Correlation between heat shock proteins, adiponectin, and T lymphocyte cytokine expression in type 2 diabetics

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Abstract

Type 2 diabetes mellitus (T2DM) features insulin resistance, hyperglycemia, dyslipidemia, overproduction of inflammatory cytokines, and systemic oxidative stress. Here, heat shock proteins Hsp70 and Hsp 90, adiponectin, and heme oxygenase-1 (HO-1, Hsp32) are profiled in peripheral blood mononuclear cells (PBMC) and serum from 25 T2DM patients and 25 healthy control subjects. Cells cultured with phorbol 12-myristate 13-acetate/ionomycin were evaluated by three-color flow cytometry for immunophenotypic biomarkers. Plasma HO-1, Hsp, and adiponectin levels were assayed by enzyme-linked immunosorbent assay (ELISA). Relative to healthy controls, T2DM patients exhibited significantly elevated plasma Hsp70, and representation of T helper immunophenotypes activated to express inflammatory cytokines, including CD4⁺ IFN- γ ⁺, CD4⁺ TNF- α ⁺, CD4⁺ IL-6⁺, CD4⁺ IL-1 β ⁺ T cells, significantly lower representation of CD4⁺ IL-10⁺ T cells, plasma adiponectin and cell-associated HO-1 expression—with no significant differences in plasma Hsp90 between T2DM and healthy controls. Plasma HO-1 and adiponectin in T2DM patients inversely correlated with TNF- α and showed inverse correlation between serum LDL and plasma HO-1. Moreover, TNF- α and Hsp90 in T2DM patients correlated positively with fasting blood glucose (FBG). These results demonstrate correlation between potentially pathogenic T cells, HO-1, and adiponectin, additionally revealing a T helper (Th)1-related character of T2DM immunopathogenesis, suggesting potential for novel T cell-related management strategies for T2DM and related co-morbidities.

Keywords Diabetes · Heat shock proteins · Inflammatory cytokines · Adiponectin · T lymphocytes · Regulatory T cells · Cytokines · Hemeoxygenase-1

Introduction

Inability to effectively metabolize glucose due to insulin resistance, which is the principal pathophysiological feature of type 2 diabetes mellitus (T2DM), results in hyperglycemia and systemically high levels of reactive oxygen metabolites (ROS). These physiologic effects promote DNA strand breaks, leading to increased activity of the nuclear enzyme poly[ADP-ribose] polymerase

(Chiu et al. 2008), in turn triggering multiple signaling cascades, resulting in further ROS production and dysregulated inflammation, with a diverse range of tissue damage (Wright et al. 2006). This process engenders inappropriately high production of proinflammatory cytokines, prominently IL-1 β , IL-6, and TNF- α , which combine to exacerbate T2DM progression through mechanisms that prominently include impaired insulin signaling, ablation of pancreatic beta islet cells (Cnop et al. 2005; Donath and Shoelson 2011), and further reduction in availability of insulin to meet normal metabolic requirements.

T cells are a major focus of the present study. T2DM is not considered to be a T cell-mediated syndrome; nevertheless, some evidence exists that the distinction between mechanisms of chronic inflammatory disease that are T cell-dependent, versus independent, are not clearly defined. For example, possibly protective cellular responses and inverse correlation between humoral and cellular beta cell autoimmunities were observed in persons with an intermediate diabetic condition,

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exhibiting features of both type 1 (autoimmune) and 2 diabetes (Mayer et al. 2007). Results of this 2007 study, suggesting an autoimmune component in T2DM, are augmented by a 2012 report that explores T cell contribution to inflammatory processes underlying T2DM (Brooks-Worrell and Palmer 2012). Other evidence suggesting a role for autoreactive T cells in T2DM is provided by studies of Kuwaiti women exposed to military toxicants as a result of the 1991 Persian Gulf War. These investigations revealed T lymphocyte activation patterns in gestational diabetes (which includes immunopathogenic features very similar to T2DM) consistent with T cell-dependent effects (Mahmoud et al. 2005, 2006). The potential contribution of T cell-regulated processes in T2DM is underscored in a 2017 report by investigators at Wuhan Polytechnic University in China and the Cardiovascular Research Institute, at Case Western Reserve University, in the USA. These investigators explore the role of T cell subpopulations in affecting adaptive immune processes associated with T2DM pathomechanisms—that offer promise for development of novel therapies for the disease (Xia et al. 2017).

Therapeutic exercise mimetics include stressors, such as hot water bath immersion, that induce expression of Hsp to levels that contribute to amelioration of T2DM without requirement for exogenous insulin or other drugs. The outcomes of these approaches have proven very encouraging (Hooper et al. 2014); however, a major question that remains to be addressed definitively, is the influence on disease severity of the cellular/tissue site at which Hsp are biologically active. Data shown in the present report, which will be discussed below, suggest that both iHsp and eHsp (including HO-1) correlate with occurrence of T2DM and levels of severity-related analytes; nevertheless, no mechanistic conclusions can be drawn on the basis of these observations. Nevertheless, the authors have previously demonstrated that levels of lymphocyte-associated heme oxygenase-1 (iHO-1), which is the form studied here, is a valid biomarker for monitoring of therapeutic effect on osteoarthritis by a topical HO-1 inducer in a phase 1 human clinical trial (Mahmoud et al. 2014).

The present study evaluates a hypothesis that T2DM patients exhibit correlations between representation of T cell populations activated to express inflammatory cytokines and plasma eHsp70, eHsp90, the inflammation-regulatory, anti-diabetic molecule adiponectin, and the regulators of diabetes-associated response to oxidative stress, including activity of leukocyte-associated iHO-1 (iHsp32) and adiponectin. Outcomes of this work demonstrate relationships between these parameters in peripheral blood of T2DM patients versus values measured in blood of healthy control subjects. This insight is expected to aid in the development of strategies for the prevention and management of T2DM, and potentially, a wide range of other chronic inflammatory diseases in which cellular energy utilization is affected.

Materials and methods

Study participants

The study included 25 T2DM patients: 13 males and 12 females (mean age 51 ± 2.1 years) in which diabetes was diagnosed for the first time with a fasting glucose test and a 75 g glucose tolerance test. Disease duration ranged between 6.1 ± 0.7 years. A control group of 25 age- and gender-matched healthy normoglycemic subjects were included (mean age 47.1 ± 1.7). All patients were being treated with regimens of glucose-lowering medication: 14 were prescribed metformin (Glucophage); 6 were receiving gliclazide (Diamicon), and 5 were being treated with glimepiride (Amaryl). All patients were controlled for hypertension. Twelve of the patients were diagnosed with coronary heart disease (CHD), and 18 manifested no co-morbidities. Patients excluded from the study included those receiving treatment with angioplastic intervention, immunomodulatory agents (e.g., steroids, azathioprine, or cyclosporine A), or diagnosed with acute respiratory or genitourinary infections or neoplastic disease. Demographic, anthropometric, and metabolic data, as well as information on diabetes complications, were extracted from the patients' medical records. Participation in the present investigation was voluntary, with informed consent, met Kuwait University's human subject protection criteria, and was additionally approved by the institution's Joint Committee for the Protection of Human Subjects in Research, with compliance responsibility assigned to the study's lead investigator, Dr. Fadia Mahmoud, Ph.D.

Phlebotomy and cell culture

A fasting blood sample was taken from each patient. Ten milliliters of peripheral venous blood was collected in heparinized vacutainer tubes for assessment of blood chemistry profile and adiponectin, and 10 ml of blood was collected in EDTA tubes for measurement of intracellular cytokines and HO-1. Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation was used to separate peripheral blood mononuclear cells (PBMC). The cells were washed and suspended in an RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) at a density of 1×10^6 cells/ml. Two hundred-microliter cultures of PBMC in 96-well plates were incubated under humidified conditions for 6 h at 37°C , along with 50 ng/ml of phorbol 12-myristate 13-acetate, 1 ng/ml of ionomycin (PMA/I, Sigma, St. Louis MO, USA), and 2 mM monensin (Sigma), a glycoprotein export inhibitor which facilitates retention of inflammatory cytokines by PBMC and improved detection.

Blood chemistry profile assessment

Assessment of fasting blood glucose (FBG), glycated (or glycosylated) hemoglobin (HbA1c), cholesterol, HDL, LDL, and

triglycerides were performed using the standard blood analyte detection equipment COBAS INTEGRA 400/700/800 biochemical analysis system (Roche Diagnostics, Indianapolis, IN, USA).

Cell staining and flow cytometry

The expression of interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), interleukin-1 beta (IL-1 β), and interleukin-10 (IL-10), by CD3+ CD4+ lymphocytes for each cell culture condition was analyzed according to the Beckman Coulter protocol. Briefly, cells harvested from each culture were first incubated for 15 min at room temperature with fluorescein-isothiocyanate (FITC) anti-human CD3 and CD4 (Dakopatts, A/S, Glostrup, Denmark, and BD PharMingen, Heidelberg, Germany) diluted with Pharmingen staining buffer, then fixed and permeabilized using the Fix-and-Perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA). Intracellular labeling of permeabilized cells for inflammatory cytokines was accomplished by 30-min incubations at room temperature, with phycoerythrin (RD1)-conjugated monoclonal antibodies to human IFN- γ , TNF- α , IL-6, IL-1 β , and IL-10. Fluorophore-labeled mouse and rat anti-human monoclonal antibodies (Ab) were suspended in phosphate-buffered saline, 0.1 M sodium azide, 2 mg/ml bovine serum albumin (BSA). The desired concentration of antibody in each staining procedure was obtained by diluting the monoclonal suspension into 1 ml of Pharmingen staining buffer containing 1 million cells. Ratios of antibody to cells used in these procedures were mouse anti-human, FITC-labeled CD3 and CD4: 1.0 μ g/million cells; mouse anti-human RD1-labeled TNF- α : 1 μ g mAb/million cells; mouse anti-human RD1-labeled IFN- γ : 0.5 μ g mAb/million cells; rat anti-human RD1-labeled IL-6: 0.5 μ g mAb/million cells; mouse anti-human RD1-labeled IL-1 β : 0.5 μ g mAb/million cells; and rat anti-human RD1-labeled IL-10: 0.5 μ g mAb/million cells. Antibody-labeled cells were washed and evaluated by three-color flow cytometry for expression of each selected cytokine, using the FC-500 flow cytometer (Beckman Coulter Corporation, Hialeah, FL, USA). Isotypic controls for the antibody used to detect cytokine expression were established for each cell preparation. Lymphocyte subpopulations in each sample were gated by forward/side scatter analysis and further gating selected CD3+ and CD4+ cells which were analyzed for cytokine expression as described above.

Positive analysis regions for cells expressing selected target antigens were set against controls and specific binding of fluorophore-conjugated antibodies was analyzed according to standard methods recommended by the manufacturer. Lymphocyte subpopulations were identified by position on forward and side scatter plots and live-gated. Peripheral blood production potential of each cytokine was calculated as percentage of the mother population. Results are reported as

percentage of T helper (CD4+) cells activated to express selected cytokines within the CD4+ population $\pm$ standard error of the mean (SEM). The software that was used for flow cytometry is CXP cytometer version 2.1.

Enzyme-linked immunosorbent assay for cell lysates and plasma content of HO-1 and heat shock proteins

Measurements of lymphocyte expression of iHO-1, and of the plasma levels of eHsp70 and eHsp90, were made using the StressXpress™ Human HO-1 enzyme-linked immunosorbent assay (ELISA) Kit, Hsp70 high-sensitivity EIA Kit, and Hsp90 α (human) ELISA Kit (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA), respectively. Briefly, lysates were made from cells harvested from each culture according to the protocol provided in the StressX kit. Cell cultures were centrifuged to pellets, then this plus the adherent layers were washed with phosphate-buffered saline (PBS) and treated with the extraction reagent provided with each kit, followed by treatment with aprotinin protease inhibitor at 1 μ g/ml and brief sonication. The resulting lysates were next centrifuged and supernatants assayed for iHO-1 content by incubation in 96-well microtiter plates coated with an immobilized anti-human HO-1 antibody. HO-1 present in the samples is captured by the immobilized antibody and is detected with an HO-1 specific, rabbit polyclonal antibody. The polyclonal antibody is subsequently bound by a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody and the related reagents provided in the StressXpress kit. For measurement of eHsp70 and eHsp90, plasma obtained from the heparin tubes were diluted as instructed by the manufacturer. The samples and standards were added to the microtiter plate coated with anti-human Hsp70 or Hsp90 antibody. The secondary antibody was then added, followed by the other kit reagents. Cell-associated iHO-1 expression and plasma eHsp70 and eHsp90 were evaluated during the absorbance of the respective developed kit reagents at 450 nm in a Biotek ELX 808 Microplate Reader. Results are reported as mean $\pm$ SEM.

Plasma adiponectin measurement

Measurement of plasma adiponectin levels were made using Adiponectin Human ELISA Kit (AdipoGen™, Liestal, Switzerland). Plasma obtained from the heparin tubes were diluted as instructed by the manufacturer. The samples and standards were added to the microtiter plate coated with anti-human adiponectin antibody. The secondary antibody was then added followed by the other provided related kit reagents. Plasma adiponectin were evaluated during the absorbance of the respective developed kit reagents at 450 nm in a Biotek ELX 808 Microplate Reader. Results are reported as mean $\pm$ SEM.

Statistical analysis

Statistical evaluation of data outcomes was performed using student's *t* test to identify significant differences in magnitude of outcomes between diabetic and healthy subject groups. The Pearson product-moment coefficients were determined in order to evaluate the statistical significance of correlations between variables. A *P* value of < 0.05 was considered statistically significant. We used SPSS for Windows statistical package (Norusis/SPSS, Inc.) version 16.

Results

Participant biodata

Biodata for participating T2DM patients and healthy control subjects, along with diabetes-related peripheral blood serum parameters evaluated at entry to the study is shown in Table 1. No significant differences were observed in the age or in the average body mass index (BMI) between diabetes patients and healthy control subjects.

Cytokine-expressing CD3+ CD4+ T cell populations in blood of diabetic and healthy subjects

Flow cytometric data showing the effects of PMA/I stimulation on inflammatory cytokine expression by PBMC cultures from blood of a single diabetic subject of the present study is shown in Fig. 1. Permeabilized cells were stained with FITC-conjugated anti CD3+ and CD4+ (designated as a mother population) and secondarily labeled with RD1-conjugated antibodies to human IFN- γ , TNF- α , IL-6, IL- β , and IL-10. Expression of CD3+ CD4+ cells from diabetic and healthy subjects displayed as dot plot histograms in Fig. 1a–j. Percentage representation within the mother population of T-helper lymphocytes (CD3+ CD4+) activated to express each selected inflammatory cytokine is displayed in the upper right quadrant of the FACS data displays (dot plot histograms) in Fig. 1. Analysis of flow cytometric data obtained from assay of cultured, PMA/I-stimulated PBMCs from T2DM and healthy control subjects are shown in Fig. 2. Relative to healthy participants, PBMCs from blood of diabetics exhibited significantly elevated representation of CD3+ CD4+ T cells activated to express the cytokines IFN- γ ($P = 0.008$), TNF- α ($P = 0.005$), IL-6 ($P = 0.049$), IL-1 β ($P = 0.030$), but significantly lower representation of IL-10 ($P = 0.046$).

Serum chemistry analysis

Relative to healthy subjects, the blood of T2DM participants also contained significantly higher levels of HbA1c and

Table 1 Participant biodata and diabetes-related peripheral blood serum parameters. BMI measurements for each subject were made during clinical screening for participation in the study. Ten milliliter of heparinized whole venous blood, was analyzed for fasting blood glucose (FBG), glycated hemoglobin (HbA1c), total cholesterol, HDL, LDL, and triglycerides were obtained using the COBAS INTEGRA 400/700/800 biochemical analysis system. Measurement outcomes are shown as mean values of each parameter $\pm$ SEM

	T2DM patients (<i>n</i> = 25)	Healthy control subjects (<i>n</i> = 25)
Sex: M/F	12:6	11:9
Age in years	50.3 $\pm$ 1.4	47.1 $\pm$ 1.7
BMI, kg/m ²	27.64 $\pm$ 0.71	26.4 $\pm$ 3.2
FBG, mmol/l	8.05 $\pm$ 0.58	5.2 $\pm$ 0.35
HbA1c, %	8.81 $\pm$ 0.22*	5.4 $\pm$ 0.58
Cholesterol, mmol/L	4.72 $\pm$ 0.16	4.0 $\pm$ 0.38
HDL, mmol/L	0.93 $\pm$ 0.05*	1.56 $\pm$ 0.2
LDL, mmol/L	2.99 $\pm$ 0.23	2.2 $\pm$ 0.3
Triglycerides, mmol/L	2.40 $\pm$ 0.21*	1.2 $\pm$ 0.25

*Significance level of difference between diabetic and healthy control subjects is $P < 0.05$, or less

triglyceride levels with significantly lower HDL and significantly higher levels of serum triglycerides ($P < 0.05$) (Table 1).

Cell-associated HO-1 and serum adiponectin

Data shown in Table 2 demonstrates that relative to healthy control subjects, peripheral blood of T2DM patients contained significantly lower levels of cell-associated HO-1 ($P = 0.035$) and serum adiponectin ($P = 0.048$) and significantly higher levels of cell-associated Hsp70 ($P = 0.010$), but no differences noted in Hsp-90 content of PBMCs ($P = 0.168$).

Blood immunoparameter and metabolite correlations

Relationships of immunoparameters and physiological metabolites in peripheral blood of T2DM patients is shown in Fig. 3 and Table 3. Data in Table 3, shows significantly inverse correlations between %CD4+ TNF- α + T cells and both cell-associated HO-1 ($r = -0.434$, $P = 0.039$) and adiponectin ($r = -0.510$, $P = 0.013$) (Fig. 3a, b respectively). Results of Pearson analyses shown in Table 3, reveals that serum LDL exhibited an inverse correlation with plasma HO-1 ($r = -0.533$, $p = 0.013$) and a significant positive correlation with %CD4+ TNF- α + T cells ($r = 0.495$, $P = 0.016$) (Fig. 3c, d respectively). Analyses of data shown in Table 3, demonstrates a significantly positive correlation between plasma Hsp90 and serum glucose ($r = 0.518$, $P = 0.014$) (Fig. 3e).

Fig. 1 Profiles of cellular immunophenotypes in cultured human PBMC for diabetic and healthy (control) subjects. Flow cytometric data shows the distribution of immunophenotypes from a representative T2DM patient and a healthy subject. Cells were treated in culture with 50 ng/ml of phorbol 12-myristate 13-acetate, and 1 ng/ml of ionomycin for 6 h at 37 °C, 5% CO₂ for 6 h to enhance expression of inflammatory cytokines, stained with anti-CD3+ and anti-CD4+ fluorescein-isothiocyanate to label T lymphocytes, then permeabilized and stained additionally with phycoerythrin-labeled antibodies to IFN- γ (1A, 1F);

TNF- α (1B, 1G); IL-6 (1C, 1H); IL- β (1D, 1I); and IL-10 (1E, 1J). CD3+ CD4+ lymphocytes were gated as a mother population and analyzed to display PE fluorescence, indicating cells expressing a particular cytokine on the vertical axes of the resulting plots. Approximate representation of cells activated to express each selected cytokine within CD3+ CD4+ mother populations were calculated from events in the upper right quadrant of each histogram, relative to events in the other three quadrants and recorded as percentages

Discussion

The present investigation was conducted with a primary purpose of evaluating correlations between T2DM-associated T cell activation patterns and blood biomarker correlates of the disorder. It is anticipated that data presented here will provide clinicians and researchers with reference information that may aid in preliminary evaluation of patients at-risk or diagnosed with disorders of glucose metabolism. Previous evaluation of correlations between the physiologic effect of heat shock proteins on T lymphocyte activity have revealed that Hsp are capable of promoting T cell proliferation in ways that affect cardiac function through mechanisms involving modulation of inflammatory cytokine expression (Molitero et al. 1995). Additionally, findings by authors of the present report, suggest that HO-1, a cytoprotective Hsp, holds promise for modulation of T2DM-associated T cell activation in ways that may lead to improved strategies for prevention and management of the disease (Mahmoud et al. 2013). Moreover, pathogenic T cell activity in rheumatoid arthritis may be abated by Hsp interaction with these cells. (Strober and Holoshitz 1990). Outcomes of this study are thus anticipated to be useful for design of basic and clinical research in diabetes. It is important to emphasize that these results are most useful when considered in context with related data from other sources and do not

constitute independently definitive descriptions of the diabetic state. It is particularly important to acknowledge, that for reasons of ethical standards by which human clinical research is conducted at the participating institutions, the investigators did not segregate T2DM patients into groups administered a single therapy and/or designate an untreated T2DM cohort. As described in “[Materials and methods](#),” all participating diabetic patients were being treated with regimens of glucose-lowering medication—and it is here acknowledged that these treatments may have acted as confounders. This notwithstanding, the inclusion and exclusion criteria for subject recruitment were established to include medications that are known to have low incidence of side effects. Moreover, literature reviews conducted as part of the experimental design phase, identified commonly used drugs that appeared to have minimal effect on clinically relevant production of heat shock protein expression—particularly HO-1, Hsp70, and Hsp90. Authors of this report observed no trends in the data suggesting segregation of results according to drugs administered, gender, or any other characteristics of the subject groups.

In comparison to cells from healthy control subjects, PMI-stimulated cells within the CD3+ CD4+ compartment from peripheral blood of T2DM patients participating in the present study, exhibited significantly greater representation of CD4+ IFN- γ ($P < 0.01$), CD4+ TNF- α

* $P < 0.05$, ** $p < 0.01$ versus healthy control subjects

Fig. 2 Percentage representation of peripheral blood CD3+ CD4+ T cells activated to express inflammatory, Th1- and Th2-associated cytokines in T2DM patients, and healthy control subject. Percentage representation in peripheral blood of T2DM patients and healthy control subjects of CD3+ CD4+ T lymphocytes activated to express interferon-gamma (CD3+ CD4+ IFN- γ), tumor necrosis factor-alpha (CD3+ CD4+ TNF- α), interleukin-6 (CD3+ CD4+ IL-6), interleukin-1 beta (CD3+ CD4+ IL-

1 β), and interleukin-10 (CD3+ CD4+ IL-10). PBMC from study participants were cultured with 50 ng/ml PMA/I, 1 ng/ml ionomycin, and 2 mM monensin, stained using internal cell fluorophore labeling, then analyzed by two-color flow cytometry. Results are reported as percentage of T helper (CD3+ CD4+) cells activated to express selected cytokines within the CD3+ CD4+ population $\pm$ SEM

($P < 0.05$), CD4+ IL-6 ($P < 0.05$), and CD4+ IL-1 β ($P < 0.05$) T cells, with significantly lower representation of T cells activated to express the anti-inflammatory/immunosuppressive cytokine, IL-10 (CD4+ IL-10 ($P < 0.05$)) (Fig. 2). These outcomes are consistent with observations in previous studies that T cells activated to express inflammatory cytokines are present at elevated levels in persons afflicted with diseases featuring dysregulated inflammatory signaling (Mahmoud et al. 2013). Expanded T cell subpopulations primed to express IL-6 (CD4+ IL-6+) and IL-1 β (CD4+ IL-1 β) are also a feature of low-grade inflammation in T2DM (Al-Shukaili et al. 2013). The present study also showed greater stimulant (PM/I)-mediated increases in these two T cell subpopulations in cells

from T2DM subjects than in cultures derived from the blood of healthy controls (Fig. 2). The significant positive correlation between CD4+ TNF- α + T cells and plasma LDL cholesterol ($r = 0.495$, $P = 0.016$ (Table 3, Fig. 3d) underscore the expected pro-atherogenic effect of proinflammatory cytokines expressed by these T cell immunophenotypes. Elevated populations of CD4+ IFN- γ T cells in blood of T2DM patients relative to healthy control subjects, further suggested that T2DM manifests a Th1-associated immune orientation, since IFN- γ is a hallmark biomarker for Th1-related disease pathogenesis (Rook and Zumla 1997).

Outcomes shown here and in previous research by the authors, demonstrating expanded populations of T cells activated to express potentially pathogenic levels of inflammatory cytokines, raise a possibility that such differences in the lymphocyte subpopulation immunophenotype representation, potentially predispose the diabetic condition to development of autoimmunity. Some descriptions of T2DM demonstrate that these patients are at elevated risk for autoimmunity of the thyroid and endocrine tissue (Elebrashy et al. 2016; Sarfo-Kantanka et al. 2017). Results described in the present report show that whereas numbers of inflammation-promoting T cells are increased in T2DM patients, with population sizes that correlate with blood analytes relevant to the disease (Table 3, Fig. 2), no data is presented here to indicate the potential of these subpopulations for pathological autoreactivity. The authors have nevertheless adapted related studies of type 1 diabetes (IDDM), which incorporate background and methodology used in the present work, to develop heat shock protein-based countermeasures to autoimmunity,

Table 2 Heme oxygenase (HO-1), HSP70, HSP90, and adiponectin levels in peripheral blood of diabetes patients and healthy control subjects. ELISA assays were performed on peripheral blood plasma to assess HSP70, HSP90, and adiponectin and on PBMC culture lysates to measure cellular content of HO-1 protein. Levels of each analyte are shown as mean values of each parameter $\pm$ SEM

	T2DM patients (n = 25)	Healthy control subjects (n = 25)
iHO-1, ng/ml	6.38 $\pm$ 0.37*	9.75 $\pm$ 1.50
eHSP70, ng/ml	0.42 $\pm$ 0.01*	0.27 $\pm$ 0.05
eHSP90, ng/ml	34.3 $\pm$ 4.9	26.1 $\pm$ 3.4
Adiponectin, μ g/ml	6.78 $\pm$ 0.88*	10.59 $\pm$ 1.10

Significance level of difference between diabetic and healthy control subjects is $P < 0.05$, or less

Table 3 Pearson product moment correlations between physiological metabolites and immunoparameters in peripheral blood of T2DM patients

Correlations between plasma heme oxygenase (iHO-1), adiponectin and percentages of CD4+ TNF- α + T cells in peripheral blood of T2DM patients	
iHO-1, ng/ml	%CD4+ TNF- α + T cells $r = -0.434, P = 0.039$
Adiponectin, μ g/ml	$r = -0.510, P = 0.013$
Correlation between plasma heme oxygenase (iHO-1), percentages of CD4+ TNF- α + T cells and levels of LDL in PBMC from T2DM patients	
%CD4+ TNF- α + T cells	LDL, mmol/L $r = 0.495, P = 0.016$
iHO-1, ng/ml	$r = -0.533, p = 0.013$
Relationship between plasma Hsp90, and serum glucose level in PBMC from T2DM patients	
eHsp90, ng/ml	Glucose mmol/l $r = 0.518, P = 0.014$

Inverse or direct correlation between variables is considered significant at $P < 0.05$

that have proven highly effective in treating a wide range of pathologies characterized by dysregulated inflammation (Al Harbi and Haines 2007, McCarty and Al-Harbi 2013).

Data shown in the present report, demonstrates that lymphocyte expression of iHO-1 and plasma adiponectin were significantly lower in T2DM patients relative to healthy

control subjects ($P < 0.05$) (Table 2), an effect that may explain the increased representation of T cells activated to express TNF- α in diabetes patients ($P < 0.01$) (Table 2). These findings serve to define some of the key mechanisms by which eHO-1 and adiponectin are involved in the development of T2DM, where increased expression of inflammatory

Fig. 3 Correlations between immunoparameters and metabolites in peripheral blood of T2DM patients. Peripheral blood freshly collected from T2DM patients was assessed for lymphocyte-associated HO-1 by ELISA analysis of PBMC lysates; eHsp90 and adiponectin by ELISA analysis of plasma; serum glucose and lipids using the COBAS INTEGRA 400/700/800 biochemical analysis system; and

representation of CD3+ CD4+ cells activated to express inflammatory cytokines by three-color flow cytometry. Outcomes are shown in blood of each T2DM patient for HO-1 and CD4+ TNF- α + (2A); HO-1 and serum LDL (2B); TNF- α + and adiponectin (2C); TNF- α + and serum LDL (2D); and plasma Hsp90 and serum glucose (2E)

cytokines contributes to the vascular pathology associated with diabetes. Moreover, data in Table 3 showing the inverse correlation between representation of CD3+ CD4+ cells activated to express inflammatory cytokines, with lymphocyte-associated iHO-1 and plasma adiponectin; and the inverse correlation between plasma LDL and iHO-1 suggest a possible therapeutic use of induction of HO-1 in management of serum cholesterol levels—which might have corollary benefits in preventing inflammatory tissue damage. The authors nevertheless here acknowledge that the correlations between immunoparameters and HO-1, adiponectin, Hsp70, Hsp90, or serum metabolites shown in Fig. 3 and Table 3 do not provide definitive support for preventive or therapeutic clinical strategies. Ongoing and future work by authors of the present report will include determination of coefficient of variability (CV) for determination of intra and inter assay variability in detection of HO-1, eHsp90, and adiponectin by ELISA and other methods to be used. The scope of the experiments described here will be further extended by evaluating between expression of the Hsp70, Hsp90, adiponectin, and HO-1 and known markers of sub-clinical inflammation such as IL-6 and C-reactive protein.

Moreover, evidence from previous studies suggesting that the present work has potential for eventual contribution to clinical medicine, particularly in prevention and management of cardiovascular disorders, is provided by a 2012 report demonstrating that the heme oxygenase inducer L-4F is an apolipoprotein A1 mimetic (the major apolipoprotein of HDL) and is associated with variation of adiponectin levels as a correlate of inflammation (Marino et al. 2012).

Additionally, an earlier investigation by L'Abbate et al. showed that induction of iHO-1 was associated with a parallel increase in the serum levels of adiponectin and contributed to the mitigation of anti-inflammatory tissue damage (L'Abbate et al. 2007). These findings may have important clinical implications in the management of type 2 diabetes. The present investigation also increased understanding of native immunoregulatory processes mediated by Hsp, which may act as countermeasures to hyperinflammatory progression in T2DM. Previous studies have shown that eHO-1 enzyme (eHsp32) provides cytoprotective effects (Panahian et al. 1999).

As described in the “Introduction” to this report, the heme oxygenase variant with most significant relevance to strategies for modulating the enzyme to prevent or treat disease, is HO-1, the inducible form which may function within cells (iHO-1), or extracellularly (eHO). Interestingly, iHO and eHO exhibit different—and in some cases, opposite modes of activity in T2DM pathogenesis, as revealed by a 2010 report which evaluated the role of plasma HO-1 concentrations as a modulator of oxidative stress in development of T2DM and comorbidities (Ban et al. 2010)—and results of an investigation of obesity-related maternal diabetes-associated with

mitochondrial impairment, reported in 2017, demonstrating that plasma eHO-1 was significantly decreased by T2DM in advance of pregnancy (Duan et al. 2017).

The immunoregulatory role of heat shock proteins, particularly heme oxygenases, constituted a significant element of focus for the present investigation for reasons described above. The authors also considered correlation between features of T2DM and the Th2-associated, major anti-inflammatory cytokine IL-10, which has potent anti-inflammatory properties. This cytokine decreases production of proinflammatory cytokines, including TNF- α and IL-1 β (O'Shea and Murray 2008). A 2010 animal study demonstrated that an IL-10/iHO-1 pathway induced adiponectin-mediated suppression of LPS-stimulated TNF- α in cultures of Kupffer cells (Mandal et al. 2010). Moreover, genetic knockdown of IL-10 expression or inhibition of iHO-1 activity in these cultures prevented the inhibitory effect of adiponectin on LPS-stimulated TNF- α expression.

The significantly higher level of glycated hemoglobin (HbA1c%) in the blood of diabetic versus control subjects (Table 1, $P < 0.05$) was expected based on the known association of this form of the molecule with the diabetic condition and is often used diagnostically as an objective index of glycemic control (Herman and Fajans 2010; Ketema and Kibret 2015). Likewise, significantly higher levels of triglycerides and lower HDL levels in blood of diabetic subjects versus controls (Table 1, $P < 0.05$) was expected, based on aberrant insulin signaling and fatty acid metabolism in T2DM patients (Chang et al. 2014).

Plasma eHsp70 was elevated in diabetics relative to healthy control subjects ($P < 0.05$) (Table 2). A previous study showed that serum level of plasma eHsp70 is significantly higher in long-term T2DM patients versus those newly diagnosed, and the higher eHsp70 levels were inversely correlated with fasting blood sugar (Nakhjavani et al. 2010). These results suggest that enhanced eHsp70 production may be an adaptive countermeasure to hyperglycemia-induced oxidative stress, and therefore may be valuable as a diagnostic biomarker of persistent metabolic derangement. Conversely, lower eHsp production levels may contribute to disrupted glucose homeostasis. These possibilities are supported by an observation that increased extracellular eHsp70 is observed to act cytoprotectively in rheumatoid arthritis by increasing expression of IL-10-producing T cells, which subsequently inhibit overproduction of inflammatory mediators by other cells, thus helping to avoid tissue damage (Wendling et al. 2000). eHsp70 is also reported to inhibit inflammatory cytokine production, through inhibition of protein kinase, and NF- κ B signal pathways (Luo et al. 2008). This heat shock protein has also been demonstrated to suppress inflammatory cytokine production via IL-10 driven downregulation of C/EBP β and C/EBP δ transcription factors in a fibroblast-like synovocyte model (Borges et al. 2013).

The biological role of Hsp70 in T2DM is a subject of ongoing investigation, which may at some point yield application to strategies for prevention and management of the syndrome. The elevated plasma levels of eHsp70 in peripheral blood of T2DM patients relative to blood of healthy control subjects, shown in Table 2 ($P = 0.010$) is likely a reflection of higher systemic levels of toxic metabolites capable of increasing expression of several major heat shock proteins, including Hsp70 (Noble and Shen 2012). Indeed, elevated Hsp70 expression appears to be a cellular adaptive response to hyperglycemia-associated oxidative stress by many cell types (Hall and Martinus 2013). The outcomes in Table 2 suggest existence of a mechanism in which eHsp70 is elevated in response to the diabetic condition, that, in future studies, may prove to be protective. Nevertheless, insufficient data are available in these experiments to draw mechanistic conclusions from this observation.

A marked upregulation of iHsp90 in skeletal muscles has been reported in type 2 diabetes patients (Venojarvi et al. 2014). In the present study, plasma levels of eHsp90 were increased, but not to a significant level relative to healthy control subjects (Table 2). However, a positive correlation between eHsp90 and fasting blood glucose was observed ($r = 0.518$, $p = 0.014$) (Fig. 3e). eHsp90 plays a physiologic role which involves contribution in maintaining the activity of protein kinase B and regulation of insulin-mediated glucose transport and glycogen synthesis (Atalay et al. 2009). The causes and consequences of increased eHsp90 levels in diabetes and, more specifically, its relationship to impaired glucose regulation, are not known. iHsp90 is the master regulator of the heat shock response and its inhibition can upregulate iHsp70 (Urban et al. 2010). Chronic dosing with Hsp90 inhibitors reversed hyperglycemia in a diabetic mouse model and improved insulin sensitivity in a diet-induced obese mouse model of insulin resistance (Lee et al. 2013).

Observations presented in Tables 2 and 3 of this report suggest that strategies which affect Hsp expression and biologic activity offer potential strategies for novel approaches to T2DM prevention and management. Specifically, control of blood FBG levels by inhibition of Hsp90 may emerge as a novel addition to therapies available to clinicians as T2DM treatment regimens. Indeed, results of studies reported in 2013 and 2014 strongly suggest that Hsp90 inhibitors improve prognosis of diabetes and neurodegenerative disease via activation of HSF1, thereby enhancing expression of the heat shock response (Hsp70, etc.)—with a caveat that significant toxicity is associated with these inhibitors (Lee et al. 2013, Chen et al. 2014).

The above findings raise the very intriguing possibility that Hsp90 inhibitors may emerge as dual-use drugs, with application to disorders of glucose metabolism—and additionally as chemopreventives and/or therapeutics for certain kinds of cancers, particularly prostate tumors. Such potential for combined

anticancer and antidiabetic properties of a single pharmacologic strategy was suggested by investigators at the Institute of Cancer Research (ICR) in London, UK, who demonstrated that Hsp90 inhibitors also inhibit expression of an androgen receptor variant (ARV) by disrupting splicing of the mRNA for this molecule, without affecting its chaperone function—and also suppressed aberrantly high AKT and GR, signaling, which are major features of prostate tumors (Ferraldeschi et al. 2016). Since growth and viability of prostate cancers is often dependent on androgen stimulation, influences that suppress expression of ARV are promising countermeasures to these tumors. Results of Hsp90 inhibition studies have proven successful in diminishing resistance to hormone treatments and are entering into the first phases of human trials at the time of this writing (Ferraldeschi et al. 2016). Data in Table 3 of the present report shows a significantly positive relationship between plasma Hsp90 and serum glucose (Table 3; Fig. 3e). These outcomes suggest that influences and pharmacological agents that suppress Hsp90 expression may simultaneously contribute to reduction in systemic inflammation and restoration of normoglycemic homeostasis. Moreover, the above-mentioned findings by Ferraldeschi et al. raise the possibility that Hsp90 inhibitors may exhibit antitumor activity as a corollary effect of T2DM treatment. Prostate cancer patients afflicted with or at-risk of T2DM would be particularly good candidates for use of Hsp90 inhibitors. Indeed, such drugs fall into the category of “Network” interventions, which target several signaling processes simultaneously to improve disease prognosis (Poornima et al. 2016). It is here acknowledged that consideration of cancer is not a major focus of the present report; however, consideration of expanded medical venues for therapeutic targets discussed here, broadens its use as background for future research design in which simultaneous treatment of diabetes and other disease are considered.

The data shown in Table 2 demonstrates an elevated representation of T lymphocytes derived from T2DM patients, activated to express inflammatory cytokines, as compared to samples from nondiabetic control subjects. This suggests a role for T cells in T2DM pathophysiology. Nevertheless, outcomes of the present report do not allow this observation to be classed specifically as “autoimmune” in nature. However, these results suggest that preventive therapeutic approaches that affect T cell activation may prove to be useful clinical tools as insight into T2DM pathomechanisms increases. Some preliminary indication of strategies by which T cell-associated countermeasures for T2DM might be pursued were suggested via the outcomes of a previous in vitro study by the authors of this report. These outcomes demonstrated that iHO-1-mediated suppression of LPS-induced activation of CD3+ CD4+ T cells, expressing inflammatory cytokines, which occurred to a significantly greater degree in PBMC derived from T2DM patients than from healthy control subjects (Mahmoud et al. 2013).

Conclusions

The observation made in the present study relative to healthy control participants, peripheral blood of T2DM patients contain larger populations of T cells activated to express the inflammatory cytokines TNF- α , IL-6, and IL1- β , but lower representation of T cells activated to express the immunosuppressive cytokine, IL-10, was expected based on the proinflammatory nature of T2DM pathophysiology. A novel result, shown here in Fig. 2, which adds insight into the immunological features of this disease, was the observation of significantly larger populations of cells activated to express the Th1-associated cytokine IFN- γ (CD4⁺ IFN- γ ⁺), but significantly lower size of T cells activated to express the Th2-associated cytokine, IL-10, in cultures from T2DM patients versus healthy control subjects. These outcomes suggest that the immunological character of T2DM manifests a Th1 character and allow improved insight for better focused strategies in future efforts to develop prevention and therapy for T2DM. It is nevertheless important to emphasize that the clinical and translational aspects presented by the present report are speculative, since broad interpretation of these results is constrained by the small participant sample number (25)—and the study does not include a mechanistic analysis of causes for the T2DM-related effects shown. Nevertheless, the correlations shown here are of potential use in design of experiments for ongoing characterization of T2DM, along with its value as reference material to healthcare professionals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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