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LPS-mediated production of pro/anti-inflammatory cytokines and eicosanoids in whole blood samples: Biological effects of +896A/G TLR4 polymorphism in a Sicilian population of healthy subjects

Carmela Rita Balistreri *, Calogero Caruso, Florinda Listì, Giuseppina Colonna-Romano, Domenico Lio, Giuseppina Candore

Immunosenescence Group, Department of Pathobiology and Medical and Forensic Biotechnologies, University of Palermo, Palermo, Italy

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ABSTRACT

Toll-like receptors (TLRs) are the principal mediators of rapid microbial recognition: the lipopolysaccharide (LPS) receptor TLR4 seems to have a paradigmatic role. Single nucleotide polymorphisms (SNPs) in the TLR4 gene, such as +896A/G, known to attenuate receptor signaling, have been described. The +896A/G SNP is significantly less frequent in patients with myocardial infarction, Alzheimer's disease or prostate cancer, whereas it is overrepresented in centenarians. To clarify and confirm the biological effects of +896A/G SNP and its role in the pathophysiology of age-related diseases and longevity, we assessed the levels of IL-6, TNF-α, IL-10 and eicosanoids (LTB4 and PGE2) in LPS-stimulated whole blood samples *in vitro* of 50 young healthy Sicilians, screened for the presence of this SNP. To evaluate the possible influence of SNPs in PTGS2 and 5-Lo genes on eicosanoid production, the enrolled individuals were also genotyped for −765G/C PTGS2 and −1708G/A 5-Lo SNPs. Both pro-inflammatory cytokines and eicosanoids were significantly lower in carriers bearing the TLR4 mutation, whereas the anti-inflammatory IL-10 values were higher. On the basis of data reported herein, some suggestions can be drawn. First, pathogen load, by interacting with the host genotype, determines the type and intensity of inflammatory responses, according to the pro-inflammatory status and tissue injury, implicated in the pathophysiology of major age-related diseases. Second, adequate control of inflammatory response might reduce the risk of these diseases, and, reciprocally, might increase the chance of extended survival in an environment with reduced antigen (that is, pathogen) load.

1. Introduction

Genetic variations in innate immune response are thought to influence the risk of several human diseases by altering host response to environmental stress (Lin et al., 2006; Goldstein, 2009; Hirschhorn, 2009; Kraft and Hunter, 2009). Good examples, under current examination, are innate immune pathways. Among these, TLR4, the key receptor for LPS, provides an ideal model. It permits the study of the consequences of its genetic variations and their relation to the function of the receptor pathway, and their implications in the risk of atherosclerosis, Alzheimer's disease (AD) and cancer (Balistreri et al., 2009; Beutler, 2002; Uematsu and Akira, 2008). A SNP, Asp299Gly or +896A/G (rs:4986790), has been identified in the TLR4 gene, encoding single amino-acid substitution in the extra-cellular receptor domain (Arbour et al., 2000). This SNP induces a blunted innate/inflammatory response to both foreign pathogens and endogenously generated inflammatory ligands (Arbour et al., 2000; Balistreri et al., 2009; Schröder and Schumann, 2005). In particular, it may influence inflammatory responses and the risk of major age-related diseases affecting the production of inflammatory mediators.

Cytokines and eicosanoids represent the key regulators of innate/inflammatory response. High serum or plasma levels of these molecules have been correlated with the major age-related diseases, confirming their role in atherosclerosis, AD and cancer pathophysiology (Akiyama et al., 2000; Boyce, 2008; Bruunsgaard et al., 2001; Candore et al., 2007a,b; Caruso et al., 2009; Krabbe et al., 2004; Leon, 2007; Rogers, 2008; Sheu et al., 2008). However, the magnitude of cytokine and eicosanoid production has been shown to vary individually and is likely based on genetic heterogeneity (Bucova et al., 2008; Candore et al., 2007a,b; Caruso et al., 2009; Grimble, 2003; Rea et al., 2006; Reyes-Gibby et al., 2008). This might determine changes in the innate/inflammatory

* Corresponding author at: Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi dell', Università di Palermo, Corso Tukory 211, Palermo 90134, Italy. Tel.: +39 0916555911; fax: +39 0916555933.
E-mail address: crbalistreri@unipa.it (C.R. Balistreri).

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response and, consequently, in tissue injury and the processes involved in the development and progression of age-related diseases (Bucova et al., 2008; Candore et al., 2007a,b; Caruso et al., 2009; Grimble, 2003; Rea et al., 2006; Reyes-Gibby et al., 2008). Hence, certain people develop diseases and others remain healthy. One or more functional SNPs in one or more genes might be responsible. Accordingly, recent studies have suggested the role of +896A/G TLR4 SNP in cytokine and eicosanoid production (Arbour et al., 2000; Ferwerda et al., 2007, 2008; Hoshino et al., 1999; Michel et al., 2003; Norata et al., 2005; Poltorak et al., 1998; von Aulock et al., 2003; Werner et al., 2003). On the other hand, an association of this TLR4 SNP with an increased risk of Gram negative infections and septic shock has been demonstrated (Agnese et al., 2002; Lorenz et al., 2002; Schröder and Schumann, 2005). However, contrasting data have been obtained in other studies (Child et al., 2003; Erridge et al., 2003; Read et al., 2001; Heesen et al., 2003; Imahara et al., 2005; Schippers et al., 2005; von Aulock et al., 2003; Yang et al., 2004).

Changes in eicosanoid levels are also correlated with SNPs in the promoter region of PTGS2 and 5-Lo genes, respectively codifying the cyclooxygenase-(Cox)-2 and 5-lipoxygenase (5-Lo), enzymes involved in arachidonic acid metabolism. Two functional (−765G/C PTGS2 and −1708G/A 5-Lo) SNPs have recently been identified and associated with the major age-related diseases (Candore et al., 2007a,b; Caruso et al., 2009; Cipollone et al., 2004; In et al., 1997; Orbe et al., 2006).

To clarify and confirm the possible pathophysiological effects of +896A/G TLR4 SNP (Balistreri et al., 2004, 2008, 2010), we analysed the levels of IL-6, TNF-α, IL-10 and eicosanoids (LTB4 and PGE2) in LPS-stimulated whole blood samples *in vitro* of 50 young healthy Sicilians, screened for the presence of +896A/G TLR4 SNP. To evaluate the possible influences of SNPs in PTGS2 and 5-Lo genes on eicosanoid production, we also screened the enrolled individuals for the −765G/C PTGS2 and −1708G/A 5-Lo SNPs. Hence, our idea was to test whether the three SNPs have a major influence on the production of inflammatory mediators, when they operate in combination to create a "risk profile," as suggested in our previous studies (Candore et al., 2007a,b; Caruso et al., 2009).

2. Materials and methods

2.1. Population studied and TLR4, PTGS2 and 5-Lo genotyping

The study included 50 Sicilian Caucasoids from Palermo and neighbourhood (age range: 25–50 years; 24 females and 26 males), in good health according to their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, proteins). They have previously been genotyped for the following SNPs: +896A/G TLR4 SNP, −765G/C PTGS2 SNP (rs:20417) and −1708 G/A 5-Lo SNP (no available rs designation) (Table 1). The procedures for detecting these SNPs have previously been described (Balistreri et al., 2005; Listì et al., 2008). Of 50 individuals, 40 were homozygous for wild-type alleles of +896A/G TLR4 SNP, and 10 had one or two +896G alleles. Furthermore, 30 out 40 were also homozygous for wild-type alleles of −765G/C PTGS2 and −1708G/A 5-Lo SNPs, and of 10 carriers with one or two +896A/G alleles, 6 also had one or two −765C PTGS2 and −1708A 5-Lo alleles.

The study received approval from local ethics committees and all participants gave their informed consent.

2.2. Whole blood assay

Venous EDTA blood samples were collected from all subjects under basal conditions at 9.00 am. Within 4 h, using aseptic handling, the first series of blood aliquots (900 μl) of each sample was placed in sterile 1.5 ml Eppendorf tubes and stimulated with 1 γ/ml of *Escherichia coli* LPS (serotype 055:B5, L6529-1MG, Sigma–Aldrich, St. Louis, MO, USA). A second series of blood aliquots (900 μl) of the same samples was not stimulated with LPS. Both series of blood aliquots were gently vortexed for 5 s and incubated with open lids for 4, 24, and 48 h at 37 °C, in 5% CO_2 and humidified air. The samples were incubated for different times (4, 24 and 48 h), because preliminary results showed different kinetics in the several assays. A different relationship between the TLR4 allele effect and times of incubation (4, 24 and 48 h) was, indeed, observed. After induction, 500 μl of RPMI-1640 (with 25 mM Hepes and L-glutamine, Gibco, Breda, The Netherlands) was added to each sample. The samples were briefly vortexed and centrifuged for 3 min at 10,000 rpm and the supernatant (900 μl) was transferred to new Eppendorf tubes. The samples were then flash frozen in ethanol and dry ice and stored at −80 °C, until the enzyme-linked immunosorbent assay (ELISA) test was performed.

2.3. LPS contaminations: Limulus Amebocyte Lysate (LAL)-chromo end-point test

To detect and quantify the probable LPS contaminations in supernatants of the cultures under baseline conditions (LPS unstimulated) from both low (+896G+) and high (+896A+) responder cells at 4, 24 and 48 h, the Limulus Amebocyte Lysate (LAL)-chromo end-point test was utilised (International PBI, Milano, Italy). The test was performed according to the manufacturer's instructions. Results were expressed as pg/ml. Detection limit in our laboratory was 0.005 pg/ml.

2.4. Cytokine and eicosanoid assays

IL-6, TNF-α, IL-10, LTB4 and PGE2 supernatant levels were determined by ELISA (R&D System, Abingdon, Oxon, UK). All tests were performed according to the manufacturer's instructions. To standardize our results, reference preparations of IL-6, TNF-α, IL-10, LTB4 and PGE2 were tested in all assays. Results were expressed as pg/ml. Detection limits in our laboratory were 1.6 pg/ml for IL-6, 2.1 pg/ml for TNF-α, 3.9 pg/ml for IL-10, 18.2 pg/ml for PGE2 and 13.7 pg/ml for LTB4.

2.5. Statistical analysis

LPS levels were expressed as median (range). To detect the possible correlations between the subliminal LPS levels and the production of cytokines, the non-parametrical Spearman correlation test was used. The quantitative values of the cytokines and eicosanoids were expressed as mean ± SD and presented on a log scale in the figures. To assess the differences in cytokine and eicosanoid production between low and high responder cells, the analysis of variance (ANOVA) test (corrected by Bonferroni) was utilised. Values of $p < 0.05$ were considered significant. To identify the possible correlations between the mediator levels and times of incubation, the non-parametrical Spearman correlation test was also used.

3. Results

3.1. LPS contamination

LPS contamination in supernatants of cell cultures at baseline conditions from both low (+896G+) and high (+896A+) responder subjects was detected at all times. LPS levels were 0.142 pg/ml (0.102–0.208). A positive correlation was observed between LPS and IL-6 and TNF-α levels in supernatants of cell cultures from subjects with high (+896A+) responder SNP ($n = 40$, $r = 0.433$, $p < 0.001$ and $r = 0.33$, $p < 0.013$, respectively, data not shown). Furthermore, a negative correlation was found between LPS and IL-10 levels in supernatants of cultures under baseline conditions from high (+896A+) responder cells, consistent with the reduced

Table 1
Genes (accession number), SNPs (accession number), and substitutions investigated in the study.

Genes	SNPs	Biological effect
TLR4 (NM-138554.1)	+896A/G (Asp299Gly; rs4986790)	Determining a single amino acid substitution in the extracellular receptor domain and, hence, a blunted innate/inflammatory response to both foreign pathogens and endogenously generated inflammatory ligands (Balistreri et al., 2009)
PTGS2 (NM-000963) (Cox-2 gene)	−765G/C (rs20417)	Located within a putative binding site for the transcription factor Sp1, determining a reduction in the expression of Cox-2 enzyme (Cipollone et al., 2004; Orbe et al., 2006)
5-Lo (NM-000698)	−1708G/A (no rs available designation)	Modifying gene transcription with an increased protein expression (In et al., 1997)

release of IL-10 observed in these high (+896A+) responder cell cultures ($n = 10$, $r = -0.203$, $p = 0.023$, data not shown). Thus, the possible reasons for this correlation might be the important contribution of biological effect of the SNP.

3.2. Data of cytokine production

Significant differences in IL-6 and TNF-α levels were obtained at all times and both at baseline conditions and after LPS stimulation between cell cultures from subjects with wild-type allele and those with the +896G allele (Fig. 1A and B). In particular, significant higher levels of both pro-inflammatory cytokines at all times and at baseline conditions and after LPS stimulation were detected in supernatants of cell cultures from individuals with the +896A wild type allele respect to those detected in the supernatant of cell cultures from subjects with +896G allele at baseline conditions and after LPS stimulation. This comparison demonstrated the following significant differences: 5.22 ± 1.5 at 4 h, 9.49 ± 1.4 at 24 h, 12.66 ± 2.1 at 48 h pg/ml IL-6 levels at baseline conditions and 631 ± 17.9 at 4 h, 823 ± 12.1 at 24 h, 887 ± 14.2 at 48 h pg/ml IL6 after LPS stimulation for +896A+ cells vs. 1.71 ± 0.5 at 4 h, 2.15 ± 0.7 at 24 h, 1.62 ± 0.2 pg/ml IL-6 levels at baseline conditions and 439 ± 22.6 at 4 h, 555 ± 23.4 at 24 h, 539 ± 21.2 at 48 h for +896G+ cells; 7.71 ± 1.27 at 4 h, 19.45 ± 3.73 at 24 h and 20.23 ± 5.1 at 48 h pg/ml TNF-α levels at baseline conditions and 779.35 ± 15.1 at 4 h, 581.82 ± 32.2 at 24 h, 480.94 ± 30 at 48 h for +896A+ cells vs. 2.46 ± 0.9 at 4 h, 6.78 ± 1.1 at 24 h, 12.34 ± 3.8 at 48 h pg/ml TNF-α levels at baseline conditions and 296.25 ± 13.8 at 4 h, 344.4 ± 28.2 at 24 h, 239 ± 17.1 at 48 h pg/ml TNF-α levels after LPS stimulation for +896G+ cells.

Furthermore, the comparison of IL-10 levels between the cultures with +896G allele and those with wild type allele at baseline conditions or after LPS stimulation showed significant differences (Fig. 1C). In particular, significant higher levels of the IL-10 anti-inflammatory cytokine were detected at all times in supernatants of cell cultures from subjects with +896G allele at baseline conditions (4.96 ± 2.21 at 4 h, 7.81 ± 1.6 at 24 h, 10.15 ± 4.9 at 48) and after LPS stimulation (9.53 ± 4.6 at 4 h, 11.32 ± 3 at 24 h and 8.04 ± 3.28 at 48 h) respect to those observed in the supernatants of the cell cultures from subjects with +896A wild type allele at baseline conditions (2.87 ± 0.5 at 4 h, 3.98 ± 2.at 24 h and 3.3 ± 1.1 at 48 h) and after LPS stimulation (3.65 ± 1.6 at 4 h, 4.06 ± 1.7 at 24 h and 3.04 ± 1.4 at 48 h).

In addition, a positive correlation was identified between the IL-6 values and the times of incubation after LPS stimulation in cell cultures with the wild type allele ($r = 0.367$, $p < 0.001$), characterised by a significant different relationship of its levels between 4 and 48 h and a critical peak at 48 h (Table 2). No significant correlations were detected between the other cytokines and the times of incubation after LPS stimulation (data not shown).

3.3. Data of eicosanoid production

The analysis of the eicosanoid levels showed significant higher PGE2 levels at all times among the cell cultures with the wild type allele both at baseline (385 ± 52 at 4 h, 652 ± 75 at 24 h, 686 ± 34 at 48) and LPS stimulated conditions (1235 ± 145.8 at 4 h, 2085 ± 238.9 at 24 h, 3188 ± 312.9). The comparison of PGE2 levels between the cultures with wild type allele and those with +896G allele at baseline conditions (155.8 ± 8 at 4 h, 229 ± 9 at 24 h, 81 ± 8.9) or after LPS stimulation (375 ± 45.8 at 4 h, 455 ± 38.9 at 24 h, 198 ± 12.9) demonstrated, indeed, significant differences (Fig. 2A). In addition, a positive correlation was observed between the PGE2 values and the times of incubation in the stimulated LPS cell cultures with the wild type allele ($r = 0.428$, $p < 0.0001$), characterised by a critical peak at 48 h (Table 2).

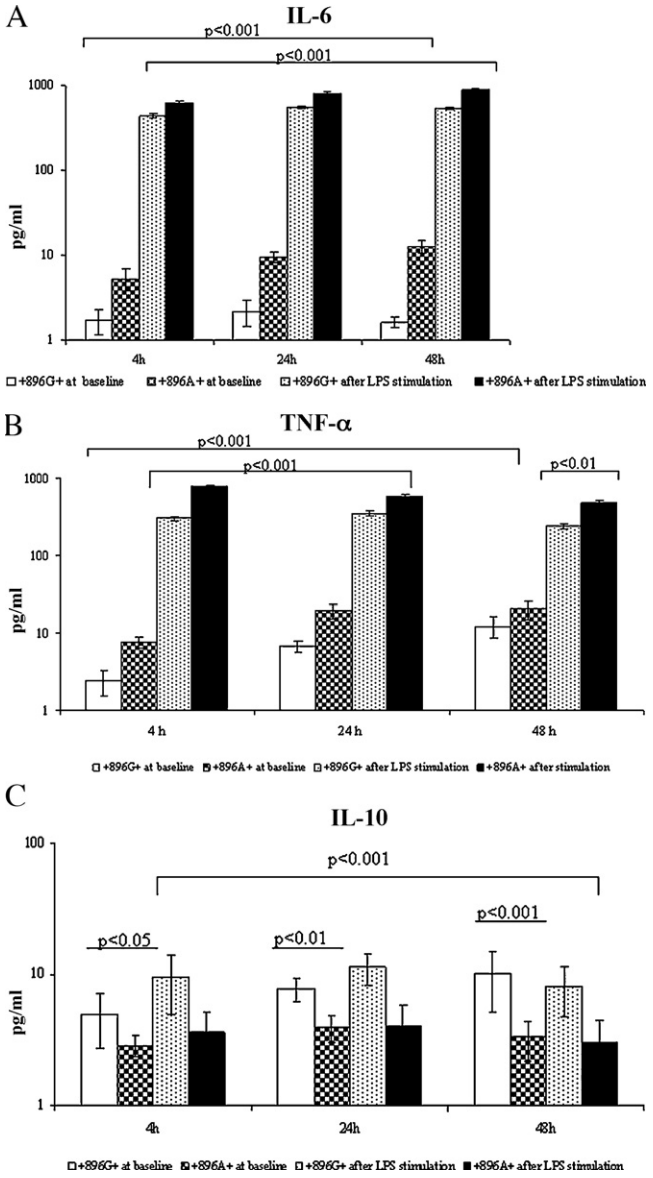

Fig. 1. (A) IL-6 levels (pg/ml) produced by cells, from subjects with the wild-type allele (+896A+) and with the +896G allele (+896G+), at baseline conditions or stimulated for 4, 24, 48 h in whole blood assay with 1 γ/ml LPS. Comparing the IL-6 values between +896G+ vs. +896A+ cells at baseline conditions and after LPS stimulation significant differences were obtained at all times ($p < 0.001$). (B) TNF-α levels (pg/ml) produced by cells, from subjects with the wild-type allele (+896A+) and with the +896G allele (+896G+), stimulated for 4, 24, 48 h in whole blood assay, with 1 γ/ml LPS or at baseline conditions. The comparison of the TNF-α values between stimulated +896G+ vs. +896A+ cells demonstrated significant differences: at 4 h and 24 h ($p < 0.001$), and at 48 h ($p < 0.01$). Comparing the TNF-α levels between the +896G+ cultures and those with +896A allele at baseline conditions, significant differences were observed at all times ($p < 0.001$). (C) IL-10 levels (pg/ml) produced by cells, from subjects with the wild-type allele (+896A+) and with the +896G allele (+896G+), stimulated for 4, 24, 48 h in whole blood assay, with 1 γ/ml LPS or at baseline conditions. Comparing its values between the stimulated +896G+ vs. +896A+ cells significant differences were obtained at all times ($p < 0.001$). Comparing values between +896G+ vs. +896A+ cells at baseline conditions, significant differences were observed: at 4 h ($p < 0.05$), at 24 h ($p < 0.001$) and at 48 h ($p < 0.01$).

Furthermore, the comparison of LTB4 values between the +896G+ (90 ± 6 at 4 h, 119 ± 45.5 at 24 h, 107 ± 41.8 at 48 h) vs. +896A+ (315 ± 45.7 at 4 h, 489 ± 52.5 at 24 h, 345 ± 64.7 at 48 h) cells showed significant differences at baseline conditions (see Fig. 2B). In contrast, the comparison of LTB4 values between the stimulated +896G+ (567 ± 44.9) vs. +896A+ (734 ± 73.8) cells

Table 2
Significant correlations of pro-inflammatory mediator levels in the subjects with wild type allele (+896A+) with the times of LPS stimulation.

LPS stimulation	Levels at 4 h	Levels at 24 h	Levels at 48 h	*r*	*p* values
IL-6	631 ± 17.9	823 ± 12.1	887 ± 14.2	0.367	<0.001
PGE2	1235 ± 145.8	2085 ± 238.9	3188 ± 312.9	0.428	<0.0001
LTB4	423 ± 25.6	505 ± 67.9	734 ± 73.8	0.167	<0.01

demonstrated significant differences only at 48 h. A positive correlation was found between the LTB4 values and the times of LPS incubation in the cultures with the wild type allele ($r = 0.167$, $p < 0.01$).

In order to evaluate the possible biological effects of SNPs in PTGS2 and 5-Lo genes on eicosanoid production, we also screened the enrolled individuals for the −765G/C PTGS2 and −1708G/A 5-Lo SNPs. Accordingly, significant higher PGE2 levels were found among the cell cultures with the −765G wild type allele at all times and both at baseline conditions (380 ± 45.8 at 4 h, 489 ± 38.9 at 24 h, 560 ± 12.9 48 h) and after LPS stimulation (3340 ± 56 at 4 h, 4590 ± 78 at 24 h, 4790 ± 67 at 48 h) respect to those with the −765C variant allele (−765C+) both at baseline conditions (56.2 ± 4.8 at 4 h, 39.9 ± 3.9 at 24 h, 128 ± 12.9 at 48 h) and stimulated (310 ± 7.8 at 4 h, 369 ± 11.9 at 24 h, 410 ± 20.8 at 48 h) (Fig. 3A). A positive correlation was observed between the PGE2 levels and the times of LPS incubation in the cell cultures with the −765G wild type allele ($r = 0.201$, $p < 0.01$).

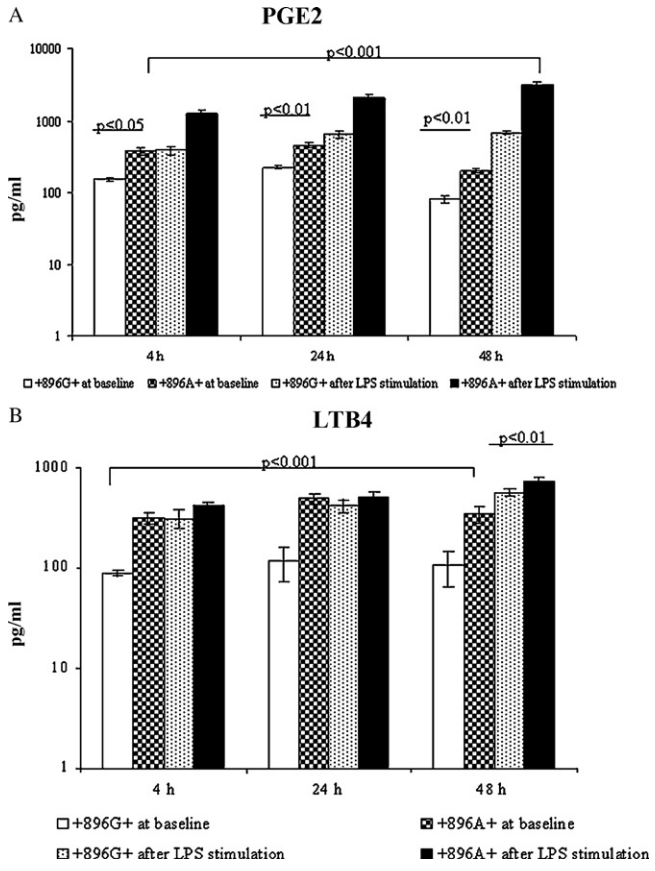

Fig. 2. (A) PGE2 levels (pg/ml) produced by cells, from subjects with the wild-type allele (+896A+) and with the +896G allele (+896G+), stimulated for 4, 24, 48 h in whole blood assay, with LPS (1 γ/ml) or at baseline conditions. The analysis of PGE2 values between stimulated +896G+ vs. +896A+ cells showed significant differences at all times ($p < 0.001$). Comparing its values between +896G+ vs. +896A+ cells at baseline conditions, significant differences were observed: at 4 h ($p < 0.05$), at 24 h ($p < 0.01$) and at 48 h ($p < 0.01$). (B) LTB4 levels (pg/ml) produced by cells, from subjects with the wild-type allele (+896A+) and with the +896G allele (+896G+), stimulated for 4, 24, 48 h in whole blood assay, with LPS (1 γ/ml) or at baseline conditions. Comparing LTB4 values between stimulated +896G+ vs. +896A+ cells, significant differences were only obtained at 48 h ($p < 0.01$). Comparing LTB4 values between +896G+ vs. +896A+ cells at baseline conditions, significant differences were found at all times ($p < 0.001$).

Furthermore, we stratified the subjects studied according to the combined presence of +896A/G TLR4 SNP with the two SNPs in PTGS2 and 5-Lo genes, to test the possible influence of the three SNPs on the eicosanoid release, creating a "risk profile". Accordingly, all possible combinations of the three SNPs have been considered. The comparison of PGE2 levels between +896A + TLR4/−765G + PTGS2 ("high responder" genotype) vs. +896G + TLR4/−765C+ ("low responder" genotype) PTGS2 cells at stimulated (815 ± 145.8 at 4 h, 1085 ± 238.9 at 24 h, 2089 ± 312.9 at 48 h vs. 298 ± 45.8 at 4 h, 397 ± 38.9 at 24 h, 165 ± 12.9 at 48 h, respectively) and baseline conditions (245 ± 52 at 4 h, 542 ± 75 at 24 h, 609 ± 34 vs. 101 ± 8 at 4 h, 193 ± 9 at 24 h, 72 ± 8.9, respectively) showed significant differences at all times (Fig. 4A). The same results were observed by comparing the LTB4 levels between +896A + TLR4/−1708A + 5-LO ("high responder" genotype) vs. +896G + TLR4/−1708G + 5-LO ("low responder" genotype) cells at stimulated (421 ± 67.9 at 4 h, 498 ± 61 at 24 h, 681 ± 44.9 at 48 h vs. 182 ± 45.7 at 4 h, 322 ± 52.5 at 24 h, 296 ± 64.7 at 48 h, respectively) and baseline conditions (212 ± 67.9 at 4 h, 318 ± 61 at 24 h, 468 ± 44.9 vs. 65 ± 6 at 4 h, 102 ± 45.5 at 24 h, 137 ± 41.8 at 48 h) (Fig. 4B).

In addition, positive correlations were observed between the PGE2 and LTB4 levels and the times of LPS stimulation ($r = 0.509$, $p < 0.0001$ and $r = 0.199$, $p < 0.01$, respectively) in the cell cultures with +896A + TLR4/-765G + PTGS2 ("high responder" genotype) and between +896A + TLR4/−1708A + 5-LO ("high responder" genotype), respectively.

4. Discussion

Current evidence supports the involvement of innate immunity and inflammation in the pathophysiology of major age-related diseases (Caruso et al., 2005; Licastro et al., 2005; Vasto et al., 2007). Different mediators, i.e. pro-inflammatory cytokines and eicosanoids, display a crucial role in atherosclerosis, neurodegeneration and carcinogenesis. Their production and release have prevalently been associated with the activation of TLR4 receptor (Balistreri et al., 2009; Candore et al., 2007; Caruso et al., 2009). In other words, the TLR4 receptor, activated by pathogens or endogenous molecules, would have the role of hub in the inflammatory responses involved in the pathophysiology of these diseases (Balistreri et al., 2009; Vasto et al., 2007). On the other hand, this suggestion is supported by a significant correlation between the +896A/G SNP and atherosclerosis, AD and prostate cancer (Balistreri et al., 2004, 2008, 2010).

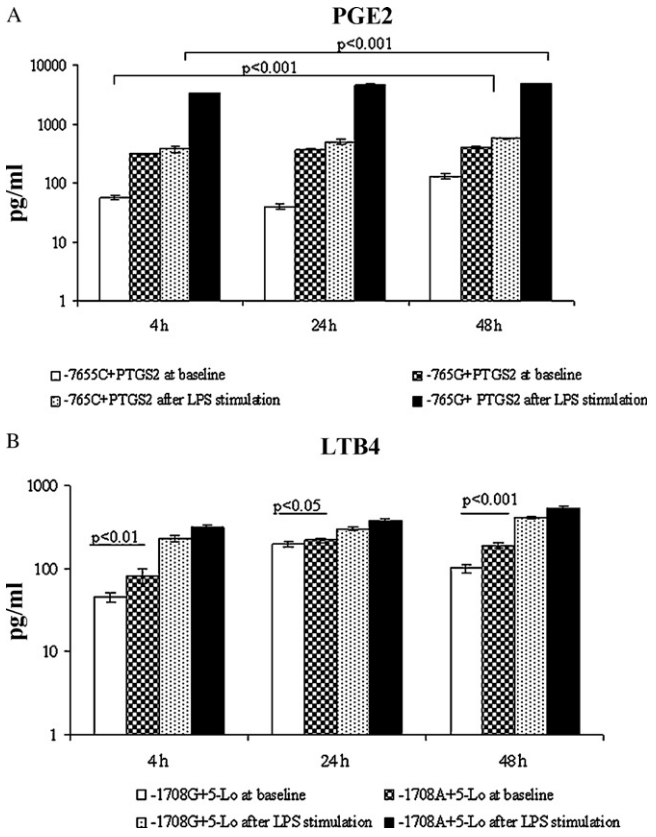

Fig. 3. (A) PGE2 levels (pg/ml) produced by cells, from subjects with the wild-type allele (−765G + PTGS2) and with the −765C allele (−765C+), stimulated for 4, 24, 48 h in whole blood assay, with LPS (1 γ/ml) or at baseline conditions. Significant differences were obtained at all times ($p < 0.001$), comparing the PGE2 values between the cultures with the wild-type −765G allele (−765G+) and those with the −765C variant allele (−765C+) both at stimulated and baseline conditions. (B) LTB4 levels (pg/ml) produced by cells, from subjects with the wild-type allele (−1708G+) and with the −1708A allele (−1708A + 5-Lo), stimulated for 4, 24, 48 h in whole blood assay, with LPS (1 γ/ml) or at baseline conditions. The comparisons of the LTB4 values between the cultures with the wild-type −1708G (−1708G+) and those with the −1708A variant allele (−1708A+) both at stimulated and baseline conditions demonstrated significant differences at all times (precisely at 4 h ($p < 0.01$), 24 h ($p < 0.05$) and 48 h ($p < 0.001$)) and only at baseline conditions.

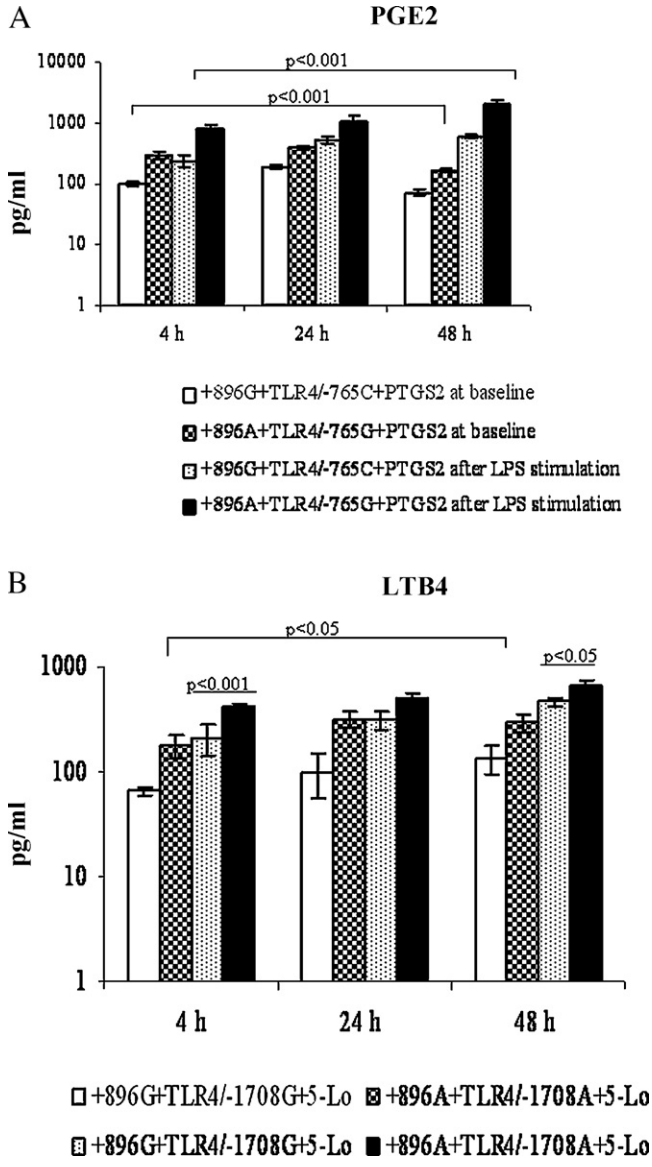

Fig. 4. (A) PGE2 levels (pg/ml) produced by cells, from subjects with the +896A + TLR4/−765G + PTGS2 "high responder" genotype (pro-inflammatory profile) and subjects with +896G + TLR4/−765C + PTGS2 "low responder" genotype (anti-inflammatory profile), stimulated for 4, 24, 48 h in whole blood assay with LPS (1 γ/ml) or at baseline conditions. Comparing the quantitative PGE2 values of cultures both at stimulated and baseline conditions from +896A + TLR4/−765G + PTGS2 vs. +896G + TLR4/−765C + PTGS2 cells, significant differences were observed at all times ($p < 0.001$). (B) LTB4 levels (pg/ml) produced by cells, from subjects with the +896A + TLR4/−1708A + 5-LO "high responder" genotype (pro-inflammatory profile) and subjects with +896G + TLR4/−1708G + 5-LO "low responder" genotype (anti-inflammatory profile), stimulated for 4, 24, 48 h in whole blood assay with LPS (1 γ/ml) or at baseline conditions. The comparison between the cultures from +896A + TLR4/−1708A + 5-LO vs. +896G + TLR4/−1708G + 5-LO cells both at stimulated and baseline conditions demonstrated significant differences at 4 h ($p < 0.001$) and 48 h ($p < 0.05$) after LPS stimulation and at all times at baseline condition ($p < 0.05$).

To further validate our data, by verifying the ability of +896A/G TLR4 SNP to modify the intensity of inflammatory responses, in this report LPS-mediated production of the pro- and anti-inflammatory cytokines (IL-6, TNF-α and IL-10) and eicosanoids (LTB4 and PGE2) has been detected in whole blood samples of 50 healthy Sicilians, genotyped for +896A/G TLR4, −765G/C PTGS2 and −1708G/A 5-Lo SNPs. Significantly higher levels of pro-inflammatory IL-6 and TNF-α cytokines have been produced by cells of individuals with the wild-type +896A TLR4 allele (+896A+). We have also observed significantly higher eicosanoid levels produced in wild-type subjects compared to +896G carriers. Significantly higher levels of LTB4 and PGE2 were also detected, by stratifying the subjects for the presence of PTGS2 and 5-Lo SNPs, or for the combined presence of +896A/G TLR4 SNP and PTGS2 and 5-Lo SNPs. On the other hand, this strengthens our suggestion that pro-inflammatory TLR4 SNPs might have a major influence on the production of inflammatory mediators, when they operate in combination to create a "risk profile" (Balistreri et al., 2008, 2010; Candore et al., 2007a,b; Caruso et al., 2009).

The data obtained confirm a strong effect of +896A/G TLR4 SNP on the production of pro-inflammatory cytokines, as well as in modulating eicosanoid production. They also confirm the important implications of PTGS2 and 5-Lo SNPs in the production of these molecules. On the whole, the data obtained on pro-inflammatory cytokines and eicosanoids are consistent with the findings showing increased levels of these molecules in vessel lesions, senile plaques and tumour tissues (Balistreri et al., 2009; Bostock et al., 2009; Caruso et al., 2009; Davila and Hibberd, 2009; Srivastava et al., 2009; Yandell et al., 2008).

Another interesting result of this study is represented by significantly higher levels of anti-inflammatory IL-10 cytokine produced by cells of carriers of the +896G TLR4 allele. This seems to

evidence the capacity of the +896G TLR4 allele to mediate a better control of inflammatory responses induced by chronic stimuli, so likely decreasing the effects of atherogenetic damage, amyloid peptide insult and prostate carcinogens (Balistreri et al., 2009; Candore et al., 2007; Caruso et al., 2009; Vasto et al., 2007). The principal function of IL-10 is to limit and ultimately to terminate the inflammatory signal. Several lines of evidence indicate a regulatory role of IL-10 in the development and progression of the major age-related diseases (Lio and Caruso, 2006). On the other hand, a higher frequency of +896G TLR4 allele has been observed in centenarians compared to patients affected by the major age-related diseases, as demonstrated in our studies (Balistreri et al., 2004, 2009, 2010). This supports the data obtained in our previous studies showing that genetic background controlling inflammation might play an opposite role in cardiovascular diseases (CVD), AD, prostate cancer and in longevity. People genetically predisposed to weak inflammatory activity, have less chance of developing CVD, AD and prostate cancer and, therefore, without any complication of serious infectious disease, have a chance of living longer (Candore et al., 2007a,b; Caruso et al., 2009; Vasto et al., 2007).

Some literature data agree with the results of our study, while others show contrasting results (Arbour et al., 2000; Child et al., 2003; Erridge et al., 2003; Heesen et al., 2003; Imahara et al., 2005; Michel et al., 2003; Norata et al., 2005; Read et al., 2001; Schippers et al., 2005; Werner et al., 2003; von Aulock et al., 2003; Yang et al., 2004). The different associations between the genotypes of +896A/G SNP and the levels of inflammatory mediators evidenced in different studies may be ascribed to some factors. The first limitation might derive from the small number of individual carriers of TLR4 SNPs enrolled in some studies, with different number of homozygous and heterozygous individuals, compared to the wild-type carriers. The heterozygous and homozygous state is necessary to determine differences in the response to LPS stimulation tested. The cause might be attributed to the reduced frequency of the TLR4 SNPs in the individuals analysed and possibly because they belong to no ethnically homogenous population. A second limitation of these studies might be the different models used. Several factors, such as CD14, LPS-binding protein (LBP) and serum HDL may modulate in the *in vitro* studies changing LPS recognition and binding to TLR4 receptor. Their inter-individual difference in levels may influence the LPS response and the production and release of inflammatory mediators. Other limitations may be ascribed to the *in vitro* method used for the LPS stimulation. The easy and reproducible technique commonly performed in the *in vitro* studies is the whole blood assay. Nevertheless, it presents unfavourable aspects, such as the impossibility of correcting the inter-individual differences in number of leukocytes (in particular of the peripheral blood mononuclear cells) and to reduce the effects of soluble factors, such as CD14, LBP, serum HDL (as above described), able to modify the LPS signaling. In addition, different serotypes of LPS are utilised in the various studies. However, it remains less clear whether they can determine a different response. In contrast, correlations between the different LPS concentrations and the production of inflammatory mediators have been observed in several studies (Erridge et al., 2003; Heesen et al., 2003; Imahara et al., 2005; Schippers et al., 2005; von Aulock et al., 2003).

On the basis of data reported herein, some suggestions can be drawn. Pathogen load, by interacting with the host genotype, determines the type and the intensity of inflammatory responses accountable for pro-inflammatory status and tissue injury, known to be implicated in the pathophysiology of the major age-related diseases, such as CVD, AD and cancer (Balistreri et al., 2009). Second, adequate control of inflammatory response might reduce the risk of these age-related diseases, and, reciprocally, it might increase the chance of extended survival in an environment with reduced antigen (that is, pathogen) load (Balistreri et al., 2009; Candore et al., 2007a; Vasto et al., 2007). Accordingly, a higher frequency of the anti-inflammatory +896G TLR4 allele has been observed in centenarians (Balistreri et al., 2004).

A long life in a healthy, vigorous, youthful body has always been one of humanity's greatest dreams. Recent progress in genetic manipulations and calorie-restricted diets in laboratory animals hold forth the promise that some day science might enable us to exert total control over our biological ageing (Jirillo et al., 2008). However, it is becoming clear that the control of inflammation plays a central role in delaying age-related inflammatory diseases and in the attainment of longevity.

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