Proteomic Insight Reveals Elevated Levels of Albumin in Circulating Immune Complexes in Diabetic Plasma*§

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A hyperglycemic condition in diabetes promotes formation of advanced glycation end products, which are known to elicit immune response and form complexes with immunoglobulins called circulating immune complexes. To investigate the involvement of advanced glycation end product (AGE)-modified proteins in the elicitation of an immune response, circulating immune complexes were isolated and proteins associated were identified and characterized. Label-free-based mass spectrometric analysis of circulating immune complexes in clinical plasma of pre-diabetic, newly diagnosed diabetes, and diabetic microalbuminurea revealed elevated levels of serum albumin in the circulating immune complexes, which were also observed to be AGE modified. Further, to examine the role of glycation, circulating immune complexes were analyzed in the streptozotocin-induced diabetic mice treated with or without aminoguanidine, a prototype glycation inhibitor. Mass spectrometric analysis of circulating immune complexes showed elevated levels of serum albumin in plasma from diabetic mice over that of control animals. Aminoguanidine-treated diabetic mice displayed decreased AGE modification of plasma albumin, accompanied by a reduced level of albumin in the circulating immune complexes. In addition, elevated levels of proinflammatory cytokines such as IL-1β, IL-2, and TNF-α were observed in diabetes, which were reduced with aminoguanidine treatment, suggesting the involvement of glycation in the immune response. Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.058008, 2011–2020, 2016.

Formation of advanced glycation end products (AGEs)1 is an inevitable process during biological aging and is accelerated in diabetes (1). AGEs play a central role in development of microvascular pathology in renal glomerulus, retina, and peripheral nerves, the serious secondary complications of diabetes known as nephropathy, retinopathy, and neuropathy, respectively (2). AGE modification of the proteins leads to structural distortion, loss of side chain charge, formation of protease-resistant aggregates, and functional impairment (3, 4). Importantly, such chemically altered protein structures act as neo-self antigens or autoantigens, leading to the formation of autoantibodies (5, 6). Elevated levels of autoantibodies against AGEs were observed in the sera of diabetic patients (7, 8). Circulating autoantibodies showed higher recognition of gluco-oxidatively modified human serum albumin (HSA) in patients with diabetic complications (9). The autoantibodies form complexes with their antigens and are referred as circulating immune complexes (CICs), which were found to affect quantification of AGE antibody titer in vivo (10). CICs are generally cleared by phagocytosis (11); however, their accumulation is implicated in pathogenesis of diabetic complications. Elevated levels of CICs of glycoxidized LDL has been reported to be associated with retinopathy in type 1 diabetes and in the pathogenesis of atherosclerosis (6, 12). In fact, modified LDL associated with the immune complexes are proinflammatory, atherogenic and accumulating evidence indicates that LDL-containing immune complexes can also serve as a biomarker for macrovascular disease in type 1 diabetes (13). Further, it is reported that a major percentage of antibodies against AGE-modified proteins were constituted by IgGs in the diabetic patients (14). Hence, identification and

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1 The abbreviations used are: AGEs, advanced glycation end products; CICs, circulating immune complexes; AMG, aminoguanidine; LC-MS5, liquid chromatography mass spectrometry to the elevated energy; IgG, immunoglobulin-G; CON, control; IGT, impaired glucose tolerance; NDD, newly diagnosed diabetes; DM, diabetes mellitus; DM-MIC, diabetes mellitus with microalbuminurea; STZ, Streptozotocin; IFN, interferon gamma; GM-CSF, granulocyte macrophage colony-stimulating factor.
characterization of proteins associated with CICs possibly provides the information about AGE-modified proteins that elicit immune response in diabetic condition.

Therefore, this study aims to analyze proteins associated with the CICs in the plasma. We analyzed IgG immune complexome using protein G Sepharose affinity chromatography followed by label-free-based mass spectrometric quantification of proteins. Elevated levels of serum albumin were observed in the CICs of prediabetic, newly diagnosed diabetic, and diabetic with microalbuminurea plasma in comparison to healthy control plasma. Serum albumin was found to be more glycated in plasma of diabetes than control, suggesting the involvement of glycation in the formation of CICs. Followed by the observations in the clinical samples, serum albumin levels in the CICs were quantified and characterized in the plasma of streptozotocin (STZ)-induced diabetic mice treated with or without AGE inhibitor aminoguanidine (AMG). The levels of serum albumin were notably increased in diabetic mice plasma CICs, and interestingly, the plasma from diabetic animals treated with AMG showed decreased levels of the albumin in CICs accompanied with reduced AGE modification in plasma. Furthermore, cytokines, the key mediators of immune response, inflammation, and disease pathogenesis, were quantified to demonstrate the possible role of hyperglycemia-induced AGE modification in inflammation and autoimmune response.

**EXPERIMENTAL PROCEDURES**

**Clinical Sample Details**—Clinical plasma samples were collected from the study subjects recruited from Dr. Mohans’ Diabetes Specialities Centre, Chennai, India. The study was carried out in accordance to the Helsinki Declaration and was approved by the institutional ethics committee of Madras Diabetes Research Foundation, and informed consent was obtained from all the subjects. Study subjects comprised of control subjects with normal glucose tolerance (CON, n = 12), subjects with prediabetes or impaired glucose tolerance (IGT, n = 12), individuals who are newly diagnosed for type 2 diabetes (NDD, n = 12), type 2 diabetes patients without complications (DM, n = 12), and diabetic patients with microalbuminurea (DM-MIC, n = 10). All clinical diagnostics were done as per the American Diabetes Association criteria. Exclusion criteria included known history of cancer, hypothyroidism, hematuria, and known history of inflammatory diseases or any infection. For the preparation of plasma, peripheral venous blood was collected in EDTA vacutainers (BD Biosciences). Biochemical parameters such as fasting blood glucose, postprandial blood sugar, glycated hemoglobin (HbA1c), oral glucose tolerance test, lipids, urea, and creatinine were analyzed immediately after the sample collection. Descriptive characters and diagnostic parameters are listed in Supplemental Table 1. Further plasma separation was carried out by centrifugation at 1500 × g for 15 min, and collected plasma was stored at −80 °C until further use. Urinary excretion of albumin of 24 h was measured to assess microalbuminurea complication. Plasma samples in equal volumes were pooled into three subgroups based on similar HbA1c. Equal amount of plasma proteins from these were further processed for isolation of CICs.

**Mice Experiment Details**—All the animal experiments were carried out at the Experimental Animal Facility, National Centre for Cell Sciences, India. Experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India, and approved by Institutional Animal Ethics committee. Diabetes was induced in BALB/c mice as described (15). Male BALB/c mice were injected with 50 mg/kg body weight of STZ in 50 mM citrate buffer, pH 4.5, for five consecutive days to induce hyperglycemia, whereas control mice were injected with 50 mM citrate buffer. The induction of diabetes was confirmed by measuring the blood glucose levels with glucometer (Bayer, Germany). The animals with blood glucose of 6.12 ± 1.7 mmol/l were considered diabetic. The glycation was monitored by glycated hemoglobin (HbA1c) measurement (Nycocard HbA1c analyzer). Further, animals were treated either with or without glycation inhibitor AMG at a dose of 1 g/l drinking water to diabetic animals for about 60 days after induction of diabetes. The animals were recorded for body weight, blood glucose, and HbA1c (Supplemental Table 2). At the end of the study, blood samples were collected in sterile tubes containing EDTA, and plasma was obtained by centrifugation at 1500 g for 10 min.

**MATERIALS AND METHODS**

All chemicals and reagents were purchased from Sigma Aldrich unless otherwise stated. MS Grade solvents (water, acetonitrile (ACN) and methanol) were procured from J T. Baker (J T. Baker, PA).

**Study Design**—Complete overview of the current study is depicted in Fig. 1. Based on the hypothesis that formation of CICs occurs by elicitation of the immune response against AGE modified proteins, we isolated CICs from clinical plasma samples and quantified and characterized them by using label-free mass spectrometry. Serum albumin levels were found to be elevated in IGT, NDD, and DM-MIC plasma in comparison to that of CON, which was also characterized

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**Fig. 1. Study overview.**

**Clinical plasma (CON, IGT, NDD, DM, DM-MIC)**

- Isolation of CICs Using Protein G
- Circulating Immune Complexes (CICs)
- In solution Trypsin Digestion
- Nano-LC-MS² Label free based identification, quantification and characterization for AGES
- Investigation of role of glycation in formation of CICs in STZ Diabetic mouse model using AGE inhibitor Aminoguanidine
- Confirmation by using western blot
- Quantification of AGES in plasma albumin
- Measurement of cytokines in Plasma

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to be AGE modified by Western blotting and mass spectrometric analysis. As a proof of concept, the observations were corroborated in STZ-induced diabetic mice treated with or without prototypic AGE inhibitor AMG.

**Measurement of Fluorescent AGEs in Plasma—**Fluorescent AGEs such as argpyrimidine, pentosidine, crocine, vesperslynine A or B, vesperslynine C, and imidazolone B, which have characteristic excitation and emission wavelengths (16), were quantified in the plasma. 100 μl of diluted plasma in PBS composition (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and the above-mentioned AGEs were quantified by using fluorescence spectrophotometer (Thermo, Varioskan Flash Multimode Reader). (Ex/Em wavelengths for each of the AGEs is provided in Supplemental Table 3.)

**Isolation of CICs—**CICs were isolated from 400 μg of plasma protein by using Protein G Sepharose (Sigma Aldrich). Manufacturer’s instructions were followed for the preparation of the resin and further steps. Briefly, protein from clinical plasma (three subgroups made based on HbA1c) or from that of mice experiment was incubated with 40 μl of protein G Sepharose. Final volume was adjusted to 600 μl with 1x IP buffer and incubated for 2 h at 4 °C on rotospin rotary mixer. After the incubation, supernatant was discarded and the beads were washed for five times using 1x IP buffer. Protein absorption of the wash fraction was measured at 280 nm to monitor the complete removal of nonspecifically interacting proteins. The bound CICs fraction was eluted using 0.1% RapiGest (Waters, Milford, MA) in 50 mM ammonium bicarbonate buffer after heating at 80 °C for 15 min and centrifugation at 12,000 g for 5 min. Protein estimation was performed using Bio-Rad Bradford kit (Bio-Rad Laboratories, CA).

To evaluate the possibility of potential nonspecific binding of HSA to protein G Sepharose or Sepharose, physiological concentrations of HSA or 400 μg of clinical CON and DM plasma were incubated with either protein G Sepharose or only Sepharose column, respectively. All the conditions were maintained as used for isolation of CICs from plasma. Unbound fraction was collected, and bound fraction was eluted using SDS-loading dye after the washes. SDS-PAGE was performed to visualize the proteins.

**Western Blotting—**Plasma proteins (10 μg) or 10 μl of the CICs were separated by 10% SDS-PAGE and transferred onto PVDF membrane. After the transfer, membranes were blocked in buffer containing 5% skimmed milk (HiMedia, India) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) by incubating at 4 °C overnight. For detection of serum albumin and AGEs, blocked membrane was probed with anti-serum albumin antibody (1:5000) for 1 h at 5 °C. After the incubation, membranes were washed once with PBS-T (PBS containing 0.05% Tween 20) and two washes with PBS, followed by incubation with secondary antibody conjugated to HRP (Bangalore Genei, India) at a dilution of 1:5000 for 1 h at 25 °C. After washing as above, bands were detected by chemiluminescence using the WesternBright™ Quantum Western blotting detection kit (Advanta, Menlo Park, CA) as per the manufacturer’s instructions. Licor Image Studio™ Lite software (Licor Biosciences) was used for the quantitative analysis of the detected bands.

**In-Solution Trypsin Digestion—**CICs (50 μl) were reduced and alkylated with 100 μm dithiothreitol at 60 °C for 15 min and 200 μm iodoacetamide for 30 min at 25 °C under dark condition, respectively. The denatured CICs was digested with trypsin (1:20) for 18 h at 37 °C. Digestion reaction was stopped, and rapigest was cleaved by using formic acid and incubating at 37 °C for 45 min. Precipitated rapigest was removed by centrifuging at 14,000 g for 15 min, and supernatant containing peptides was collected.

**Liquid Chromatography-Mass Spectrometry Analysis—**Digested peptide was diluted in 3% ACN containing 0.1% FA in 1:3 ratio prior to LC-MS² (liquid chromatography mass spectrometry at elevated energy) analysis. 100 fmol of yeast alcohol dehydrogenase was used as an internal standard. Mass spectra were acquired in three technical replicates by using Nano Acuity UPLC system coupled to SYNAPT-HDMS (Waters). The binary solvent system comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). Injected peptides were first pre-concentrated and desalted online using a Symmetry C18 trapping column (180 μm x 2 cm) (Waters Corporation) with a 0.1% mobile phase B at a flow rate of 5 μl/min. After the nano-LC separation using a Ethylene Bridged Hybrid (BEH)-C18 (1.7 μm x 75 μm x 250 mm) column (Waters Corporation) peptides were eluted into the Nano-LockSpray ion source at a flow rate of 250 nl/min using a gradient of 3 to 40% B for 95 min. All the MS runs were performed in a positive V-mode at resolution of about 9000 full width half maximum with a scan time of 0.75 s in a mass range of 50–2000 m/z with alternating low (4 eV) and high (15 – 40 eV) collision energy. The mass spectrometer was calibrated with MS/MS spectra of Glu-fibrinopeptide B (m/z 785.8426) (500 fmol/μl), and every 30s, the lock mass correction was done by the same peptide.

The LC-MS² data were analyzed by Protein Lynx Global Server 2.5.1 (PLGS; Waters Corporation) software. The identification of the proteins and quantification was performed using reviewed human database (UniProt release 2013.09, 42, 897 entries) or reviewed mouse database (UniProt release 2014_08, 17,023 entries) downloaded from UniProt, to which alcohol dehydrogenase 1 (P00330) protein sequence of Saccharomyces cerevisiae was appended for quantification purpose. The preliminary search was done using ion accounting parameters where in precursor and product ion tolerance was set to automatic, minimum number of fragment ion matches per peptide three, minimum number of fragment ion matches per protein five, and minimum peptide matches per protein was one. The number of missed cleavage sites was set to two, along with carbamidomethylated Cys (C) residues as fixed and Met (M) oxidation as variable modifications. The false positive rate was 4%. Ion intensity threshold was kept at 500 counts. The label-free protein quantification was performed as described earlier (17). PLGS quantification was used for further calculating microgram per milliliter of plasma of protein by using dilution factor.

**AGE Modification Analysis by LC-MS²—**AGE modification analysis for human serum albumin (HSA) from clinical CICs was performed as described earlier (18). Briefly, a targeted search was performed involving variable glycation modifications, namely amadori (+162.0528) at lysine or arginine, carbamoylmethyl lysine (+58.0055) and carboxymethyl lysine (+72.0211) at lysine, methyl-glyoxal-induced hydroimidazolone-1 (MGH1) (+54.0106) at arginine, and oxidation at Met and fixed carbamidomethylation of Cys residues. The ion accounting parameters used for search included precursor and product ion mass tolerance of 30 ppm and 300 ppm, respectively, and the ion intensity threshold for precursor and fragments were 500 and 10 counts, respectively. Fragment matches per peptide were kept to three, and two missed cleavages were allowed. The false positive rate was set to 1%.

The PLGS identified AGE-modified peptides observed in duplicates out of triplicate MS runs were manually inspected for the following criteria: 1) missed cleavage at the modified residue; 2) the accurate mass increase corresponding to AGE modification; 3) presence of unmodified peptide with matching fragments for each modified peptide; 4) presence of fragment ions retaining modification; 5) if modification is at the N terminus, then presence of b-ions retaining modification and unmodified y-ions; 6) if modification is at the middle position, then presence of b- or y-ions retaining modification; 7) presence of at least a few consecutive b- or y-ions; and 8) presence of complementary b- or y-ions. AGE-modified peptides following the above criteria were further processed to remove noise and un-
matched peaks. A similar approach was used for the determination of AGE modifications in the proteins associated with CICs from mice plasma.

Further, the relative quantification of AGE modifications in plasma albumin from CON, DIAB, and D-AMG mice plasma was performed. Intensity of modified peptides containing previously reported amino acid residues sensitive for glycation modification were monitored (15, 19). To rule out the possibility of variations in loading the samples, modified peptide intensities were normalized with the highest total ion count of all the mass spectral acquisitions. Peptides that were consistently observed in two replications out of triplicate MS runs were considered for calculating average intensity.

Cytokine Assay—The levels of eight cytokines (IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-gamma, and TNF-alpha) were measured in plasma from mice using the Bio-Plex Pro Mouse Cytokine Group I Panel 8-Plex (Bio-Rad). The assay protocol was performed according to manufacturer’s instructions. Briefly, 50 μl of the solution containing microbeads labeled with specific antibodies for each of the cytokines to be measured was added into each well, washed thrice (wash step) with 100 μl of Bio-Plex wash buffer. 50 μl of plasma aliquots diluted to 1:4 in Bio-Plex sample diluent was added to each well and incubated for 30 min at 25 °C on constant shaking at 800 rpm. After the wash step as above, 25 μl of the detection antibody premix were added and incubated for 30 min at 25 °C followed by incubation with 50 μl of the streptavidin–phycoerythrin solution for 10 min at 25 °C. After another wash step, the beads were resuspended with 125 μl of the assay buffer. The signal was recorded using a Bio-Plex MAGPIX (Bio-Rad) multiplex reader. A simultaneously constructed standard curve for each cytokine using Bio-Plex Manager version 6.2 software was used for the determination of concentration of the cytokines in plasma.

Statistical Analysis—All the experiments were performed in triplicates. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used to determine statistical significance. All the data are expressed as mean ± S.E. A p value <0.05 was considered as statistically significant.

RESULTS

The present study deals with isolation, quantification, and characterization of CICs and investigation of role of glycation in immune complex formation. Two major experiments were performed, (1) CICs were isolated from clinical plasma samples that included CON, IGT, NDD, DM, and DM-MIC, and the proteins associated with immunoglobulins were identified, characterized, and quantified. This study revealed elevated levels of serum albumin in the CICs from IGT, NDD, and DM-MIC with respect to CON, and (2) The elevated level of serum albumin in the immune complex was also observed in STZ-induced diabetic mice, and the role of glycation of formation of immune complex was demonstrated using AMG, a well-established glycation inhibitor.

Elevated Human Serum Albumin in CICs of Plasma from Prediabetic, Newly Diagnosed Diabetic and Diabetic Microalbuminuria—A total of 40 proteins were identified in the CICs, including mainly IgGs and immune-related proteins such as compliment factors C1, C3, and C4. Apart from these, serum albumin was observed to be present in all the isolated CICs. Relative fold change of proteins identified from the CICs in different conditions with respect to healthy control is listed in Supplemental Table 4. Levels of human serum albumin were found to be significantly increased in the CICs from IGT (1.7-fold), NDD (1.7-fold), and DM-MIC (1.45-fold) when compared with CICs from CON (Fig. 2A). There was slight increase in CIC albumin levels in DM, even though it was not found to be statistically significant. Western blot analysis also revealed a similar trend in the levels of serum albumin in the CICs (Fig. 2B).

As serum albumin is the most abundant plasma protein and is known to interact with various protein and ligands, it can perhaps bind nonspecifically to protein G Sepharose or Sepharose. This possibility was ruled out as HSA did not bind to protein G Sepharose nonspecifically, and even plasma proteins from control and diabetes did not show binding upon incubation with only Sepharose, suggesting the albumin present in the CICs was not due to a nonspecific interaction (Supplemental Fig. 1). Furthermore, the serum albumin in the
CICs was characterized to be AGE modified by Western blotting (Fig. 2C) and mass spectrometric analysis (Supplemental Data 1 and 2). Spectrophotometric measurement of fluorescent AGEs (Fig. 3A) and Western blotting using anti-AGE antibodies (Fig. 3B) showed increased AGEs in the plasma of IGT, NDD, DM, and DM-MIC in comparison to CON.

Aminoguanidine Decreased the Levels of Serum Albumin in CICs from Diabetic Plasma—To determine the role of glycation in the formation of immune complex, STZ-induced diabetic mice were treated with or without AMG. AMG decreased HbA1c and plasma AGEs in diabetic mice, as evidenced by fluorescence spectrometric, Western blotting and mass spectrometric analysis (Supplemental Table 2, Figs. 4A, 4B, and 4C). The annotated spectra of AGE-modified peptides from mouse serum albumin and corresponding intensities used to generate the heatmap are provided in Supplemental Data 3 and 4. Mass spectrometric analysis of CICs showed elevated levels of serum albumin in diabetic mice plasma compared to nondiabetic mice plasma. AMG treatment reduced the albumin levels in the CICs of diabetic mice plasma (Fig. 5A). This observation was also evident by Western blotting with antialbumin antibodies (Fig. 5B). Albumin in the CICs was AGE modified as observed by Western blotting and mass spectrometric analysis (Fig. 5C) (Supplemental Data 5 and 6). The elevated levels of serum albumin in CICs of diabetic mice were accompanied by a decline in plasma albumin levels. However, the decreased plasma albumin levels in diabetes were not restored by AMG treatment (Fig. 5D). In addition to serum albumin, the levels of apolipoprotein E (Apo E) (1.6), carboxylesterase 1C (1.6), and alpha 2 macroglobulin (1.4) were found to be increased in diabetic CICs. Alpha 1 antitrypsin and apolipoprotein A1 (Apo A1) were observed only in the CICs from diabetic mice plasma but not in CICs from plasma of control mice or diabetic mice treated with AMG. The relative fold change of proteins is depicted in Supplemental Fig. 2. Mass spectrometrically identified AGE-modified peptides of the above CIC-associated proteins are listed in Supplemental Data 7 and annotated spectra are represented in Supplemental Data 8.

Cytokines in Plasma—Inflammatory cytokines and associated inflammation plays a determinant role in the development of microvascular complications of diabetes such as nephropathy, neuropathy, and retinopathy (20). Bio-Plex analysis of cytokines in the plasma showed marked increase in the
level of proinflammatory cytokines TNF-alpha, IL-1b, and IL-2 in diabetic mice compared with that of control mice. Diabetic mice treated with AMG showed decreased levels of these proinflammatory cytokines in the plasma. However, we observed no significant change in the level of GM-CSF, which is also an important proinflammatory cytokine (21). IL-10 was found to be decreased in diabetic mice plasma, and it was increased in the diabetic mice treated with AMG. However, we observed increase in the level of IL-5 in diabetic plasma in comparison to that of control (Fig. 6). IL-4 levels were found to be lower than detection limits.

**DISCUSSION**

We investigated IgG immune complexome using protein G Sepharose affinity chromatography followed by label-free-based mass spectrometric quantification of proteins to analyze proteins associated with the CICs in the plasma. A major finding of this study is that elevated levels of albumin were observed in the CICs of IGT, NDD, and DM-MIC compared with healthy CON. However, there was no significant difference in the CIC serum albumin level in the DM group, which could possibly be due to ongoing treatment and management of diabetes. Western blotting of CICs suggested that the albumin was AGE modified and plasma albumin from diabetic conditions was modified to a greater extent compared with healthy control.

To corroborate our findings of clinical study, serum albumin levels in the CICs were quantified and characterized in the plasma of STZ-induced diabetic mice treated with or without AGE inhibitor AMG. The levels of albumin were notably increased in diabetic mice plasma CICs, and interestingly, the plasma from diabetic animals treated with AMG showed decreased levels of the albumin in CICs accompanied with reduced AGE modification in plasma. Furthermore, cytokines, the key mediators of immune response, inflammation, and disease pathogenesis, were quantified to demonstrate possible role of hyperglycemia-induced AGE modification in inflammation and autoimmune response. Serum albumin is the most
abundant plasma protein (22) and preferentially gets glycated (23). Amadori-modified albumin is reported to be independently associated with nephropathy in type 1 diabetic patients (24), and in our previous study, AGE-modified serum albumin was detected in the kidney of STZ-induced diabetic rats (25). Glycated albumin levels are reported to be elevated significantly in type 2 diabetic patients with coronary artery disease (26). Glycation of albumin also induces conformational change and increases amyloid beta cross sheets (27), which may facilitate protein cross-linking and aggregation, and make it immunogenic. Autoantibodies for glycated albumin were also observed in type 1 diabetes (28). Further, as proof of concept to investigate the role of glycation in elicitation of immune response, CICs were quantified from plasma of STZ-induced diabetic mice treated with/without glycation inhibitor AMG. The protective role of AMG attributed to its AGE inhibition activity (29, 30) was exploited in our present study to evaluate the role of AGE-modified proteins in elicitation of CICs. AMG treatment reduced plasma albumin AGE modification; this was reflected in reduced albumin levels in CICs, suggesting the involvement of AGE formation in eliciting immune response and formation of CICs. In many previous studies, it has been shown that AMG treatment reduces the in vivo AGE formation and prevents diabetic complications in STZ-induced diabetic rat models (31, 32). The antibodies directed against AGE-modified proteins and the CICs formed are known to get deposited in the glomerular basement membrane and hence implicated in the progression of diabetic nephropathy (33). It is well established that AMG alleviates the symptoms of diabetic nephropathy by preventing the formation of AGEs (34). Apart from albumin, elevated levels of Apo E, carboxylesterase 1C, and alpha 2 macroglobulin were observed in diabetic mice CICs when compared with control. Alpha 1 antitrypsin and Apo A1 were observed only in the CICs from diabetic mice plasma but not in CICs from plasma of control mice or diabetic mice treated with aminoguanidine. Previously, it has been reported that Apo E, Apo A1, alpha 2 macroglobulin, and alpha 1 antitrypsin are glycated in diabetic conditions (24), and in present study, we report MS identified probable AGE modification sites in these proteins. Enhanced

![Fig. 5. Validation of clinical observations of elevated CIC albumin in mice model.](image)

(A) Bar graph depicts quantity of serum albumin in CICs from mice samples. Label-free-based MS quantification revealed increased CIC albumin in DIAB mice compared with that of CON, which was reduced in the diabetic mice treated with AMG (D-AMG) (n = 4; technical replicates). Significant difference indicated by *** (at p < 0.0001) was calculated by one-way ANOVA analysis. (B) Western blotting analysis of CICs using anti-serum albumin antibodies. (n = 3). Bar graph was plotted and fold change was calculated by the antibody signal is represented with respect to control (considered as 1). Values are mean ± S.E. Statistical significance of p < 0.01 is represented by * as calculated by one-way ANOVA. (C) Anti-AGE Western blot of CICs from mice plasma (n = 3). (D) Graphical representation of total plasma albumin estimated by BCG method (n = 4 biological replicates in three technical replications). Values are mean ± S.E. Statistical significance of p < 0.05 is represented by * as calculated by one-way ANOVA.
glycation of Apo E is reported to impair its function and may lead to accelerated atherogenesis in diabetic patients with poor glycemic control (35). Apo A1 is a major component of high density lipoproteins and glycation of Apo A1 is shown to hamper its key functions and is associated with proatherogenic events leading to coronary artery disease in diabetic patients (36, 37). In the present study, both Apo E and Apo A1 were found to be AGE modified in CICs. Even though previous studies have reported glycoxidatively modified LDL in the immune complexes (12, 38), in the present study, we did not identify any LDL protein in the CICs. This could possibly be due to the duration of the diabetes and a different experimental approach followed in the current study. Alpha 1 antitrypsin is an important serine protease inhibitor in plasma known to be involved in preventing inflammatory and proangiogenic events. Impaired function of alpha 1 antitrypsin in plasma is attributed to excessive glycation in diabetes, and the levels of alpha 1 antitrypsin were reported to decrease in nonobese diabetic mice (39).

Cytokines are low molecular weight polypeptides that regulate inflammatory immune response. Previous studies have shown that elevated inflammatory cytokines IL-1b and IL-6 together impose risk for developing type 2 diabetes (40), and also proinflammatory cytokines that are generated by the reaction of AGEs with their receptors play key role in development of diabetic complications by inducing inflammation (20). In the present study, we observed an increase in the level of proinflammatory cytokines TNF-alpha, IL-1b, and IL-2 in diabetic mice compared with that of control, and treatment of AMG decreased the level of proinflammatory cytokines. IL-10 is a strong operating anti-inflammatory cytokine, and low production capacity of IL-10 is reported to be associated with metabolic syndrome and type 2 diabetes (41). The IL-10 levels were decreased in diabetic mice compared with the control mice, and its levels were increased in plasma of diabetic mice treated with AMG. IL-5 is a type 2 cytokine that can induce pathogenic activity or can be protective to a host (42). In our analysis, IL-5 was found to be decreased in diabetic mice in comparison to control and diabetic mice treated with AMG.

To the best of our knowledge, ours is the first mass-spectrometry-based study to report elevated albumin levels in CICs from subjects with different levels of glucose intolerance and diabetic complications and to show they are also AGE modified. Our finding is consistent with the mice work wherein we observed increased albumin levels in CICs from diabetic mice, which in turn was reduced when diabetic mice were treated with aminoguanidine. It has also been reported previously that albumin transcription is reduced in diabetes mellitus (43) and reduced serum albumin levels were reported in subjects showing heavy proteinuria (44). Therefore, it is possible that, AGE-modification-induced formation of CICs acts as a major contributor to the reduction in serum albumin levels in addition to proteinuria over long-standing hyperglycemic conditions. Further, low albumin levels are associated with increased plasma AGEs and HbA1c (45, 46). Recently, immunization of AGE-modified albumin has been shown to inhibit diabetic nephropathy progression in diabetic mice (47), emphasizing the need for the development of diabetic nephrop-
athy-based immunotherapy with AGE immunization as a potential candidate. Therefore, elevated and AGE-modified albumin in CICs in our study, particularly in subjects with IGT and DM-MIC, imply that they could serve as a reliable biomarker or risk predictor for not only the genesis of type 2 diabetes but also diabetic complications such as nephropathy. Further, we propose that, a longitudinal, prospective cohort study in the clinical diabetes setting might give deeper insights about AGE-modified proteins, including HSA, and their association with elicitation of adverse immune responses and development of diabetic complications. In conclusion, this study reports that elevated levels of serum albumin in CICs of clinical diabetic plasma, which was found to be AGE modified. These findings were corroborated in STZ-induced diabetic mice model. Further, AMG treatment regulates the albumin levels in the CICs of diabetic mice, suggesting the involvement of glycation in elicitation of autoimmune response and formation of CICs.

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REFERENCES

1. Van Boekel, M. A. (1991) The role of glycation in aging and diabetes mellitus. Mol. Biol. Rep. 15, 57–64
2. Brownlee, M., Cerami, A., and Vlassara, H. (1988) Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. New Eng. J. Med. 318, 1315–1321
3. Ahmed, N., Dobler, D., Dean, M., and Thorlalley, P. J. (2005) Peptide mapping identifies hotspot site of modification in human serum albumin by methylglyoxal involved in ligand binding and esterase activity. J. Biol. Chem. 280, 5724–5732
4. Bansode, S. B., Chougale, A. D., Joshi, R. S., Giri, A. P., Bodhankar, S. L., Hansukar, A. M., and Kulkarni, M. J. (2013) Proteomic analysis of protein resistant proteins in the diabetic rat kidney. Mol. Cell. Proteomics 12, 228–236
5. Araki, N., Ueno, N., Chakrabarti, B., Morino, Y., and Horiuchi, S. (1992) Immunochenmical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. J. Biol. Chem. 267, 10211–10214
6. Virella, G., Thorpe, S. R., Alderson, N. L., Stephan, E. M., Atchley, D., Wagner, F., and Lopes-Virella, M. F. (2003) Autoimmune response to advanced glycosylation end-products of human LDL. J. Lipid Res. 44, 487–493
7. Fredrikson, G. N., Anand, D. V., Hopkins, D., Corder, R., Alm, R., Bengtsson, E., Shah, P. K., Lahiri, A., and Nilsson, J. (2009) Associations between autoantibodies against apolipoprotein B-100 peptides and vascular complications in patients with type 2 diabetes. Diabetologia 52, 1426–1433
8. Nicoloff, G., Baydannoif, S., Petrova, Ch., and Christova, P. (2002) Antibodies to advanced glycation end products in children with diabetes mellitus. Vasc. Pharmacol. 39, 39–45
9. Khan, M. W., Qadrie, Z. L., and Khan, W. A. (2010) Antibodies against gluco-oxidatively modified human serum albumin detected in diabetes-associated complications. Int. Arch. Allergy Immunol. 153, 207–214
10. Turk, Z., Ljubic, S., Turk, N., and Benko, B. (2001) Detection of autoantibodies against advanced glycation endproducts and AGE-immune complexes in serum of patients with diabetes mellitus. Clin. Chim. Acta 303, 105–115
11. Cornacoff, J. B., Hebert, L. A., Smead, W. L., VanAman, M. E., Birmingham, D. J., and Waxman, F. J. (1983) Primate erythrocyte-immune complex clearing mechanism. J. Clin. Invest. 71, 236–247
12. Lopes-Virella, M. F., Baker, N. L., Hunt, K. J., Lyons, T. J., Jenkins, A. J., and Virella, G. (2012) High concentrations of AGE-LDL and oxidized LDL in circulating immune complexes are associated with progression of retinopathy in type 1 diabetes. Diabetes Care 35, 1333–1340
13. Orekhov, A. N., Bobryshev, Y. V., Soberini, I., Melnichenko, A. A., and Korwar, A. M., Vannuruamary, G., Jagadeeshprasrad, M. G., Deshmukh, A. B., Patil, H. S., Bhosale, S. D., Shaikh, M. L., Thulasiram, H. V., Boppana, R., and Kulkarni, M. J. (2013) Proteome wide reduction in AGE modification in streptozotocin induced diabetic mice by hydralazine mediated transglycation. Scient. Rep. 3, 2941
14. Butiko, M., Pallat, H., Cordoba, A., and Yu, X. C. (2014) Recombinant antibody color resulting from advanced glycation end product modifications. J. Am. Chem. Soc. 136, 9816–9825
15. Silva, J. C., Goreinstein, M. V., Li, G.-Z., Vissers, J. P., and Geromano, S. J. (2008) Absolute quantification of proteins by LCMS/MS a virtue of parallel MS acquisition. Mol. Cell. Proteomics 5, 144–156
16. Bhonsle, H. S., Korwar, A. M., Kesavan, S. K., Bhosale, S. D., Bansode, S. B., and Kulkarni, M. J. (2012) “Zoom-in” — A targeted database search for identification of glycation modifications analyzed by untargeted tandem mass spectrometry. J. Mass Spectr. 18, 475–481
17. Schalkwijk, C. G., and Miyata, T. (2012) Early and advanced non-enzymatic glycation in circulating immune complexes are associated with progression of retinopathy in type 1 diabetes. J. Clin. Invest. 1426–1433
18. Corbett, L., Zadour, E., and Mora-Fernández, C. (2008) The role of inflammatory cytokines in diabetic nephropathy. J. Am. Soc. Nephrol. 19, 433–442
19. Inaba, T., Adefule, A. D., Gutasavea, D. R., Parker, J. B., Yerigenahtly, S. D., Clair, B., Kutlar, A., Odo, N., and Head, C. A. (2011) The proinflammatory cytokine GM-CSF downregulates fetal hemoglobin expression by attenuating the AMP-dependent pathway in sickle cell disease. Blood Cells. Mol. Disease 47, 235–240
20. Evans, T. W. (2002) Review article: albumin as a drug — Biological effects of albumin unrelated to oncotic pressure. Alimentary Pharmacol. Ther. 16, 6–11
21. Bhonsle, H. S., Singh, S. K., Srivastava, G., Boppana, R., and Kulkarni, M. J. (2008) Albumin competitively inhibits glycation of less abundant proteins. Protein Peptide Lett. 15, 663–667
22. Schalkwijk, C. G., and Miyata, T. (2012) Early and advanced non-enzymatic glycation in diabetic vascular complications: The search for therapeutics. Amino Acids 42, 1193–1204
23. Chougale, A. D., Bhat, S. P., Bhujbal, S. V., Zambare, M. R., Puntambekar, S. D., Clair, B., Kutlar, A., Odo, N., and Head, C. A. (2011) The proinflammatory cytokine GM-CSF downregulates fetal hemoglobin expression by attenuating the AMP-dependent pathway in sickle cell disease. Blood Cells. Mol. Disease 47, 235–240
24. Hans, S. R., Riz, R., and Ghafoor, F. (2012) Value of serum glycation albumin in prediction of coronary artery disease in type 2 diabetes mellitus. Public Health Res. 2, 37–42
25. Bouma, B., Kroon-Batenburg, L. M., Wu, Y.-P., Brünjes, B., Posthuma, G., Kranenburg, O., de Groot, P. G., Voest, E. E., and Geelink, M. F. (2003) Glycation induces formation of amyloid cross-beta structure in albumin. J. Biol. Chem. 278, 41810–41819
28. Arif, B., Ashraf, J. M., Moinuddin Ahmad, J., Arif, Z., and Alam, K. (2012) Structural and immunological characterization of Amadori-rich human serum albumin: Role in diabetes mellitus. *Arch. Biochem. Biophys.* 522, 17–25

29. Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P., and Cerami, A. (1986) Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking, *Science* 232, 1629–1632

30. Nicholls, K., and Mandel, T. E. (1989) Advanced glycation end-products in experimental murine diabetic nephropathy: effect of islet isografting and of aminoguanidine. *Lab. Invest.* 60, 486–491

31. Soulis-Liparota, T., Cooper, M., Papazoglou, D., Clarke, B., and Jerums, G. (1991) Retardation by aminoguanidine of development of albuminuria, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. *Diabetes* 40, 1328–1334

32. Cameron, N. E., Cotter, M. A., Dines, K., and Love, A. (1992) Effects of aminoguanidine on peripheral nerve function and polyol pathway metabolites in streptozotocin-diabetic rats. *Diabetologia* 35, 946–950

33. Velez, M. G., and Bhalla, V. (2012) The role of the immune system in the pathogenesis of diabetic nephropathy. *J. Nephrol. Ther.* 10, 362–369

34. Sugimoto, H., Shikata, K., Wada, J., Horiiuchi, S., and Makino, H. (1999) Advanced glycation end products-cytokine-nitric oxide sequence pathway in the development of diabetic nephropathy: Aminoguanidine ameliorates the overexpression of tumour necrosis factor-alpha and inducible nitric oxide synthase in diabetic rat glomeruli. *Diabetologia* 42, 878–886

35. Shuvaev, V. V., Fujii, J., Kawasaki, Y., Itoh, H., Hamaoka, R., Barbier, A., Ziegler, O., Siest, G., and Taniguchi, N. (1999) Glycation of apolipoprotein E impairs its binding to heparin: Identification of the major glycation site. *Biochim. Biophys. Acta* 1454, 296–308

36. Hedrick, C. C., Thorpe, S. R., Fu, M.-X., Harper, C. M., Yoo, J., Kim, S.-M., Wong, H., and Peters, A. L. (2000) Glycation impairs high-density lipoprotein function. *Diabetologia* 43, 312–320

37. Pu, L. J., Lu, L., Zhang, R. Y., Du, R., Shen, Y., Zhang, Q., Yang, Z. K., Chen, Q. J., and Shen, W. F. (2013) Glycation of apoprotein AI is associated with coronary artery plaque progression in type 2 diabetic patients. *Diabetes Care* 36, 1312–1320

38. Virella, G., and Lopes-Virella, M. F. (2012) The pathogenic role of the adaptive immune response to modified LDL in diabetes. *Frontiers Endocrinol.* 3, 7–14

39. Ortiz, G., Salica, J. P., Chuluyan, E. H., and Gallo, J. E. (2014) Diabetic retinopathy: Could the alpha-1 antitrypsin be a therapeutic option? *Biol. Res.* 47, 1–9

40. Spranger, J., Kroke, A., Möhlig, M., Hoffmann, K., Bergmann, M. M., Ristow, M., Boeing, H., and Pfeiffer, A. F. (2003) Inflammatory cytokines and the risk to develop type 2 diabetes results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* 52, 812–817

41. van Exel, E., Gussekloo, J., de Craen, A. J., Frolich, M., Bootman-van der Wiel, A., and Westendorp, R. G. (2002) Low production capacity of interleukin-10 associates with the metabolic syndrome and type 2 diabetes the Leiden 85-Plus Study. *Diabetes* 51, 1088–1092

42. Wynn, T. A. (2015) Type 2 cytokines: Mechanisms and therapeutic strategies. *Nat. Rev. Immunol.* 15, 271–282

43. Barrera-Hernandez, G., Wanke, I. E., and Wong, N. C. (1996) Effects of diabetes mellitus on hepatocyte nuclear factor 1 decrease albumin gene transcription. *J. Biol. Chem.* 271, 9969–9975

44. Viswanathan, V., Snehalatha, C., Kumutha, R., Jayaraman, M., and Ramachandran, A. (2004) Serum albumin levels in different stages of type 2 diabetic nephropathy patients. *Ind. J Nephrol.* 14, 89–92

45. Bhonsle, H. S., Konwar, A. M., Kote, S. S., Golegaonkar, S. B., Chougale, A. D., Shaik, M. L., Dhonde, N. L., Giri, A. P., Sheikkar, K. M., Boppana, R., and Kulkarni, M. J. (2012) Low plasma albumin levels are associated with increased plasma protein glycation and Hba1c in diabetes. *J. Proteome Res.* 11, 1391–1396

46. Tiwari, S., Bothale, M., Hasan, I., Kulkarni, M. J., Sayyad, M. G., Basu, R., Basu, A., and Unnikrishnan, A. G. (2015) Association between serum albumin and glycated hemoglobin in Asian Indian subjects. *Ind. J. Endocrinol. Metabolism* 19, 52–55

47. Mashitah, M. W., Azizah, N., Samsu, N., Indra, M. R., Bilal, M., Yunisa, M. V., and Arisanti, A. D. (2015) Immunization of AGE-modified albumin inhibits diabetic nephropathy progression in diabetic mice. *Diabetes, Metabol. Synd. Obesity* 8, 347–355