remained diabetes-free) had significantly higher haptoglobin levels compared with untreated controls (Figure 5b, $P < 0.05$).

Discussion

The need to identify a biomarker that can predict virus-induced T1D in humans prompted us to search for such a marker in the serum proteome of a virus-induced rodent model of the disease. Causal association between viruses and T1D has been definitively established in rodent models, including the BBDR rat that becomes diabetic following infection with KRV. Importantly, T1D induced by pIC + KRV in BBDR rats shares several key similarities with human T1D, including association with susceptible MHC/HLAs, lack of a gender bias among diabetics and the risk of life-threatening disease complications, including diabetic ketoacidosis. In this report, we used 2D gel electrophoresis in conjunction with mass spectrometry

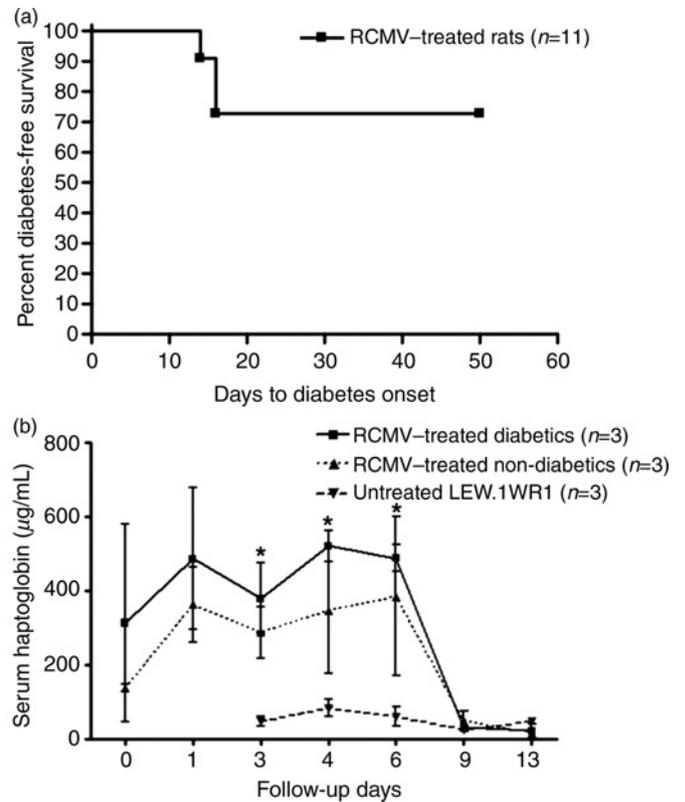


Figure 5 Diabetes incidence and serum haptoglobin levels in LEW1.WR1 rats treated with rat cytomegalovirus (RCMV) alone. (a) Kaplan-Meier survival curve depicts the percent of rats exhibiting diabetes-free survival; $n = 11$ RCMV-treated rats, three developed diabetes. (b) Rats were tail bled on the indicated days and serum haptoglobin levels were determined by ELISA; $n = 3$ for each group (3 of 8 serum samples from RCMV-treated non-diabetic rats were analyzed). Serum haptoglobin levels were significantly higher ($P < 0.05$) in diabetic RCMV-treated rats compared with untreated rats. RCMV, rat cytomegalovirus; ELISA, enzyme-linked immunosorbent assay

to identify haptoglobin as an early biomarker in BBDR rat sera of pIC + KRV-induced T1D. As confirmed by both Western and ELISA analyses, haptoglobin remained elevated in pIC + KRV-treated rats, but not in those that remained diabetes-free following treatment with pIC alone or in conjunction with a non-diabetogenic virus. Although haptoglobin (and other acute phase proteins) may be expected to increase ‘non-specifically’ following exposure to any infectious agent, its clinical utility as a predictor specific to T1D would be in those individuals with a known genetic component for diabetes susceptibility.

Haptoglobin is an acute phase and hemoglobin scavenger protein that normally circulates in the serum. Although haptoglobin is secreted both as an $\alpha_2\beta_2$ tetramer and also as dimerized pro-haptoglobin, it is the $\alpha_2\beta_2$ tetrameric form that sequesters free hemoglobin, which may be released into the circulation as a result of trauma or inflammation.^{24,25} Such trauma or inflammation can additionally stimulate increased synthesis of haptoglobin precursor protein from hepatocytes, with subsequent cleavage by serum proteases to the active $\alpha_2\beta_2$ tetrameric structure.^{24,25} In addition to haptoglobin, our initial 2D gel analysis identified many acute-phase proteins known to be involved in heme degradation, including hemopexin and biliverdin

reductase. Alpha and beta-hemoglobin were also identified, pointing to the engagement of the hemolytic pathway in our samples. Other proteins identified included alpha 1 inhibitor III, spin 2a and alpha 2 macroglobulin. These proteins belong to the serine protease inhibitor (serpin) superfamily and play key roles in controlling inflammation.²⁶ Of the identified proteins, the most significant differences in protein quantity occurred in the variants of mature haptoglobin, which increased up to nine-fold in both pIC alone and pIC + KRV-treated rats relative to rats treated with PBS alone. Yet, when comparing the pIC alone and pIC + KRV-treated groups, rats that were co-treated with KRV had significant *decreases* in the serum protein levels of haptoglobin precursors, which may reflect an enhanced cleavage of haptoglobin to the activated α - and β -components. Indeed, this is consistent with the enhanced expression of the cleaved α - and β -forms of haptoglobin shown by Western analysis (Figure 1) in most of the pIC + KRV-treated rats.

A variety of infections, including parvoviral infection, can induce RBC hemolysis,²⁷ releasing potentially toxic-free hemoglobin into the serum. Activation of haptoglobin from its pro form allows free hemoglobin molecules to be sequestered in hemoglobin:haptoglobin complexes and cleared from the circulation.²⁸ Our native gel data demonstrate that the elevation in serum haptoglobin in response to pIC treatment observed on day 0 corresponds with an elevation in the hemoglobin:haptoglobin complex formation in the serum, with a subsequent disappearance of this complex corresponding with the predicted clearance of pIC from the serum. However, in pIC + KRV treatment, the elevation in serum hemoglobin:haptoglobin complexes persisted at least through day +6, the time point when it was last tested. These results suggest that the observed increase in serum haptoglobin levels with pIC + KRV treatment, as quantified by ELISA, has a functional consequence of sequestering free hemoglobin in the serum.

The further evaluation of haptoglobin as a biomarker was of particular interest due to its biological properties. Haptoglobin, an acute phase reactant, has been reported to inhibit hemoglobin-induced oxidative damage in cells, and possesses anti-inflammatory and immunomodulatory properties. It has been demonstrated to inhibit prostaglandin synthesis, dampen the inflammatory capacity of neutrophils, monocytes and macrophages,²⁹ and interfere with B- and T-cell mitogenic responses *in vitro*.³⁰ Additionally, in humans, mutations in the haptoglobin gene are associated with increased risk of diabetic complications such as retinopathy, nephropathy and cardiovascular disease.^{31,32} In our rat studies, however, haptoglobin was identified very early following virus infection, well prior to the development of diabetes or its complications, and thus may represent a biomarker for the *pathogenesis* of T1D. Accordingly, we were interested to determine if elevations in haptoglobin in our diabetes induction models might be a potential marker for the autoreactive inflammatory response in genetically susceptible BBDR and LEW1.WR1 rats.

We performed several validation studies to determine if haptoglobin levels were sensitive in detecting T1D

development, including in virus models in which T1D expression is variable. In our study, KRV alone (without pretreatment with a TLR ligand) caused diabetes in 42% of infected BBDR rats. However, although serum haptoglobin levels were higher in KRV-treated rats than in untreated controls, the absolute serum levels of haptoglobin did not distinguish diabetes outcome within KRV-treated animals. In contrast, serum haptoglobin levels were significantly higher in RCMV-treated LEW1.WR1 rats that developed diabetes (but not those that remained diabetes-free) compared with untreated controls.

In this report, we provide evidence of haptoglobin as a biomarker for pIC + KRV-induced T1D, and its sustained serum elevations in this model may reveal insights into the pathogenesis of the disease. KRV is known to be a strong inducer of innate immune-mediated proinflammatory cytokines such as interferon- γ and interleukin (IL)-12, and has been reported to alter lymphocyte responses and cytotoxic lymphocyte activity *in vitro*.²² A closely related virus, H-1, despite sharing 80–90% sequence homology, does not induce proinflammatory cytokines to the same degree.²⁰ Five days following infection, serum IL-20p40 levels, for example, are highest in KRV-treated rats when compared with rats treated with pIC alone or H-1 virus alone.²⁰ The kinetics of cytokine secretion corresponds with the kinetics with which we observed significant increases in the serum haptoglobin levels in pIC + KRV-treated rats.

In addition to T- and B-cells, KRV is known to infect hepatocytes, and both KRV and H-1 are particularly pathogenic to the developing liver and cerebellum.^{33,34} Haptoglobin is an acute-phase response protein secreted primarily by the liver in response to proinflammatory cytokines.³⁵ Sustained elevations in serum haptoglobin in the pIC + KRV-treated rats may be attempts by the body, albeit ineffective, to guard against excessive inflammation and cytotoxicity generated by this perturbation. Indeed, the length of time and degree to which haptoglobin levels are elevated may reveal a chronic inflammatory response to an environmental perturbant that can eventually precipitate autoimmunity in a susceptible host. Such virus-plus-susceptibility gene interactions have recently been proposed to mediate the severity of disease phenotype in a mouse model of Crohn's disease.³⁶ Similarly, identification (in rodent models) and validation (in human studies) of differentially expressed serum proteins, such as haptoglobin, may reveal a 'panel' of biomarkers that occur early in the development of an environmentally initiated autoimmune diabetes. In conjunction with autoantibodies, these early biomarkers should provide a more robust – and earlier – predictor of T1D. Such biomarkers are critical for identifying children who may benefit from treatments to prevent or slow the progression of diabetes.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; AJK and CY designed experiments, collected serum samples and performed ELISA analyses. KY prepared the virus, and AJK, SWT, DH, JEE, KMG and JL performed proteomics analyses and

analyzed data. AJK, RB and DLG wrote the manuscript, and JPM, KY, DLG, AAR and RB contributed to design of the studies and reviewed the manuscript.

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