I Title

Increased spontaneous CCL2 (MCP-1) and CCL5 (RANTES) secretion *in vitro* in LADA compared to type 1 diabetes and type 2 diabetes: Action LADA 14

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II Abstract

Aims

Immune-mediated type 1 diabetes (T1D) in adulthood and latent autoimmune diabetes in adults (LADA) share similar pathological mechanisms but differ clinically in disease progression. The aim of this study was to acquire insights into spontaneous and stimulated chemokine secretion of immune cells in different diabetes types.

Materials and methods

We investigated *in vitro* spontaneous, mitogen (PI) and antigen (HSP60, p277, pGAD, pIA2) stimulated chemokine secretion of leucocytes from patients with T1D (n=32), LADA (n=22), type 2 diabetes (T2D) (n=49), and glucose-tolerant individuals (n=13). Chemokine concentration in supernatants was measured for CCL2 (MCP-1), CXCL10 (IP10) and CCL5 (RANTES) using a multiplex bead array assay.

Results

Spontaneous secretion of CCL2 and CCL5 were higher in LADA compared to T1D and T2D (all $p<0.05$) while CXCL10 was similar in the groups. Mitogen stimulated secretion of CCL2 in LADA was lower compared to T1D and T2D (all $p<0.05$) while CXCL10 and CCL5 were similar in all groups. Upon stimulation with pIA2 the secretion of CCL2 in LADA was lower compared to T2D ($p<0.05$). Spontaneous CXCL10 secretion in LADA was positively associated with BMI ($r^2=0.35$; $p=0.0035$) and C-peptide ($r^2=0.30$; $p=0.009$).

Conclusions

In conclusion, chemokine secretion is altered between different diabetes types. Increased spontaneous secretion of CCL2 and CCL5 and decreased secretion of CCL2, upon stimulation with PI and pIA2, in LADA compared to T1D and T2D could reflect altered immune responsiveness in LADA patients in association with their slower clinical progression compared to insulin dependence.

Keywords: latent autoimmune diabetes in adults; type 1 diabetes; type 2 diabetes; CCL2; CXCL10; CCL5

III Introduction

Diabetes is a heterogeneous group of metabolic disorders sharing hyperglycaemia as a common feature resulting from insufficient insulin secretion, insulin action or both [1]. The two major forms of diabetes are type 1 diabetes (T1D) with a lack of insulin secretion due to immune-mediated ß-cell destruction and type 2 diabetes (T2D) with a combination of decreased insulin secretion and action [2]. Latent autoimmune diabetes in adults (LADA) is classified as a subtype of T1D and at diagnosis characterized as clinical T2D in people who are positive for T1D associated autoantibodies, whereby the age of onset of LADA seems to be related to the clinical resemblance to T2D [3]. The common criteria for the diagnosis of LADA are adult age at onset, detection of autoantibodies, and no need for insulin treatment for 3-6 months after diagnosis [4].

Immunological reactions play a role in the pathogenesis in T1D, LADA, and T2D, but it remains to be resolved how pathological and clinical differences between these types of diabetes can be ascribed to different immunological reactivity [5–8]. Patients with T1D and LADA are positive for islet antibodies, while T2D patients are antibody negative. T1D is known to result from ß-cell directed cellular autoimmunity that is associated with insulitis and altered innate immunity [9, 10]. LADA as a subtype of T1D supposes to be a milder form of autoimmune diabetes with a slower disease progression showing fewer immune cell infiltrated islets with a lower pro-inflammatory cytokine gene expression [11]. Several attempts have been made to ameliorate disease progression by immunopreventive therapies including vitamin D [12, 13]. Furthermore, regarding cellular immunological changes LADA has overlapping features with both T1D and T2D showing equal numbers of regulatory B-cells like T2D and natural killer cells like T1D [14]. T2D is associated with increased systemic inflammation and some islet inflammation [15, 16].

Chemokines might have a role in chemoattracting immune cells and based on mouse models a role in the process of ß-cell destruction for CCL2 (MCP-1), CXCL10 (IP-10), and CCL5

(RANTES) have been shown [17–20]. *In vivo* CCL2 (MCP-1) and CXCL10 (IP-10) are secreted amongst others by monocytes and endothelial cells and CCL5 (RANTES) predominantly by T-lymphocytes [19, 20].

There are few systematic studies comparing immune responses in adulthood in recent onset T1D, T2D, and LADA. In one study, comparing cytokine secretion by peripheral mononuclear cells, a higher secretion of IL-17 upon stimulation with insulin and a higher secretion of IL-10 upon stimulation with insulin or GAD65 were found in LADA compared to T1D [21]. Another study comparing adaptive immunity, e.g. T-cell reactivity upon stimulation with mitogens and antigens in T1D, LADA, T2D, and healthy controls, did not find differences for cytokine secretion between groups while response to stimulation with autoantigens in general was low [22]. Increased systemic concentrations of adhesion molecules but not chemokines were found in sera of patients with T2D compared to patients with T1D and LADA [23]. Likewise, systemic levels of cytokines were increased in T2D, despite substantial overlap in T1D, LADA, and T2D [24]. An inverse relationship between organ-specific autoantibodies and systemic immune mediators in T1D and T2D including LADA have been described pointing out the association of humoral and systemic immunity [25]. Taken together, differences in the immune responses of LADA versus T1D and T2D are mostly related to islet antibodies, that are characteristic for T1D and LADA, and increased systemic inflammation mainly in T2D. Not much is known about innate immune response and stimulated chemokine secretion of leucocytes in recent onset LADA versus adult T1D and T2D.

In the present study we investigated *in vitro* the spontaneous, mitogen, and islet antigen peptide stimulated chemokine secretion (CCL2 (MCP-1), CXCL10 (IP-10), CCL5 (RANTES)) of freshly isolated leucocytes from individuals with T1D, LADA, T2D, and glucose-tolerant individuals to gain more insight into the immunological role of chemokines in diabetes and furthermore to differentiate between the types of diabetes on basis of chemokine secretion.

IV Material and Methods

2.1. Study population

The study population consisted of 32 patients with T1D, 49 patients with T2D, 22 patients with LADA, and 13 healthy glucose tolerant control individuals from Germany. All individuals enrolled in this study in multiple centers in Germany were a German subgroup of participants of the Action LADA study which has been described in detail before [25, 26]. Diagnosis of T1D and T2D was determined by investigators according to international criteria [1]. According to the study protocol, criteria for the definition of LADA were the occurrence of diabetes between the age of 30 and 70 years, no insulin treatment for the first 6 months after diagnosis, and positivity for glutamic acid decarboxylase 65 autoantibody (GADA). Age, gender, duration of diabetes and BMI were recorded and fasting levels of blood glucose, serum triglycerides, C-peptide levels and HbA1c were determined. The ethical committee of the Heinrich-Heine-University Duesseldorf approved the study protocol in accordance with the declaration of Helsinki and informed consent was obtained from all patients (ethic vote number 2279).

2.2. Immune cell stimulation

Freshly isolated human leucocytes were stimulated as described before while recommendations of the position statement of the T-Cell workshop committee of the immunology of diabetes society were followed [22, 27–29]. In brief, peripheral venous blood was drawn into K^+-EDTA tubes and stored for 24h at room temperature. Peripheral blood mononuclear cells (PBMC) were then isolated from whole blood by Ficoll separation tubes and viability of cells was ensured. 3.5 million PBMCs were incubated with mitogens and antigens in 0.5ml medium RPMI in 24 well plates as described before [22]. Cells were stimulated with mitogen phorbol-myristate-acetate/ionomycin (PI) (200 pg/ml) (Sigma, Germany) as positive control and antigens heat shock protein 60 (HSP60) (10 ng/ml) (Peptor, Israel), peptide p277 (p277) (200 ng/ml) (Peptor, Israel), peptides of glutamic acid decarboxylase 65 [aa 270 to 292; 554 to 575;

335 to 352] (pGAD) (20 ng/ml) or peptides of protein-tyrosine-phosphatase-like-antigen [aa 831 to 850; 841 to 860]) (pIA-2) (200 ng/ml) (all peptides were synthesized at Leiden University Medical Center, the Netherlands). Culture medium alone was used to assess background chemokine secretion (BG). After incubation for 18h at 37°C 0.5ml medium RPMI 1640 containing 10% human AB serum was added to each well and cells were incubated for additional 22h. Then, 1ml of supernatants were collected and stored at –20°C until analysis of chemokine concentration.

2.3. Chemokine measurement

Supernatants were analysed in duplicate for CCL2 (MCP-1), CXCL10 (IP10), and CCL5 (RANTES) by a multiplex bead array assay (LUMINEX 100 MBAA platform, Luminex, Austin, USA). Quantification limits were 61.73 pg/ml for CCL2, 20.58 pg/ml for CXCL10, and 20.58 pg/ml for CCL5. For cytokine concentrations lower than the quantification limit a value half of the quantification limit was assigned. The determined inter-assay variation was <10% and intra-assay variation was <20%.

2.4. Statistical analysis

Statistical analysis was performed using SAS Enterprise Guide v4.1 software (SAS Institute, USA) and GraphPad Prism v4 software (GraphPad Software, USA). Stimulation indices (SI) were calculated as ratio of chemokine concentration with and without stimulation (spontaneous secretion). Comparison between groups for anthropometric and clinical characteristics was performed with Freeman-Halton-Test for testing all groups and Fisher-Exact-Test for pairwise testing of two groups for categorial data and with Kruskal-Wallis-Test for testing all groups and Mann-Whitney-Test for pairwise testing of two groups for metrical data. All groups were compared for cytokine secretion using Kruskal-Wallis-Test and in case of significance, Mann-Whitney-Test was used to compare the groups pairwise. Comparisons between groups were adjusted with model 1 for sex, age, BMI, and diabetes duration and model 2 for plasma glucose,

HbA1c, serum triglycerides, and C-peptide using multiple linear regression analysis for testing of all groups and in case of significance with Tukey-Kramer-Post-Hoc-Test for testing of two groups. For correlation analysis between BMI, C-peptide and HbA1c and chemokine secretion Spearman correlation coefficient was calculated. If a correlation was significant, multiple linear regressions analysis with adjustment for sex, age and diabetes duration or sex, age and BMI was performed. Regression analysis was performed with and without outliers yielding equal results. For all statistical analyses, $p < 0.05$ was considered statistically significant.

V Results

3.1. Patients characteristics

Comparison of the groups for anthropometric and clinical characteristics showed differences for BMI, ambient plasma glucose, HbA1c, serum triglycerides, and fasting C-peptide (all p<0.05) (table 1). For BMI and serum triglycerides, T2D showed higher values compared with T1D and controls (all p<0.05) while ambient plasma glucose and HbA1c were obviously higher in all diabetes groups compared to controls (all p<0.05). Fasting C-peptide was higher in T2D compared with T1D and LADA (all p<0.05). More males were recruited for T1D and T2D compared with people with LADA and controls (all p<0.05). Diabetes duration was similar in diabetes groups and age was similar between all groups (table 1).

3.2. Differences between groups for spontaneous chemokine secretion

Spontaneous secreted or background (BG) CCL2 (MCP-1) and CCL5 (RANTES) concentration differed among the groups (table 2 and figure 1). CCL2 (MCP-1) concentration in supernatants without mitogen or antigenic stimulus was increased in people with LADA compared with those with T1D (714.25 pg/ml vs. 129.31 pg/ml; unadjusted p<0.05; model 1 adjusted p<0.05; model 2 adjusted p<0.01) and T2D (714.25 pg/ml vs. 111.99 pg/ml; unadjusted p<0.01; model 1 adjusted p<0.01; model 2 adjusted p<0.01). Similarly, CCL5 (RANTES) concentration in supernatants without stimulation was increased in people with LADA compared with those with T1D (1785.60 pg/ml vs. 1371.50 pg/ml; unadjusted p<0.05; model 1 adjusted ns; model 2 adjusted ns) and T2D (1785.60 pg/ml vs. 1223.50 pg/ml; unadjusted p<0.05; model 1 adjusted ns; model 2 adjusted ns). No differences between groups were seen for spontaneous CXCL10 (IP10) secretion (table 2).

3.3. Differences between groups for stimulated chemokine secretion

Upon stimulation with mitogen or pIA2, lower CCL2 (MCP-1) stimulation indices in LADA

were observed (table 2 and figure 1). CCL2 (MCP-1) stimulation indices upon stimulation with mitogen were lower in LADA compared to T1D (stimulation indices 1.12 vs 6.47; unadjusted $p < 0.05$; model 1 adjusted ns; model 2 adjusted ns) and T2D (stimulation indices 1.12 vs 7.56; unadjusted $p < 0.01$; model 1 adjusted ns; model 2 adjusted $p < 0.05$). In general, responses to antigen stimulation were low. CCL2 (MCP-1) stimulation indices upon stimulation with pIA2 were lower in LADA compared to T2D (stimulation indices 1.17 vs 2.60; unadjusted ns; model 1 adjusted ns; model 2 adjusted $p < 0.05$). CCL2 (MCP-1) stimulation indices were similar in all groups upon stimulation with HSP60, p277, and pGAD. Similarly, CXCL10 (IP10) and CCL5 (RANTES) stimulation indices were similar between groups upon stimulation with different antigens and mitogen.

3.4. Association of chemokines and BMI, C-peptide and HbA1c

For LADA, spontaneous secretion of CXCL10 (IP10) was positively associated with BMI ($r^2 = 0.35$, $p < 0.05$) and C-peptide ($r^2 = 0.30$, $p < 0.05$) (figure 2) and for BMI remained significant after adjustment for sex, age and diabetes duration. For healthy controls CCL5 (RANTES) stimulation indices upon PI stimulation were directly associated with HbA1c ($r^2 = 0.47$, $p < 0.05$) and C-peptide ($r^2 = 0.33$, $p < 0.05$). Both associations remain significant after adjustment for sex, age and BMI. These associations were not found for other groups. No significant association was observed for CCL2 (MCP-1) with C-peptide, BMI and HbA1c.

VI Discussion

We investigated chemokine secretion of leucocytes in adult onset T1D patients, LADA patients, T2D patients, and glucose-tolerant individuals to better understand their potential impact on the different clinical progression rate in the major types of diabetes.

People with LADA differed from those with T1D and T2D, both by increased spontaneous secretion of CCL2 (MCP-1) and CCL5 (RANTES) and, for CCL2 (MCP-1) lower responses on stimulation with mitogen and pIA2. CCL2 (MCP-1) and CCL5 (RANTES) are chemokines and play a role in activation and migration of immune cells like monocytes, NK-cells, T-cells, dendritic cells and granulocytes [30, 31]. Analyses of serum samples from the European Action LADA 5 study showed that LADA has similar serum concentrations of CCL2 (MCP-1), CCL3 and CCL4 compared to T1D and T2D [23]. However, not all LADA cases progress to insulin dependence. Therefore, it is interesting that CCL2 (MCP-1) was higher than controls both in children with T1D and in first degree relatives of T1D cases [32, 33]. Similarly pre-T1D children had higher CCL2 (MCP-1) levels than those with diabetes autoantibodies that did not progress [34]. By inference, serum CCL2 (MCP1) could be a marker of progression towards clinical T1D. Furthermore, blockade of CCL2 (MCP-1) improved pancreatic islet engraftment and survival in mice emphasizing a proinflammatory and disease progressive role of CCL2 (MCP-1) [35]. If extrapolated to our study in adults, the increased CCL2 (MCP-1) secretion might also reflect the differential disease progression in people with LADA compared to both T2D and T1D.

In the NOD mouse model it has been shown that CCL2 (MCP-1) unexpectedly prevents diabetes development probably mediated by tolerogenic dendritic cells [36]. These findings support an unexpected beneficial role for CCL2 (MCP-1) in T1D implicating the CCL2/CCR2 axis in human autoimmunity. By contrast, upon stimulation with mitogen and islet auto antigenic peptide IA-2, we found CCL2 (MCP-1) secretion was decreased in LADA though the clinical impact of such an effect is unclear.

Spontaneous CCL5 (RANTES) secretion was increased in people with LADA compared to T1D in this study and increased CCL5 (RANTES) serum concentrations in non-remitting T1D patients have been shown elsewhere [37]. In the NOD mouse, CD4+ and CD8+ T cells secrete CCL5 (RANTES) and other cytokines (IL-2, IL-6, IL-10, CCL3), each of which showed a dose dependent effect on ß-cell proliferation, therefore being potential therapeutic targets [38]. In this context, increased CCL5 (RANTES) in people with LADA found here could reflect reduced progression of islet destruction.

Another recent study in NOD mice showed that chemokines CCL5 (RANTES) and CCL8 were persistently elevated in inflamed islets and the influx of CD11c+ cells was partially dependent on their receptor CCR5 [39]. Treatment with islet Ag-specific regulatory T cells led to a marked decrease of CCL5 (RANTES) and CCL8. In this setting islet CCL5 (RANTES) was associated with destructive insulitis. In our study increased CCL5 (RANTES) in people with LADA could indicate an increased proinflammatory status and islet destruction compared to T2D patients. While our study is the first reporting upregulated CCL2 (MCP-1) and CCL5 (RANTES) secretion in people with LADA, compared with adult onset T1D and T2D, we do not have access to human islet histology.

In considering whether the chemokine secretion is related to metabolic features including BMI, HbA1c or C-peptide, we observed associations between spontaneous or stimulated chemokine secretion and clinical characteristics suggesting that immune reactions are influenced by metabolic and anthropometric factors. In the LADA group, spontaneous secretion of CXCL10 (IP10) was positively associated with BMI and C-peptide in line with elevated fat mass triggering a low-grade inflammation [40]. In pancreatic islets of T1D patients, insulitic lesions were characterized by elevated levels of the chemokine CXCL10 (IP10) and infiltration of lymphocytes expressing the corresponding chemokine receptor CXCR3 [41]. While there was a positive association of spontaneous secretion of CXCL10 (IP10) and C-peptide in the LADA group, we did not see a difference of CXCL10 (IP10) secretion in people with LADA versus T1D or T2D. That result contrasts with two studies showing increased serum CXCL10 (IP10):

one in pediatric non-diabetic islet antibody positive children, another in both newly diagnosed T1D patients and subjects at increased disease-risk being positive for autoantibodies ICA and GAD [34, 42]. The positive association of CXCL10 (IP10) with BMI could be the result of increased inflammation in more obese people, while the positive association of CXCL10 (IP10) with C-peptide could reflect an increased inflammatory response in association with increased ß-cell mass and function that could either perpetuate the immune response.

Our data shows that LADA patients, compared with T1D and T2D patients, have an increased spontaneous secretion of CCL2 (MCP-1) and a decreased response of CCL2 (MCP-1) from *in vitro* stimulation of fresh leucocytes with both PI and autoantigenic peptide pIA2. These results complement other studies showing altered inflammatory markers between the major types of diabetes and within autoimmune diabetes [43]. The increased spontaneous secretion of CCL2 (MCP-1) in LADA patients could contribute to the ongoing immune-mediated ß-cell destruction in LADA by maintaining an inflammatory milieu in pancreas islets, whereby an explanation of the mechanisms requires further studies. However, regarding limitations of this study, the number of participants in each group was small, multiple testing was applied and analysis was cross-sectional, not longitudinal. In addition, duration of diabetes was hetero-genous on a certain degree in groups and might limit direct comparability. However date were adjusted for diabetes duration to compensate for that. Future longitudinal studies are needed to further elucidate cytokine profiles of diabetes groups.

The altered (proinflammatory) chemokine response we describe is associated with C-peptide. However, it is unclear if that immune response impacts the variable rates of ß-cell deterioration in the major types of diabetes. These exploratory findings are not sufficient to recommend potential therapeutical targets and require further studies given the potential clinical utility of altering ß-cell deterioration through immune modulation.

VII Acknowledgements

Conflict of Interest

NCS is currently employed by Lilly Deutschland GmbH, Bad Homburg, Germany and is member of the medical faculty of the Heinrich-Heine-University Duesseldorf. The authors declare no competing financial interests.

Author Contribution

MO: experimental work, data curation, formal analysis, writing – original draft, AS: supervision, writing - review & editing, KS: supervision, writing - review & editing, BM: experimental work, writing - review & editing, RDL: writing - review & editing, NCS: conceptualization, supervision, writing - review & editing. All authors have read and approved the final manuscript.

Data availability statement

Data are further under analysis und are available on request from the author.

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Appendix

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Tables

Table 1 Baseline characteristics of the study population

	Control	T1D	LADA	T2D	*p*-value
Participants (male/female)	13 (4/9)	32 (23/9)[†]	22 (6/16)[‡]	49 (35/14)[†,§]	<0.05
Age (years)	49.0 (12.9)	44.5 (12.3)	48.4 (16.8)	48.9 (18.4)	ns
BMI (kg/m^2)	22.2 (4.7)	24.5 (6.0)	26.7 (7.4)[†]	30.3 (6.3)[†,‡]	<0.05
Diabetes duration (years)	-	0.87 (1.85)	1.84 (2.57)	1.01 (2.43)	ns
Plasma glucose (mg/dl)	84 (20)	130 (52)[†]	127 (60)[†]	123 (52)[†]	<0.05
HbA1c (%)	3.7 (0.4)	7.2 (4.6)[†]	6.7 (3.2)[†]	6.5 (3.0)[†]	<0.05
Serum triglycerides (mg/dl)	112 (99)	100 (89)	120 (65)	168 (141)[†,‡]	<0.05
Serum C-peptide (ng/ml)	1.80 (1.30)	1.35 (0.99)	1.40 (2.00)	2.50 (1.85)[‡,§]	<0.05

Data presented as numbers or median (with difference between first and tertiary quartile). Column "*p*-value" shows the comparison of all groups using Freeman-Halton-Test for categorical data or Kruskal-Wallis-Test for metrical data. Group comparison pairwise was performed using Fisher-Exact-Test for categorical data and Mann-Whitney-Test for metrical data: [†]p<0.05 vs Control; [‡]p<0.05 vs T1D; [§]p<0.05 vs LADA. Abbreviations: Control – healthy individuals; LADA – latent autoimmune diabetes in adults; T1D – type 1 diabetes; T2D – type 2 diabetes; ns – not significant

Table 2 Testing for differences between groups for chemokine secretion (pg/ml or stimulation index (SI))

BG/Stimuli	T1D	LADA	T2D	Control	p-value unadj.	adj.M1	adj.M2
CLL2 (MCP-1)							
BG	129.31 (511.72)	714.25 (2916.80)	111.99 (470.75)	132.10 (675.61)	**0.0196**	**0.0222**	**0.0020**
PI	6.47 (24.67)	1.12 (3.90)	7.56 (18.02)	5.70 (7.48)	**0.0194**	0.1143	**0.0147**
HSP60	1.00 (1.03)	0.66 (0.95)	1.05 (2.05)	0.88 (0.95)	0.0619	0.1010	0.1328
p277	1.16 (3.96)	0.95 (1.08)	1.00 (1.13)	0.91 (1.46)	0.0920	0.1864	0.2734
pGAD	1.73 (5.01)	1.13 (4.10)	1.07 (1.83)	0.59 (1.97)	0.3094	0.1024	0.5138
pIA2	2.87 (5.45)	1.17 (5.90)	2.60 (14.51)	2.27 (54.87)	0.1693	0.2045	**0.0323**
CXCL10 (IP10)							
BG	638.13 (1303.40)	1723.90 (1720.00)	1011.30 (1863.20)	984.97 (3087.00)	0.2210	0.6017	0.3834
PI	1.77 (2.82)	0.96 (1.78)	1.43 (1.60)	1.55 (2.19)	0.2452	0.4011	0.2673
HSP60	0.94 (0.27)	0.93 (0.18)	0.97 (0.25)	0.95 (0.16)	0.5415	0.9930	0.9235
p277	0.96 (0.23)	0.90 (0.18)	0.93 (0.27)	1.00 (0.13)	0.7752	0.8335	0.9512
pGAD	1.02 (0.41)	0.98 (0.33)	1.09 (0.26)	1.04 (0.21)	0.6273	0.8131	0.7976
pIA2	0.54 (0.57)	0.23 (0.65)	0.75 (0.69)	0.77 (0.28)	0.0731	0.1668	0.1863
CCL5 (RANTES)							
BG	1371.50 (1015.70)	1785.60 (873.72)	1223.50 (944.91)	946.19 (1025.10)	**0.0331**	0.4230	0.0689
PI	7.18 (7.40)	6.34 (3.03)	6.16 (8.32)	8.86 (10.32)	0.5091	0.9201	0.6861
HSP60	1.08 (0.49)	1.02 (0.62)	1.17 (0.28)	1.21 (0.42)	0.5160	0.6071	0.6819
p277	1.00 (0.52)	1.09 (0.68)	1.13 (0.55)	1.00 (0.35)	0.9614	0.8158	0.9831
pGAD	1.01 (0.43)	1.10 (0.52)	1.01 (0.37)	0.82 (0.23)	0.2956	0.4446	0.5669
pIA2	1.13 (0.59)	1.24 (0.74)	1.10 (0.60)	1.00 (0.33)	0.5000	0.9156	0.5157

Data are presented as median (with difference between first and tertiary quartile) of spontaneous cytokine concentrations [pg/ml] (BG) or median stimulation indices (with difference between first and tertiary quartile) (SI) (PI; HSP60; p277; pGAD; pIA2); unadjusted p-values are corresponding to the comparison of all groups with Kruskal-Wallis-Test; adjusted p-value<0.05 corresponding to comparison of all groups with multiple linear regression: M1 adjusted for sex, age, BMI and diabetes duration; M2 adjusted for plasma glucose, HbA1c, serum triglycerides and C-peptide; significant p-values are presented in bold; Abbreviations: T1D – type 1 diabetes; LADA – latent autoimmune diabetes in adults; T2D – type 2 diabetes; Control – healthy individuals; BG=spontaneous secretion
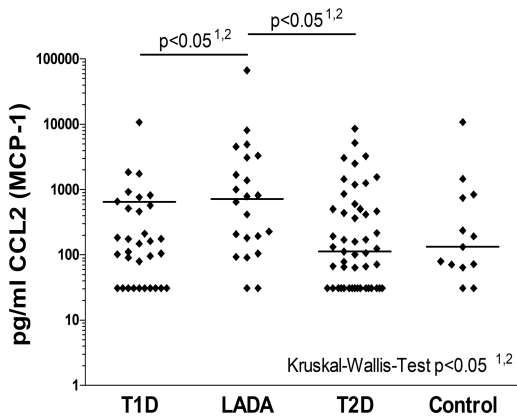
Legends to figures

Fig. 1 CCL2 and CCL5 secretion in people with type 1 diabetes (T1D), latent autoimmune diabetes in adults (LADA), type 2 diabetes (T2D) and glucose tolerant control individuals

Each dot corresponds to spontaneous chemokine concentration or stimulation index (SI; ratio of stimulated cytokine secretion and spontaneous secretion) of an individual. The horizontal lines show the median. Comparison of all groups with Kruskal-Wallis-Test and pairwise comparison of two groups with Mann-Whitney-Test; comparison of all groups with multiple linear regression: [1]$p<0.05$ (adjusted for sex, age, BMI, and diabetes duration); [2]$p<0.05$ (adjusted for plasma glucose, HbA1c, serum triglycerides, and C-peptide); Abbreviations: T1D – type 1 diabetes; LADA – latent autoimmune diabetes in adults; T2D – type 2 diabetes; Control – healthy individuals; BG=spontaneous secretion; ns – not significant
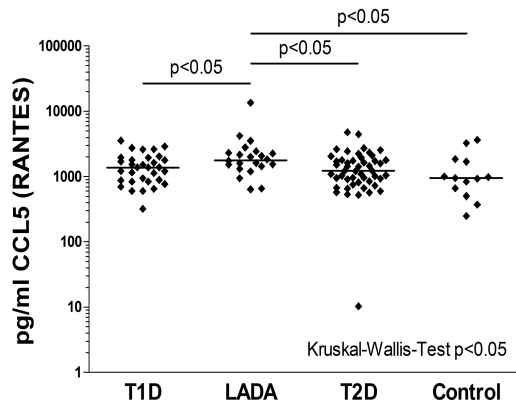
Fig. 2 Association of chemokines secretion with anthropometric and clinical data

Each dot represents an individual. The line shows the association between two parameters calculated using linear regression. r^2 and p-value correspond to multiple linear regression model. [1]$p<0.05$ after adjustment for sex, age, and diabetes duration, [2]$p<0.05$ after adjustment for sex, age, and BMI. Abbreviations: SI – ratio of stimulated chemokine secretion and spontaneous secretion; LADA – latent autoimmune diabetes in adults; Control – healthy individuals

CCL2 (MCP-1) spontaneous secretion

p<0.05[1,2]   p<0.05[1,2]

Kruskal-Wallis-Test p<0.05 [1,2]

X-axis: T1D, LADA, T2D, Control
Y-axis: pg/ml CCL2 (MCP-1)

CCL5 (RANTES) spontaneous secretion

p<0.05   p<0.05   p<0.05

Kruskal-Wallis-Test p<0.05

X-axis: T1D, LADA, T2D, Control
Y-axis: pg/ml CCL5 (RANTES)

CCL2 (MCP-1) PI stimulated secretion

p<0.05   p<0.05[2]

Kurskal-Wallis-Test p<0.05 [2]

X-axis: T1D, LADA, T2D, Control
Y-axis: SI CCL2 (MCP-1)
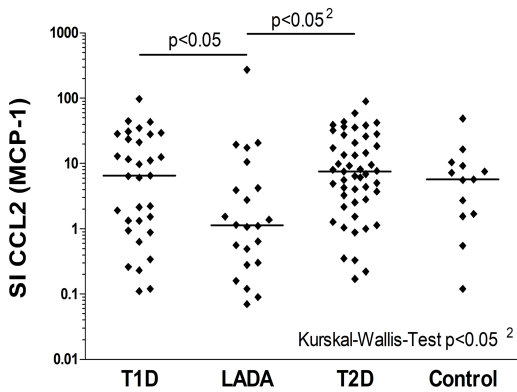
CCL2 (MCP-1) pIA2 stimulated secretion

ns[2]

Kruskal-Wallis-Test ns [2]

X-axis: T1D, LADA, T2D, Control
Y-axis: SI CCL2 (MCP-1)

LADA spontaneous secretion

pg/ml CXCL10 (IP10) vs BMI

r^2 0.35 ($p=0.0035$)[1]

LADA spontaneous secretion

pg/ml CXCL10 (IP10) vs C-Peptide

r^2 0.30 ($p=0.0088$)

Control PI stimulated secretion

SI CCL5 (RANTES) vs HbA1c

r^2 0.47 ($p=0.0145$)[2]

(for HbA1c one value w as missing)

Control PI stimulated secretion

SI CCL5 (RANTES) vs C-Peptide

r^2 0.33 ($p=0.0409$)[2]