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Original article

TLR4 and RAGE: Similar routes leading to inflammation in type 2 diabetic patients

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Abstract

Aim. – The present study investigates the interaction of TLR4 and RAGE with their respective ligands as inducers of the inflammatory markers IL-6 and TNF-α. Also, the reactivity of peripheral blood mononuclear cells (PBMNC) from type 2 diabetic (T2D) patients and non-diabetic healthy controls (ND) were comparatively studied.

Methods. – Concentrations of IL-6 and TNF-α were measured by sandwich Elisa, using kits supplied by Assay Designs (Ann Arbor, MI, USA). PBMNC from T2D and ND were incubated in the presence or absence of LPS, anti-TLR4 or anti-RAGE for 72 hours at 37° C under 5% CO_2. The final volume was adjusted to 300 μL in DMEM supplemented with 10% fetal bovine serum. After incubation, the cells were centrifuged, the supernatant collected and the cytokines measured.

Results. – PBMNC from T2D were more sensitive to innate immune stimulation with LPS and monoclonal agonist anti-TLR4 than were cells from ND. The actions of LPS, anti-TLR4 and anti-RAGE potentiated the production of IL-6 and TNF-α in both groups. The simultaneous activation of monoclonal anti-RAGE and anti-TLR4 suggests that both antibodies used different receptors on the cell surface, but converged on the same PBMNC signaling metabolic pathways. This simultaneous activation induced a higher production of IL-6 and TNF-α in PBMNC from the T2D patients than from the ND subjects.

Conclusion. – Our results clearly show an exacerbation of innate immunity in PBMNC with T2D that was possibly hyperglycaemia-induced. These data, when analyzed together, suggest the importance of innate immunity in the pathogenesis of T2D.

Keywords: Type 2 diabetes; TLR4; RAGE; Proinflammatory cytokines; IL-6; TNF-α; Peripheral blood mononuclear cells

Résumé

TLR4 et RAGE : induction de l'inflammation par des voies similaires chez les diabétiques de type 2.

Objectif. – Cette étude avait pour objectif d'examiner l'interaction de TLR4 et RAGE avec leurs ligands respectifs comme inducteurs des marqueurs de l'inflammation, IL-6 et TNF-α. La réactivité des cellules mononucléées du sang périphérique de patients atteints de diabète de type 2 (DT2) a été comparée à celle de cellules mononucléées de témoins non-diabétiques (ND).

Méthodes. – Les concentrations d'IL-6 et TNF-α ont été mesurées par méthode Elisa. Les cellules mononucléées issues des DT2 et des témoins ND ont été incubées en présence ou en absence de LPS, d'anti-TLR4 et/ou d'anti-RAGE pendant 72 heures à 37 °C avec 5 % de CO_2. Le volume final a été ajusté à 300 μL avec DMEM supplémenté avec 10 % de sérum de veau fœtal. Après incubation, les cellules ont été centrifugées, le surnageant récupéré et les cytokines ont été mesurées.

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Résultats. – Les cellules mononucléées des DT2 étaient plus sensibles à la stimulation immunitaire par les LPS et l'agoniste monoclonal anti-TLR4 que les cellules des témoins ND. L'action des LPS, des anti-TLR4 ou anti-RAGE a potentialisé la production d'IL-6 et de TNF-α dans les deux groupes. L'effet de l'activation simultanée par les anticorps monoclonaux anti-RAGE et anti-TLR4 suggère que si les deux anticorps ont utilisé différents accepteurs sur la surface de la cellule, leurs effets convergent vers les mêmes voies de signalisation métaboliques. Cette activation simultanée a induit une augmentation de la production d'IL-6 et TNF-α plus importante dans les cellules mononucléées des diabétiques que celle observée dans les cellules des témoins ND.

Conclusions. – Les résultats montrent clairement une augmentation de l'immunité innée dans les cellules mononucléées des DT2 PBMC, peut-être induite par l'hyperglycémie. Ces données suggèrent l'importance de l'immunité innée dans le diabète de type 2.

Mots clés : Diabète de type 2 ; TLR4 ; RAGE ; Cytokines pro-inflammatoires ; IL-6 ; TNF-α ; Cellules mononucléées du sang périphérique

1. Introduction

Diabetes mellitus is a multifactorial disease associated with alterations and adaptations of glucose metabolism [1]. Chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of type 2 diabetes (T2D) [2,3]. Toll-like receptors (TLRs), especially TLR4, mediate the link between the metabolic and immune systems, thereby leading to the production of proinflammatory cytokines and the deleterious effects of diabetes [4–13].

In T2D, advanced glycation end-products (AGEs) are inducers of inflammatory responses. The interaction of AGEs with their respective receptor (RAGE) on the cell surface leads to the production of proinflammatory cytokines [1]. It has been suggested that RAGE most probably acts like a TLR [14] and, thus, leads to the production of proinflammatory cytokines that, in turn, activate nuclear factor (NF)-kappa-B, resulting in the overexpression of genes for cytokines, growth factors and adhesion molecules [15–17]. It is also known that diabetic patients have higher levels of AGEs, and that chronic or acute hyperglycaemia and oxidative stress contribute to AGE formation and accumulation in tissues [18,19].

The present study is an investigation of the role of TLR4 and RAGE activation with agonist monoclonal antibodies and lipopolysaccharides (LPS) in terms of proinflammatory cytokines in peripheral blood mononuclear cells (PBMNC) from T2D patients in comparison to cells from healthy, non-diabetic subjects (ND).

2. Material and methods

2.1. Diabetic patients and healthy volunteers

The ethics committee of the Santa Casa Hospital of Belo Horizonte, Brazil, approved this study, and informed consent was obtained from all participants. Patients with T2D (diagnosed according to the criteria of the American Diabetes Association) and healthy volunteers, all within the age range of 30–80 years, were recruited from the endocrinology department of the Santa Casa Hospital. Diabetic patients were being treated with statins and beta-blockers in addition to hypoglycaemic drugs. All volunteers were subjected to a detailed physical examination, and evaluation of medical history and laboratory data (Table 1), before entering the study. Subjects were excluded if they presented with one or more of the following conditions or pathologies: current smokers; pregnancy; alcoholism; dementia; inflammation; malignant disease; and infection.

Table 1
Characteristics of the studied healthy subjects (ND) and type 2 diabetes patients (T2D).

Parameters	Means ± SE		
	ND	*P*	T2D
Age (years)	51.6 ± 2.9	ns	57.9 ± 2.4
Body mass index (kg/m^2)	26.8 ± 0.9	< 0.05	30.8 ± 1.3
Fasting glucose (mg/dL)	90.3 ± 1.8	< 0.05	148.4 ± 7.2
Abdominal circumference (cm)	85.3 ± 3.8	< 0.05	107.0 ± 5.9
HbA_{1c} (%)	5.9 ± 0.14	< 0.05	8.2 ± 0.3
Triglycerides (mg/dL)	116.8 ± 9.9	ns	143.4 ± 13.6
Total cholesterol (mg/dL)	194.4 ± 7.6	ns	183.8 ± 9.5
LDL cholesterol (mg/dL)	119.5 ± 5.8	ns	105.2 ± 5.7
HDL cholesterol (mg/dL)	54.7 ± 2.8	ns	48.0 ± 2.2
Creatinine (mg/dL)	0.82 ± 0.07	< 0.05	1.21 ± 0.15
Urea (mg/dL)	33.9 ± 2.2	ns	43.2 ± 5.8

LDL/HDL: low-/high-density lipoprotein; ns: non-significant.

2.2. Reagents

LPS from *Escherichia coli* (catalogue number: L4391) and the monoclonal antibodies anti-TLR4 (catalogue number: WH0007099M1) and anti-RAGE (catalogue number: R5278) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Antibodies were endotoxin-free.

2.3. Separation of peripheral blood mononuclear cells

PBMNC were purified from 10.0 mL of heparinized venous blood, using a Ficoll-Hypaque gradient as described by Bicalho et al. [20], but with slight modifications. Cellular viability of each sample was always greater than 95%, as determined by the trypan blue exclusion test.

2.4. Quantification of interleukin-6 and tumour necrosis factor-alpha

Aliquots (100 μL) of a suspension of PBMNC (1×10^6/1 mL) from T2D patients and ND in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), were incubated in the presence or absence of LPS (50 μL/50 μg), anti-TLR4 (100 μL/100 ng)

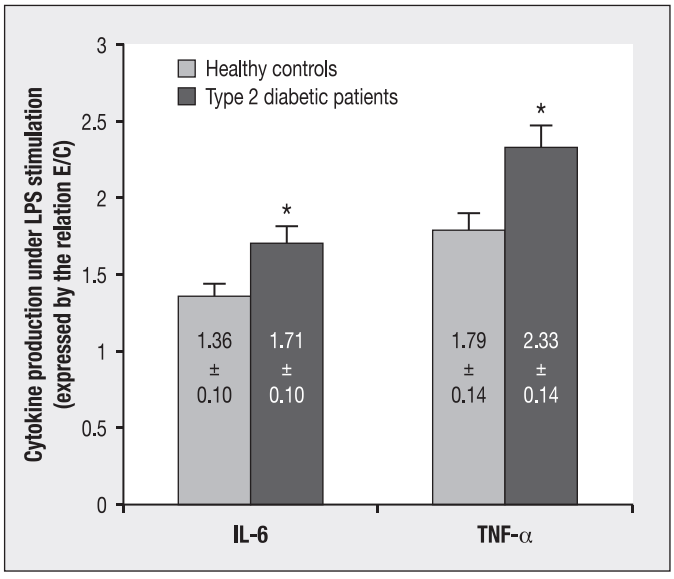

Fig. 1. Peripheral blood mononuclear cells (PBMNC) from patients with type 2 diabetes produce more interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) with lipopolysaccharide (LPS; from *Escherichia coli*) activation than do PBMNC from healthy controls. *$P < 0.05$ *vs* healthy controls. Baseline levels of cytokine production are expressed as pg/mL ± SE: IL-6, diabetics = 903 ± 114, controls = 969 ± 131; TNF-α, diabetics = 126 ± 9, controls = 181 ± 14; $n = 20$ for each group.

or anti-RAGE (100 μL/100 ng) for 72 hours at 37° C under 5% CO_2. The final volume was adjusted to 300 μL in DMEM supplemented with 10% FBS. After incubation, the cells were centrifuged and the supernatant collected. Concentrations of interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) were measured, using sandwich enzyme-linked immunosorbent assay (ELISA) kits (Assay Designs, Ann Arbor, MI, USA). Plates were read at 450 nm on an automated ELISA plate reader, and levels of cytokines were determined using standard curves constructed with recombinant IL-6 and TNF-α.

2.5. Statistical analyses

Data were expressed as E/C [where E = supernatant of cell culture in the presence of activators (LPS, anti-TLR4 or anti-RAGE) divided by C (supernatant of cell culture in the absence of activators)]. Mean values ± standard error (SE) were used in the figures and legends. Comparison of data between groups was performed with Origin 6.0 software (Microcal Software Inc., Northampton, MA, USA), using unpaired Student's *t* tests in the analyses. In each case, $P < 0.05$ was considered significant.

3. Results

3.1. PBMNC from T2D are more sensitive to LPS than those from ND

As shown in Fig. 1, LPS activated the secretion of IL-6 and TNF-α in PBMNC from both T2D and ND. However, activation of cytokine production was higher in T2D ($P < 0.05$) than in ND. The results, expressed as E/C ± SE were: IL-6, ND: 1.36 ± 0.10, T2D: 1.71 ± 0.10; TNF-α, ND: 1.79 ± 0.14, T2D: 2.33 ± 0.14.

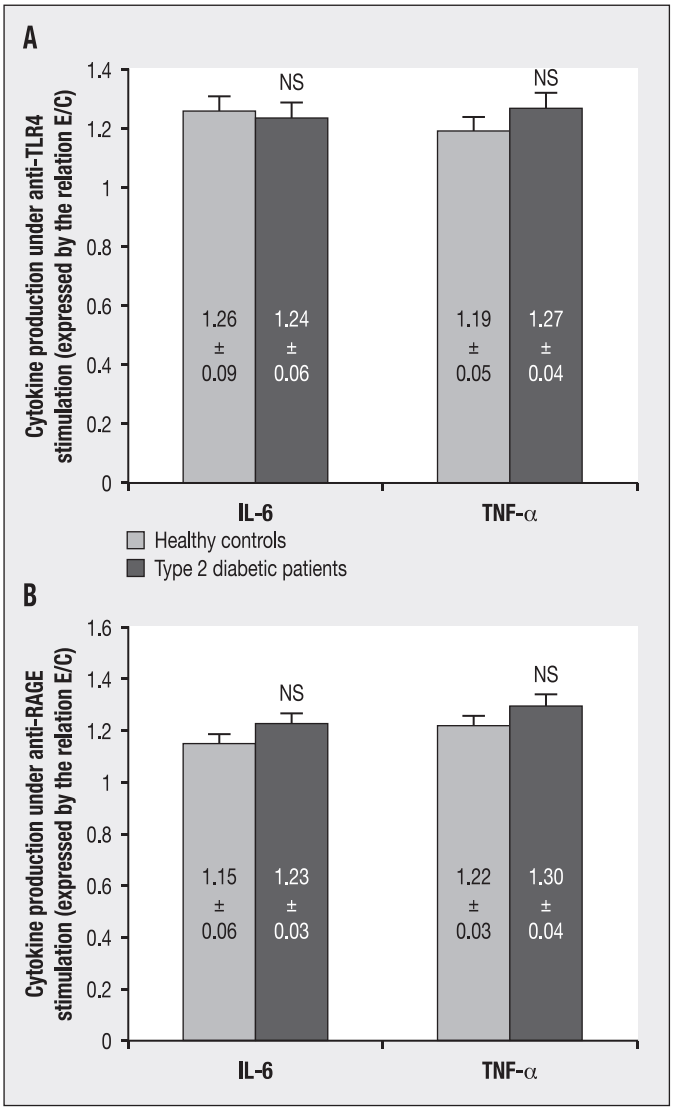

Fig. 2. Monoclonal anti-TLR4 and anti-RAGE antibodies similarly activate cytokine production in peripheral blood mononuclear cells (PBMNC) from type 2 diabetics and healthy controls. A. Activation with anti-TLR4. B. Activation with anti-RAGE; ns: non-significant ($P > 0.05$); IL-6: interleukin-6; TNF-α: tumour necrosis factor-alpha. Baseline levels of cytokine production are expressed as pg/mL ± SE: IL-6, diabetics = 1286 ± 121, healthy controls = 1508 ± 153; TNF-α, diabetics = 113 ± 11, controls = 174 ± 15; $n = 20$ for each group.

3.2. Anti-TLR4 and anti-RAGE similarly activate cytokine production of PBMNC from T2D and ND

Fig. 2 shows that anti-TLR4 (A) and anti-RAGE (B) activated IL-6 and TNF-α production of similar intensity in PBMNC from both T2D and ND ($P > 0.05$). The results, expressed as E/C ± SE were: (A) anti-TLR4 (IL-6), ND: 1.26 ± 0.09, T2D: 1.24 ± 0.06 ($P > 0.05$); (TNF-α), ND: 1.19 ± 0.05, T2D: 1.27 ± 0.04 ($P > 0.05$); (B) anti-RAGE (IL-6), ND: 1.15 ± 0.06,

T2D: 1.23 ± 0.03 ($P > 0.05$); (TNF-α), ND: 1.22 ± 0.03, T2D: 1.30 ± 0.04 ($P > 0.05$).

3.3. Differential effects of anti-TLR4 and anti-RAGE with LPS on cytokine production by PBMNC from T2D and ND

Fig. 3 shows that LPS activated IL-6 and TNF-α production independent of the presence of antibodies (anti-TLR4 and anti-RAGE). However, anti-TLR4 (A and B), but not anti-RAGE (C and D), together with LPS showed a significant difference between T2D and ND ($P < 0.05$).

3.4. PBMNC from T2D are more sensitive to anti-TLR4 and anti-RAGE activation than PBMNC from ND

The results with simultaneous activation of anti-TLR4 and anti-RAGE are shown in Fig. 4A and B. PBMNC from ND activated one receptor at a time whereas, in T2D, there was synergy between anti-TLR4 and anti-RAGE when added at the same time. The results, expressed as E/C ± SE were: (IL-6), ND: 1.10 ± 0.09, T2D: 1.42 ± 0.05; (TNF-α), ND: 1.22 ± 0.04, T2D: 1.52 ± 0.06. There was a statistically significant difference ($P < 0.05$) between T2D and ND with simultaneous activation of both antibodies.

4. Discussion

Our results suggest similar metabolic responses for both TLR4 and RAGE in inducing proinflammatory cytokine secretion by PBMNC. The production of IL-6 and TNF-α were both increased in the presence of LPS, although it was significantly greater in cultured PBMNC from T2D than from ND subjects (Fig. 1). LPS is one of the best studied immunostimulatory components of bacteria, and a known cause of systemic inflammation [21,22], inducing the expression of TNF-α, IL-1, IL-6, inducible nitric oxide synthase (iNOS) and others that are target genes of NF-kappa-B in the TLR4 pathway [23].

TLR expression is increased in many inflammatory disorders, including atherosclerosis and diabetes [24–27]. Dasu et al. [28] reported an increase in TLR2 and TLR4 mRNA in diabetic patients. Buraczynska et al. [29] reported an association between polymorphisms in the TLR4 gene and early onset of diabetic retinopathy in T2D patients. Ghanin et al. [30] demonstrated that insulin exerts an anti-inflammatory effect by suppressing TLR expression. Thus, the insulin-resistant state that occurs in T2D can promote an increase in TLR expression and facilitate TLR-mediated signal transduction pathways. Our present results demonstrate significantly increased IL-6 and TNF-α production ($P < 0.05$) in the anti-TLR4-induced supernatant of PBMNC from either T2D or ND, but we were not able to discriminate between groups (Fig. 2A). However, the simultaneous activation of LPS and anti-TLR4 (Fig. 3A and B) was able to discriminate between T2D and ND. PBMNC from the former were more sensitive to both stimuli in comparison to ND ($P < 0.05$). These results are in agreement with Dasu et al. [31], who demonstrated exacerbation of TLR4 from cells under hyperglycaemic conditions, which suggests an important role for hyperglycaemia in the exacerbation of proinflammatory cytokine secretion, and in the synergistic effects of LPS and TLR4.

Our present results with simultaneous activation of LPS and anti-TLR4 (Fig. 3A and B) suggest that LPS uses the TLR4 epitope in addition to others, as the activation seen with anti-TLR4 (before adding LPS) was similar to that with LPS alone. The anti-TLR4 antibodies were not able to block activation-induced LPS, possibly due to the multiligand property of the LPS molecule, compared with the monoclonal antibody against TLR4, which binds to a specific epitope receptor, although both bind to the same TLR4 molecule. However, there is no direct evidence to support this hypothesis. Triantafilou et al. [32] suggested that LPS, which is recognized by TLR4, may also be recognized by other receptors. Our present results may also be explained by a polymorphism in the TLR4 gene. Arbour et al. [33] observed that patients, whether heterozygous or homozygous for two different single nucleotide polymorphisms ($Asp^{299}Gly$ and $Thr^{399}Ile$) of TLR4, are hyporesponsive to a challenge with LPS. In addition, Shi et al. [6] observed raised TLR4 mRNA expression in the adipose tissue of obese diabetic mice.

Lin [14] suggested that RAGE functions as a "non-canonical Toll" that binds AGEs and other endogenous pattern-recognition ligands, thus triggering inflammation. On this basis, PBMNC were cultured using anti-RAGE instead of anti-TLR4. RAGE can induce a range of transduction pathways that lead to translocation of transcription factor NF-kappa-B [34–37], and also appears to modulate the behaviour of molecules linked to the inflammatory response [36]. Our present results strongly suggest that anti-RAGE may act as a TLR, activating several signaling pathways and NF-kappa-B to produce proinflammatory cytokines. The results (Fig. 2B) are similar to those observed with anti-TLR4 (Fig. 2A). Also, anti-RAGE activated IL-6 and TNF-α production by PBMNC from T2D and ND in a similar manner ($P > 0.05$). These results are in agreement with Lotze and Tracey [38], who reported that RAGE and TLR4 have common signaling pathways.

Simultaneous activation with anti-RAGE and LPS (Fig. 3C and D) showed the same response as with anti-TLR4 and LPS. In both cases, LPS activated IL-6 and TNF-α production independent of the presence of the antibodies (anti-TLR4 and anti-RAGE). However, simultaneous activation with anti-RAGE and LPS (Fig. 3C and D) was not able to discriminate T2D from ND, as was observed with anti-TLR4 (Fig. 3A and B).

To evaluate both signaling pathways (RAGE and TLR4), an experiment using simultaneous activation with both anti-TLR4 and anti-RAGE was also performed. The results, shown in Fig. 4A and B, reveal the absence of activation of the TLR4 complex in ND, and a synergistic effect of both antibodies on cells from T2D that was able to discriminate between groups. The exacerbated response seen in T2D may be explained by hyperglycaemia-induced cellular metabolic adaptation or due to higher levels of endotoxin in their plasma, as reported by Dasu et al. [28]. High levels of endotoxin have also been reported by others [39,40], who observed an increase in LPS, and LPS-binding protein (LBP) concentrations and TLR4 expression, after ingestion of high-fat, high-carbohydrate meals or saturated fat by healthy subjects. In this context, an increase in free fatty

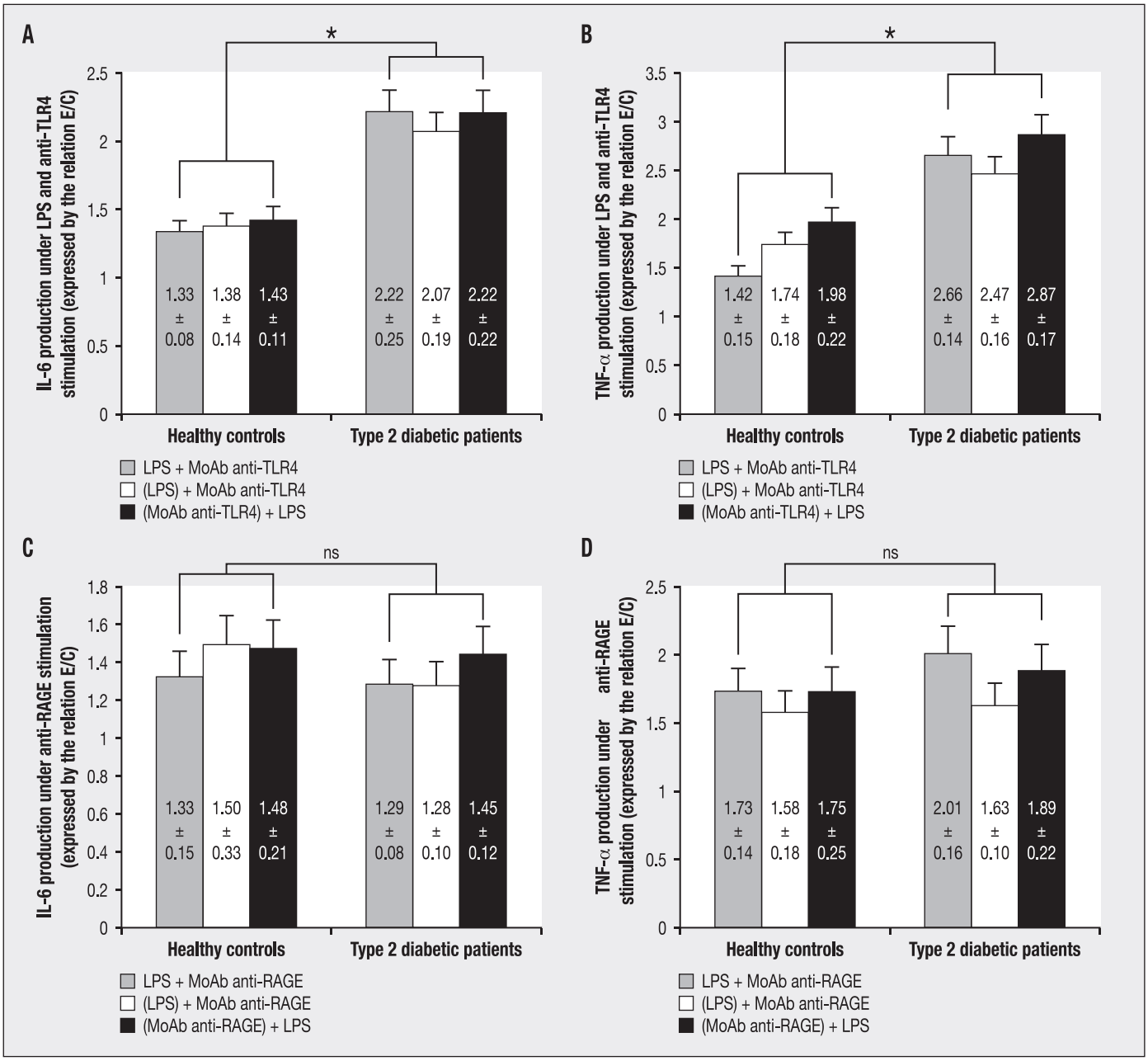

Fig. 3. Anti-TLR4 and anti-RAGE antibodies do not block lipopolysaccharide (LPS; from *Escherichia coli*)-induced cytokine production. IL-6: interleukin-6; TNF-α: tumour necrosis factor-alpha; MoAb: monoclonal antibody. LPS + antibody (anti-TLR4 or anti-RAGE): LPS and antibody were added to cell culture simultaneously; (LPS) + antibody (anti-TLR4 or anti-RAGE): LPS was added to cell culture 30 min before the antibody; (antibody; anti-TLR4 or anti-RAGE) + LPS: antibody was added to cell culture 30 min before LPS. A/C. IL-6 production; (B/D): TNF-α production; *$P < 0.05$, diabetics *vs* controls; ns: non-significant ($P > 0.05$), diabetics *vs* controls. Baseline levels of cytokine production expressed as pg/mL ± SE: (A) IL-6, diabetics = 1237 ± 144, controls = 1200 ± 210; (B) TNF-α, diabetics = 113 ± 11, controls = 174 ± 15; (C) IL-6, diabetics = 1033 ± 181, controls = 1340 ± 156; (D) TNF-α, diabetics = 167 ± 18, controls = 242 ± 30; $n = 10$ for each group.

acids in plasma results in an acute increase in the generation of intranuclear and total NF-kappa-B and reactive oxygen species (ROS), thereby inducing inflammation [41]. Indeed, Ghanim et al. [42] demonstrated that free fatty acids are modulators of inflammation; they also observed that the PBMNC from obese individuals are in a proinflammatory state.

Many authors have focused on the high mobility group box family of proteins (HMGB), which comprises four groups: HMGB1; HMGB2; HMGB3; and HMGB4 [43]. HMGB1 was recently revealed to be an alarmin that alerts the immune system to initiate host defenses or tissue repair [44]. It also stabilizes nucleosome formation and acts as a transcription-factor-like protein that regulates the expression of several genes [45–47]. Interaction of HMGB1 with TLR2 and TLR4 might therefore enable HMGB1 to promote inflammatory responses similar to those of LPS. There is a partial overlap in genes induced after treatment of neutrophils with either HMGB1 or LPS, suggesting that they may be sharing the same receptor—TLR4 [47,48]. Moreover, various activation signals (such as LPS) and proinflammatory stimuli can activate monocytes and tis-

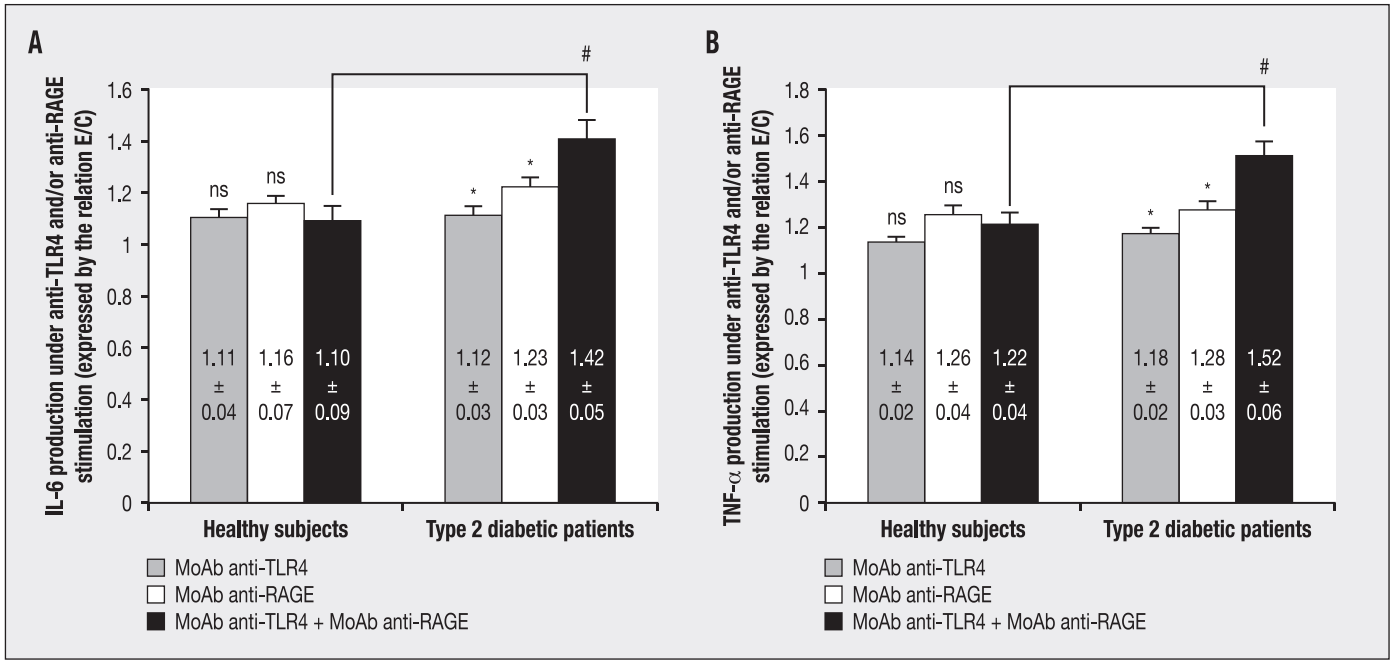

Fig. 4. Effect of anti-TLR4 and anti-RAGE antibodies on (A) interleukin-6 (IL-6) and (B) tumour necrosis factor-alpha (TNF-α) production by peripheral blood mononuclear cells (PBMNC) from type 2 diabetics and healthy controls. MoAb: monoclonal antibody (anti-TLR4, anti-RAGE). *$P < 0.05$ *vs* using both antibodies (anti-TLR4 + anti-RAGE); ns: non-significant ($P > 0.05$) *vs* using both antibodies (anti-TLR4 + anti-RAGE). Baseline levels of cytokine production expressed as pg/mL ± SE: IL-6, diabetics = 1577 ± 162, controls = 1325 ± 314; TNF-α, diabetics = 206 ± 27, controls = 354 ± 15; $n = 8$ for each group.

sue macrophages to secrete HMGB1 [49]. The presence of NF-kappa-B binding sites in the RAGE promoter creates a positive-feedback loop through increasing RAGE expression [15]. As such, HMGB1-mediated stimulation of RAGE may amplify this response by up-regulating RAGE expression [37]. These results, in conjunction with our present data (Fig. 4), reinforce our observation of a greater response in cells from T2D patients. If LPS does indeed mimic HMGB1, it could activate TLR4 and RAGE simultaneously. As shown in Fig. 4, cells from T2D appear to activate both receptors, thus producing more proinflammatory cytokines. However, this did not occur with ND. Does this mean that HMGB1 is the physiological activator of TLR4–RAGE complexes *in vivo*?

5. Conclusion

Our results suggest that TLR4 and RAGE are distinct receptors that use similar metabolic pathways for proinflammatory cytokine production. PBMNC from T2D patients have alterations in their reactivity that are possibly hyperglycaemia-induced, being more sensitive to LPS and anti-TLR4. It may also suggest exacerbated innate immunity with consequences for the chronic inflammation seen with T2D.

Conflicts of interest statement

The authors have not declared any conflicts of interest.

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