Assessment of the alterations of immune regulation and the incretin axis in type 1 diabetes

Ph.D. Thesis

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List of abbreviations

ANOVA: analysis of variance

ATPO: thyroperoxidase (TPO) autoantibody

CNTRL: control subject

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

CXCR: CXC chemokine receptor

DPP4: dipeptidyl peptidase 4

ELISA: enzyme-linked immunosorbent assay

Foxp3: forkhead box P3

GADA: glutamic acid decarboxilase autoantibody

GLP-1: glucagon-like peptide-1

HbA1c: glycated hemoglobin

ICA: islet cell autoantibody

IL: interleukin

IL2RA: IL-2 receptor α chain

MFI: median fluorescent intensity

PTPN2: protein tyrosine phosphatase, non-receptor type 2

SNP: single nucleotid polymorphism

T1DM: type 1 diabetes mellitus

T_{reg}: regulatory T-cell

1. Introduction

Although insulitis as the pathophysiologic basis of type 1 diabetes (T1DM) has been known for decades and the pool of known features of the autoimmune process is dinamically growing, many aspects of β-cell loss still remain elusive. Beyond their role in the regulation of the postprandial insulin response and β-cell survival incretin hormones, particularly GLP-1 (and also DPP4 via its inactivation by cleavage), also have a role in the regulation of immune processes. A growing body of evidence underlines the relevance of the alterations of the DPP4-incretin axis in T1DM. Previously our study group reported elevated serum DPP4 enzymatic activity in T1DM patients. The importance of this parameter is also underlined by the results of pilot studies and the broad, immunologically relevant substrate pool of DPP4, furthermore the known immunomodulatory effects of both the native and also the cleaved form of GLP-1. Our view of autoimmunity is also changing, the presence of autoantibodies and autoreactive T-cells cannot be considered pathologic any more. Instead of this view the alterations of the regulatory mechanisms might be the basis of the manifestation of autoimmune diseases. Although immunoregulation might be disturbed at many points, their role in the development of certain diseases, including T1DM, is not fully clarified. The role of $Foxp3^+$ T_{reg} cells is crucial in the limitation of cellular immune processes. Some research results also suggest that besides the active $CD4^+Foxp3^+CD25^+$ T_{reg} cells a $CD4^+Foxp3^+CD25^{-/low}$ subset also exists and represents a reserve pool, which might gain an active regulatory function after activation. The role of reserve T_{reg}s in the development of T1DM is not yet clarified. Furthermore the role of numerous genetic factors had already been identified, many of which affect immunologically relevant targets.

2. Aims

2.1 To assess the associations between the higher serum DPP4 activity in T1DM patients previously reported by our workgroup, GLP-1 plasma levels and certain immunologic parameters.

2.2 To estimate the proportions of $CD25^{+}$ (active) and $CD25^{-/low}$ (reserve) T_{reg} cells within the $CD4^{+}Foxp3^{+}$ group and their inhibitory (CTLA-4) and chemotactic (CXCR3) capacity in the peripheral blood of T1DM patients and healthy control subjects.

2.3 To further clarify the role of the active (7-36) and the cleaved (9-36) form of GLP-1 in immune regulation in T1DM and healthy subjects.

2.4 To assess the genotypes of the study participants for certain polymorphisms of genes with gene product fundamentally involved in T_{reg} function and a known common polymorphism of *DPP4* (SNPs: *DPP4* rs6741949, *CTLA4* rs3087243, *CD25* rs61839660, *PTPN2* rs2476601) and their potential effect on the incretin response, serum DPP4 activity and the selected immunologic parameters.

3. Methods

3.1 Subjects

We analyzed the samples of age, gender and BMI matched T1DM (39 clinically diabetic, ICA/GADA autoantibody positive and/or C-peptide negative patients) and non-diabetic control subjects (36 volunteers). For the determination of lymphocyte subset proportions we used all samples (39 T1DM and 36 CNTRL). For analysis of the DPP4-incretin axis and its associations we excluded those with a significantly impaired carbohydrate metabolism, thus the number of subjects included were 34 (T1DM) and 35 (CNTRL) in this setting. When analyzing the genotype-phenotype associations the numbers of subjets were 33 (T1DM) and 34 (CNTRL). The causes of the further decrease in subject numbers were invalid measurement results.

3.2 Assessment of certain parameters of the DPP4-incretin axis

3.2.1 Measurement of the serum DPP4 enzymatic activity

DPP4 enzymatic activity was not associated with prandial status in our previous studies. Fasting serum DPP-4 activity was determined in a continuous monitoring assay using Gly-Pro-pNA as substrate. The samples were stored at -20 C˚, followed by long-term storage at -80 C˚. Enzyme activity is expressed in nmol/ml/min (U/L) of pNA hydrolyzed during the 30 min assay process. We determined the quantity of the pNA at 405 nm with Varioskan Flash microplate reader.

3.2.2 Assessment of GLP-1 plasma concentrations

We used anticoagulated (EDTA) prandial plasma samples taken 45 min after a test meal containing 50 grams of carbohydrate, 22 grams of protein and 9 grams of fat. Active ($GLP\text{-}1^{7\text{-}36}$) and total GLP-1 ($GLP\text{-}1^{7\text{-}36}$ and $GLP\text{-}1^{9\text{-}36}$) were assessed separately with ELISA method. Cleaved GLP-1 (9-36) plasma concentrations were calculated.

3.3 Flow cytometric measurements

EDTA-anticoagulated full peripheral blood samples were used on the day of sampling. Three tubes were set from all samples: in the first tube with complete fixation and permeabilization without staining; in the seccond and third tubes we stained the assessed surface (CD3, CD4, CD8, CD25, CD26, CD44, CXCR3) and intracellular (CTLA-4, Foxp3) markers. The expression was determined with a Beckman Coulter Navios flow cytometer and Beckman Coulter Kaluza software.

3.4.1 Gating strategy

Lymphocytes were gated according to their forward and side scatters. Within the group of $CD3^+$ T-cells we gated the $CD4^+$ helper and $CD8^+$ cytotoxic T-lymphocytes. Among the $CD3^+CD4^+$ group we separated T_{reg}s according to their Foxp3 expression. We assessed their CD25, CD26 and CTLA-4 expression and gated the $CD25^+$ and $CD25^{-/low}$ T_{reg} subpopulations. The cutoff values for CD25 and Foxp3 positivity were set according to the autofluorescence in the unstained tube. CD25 and CTLA-4 expressions were also expressed in median fluorescence intensity (MFI).

3.5 Assessment of further laboratory parameters

Routine laboratory (white blood cell counts, plasma glucose, HbA1c, creatinine, liver enzymes, serum cholesterol), immunologic (specific autoantibodies: ATPO, ICA, GADA, perietal cell autoantibody) and endocrine (C-peptide, TSH, B12-vitamine, chromogranine) were determined in the Central Laboratory of Semmelweis University and the Endocrine Laboratory of the 2nd Department of Internal Medicine, Semmelweis University, with standard methods.

3.6 Genotype-phenotype associations

Common polymorphisms of three genes (*CTLA4*, *CD25*, *PTPN2*) coding products involved in immunoregulation and a known common polymorphism of *DPP4* were assessed. Their main characteristics are summarized in the following table:

Gene	Region and position	Risk SNP	MAF 1000 genomes	Function of the gene product
IL2RA (CD25)	10p15.1 intronic	rs61839660	0,07 (*T*)	Marker and mediator of T-cell activation
CTLA4	2q33.2 near gene	rs3087243	0,47 *(A*)	Analog of CD28, inhibitor of T-cell activation
PTPN2	18p11.21 intergenic	rs2542151	0,14 (*G*)	Inhibits β-cell apoptosis and IL-2 signaling
DPP4	2q24.2 intronic	rs6741949*	0,41 (*C*)	Cleavage of incretins and cytokines

Genomic DNA was isolated by a magnetic bead based method. Genotyping was carried out by TaqMan assays capable of biallelic discrimination in an ABI 7500 Fast Real-Time PCR-System in the Endocrine Laboratory of the 2nd Department of Internal Medicine, Semmelweis University.

3.7 Statistical analysis

Statsoft Statistica software was applied. Kolmogorov-Smirnov and Shapiro-Wilks tests were used to assess normality for the continuous parameters measured. The following tests were used to compare the results of the T1DM and control groups: unpaired two-tailed T-test and Mann-Whitney U-test (independent variables), oneway ANOVA and Kruskal-WAllis ANOVA (multiple independent variables), Friedman-ANOVA (multiple dependent variables), Spearman and Pearson-tests (correlation). Genotype distributions were assessed using the Hardy-Weinberg equilibrium (HWE) test.

4. Results

Here we only summarize the results which are statistically significant, and in addition, have sufficient statistical power (>80%) and/or, according to our present knowledge, might have clear clinical or pathophysiologic relevance.

4.1 Associations between serum DPP4 activity and plasma GLP-1 levels

Consistently with our previous results, we observed higher serum DPP4 activity in T1DM patients (T1DM: 44.13 U/l vs. CNTRL: 40.29 U/l, p=0.025). We found an inverse correlation between the serum DPP4 activity and the active GLP-1^{7-36} plasma concentrations (r=-0.53, p=0.002) in T1DM patients.

4.2 Associations between the selected parameters of the incretin axis and hematologic parameters

Plasma concentrations of the cleaved GLP-1^{9-36} showed direct correlation with lymphocyte counts (G/l) (r=0.58; p=0.0003) in healthy controls. This correlation was totally absent in T1DM patients.

4.3 CD25 expression of T_{helper} and T_{reg} cells

The CD25 expression of $CD4^+$ T-cells (MFI) was significantly lower in T1DM patients (T1DM: 0,74 vs. CNTRL: 1,21, $p<10^{-4}$). The proportion of $CD25^+$ cells ($CD25^+/CD4^+$) was also lower in the T1DM group (T1DM: 34.68% vs. CNTRL: 48.3%, $p<10^{-4}$). The proportion of T_{reg}s was similar in the study groups. However, the CD25 expression (MFI) of T_{reg} cells was significantly lower in the T1DM study group (T1DM: 1.89 vs. CNTRL: 2.22, p=0.007) as well as the proportion of $CD25^+$ cells ($CD25^+/CD4^+Foxp3^+$) among T_{reg}s (T1DM: 63.48% vs. CNTRL: 73.2%, p<0.001).

4.4 CTLA-4 and Foxp3 expression of T-cell subsets

We observed significantly decreased CTLA-4 expression (MFI) in the non-cytotoxic T-cells (T_{nc}: $CD3^{+}CD8^{neg}$) of T1DM subjects (T1DM: 1.49 vs. CNTRL 1.89, p=0.002). The CTLA-4 and Foxp3 expressions of the $CD25^{+}$ and $CD25^{-/low}$ T_{reg} subpopulations were similar in the study groups, however, a stepwise increase ($T_{helper}/T_{nc}<CD25^{-/low}$ $T_{reg}<CD25^{+}$ T_{reg}) was observed in both the CTLA-4 and Foxp3 expressions in the different T-cell subpopulations of the entire study group.

4.5 CXCR3 expression of T_{reg} cells

The proportion of $CXCR3^{+}$ cells was significantly lower among $CD8^{neg}Foxp3^{+}CD25^{-/low}$ T_{reg} cells than among $CD8^{neg}Foxp3^{+}CD25^{+}$ T_{reg}s (53.72% vs. 39.89 $p<10^{-5}$). However, no difference could be observed between the T1DM and the healthy control study group.

4.6 Associations between genetic polymorphisms and the assessed endocrine and immunologic parameters

4.6.1 Association of the rs3087243 SNP with CTLA-4 expression in $CD25^{+}$ T_{reg} cells

CTLA-4 expressions (MFI) were higher in the $CD8^{neg}Foxp3^{+}CD25^{+}T_{reg}$ cells isolated from individuals who carry a minor ("*A*") compared to individuals homozygous for the *G* allele (rs3087243-*A* allele carriers: 2.91 vs. *G/G* homozygous individuals: 2.52, p=0.017).

4.6.2 Association between the CTLA4 rs3087243 genotype and the postprandial GLP-1 plasma levels

The 45' min prandial plasma total GLP-1 levels were significantly lower in the entire study population in the peripheral blood of individuals with the CTLA4 rs3087243-*G/G* genotype than in carriers of the minor (*A*) allele (rs3087243-*G/G* genotype: 12.5 pmol/L vs. *A* allele carriers: 15.62 pmol/L, p=0.0008). This difference could also be observed in the T1DM and the healthy control groups when analyzed separately. The difference between homozygous and heterozygous *A* allele carriers was not statistically significant. Carrying the A allele of rs3087243 was related to a 3.11 pmol/L increase in the prandial total GLP-1 plasma levels (p=0.002) in the entire study population.

4.6.3 Association between the *DPP4* rs6741949 *genotype and serum DPP4 enzymatic activity*

Fasting serum DPP-4 enzymatic activity was higher in individuals with the *DPP4* rs6741949-*G/G* genotype than in those who carry at least one *C* allele (rs6741949-*G/G* genotype: 45.34 U/L vs. *C* allele carriers: 40.7 U/L, p=0.037). Carrying the C allele was related to a 4.6U/L decrease in serum DPP-4 enzymatic activity (p=0.037).

5. Conclusions

- The elevated serum DPP4 enzymatic activity we observed in T1DM patients is a stable parameter, which, in addition to its effect on the incretin axis, might have further relevance in the regulation of immunological processes:

  - We could confirm the previous results of our workgroup regarding the higher serum DPP4 enzymatic activity in T1DM patients. Furthermore, we found it to be inversely correlated with prandial plasma active GLP-1^{7-36} levels. This suggests the true pathophysiologic relevance of our findings, but also underlines that, coherently with the known bibliographic data, the soluble form of DPP4 might only be responsible for the inactivation of 15% of the total GLP-1 secreted, its majority is cleaved by the membrane bound form of DPP4.

  - The direct correlation between the plasma levels of the cleaved ("inactive") GLP-1^{9-36} and lymphocyte count is a novel finding. In the view of the dual receptor hypothesis (a theory explaining the GLP-1^{7-36} receptor-independent effects GLP-1^{9-36}) this might indicate dysfunctional $GLP1^{9-36}$ signaling in T1DM patients.

- According to our knowledge, we first studied the $CD4^{+}Foxp3^{+}CD25^{-/low}$ "reserve" T_{reg} cells in T1DM and described their elevated proportion within the total Treg population. Our finding might suggest that an impaired peripheral activation of T_{reg} cells might be part of the immune dysregulation in T1DM.

- Furthermore we described two characteristics of the $Foxp3^{+}CD25^{-/low}$ T_{reg} cells, which are not specific for T1DM, although through the altered proportion of T_{reg} subpopulations they might gain relevance in the pathogenesis:

  - $CD25^{-/low}$ T_{reg} cells are characterized by lower CTLA-4 expression when compared to active T_{reg}s, which might suggest a decreased inhibitory capacity.

  - The higher CXCR3 expression on $CD25^{-/low}Foxp3^{+}$ compared to $CD25^{+}Foxp3^{+}$ T_{reg} cells, according to our knowledge, is a novel finding and suggests the increased chemotactic capacity of this subpopulaton. In the view of the known signaling pathways, our finding may suggest that this T_{reg} subpopulation is waiting for orientational chemotactic stimuli in a „standby mode".

- Our results suggest that the active ($CD25^{+}$) T_{reg} cells of homozygous *CTLA4* rs3087243-*G* allele carriers express CTLA-4 at lower levels, which might refer to diminished suppressive capacity. This might in part be the reason for the higher risk observed in GWAS studies for T1DM and other autoimmune diseases.

- According to our knowledge, we first reported the association between a common genetic variant and reduced prandial GLP-1 response, namely the significantly decreased 45 min. prandial plasma GLP-1 levels in individuals with *CTLA4* rs3087243-*G/G* genotype with sufficient statistical power independently of the presence of T1DM. Based on its prevalence in the population the *CTLA4* rs3087243 might be potentially considered as a candidate gene variant for future trials tailoring the incretin response/therapy. Given that this gene variant is located in a non-coding region (near gene) further studies are needed to understand the

exact mechanisms resulting in decreased gene product expression as well as the possibility of an immune mediated L-cell damage which might be coherent with our current knowledge regarding the *CTLA4* gene.

- According to our knowledge we first described that the *DPP4* rs6741949-*G/G* genotype is related to higher serum DPP4 activity. Further probably time-consuming studies could clarify the way, how this intronic SNP might lead to higher DPP4 activity.

6. Publications

Full-length published original research articles related to the Ph.D. thesis

Zóka A, Barna G, Hadarits O, Al-Aissa Z, Wichmann B, Műzes G, Somogyi A, Firneisz G. (2015) Altered crosstalk in the dipeptidyl peptidase-4-incretin-immune system in type 1 diabetes: A hypothesis generating pilot study. Hum Immunol, 76: 667-72. doi: 10.1016/j.humimm. 2015.09.018. IF: 2,127

Zóka A, Barna G, Somogyi A, Műzes G, Oláh Á, Al-Aissa Z, Hadarits O, Kiss K, Firneisz G. (2015) Extension of the $CD4^{+}Foxp3^{+}CD25^{-/low}$ regulatory T-cell subpopulation in type 1 diabetes mellitus. Autoimmunity, 48: 289-97. doi: 10.3109/08916934.2014.992518. IF: 2,917

Further full-length published original research articles

Hadarits O*, *Zóka A**, Barna G, Al-Aissa Z, Rosta K, Rigó J Jr, Kautzky-Willer A, Somogyi A, Firneisz G. (2016) Increased Proportion of Hematopoietic Stem and Progenitor Cell Population in Cord Blood of Neonates Born to Mothers with Gestational Diabetes Mellitus. Stem Cells Dev, 25: 13-7. doi: 10.1089/scd.2015.0203. IF: 3,777 (*equal contribution)

Al-Aissa Z, Rosta K, Hadarits O, Harreiter J, *Zóka A*, Bancher-Todesca D, Patócs A, Kiss K, Sármán B, Pusztai P, Sziller I, Rigó J, Rácz K, Somogyi A, Kautzky-Willer A, Firneisz G. (2015) Cord Serum Dipeptidyl-Peptidase 4 Activity in Gestational Diabetes. Eur J Clin Invest, 45: 196-203. doi: 10.1111/eci.12397. IF: 2,687

Full-length original research article related to the Ph.D. thesis, accepted for publication

Zóka A, Barna G, Nyírő G, Molnár Á, Németh L, Műzes G, Somogyi A, Firneisz G. Reduced GLP-1 response to meal is associated with the CTLA4 rs3087243 G/G genotype. Cent Eur J Immunol. Accepted for publication: 2017.05.22. IF: 0,776

Further articles related to the Ph.D. thesis

Firneisz G, *Zóka A*. (2017) Elevation of serum dipeptidyl peptidase-4 activity in type 1 diabetes: Potential explanations and implications. Diabetes Res Clin Pract, 127: 291-2. doi: 10.1016/j.diabres.2016.08.005.

Zóka A, Műzes G, Somogyi A, Varga T, Szémán B, Al-Aissa Z, Hadarits O, Firneisz G. (2013) Altered immune regulation in type 1 diabetes. Clin Dev Immunol, 2013:254874. doi: 10.1155/2013/254874. IF: 2,934

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