Figure 3.4 manually annotated AGE modified peptide of MSA, identified from mice plasma. Where A is the modified peptide with AMADORI modification at K-212. B is corresponding unmodified peptide sequence and C shows zoomed spectra at modified residue with increase in mass corresponding to modification (AMADORI).
3.3.3. Aminoguanidine treatment decreased the level of serum albumin in plasma CICs

After the findings of elevated and AGE modified human serum albumin in the CICs from diabetic plasma of clinical subjects, to establish the role of AGE modified proteins in the elicitation of autoimmune response and formation of CICs, STZ induced diabetic mice model was used with or without treatment of prototypic AGE inhibitor, AMG. Label free nano LC-MS quantification of CICs showed elevated levels of serum albumin in the CICs from diabetic mice. Whereas, treatment of diabetic mice with AMG reduced the levels of serum albumin in the CICs (Fig 3.5).

![Bar graph depicting serum albumin in CICs from mice plasma samples](Fig 3.5)

**Figure 3.5** Bar graph depicts quantity of serum albumin in CICs from mice plasma 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) biological replicates and technical triplicates). Significant difference indicated by *** (at p < 0.0001) was calculated by one-way ANOVA analysis.

The concentration of serum albumin in the CICs was also determined by western blotting technique using anti-serum albumin antibodies and the trend remained same as observed by mass spectrometric quantification and is represented in Figure 3.6a. The AGE modification of serum albumin was evaluated using anti-AGE albumin western blotting (Figure 3.6b) and by mass spectrometric analysis. Representative, manually annotated spectra are shown in Fig 3.7.
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**Figure 3.6a** 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.

**Figure 3.6b** Anti-AGE Western blot of CICs from mice plasma (n=3).
Figure 3.7 manually annotated AGE modified peptide of MSA identified in CICs. Where A is the modified peptide with CML modification at K-97. B is corresponding unmodified peptide sequence and C shows zoomed spectra at modified residue with increase in mass corresponding to modification (CML).
The characteristic peptide information and all the annotated peptides data are given in (Supplementary data No 3.2). The AGE inhibition property of AMG is the main protective factor in diabetic complications (Brownlee et al., 1986; Nicholls et al., 1989). Several lines of evidences from the previous studies have confirmed that AMG treatment prevents the complications of diabetes by reducing the \textit{in vivo} accumulation of AGEs (Liparota et al., 1991; Cameron et al., 1992). AMG prevents the symptoms of diabetic nephropathy by inhibiting the formation of AGEs (Sugimoto et al., 1999). We exploited this property of AMG to evaluate whether the inhibition of AGE formation will have an effect on the CIC albumin level in diabetic plasma. The treatment with AMG decreased the AGE levels in plasma albumin and it also reflected in the decreased level of albumin in the CICs.

Furthermore, along with serum albumin, alpha 2 macroglobulin (1.4), carboxylesterase 1C (1.6), apolipoprotein E (Apo E) (1.6) were found to be elevated in CICs from diabetic mice plasma compared to that of control plasma or plasma of diabetic animals treated with AMG. Apolipoprotein A1 and Alpha 1 antitrypsin were observed exclusively in the CICs from diabetic plasma, but were not observed in CICs from control or diabetic animals treated with AMG. The relative fold change as calculated after mass spectrometric analysis is represented in (Fig 3.8).
Figure 3.8 Relative fold change of CIC associated proteins in DIAB mice plasma treated with or without AMG, in comparison to CICs from CON mice plasma.

In addition to the relative fold change AGE modification analysis was also performed and the software identified AGE modified peptides of CIC associated proteins were manually validated and are represented in Figure 3.9.
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A.

\[ \Delta M (\gamma 6) = 161.968 \text{ Da} \]

Sequence: 174-186 VINDFVEKGTQGK AMADORI (8)

**Figure 3.9a** Manually annotated AGE modified peptide of Alpha 1 antitrypsin associated with CICs. B is the peptide with modification at K-181 with AMADORI. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (AMADORI).
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Figure 3.9b Manually annotated AGE modified peptide of Apolipoprotein A1 associated with CICs. B is the peptide with modification at K-129 with CML. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (CML).
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Figure 3.8c Manually annotated AGE modified peptide of Apolipoprotein E associated with CICs. B is the peptide with modification at K-86 with CML. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (CML).
Figure 3.8d Manually annotated AGE modified peptide of Carboxylesterase C associated with CICs. B is the peptide with modification at K-257 with CEL. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (CEL).
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Figure 3.8e Manually annotated AGE modified peptide of Alpha 2 macroglobulin associated with CICs. B is the peptide with modification at R-1315 with AMADORI. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (AMADORI).
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The entire list of identified AGE peptides and their annotated spectra are given in supplementary data 3.3. Earlier researchers have reported the glycation of Apo E, Apo A1, alpha 2 macroglobulin, and alpha 1 antitrypsin in diabetic conditions (Schalkwijk et al., 2012). In this study we identified these proteins to be associated with CICs in the plasma and have been found to be AGE modified. Apo A1 is a major constituent of high density lipoproteins, which is reported to be proatherogenic when glycated, leading to coronary artery disease in diabetic patients (Hedrick et al., 2000). Even though LDL are very well known to get glycoxidatively modified and to be associated with immune complexes leading to diabetic complications (Virella et al., 2003; Virella et al., 2012), in our present study we did not identify LDL in the CICs. This may be owing to the fact that short duration of diabetes and the different experimental approach used in the current study. Further, alpha 1 antitrypsin is a well known circulating serine protease inhibitor, inhibiting proteases like trypsin, elastase, thrombin and proteinase-3 (Korkmaz et al., 2010). These serine proteases activate receptors known as protease activated receptors (PARs) on the immune cells such as neutrophils, eosinophils and macrophages, which is an essential step in inflammatory responses (Shpacovtc et al., 2008). Hence inhibition of protease inhibitors, in turn, contributes to decrease in the inflammatory proangiogenic processes. During diabetes glycation of alpha 1 antitrypsin is reported to impair its function and also the plasma level is said to be decreased in nonobese diabetic mice (Ortiz et al., 2014). Yet another protein identified in CICs and found to be AGE modified was Carboxylesterase 1C. Carboxylesterases are mainly involved in detoxification (Potter et al., 2006) and drug metabolism (Laizure, et al 2013). The esterase is linked to diabetes since the activity of the lens esterase is decreased with normal ageing associated senile cataract (Kamei, 1996) and in diabetic patients (Solerte et al 1986; Aoyagi, et al 1985). Carnosine, an endogenous dipeptide containing histidine and beta alanine was shown to prevent the inactivity of esterase caused due to glycation in vitro (Yan et al., 2005).
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3.3.4. Plasma albumin was decreased in diabetic plasma

BCG method for the quantification of albumin is a classical method being used (Doumas et al., 1971). Plasma prepared with EDTA can be used where as heparin interferes with the assay (Bonvicini et al., 1979). The interferences like hemolysis do not affect the estimation since the complex formed has the distinct absorption maxima. The estimation showed the significantly decreased level of total plasma albumin in diabetic mice plasma condition to that of control mice. However, regardless of the AMG treatment we did not observe any increased albumin level in the AMG treated diabetic mice group (Figure 3.9).

Figure 3.9 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.

In a previous study, AMG prevented the development of albuminuria, mesangial expansion and kidney tissue AGE fluorescence in STZ induced diabetic rats when treated for 32 weeks (Liparota et al., 1991).
3.3.5. Proinflammatory cytokines were increased in diabetic mice plasma and which were decreased with AMG treatment

The proinflammatory cytokines TNF-alpha, IL-1β, and IL-2 were increased markedly in diabetic mice compared to that of control mice, as analyzed by Bio-plex assay. The plasma from diabetic mice treated with AMG showed decreased level of these proinflammatory cytokines. Cytokines are short polypeptides with low molecular weight involved in regulation of the immune response. Previous reports suggest that the elevation in the level of inflammatory cytokines such as IL-1β and IL-6 together cause subclinical inflammation and precede the development of T2DM (Spranger et al., 2003) and also proinflammatory cytokines induced by the AGE interaction with the receptors play a determinant role in development of microvascular complications of diabetes (Navarro-González et al., 2008). GM-CSF is an important proinflammatory cytokine (Ikuta et al., 2011), however, in this study, we did not observe any significant change in its level. The levels of IL-10 were observed to be decreased in diabetic mice compared to control and the AMG treatment increased the levels of IL-10 in our study. The low production of IL-10, a strong operating anti-inflammatory cytokine is shown to be associated with metabolic syndrome and type 2 diabetes (Van Exel et al., 2002). IL-5 is one of the type 2 cytokines, which can be host protective or can drive pathogenicity when dysregulated (Wynn, 2015). In the present study IL-5 levels were decreased in diabetic mice to that of control mice or diabetic mice treated with AMG. The bar graph showing different cytokines in the analysis is depicted in Figure 3.10.
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Figure 3.10 Measurement of cytokines in mice plasma. Bar graph depicting cytokines in plasma samples from CON, DIAB, and D-AMG mice (*n = 3* biological replicates in technical triplicates. Values are mean ± S.E. Significant difference at *p < 0.01* is indicated by ** and *p < 0.05* is indicated by *.

3.4. Conclusion

In this chapter we discussed the role of glycation in the elicitation of autoantibodies and the formation of CICs in STZ induced diabetic mice model treated with or without AGE inhibitor AMG. AMG reduced the plasma AGEs and specifically AGE modification of serum albumin. Serum albumin levels were significantly increased in diabetic mice plasma CICs and also it was characterized to be AGE modified, which corroborated our initial findings of elevated HSA in the clinical diabetic plasma CICs. Additionally treatment with AGE inhibitor AMG decreased the serum albumin levels in the CICs in the plasma. We have also observed decreased serum albumin levels in the plasma of diabetic mice. Previous studies have reported the decreased transcription of
albumin gene during DM (Barrera-Hernandez et al., 1996) and also there are reports showing the decreased levels of serum albumin during conditions of heavy proteinuria of diabetic nephropathy (Viswanathan et al., 2004). Considering the previous reports and the observations of the current study, one can hypothesize that immune response against the AGE modified albumin may partly contribute to the reduced levels of plasma levels of albumin along with the other contributing factors such as decreased albumin transcription and proteinuria of chronic hyperglycemic conditions. In conclusion, this study suggests that AMG mediated AGE inhibition regulates the serum albumin levels in CICs of diabetic mice confirming the role of glycation and subsequent AGE modification in the elicitation of autoimmune response and the formation of CICs. The entire clinical study and animal model study design for the CICs analysis is depicted in a combined way in Figure 3.11.
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Clinical plasma (CON, IGT, NDD, DM, DM-MIC)

AGE-Protein

Isolation of CICs Using Protein G

Circulating Immune Complexes (CICs)

In solution Trypsin Digestion

Nano-LC-MS\(^2\) 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

Figure 3.11 Study overview.
Table 3.1 Physiological data.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment</th>
<th>Body Weight (gms)</th>
<th>Blood Glucose (mg/dL)</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CON (n=6)</td>
<td>26.28 ±2.27</td>
<td>102.5± 7.5</td>
<td>4.2±0.17</td>
<td></td>
</tr>
<tr>
<td>2. DIAB (n=6)</td>
<td>20.4 ±1.74</td>
<td>550 ± 44</td>
<td>8.15 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>3. D-AMG (n=6)</td>
<td>22.0 ± 0.89</td>
<td>284.8 ± 56</td>
<td>6.8 ± 0.47</td>
<td></td>
</tr>
</tbody>
</table>