TLR2 and TLR4 expression on CD14⁺⁺ and CD14⁺ monocyte subtypes in adult-onset autoimmune diabetes

Pavlina Cejkova^{a,b}, Iva Nemeckova^a, Jan Broz^c, Marie Cerna^b

Background. Peripheral blood monocytes are key effectors of innate immunity. Dysfunction, changes in their counts or altered expression of cytokines and pattern-recognition receptors on monocytes may contribute to the development of the autoimmune type of diabetes mellitus (AD).

Aims. We aimed to analyze the counts and proportions of the two main subtypes of monocyte cells, CD14⁺⁺ and CD14⁺, and to look for potential changes in the expression of toll-like receptors 2 (TLR2) and 4 (TLR4) as well as cytokine prolactin (PRL) in adult-onset AD, including diabetes mellitus type 1 (T1DM) and latent autoimmune diabetes in adults (LADA).

Methods. We examined 21 T1DM patients, 9 patients with LADA, 16 control patients with type 2 diabetes mellitus (T2DM) and 24 healthy individuals. All diabetic patients were diagnosed after the age of 18 years. Expression at the mRNA level was determined by quantitative PCR. Flow cytometry was used to ascertain membrane expression and cell counts.

Results. T1DM patients had fewer CD14⁺⁺ ($P < 0.01$) and CD14⁺ ($P < 0.0001$) monocytes whereas T2DM subjects showed decreased counts of CD14⁺ monocytes compared to healthy controls ($P < 0.001$). TLR2 protein expression was significantly increased in T1DM CD14⁺ monocytes compared to healthy controls ($P < 0.05$), while TLR4 expression in T1DM CD14⁺⁺ cells was significantly lower ($P < 0.0001$). There was no significant difference between the groups in terms of PRL mRNA expression in monocytes.

Conclusions. The observed changes in the proportions of both immune cell types and in the expression of functional pattern-recognition receptors on monocytes in the subjects examined may arise as a consequence of chronic inflammation that accompanies long-term diabetes.

Key words: adult-onset autoimmune diabetes, CD14 monocyte, inflammation, prolactin, toll-like receptor

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^aDepartment of Anthropology and Human Genetics, Faculty of Science, Charles University in Prague, Czech Republic

^bDepartment of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague, Czech Republic

^c2nd Department of Internal Medicine, Diabetes Center, Third Faculty of Medicine, Charles University in Prague, Czech Republic

Corresponding author: Pavlina Dankova (Cejkova), e-mail: Pavlina.Dankova@natur.cuni.cz

INTRODUCTION

It has long been recognized that innate immunity and monocytes play a crucial role in the pathogenesis of autoimmune disorders including type 1 diabetes mellitus (T1DM). Monocytes are multifunctional cells with roles in homeostasis, immune defence and tissue repair; they are also known to express a very diverse transcriptome¹. At least two types of monocytes can be distinguished based on the level of CD14 expression^{2,3}: (1) classical monocytes, which are strongly positive for the CD14 cell surface molecule (CD14⁺⁺ CD16⁻ monocytes), and (2) more mature, non-classical monocytes, which co-express the CD16 molecule (CD14⁺ CD16⁺ monocytes). The latter share certain phenotypic characteristics with classical monocytes but resemble tissue macrophages by expressing many macrophage-like surface antigens.

Variation in the proportions of individual monocyte subsets has been observed in several pathological states such as rheumatoid arthritis⁴ or type 1 diabetes with juvenile-onset⁵, although some studies focused on long-term juvenile-onset autoimmune diabetes⁶ or chronic inflamma-

tory conditions (e.g. systemic juvenile idiopathic arthritis) (ref.⁷), have not confirmed any differences in the absolute numbers or proportions of monocyte subpopulations. Still, a wealth of gene and protein expression data points to increased monocytic activity in type 1 diabetic patients compared to healthy controls^{5,8-10}.

Monocytes exert their crucial role in immune defence through toll-like receptors (TLRs), a type of pattern recognition receptors. Activation of TLRs results in the secretion of anti-bacterial peptides and cytokines. These initiate an inflammatory response that further leads to the recruitment of cells responsible for adaptive immunity. Activation of TLRs has been noticed in a number of inflammatory diseases¹¹⁻¹³ (also reviewed in ref.¹⁴). It has been suggested, and supported by studies on animal models, that TLRs can cause autoimmune imbalances, including T1DM (ref.¹⁴⁻¹⁶). TLRs also seem to participate in the pathogenesis of type 2 diabetes mellitus (T2DM) and the metabolic syndrome^{17,18}. The TLR up-regulation observed in T1DM patients is hypothesized to be induced by hyperglycaemia¹⁹.

Upon stimuli, monocytes are capable of producing

many cytokines and chemokines, including prolactin (PRL), a stress and immunostimulatory molecule²⁰. Impaired PRL production leads to insufficient activation of the immune response to infection in sepsis, which can eventually cause death^{21,22}. On the other hand, serum hyperprolactinaemia has been detected in several autoimmune diseases and is believed to contribute to the pathogenesis of these illnesses^{23,24}.

The aim of this study was to identify differences concerning the innate component of immunity that may reflect immune and/or inflammatory processes taking place in adult-onset autoimmune diabetes (AD), including T1DM and latent autoimmune diabetes in adults (LADA). All diabetic patients included in the study were diagnosed after the age of 18 years. T2DM patients and healthy individuals served as controls. We analyzed the proportions and absolute numbers of monocytes, measured monocytic TLRs, and verified the potential relationship between the molecular markers studied and clinical markers of inflammation. Our ultimate objective was to describe the role of monocytic PRL in the immune process leading to adult-onset autoimmune diabetes.

MATERIAL AND METHODS

Subjects

Seventy subjects were enrolled in total. Forty-six were diabetic patients of the 2nd Department of Internal Medicine at the Faculty Hospital Kralovske Vinohrady in Prague diagnosed after the age of 18 years: 21 T1DM (mean age 43, SD 18.78; 7 female), 9 LADA (mean age 61, SD 13.68; 2 female), and 16 T2DM (mean age 65.25, SD 14.52; 7 female) as diabetic controls. The diagnosis of T1DM was made in accordance with the American Diabetes Association Criteria²⁵. The diagnosis of LADA required that the condition did not necessitate insulin administration for 6 months after the initial diagnosis. The healthy control group, CO (mean age 24.5, SD 3.659), comprised 24 healthy volunteers (21 female). EDTA-blood samples were collected, and the medical history of each subject was noted upon his or her informed consent. In case that no difference between T1DM and LADA patients could be seen during data evaluation, the two groups were merged into a single autoimmune diabetes group (AD). Due to limited cell counts, all samples were tested for expression at the mRNA level; some of the samples, however, could not be tested for other parameters such as monocyte subtypes and TLR protein expression (applies especially to LADA group), which represents certain limitation of the study. The study was approved by the ethical committee of the Faculty of Science, Charles University in Prague (Institutional Review Board, Faculty of Science, Charles University in Prague, Vinicna 7, Prague 2, Czech Republic).

Monocyte isolation and RNA extraction

Peripheral blood monocytes were separated using CD14 positive immunomagnetic beads (Dyna, Invitrogen,

Life Technologies Corporation, Carlsbad, CA, USA) following the recommended protocol. Separated monocytes were subjected to RNA isolation with the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), with minor modification. The total amount and purity of RNA were determined spectrophotometrically.

Quantitative real-time PCR

To test the expression of *PRL*, *TLR2* and *TLR4* genes in monocytes, an ABI 7000 SDS real-time PCR instrument (Applied Biosystems, Foster City, California, USA) was used with the following TaqMan-based Assay-on-Demand gene expression probes (Life Technologies Corporation, Carlsbad, CA, USA): Hs00168730_m1 (*PRL*), Hs00152932_m1 (*TLR2*), Hs00152939_m1 (*TLR4*). The human *PGK1* (phosphoglycerate kinase-1) gene served as an internal control. The 2^{-ΔCt} method was applied for relative quantification, and detected mRNA values were expressed in arbitrary units (AU). To present the acquired data in an uncluttered manner and with better resolution, values from the *PRL* assay were multiplied by 10⁶, and values from the *TLR2* and *TLR4* assays were multiplied by 10³.

Flow cytometry (FCM)

Expression of CD14, TLR2 and TLR4 receptors on the cell surface was detected by flow cytometry. Peripheral blood mononuclear cells (PBMC) were isolated from the whole blood by Histopaque-1077 (Sigma-Aldrich, Prague, Czech Republic) gradient separation, washed with PBS (Gibco, Life Technologies Corporation, Carlsbad, CA, USA), fixed with 3.8% formaldehyde and incubated in the dark at 4°C for 30 min with the following monoclonal antibodies: mouse monoclonal to CD14 stained with the fluorescein isothiocyanate (FITC) (Exbio, Prague, Czech Republic), mouse monoclonal to TLR2 stained with allophycocyanin (APC) and mouse monoclonal to TLR4 also stained with APC (both eBioscience, USA). Typically, 30000 events per sample were acquired from the whole PBMC population. Target markers were detected on a FACSCalibur flow cytometer (BD Biosciences, USA). The results were analysed using CellQuestPro software (BD Biosciences, USA). The region containing monocytes was delimited based on their characteristic size and internal complexity in light scatters (forward scatter (FSc)/side scatter (SSc) parameters, "monocyte gate"), followed by gating on double positivity for CD14 and TLR2/4. Monocytes strongly positive for CD14 (CD14⁺⁺) and monocytes weakly positive for CD14 (CD14⁺) were distinguished. Absolute monocyte counts were obtained from the whole PBMC population. Expression of the markers under study was evaluated as both the number of positive cells (presence or absence of selected marker) and mean fluorescence intensity (MFI, level of expression of the given marker on the surface of each detected cell).

Statistical analysis

Data were statistically evaluated using Epi Info (Version 3.3, October 2004, Atlanta, Georgia) and

GraphPad Prism software. The D'Agostino-Pearson normality test was used to check normality. Data are presented as medians with 25% and 75% percentiles, respectively. Data involving two groups were compared using the Mann-Whitney rank-sum test for unpaired values or the Wilcoxon test for paired observations. Data involving three or more groups were compared using the Kruskal-Wallis test or the Friedman test for repeated measures. Significance was accepted at $P < 0.05$.

RESULTS

Baseline characteristics of subjects under study

Clinical and biochemical characteristics of diabetic patients included in the study are detailed in Table 1. The age of patients differed at the time of testing. T1DM individuals were significantly younger than LADA ($P < 0.05$) and T2DM subjects ($P = 0.0001$). Moreover, healthy individuals had significantly lower age at time of testing when compared to all diseased groups ($P < 0.0001$). Gender distributions differed in healthy controls in comparison to diabetic patients ($P < 0.01$) but not among diabetic patients themselves. Therefore, significant findings observed in this study had to be adjusted in order to exclude

the effect of age and gender. There were no significant differences in age at disease manifestation, BMI, TSH, lipid profile (HDL, serum triglycerides and cholesterol) or markers of microalbuminuria (serum albumin, albumin in urea, serum creatinine, creatinine in urea, ALT and AST). C-peptide levels in the T1DM group were decreased compared to LADA (statistically significant) and T2DM patients (statistically insignificant). Markers of glucose metabolism (serum glucose and glycosylated haemoglobin) did not differ among the groups tested. Similarly, there were no detectable differences in CRP levels among the three diabetic groups.

Numbers of monocytes differ significantly between diabetic patients and healthy subjects

The levels of CD14 surface expression detected by fluorescence-based FCM allowed the distinction between monocytes characterized by a high presence of the CD14 marker on the cell surface (CD14⁺⁺) and cells with decreased amounts of membrane-bound CD14 (designated CD14⁺). Although counts of monocytes defined by physical parameters, such as size and cellular complexity (monocyte gate), did not significantly differ among the groups under study (data not shown), fluorescence-based FCM revealed changes in the spectrum of monocytes clas-

Table 1. Clinical and biochemical characteristics of patients.

	T1DM	LADA	T2DM
n (F; %)	21 ^a (7; 33%)	9 ^a (2; 22%)	16 ^a (7; 44%)
Age at testing (years)	43 (18.78) *,#	61 (13.67)	65.25 (14.52)
Age at onset (years)	45.25 (19.74)	51.5 (6.9)	61
Duration of diabetes < 1 year	4	5	NA
Duration of diabetes < 5 years	0	1	NA
Duration of diabetes > 5 years	15	2	NA
BMI	25.48 (21.38 - 27.03)	26.21 (24.09 - 30.06)	28.57 (24.86 - 32.75)
CP (pmol/l)	37 (18.5 - 111) §	229 (124 - 460)	260 (24 - 507)
S-Glucose (mmol/L)	10.7 (7.29 - 13.4)	9.06 (7.32 - 12.1)	10.2 (7.38 - 17.7)
HbA1C (mmol/mol)	9.1 (7.65 - 11.2)	7.6 (5.3 - 12.1)	10.5 (8.4 - 12.1)
S-Albumin (g/L)	37.5 (34.4 - 39.33)	39.25 (38 - 40.5)	36.05 (31.5 - 40.48)
U-Albumin (mg/L)	7 (3 - 59)	10.3 (1.5 - 1833)	37.2 (12 - 141)
S-Creatinine (umol/L)	70.5 (59 - 83.75)	80.5 (67.5 - 100.8)	73 (58 - 85)
U-Creatinine (mmol/L)	6.6 (5.4 - 9.8)	5.25 (4.525 - 11.68)	9.65 (5.825 - 13.33)
ALT (ukat/L)	0.48 (0.39 - 0.69)	0.345 (0.2825 - 0.5875)	0.51 (0.335 - 1)
AST (ukat/L)	0.43 (0.37 - 0.6)	0.315 (0.24 - 0.45)	0.44 (0.315 - 0.865)
HDL (mmol/L)	1.205 (1.015 - 1.565)	1.17 (0.795 - 1.478)	1.02 (0.77 - 1.545)
S-triglycerides (mmol/L)	1.54 (1.035 - 2.66)	1.525 (0.7325 - 2.618)	1.38 (0.99 - 2.24)
S-cholesterol (mmol/L)	4.93 (4.233 - 5.423)	5.245 (3.983 - 6.238)	3.97 (2.735 - 5.26)
TSH (mIU/L)	1.45 (0.6925 - 2.69)	1.52 (0.69 - 5.82)	1.605 (0.4375 - 12.65)
CRP (mg/L)	2.7 (1.425 - 4.725)	3.505 (1.588 - 50.48)	6.5 (3.4 - 31.05)

Values are means (SD) or medians (interquartile range), unless indicated otherwise. Values without standard deviation indicate only one subject with a known value within the group. T1DM, type 1 diabetes mellitus; LADA, latent autoimmune diabetes in adults; T2DM, type 2 diabetes mellitus; F, female; NA, not available; BMI, body mass index; CP, C-peptide; HbA1C, glycosylated hemoglobin; ALT, alanine amino transferase; AST, aspartate amino transferase; HDL, high density lipoprotein; TSH, thyroid stimulating hormone; CRP, C-reactive protein; S-, serum levels; U-, urea levels.

^a Due to limited cell numbers, not all samples were tested for all molecular parameters.

* $P < 0.05$ (compared to LADA; one-way ANOVA)

$P = 0.0001$ (compared to T2DM; one-way ANOVA)

§ $P < 0.05$ (compared to LADA; Mann Whitney Test)

sified according to the level of CD14 expression. T1DM patients had 1.7 times less and AD subjects 1.5 times less CD14⁺⁺ monocytes than healthy controls (T1DM: median count 1828, 1124–2595, $P < 0.01$; AD: median 2084, 1267–2718, $P < 0.01$; CO: median 3131, 2343–4047). Similarly, the T2DM group of patients showed a trend towards decreased counts of CD14⁺⁺ monocytes when compared to the healthy control group (T2DM: median 1965, 588–2839; $P = \text{NS}$; Fig. 1a). Interestingly, a similar but even more profound pattern could be observed in CD14⁺ monocytes. There was a significant threefold decrease in CD14⁺ counts in T1DM patients compared to healthy controls (T1DM: median 300, 232.5–505.3; CO: median 893, 570–1259; $P < 0.0001$). There were also 3.2 times less CD14⁺ monocytes in AD patients (median 281, 234–446, $P < 0.0001$) and 3.4 times less CD14⁺ monocytes in T2DM patients (median 259, 175–349, $P < 0.001$) compared to healthy subjects, and a trend towards a drop-off in CD14⁺ cells in LADA subjects (median 257, 242–332; $P = \text{NS}$; Fig. 1b). Between the AD group and the T2DM control group, there were no differences in absolute numbers or percentages of either monocyte subtype.

TLR2 protein expression on CD14⁺⁺ monocytes and CD14⁺ monocytes

Membrane expression of TLR2 proteins on monocytes defined by physical parameters was the same among T1DM, LADA, T2DM control and healthy control subjects. However, when the cells were classified based on their CD14 antigen expression, CD14⁺ cells exhibited a significant increase in the level of the TLR2 protein within the AD group (AD: MFI 117.6, 83.98–142.3; CO: MFI 83.08, 59.63–98.23; $P < 0.05$, increase 1.4 times) and a trend towards higher TLR2 cell surface expression in the LADA group when compared to healthy controls (MFI 119.8, 117.6–140.7; $P = \text{NS}$), but not in T1DM or T2DM patients (Fig. 2a). There was no difference in

TLR2 protein expression on CD14⁺⁺ monocytes among all the groups.

TLR4 protein expression on CD14⁺⁺ monocytes but not CD14⁺ monocytes is down-regulated in patients with autoimmune diabetes

The FCM analyses revealed a significant decrease of the TLR4 protein level in the groups with AD when compared to healthy controls, but not when compared to T2DM controls. Membrane expression of TLR4 proteins on monocytes defined by physical parameters was twice lower in T1DM patients than in healthy individuals (T1DM: MFI 39.71, 29.42–42.97; CO: MFI 80.80, 62.51–91.44; $P < 0.0001$). Likewise, TLR4 surface expression was decreased by half in the merged AD group (MFI 39.81, 31.18–45.12) when compared to healthy controls ($P < 0.0001$). Similarly, CD14⁺⁺ monocytes from T1DM patients had half the TLR4 cell surface expression compared to CD14⁺⁺ cells from healthy subjects (T1DM: MFI 52.86, 46.09–75.60; CO: MFI 107, 83.29–131.1; $P < 0.0001$). TLR4 expression on membranes of CD14⁺⁺ monocytes of all AD patients (MFI 55.3, 46.37–70.38) was almost twice lower (1.93 $\times$) than that of healthy controls ($P < 0.0001$; Fig. 2b). In contrast to TLR2, there were no apparent changes in TLR4 levels on the cell membrane of CD14⁺ monocytes.

Comparison of TLR2 and TLR4 protein expression between CD14⁺⁺ and CD14⁺ monocytes

In healthy subjects, CD14⁺⁺ monocytes expressed 1.7 times more TLR2 proteins on their surface ($P < 0.0001$) and even have 2.3 times up-regulated amounts of TLR4 ($P < 0.0001$) than CD14⁺ cells. By contrast, no differences in TLR2 and TLR4 membrane antigen levels on CD14⁺⁺ compared with CD14⁺ cells were evident in diabetic patients (Fig. 3a, 3b).

Fig. 1. CD14⁺⁺ (a) and CD14⁺ (b) monocyte counts in diabetic patients and healthy controls.

Absolute counts of CD14⁺⁺ (a) and CD14⁺ cells (b) were obtained from 30000 events representing the whole PBMC population. The horizontal bars correspond to group medians. T1DM, type 1 diabetes mellitus (N = 18); LADA, latent autoimmune diabetes in adults (N = 3); AD, pooled autoimmune diabetes (N = 21); T2DM, type 2 diabetes mellitus (N = 8); CO, healthy control group (N = 24); PBMC, peripheral blood mononuclear cells.

Fig. 2. Expression of TLR2 on CD14⁺ (a) and TLR4 on CD14⁺⁺ (b) monocyte surface.

TLR2 protein expression (a) is significantly increased on CD14⁺ monocytes from AD patients when compared to healthy controls; TLR4 antigen expression (b) is significantly lower on the CD14⁺⁺ monocyte surface from T1DM and AD patients in comparison to healthy individuals. Horizontal bars represent group medians. T1DM, type 1 diabetes mellitus (N = 18); LADA, latent autoimmune diabetes in adults (N = 3); AD, pooled autoimmune diabetes (N = 21); T2DM, type 2 diabetes mellitus (N = 8); CO, healthy control group (N = 24); MFI, mean fluorescent intensity.

Fig. 3. Comparison of TLR2 (a) and TLR4 (b) antigen expression between CD14⁺⁺ and CD14⁺ monocytes.

CD14⁺⁺ monocytes from healthy subjects, but not from the diseased groups, show significantly higher TLR2 (a) and TLR4 (b) expression than CD14⁺ monocytes. Horizontal bars represent group medians. T1DM, type 1 diabetes mellitus (N = 18); LADA, latent autoimmune diabetes in adults (N = 3); AD, pooled autoimmune diabetes (N = 21); T2DM, type 2 diabetes mellitus (N = 8); CO, healthy control group (N = 24); MFI, mean fluorescent intensity

TLR2 and TLR4 mRNA expression in peripheral blood monocytes

To determine whether the changes in monocyte TLR2 and TLR4 surface expression in the groups under study resulted from altered mRNA expression, we investigated TLR2 and TLR4 mRNA levels in cells isolated from peripheral blood by CD14-positive immunomagnetic separation. Consistently with trends observed at the protein level in CD14⁺ cells from the AD group, we found a tendency towards a twofold increase in TLR2 mRNA levels in all diabetic groups compared to the control group (T1DM: median 460.1 AU, 178.1–648.7; LADA: median 395.1 AU, 81.3–768.2; T2DM: median 504.7 AU, 176.9–657.9; CO: median 193.9 AU, 177.2–246; $P = \text{NS}$; raw data multiplied by 10^3).

In the case of TLR4, significant reduction in protein expression observed on the cell surface of all monocytes and CD14⁺⁺ cells from the T1DM and AD groups in comparison to the healthy control group was not detected at the mRNA level (T1DM: median 130.3 AU, 70.93–212; LADA: median 160.4 AU, 70.54–216.6; T2DM: median 141.2 AU, 61.74–188.3; CO: median 163.8 AU, 135.9–202.5; $P = \text{NS}$).

PRL mRNA expression in peripheral blood monocytes

Though physiological expression of extrapituitary prolactin is generally very low (CO median 181.9 AU, 118.2–256.6; raw data were multiplied by 10^6), we could observe a trend towards decreased PRL mRNA levels in both T1DM and T2DM subjects (T1DM: median 117.3

AU, 50.86–208.3; T2DM: median 133.1 AU, 82.23–188; $P = \text{NS}$). On the contrary, patients diagnosed with LADA showed no changes in PRL mRNA expression compared to healthy individuals (LADA: median 192.3 AU, 45.36–267.4; $P = \text{NS}$).

Relationship between TLR expression and biochemical parameters

Several relationships between biochemical parameters and molecular data have been observed. In all diabetic patients, there was a significant negative correlation between TLR2 surface expression on total monocytes (defined by FSc and SSc) and serum glucose ($r = -0.4803$, $P < 0.01$), and similarly, a significant negative correlation between TLR4 protein expression on total monocytes and serum glucose ($r = -0.5617$, $P < 0.01$). Nevertheless, neither of these relationships was apparent within each single monocyte subtype. Further, absolute numbers of CD14⁺ monocytes expressing TLR2 correlated significantly with HDL levels ($r = 0.529$, $P < 0.05$) and negatively with serum triglycerides ($r = -0.533$, $P < 0.05$).

DISCUSSION

Much of the present understanding about the biology of monocytes in T1DM stems from studies performed on diabetes with childhood onset. It is therefore difficult to compare and correctly evaluate all available data together and thus paint a complete picture of the immune processes taking place in the background of this disease. Various regulatory mechanisms are known to be involved, and diverse genetic risk factors affect children and adult patients^{26,27}.

The distinct roles of particular monocyte subtypes in the pathogenesis of T1DM are indisputable. Our study revealed a slight but significant decrease in absolute numbers of CD14⁺⁺ monocytes and a more profound, threefold reduction in CD14⁺ monocytes in both T1DM and T2DM patients diagnosed in adulthood. The drop in both subsets of monocytes may suggest deficient activation of the immune system in diabetic patients. This effect is independent of age and gender and may be related to changes of the immune milieu in long-term diabetes, as 63% of our patients had suffered from autoimmune diabetes for more than 5 years. Immune processes that accompany initial stages of the disease at the time of its first manifestation are known to differ from those observed after several months or years of progression¹⁶. Consistently with this observation and our data, monitoring of percentages and absolute numbers of monocytes in new-onset T1DM in comparison to long-term diabetic patients diagnosed in childhood revealed increased monocyte counts in newly diagnosed patients, but unchanged percentages and even lower monocyte counts in long-term patients²⁸.

As regards diminished counts of monocytes in long-term diabetic patients diagnosed in adulthood, there is substantial evidence that chronic inflammation can induce activation of the immune system and eventually cause exhaustion of some of its cell components, the

ability to respond to stimulation or both²⁹. In T1DM, autoimmune destruction of pancreatic β cells, which manifests as an inflammatory process, may persist for months or even years before being diagnosed. Though T2DM is considered a metabolic disorder, chronic low-grade inflammation (so-called metaflammation, an inflammatory response to metabolic stress, excessive nutrient intake and obesity), has been clearly demonstrated (reviewed in ref.³⁰). Median BMI in all three patient groups indicates excess weight and the 75th percentile indicates obesity in the LADA and T2DM groups. It thus seems that inflammatory processes in diabetes may influence peripheral blood leukocyte counts negatively.

Changes and defects in the expression and function of toll-like receptors might contribute to altered recruitment of antigen-presenting cells, resulting either in suboptimal adaptive immune responses leading to increased frequency of illness related to infection or in excessive adaptive immune reactions causing autoimmune disorders or allergies. Higher levels of TLR2 and lower levels of TLR4 in *ex vivo* conditions encountered in this study indicate that monocyte phenotypes in diabetes are not equivalent to monocytes in healthy individuals. Though we did not observe any significant decline in TLR4 mRNA expression in any of the diseased groups compared to the healthy control group, diabetic patients had significantly lower levels of TLR4 membrane antigen on their monocytes, especially CD14⁺⁺ monocytes. The effect of aging on the immune system functioning is widely accepted; study on mice has reported higher age as a cause of depletion of murine TLRs on macrophages³¹. We therefore aimed to determine whether the down-regulation of TLR4 expression on CD14⁺⁺ monocytes from T1DM patients is attributable to their higher age compared to the age of healthy individuals included in the study. After the age adjustment, we observed the same drop (twice in monocytes characterized both by light scatter parameters and CD14 expression) as before the adjustment. We thus confirm that the reduction of TLR4 expression on monocytes observed in our study, especially on CD14⁺⁺ monocytes, is age-independent and can therefore be ascribed to type 1 diabetes with late onset. Alternatively, it may be part of some metabolic regulatory mechanism, as recently suggested by Pierre et al.³². In that study, TLR4^{-/-} mice turned out to be protected from gaining body weight and developing glucose intolerance induced by a high-fat diet (HFD), and the reduced fat storage in TLR4^{-/-} mice was explained by the absence of ER stress after HFD feeding³².

Intriguingly, a situation different from TLR4 can be seen in TLR2 expression. Our study shows mild but significant up-regulation of TLR2 production by CD14⁺ monocytes from patients with AD. A trend towards increased amounts of TLR2 in monocytes from T2DM patients is also observable. These findings are also supported by mRNA data, which revealed an increased, albeit non-significantly, TLR2 mRNA level in total monocytes from diabetic patients. In contrast to CD14⁺ monocytes, CD14⁺⁺ cells did not exhibit any changes regarding expression of the TLR2 molecule on the cell surface. It has been suggested that substantially higher TLR2 and TLR4

expression in monocytes from diabetic subjects reflects poor glycaemic control^{17,19,33,34}. Although all participating diabetic groups showed markers of hyperglycaemia, we were unable to confirm any relationship between the expression of TLR and HbA1c. Instead, the surface expression of both TLR2 and TLR4 on total monocytes was negatively correlated with serum glucose levels. This finding corroborates results obtained by Stechova et al. (ref.³⁵) regarding the influence of maternal hyperglycaemia on cord blood mononuclear cells, which showed that glucose may have a decreasing effect on the inflammatory reaction of the organism.

Because there is a known connection between lipopolysaccharides and serum lipoproteins such as HDL (ref.³⁶), and since the ability of serum lipids to attenuate proinflammatory activity of bacterial lipoproteins through binding to toll-like receptors has already been confirmed³⁷, we evaluated the potential effect of HDL, triglycerides and cholesterol on the spectrum and numbers of monocytes, and their TLR expression in diabetic subjects. We nevertheless could not see any relationship and found no correlation with levels of TLR2 or TLR4 expression on either monocyte subtype. Moreover, absolute numbers of CD14⁺ monocytes expressing TLR2 correlated significantly with HDL levels and negatively with serum triglycerides. One might speculate that, in diabetic patients, elevated serum levels of HDL accompanied by increased numbers of CD14⁺/TLR2⁺ cells might protect against bacterial infections and lipoprotein toxicity, as previously demonstrated in mice and rabbits^{38,39}. Elevated HDL levels and increased CD14⁺/TLR2⁺ cell counts might play a part in compensatory mechanisms activated in response to decreased CD14⁺⁺ monocyte counts and TLR4 expression.

In other words, CD14⁺⁺ monocytes of healthy subjects examined in our study express 1.7 times more TLR2 receptors on their surface and even have 2.3 times up-regulated TLR4 antigen levels than CD14⁺ cells, which is apparently a physiological reflection of different levels of maturity associated with different functions. By contrast, no significant differences in the expression of TLR2 and TLR4 proteins on CD14⁺⁺ compared to CD14⁺ cells could be seen in diabetic subjects. The minimization of differences in TLR4 membrane expression seems to be mainly caused by decreased TLR4 expression on CD14⁺⁺ cells in both T1DM and, to a lesser extent, T2DM patients, perhaps pointing to dysregulation of innate immunity in both types of diabetes, which might be caused by hyperglycaemia, fat storage or other metabolic factors³².

Our findings show that adult-onset autoimmune diabetes is accompanied by changes in absolute numbers and proportions of immune cells as well as altered expression of functional innate receptors on monocytes and that these changes differ from those reported in juvenile AD. On the other hand, overstimulation of the immune system by chronic low-grade inflammation in long-term diabetic patients does not seem to have a significant impact on *PRL* gene transcription activity. We nevertheless cannot rule out the possibility that extrapituitary prolactin regu-

lated at the protein level plays a role in the pathogenesis or progression of AD.

ABBREVIATIONS

AD, Autoimmune diabetes; ALT, Alanine amino transferase; APC, Allophycocyanine; AST, Aspartate amino transferase; AU, Arbitrary unit; BMI, Body mass index; CD, Ccluster of differentiation; CO, Healthy controls; CP, C-peptide; CRP, C-reactive protein; EDTA, Ethylenediaminetetraacetic acid; ER, Endoplasmic reticulum; F, Female; FITC, Fluorescein isothiocyanate; FSc, Forward scatter; HbA1C, Glycosylated hemoglobin; HDL, High density lipoprotein; HFD, High fat diet; LADA, Latent autoimmune diabetes in adults; MFI, Mean fluorescence intensity; mRNA, Messenger ribonucleic acid; NA, Not available; PBMC, Peripheral blood mononuclear cell; PGK1, Phosphoglycerate kinase-1; PRL, Prolactin; S-, Serum levels; SD, Standard deviation; SSc, Side scatter; T1DM, Type 1 diabetes mellitus; T2DM; Type 2 diabetes mellitus; TLR2(4), Toll-like receptor 2 (4); TSH, Thyroid stimulating hormone; U-, Urea levels.

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REFERENCES

1. Wells CA, Chalk AM, Forrest A, Taylor D, Waddell N, Schroder K, Himes SR, Faulkner G, Lo S, Kasukawa T, Kawaji H, Kai C, Kawai J, Katayama S, Carninci P, Hayashizaki Y, Hume DA, Grimmond SM. Alternate transcription of the Toll-like receptor signaling cascade. *Genome Biology* 2006;7(2).
2. Ziegler-Heitbrock L. The CD14+CD16+ blood monocytes: their role in infection and inflammation. *Journal of Leukocyte Biology* 2007;81:584-92.
3. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJM, Liu YJ, MacPherson G, Randolph GJ, Scherberich J, Schmitz J, Shortman K, Sozzani S, Strobl H, Zembala M, Austyn JM, Lutz MB. Nomenclature of monocytes and dendritic cells in blood. *Blood* 2010;116:E74-E80.
4. Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14brightCD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis and Rheumatism* 2012;64:671-7.
5. Mysliwska J, Smardzewski M, Marek-Trzonkowska N, Mysliwiec M, Raczynska K. Expansion of CD14(+)CD16(+) monocytes producing TNF-alpha in complication-free diabetes type 1 juvenile onset patients. *Cytokine* 2012;60:309-17.
6. Krankel N, Armstrong SP, McArdle CA, Dayan C, Madeddu P. Distinct Kinin-Induced Functions Are Altered in Circulating Cells of Young Type 1 Diabetic Patients. *Plos One* 2010;5(6).

7. Macaubas C, Nguyen KD, Peck A, Buckingham J, Deshpande C, Wong E, Alexander HC, Chang SY, Begovich A, Sun Y, Park JL, Pan KH, Lin R, Lih CJ, Augustine EM, Phillips C, Hadjinicolaou AV, Lee T, Mellins ED. Alternative activation in systemic juvenile idiopathic arthritis monocytes. *Clinical Immunology* 2012;142:362-72.
8. Devaraj S, Dasu MR, Rockwood J, Winter W, Griffen SC, Jialal I. Increased toll-like receptor (TLR) 2 and TLR4 expression in monocytes from patients with type 1 diabetes: further evidence of a pro-inflammatory state. *J Clin Endocrinol Metab* 2008;93:578-83.
9. Devaraj S, Glaser N, Griffen S, Wang-Polagruto J, Miguelino E, Jialal I. Increased monocytic activity and biomarkers of inflammation in patients with type 1 diabetes. *Diabetes* 2006;55:774-9.
10. Devaraj S, Cheung AT, Jialal I, Griffen SC, Nguyen D, Glaser N, Aoki T. Evidence of increased inflammation and microcirculatory abnormalities in patients with type 1 diabetes and their role in microvascular complications. *Diabetes* 2007;56:2790-6.
11. Hausmann M, Kiessling S, Mestermann S, Webb G, Spottl T, Andus T, Scholmerich J, Herfarth H, Ray K, Falk W, Rogler G. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* 2002;122:1987-2000.
12. Shao L, Zhang P, Zhang Y, Lu Q, Ma A. TLR3 and TLR4 as potential clinically biomarkers of cardiovascular risk in coronary artery disease (CAD) patients. *Heart Vessels* 2014;29(5):690-8.
13. Mudaliar H, Pollock C, Komala MG, Chadban S, Wu H, Panchapakesan U. The role of Toll-like receptor proteins (TLR) 2 and 4 in mediating inflammation in proximal tubules. *Am J Physiol Renal Physiol* 2013;305:F143-F154.
14. Kaizer EC, Glaser CL, Chaussabel D, Banchereau J, Pascual V, White PC. Gene expression in peripheral blood mononuclear cells from children with diabetes. *Journal of Clinical Endocrinology & Metabolism* 2007;92:3705-11.
15. Zipris D. Innate immunity and its role in type 1 diabetes. *Curr Opin Endocrinol Diabetes Obes* 2008;15:326-31.
16. Wang XJ, Jia S, Geoffrey R, Alemzadeh R, Ghosh S, Hessner MJ. Identification of a molecular signature in human type 1 diabetes mellitus using serum and functional genomics. *Journal of Immunology* 2008;180:1929-37.
17. Dasu MR, Devaraj S, Park S, Jialal I. Increased Toll-Like Receptor (TLR) Activation and TLR Ligands in Recently Diagnosed Type 2 Diabetic Subjects. *Diabetes Care* 2010;33:861-8.
18. Sherry CL, O'Connor JC, Kramer JM, Freund GG. Augmented lipopolysaccharide-induced TNF-alpha production by peritoneal macrophages in type 2 diabetic mice is dependent on elevated glucose and requires p38 MAPK. *Journal of Immunology* 2007;178:663-70.
19. Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I. High Glucose Induces Toll-Like Receptor Expression in Human Monocytes Mechanism of Activation. *Diabetes* 2008;57:3090-8.
20. Ben Jonathan N, Mershon JL, Allen DL, Steinmetz RW. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. *Endocr Rev* 1996;17:639-69.
21. Felmet KA, Hall MW, Clark RS, Jaffe R, Carcillo JA. Prolonged lymphopenia, lymphoid depletion, and hypoprolactinemia in children with nosocomial sepsis and multiple organ failure. *J Immunol* 2005;174:3765-72.
22. Cejkova P, Chroma V, Cerna M, Markova M, Marek J, Lacinova Z, Haluzik M. Monitoring of the Course of Sepsis in Hematooncological Patients by Extrapituitary Prolactin Expression in Peripheral Blood Monocytes. *Physiological Research* 2012;61:481-8.
23. Leanos-Miranda A, Cardenas-Mondragon G. Serum free prolactin concentrations in patients with systemic lupus erythematosus are associated with lupus activity. *Rheumatology (Oxford)* 2006;45:97-101.
24. Neidhart M, Gay RE, Gay S. Prolactin and prolactin-like polypeptides in rheumatoid arthritis. *Biomed Pharmacother* 1999;53:218-22.
25. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2008;31:S55-S60.
26. Cerna M, Kolostova K, Novota P, Romzova M, Cejkova P, Pinterova D, Pruhova S, Treslova L, Andel M. Autoimmune diabetes mellitus with adult onset and type 1 diabetes mellitus in children have different genetic predispositions. *Ann N Y Acad Sci* 2007;1110:140-50.
27. Sabbah E, Savola K, Ebeling T, Kulmala P, Vahasalo P, Ilonen J, Salmela PI, Knip M. Genetic, autoimmune, and clinical characteristics of childhood- and adult-onset type 1 diabetes. *Diabetes Care* 2000;23:1326-32.
28. Vuckovic S, Withers G, Harris M, Khalil D, Gardiner D, Flesch I, Tepes S, Greer R, Cowley D, Cotterill A, Hart DNJ. Decreased blood dendritic cell counts in type 1 diabetic children. *Clinical Immunology* 2007;123:281-8.
29. Sousa CRE, Yap G, Schulz O, Rogers N, Schito M, Aliberti J, Hieny S, Sher A. Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology. *Immunity* 1999;11:637-47.
30. Gregor MF, Hotamisligil GS. Inflammatory Mechanisms in Obesity. *Annual Review of Immunology*, Vol 29 2011;29:415-45.
31. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: Impaired toll-like receptor expression and function in aging. *Journal of Immunology* 2002;169:4697-701.
32. Pierre N, Deldicque L, Barbe C, Naslain D, Cani PD, Francaux M. Toll-Like Receptor 4 Knockout Mice Are Protected against Endoplasmic Reticulum Stress Induced by a High-Fat Diet. *Plos One* 2013;8(5).
33. Mohammad MK, Morran M, Slotterbeck B, Leaman DW, Sun YP, von Grafenstein H, Hong SC, McInerney MF. Dysregulated Toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse. *International Immunology* 2006;18:1101-13.
34. Ururahy MAG, Loureiro MB, Freire-Neto FP, de Souza KSC, Zuhl I, Brandao-Neto J, Hirata RDC, Doi SD, Arrais RF, Hirata MH, Almeida MD, de Rezende AA. Increased TLR2 expression in patients with type 1 diabetes: evidenced risk of microalbuminuria. *Pediatric Diabetes* 2012;13:147-54.
35. Stechova K, Spalova I, Durilova M, Bartaskova D, Cerny M, Cerna M, Pithova P, Chudoba D, Stavikova V, Ulmannova T, Faresjo M. Influence of Maternal Hyperglycaemia on Cord Blood Mononuclear Cells in Response to Diabetes-associated Autoantigens. *Scandinavian Journal of Immunology* 2009;70:149-58.
36. Wurfel MM, Kunitake ST, Lichenstein H, Kane JP, Wright SD. Lipopolysaccharide (Lps)-Binding Protein Is Carried on Lipoproteins and Acts As A Cofactor in the Neutralization of Lps. *Journal of Experimental Medicine* 1994;180:1025-35.
37. Bas S, James RW, Gabay C. Serum lipoproteins attenuate macrophage activation and Toll-Like Receptor stimulation by bacterial lipoproteins. *Bmc Immunology* 2010;11(46).
38. Levine DM, Parker TS, Donnelly TM, Walsh A, Rubin AL. In-Vivo Protection Against Endotoxin by Plasma High-Density-Lipoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 1993;90:12040-4.
39. Cue JI, Dipiro JT, Brunner LJ, Doran JE, Blankenship ME, Mansberger AR, Hawkins ML, Peck M. Reconstituted High-Density-Lipoprotein Inhibits Physiological and Tumor-Necrosis-Factor-Alpha Responses to Lipopolysaccharide in Rabbits. *Archives of Surgery* 1994;129:193-7.