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Modulation of Immune Response by RAGE and TLR4 Signalling in PBMCs of Diabetic and Non-Diabetic Patients

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Abstract

Diabetes is associated with increased glucose levels and accumulation of glycated products. It is also associated with impairment in the immune response, such as increased susceptibility to infections. In this study, we assessed the possible interactions between TLR4 and RAGE signalling on apoptosis and on the expression of inflammatory cytokines in PBMC from individuals with and without diabetes. PBMCs were isolated from seven diabetic patients and six individuals without diabetes and stimulated *in vitro* with bacterial LPS (1 μg/ml) associated or not with BSA-AGE (200 μg/ml). This stimulation was performed for 6 h, both in the presence and in the absence of inhibitors of TLR4 (*R. sphaeroides* LPS, 20 μg/ml) and RAGE (blocking monoclonal antibody). Apoptosis at early and late stages was assessed by the annexin-V/PI staining using flow cytometry. Regulation of TNF-α and IL-10 gene expression was determined by RT-qPCR. PBMCs from diabetes patients tended to be more resistant apoptosis. There were no synergistic or antagonistic effects with the simultaneous activation of TLR4 and RAGE in PBMCs from either diabetes or non-diabetes group. Activation of TLR4 is more potent for the induction of TNF-α and IL-10; RAGE signalling had a negative regulatory effect on TNF-α expression induced by LPS. TLR and RAGE do not have relevant roles in apoptosis of PBMCs. The activation of TLR has greater role than RAGE in regulating the gene expression of IL-10 and TNF-α.

Introduction

The process of apoptosis or programmed cell death is essential for maintaining the development of living beings, acting as part of a mechanism of 'quality control' and repair, compensating stochastic genetic errors or errors of development [1]. *In vitro* studies have associated the induction of apoptosis to hyperglycaemia in diabetes mellitus [2–4]. However, most of the evidence is focused on the vascular complications, as most studies used endothelial cells of the retina, kidney, myocardial and endothelial cells from human umbilical vein. There is a relative paucity of information regarding the modulation of apoptosis associated with diabetes in immune cells, which may be one mechanism involved in the disturbance of the immune system that is inherent to diabetes.

A hallmark of diabetes is chronic hyperglycaemia, which leads to the formation of advanced glycation end products (AGEs). These AGEs include various biochemical compounds that can exert numerous biological effects. Accumulation of AGEs was demonstrated in the kidneys [5], atherosclerotic plaques [6], heart [7] and other sites affected by diabetes-associated complications. AGEs exert their effects by binding to its single-pass transmembrane receptor RAGE (receptor for AGEs). This receptor is able to interact with multiple ligands acting as a pattern-recognition receptor, similar to the receptors involved in the innate immune response [8]. AGE–RAGE interaction on monocytes has been shown to induce chemotaxis and haptotaxis and activation of the transcription factor nuclear factor-κB (NF-κB), which is involved in the regulation of inflammatory cytokines such as TNF-a, IL-1b and IL-6 [9]. Moreover, expression of functional RAGE has also been shown in T cells, in which its activation plays a role in priming for promoting antigen-specific expansion [10]. The connection between RAGE signalling and cytokine expression has been assessed as a therapeutic strategy to

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dampen inflammation. In type 2 diabetes, the association of sRAGE levels was positively associated with markers of inflammation in the plasma, such as MIP-1α and TNF-α. *In vivo* studies revealed that administration of soluble RAGE (sRAGE), the extracellular ligand-binding domain of RAGE, diminished vascular permeability and attenuated the atherosclerotic process in rodents with diabetes [9]. In spite of increased inflammation, diabetes is also associated with increased susceptibility to infection and sepsis [11, 12]. Toll-like receptors (TLR) recognize microbial-associated molecular patterns initiating and modulating the immune response. Interestingly, TLR signalling also activates NF-κB, which suggests that there may be an interaction between RAGE and TLR4, as the signal generated by both receptors funnels to the same pathway. In fact, the mechanisms associated with diabetes-associated modulation of immune response to infections may include regulation of apoptosis and gene expression by the immune cells resulting from a synergistic or antagonistic interaction between RAGE and TLR4. This hypothesis is supported by a study demonstrating that simultaneous activation of these receptors induced a higher production of IL-6 and TNF-α in PBMC from type 2 diabetes patients [13]. In this study, we assessed the role of TLR4 and RAGE signalling and the possible interactions between these receptors on apoptosis and on the expression profile of inflammatory cytokines in PBMC from individuals with and without diabetes.

Materials and methods

Diabetes patients and healthy volunteers. PBMCs were isolated from total venous blood collected from six volunteers without diabetes and seven individuals with type 2 diabetes. The diabetes individuals (age range 38–51 years) were diagnosed for more than 5 years and presented poor metabolic control (HbA1c > 8.5%). Although not exactly matched, there were no differences between the groups with or without diabetes regarding demographic characteristics, including age, gender and smoking status (data not shown). Additional inclusion criteria for the selection of diabetes volunteers were as follows: the absence of dyslipidemia and no medications other than insulin or other drugs to manage blood sugar levels in the previous 3 months. The experimental protocol was approved by the institutional Human Subjects Research Ethics Committee (protocol #35/09).

On average 24 ml of peripheral blood was collected from each patient, and the PBMCs were separated by density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare Life Sciences, Piscataway, NJ, USA) at 900 × *g* for 40 min at +18 °C (acceleration set to '2' and no brake). PBMCs were washed in RPMI medium supplemented with 10% FBS, resuspended in low serum (1% v:v) RPMI medium, counted and immediately plated for the experiments.

Preparation of glycated serum albumin (BSA-AGE). Bovine serum albumin – fraction V (BSA) was diluted in PBS (pH 7.4) at a concentration of 50 mg/ml and subsequently incubated at 37 °C for 8 weeks with 0.5 M glucose in 0.2 M phosphate buffer (PBS) pH 7.4 containing 0.5 mM EDTA. The free glucose remaining after this incubation was removed by extensive dialysis (12 h) in PBS free of Ca and Mg, pH 7.4. The BSA control has been prepared in parallel using the same protocol, except for the addition of 0.5 M glucose. The BSA-AGE and BSA control were aliquoted and stored in refrigerator (+4 °C) after confirmation of glycation by the determination of absorbance at 405 nm on a spectrophotometer. Absorbance values over 3.0 in the samples of BSA-AGE and <0.1 in samples of BSA control confirmed the extensive glycation.

Reagents. We used BSA-AGE (prepared as previously described) as a ligand for RAGE and LPS from *Escherichia coli* (cat# L6529; Sigma-Aldrich Inc., St. Louis, MO, USA) as a ligand for TLR4. Monoclonal blocking antibody for RAGE was from Abcam, Cambridge, MA, US and the TLR4 inhibitor (*Rhodobactersphaeroides* LPS) was from InvivoGen (Cayla, France). Phorbol 12-myristate 13-acetate (PMA) was from Sigma-Aldrich. Culture medium and supplements were obtained from Life Technologies (Invitrogen, Life Technologies, Foster City, CA, USA).

Apoptosis assay. PBMCs were stimulated with LPS (1 μg/ml) and AGE-BSA (200 μg/ml) both alone and simultaneously for 6 h. Protein transport inhibitor brefeldin A was added to cultures (10 mg/ml) in the last 3 h of the experimental period of 6 h for the stimuli. These experiments were performed in the presence and in the absence of RAGE (abRAGE) and TLR4 (RS-LPS) inhibitors. The cells were collected by centrifugation (250 *g*, 5 min) and slowly frozen to −80 °C (−1 °C/min rate) in a solution of 90% FBS/10% DMSO until ready to perform the test, when they were rapidly thawed in a water bath at 37 °C and resuspended in prewarmed RPMI supplemented with 10% FBS for labelling with FITC-conjugated annexin-V and propidium iodide (PI), according to the instructions of the supplier of the reagents used (BD Biosciences). Data acquisition was performed in a flow cytometer (FACS Canto; BD Biosciences San Jose, CA, USA) at the Laboratory for Applied Immunology, FMRP-USP and subsequently analysed with FLOWJO software (Treestar Inc., Ashland, OR, USA). Apoptosis was assessed by flow cytometry, according to the use of the kit (FITC-Annexin II V Apoptosis Detection Kit; BD Biosciences) according to the manufacturer's instructions.

Regulation of gene expression. For these experiments, PBMCs were plated in low serum RPMI at 1 × 10^6 cells per well in 48-well plates. After a de-inducing period of four hours, the cells were stimulated with *E. coli* LPS (1 μg/ml) and BSA-AGE (200 μg/ml), both alone and associated for 6 h. This period corresponded to the peak of target gene induction after LPS stimulation of PBMCs

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from systemically healthy individuals (data not shown). These stimulations were performed both in the presence and the absence of a 40-min pretreatment with inhibitors of TLR4 (RS-LPS, 20 μg/ml) and RAGE (abRAGE, 4 μg/ml). PMA (50 ng/ml) was also used as a positive control. Cells were collected by centrifugation and total RNA was extracted using an affinity column system, according to the instructions of the supplier (RNAqueous® Kit; Ambion, Life Technologies, Foster City, CA, USA) and the concentration determined on a microvolume spectrophotometer (NanoVue; GE Healthcare). Samples presenting an A260/A280 ratio between 1.8 and 2.1 were considered acceptably free from contaminants. cDNA was synthesized using random hexamer primers and moloney leukaemia virus reverse transcriptase, according to the manufacturer's instructions (High Capacity Reverse Transcription kit; Applied Biosystems). Real-time PCR was performed using TaqMan reagents (Applied Biosystems) and predesigned and optimized pairs of primers and probe (TaqMan Gene Expression Assays; Applied Biosystems). Selected target genes were IL-10 and TNF-α, and GAPDH was used as a housekeeping gene for normalization of the results. This reaction was performed in a STEPONE PLUS thermocycler (Applied Biosystems) and the results analysed by $\Delta(\Delta C_t)$ the method using the thermocycler's software.

Statistical analysis. Means and standard deviations were obtained from the data of replicate experiments using cells from different individuals, according to the presence of diabetes. These averages were compared within the same group (between different stimuli and experimental conditions) or between groups (in the same experimental condition/stimulus) using unpaired *t*-tests. For these comparisons, each experimental condition (stimulus) and the results after the same stimulus in the cells of the groups with and without diabetes were considered as independent events. These comparisons and graphs were performed with GRAPHPAD PRISM 5, using a significance level of 95% ($P < 0.05$).

Results

TLR4 and RAGE signalling and apoptosis of PBMCs

The experimental protocol including freezing of the PBMCs induced a significant amount of late-stage apoptosis (approximately 30–40%) and, interestingly, PBMC from individuals with diabetes were more resistant to apoptosis (42.5 ± 7.43% versus 26.9 ± 13.83% of PBMCs in late-stage apoptosis from 'controls' and 'diabetics', respectively) associated with the experimental protocol; however, this difference did not reach statistical significance ($P = 0.13$). Interestingly, PBMCs from diabetic individuals were generally more 'prone' to cell death based on the percentage of annexin V-positive and PI-negative (early apoptosis) in unstimulated cells

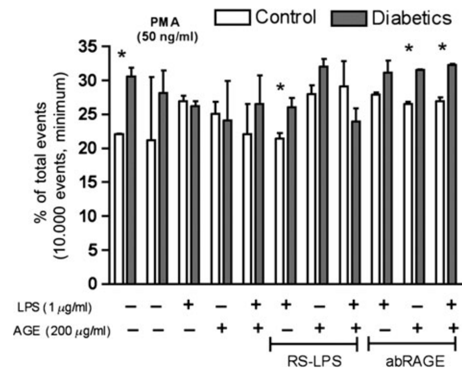

Figure 1 Modulation of apoptosis by RAGE and TLR4 ligands in PBMC from systemically healthy volunteers (control) and from individuals with type 2 diabetes (diabetes). Evaluation of the percentage of cells in early-stage apoptosis (annexin V-positive/PI-negative) in the different experimental conditions: unstimulated control, PMA (50 ng/ml), *Escherichia coli* LPS (1 μg/ml) and AGE-BSA (200 μg/ml) isolated or associated, in the presence or absence of inhibitors TLR4 (LPS-RS, 20 μg/ml) and RAGE (neutralizing antibody abRAGE, 4 μg/ml).

($P = 0.008$, Fig. 1). This trend was consistent in most experimental conditions, except for the LPS- and AGE-stimulated cells, which reduced the percentage of annexin V-positive/PI-negative cells in PBMCs from diabetic individuals, suggesting that TLR or RAGE activation promoted survival of these cells. Blocking RAGE with the neutralizing antibody and blocking TLR with RS-LPS reversed this effect.

However, upon PMA stimulation, there was a greater induction (*$P = 0.05$) of late/final stage apoptosis (annexin V-positive/PI-positive cells, Fig. 2) in PBMC from diabetes group and the overall percentage of late-stage apoptotic cells in PBMCs from diabetics was lower than that of PBMCs from non-diabetics ('control'), albeit without statistical significance. Stimulation of RAGE with AGE-BSA and TLR4 stimulation with *E. coli* LPS did not result in a significant modulation of apoptosis, and there was no synergistic effect when these ligands were used simultaneously. Inhibition of RAGE or TLR4 also did not affect the results, indicating that these receptors did not play a relevant role on late-stage apoptosis of (Fig. 2); however, activation of RAGE by AGE-BSA in PBMCs of diabetics (but not on PBMCs from 'controls') had a discrete effect of promoting cell survival, particularly as blocking of this receptor reversed this discrete effect (Fig. 1).

Role of RAGE and TLR4 signalling on cytokine gene expression (mRNA)

TNF-α

TNF-α mRNA was more potently induced by the activation of TLR4 in PBMCs from patients with and without diabetes. Interestingly, activation of RAGE had a

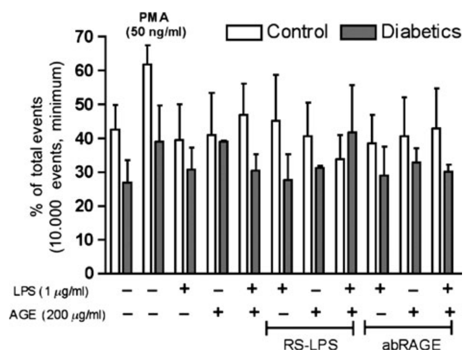

Figure 2 Modulation of apoptosis by RAGE and TLR4 ligands in PBMC from systemically healthy volunteers (control) and from individuals with type 2 diabetes (diabetes). Evaluation of the percentage of cells in late-stage apoptosis (annexin V-positive/PI-positive) in the different experimental conditions: unstimulated control, PMA (50 ng/ml), *Escherichia coli* LPS (1 μg/ml) and AGE-BSA (200 μg/ml) isolated or associated, in the presence or absence of inhibitors TLR4 (LPS-RS – 20 μg/ml) and RAGE (neutralizing antibody abRAGE, 4 μg/ml).

discrete stimulatory effect on TNF-α expression only in PBMC from subjects without diabetes. In fact, the dependence of the TLR4 signalling pathway is confirmed by a significant reduction on TNF-α mRNA when cells were pretreated with RS-LPS. Inhibition of RAGE resulted in contrasting effects in PBMCs of diabetes group (increased TNF-α mRNA expression upon LPS stimulation) and of non-diabetes group (decreased expression of TNF-α mRNA upon LPS stimulation). Only in PBMC from subjects without diabetes, the simultaneous activation of TLR4 and RAGE attenuated TNF-α expression in comparison with the activation of TLR4 alone. RAGE signalling alone was a weak inducer of TNF-α, particularly in PBMCs from diabetes volunteers; however, these cells from these subjects were more sensitive to induction of TNF-α with PMA stimulation (Fig. 3).

Il-10

Similar to TNF-α, IL-10 mRNA expression by PBMC was primarily induced by TLR4 signalling in PBMC of both individuals with and without diabetes. Activation of RAGE did not induce IL-10 expression. The role of these receptors is supported by the finding of a significant decrease on IL-10 mRNA with inhibition of TLR4, whereas blocking RAGE had no effect. There was no synergistic or antagonistic effect between RAGE and TLR4. No significant differences on IL-10 gene expression were noted between PBMCs from diabetes and non-diabetes groups (Fig. 4).

Discussion

There is evidence indicating that activation of RAGE in innate immune cells and lymphocytes has a functional modulating role, affecting cytokine gene expression, activation of adaptive immunity and even T cell polarization [10, 14]. However, there is scarce information on the role of RAGE, and particularly on the possible interaction between RAGE and TLR signalling, on apoptosis and cytokine expression by immune cells. This information is relevant considering that reduced resistance to infection is a major complication in diabetes and both TLR and RAGE activate some downstream common signalling pathways involved with both cell survival and cytokine expression.

Our results show that PBMCs from individuals with type 2 diabetes were more resistant to apoptosis associated

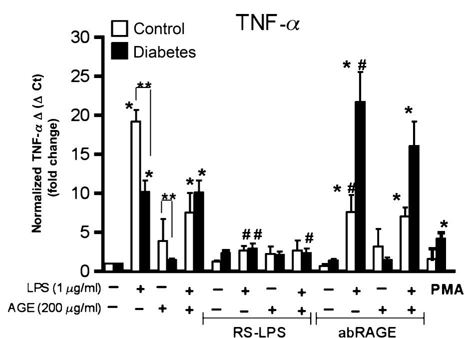

Figure 3 Regulation of TNF-α gene expression by RAGE and TLR4 signalling in PBMC from systemically healthy volunteers (control) and from individuals with type 2 diabetes (diabetes). Activation of TLR4 and RAGE was performed with *Escherichia coli* LPS (1 μg/ml) and BSA-AGE (200 μg/ml), respectively, both in the presence and absence of inhibitors (TLR4, RS-LPS 20 μg/ml and RAGE, abRAGE, 4 μg/ml). *Significant difference compared to unstimulated control within the same group ('Control' or 'Diabetes'). #Significant difference when compared to isolated stimulation within the same group. **Significant difference between control and diabetes groups.

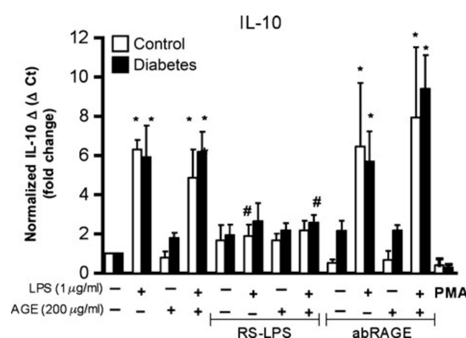

Figure 4 Regulation of IL-10 gene expression by RAGE and TLR4 signalling in PBMC from systemically healthy volunteers (control) and from individuals with type 2 diabetes (diabetes). Activation of TLR4 and RAGE was performed with *Escherichia coli* LPS (1 μg/ml) and BSA-AGE (200 μg/ml), respectively, both in the presence and the absence of inhibitors (TLR4, RS-LPS 20 μg/ml and RAGE, abRAGE, 4 μg/ml). *Significant difference compared to unstimulated control within the same group ('Control' or 'Diabetes'). #Significant difference when compared to isolated stimulation within the same group. **Significant difference between control and diabetes groups.

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with freeze-thawing in the experimental protocol used and also subsequent to PMA stimulation in comparison with PBMCs from individuals without diabetes. Activation of RAGE and TLR4 did not play a relevant role in the induction of apoptosis in these cells. A similar finding is reported in another study [15] that demonstrated that a smaller proportion of PI-positive in PBMCs of patients with type 2 diabetes. These authors mention that modulation of enzymatic activity due to hyperglycaemia, which alters the intracellular metabolic tolerance and reduces membrane permeability (increased stiffness). These phenomena may be part of a complex adaptive response mechanism associated with diabetes-induced changes. However, there may be differences associated with the microenvironment, as increased apoptosis of macrophages in atherosclerotic plaques of deceased diabetic patients was correlated with increased expression of RAGE and RAGE ligands [16]. Literature reports on immune modulation in diabetes differ widely and this may be due to the dynamic changes and great variation in severity of the metabolic imbalance in diabetics, insufficient number of samples, and to in consistencies in the methods used. There are studies associating the increased oxidative stress in type 2 diabetes to apoptosis of lymphocytes via intrinsic and extrinsic pathways [17]. Other studies [18–20] also show similar results regarding lymphocyte apoptosis in diabetes. In this study, we did not evaluate the biological mechanisms related to intrinsic and extrinsic pathways of apoptosis, but our results suggest that it will be interesting to assess mechanisms related to cell survival such as Akt activation, production of reactive oxygen species (ROS) and protein expression of anti-apoptotic Bcl-2 and Bcl-xL after stimulation with LPS and BSA-AGE.

We also studied the role of RAGE and TLR4 signalling on the regulation of candidate inflammatory and anti-inflammatory cytokines. It is important to consider the characteristics of the experimental approach used, which has both advantages and disadvantages: the short period of stimulation (6 h) is adequate for the evaluation of early-response genes that are rapidly transcribed in response to external stimulation, and it also minimizes possible changes on the phenotype of PBMC obtained from individuals with and without diabetes caused by the standard conditions of *in vitro* culture, including pH, nutrients, amino acids and glucose levels. However, we cannot speculate that the effects observed at this period are actually sustained in the steady state of equilibrium between mRNA synthesis and degradation, particularly of genes that are also regulated at the post-transcriptional level as IL-10 and TNF-α [21]. This short period of stimulation also precludes us from making any inferences regarding processes that involve antigen processing by innate immune cells and antigen presentation involving interactions between antigen-processing cells and lymphocytes, but allows us to assess the direct effect of RAGE/TLR signalling on various immune cells. Moreover, there is also the limitation of evaluating gene expression only at the mRNA level, precluding comparison with results in the literature evaluating gene regulation at the protein level. The use of the mixed population of PBMCs has also a limitation of not providing information on the specific responses of the different immune cell types (lymphocytes, monocytes and neutrophils), but using PBMCs, we preserved the interactions between innate (monocytes) and adaptive (lymphocytes) cells by soluble mediators secreted.

IL-10 and TNF-α gene expression were induced more potently upon TLR4 activation by LPS in PBMC of both diabetic and non-diabetic individuals; however, TNF-α expression was significantly higher in PBMC of non-diabetic patients. Even though RAGE activation alone was not a potent inducer of either IL-10 or TNF-α, activation of RAGE may play a dampening role on the expression of TNF-α in PBMC of patients with diabetes, as the inhibition of RAGE activation resulted in a marked increase on the expression of this gene. This attenuation effect or negative regulation of TNF-α expression by RAGE signalling is supported by the reduced expression of TNF-α by PBMC of patients without diabetes when both RAGE and TLR4 were activated as opposed to the activation of TLR4 alone by bacterial LPS. This was the only instance that suggested interaction between RAGE and TLR4.

The overall lack of interaction between RAGE and TLR4 on apoptosis is interesting and unanticipated by us, as the activation of these different cell surface receptors by their ligands can converge to the same intracellular signalling pathways, most notably NF–κB [22]. NF-κB is the main signalling pathway activated by TLR activation and its relevance to the expression of the selected target genes is demonstrated by TLR4 receptor blockade. Gene expression depends on the net effect of DNA-binding proteins, especially in the promoter region of genes. Although NF-κB pathway is a convergence point for the signals generated by TLR and RAGE activation, many other signalling pathways (and hence, transcription/repression factors and other DNA-binding proteins) can be mobilized. It is tempting to speculate that activation of RAGE, which is constitutively higher in PBMC of diabetes group, activates a transcription repressor that reduces the expression of TNF-α. There is also the consideration regarding the nature of RAGE ligand used, as the affinity and the downstream effects may differ with different ligands, including damage-associated molecular patterns (HMGB1), S100/Calgranulins and other glycated compounds (e.g. BSA-AGE) [23, 24]. It is important to note that as we did not use the inhibitors of TLR4 and RAGE simultaneously, we cannot rule out the possibility of a compensatory effect, in which inhibition of one receptor is compensated by the preserved activity of the other.

Accounting for this possible compensatory effect, however, does not negate the lack of synergistic effect observed when both receptors were activated simultaneously.

Conclusions

In summary, our study demonstrated that activation of RAGE and TLR4, alone or simultaneously, did not induce apoptosis in PBMCs. PBMC from type 2 diabetes mellitus individuals tend to be more resistant to apoptosis. We also showed that activation of TLR4 has a more important role than RAGE in the induction of IL-10 and TNF-α gene expression. Our results also suggest that RAGE signalling may have a negative regulatory effect on LPS-induced TNF-α expression in PBMCs.

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References

1 van den Oever IA, Raterman HG, Nurmohamed MT, Simsek S. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. *Mediators Inflamm* 2010;2010:792393.
2 Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J, Gessl A, Waldhausl W. High-glucose–triggered apoptosis in cultured endothelial cells. *Diabetes* 1995;44:1323–7.
3 Du XL, Sui GZ, Stockklauser-Farber K *et al.* Introduction of apoptosis by high proinsulin and glucose in cultured human umbilical vein endothelial cells is mediated by reactive oxygen species. *Diabetologia* 1998;41:249–56.
4 Lorenzi M, Cagliero E. Pathobiology of endothelial and other vascular cells in diabetes mellitus. Call for data. *Diabetes* 1991; 40:653–9.
5 Oldfield MD, Bach LA, Forbes JM *et al.* Advanced glycation end products cause epithelial-myofibroblast transdifferentiation via the receptor for advanced glycation end products (RAGE). *J Clin Invest* 2001;108:1853–63.
6 Forbes JM, Yee LT, Thallas V *et al.* Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. *Diabetes* 2004;53:1813–23.
7 Candido R, Forbes JM, Thomas MC *et al.* A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. *Circ Res* 2003;92:785–92.
8 Neeper M, Schmidt AM, Brett J *et al.* Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* 1992;267:14998–5004.
9 Lalla E, Lamster IB, Feit M *et al.* Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. *J Clin Invest* 2000;105:1117–24.
10 Moser B, Desai DD, Downie MP *et al.* Receptor for advanced glycation end products expression on T cells contributes to antigen-specific cellular expansion in vivo. *J Immunol* 2007;179:8051–8.
11 Koh GC, Peacock SJ, van der Poll T, Wiersinga WJ. The impact of diabetes on the pathogenesis of sepsis. *Eur J Clin Microbiol Infect Dis* 2012;31(4):379–88.
12 Vallerskog T, Martens GW, Kornfeld H. Diabetic mice display a delayed adaptive immune response to Mycobacterium tuberculosis. *J Immunol* 2010;184:6275–82.
13 Veloso CA, Fernandes JS, Volpe CM *et al.* TLR4 and RAGE: similar routes leading to inflammation in type 2 diabetic patients. *Diabetes Metab* 2011;37(4):336–42.
14 Chen Y, Akirav EM, Chen W *et al.* RAGE ligation affects T cell activation and controls T cell differentiation. *J Immunol* 2008;181:4272–8.
15 Graber R, Farine JC, Fumagalli I, Tatti V, Losa GA. Apoptosis and oxidative status in peripheral blood mononuclear cells of diabetic patients. *Apoptosis* 1999;4:263–70.
16 Burke AP, Kolodgie FD, Zieske A *et al.* Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol* 2004;24:1266–71.
17 Khan S, Raghuram GV, Bhargava A *et al.* Role and clinical significance of lymphocyte mitochondrial dysfunction in type 2 diabetes mellitus. *Transl Res* 2011;158:344–59.
18 Arya AK, Pokharia D, Tripathi K. Relationship between oxidative stress and apoptotic markers in lymphocytes of diabetic patients with chronic non healing wound. *Diabetes Res Clin Pract* 2011;94: 377–84.
19 Nakagami H, Morishita R, Yamamoto K *et al.* Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and AKT in endothelial cells. *Hypertension* 2001;37: 581–6.
20 Ido Y, Carling D, Ruderman N. Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. *Diabetes* 2002;51:159–67.
21 Rajasingh J, Bord E, Luedemann C *et al.* IL-10-induced TNF-alpha mRNA destabilization is mediated via IL-10 suppression of p38 MAP kinase activation and inhibition of HuR expression. *FASEB J* 2006;20:2112–4.
22 Lin L. RAGE on the Toll Road? *Cell Mol Immunol* 2006;3:351–8.
23 Donato R. RAGE: a single receptor for several ligands and different cellular responses: the case of certain S100 proteins. *Curr Mol Med* 2007;7:711–24.
24 Valencia JV, Mone M, Zhang J, Weetall M, Buxton FP, Hughes TE. Divergent pathways of gene expression are activated by the RAGE ligands S100b and AGE-BSA. *Diabetes* 2004;53:743–51.

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