Research article

Inflammatory responses associated with acute coronary syndrome up-regulate IRAK-M and induce endotoxin tolerance in circulating monocytes

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Acute coronary syndrome (ACS) groups different cardiac diseases whose development is associated with inflammation. Here we have analyzed the levels of inflammatory cytokines and of members of the TLR/IRAK pathway including IRAK-M in monocytes from ACS patients classified as either UA (unstable angina), STEMI (ST-elevation myocardial infarction) or NSTEMI (non-ST-elevation myocardial infarction). Circulating monocytes from all patients, but not from healthy individuals, showed high levels of pro-inflammatory cytokines, TNF-α and IL-6, as well as of IRAK-M and IL-10. TLR4 was also up-regulated, but IRAK-1, IRAK-4 and MyD88 levels were similar in patients and controls. Further, we investigated the consequences of cytokines/IRAK-M expression on the innate immune response to endotoxin. *Ex vivo* responses to LPS were markedly attenuated in patient monocytes compared to controls. Control monocytes cultured for 6 h in supplemented medium (10% serum from ACS patients) expressed IRAK-M, and LPS stimulation failed to induce TNF-α and IL-6 in these cultures. Pre-incubation of the serum with a blocking anti-TNF-α antibody reduced this endotoxin tolerance effect, suggesting that TNF-α controls this phenomenon, at least partially. We show for the first time that inflammatory responses associated with ACS induce an unresponsiveness state to endotoxin challenge in circulating monocytes, which correlates with expression of IRAK-M, TLR4 and IL-10. The magnitude of this response varies according to the clinical condition (UA, STEMI or NSTEMI), and is regulated by TNF-α.

Keywords: Acute coronary syndrome, IRAK-M, cytokines, endotoxin tolerance, inflammation, monocytes

INTRODUCTION

Acute coronary syndrome (ACS) groups together diverse diseases such as unstable angina (UA), ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI) and, according to some authors, ischemic sudden death. About two-thirds of ACS cases evolve from atherosclerotic plaques that are minimally or mildly obstructive of the lumen.[1,2] In this context, inflammation plays an essential role in plaque disruption by enhancing protease activity and apoptosis of matrix-synthesizing smooth muscle cells.[2–4] Plaque rupture is favored by inflammatory cytokines, as they reduce matrix synthesis and/or stimulate its degradation. Histological analysis of atherosclerotic coronary

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arteries taken from patients who died of ACS show activated macrophages as well as T lymphocytes and mast cells infiltrated in unstable or ruptured atherosclerotic plaques, thus suggesting on-going local inflammation.[1,5,6] In addition, systemic inflammation has been reported in ACS patients.[7] Among others, they express high levels of interleukin (IL)-6 and tumor necrosis factor α (TNF-α),[8,9] and the inflammatory process continues despite the resolution of clinical symptoms.[10]

In the last years, infectious agents have been repeatedly linked to the initiation and progression phases of atherosclerosis.[11–14] Of note, both *Chlamydia pneumoniae* and cytomegalovirus have been isolated from atherosclerotic lesions.[15–17] However, it remains to be shown whether these and other pathogens are a direct cause of coronary diseases or just accumulate in atherosclerotic plaques because of impaired immune response or favorable growth conditions.

The link between inflammatory responses and ACS has provoked several studies to unravel the molecular basis of this process, which have revealed crucial roles for Toll-like receptors (TLRs) and IL-1 receptor-associated kinases (IRAKs).[1–4,18–20] While ectodomains of TLRs recognize pro-inflammatory pathogen-associated molecular patterns such as lipopolysaccharide (LPS), IRAKs mediate the intracellular signal transduction process. Following TLR engagement, the receptor binds intracellular adapter proteins such as myeloid differentiation factor 88 (MyD88), which in turn recruit members of the IRAK family (*e.g.* IRAK-1 and IRAK-4) and other proteins implicated in inflammation signaling.[21–29] The resulting complexes convert the extracellular stimulus into a potent transcriptional response through activation of mitogen-activated protein kinases (MAPKs) and nuclear factor kappaB (NF-κB) pathways.[21–32] Of particular note, the Asp299Gly polymorphism in TLR4 is associated with a significant reduction in vascular inflammation.[33] Furthermore, down-regulation of inflammatory pathways induces cardioprotection during infarct via decreased association of TLR4 with MyD88, inhibition of IRAK-1 and IKK activity, and subsequent impairment of NF-κB-mediated transcription.[34]

In contrast to IRAK-1 and IRAK-4, the catalytically defective IRAK-M negatively regulates the inflammatory signaling responses.[35–39] We and others could show that this pseudo-kinase is involved in the control of endotoxin tolerance in monocytes as well as in deactivation of tumor-infiltrating macrophages and osteoporosis.[25,35,37–40] Interestingly, we have also found that human monocytes treated with a nitric oxide donor express large amounts of IRAK-M, apparently under the control of TNF-α.[41]

On the basis of these findings, we hypothesized that systemic inflammation during ACS might induce IRAK-M expression in circulating monocytes. We tested this hypothesis in monocytes from 34 ACS patients classified either as UA, STEMI or NSTEMI. We also studied the consequences of IRAK-M expression on the innate immune response to endotoxin challenge in circulating monocytes from these patients.

PATIENTS AND METHODS

Reagents

All reagents were of the highest quality available and were obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich (St Louis, MO, USA). LPS from *Salmonella abortus* was provided by Dr Galanos. For cell culture, we used Dulbecco's MEM (DMEM) from Gibco (Paisley, UK). The rabbit anti-IRAK-M polyclonal antiserum was obtained from Chemicon International (Temecula, CA, USA). Mouse anti-β-actin was from AL Immunotools (Santa Cruz, CA, USA). The anti-TNF-α blocking polyclonal antibody and isotype control were from Santa Cruz. CD-14-FITC was from Cymbus Biotechnology (Chandlers Food, UK). The LS Separation Columns system and anti-CD14 magnetic beads were from Miltenyi Biotech (Bergisch Gladbach, Germany). TNF-α, IL-6 and IL-10 were obtained from Sigma-Aldrich.

Patients and healthy volunteers

We studied 34 patients (age 52 ± 12 years, mean ± SD) consecutively admitted to the Department of Internal Medicine at the Hospital 'La Paz', and diagnosed with ACS. They belong to three groups: UA (n = 12), STEMI (n = 11) and NSTEMI (n = 11). All patients were admitted within 1–3 h of their arrival at the hospital, and blood samples were taken before any treatment was started. None of the admitted patients was under previous treatment. Ten sex- and age-matched healthy volunteers without no personal history of either coronary disease or cardiovascular risk factors were included as controls.

Patients were classified following the standard clinical criteria explained in supplemental data (see Supplemental Fig. 1). Levels of C-reactive protein and coronary angiograms were also taken into account for patient classification (data not shown). None of the admitted patients was suffering from infectious diseases at the time of inclusion; microbial tests were negative in all cases.

Blood samples were taken from patients and healthy donors, from which $CD14^+$ monocytes were purified. Total RNA was isolated from these monocytes and used to synthesize cDNA. Plasma from patients and healthy donors was also used for other assays (see below). Written informed consent was obtained from all subjects

(patients and healthy volunteers). This study was approved by the local Ethics Committee.

Isolation of CD14⁺ monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of patients and healthy donors by centrifugation on Ficoll–Hypaque Plus (Amersham Biosciences, The Netherlands). Then, human monocytes were isolated on a LS positive separation columns using an anti-CD14 antibody conjugated to magnetic beads, following the manufacturer's recommendations. $CD14^+$ monocytes were used for cell culture as well as for mRNA and/or protein purification.

Cell cultures

$CD14^+$ blood monocytes from patients and healthy volunteers were cultured to 10^6 cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and 10% heat-inactivated normal serum, in the presence or absence of 10 ng/ml LPS for 1 h. In other experiments, $CD14^+$ monocytes from healthy volunteers (control monocytes) were cultured in medium containing 0%, 1% or 10% serum from patients or healthy volunteers for the indicated periods of time. In some experiments, control monocytes, cultured for 16 h in medium containing 10% plasma from patients or healthy volunteers, were stimulated with 10 ng/ml LPS for the indicated times. $CD14^+$ monocytes from healthy volunteers were also stimulated with TNF-α, IL-6 or IL-10 at the indicated concentrations and for various periods of time. All solutions and culture media used were endotoxin free, as tested by a *Limulus* amebocyte lysate kit (PYROGENT, Cambrex, Walkensville).

RNA and protein isolation

$CD14^+$ monocytes were washed once with PBS and RNA was isolated using TRIReagent (Roche, Palo Alto, CA, USA). The purified RNA was treated with RNAse-free DNAse I (Amersham Biosciences), and cDNA was obtained by reverse transcription of 1 μg of this RNA using a poly(dT) oligonucleotide primer (Roche). Protein fractions were isolated as recommended, and the final pellets were dissolved in a solution containing 1% SDS.

mRNA quantification

The expression levels of TNF-α, IL-6, IL-10, TLR4, IRAK-1, IRAK-4, IRAK-M, MyD88, and 18S were analyzed by real-time Q-PCR (LightCycler; Roche

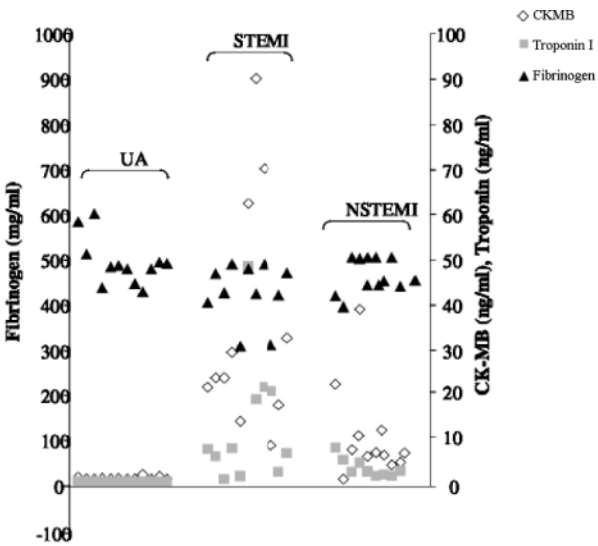
A

UA	Chest discomfort at rest with ST-segment depression of at least 0.1 mV or T-wave inversion in two or more contiguous electrocardiography leads, a creatine kinase MB fraction and troponin that were within normal limits, and angiographically confirmed coronary artery disease.
STEMI	Prolonged chest pain accompanied by new or presumed new ST-segment elevation > 1 mm seen in any location or new left bundle branch block on the index or qualifying electrocardiogram with at least one positive cardiac biochemical marker of necrosis (creatine kinase MB fraction more than twice the upper limit of the normal range or troponin I > 0.5 ng/ml).
NSTEMI	At least one positive biochemical marker of necrosis without new ST-segment elevation in the electrocardiogram.

Exclusion criteria:

Active tumoral disease, chronic inflammatory disease, local or systemic infection at the moment of inclusion, positive microbial test, kidney failure (creatinine > 100 mg/l), liver failure (serum aspartate aminotransferase and/or alanine aminotransferase level > 100 IU/l, prothrombin time < 60%, total bilirubin level > 60 μmol/l), overt heart failure NYHA class II or higher, myocardial infarction or major surgery including arteriography/stent or cardiovascular surgery, trauma or treatment with corticoids or immunosuppressive drugs within the previous month, AIDS, viral B or C hepatitis, gestation or age > 70 years.

B



Supplemental Fig. 1. Patient selection. (A) Clinical criteria followed. (B) Serum samples from patients (classified as UA, STEMI or NSTEMI) were analyzed for CKMB, troponin I and fibrinogen. Individual values are represented.

Table 1. Sequences of oligonucleotides used for Q-PCR

Gene	Sense primer (5′→3′)	Antisense primer (5′→3′)
TNF-α	GCCTCTTCTCCTTCCTGATCGT	CTCGGCAAAGTCGAGATAGTCG
IL-6	CAAAGAATCTAGATGCAATAA	GCCCATTAACAACAACAATCTG
IL-10	CCCCAAGCTGAGAACCA	TCTCAAGGGGCTGGGTCAGC
IRA K-1	AAAGGAGGCCTCCTATGACC	ATGATGCAGAGCTGCCAAG
IRAK-4	CAAGTGATGGAGATGACCTCTGCTTAGT	TCTAGCAATAACTGAGGTTCACGGTGTT
IRAK-M	TTTGAATGCAGCCAGTCTGA	GCATTGCTTATGGAGCCAAT
TLR4	AGTTTCCTGCAATGGATCAAGG	GGACCGACACACCAATGATG
MyD88	GCCGCCTGTCTCTGTTCTTGA	CTGCTGGTCCTTCTTAGTCTC

Diagnostics, Indianapolis, IN, USA), using cDNA obtained as described above. Real-time Q-PCR was performed using a Fast-Start DNA master SYBR Green system (Roche) and specific primers (listed in Table 1). All results were normalized to the expression of the 18S gene, and the cDNA copy number of each gene of interest was determined using a seven-point standard curve. Standard curves were run with each set of samples, the correlation coefficients r^2 being > 0.99. To confirm the specificity of the reaction products in each experiment, the melting profile of each sample was analyzed using the LightCycler. The melting profile was determined by maintaining the reaction at 80°C for 10 s and then increasing the temperature to 95°C at a linear rate of 0.1°C/s while measuring the emitted fluorescence. Analysis of the melting curves demonstrated that each pair of primers amplified a single product. These PCR products were separated in agarose gels and stained with ethidium bromide (0.5 μg/ml) to confirm that, in each case, a single fragment of the predicted size had been amplified (see Table 2).

For 18S mRNA detection, we used the primers of QuantumRNA Classic 18S provided by Ambion, USA. All primers were synthesized, desalted and purified by IZASA (Barcelona, Spain), and their sequences are given in Table 1.

ELISA for TNF-α and IL-6

The levels of soluble TNF-α were measured using an immunoassay kit purchased from CLB (Amsterdam, The Netherlands); soluble IL-6 was determined using an immunoassay kit purchased from Bender MedSystems (Vienna, Austria).

Western blot analysis

Cell extracts were boiled in Laemmli buffer, resolved on 12% SDS–polyacrylamide gels in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS), and transferred to Immun-Blot PVDF membranes (Bio-Rad, CA, USA) at 300 mA for 1.5 h at 4°C. After blocking for 1 h in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20 (TBS-T) containing 5% non-fat milk, membranes were washed three times in TBS-T alone and probed for 20 h with antibodies diluted in TBS-T. Following washing in TBS-T, membranes were incubated for 30 min with secondary HRP-conjugated antibodies (diluted 1:2000), and finally washed three times with TBS-T. The bound antibodies were detected using ECL Plus reagents according to the manufacturer's instructions (Amersham-Pharmacia Biotech, The Netherlands).

Table 2. PCR conditions for mRNA quantitation

Gene	Tm (°C)	Cycles
TNF-α	64	95°C for 10 s, 64°C for 10 s, 72°C for 19 s
IL-6	50	95°C for 10 s, 50°C for 10 s, 72°C for 10 s
IL-10	68	95°C for 10 s, 68°C for 10 s, 72°C for 10 s
IRAK-1	60	95°C for 10 s, 60°C for 10 s, 72°C for 10 s
IRAK-4	61	95°C for 10 s, 61°C for 10 s, 72°C for 10 s
IRAK-M	59	95°C for 10 s; 59°C for 10 s 72°C for 19 s
TLR4	64	95°C for 10 s, 64°C for 10 s, 72°C for 10 s
MyD88	61	95°C for 10 s, 61°C for 10 s, 72°C for 10 s

Data analysis

The number of experiments analyzed is indicated in each figure. Data were collected from a minimum of three experiments to calculate the mean ± SD. Statistical significance was calculated using the unpaired Student's *t*-test; results were considered significant at *P* values < 0.05.

RESULTS

TNF-α and IL-6 are overexpressed in circulating monocytes from ACS patients

For all 34 ACS patients included in this study (UA, *n* = 12; STEMI, *n* = 11; and NSTEMI, *n* = 11), we determined the serum concentrations of troponin, creatine kinase MB

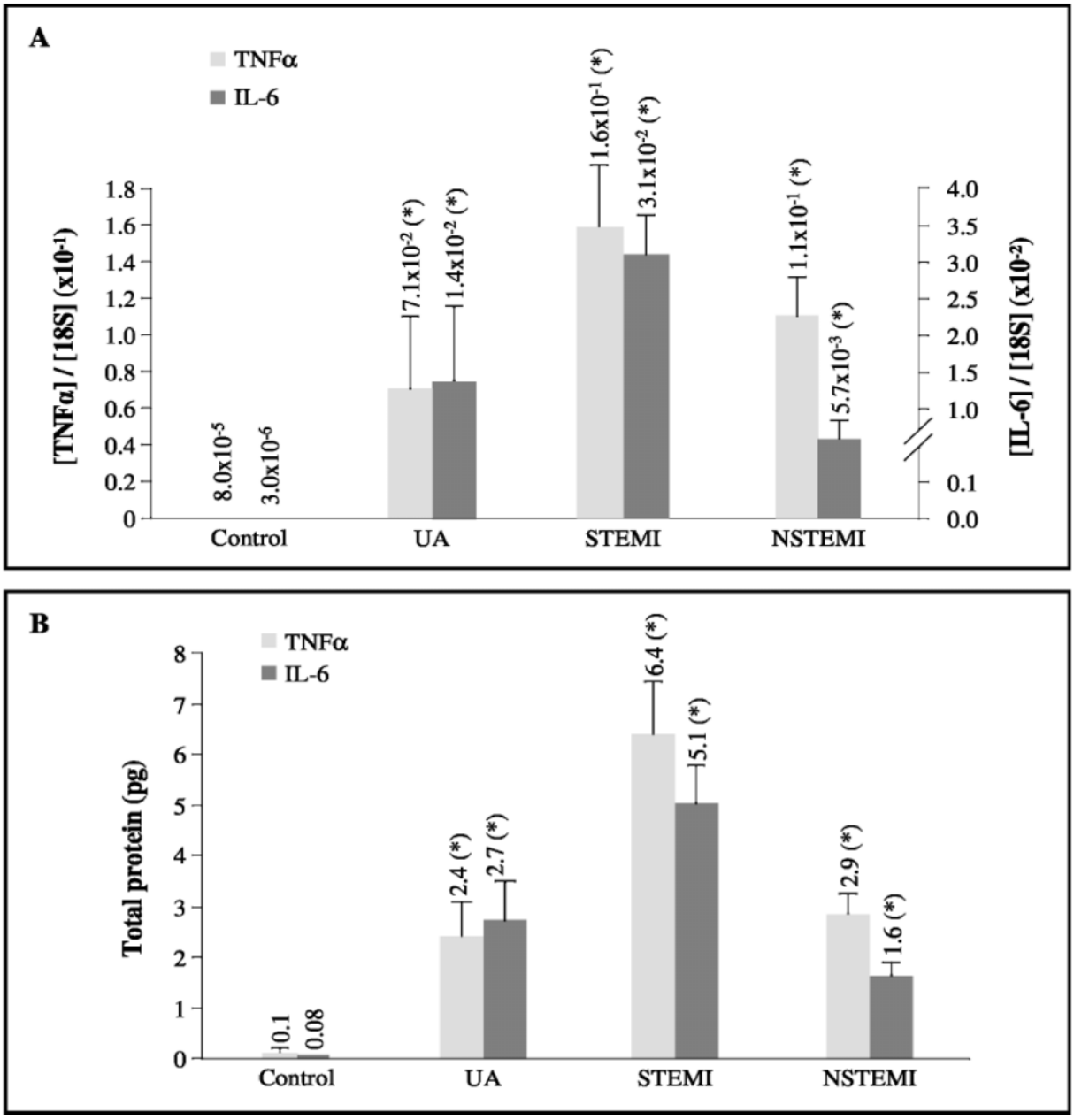


Fig. 1. Inflammation markers are up-regulated in monocytes and serum from ACS patients. (A) $CD14^+$ monocytes from UA (*n* = 12), STEMI (*n* = 11) and NSTEMI (*n* = 11) patients as well as from healthy volunteers (control, *n* = 10) were isolated. mRNA expression levels of TNF-α and IL-6 were determined by real-time Q-PCR using a LightCycler system. (B) Protein levels of TNF-α and IL-6 from serum of ACS patients and healthy individuals were analyzed using a commercial ELISA (minimum detectable levels: 0.43 pg TNF-α and 0.27 pg IL-6, respectively). The [TNF-α]/[18S] and [IL-6]/[18S] ratios (A; light and dark gray bars, respectively), as well as TNF-α and IL-6 protein levels (B; light and dark gray bars, respectively) are depicted. **P* < 0.05 versus control group.

and fibrinogen (Supplemental Fig. 1). Healthy volunteers (control group, $n = 10$) were also evaluated, and showed regular levels of all three markers.

To study the innate immune responses during ACS, we isolated and cultured monocytes from all patients and healthy individuals. On average, 91% of isolated cells expressed the specific surface marker CD14 and did not exhibit significant apoptosis (not shown). TNF-α and IL-6 mRNA levels in these monocytes were evaluated by Q-PCR. Whilst monocytes from healthy volunteers did not express the major pro-inflammatory cytokines, TNF-α and IL-6, mRNA levels of these two cytokines were high in all patients (Fig. 1A). Interestingly, we observed marked differences between the three ACS groups. In particular, STEMI patients showed significantly higher levels of TNF-α and IL-6 mRNA than both NSTEMI and UA patients.

High concentrations of TNF-α and IL-6 are found in serum of ACS patients

To determine whether increased transcription of *TNF-α* and *IL-6* genes resulted in higher protein expression, serum levels of the two cytokines were measured using commercially available immunoassay kits. Indeed, concentrations of both cytokines were much higher in serum from patients compared to healthy individuals (up to 64-fold; Fig. 1B). In addition, these concentrations correlated remarkably well with mRNA expression levels in isolated monocytes (compare Fig. 1A and B); although we stress that other cell types (*e.g.* endothelial cells and cardiac myocytes) could be additional sources of these circulating cytokines. Our findings corroborate previous reports of systemic inflammation during ACS,[1,4,9] and suggest for the first time that this process is stronger in STEMI than in UA and NSTEMI patients.

IRAK-M is expressed in circulating monocytes from ACS patients

Previously, we demonstrated that TNF-α induces IRAK-M expression in human monocytes,[41] which eventually elicits a refractoriness state against both endotoxin and tumor cells.[37,39,41] Given the high levels of pro-inflammatory cytokines in our patients, we wondered whether the *IRAK-M* gene was expressed in their circulating monocytes. Indeed, monocytes from ACS patients showed considerable levels of IRAK-M mRNA (Fig. 2A,B). Again, these levels were significantly higher in NSTEMI, but in particular in STEMI compared to UA

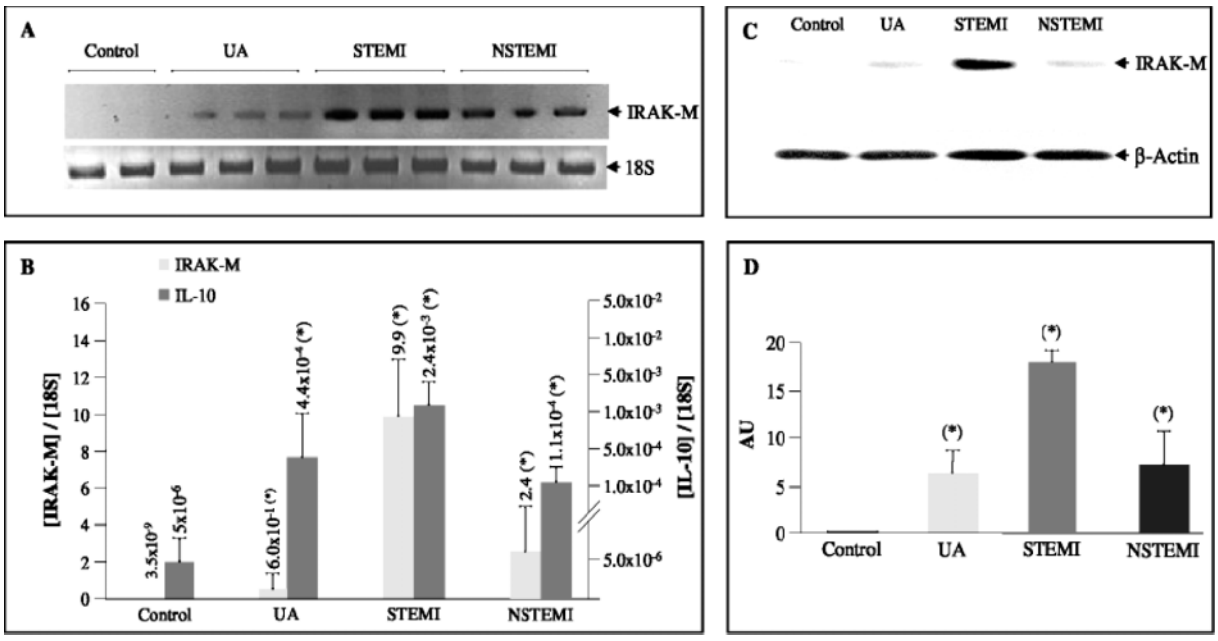


Fig. 2. IRAK-M and IL-10 are expressed in monocytes from ACS patients. (A) IRAK-M Q-PCR end products were separated by agarose gel electrophoresis and stained with ethidium bromide. (B) IRAK-M and IL-10 mRNA levels in $CD14^+$ monocytes from ACS patients and healthy volunteers were determined by Q-PCR (real time, LightCycler system) and are shown normalized against 18S. Number of samples as in Figure 1; $^*P < 0.05$ versus control group. The data shown correspond to a representative experiment ($n = 3$). (C) Expression of IRAK-M protein analyzed by a Western blot using 20 μg total protein from $CD14^+$ monocytes and an anti-IRAK-M polyclonal antiserum. An anti-β-actin monoclonal antibody was used as a control of loading and transfer. A representative experiment is shown ($n = 4$). (D) Densitometric analysis of data presented in (C). Relative intensities of stained protein bands are given in arbitrary units (AU); $^*P < 0.05$ versus control group.

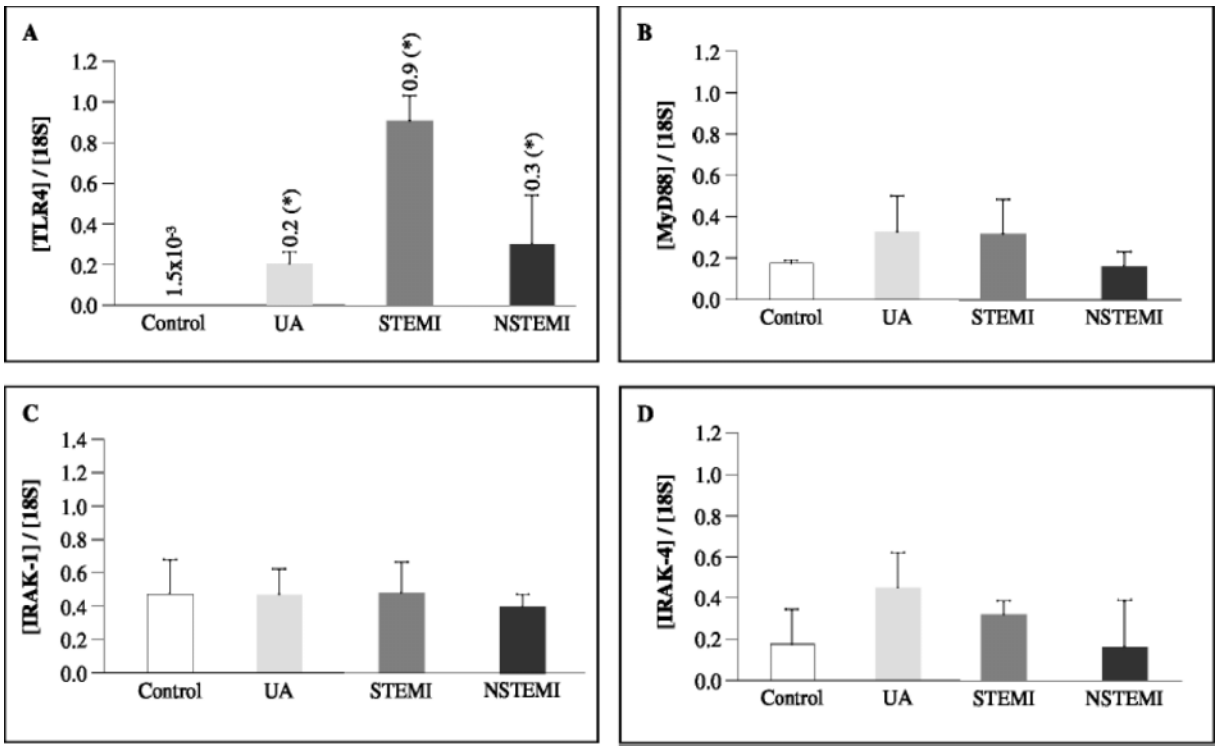


Fig. 3. TLR4 but not MyD88, IRAK-1 or IRAK-4 are overexpressed in monocytes from ACS patients. Relative amounts of TLR4 (A), MyD88 (B), IRAK-1 (C) and IRAK-4 mRNA (D) in $CD14^+$ monocytes isolated from UA, STEMI and NSTEMI patients and from controls were determined by Q-PCR. Number of samples as in Figure 1; $*P < 0.05$ versus control group.

patients. Thus, expression of the *IRAK-M* gene directly correlates with TNF-α levels in serum. We also note that IL-10 mRNA levels are elevated in ACS patients (Fig. 2B). Overexpression of both molecules thus suggests a possible unresponsiveness state to endotoxin in our patients (see below). In line with its increased mRNA levels, IRAK-M protein accumulates in monocytes from ACS patients (Fig. 2C,D) while, as we showed previously, control monocytes do not express this pseudo-kinase.[37,39,41]

Although none of the admitted patients were under treatment, we excluded that the observed effects were attributable to drugs commonly used to treat their pathologies. No significant expression of IRAK-M (mRNA or protein) was induced in cultures of control monocytes stimulated with pharmacological concentrations of atenolol, spironolactone or aspirin (not shown).

Expression of other members of the TLR/IRAK pathway

Although the exact nature of multiprotein complexes within the TLR/IRAK pathway remains to be established, it is plausible that a balance between active and catalytically defective members of the IRAK family regulates innate immune responses. Having established that circulating monocytes from ACS patients express IRAK-M, we wished to compare mRNA levels of the two functional kinases of the family, IRAK-1 and IRAK-4, in monocytes from patients and healthy volunteers. In addition, we determined transcription levels of the LPS receptor, TLR4, and of its adaptor, MyD88.

TLR4 was only minimally transcribed in the control group whilst all patients showed significant levels of this LPS receptor (Fig. 3A). The highest expression was observed in STEMI patients; however, differences between groups were not significant. In contrast, we did not detect differences in the mRNA levels of MyD88, IRAK-1 and IRAK-4 in ACS patients compared to healthy volunteers, or within ACS subgroups (Fig. 3B–D, respectively).

LPS rapidly up-regulates IRAK-M in monocytes from ACS patients but not from healthy individuals

To study innate immune responses in monocytes from ACS patients, we cultured these cells in the presence or absence of LPS. IRAK-M mRNA was significantly up-regulated in monocytes from all three classes already 1 h after LPS treatment (Fig. 4A). As we previously reported, this short-term stimulation does not up-regulate *IRAK-M* in

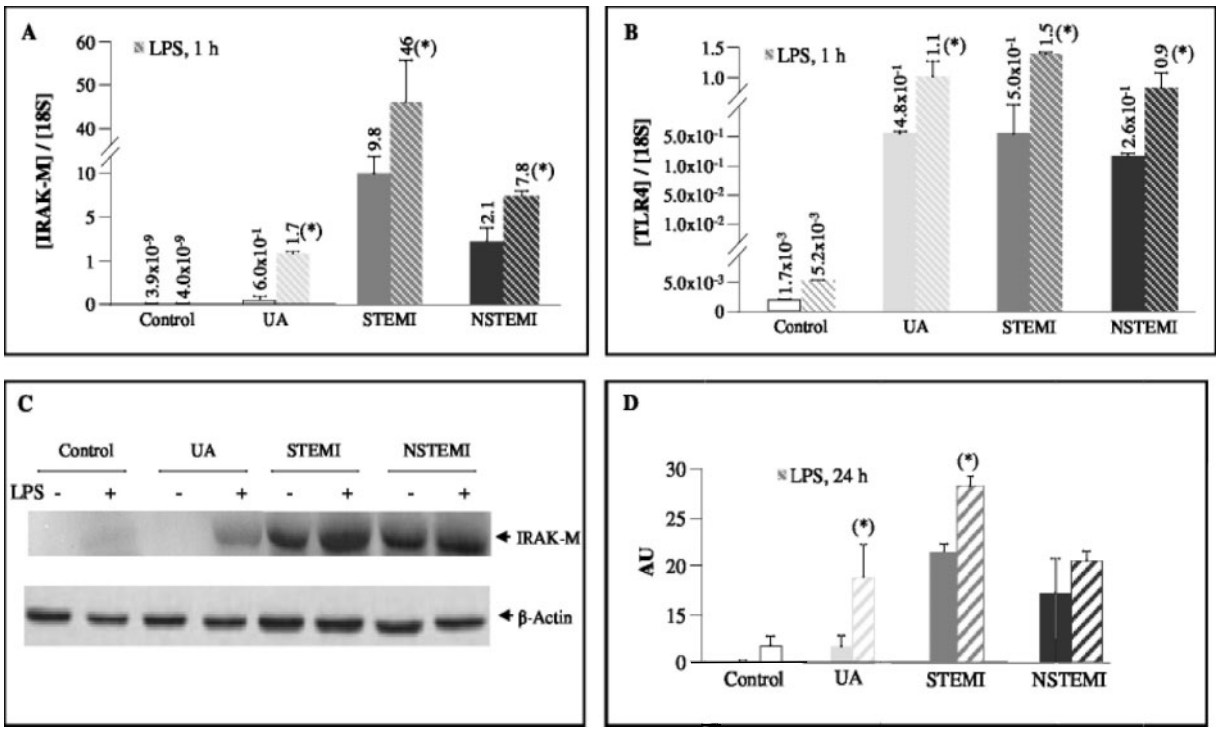


Fig. 4. Monocytes from ACS patients rapidly up-regulate IRAK-M and TLR4 in response to endotoxin challenge. Relative levels IRAK-M mRNA (A) and TLR4 mRNA (B) in $CD14^+$ monocytes from UA, STEMI and NSTEMI patients and from healthy volunteers cultured for 1 h in the presence (hatched bars) or absence (regular bars) of 10 ng/ml LPS. Number of samples as in Figure 1; $*P < 0.05$ versus no LPS treatment. (C,D) Expression of IRAK-M protein analyzed by Western blot using 20 µg total protein from $CD14^+$ monocytes from UA, STEMI and NSTEMI patients and from healthy volunteers cultured for 24 h in the presence (hatched bars) or absence (regular bars) of 10 ng/ml LPS. An anti-β-actin monoclonal antibody was used as a control of loading and transfer. A representative experiment is shown ($n = 4$) (C). The results from densitometric analysis (relative intensities of stained protein bands) are given in (D); AU, arbitrary units; $*P < 0.05$ versus no LPS-treatment.

control monocytes, for which maximal levels of *IRAK-M* mRNA are reproducibly reached about 6 h after LPS stimulation.[37,41] This finding was also verified in the current investigation (not shown). Of note, while IRAK-M was induced to significantly higher levels in patients (4-fold) than in healthy volunteers (1-fold; Fig. 4A) at the mRNA level, TLR4 mRNA increased similarly in both patients and controls (about 3-fold; Fig. 4B). In absolute terms, however, levels of IRAK-M and the endotoxin receptor were significantly higher in cells isolated from ACS patients than in the control group, thus similar to the situation observed before LPS challenge (see above and Figs 2B and 3A). Finally, we could also detect up-regulation of IRAK-M at protein level in monocytes from ACS patients following LPS challenge (Fig. 4C,D).

It is known that endotoxin stimulation generates an immune response in human monocytes through up-regulation of several cytokines (*e.g.* TNF-α, IL-6[42]). Monocytes from both, ACS patients and healthy volunteers rapidly overexpressed these genes upon LPS treatment, and we detected significant mRNA levels already 1 h after challenge (Fig. 5A,B). However, we observed significantly different responses to LPS in the two groups. Whilst cultures from healthy volunteers increased more than 27,000- and 8000-fold their basal levels of TNF-α and IL-6 mRNA, respectively, cells from ACS patients up-regulated the expression of these genes by about 200-fold, or two orders of magnitude less (Fig. 5A,B). We observed similar effects on the amounts of released TNF-α and IL-6 when cells were stimulated with LPS for 24 h (Fig. 5C,D, respectively).

It is known that during the endotoxin-tolerance frame, human monocytes exposed to LPS are unable to rapidly express the *TNF-α* and *IL-6* genes.[37,43] In this refractoriness state, up-regulation of *IRAK-M* is accelerated, and LPS stimulation for 1 h generates high levels of this pseudo-kinase.[37] Thus, our current data strongly suggest that monocytes from ACS patients are 'locked' in an endotoxin-tolerance state.

IRAK-M but not TLR4 is up-regulated in human monocytes cultured in medium supplemented with serum from ACS patients

To determine whether soluble factors present in serum of ACS patients were responsible for IRAK-M up-regulation, monocytes from healthy controls were cultured for

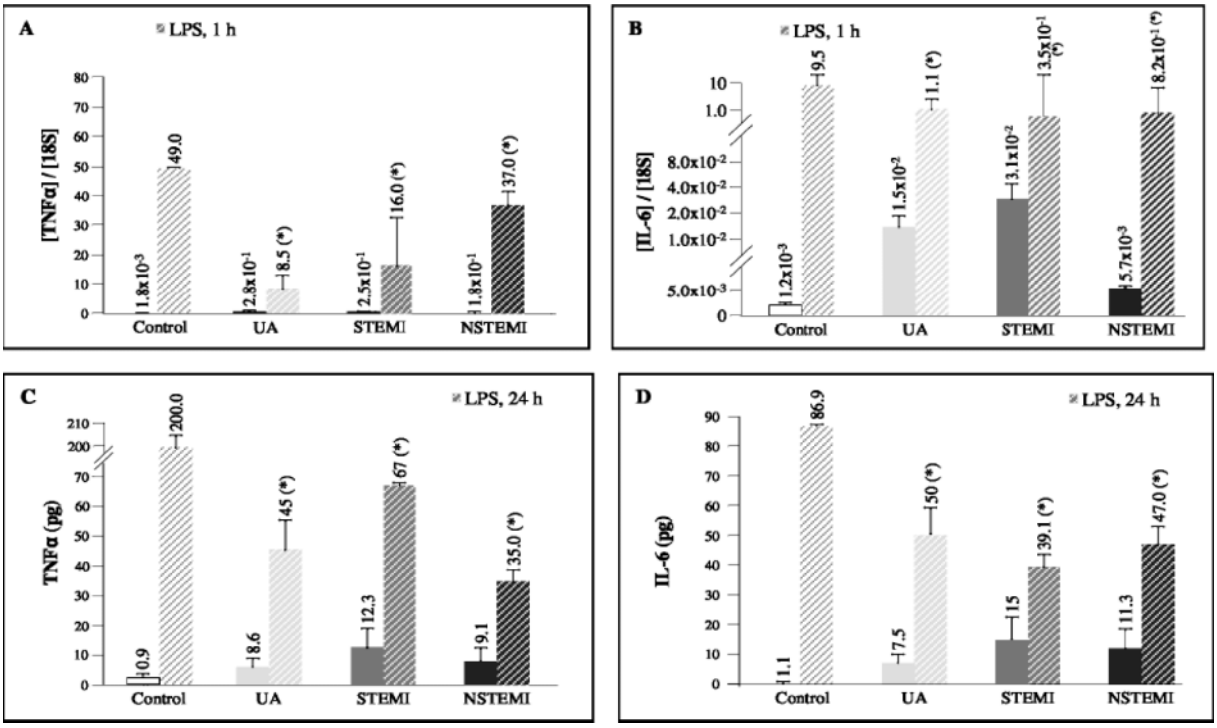


Fig. 5. Pro-inflammatory responses to endotoxin are impaired in monocytes from ACS patients. Upper panels: relative mRNA levels of TNF-α (A) and IL-6 (B) after 1 h incubation of $CD14^+$ monocytes from ACS patients and from healthy volunteers in the presence or absence of 10 ng/ml LPS. Lower panels: protein levels of TNF-α (C) and IL-6 (D) in the supernatant of monocytes cultured for 24 h in the presence or absence of 10 ng/ml LPS. Number of samples as in Figure 1; **P* < 0.05 versus the same condition of control group.

6 h in the presence of supplemented medium containing 1% or 10% filtered serum from either UA (*n* = 5), STEMI (*n* = 5) or NSTEMI patients (*n* = 5), or from healthy volunteers (control, *n* = 5; samples used for this study were randomly selected from the subgroups described above). Indeed, *IRAK-M* was induced in a dose-dependent manner in cultures supplemented with both 1% and 10% serum from ACS patients, but not from healthy individuals (Fig. 6A). We also verified increased IRAK-M protein levels in monocytes treated with ACS serum (not shown). This finding strongly suggests that a soluble factor present in the serum of ACS patients induces the expression of IRAK-M in monocytes. In agreement with the differences in TNF-α and IRAK-M levels among ACS patients discussed above, serum from STEMI patients showed the highest stimulatory activity. By contrast, TLR4 levels were not affected by the presence of serum from ACS patients (Fig. 6B).

Human monocytes cultured in medium supplemented with serum from ACS patients do not express high levels of TNF-α upon LPS treatment

Given the influence of soluble factors from serum of ACS patients in IRAK-M expression, we also analyzed their possible role on the endotoxin tolerance response characterized above (Figs 4 and 5). Monocytes were cultured for 16 h in the presence of 10% filtered serum from patients or healthy volunteers. After this pretreatment, we stimulated these cultures with 10 ng/ml LPS for 1 h or 24 h, and analyzed TNF-α expression (mRNA and protein). Pretreatment with medium supplemented with serum from healthy individuals did not interfere with LPS-mediated induction of TNF-α (Fig. 7A). Furthermore, high levels of TNF-α were detected in the supernatants of these cultures (Fig. 7B). In contrast, monocytes pretreated with medium supplemented with serum from ACS patients produced much lower levels of TNF-α upon LPS-stimulation, and induction of IL-6 was also blocked in these cultures (not shown).

IRAK-M expression and endotoxin tolerance are TNF-α-dependent processes

We have previously shown that TNF-α induces IRAK-M up-regulation in human monocytes.[41] Indeed, in the current investigation, we have measured significant concentrations of this cytokine in the serum of ACS patients (Fig. 1), and these levels correlated with IRAK-M expression in circulating monocytes (Fig. 2B). On the

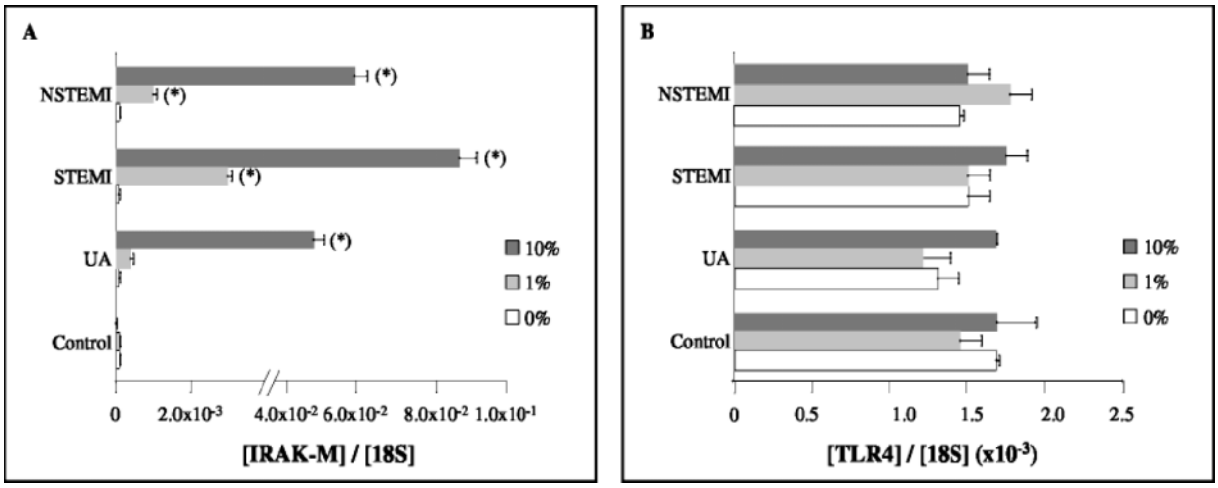


Fig. 6. Soluble factors present in serum of ACS patients up-regulate IRAK-M but not TLR4 in human monocytes. Human monocytes were cultured for 6 h in the presence of medium supplemented with 0%, 1% or 10% filtered serum, pooled from UA (n = 5), STEMI (n = 5), or NSTEMI patients (n = 5) or from healthy volunteers (control, n = 5). IRAK-M and TLR4 mRNA expression levels were determined by real-time Q-PCR. Relative levels of IRAK-M and TLR4 are shown in (A, B), respectively (0%, white bars; 1%, light bars; 10%, solid bars). The results shown are from a representative experiment (n = 3) and *P < 0.05 versus the same condition of control group.

other hand, monocytes from ACS patients also expressed IL-6 and IL-10 (Figs 1 and 2, respectively). To investigate the mechanism by which IRAK-M expression and subsequent endotoxin tolerance occur, we analyzed possible effects of TNF-α, IL-6 and IL-10 on IRAK-M induction. TNF, but neither IL-6 nor IL-10, potently up-regulated IRAK-M in human monocytes (Fig. 8A). In addition, no IRAK-M induction was observed upon incubation with much higher, supra-physiological concentrations of the latter two interleukins (not shown). To analyze monocyte responses to TNF-α in more detail, we next studied IRAK-M expression in the presence of varying concentrations of this cytokine. As shown in Figure 8B, the response was saturable at very low TNF-α concentrations (~3 pg/ml, ID_{50} 3.5 pg/ml). Note that all solutions used in our assays were tested for endotoxin contamination by *Limulus* test (data not shown). Thus, we hypothesized that circulating TNF-α directly induces IRAK-M expression in monocytes from ACS patients.

To corroborate this hypothesis, we analyzed IRAK-M mRNA levels in human monocytes cultured for 6 h in the presence of 10% filtered serum from patients or healthy volunteers, pre-incubated or not with a blocking antibody against TNF-α. As observed before, serum from ACS patients induced IRAK-M expression in human monocytes. However, co-incubation with the anti-TNF-α antibody, also tested for endotoxin contamination by *Limulus* test (data not shown), significantly

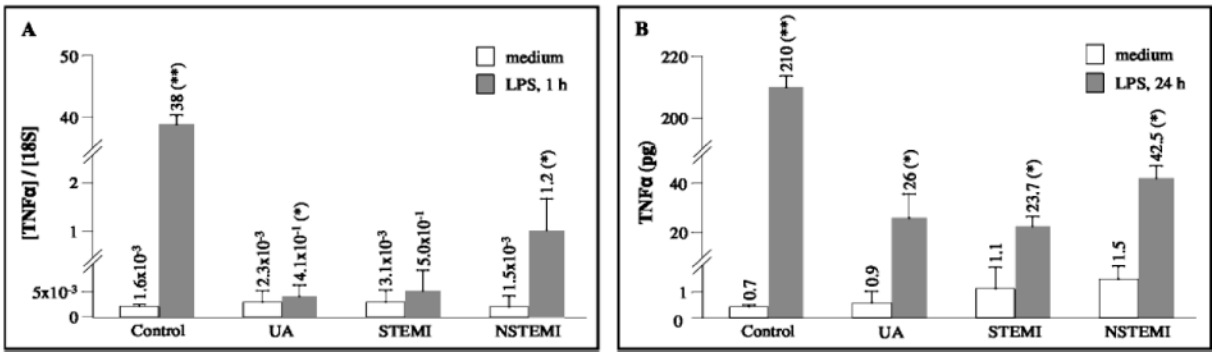


Fig. 7. Soluble factors present in serum of ACS patients induce endotoxin tolerance in human monocytes. Monocytes from healthy volunteers were cultured for 16 h in the presence of medium supplemented with 10% filtered serum pooled from ACS patients or from healthy volunteers (as in Fig. 6). After this period, cells were washed twice with PBS and stimulated or not with 10 ng/ml LPS for 1 h or 24 h. (A) Relative levels of TNF-α mRNA were determined by real-time Q-PCR. (B) Soluble TNF-α levels in the supernatants of 24-h cultures. Typical results are shown (n = 2) and *P < 0.05, **P < 0.01 versus no LPS stimulation.

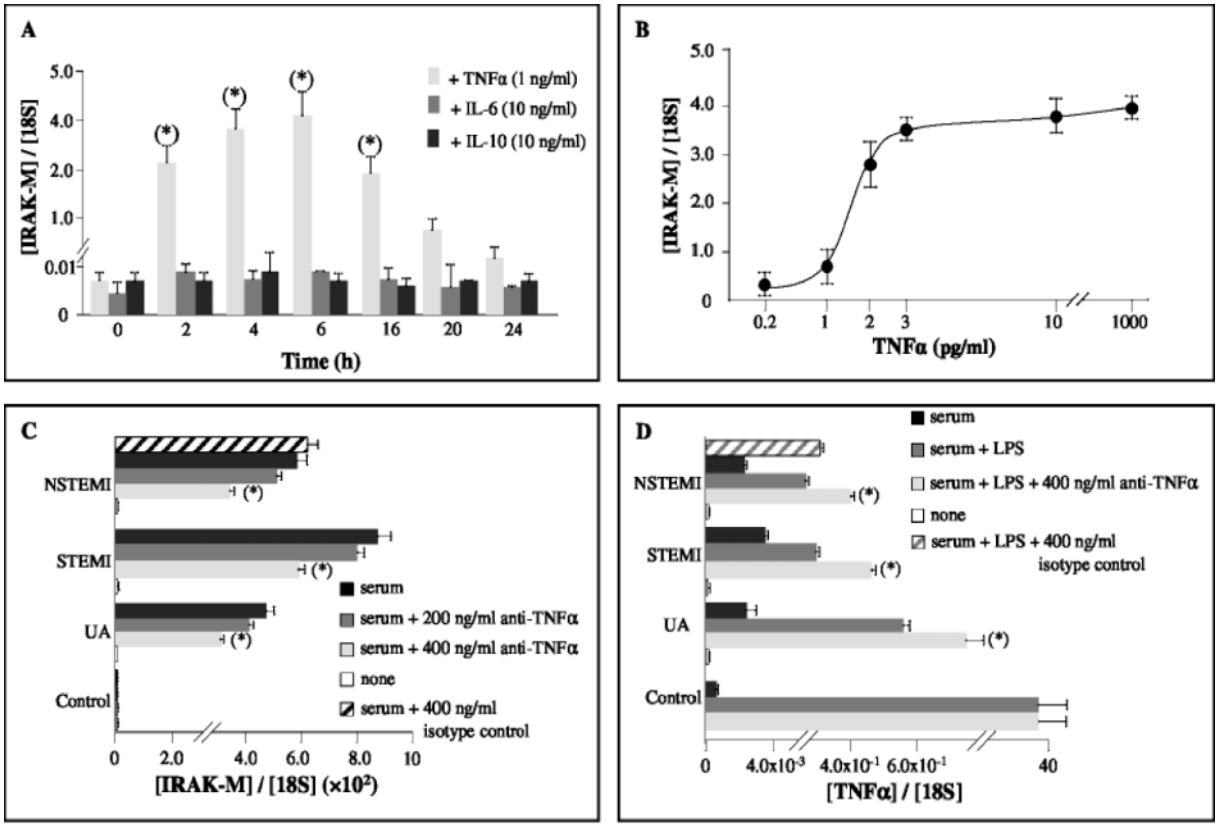


Fig. 8. TNF-α but not IL-6 or IL-10 up-regulate IRAK-M in human monocytes. Upper panels: (A) Human monocytes from healthy volunteers were cultured for the indicated periods of time in the presence of TNF-α (light bars), IL-6 (gray bars) or IL-10 (solid bars). After this, cells were harvested and levels of IRAK-M and 18S mRNA were determined by Q-PCR. Relative levels of IRAK-M are shown. **P* < 0.05 versus control. (B) Relative levels of IRAK-M mRNA in human monocytes from healthy volunteers were determined after 6 h incubation with indicated TNF-α concentrations. Lower panels: an anti-TNF-α blocking antibody reverted the effect of serum from ACS patients. (C) Human monocytes were cultured for 6 h in the presence of medium supplemented with 10% filtered serum from ACS patients or from healthy volunteers (as in Fig. 6) pre-incubated with 0, 200 or 400 ng/ml anti-TNF-α antibody. Relative expression levels of IRAK-M mRNA were determined using real-time Q-PCR. **P* < 0.05 with respect to stimulation with the corresponding serum. (D) Human monocytes were cultured for 16 h in the presence of medium supplemented with 10% filtered pooled serum from ACS patients pre-incubated or not with a blocking anti-TNF-α antibody. After this period, cells were washed twice in PBS and stimulated or not with 10 ng/ml LPS for 1 h. Levels of TNF-α and 18S mRNA were analyzed by real-time Q-PCR. **P* < 0.05 with respect to stimulation with the corresponding serum plus LPS. Typical results are shown (*n* = 3).

reduced IRAK-M expression (Fig. 8C). In other experiments, serum-pretreated cultures (± anti-TNF-α antibody for 16 h) were finally stimulated with 10 ng/ml LPS for 1 h before measuring TNF-α mRNA levels. As shown in Figure 8D, the anti-TNF-α antibody reversed the clear down-regulation of LPS-induced TNF-α expression due to pretreatment of human monocytes with serum from ACS patients. Altogether, our findings suggest that TNF-α critically regulates the observed phenomenon of endotoxin tolerance in human monocytes.

DISCUSSION

We and others have previously demonstrated a role for IRAK-M in de-activating the inflammatory responses mediated by Toll-like receptors.[25,35–41] In particular, we have shown that IRAK-M is rapidly up-regulated in human monocytes in the presence of LPS, within the framework of endotoxin tolerance,[39] and that this pseudo-kinase also plays an important role in the development of cancer tolerance.[49] Here, we show that monocytes from patients of acute coronary syndrome express appreciable amounts of IRAK-M at both the mRNA and protein levels. Interestingly, the magnitude of IRAK-M mRNA expression in UA, STEMI and NSTEMI patients differs markedly, and correlates with serum TNF-α concentration. These differences in *IRAK-M* expression appear to result from the magnitude of their basal inflammatory responses. Individuals with UA expressed the lowest levels of IRAK-M and their systemic TNF-α and IL-6 concentrations were also the lowest; similar

correlations were observed for STEMI and NSTEMI patients. These findings are in line with our previous report demonstrating *ex-vivo* up-regulation of IRAK-M mediated by TNF-α.[41]

The presence of IRAK-M in circulating monocytes from ACS patients strongly suggested the development of a refractory state to further endotoxin challenge. We have verified this point by showing that isolated monocytes from these patients do not respond to *ex vivo* short-time LPS challenges as those from healthy individuals. In these experiments, we observed a rapid up-regulation of IRAK-M in patients' monocytes, while TNF-α and IL-6 levels were not increased. In marked contrast, cultures from healthy volunteers did up-regulate both cytokines, and IRAK-M was not rapidly expressed. In this regard, we have previously reported that tolerant human monocytes up-regulate their IRAK-M levels upon short-time LPS stimulation,[37] while endotoxin treatment does not up-regulate TNF-α in these monocytes.[37,43,44] In addition, we note that IL-10 is also up-regulated in monocytes from ACS patients; the presence of this cytokine has also been related to endotoxin tolerance in several situations.[45] Thus, our current findings clearly demonstrate that monocytes from ACS patients are 'locked' in an endotoxin tolerance state.

Other members of the TLR/IRAK pathway (MyD88, IRAK-1 and IRAK-4) were expressed at similar levels in both, patients and healthy individuals. Although TLR4 was significantly up-regulated in ACS patients, suggesting that ACS patients were more susceptible to LPS reception than control individuals, we have found no further evidence implicating TLR4 up-regulation in the observed endotoxin tolerance mechanism. Its increase might simply result from a feed-back loop that counteracts the inhibitory effect of IRAK-M overexpression. In this regard, a soluble factor(s) present in the ACS patients' serum is able to induce IRAK-M, but not TLR4, expression and concomitant endotoxin tolerance in control monocytes.

Pre-incubation of the serum from ACS patients with a blocking anti-TNF-α antibody significantly reduced IRAK-M expression and the observed endotoxin tolerance effect in control monocytes. By contrast, neither IL-6 nor IL-10 had an effect on this phenomenon. Thus, and although we can not rule out the contribution of other soluble factors, our combined findings suggest TNF-α as likely candidate for triggering IRAK-M overexpression and a concomitant endotoxin-tolerant state in monocytes from ACS patients.

According to our findings, ACS patients have higher levels of systemic inflammation, and the innate immune response to endotoxin mounted by their circulating monocytes is markedly attenuated. These results suggest that ACS patients could have difficulties to clear Gram-negative infections. In fact, several studies have demonstrated the presence of *Chlamydia pneumoniae* in patients with ACS.[17,46,47] Some authors even propose these infections as direct cause of the cardiovascular disease in ACS patients. Nevertheless, no direct relationship has been demonstrated so far between the occurrence of future acute coronary events and the levels of anti-*C. pneumoniae* antibodies after the first acute coronary episode.[17] Moreover, antichlamydial antibiotics tested to date have failed to prevent secondary coronary events.[48] Other authors have shown that *C. pneumoniae* infection is associated with coronary artery disease, but no serological difference was observed between patients with unstable and stable angina.[49] Thus, our observations suggest an alternative hypothesis, namely that the opportunist pathogen preferentially colonizes ACS patients due to their impaired innate immune responses.

On the other hand, we note that the endotoxin tolerance observed in these patients could be beneficial. Indeed, high levels of IRAK-M expression would reduce inflammatory responses in ACS patients, thus potentially delaying or avoiding occurrence of future ischemic episodes. Thorough studies focused on the evolution of larger numbers of ACS patients will be needed to establish the clinical relevance of high IRAK-M levels for the progress of cardiovascular disease.

Conclusions

The high levels of IRAK-M found in circulating monocytes from ACS patients strongly suggest that the innate immune response against a new inflammatory stimulus such as an infectious agent could be impaired in these patients. We propose that this information should be considered while developing new anti-ACS therapies.

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