Autoantibodies Against the Glial Glutamate Transporter GLT1/EAAT2 in Type 1 Diabetes Mellitus. Clues to novel immunological and non-immunological therapies

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Abstract

Islet cell surface autoantibodies were previously found in subjects with type 1 diabetes mellitus (T1DM), but their target antigens and pathogenic mechanisms remain elusive. The glutamate transporter solute carrier family 1, member 2 (GLT1/EAAT2) is expressed on the membrane of pancreatic β-cells and physiologically controls extracellular glutamate concentrations thus preventing glutamate-induced β-cell death. We hypothesized that GLT1 could be an immunological target in T1DM and that autoantibodies against GLT1 could be pathogenic. Immunoprecipitation and ELISA experiments showed that sera from T1DM subjects recognized GLT1 expressed in brain, pancreatic islets, and GLT1-transfected COS7-cell extracts. We validated these findings in two cohorts of T1DM patients by quantitative immunofluorescence assays. Analysis of the combined data sets indicated the presence of autoantibodies against GLT1 in 32 of the 87 (37%) T1DM subjects and in none of healthy controls (n=64) ($p < 0.0001$). Exposure of pancreatic βTC3 cells and human islets to purified IgGs from anti-GLT1 positive sera supplemented with complement resulted in plasma membrane ruffling, cell lysis and death. The cytotoxic effect was prevented when sera were depleted from IgGs. Furthermore, in the absence of complement, 6 out of 16 (37%) anti-GLT1 positive sera markedely reduced GLT1 transport activity in βTC3 cells by inducing GLT1 internalization, also resulting in β-cell death. In conclusion, we provide evidence that GLT1 is a novel T1DM autoantigen and that anti-GLT1 autoantibodies cause β-cell death through complement-dependent and independent mechanisms. GLT1 seems an attractive novel therapeutic target for the prevention of β-cell death in individuals with diabetes and prediabetes.

Keywords: autoantibody, Type 1 diabetes mellitus, EAAT2/GLT1, complement pathway, glutamate toxicity.

1 Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by selective and progressive destruction of β-cells of the islets of Langerhans in genetically predisposed subjects [1–3]. It is commonly believed that T1DM autoimmunity is initiated by β-cell insults, leading to abnormal β-cell immunogenicity and consequent activation of autoreactive T- and B-lymphocytes. The nature of the primary insult to the β-cell is unclear, although viral infection, inflammation and endoplasmic reticulum stress have been implicated [4,5]. Since the discovery of islet cell autoantibodies (ICAs) in the sera of patients with T1DM [6], several targets have been identified, including insulin (IA), glutamic acid decarboxylase (GAD), protein tyrosine phosphatase-2 (IA-2 or ICA 512), chromogranin, zinc transporter 8 (ZnT8) and tetraspanin 7 [7–14]. Most of these are intracellular proteins, and none of the respective autoantibodies (IAA, GADA, IA-2A) is directly involved in the pathogenesis of diabetes. Yet, islet cell surface autoantibodies (ICSAs) with β-cell cytotoxic activity have been reported early on after the discovery of ICAs [9,15,16], even though the identity of their target protein(s) remained elusive.

Pancreatic endocrine cells and GABA-ergic neurons share many biological and physiological similarities, including the expression of GAD, one of main autoantigens in subjects with T1DM and those with stiff man syndrome [7,17–19]. Moreover, as glutamatergic neurons, pancreatic β-cells employ glutamate as molecular signal to regulate their own and neighbouring cell activity. In the mammalian central nervous system (CNS), glutamate at elevated concentrations is toxic to neurons. Thus, glutamate must be removed rapidly from the synaptic space and this is accomplished by three distinct excitatory glutamate transporters: EAAT3/EAAC1, EAAT2/GLT1, and EAAT1/GLAST [20–24]. The loss of each of these three transporters results in neurodegeneration [25,26].

In the islet of Langerhans, glutamate is co-secreted with glucagon by the α-cells [27], and it can potentially reach high extracellular concentrations that may be toxic to the β-cells [28–31]. We have shown previously that glutamate-induced β-cell death can be prevented by the glutamate transporter EAAT2/GLT1 (thereafter GLT1), which is expressed on the β-cell membrane [29]. Similar to what occurs in the CNS, normal GLT1 function is critical for β-cell survival, as confirmed by the evidence that GLT1 downregulation or pharmacological inhibition induces β-cell death in human islets of Langerhans [28,29]. Since immunoglobulins (IgG and IgM) targeting membrane proteins are pathogenic, we hypothesized that GLT1 could be an autoantigen in subjects with T1DM. We tested this hypothesis by searching for the presence of autoantibodies against GLT1 in the sera of individuals with T1DM. We also explored the mechanisms by which anti-GLT1 autoantibodies could induce pancreatic β-cell death.

2 Materials and Methods

2.1 Patients

Two independent cohorts of patients were analysed. The first cohort comprised 43 subjects with a diagnosis of T1DM (mean age 28.25±19.49 years; mean disease duration 3.95±4.19 years) followed at the Ospedale San Raffaele (OSR), Milan, Italy (Supplementary Tables S1 and S2). The control group consisted of 35 age-matched (mean age: 29.25±20.04 years) healthy donors (Supplementary Tables S1). The second cohort comprised 44 subjects with a recent diagnosis of T1DM (mean age 8.75±4.66 years; mean disease duration 0.30±0.31 years) and 29 age-matched (mean age 9.45±4.21 years) healthy controls who were seen at the Policlinico di Tor Vergata, Rome, Italy (Supplementary Tables S1 and S3). Autoantibodies were tested in the sera of all non-diabetic controls and were absent. Serum samples were obtained from these individuals after written informed consent, according to

the guidelines of the OSR, Università degli Studi di Milano and Policlinico di Tor Vergata Ethical board committees. The clinical features of subjects involved in this study are provided in Tables S1-S3 of the supplementary appendix. The study was approved by the University of Milano Ethical Committee (*February 27th 2009*).

2.2 Immunoprecipitation and Western Blotting

Immunoprecipitation and western blotting were performed with T1DM and control sera on lysates obtained from human isolated islets, P2 fraction (total membrane enriched) of brain extracts, and GLT1-transfected COS7. Cells growing conditions and transfection procedures are reported in supplementary methods. Tissue and cells were extracted in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Merck). The insoluble material was removed by centrifugation at 10.000 g x 30 minutes, and the supernatant was used for either immunoprecipitation or SDS-PAGE electrophoresis followed by Western blotting. The P2 fraction of brain extract was obtained as previously described [22,29] and human islets were isolated from cadaveric multiorgan donors conforming to the ethical requirements approved by the Niguarda Cà Granda Ethics Board. For immunoprecipitation, 50 µg of brain P2 membrane fraction or 20 µg of COS7 cell whole lysate were pre-cleared with protein A-Sepharose resin, then they were overnight incubated at 4°C with 2 µg of rabbit IgG (Sigma), 2 µg of affinity purified anti-GLT1 antibody (3) or 7 µL of human serum samples. The immunocomplexes were recovered by incubation with 20 µL of protein A-Sepharose resin (GE Healthcare) for 2 hours on ice, and then extensively washed with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, protease inhibitor cocktail. Proteins bound to the resin were eluted in protein sample buffer and loaded onto a 9% SDS-PAGE. Western blotting analyses were performed in standard conditions as previously described and the rabbit anti-GLT1 antibody (4) was

diluted 1/500. X-ray films were analysed by densitometry and the quantifications were performed using NIH Image 1.59 software. The band intensity was expressed as Arbitrary Units.

2.3 GFP-GLT1 In Vitro Binding Assay

The assay was performed by testing the binding of T1DM or control sera to a GFP-GLT1 recombinant protein immobilized onto anti-GFP coated multiwells (GFP-Trap). Lysates from GFP-GLT1 or GFP expressing MDCK cells (snap frozen, 200 µg) containing equal amount of recombinant protein (measured spectrophotometrically; Ex/Em: 485/ 535 nm) were diluted to 100 µL with binding buffer and added to ELISA plates pre-coated with anti-GFP antibodies (gtp-96, ChromoTeck). Plates were left overnight at 4°C on a rotary shaker. After coating, plates were washed twice with PBS and incubated at RT for 2 hours with serum samples (10% in binding buffer) from healthy controls (a pool of three distinct CTR sera) or T1DM patients (a pool of three distinct GLT1-positive sera). Anti-GLT1 rabbit serum [22] and the rabbit preimmune serum were used as positive and negative controls, respectively. After 1 hour incubation with HRP-conjugated anti-human or anti-rabbit IgG, they were incubated for 30 minutes with the substrate (Pierce) before the reaction was developed.

2.4 Quantitative immunofluorescence assay

EGFP-GLT1 or EGFP transfected COS7 or MDCK cells (supplementary methods) were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. The cells were incubated with 10-20% serum samples in buffer for 2 hours. Immunostaining with the primary antibodies was followed by incubation for 1 hour with Rhodamine-conjugated anti-human IgG (Jackson Laboratories). Up to five different images were acquired for each sample by confocal microscope (BIO-RAD or Zeiss) in two different experiments and analyzed. Z series of confocal sections were acquired separately using appropriate filters. The laser power and gain were adjusted to maintain the signal below saturation levels.

The Pearson's correlation coefficient (PCC) was used to quantify the degree of colocalization between GLT1 and human IgG stainings. Single channel images obtained with the confocal microscope (GLT1: channel 1/green; Human IgG antisera: channel 2/red) were opened in Image Pro-Plus, background subtracted, and analyzed using the automated correlation plug-in for quantitative colocalization. 0 indicates no significant correlation, 1 indicates maximal correlation. The calculated PCC for control sera was 0.24 ± 0.09 and 0.15 ± 0.08 in Group 1 and 2, respectively. The cut-off value for the GLT1 serum reactivity was set to mean + 3SD of the healthy control value. This threshold value corresponds to 100% specificity and 40% sensitivity in a ROC (receiver operating characteristic) curve generated using GraphPad Prism9 software and imposing 99% confidence intervals for sensitivity and specificity.

2.5 Immunoglobulin and Complement Mediated Cell Lysis Assay

GLT1-transfected COS7 cells or βTC3 cells [29,32] were incubated in Krebs buffer with 10% serum from healthy controls or type 1 diabetic subjects at 4°C for 30 minutes, followed by 90 minutes of incubation at 37°C in the same solution supplemented with 10% fresh or heat-inactivated human complement (Sigma). To quantify complement-mediated cell lysis, the cells were then incubated for 15 minutes in a buffer containing 0.5 μg of Propidium iodide (PrI) (Sigma), fixed in ice-cold methanol, and counterstained with DAPI (81845, Merck). PrI-positive cells were counted by three independent blinded observers, using a 20X objective from 10 randomly selected fields per coverslip.

2.6 mRNA expression analysis in pancreatic islets from control and diabetic subjects

Laser captured islets were obtained from deceased organ donors of non-diabetic subjects, Type 2 diabetic and Type 1 diabetic patients with residual insulin-containing islets (Network for Pancreatic Organ Donors with Diabetes, nPOD) and were provided by Prof. Ivan Gerling [33]. mRNA expression was measured with an Affymetrix Human Gene 2.0 ST array analysis. Data were expressed as fold

change as respect to gene expression of healthy subject samples. Sample characteristics are reported in [33].

2.7 [^{3}H]D-Aspartic and [^{3}H]D-glutamic Acid Uptake

GLT1 transport activity was assessed by measuring the uptake of [^{3}H]D-aspartic or [^{3}H]D-glutamic acid. After incubation of βTC3 cells with 10% serum from healthy controls or T1DM subjects at 37°C for 2 hours in Krebs buffer, cells were maintained for 10 minutes in 200 µL of Na^+-dependent (150 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes pH 7.5) uptake solution containing 5 µCi/mL of [^{3}H]D-Aspartic or [^{3}H]D-glutamic acid (specific activity 37 Ci/mmol; Amersham Biosciences). The amino acid uptake was stopped by washing the cells twice in ice-cold sodium-free solution. Cells were finally lysed in 150 µL of SDS 1% for liquid scintillation counting.

2.8 Statistical Analyses

Statistical analysis software GraphPad Prism 9.00 (San Diego) was used for all statistical analyses. Comparison between two groups was determined by unpaired, two tailed, Student's t-test or by two-sided Fisher's exact test. Comparison among groups was determined by one-way or two-way ANOVA, followed by Tukey's tes. Hierarchical cluster analysis and Partial Least Squares - Discriminant Analysis (PLS-DA) were performed using R software on data reported in Table S2 and S3 after normalization and scaling. P values less than 0.05 were considered to indicate statistical significance.

3 Results

3.1 Identification of GLT1 as a Target of Serum IgGs in a Subset of Patients with Type 1 Diabetes Mellitus

Immunoprecipitation experiments performed with the anti-GLT1 antibody demonstrated that both human islets of Langerhans and rat brain P2 fraction expressed GLT1, although at different levels

(Figure 1A). Immunostaining of a human pancreatic section with an anti-GLT1 antibody highlighted its expression on the plasma membrane of insulin-positive β-cells (Figure 1B).

To test the presence of anti-GLT1 autoantibodies, we first performed double immunofluorescence experiments on human pancreas frozen sections with a rabbit anti-GLT1 antibody and IgGs purified from the serum of a T1DM patient who was ICA-positive but negative for GAD autoantibodies. A partial colocalization of IgGs and GLT1 staining at the plasma membrane and in intracellular structures of islets was detected with the T1DM serum, suggesting that a subset of ICAs may recognize GLT1 as an autoantigen (Figure 1C). The quantitative analysis supported colocalization of GLT1 and IgGs in T1DM but not in the control serum (Figure 1D and S1A). Data were confirmed by a double immunofluorescence staining performed on mouse cortical astrocyte-neuron cultures, which are enriched in the GLT1 transporter (21). IgGs from the T1DM patient colocalized with GLT1 naturally expressed in astrocytes, whereas IgGs purified from healthy controls did not show any detectable binding (Figure S1B).

The immunoreactivity was not due to cross-reactivity of the anti-GLT1 antibody with GAD, an important T1DM autoantigen also expressed in the CNS, because 2D electrophoresis experiments performed on human islets demonstrated that the anti-GLT1 antibody selectively recognized a protein with identical electrophoretic mobility and isolelectrical point of GLT1 expressed in COS7 cells and distinct from that recognized by the anti-GAD antibody (Figure S1C). Furthermore, no cross-reactivity was observed between the anti-GLT1 antibody and other high affinity glutamate transporters (GLAST; EAAC1) (Figure S1D).

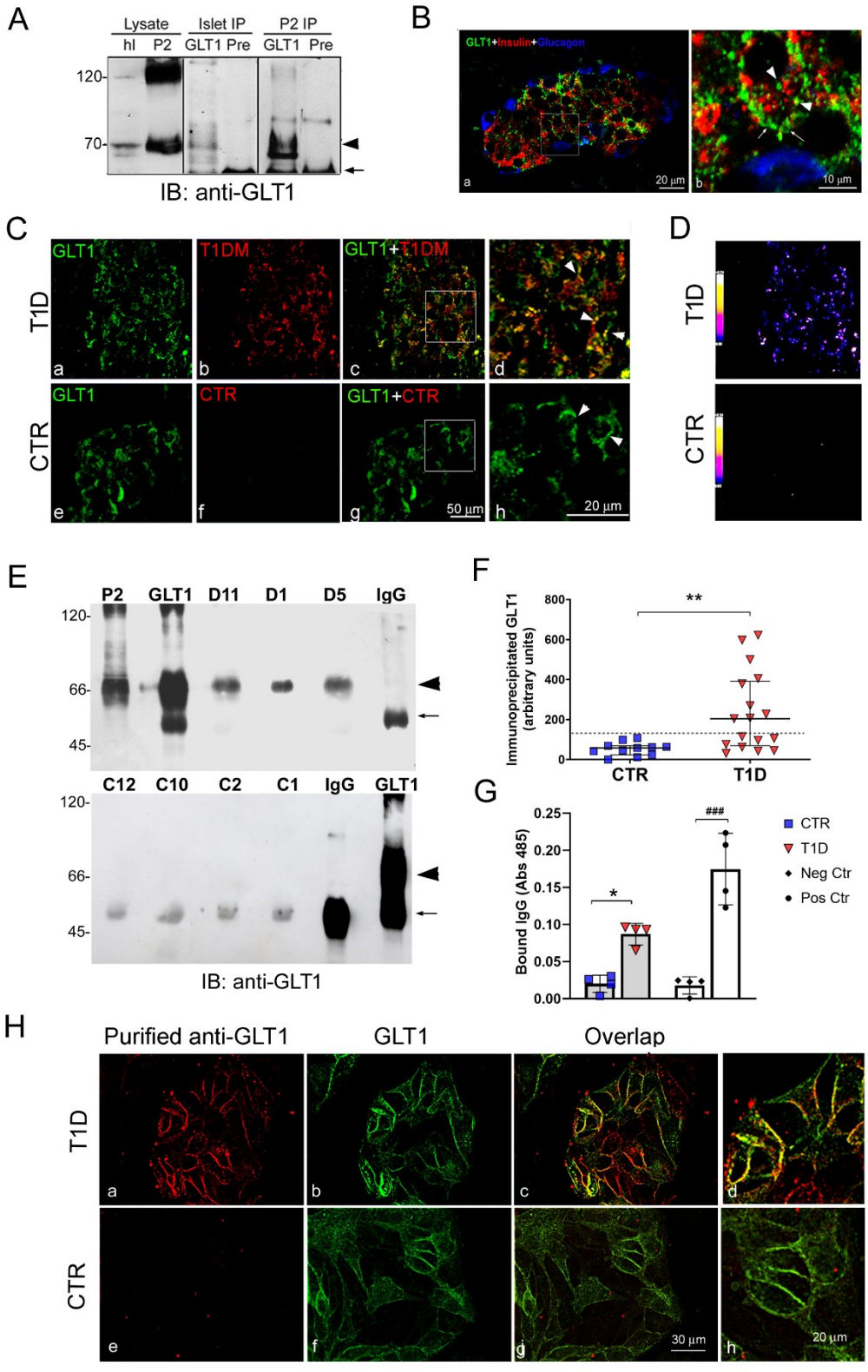

Figure 1 IgGs from a cohort of T1DM serum samples recognize GLT1 as a target. (A) GLT1 immunoprecipitation from rat P2 brain and human islet of Langerhans lysates. Arrowhead indicates GLT1 monomer, arrow indicates IgG. (B) Triple immunofluorescence labelling of human pancreatic sections with rabbit anti-GLT1 (green), anti-

insulin (red) and anti-glucagon (blue). In b a particular is shown at higher magnification (2X). Arrowheads indicate GLT1 expression at the plasma membrane. Arrows indicate GLT1 expression in vesicular structures. (C) Double immunofluorescence labelling of frozen human pancreatic sections with rabbit anti-GLT1 (green) and IgGs (red) from a ICA-positive/GAD-negative T1DM patient (#D19) (a-d) or a healthy control subject (#C5) (e-h). In panels d and h, a particular of the overlay is shown at higher magnification (2X). Arrowheads indicate colocalization (yellow) between GLT1 and T1DM IgGs at the plasma membrane and in intracellular structures. Serum characteristics are reported in Table S2. (D) Intensity Correlation Analysis of single channel images from human islets reported in figure 1B supports colocalization between GLT1 and serum sample from T1DM patient. The Product of the Differences of pixel intensity from the Mean intensity (PDM) values are presented in pseudocolored images: blue indicates moderate colocalization, white indicates maximal colocalization. (E) Immunoprecipitation of 20 µg of P2 brain membrane fractions with sera from healthy subjects (Cn) or T1DM patients (Dn), rabbit anti-GLT1 antibody (GLT1, positive control) and a rabbit serum (IgG negative control). P2 5 µg of lysate protein, (input). Representative blots are shown. Arrowheads indicate GLT1 monomer. Arrows indicate IgG. Serum characteristics are reported in Table S2. (F) Quantification of immunoprecipitated GLT1 by densitometric analysis. Band intensity is expressed as Arbitrary Units. The median values are shown for each group. The shaded line represents the cut-off (mean + 3SD of healthy controls) above which the results are considered positive. (**$p < 0.01$, unpaired t-test). (G) A cell free enzyme-linked immunosorbent assay demonstrated the selective binding of T1DM sera to a GFP-GLT1 protein immobilized onto anti-GFP coated multiwells. Positive control rabbit anti-GLT1 antibody, negative control rabbit IgGs (n = 4, in duplicate). (*$p < 0.05$ T1DM vs CTR; ###$p<0.005$ neg Ctr vs pos Ctr; one-way ANOVA). (H) (a-d), affinity purified anti-GLT1 IgG fraction from a pool of GLT1-positive T1DM sera (red) selectively recognises GFP-GLT1 (green) expressed in MDCK cells. Colocalization between the two stainings is shown in yellow in the overlay. No staining was detected whit a pool of control sera (e-h). In panels d and h, a particular of the overlay is shown at higher magnification (2X).

To investigate whether GLT1 might be a target of T1DM serum IgGs, immunoprecipitation experiments were performed with serum samples from 17 T1DM and 11 healthy control subjects incubated with equal protein amounts of the P2 fraction extracts [22] (Figure 1E). Nine out of 17 T1DM sera (47%) immunoprecipitated a protein of ~ 60 kDa that co-migrated with GLT1 and that was recognized by the rabbit anti-GLT1 antibody; this protein was not precipitated in the 11 healthy controls. The quantification of the band intensity showed a significant difference between the two groups (P = 0.01) (Figure 1F). The specificity of GLT1 as a target of serum IgG from T1DM subjects was confirmed by immunoprecipitation assays on GLT1-transfected COS7 cells. Two GLT1-positives T1DM sera immunoprecipitated GLT1 from GLT1-transfected COS7 cells, whereas control serum did not (Figure S2).

To further confirm GLT1 as T1DM antigen, a cell free enzyme-linked immunosorbent assay was developed. The pool of three GLT1-positive T1DM sera bound the plate covered with GFP-GLT1

recombinant protein more avidly than the pool of three control sera (Figure 1G). No reactivity was detected with GFP-alone. Furthermore, affinity purified anti-GLT1 antibodies from the same GLT1-positive T1DM sera, but not from control sera, selectively recognized GFP-GLT1 over-expressed in COS7 cells (Figure 1H).

3.2 GLT1 Expressed in Transfected COS7 Cells is Recognized by Type 1 Diabetes Sera

A quantitative cell-based immunofluorescence assay confirmed the specificity of immunoprecipitation results (Figure 2A). A subset of T1DM serum samples selectively labelled the cell surface of GFP-GLT1 transfected COS7-cells (Figure 2Ad-f). The line plot (Figure 2Bb) and the scatter plot (Figure 2Cb) supported colocalization between T1DM IgG and the GFP-GLT1 signals at the plasma membrane and in intracellular vesicular structures (PCC = 0.78). Staining was specific for GLT1, since it was undetectable in cells expressing EAAC1, a different glutamate transporter subtype (PCC = 0.01) (Figures S3Aa-c and S3Ba). No staining was observed in GFP-GLT1 and GFP-EAAC1 transfected COS7 cells incubated with the serum of a control subject (Figure 2Aa-c, 2Ba and 2Ca - PCC = 0.21; Figures S3Ad-f and S3Bb, PCC = 0.11). An example of GLT1-negative T1DM serum is also reported (Figure 2Ag-i, 2Bc and 2Cc - PCC = 0.22). Immunoprecipitation and quantitative immunofluorescence assays results were strongly correlated ($r = 0.68$, $p<0.005$; Figure S3C).

As only assays based on cell expressing the natively folded GLT1 protein in the cell membrane can preserve both conformational and non-conformational epitopes, we used this quantitative immunofluorescence assay to screen sera from two independent cohorts of T1DM subjects (serum sample characteristics are reported in Table S2 and S3 of the Supplementary Appendix). Analysis of the PCC indexes showed a significant difference between control and T1DM groups ($P < 0.0001$) (Figure 2D). Similar results were obtained with a different analysis of colocalization (mean fluorescence intensity (MFI) ratio of the IgG staining obtained with sera in GFP-GLT1 transfected

versus non-transfected cells; Figure S3D). The PCC index and IgG MFI ratio values were strongly correlated ($r = 0.84$, $p<0.0001$; Figure S3F). We also tested the anti-GLT1 immunoreactivity in a cohort of T2DM subjects (Supplementary Figure S3G and S3H) and we did not find any significant difference in the PCC or the MFI ratio between T2DM and control healthy subjects, thus indicating the specificity of the reactivity.

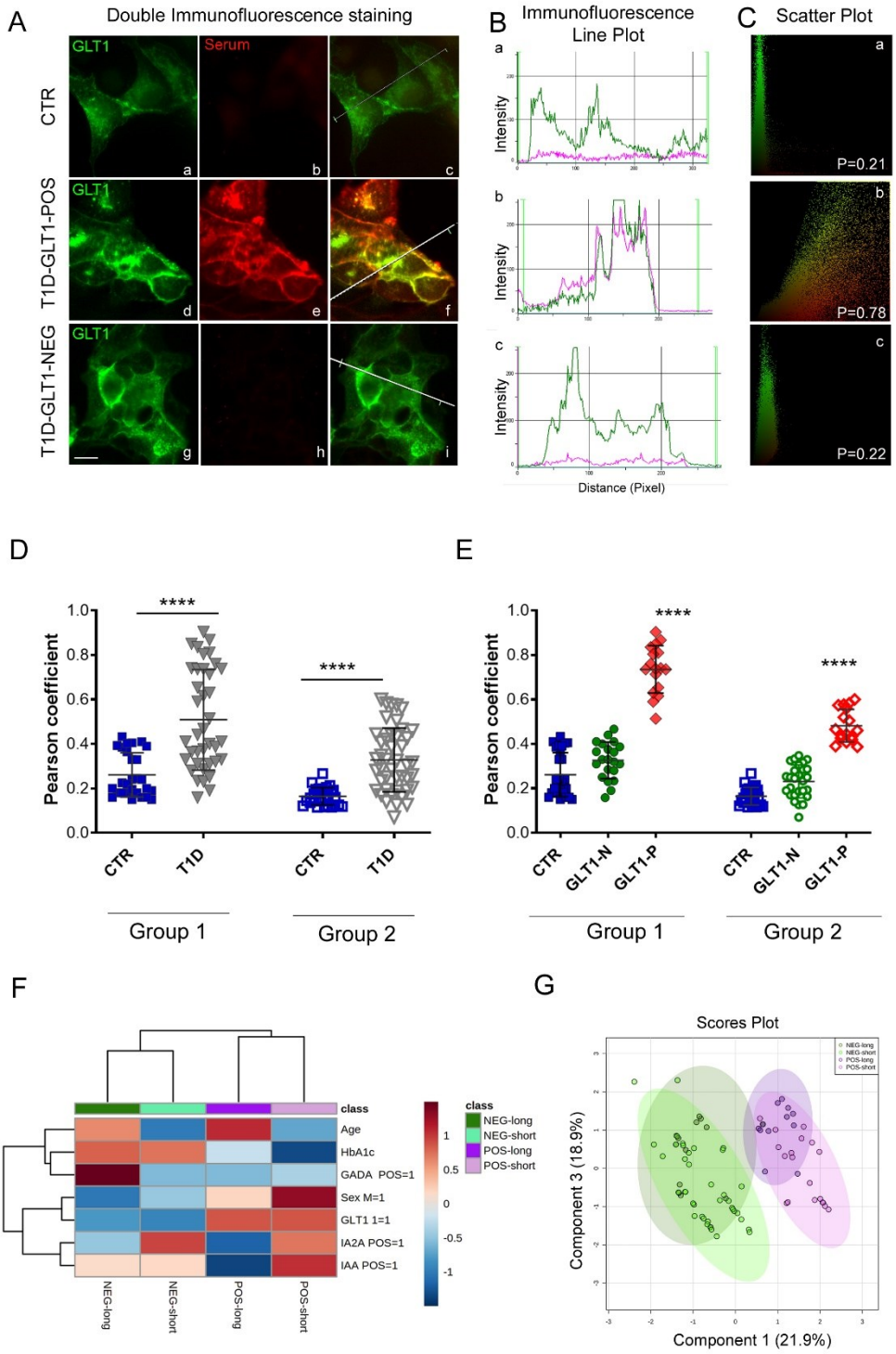

Figure 2: Validation of GLT1-immunoreactivity in serum samples from T1DM patients. (A) COS7 cells expressing the GFP-GLT1 (green) are stained with serum samples from healthy subjects (a-c) or T1DM patients (d-i) (red). The yellow staining in the overlay indicates colocalization between GFP-GLT1 and IgG labelling and is indicative of the presence of anti-GLT1 antibodies in serum samples. A total of 64 healthy controls and 87 T1DM subjects in two different cohorts were screened. Representative images obtained with serum samples from control subjects (#C1) and T1DM patients (#D5, D8) are shown (serum characteristics are reported in

Table S2 and S3). (B) Immunofluorescence intensity profiles of red and green stainings in correspondence of the white line reported in c, f and i. (C) Scatter plot of red and green stainings. The calculated PCC is reported on the right. (D) Results of the quantitative immunofluorescence analysis (Pearson's correlation coefficient, PCC) in Group 1 (full symbols) and Group 2 (empty symbols). Each symbol indicates the PCC value calculated of a single control (blue square) or T1DM (grey triangle) subject. The median values are shown for each group. (**** $p<0.0001$, unpaired t test with Welch's correction). (E) The mean PCC value + 3SD of healthy controls was used as cut-off threshold for the GLT1 serum positivity (red diamonds). GLT1-negative (green circles). (****$p<0.0001$, one-way ANOVA). (F) Hierarchical clustering analysis. In the heatmap, rows report the clinical feature and the autoantibody-positivity of T1DM patients and columns represent the patients' samples stratified according to GLT1-positivity and disease duration (short ≤ 1 year; long >1 year). (G) PLS-DA performed on data reported in Table S2 and S3. The graph shows the scores plot of the two most important components (PC) of the analysis. The explained variances are shown in brackets.

Considering the mean + 3SD of healthy control values as the cut-off threshold for the GLT1 serum positivity, sixteen of 43 T1DM sera (38%) specifically labelled GLT1-transfected COS7 cells (Figure 2E). Data were confirmed in the second cohort consisting of 44 T1DM subjects with a recent diagnosis of T1DM (0.30±0.31 years) and 30 age and sex matched healthy controls. Sixteen of the 44 T1DM sera (36%) also colocalized with the anti-GLT1 antibody, which was absent in controls (Figure 2D and 2E). Similar results were obtained considering the IgG immunofluorescence intensity ratio analysis (Figure S3D - S3E).

Altogether, these data indicate that GLT1 is a novel autoantigen and that anti-GLT1 autoantibodies are present in approximately 37% of patients with type 1 diabetes mellitus. Hierarchical cluster analysis performed on data reported in Table S2 and S3 revealed that GLT1-immunoreactivity is present in both short- (≤ 1 year disease duration; 30% of subjects) and long-term (> 1 year disease duration 50% of subjects) T1DM patients and inversely correlates with Hb1Ac (Figure 2F and S3G).

3.3 Anti-GLT1 Autoantibodies Induce Complement-Mediated β-cell Death *in vitro*

Complement-fixing islet cell autoantibodies, which should be able to activate the terminal complement complex cascade and cause cell damage, have been described in T1DM subjects as well as in individuals at high risk for developing it [34–36]. To test the possibility that GLT1 could, in fact,

be one of the previously unidentified autoantigens, we also explored the biological effects of anti-GLT1 IgGs on cells expressing the transporter by *in vivo* cell-imaging. When exposed to the T1DM serum and active complement, GLT1-expressing cells underwent progressive membrane damage and swelling. The process was particularly evident at the cell periphery, in filopodia-like structures, where GLT1 was enriched (Figure 3A). After 2 hours of incubations, several GLT1 expressing cells were severely injured and vesicular-like structures highly positive for GLT1 expression were distinctly evident in the medium (Figure 3B). No visible changes were detected in the presence of inactive complement or after exposure to control serum (Figure 3A, 3B and Movies in the Supplementary Appendix).

To verify the membrane integrity of β-cells exposed to T1DM sera in the presence of active complement, we quantified the number of cells permeable to propidium iodide (PrI) (Figure 3C and S4). The exposure of βTC3 cells to a subset of GLT1-positive sera supplemented with active complement significantly increased the PrI uptake, indicating the disruption of membrane integrity (3.7 ± 0.7 folds increase; damaged β-cells, 17 ± 5% and 3.6 ± 0.2% in GLT1-positive and control sera, respectively; $P < 0.01$). No change in PrI permeability was detected after incubation with heat-inactivated complement or GLT1-negative sera.

The change in permeability was mediated by the selective binding of IgGs to GLT1, because PrI uptake was detected only in GLT1-expressing cells exposed to GLT1-positive serum and active complement (Figure 3Dd) but not to control or GLT1-negative T1DM sera (Figure 3Da-c and e-h).

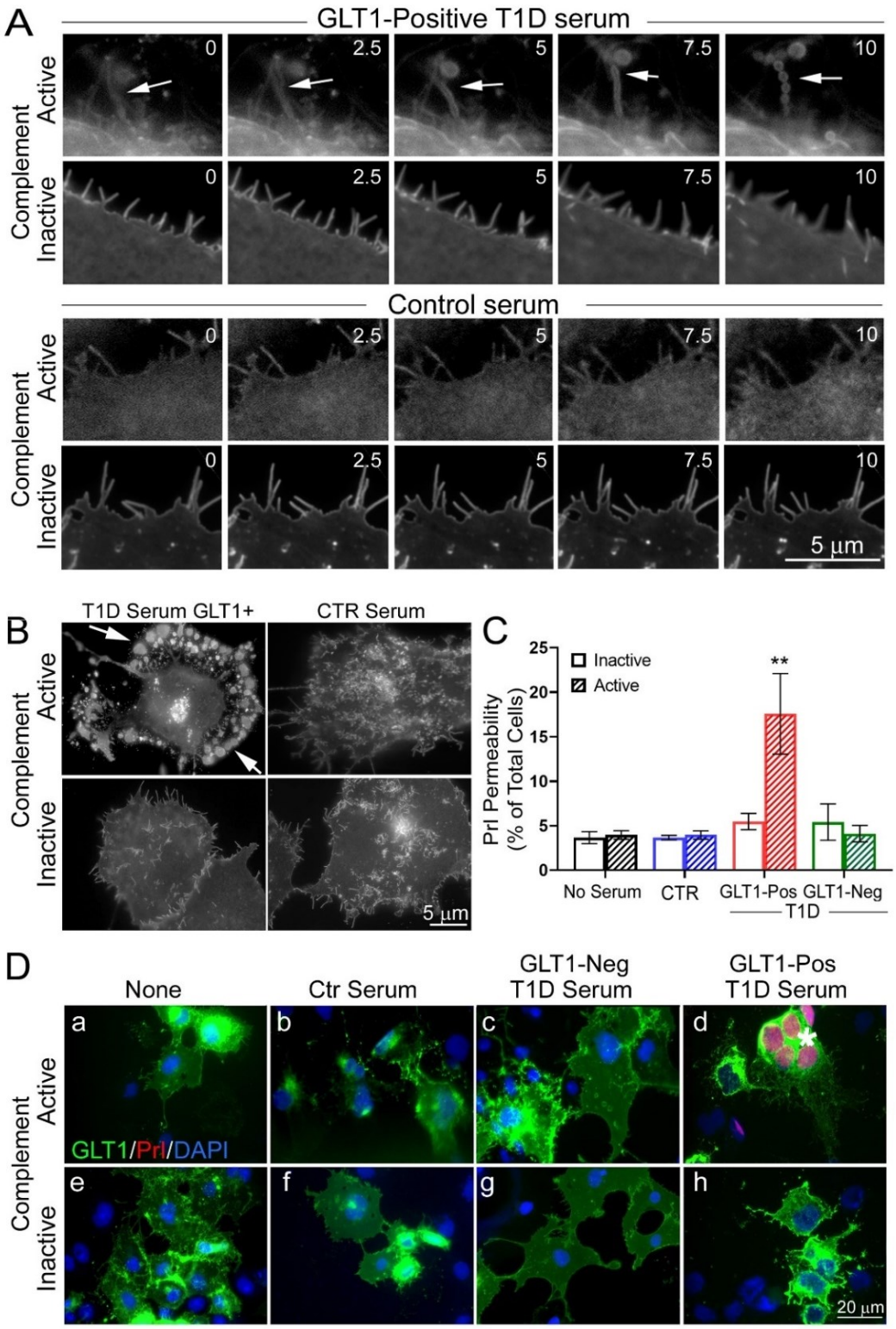

Figure 3. GLT1-positive T1DM sera cause complement-mediated β-cell toxicity. (A) Time-lapse imaging of GFP-GLT1-transfected COS7 cells exposed to an anti-GLT1-positive T1DM or control serum and complement, as indicated. Representative image sequences of cells, 10 min after the addition of control (#C5), and T1DM serum samples (#D11) are shown. Note the progressive "blebbing" of filopodia (arrows) only in the presence of the GLT1-positive serum and active complement. The time (minutes) is indicated in each photogram. (B) GFP-GLT1 transfected COS7 cells after 2 hours incubation with serum and complement, as indicated. Arrows

point to patchy membrane fragments at the cell periphery after incubation with active complement and GLT1-positive sera. Representative images are shown. (C) Quantification of membrane lysis assessed by Propidium Iodide (PrI) uptake in βTC3 cells exposed to 10% serum samples and 10% fresh (active) or heat-inactivated (inactive) complement, as indicated. The number of PrI-positive cells was counted, and data are presented as percentage of total cells (n = 3, in duplicate). Sera from three to six subjects from control and T1DM groups were separately tested and the average value ± SD for each group in the presence of active or inactive complement are reported. (** p< 0.01 Active vs Inactive; two-way ANOVA). (D) PrI permeability in COS7 cells transfected with GFP-GLT1 and exposed to 10% serum and 10% active (a-d) or inactive (e-h) complement for 90 minutes, as indicated. Nuclei were stained with DAPI (blue). Representative images obtained with control (b, f: #C5 serum), anti-GLT1-positive (d, h; #D4 serum) and anti-GLT1-negative (c, g; #D6 serum) T1DM serum samples are shown. Serum characteristics are reported in Table S2

3.4 Incubation of human islets of Langerhans with anti-GLT1 IgGs causes complement deposition and cell toxicity.

Given the possible clinical implications, data were confirmed in human isolated islets *in vitro*. GLT1-positive T1DM sera caused a significant increase in the number of islet cells positive for the C3 complement deposition (Figure 4A and 4B). C3 positivity was absent in islet cells incubated with sera from T1DM patients pre-absorbed onto a sepharose-A column, indicating that the process is specific and mediated by immunoglobulins. In line with a cytolytic effect of anti-GLT1 T1DM IgGs, a significant increase in the amount of released LDH was detected in the medium of islet cells incubated with active complement and IgG purified from three different GLT1-positive sera compared to control subjects (Figure 4C).

Interestingly, Affymetrix analysis, performed on laser captured islets from nPOD samples, revealed increased mRNA expression for C3 and C4A, C4B complement proteins in islets from T1DM subjects than healthy and T2D subjects, thus suggesting the possibility of increased activation of the complement cascade in T1DM (Figure 4D).

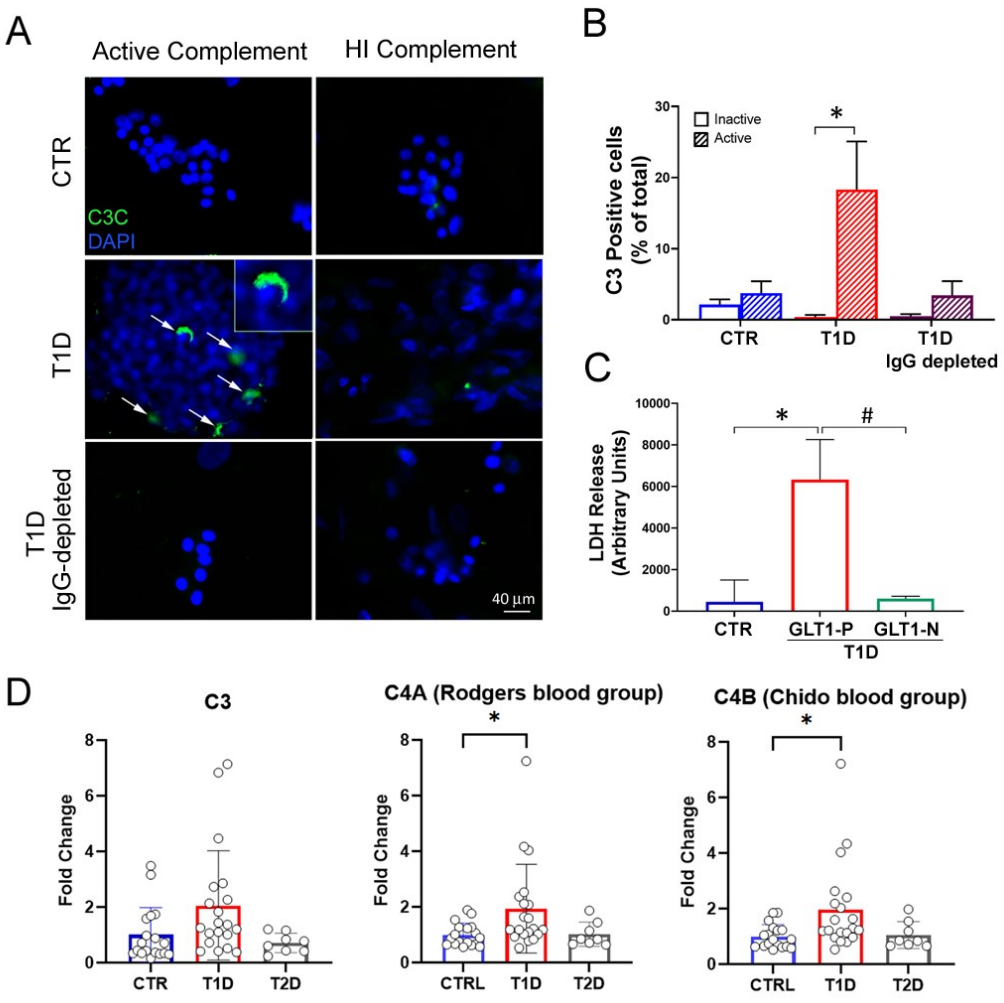

Figure 4. Incubation of human islets of Langerhans with IgG purified from GLT1-positive sera causes complement deposition and cell toxicity. (A) C3 (green) complement deposition in human islets incubated with a pool of T1DM sera (n=4) or CTR sera (n=4), in the presence of active complement. C3 deposition was lost in T1DM sera after absorption of IgGs onto Protein G sepharose beads. Nuclei were stained with DAPI (Blue). (B) Quantification of C3 positive cells in the presence of active or inactive complement, as indicated. Data are expressed as percentage of total cells in each field and are the mean ± SD of three different experiments performed in duplicate. (* $p<0.05$ active vs inactive; two-way ANOVA). (C) Cytotoxicity assessed by measurement of LDH release in the medium of human islets exposed to IgGs and active complement, as indicated. IgGs were isolated from two distinct control, anti-GLT1-positive (GLT1-P) and anti-GLT1 negative (GLT1-N) T1DM subjects and pooled. Data (difference between LDH measurements in the presence of active or heat-inactivated serum) are presented as Arbitrary Units (n=4, in duplicate). (* $p<0.05$ GLT1-P vs CTR; # $p<0.05$ GLT1-P vs GLT1-N; one-way ANOVA). (D) Evaluation of C3, C4A (Rodgers blood group), C4B (Chido Blood groups) complement mRNA expression by Affymetrix in islets from CTR, T1DM and T2DM subjects (NPOD samples). Data are expressed as fold enrichment and are the mean ± SD (*$p<0.05$ vs CTR; One-way ANOVA).

3.5 A Subset of Type 1 Diabetes Sera with GLT1 Autoantibodies Inhibits GLT1 Transport Activity and Increases β-cell Death in the Absence of Complement

The main function of GLT1 is to transport glutamate in the cell, to test the possibility that autoantibodies directed against GLT1, by binding to the protein, could also exert direct inhibitory

effect, we measured the uptake of [^{3}H]D-glutamate, a non-metabolizable GLT1 transporter substrates [20], in βTC3 cells incubated with control and T1DM sera (Figure 5A). The mean GLT1 activity measured in the presence of different sera showed a statistically significant difference between the GLT1-positive and the GLT1-negative groups ($P < 0.05$, one-way ANOVA). In particular, we found that 6 of 16 (37.5%) GLT1-positive diabetic sera drastically inhibited the uptake of [^{3}H]D-glutamate (more than 50%), in contrast to only 2 of 35 (5.7%) and 1 of 27 (3.6 %) in control and GLT1-negative groups, respectively. The inhibition was specific and due to anti-GLT1 antibodies because the effect was lost when T1DM sera were pre-absorbed over GFP-GLT1 expressing cells, but not GFP-expressing cells (Figure 5B). Interestingly, sera with inhibitory activity not necessarily showed complement fixing activity, indicating IgGs heterogeneity.

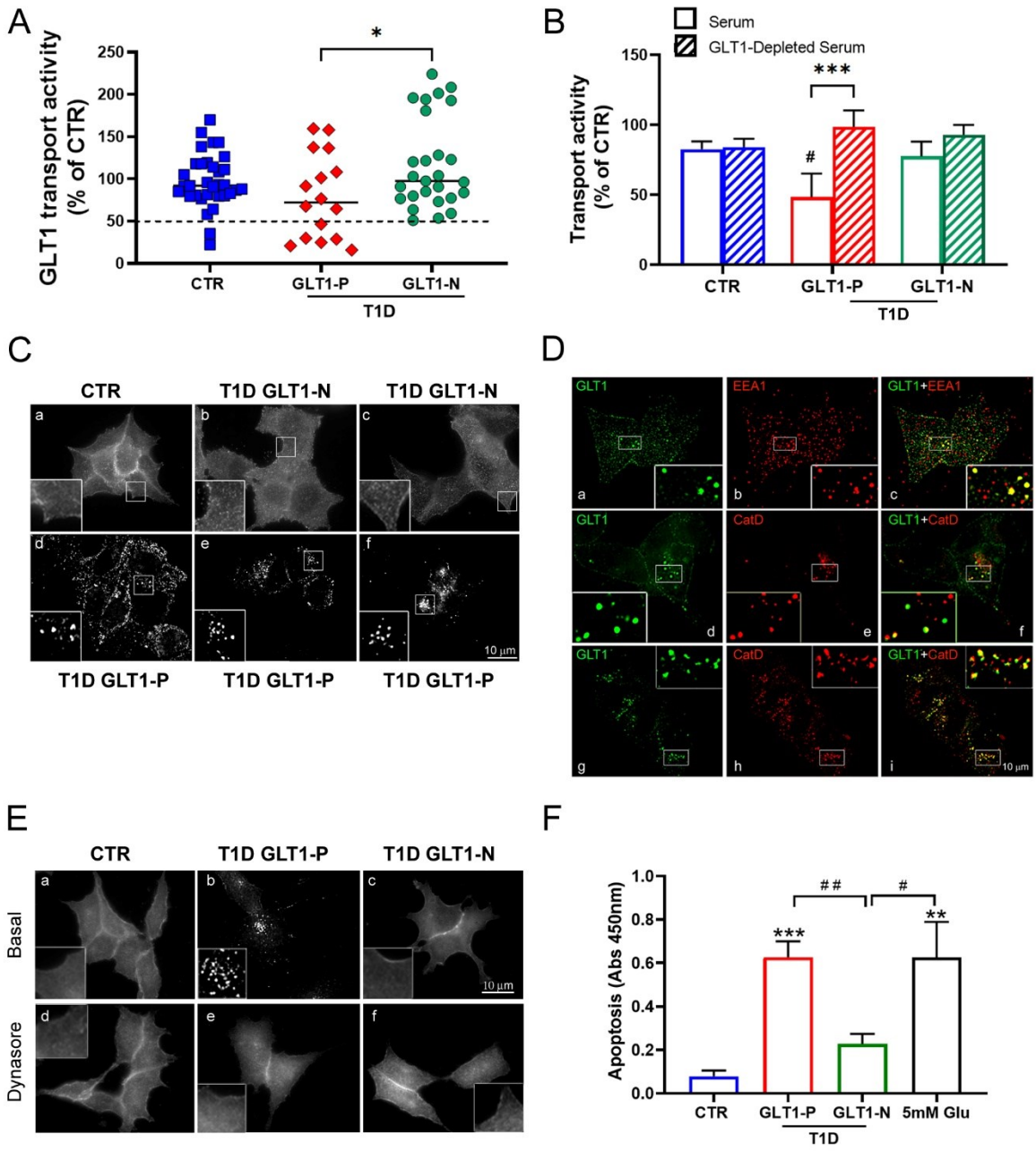

Figure 5. Incubation of βTC3 cells or human islets of Langerhans with a subset of anti-GLT1-positive sera downregulates GLT1 activity and induces cell apoptosis. (A) [^{3}H]-D-glutamate uptake experiments in βTC3 cells pre-incubated for 2 hrs with 10% control (CTR; n = 35), anti-GLT1-positive (GLT1-Pos; n = 16), or anti-GLT1-negative (GLT1-Neg; n = 27) T1DM serum samples. Data ([^{3}H]-D-glutamate uptake) are expressed as a percentage of control sera. (n = 3, in triplicate). Each serum is shown as a single point. The median values for each group are shown as gray lines. The dotted line indicates 50% inhibition of uptake measurements. (B) [^{3}H]-D-Glutamate uptake experiments after preincubation of βTC3 cells with 10% control (CTR; n = 3), anti-GLT1-positive (GLT1-P; n = 3), or anti-GLT1-negative (GLT1-N; n = 3) diabetic serum samples pre-adsorbed onto GFP (control sera, empty bars) or GFP-GLT1 coated wells (anti-GLT1 depleted sera, hatched bars) (* $p<0.05$; two-ways ANOVA). (C) GLT1 immunolocalization in βTC3 cells preincubated with 10% control or T1DM serum samples at 37°C for 2 hrs. The GLT1 internalization in intracellular vesicular compartments is induced after incubation with anti-GLT1 positive T1DM sera (d-f) but not with control sera or anti-GLT1 negative T1DM sera

(a-c). Inset, 2.5X magnification of the particular indicated in the figure. (D) βTC3 cells were preincubated for 2 hours at 37°C with 10% anti-GLT1 positive sera, fixed and double stained with GLT1 (green) and EEA1 or Cathepsin D (red). The double immunofluorescence staining reveals colocalization (yellow colour in the overlay) between internalized GLT1 and markers of the early endocytic pathway (a-c) or lysosomes (CatD) (d-i). Insets are 2.5X magnifications of the particular indicated in the figure. (E) Incubation of βTC3 cells with 60 μM dynasore prevents the GLT1 internalization induced by application of anti-GLT1 positive T1DM sera at 37°C. Insets are 2.5X magnifications of a particular of the figure. (F) Quantification of apoptosis in βTC3 cells preincubated for 24 hrs with 10% serum from control or T1DM patients. Four to seven sera from control (blue bar), anti-GLT1-negative (green bar), and anti-GLT1-positive (red bar) subjects were separately tested, and the mean value for each group was calculated. The apoptosis in the presence of 5 mM glutamate (gray bar) is shown as a positive control. Data are reported as absorbance value (ABS) measured at 450 nm (n = 3, in duplicate), (*** $p < 0.005$, ** $p < 0.01$ vs CTR; # $p < 0.05$, ## $p < 0.01$ vs GLT1-N; one-way ANOVA).

We finally evaluated the molecular mechanisms responsible for the downregulation of [^{3}H]D-aspartate uptake. *In vivo* incubation of β-cells with GLT1-positive diabetic sera with inhibitory activity caused the disappearance of GLT1 expression from the plasma membrane and its concomitant accumulation in intracellular vesicular structures (Figure 5Cd-f, insets). In contrast, GLT1 retained its plasma membrane distribution when cells were incubated in the same conditions with control sera or GLT1-negative diabetic sera (Figure 5Ca-c). Double immunofluorescence experiments indicated that anti-GLT1 autoantibodies caused the GLT1 internalization into Early Endosome Antigen 1 (EEA1)-positive and Cathepsin D-positive endo-lysosomal compartments, where GLT1 cannot perform its transport activity (Figure 5D). The internalization of GLT1 in intracellular compartments was prevented by incubation with dynasore, an inhibitor of the clathrin-dependent GLT1 endocytosis [37], thus confirming the mechanism of action of anti-GLT1 autoantibodies (Figure 5E).

Within the islet of Langerhans, a function of GLT1 is to control the extracellular glutamate concentration, thereby preventing glutamate-induced β-cell death [27,29]. We therefore tested the effects of autoantibody-mediated GLT1 inhibition on β-cell apoptosis. In the absence of active complement, a 24-hour incubation of βTC3 cells with 10% of GLT1-positive diabetic sera significantly increased cell apoptosis in comparison to control and GLT1-negative diabetic sera (Figure 5F) (2.66-fold increase; $P < 0.001$ vs CTR; $P < 0.005$ GLT1-P vs GLT1-N). Altogether, these data show that a

subgroup of T1DM sera binds to GLT1 and can interfere with its normal localization and function, by blocking the GLT1 transport activity or enhancing its internalization in endo-lysosomal compartments.

4 Discussion

After the discovery of islet cell autoantibodies (ICAs) in subjects with type 1 diabetes mellitus, several target proteins have been identified (7,8,10,12,13). Of note, none of these autoantigens reside on the plasma membrane of the β-cell as GAD65 is primarily located in the cytosol while IA-2 and ZnT8 on the insulin secretory granule. Our data show that GLT1 is the first plasma cell membrane autoantigen identified in T1DM and that autoantibodies directed against it are pathogenic. Our findings are consistent with previous reports describing the presence of cytotoxic islet cell surface autoantibodies (ICSAs) in T1DM [6,15,16]. Thus, we hypothesize that β-cell loss in subjects with T1DM may be caused by autoreactive T-cells [1–3], but also autoantibodies directed against membrane GLT1.

Autoantibodies against GLT1 were detected in 37% of patients with T1DM and in none of healthy control subjects. Cluster analysis reveals that GLT1-immunoreactivity identifies a subgroup of subjects with T1DM, distinct from those identified by GADA, IA-2A, and IAA, independent of age (Figures 2F-G and S3I). The identification of this new autoantigen provides a novel predictive biomarker for the underlying autoimmunity and new mechanisms of β-cell damage. More important, the identification of a new target of T1DM autoimmunity provides a new instrument for the development of antigen-specific immunotherapies; this is of particular interest, since anti-GLT1 antibodies exert a direct cytotoxic effect on β-cells. Pathogenic autoantibodies have already been demonstrated in endocrine [38–40], cardiac and neurologic diseases [41,42], in which they bind to cell-surface proteins, such as receptors and channels, and impair their function directly or

indirectly by inducing endocytosis and degradation [41–43]. Examples of pathogenic autoantibodies are those present in type B insulin resistance syndromes [38], premature ovarian failure [40] and myasthenia gravis-Eaton Lambert syndrome [44,45]. In type B insulin resistance syndromes, autoantibodies to the insulin receptor block insulin action, with resultant severe hyperglycemia, hypercatabolism, acanthosis nigricans, and hyperandrogenism in women. Pathogenic autoantibodies also may induce complement-mediated inflammation and cytotoxicity [42] as those directed against the skeletal muscle nicotinic acetylcholine receptor (AChR), responsible for myasthenia gravis [46].

Our data provide direct evidence that the sera from subjects with T1DM containing GLT1 autoantibodies can cause β-cell death through both complement-dependent and independent mechanisms. Cell-based assays demonstrate the binding of T1DM IgGs to GLT1, a process that, in the presence of active complement, initiates complement activation and induces plasma membrane lysis. The process is mediated by anti-β-cells IgGs, because it is prevented by IgG depletion from T1DM sera and is specific for anti-GLT1 antibodies as it is induced only in COS7 cells expressing the transporter. Of note, GLT1-immunoreactivity was detected in approximately 40% of ICA-positive sera, which is consistent with previous reports on complement-fixing ICAs [34–36], in at risk individuals.

Further studies will be necessary to identify the complement pathway involved in the phenomenon (classical vs alternative vs lectin) and to confirm the activation of the complement cascade in β-cells *in vivo*. The membrane fragmentation induced by complement activation also generates small vesicle-like structures reminiscent of exosomes that may provide new antigens and amplify the immunoreactive cascade [47–49]. In line with this possibility, β-cell derived

exosomes have been described in T1DM patients and proposed to have a role in the initiation of autoimmune responses toward intracellular epitopes [50,51].

Increased complement proteins expression has been detected at transcriptomic and proteomic levels in islets from T1DM subjects, early in disease pathogenesis [52], thus suggesting a possible role of complement activation in increasing or perpetuating the β-cell damage. This possible pathogenic mechanism clearly needs to be further clarified *in vivo* but suggests an opportunity for pharmacological intervention. This may be particularly relevant given that therapeutic agents (monoclonal antibodies, fusion proteins and peptidomimetics) targeting molecules active in the complement cascade are already available or in development for other autoimmune disease [53].

We found that complement-mediated cell death is not the only mechanism: a subset of anti-GLT1 antibody positive sera induce β-cell death in the absence of complement, through the direct inhibition of GLT1 activity and/or its internalization in endo-lysosomal compartments (Figures 3-5). Through these mechanisms, which will down regulate GLT1 at the plasma membrane, the islet ability to control the local extracellular glutamate concentration will be reduced and glutamate could reach concentrations toxic to β-cells [28,29]. This can be particularly relevant to disease development, as increased glutamate levels have been detected in sera of non-obese diabetic mice, before serum conversion and in patients with T1DM, before disease onset [54,55]. Further experiments will be necessary to prove the antigen modulation ability of anti-GLT1 antibodies and to text the possible contribution of glutamate-toxicity to β-cells in T1DM.

Lastly, the presence of pathogenic autoantibodies in a subset of T1DM subjects helps to explain the heterogeneity of the clinical course [56] and islet pathology in subjects with this disease [33,57–60].

5 Conclusions

In conclusion, our data provide evidence that GLT1 is a novel membrane autoantigen in a subset of patients with Type 1 diabetes mellitus. Autoantibodies to GLT1 are pathogenic by complement-mediated β-cell membrane lysis and death, and also by downregulating GLT1 protein and function. Studies in animals will be necessary to confirm *in vivo* the pathogenicity of anti-GLT1 antibodies. We do not know whether the GLT1-humoral autoreactivity is involved in the initiation of β-cell death, but it probably plays a role in the amplification of islet autoimmunity. Future studies should also address the predictive value of humoral and cellular autoreactivity against GLT1 and the correlation with the β-cell secretory reserve in subjects with new onset and long-lasting T1DM as well as in those who are at high risk of developing the disease. Finally, GLT1 could be an attractive therapeutic target for the prevention of β-cell death in individuals with diabetes.

6 Conflict of Interest

E.S.D.C., A.M.D., F.F. and C.P. are inventors in a PCT application (US 8,722,343 B2). The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The work has been presented in abstract form at the 48th EASD annual meeting 2012.

7 Author Contributions

Eliana Sara Di Cairano, Alessandra Galli, Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft & editing, Visualization. **Stefania Moretti**: Validation, Investigation, Writing – review & editing. **Bazzigaluppi Elena, Frolich Centonze Victoria:** Investigation, Resources. **Assi Emma:** Investigation, Data analysis, Writing – original draft. **Amalia Gastaldelli:** Data analysis, Writing – review & editing. **Federici Massimo, Ottavia Porzio, Federico**

Bertuzzi: Resources, Writing – review & editing. **Fiorina Paolo:** Resources, Writing – review & editing. **Davalli Alberto**: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision. **Folli Franco, Perego Carla**: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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