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Circulating Monocytes Exhibit an Endotoxin Tolerance Status after Acute Ischemic Stroke: Mitochondrial DNA as a Putative Explanation for Poststroke Infections

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Patients with acute ischemic stroke (AIS) suffer from infections associated with mortality. The relevance of the innate immune system, and monocytes in particular, has emerged as an important factor in the evolution of these infections. The study enrolled 14 patients with AIS, without previous treatment, and 10 healthy controls. In the present study, we show that monocytes from patients with AIS exhibit a refractory state or endotoxin tolerance. The patients were unable to orchestrate an inflammatory response against LPS and expressed three factors reported to control the evolution of human monocytes into a refractory state: IL-1R–associated kinase-M, NFkB2/p100, and hypoxia-inducible factor-1α. The levels of circulating mitochondrial DNA (mtDNA) in patients with AIS correlated with impaired inflammatory response of isolated monocytes. Interestingly, the patients could be classified into two groups: those who were infected and those who were not, according to circulating mtDNA levels. This finding was validated in an independent cohort of 23 patients with AIS. Additionally, monocytes from healthy controls, cultured in the presence of both sera from patients and mtDNA, reproduced a refractory state after endotoxin challenge. This effect was negated by either a TLR9 antagonist or DNase treatment. The present data further extend our understanding of endotoxin tolerance implications in AIS. A putative role of mtDNA as a new biomarker of stroke-associated infections, and thus a clinical target for preventing poststroke infection, has also been identified. ***The Journal of Immunology*****, 2017, 198: 2038–2046.**

After focal brain ischemia, some patients show a favorable recovery, whereas others develop infectious complications that lead to a poorer functional outcome or even death (1). A number of studies have indicated that patients with acute ischemic stroke (AIS) are colonized by pathogens after ischemia (1, 2), suggesting that a deregulation of the innate immune response could be taking place. A number of predictors of poststroke infection have been reported (e.g., stroke severity, older age, larger area of infarct) (1–4). In this regard, other authors have analyzed the potential role of monocytes in the development of this pathology (5). Most have postulated that the response of the immune system to stroke is biphasic, with early transient activation, followed by a systemic immunosuppressive status, also known as brain-induced immunosuppression (6–8). Several studies have indicated that due to the breakdown of tissue, numerous danger-associated molecular patterns (DAMPs) are generated by the brain to induce this type of immunosuppression, which eventually results in stroke-associated infection (SAI) with increased mortality (4). Curiously, this clinical status could match with the phenomenon known as endotoxin tolerance (ET), the relevance of which has increased in recent years (9–15).

Although ET is considered a protective mechanism against septic shock and ischemia, its incidence is also associated with a high risk of secondary infections in which monocytes/macrophages play a crucial role (11, 14). After an ex vivo endotoxin challenge, the monocytes locked into an ET state are unable to orchestrate an inflammatory response and thus diminished the HLA-DR expression. This set of features defines a refractory state (14). External and internal insults are recognized by the innate immune system, thereby inducing an ET state and subsequently increasing the risk for infection (9, 10, 12, 14). Mechanistically, several studies indicate that hypoxia-inducible factor-1α (HIF1α) drives ET in human monocytes, with the crucial participation of other factors such as IL-1R–associated kinase-M (IRAK-M) and NFkB2/p100 (p100) (16–18).

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Abbreviations used in this article: AIS, acute ischemic stroke; BBB, blood–brain barrier; CBA, cytometric bead array; DAMP, danger-associated molecular pattern; ET, endotoxin tolerance; HIF1α, hypoxia-inducible factor-1α; HV, healthy volunteer; IRAK-M, IL-1R–associated kinase-M; MI, myocardial infarction; mtDNA, mitochondrial DNA; ODN, oligodeoxynucleotide; p100, NFkB2/p100; pMCAO, permanent right middle cerebral artery occlusion; qPCR, quantitative PCR; SAI, stroke-associated infection.

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Remarkably, ET has been described in a number of clinical contexts, including sepsis (14), cancer (9), myocardial infarction (MI) (10), and cystic fibrosis (12).

As noted above, one of the illnesses in which ET occurs is MI (10). The absence of previous infections in these patients suggests the presence of DAMPs that trigger a tolerant state. Owing to the breakdown of tissue, a number of DAMPs, such as those from mitochondria, could spread. In fact, mitochondrial DNA (mtDNA) induces a tolerant state in human monocytes, and higher levels of this DAMP have been detected in patients with MI during ET (10). Several authors have indicated that mitochondria are evolutionary endosymbionts derived from bacteria; they contain DNA similar to bacterial DNA (19).

In the present study, we analyzed the status of monocytes isolated from patients with AIS regarding their potential response to bacterial endotoxins. Additionally, the putative role of mtDNA as a new biomarker and clinical target for poststroke infections was studied.

Materials and Methods

Patients

The study enrolled 14 patients with AIS and without previous treatment, in the neurology department of La Paz University Hospital from October through December 2014 (Table I). Inclusion criteria included age 18–85 y and clinical diagnosis of nonlacunar AIS (<24 h). Neuroimaging had previously ruled out intracraneal hemorrhage, <24 h since stroke onset, and the patients or their legally authorized representatives were able to provide valid informed consent to enroll. Patients were excluded who had a transient ischemic attack, coma (National Institutes of Health Stroke Scale > 1 point on item 1-a), prior functional dependency (modified Rankin Scale ≥ 2), concomitant disease with a life expectancy <3 mo, investigational drugs received within 30 d, acute or chronic inflammatory diseases, immune suppression, active acute or chronic infectious diseases, a history of renal or hepatic diseases, poorly controlled diabetes (hemoglobin A1c ≥ 7 g/dl) or malignancies, those who had undergone a surgical procedure in the preceding 3 mo, and those who were being treated with immunosuppressive drugs. Stroke-associated infection was determined to be a body temperature >37.8°C in patients with clinical symptoms (pleuritic pain, cough, dyspnea or urinary tract symptoms), WBC count of >11,000/ml or <4,000/ml, pulmonary infiltrate on chest x-rays, or cultures positive for a pathogen during the first 7 d after the vascular event. Specialists in infectious diseases from La Paz University Hospital validated SAI. In our cohort, the infections occurred in six (42%, SAI) patients at a mean of 60 h after symptom onset and included pneumonia (n = 3) and urinary tract infection (n = 3).

Additionally, we included an independent 23-sera cohort from patients after 72 h of AIS with and without SAIs. The clinical classifications are similar to our recruited cohort (see the clinical details in Table II).

Ten age- and sex-matched healthy volunteers (HV) without a history of AIS or other significant disease were included as controls. The study protocol adhered to the ethical guidelines of the 1975 Declaration of Helsinki and received approval from the Ethics Committee of La Paz University Hospital in Madrid. All participants or their legal representatives provided written informed consent to participate in the study, and the Ethics Committee approved this consent procedure.

Permanent middle cerebral artery occlusion model in rats

All experiments were designed to minimize animal suffering in compliance with, and were approved by, the Ethics Committee of La Paz University Hospital for the Care and Use of Animals in Research according to the Spanish and European Union rules (86/609/CEE, 2003/65/CE, 2010/63/EU, RD1201/2005, and RD53/2013).

A total of 22 male Sprague-Dawley rats weighing 250–320 g (Charles River Laboratories, L'Arbresle, France) were used. A small craniectomy was made above the rhinal fissure over the branch of the right middle cerebral artery. After the craniectomy, permanent right middle cerebral artery occlusion (pMCAO) was performed, ligating with a 9-0 suture just before its bifurcation into the frontal and parietal branches. Both common carotid arteries were then occluded for 60 min as previously described (20, 21). Body temperature was maintained at 37 ± 0.5°C with a heat lamp throughout the surgery and occlusion period.

The animals were assigned to the following groups: 1) the sham^{LPS} group (n = 9), animals that were subjected to surgery without pMCAO that received an i.p. injection of 25 mg/kg LPS; and 2) the pMCAO^{LPS} group (n = 11), animals that were subjected to surgery for stroke that received an i.p. injection of 25 mg/kg LPS 24 h after surgery. Sera were collected from all the animals before and 1 h after LPS injection.

Reagents

The following Abs were used: anti-CD14, anti–MHC class II-DR (Immunostep, Salamanca, Spain), anti-CD16, and anti–TNF-α (Miltenyi Biotec). The medium used for the cell culture was DMEM from Invitrogen. The LPS

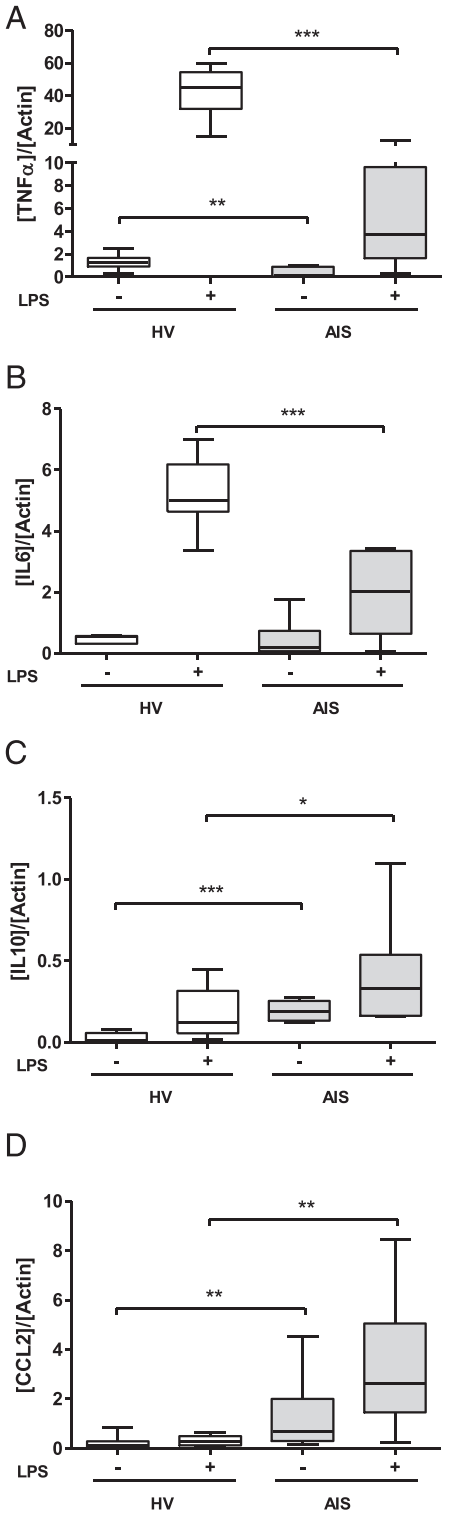

FIGURE 1. Transcriptional profile of monocytes from patients with AIS after LPS challenge. Isolated monocytes from HV (n = 10, open boxes) or patients with AIS after 24 h (n = 14, filled boxes) were treated with or without LPS (1 h, 10 ng/ml), and total mRNA was isolated and cDNA synthesized. Real-time qPCR analyses of (**A**) TNF-α, (**B**) IL-6, (**C**) IL-10, and (**D**) CCL2 were performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ AIS versus HV using the Mann–Whitney test.

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Table I. Baseline, demographic data, risk factors, stroke etiology, disease severity, and 90-d outcome in humans

	HV (n = 10)	SAI (n = 6)	Non-SAI (n = 8)	p Value
Men, n (%)	6 (60)	3 (50)	4 (50)	ns
Median age, y (SD)	76 (14.3)	82.5 (11.4)	77.5 (12.2)	ns
Systolic blood pressure on admission; median (SD)	122.8 (10.5)	182.5 (42.4)	155 (20.8)	<0.01
Blood glucose on admission, mmol/l; median (SD)	5.2 (0.46)	144.5 (62.5)	103.5 (42.8)	<0.001
Leukocytes, $\times 10^9$ cells/ml; median (SD)	7.5 (1.46)	8.4 (3.48)	7.8 (2.46)	ns
Hypertension, n (%)	0	4 (66.7)	2 (25)	ns
Diabetes mellitus, n (%)	0	1 (16.7)	0 (0)	ns
Hyperlipidemia, n (%)	0	3 (50)	3 (37.5)	ns
Atrial fibrillation, n (%)	0	1 (16.7)	6 (75)	ns
Current smoking, n (%)	0	1 (16.7)	1 (12.5)	ns
Alcohol abuse, n (%)	0	2 (33.3)	0 (0)	ns
Coronary heart disease, n (%)	0	3 (50)	2 (25)	ns
Peripheral arterial disease, n (%)	0	1 (16.7)	0 (0)	ns
Stroke etiology				
Large artery atherosclerosis, n (%)	0	2 (33.3)	0 (0)	ns
Cardioaortic embolism, n (%)	0	2 (33.3)	6 (75)	ns
Small artery occlusion, n (%)	0	0 (0)	0 (0)	ns
Other causes, n (%)	0	1 (16.7)	0 (0)	ns
Undetermined causes, n (%)	0	1 (16.7)	2 (25)	ns
NIHSS score on admission; median (IQR)	—	15.5 (10)	17 (11)	N/A
90-d outcome				
mRS 0–3, n (%)	—	2 (33.3)	7 (87.5)	N/A
mRS 4–6, n (%)	—	4 (66.7)	1 (12.5)	N/A

Comparisons between groups were performed by ANOVA or a χ^2 test.
IQR, interquartile range; mRS, modified Rankin Scale. N/A, not applicable; NIHSS, National Institutes of Health Stroke Scale; ns, not significant.

from *Salmonella abortus* was a gift from Dr. C. Galanos (Max Planck Institute of Immunobiology and Epigenetics). oligodeoxynucleotide (ODN) 5′-TTAGGG-3′ was used as the TLR9 antagonist (InvivoGen). The mtDNA was removed by DNase I (Applied Biosystems). All the reagents used for the cell cultures were endotoxin-free and were assayed with the *Limulus* amebocyte lysate test (Cambrex).

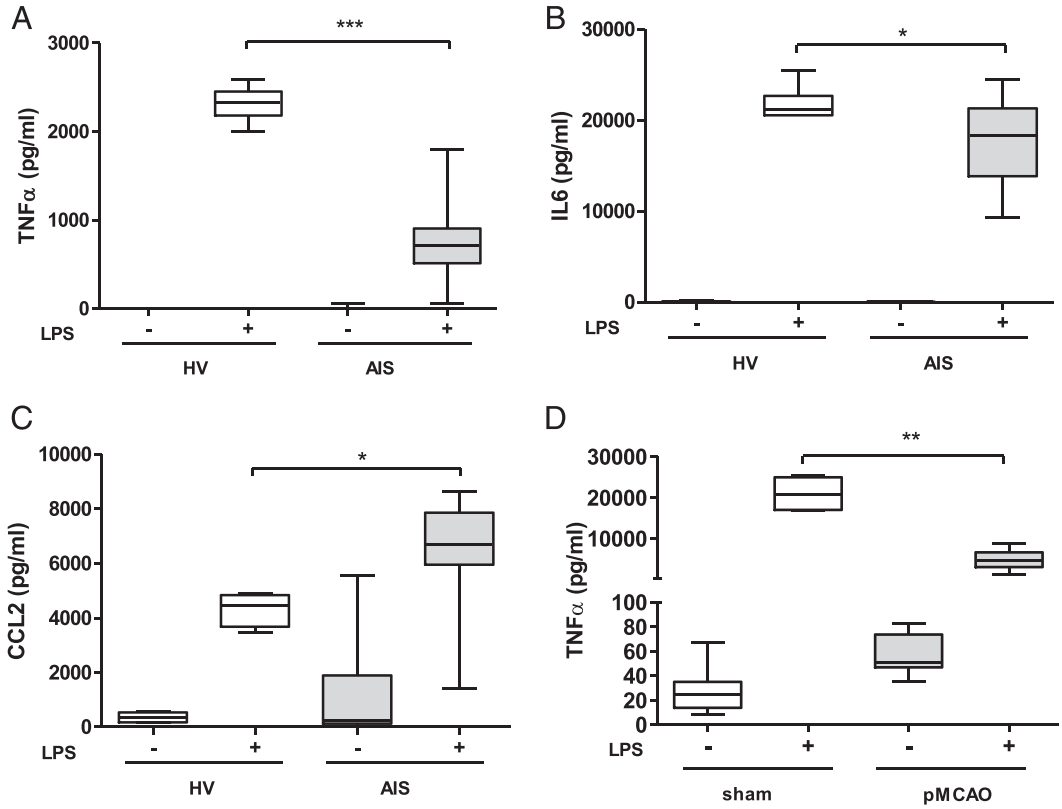

FIGURE 2. Protein levels of human monocytes from patients with AIS and in a pMCAO rat model after LPS challenge. Monocytes were isolated from HV (n = 10, open boxes) or patients with AIS (n = 14, filled boxes) with or without LPS (16 h, 10 ng/ml). Next, the cytokine levels in the culture supernatants were determined using a CBA. The protein levels of TNF-α (**A**), IL-6 (**B**) and CCL2 (**C**) are shown. $*p < 0.05$, $***p < 0.001$ AIS versus HV using the Mann–Whitney U test. Animals were subjected to a surgery for stroke (pMCAO) and received an i.p. injection of 25 mg/kg LPS 24 h after surgery. Sera were collected from all animals before and 1 h after LPS injection (**D**) The protein TNF-α levels were analyzed in sham (n = 9, open boxes) and pMCAO (n = 11, filled boxes) animal groups before and after 1 h of LPS treatment (25 mg/kg) using CBA. $**p < 0.01$ Sham versus pMCAO using the Mann–Whitney U test.

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mtDNA from clinical material

The primers for the real-time PCR analysis of mtDNA were as follows: 5′-CCACGGGAAACAGCAGTGAT-3′ and 5′-CTATTGACTTGGGTTAATC-GTGTGA-3′. The TaqMan probe (6FAM–5′-TGCCAGCCACCGCG-3′–MGB) was labeled at the 5′ end with a fluorescent reporter, 6FAM. The 20-μl PCR reaction contained 1× TaqMan universal PCR master mix (Applied Biosystems, P/N 4304437), 112 nM of each mtDNA primer, 125 nM mtDNA TaqMan probe, and 5 μl of DNA extract. The PCR conditions were 2 min at 50˚C and 10 min at 95˚C, followed by 40 denaturation cycles of 15 s at 95˚C and 60 s of annealing/extension at 60˚C. Calibration curves were used to quantify mtDNA as previously described (22).

Preparation of mtDNA

Human mtDNA was isolated by a mitochondrial isolation kit (Miltenyi Biotec) following the the manufacturer's instructions. mtDNA was extracted using the QIAamp DNA mini kit (Qiagen). Mitochondrial concentration was determined from the DNA with a NanoDrop spectrophotometer (Thermo Scientific): no protein contamination was found. All the extracts were endotoxin free, as assayed with the Sigma E-Toxate reagent from *Limulus polyphemus* (Sigma). The mtDNA purity (average 99%) was verified by quantitative PCR (qPCR).

Monocyte isolation from PBMCs

Twenty milliliters of blood was taken from a peripheral vein at 24 h (24 ± 2 h) and 72 h (72 ± 2 h) after hospital admission for AIS. The PBMCs were isolated from all the participants by Ficoll-Plus gradient (GE Healthcare Bio-Sciences) (23). The monocytes were obtained by adherence, as previously described (23). The purity of the monocyte cultures was tested by CD14 labeling and flow cytometry analysis (average 89% of $CD14^+$ cells, data not shown). Other cell surface markers were also tested (CD1a, 3.8%; CD89, 91%, data not shown). The same protocol was used to obtain monocytes from all the patients.

Flow cytometry analysis

For marker staining, the cells were labeled with the following mAbs: allophycocyanin-conjugated anti-human CD14, PE-conjugated anti-human CD16 (B73.1), FITC-conjugated anti–HLA-DR (all from Immunostep), FITC-conjugated anti-human CD14 (Miltenyi Biotec), and allophycocyanin-conjugated anti-human TNF-α. Matched isotype Abs were used as negative controls. The cells were incubated for 30 min at 4˚C in the dark. The data were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences). The data were analyzed with FlowJo software (Tree Star).

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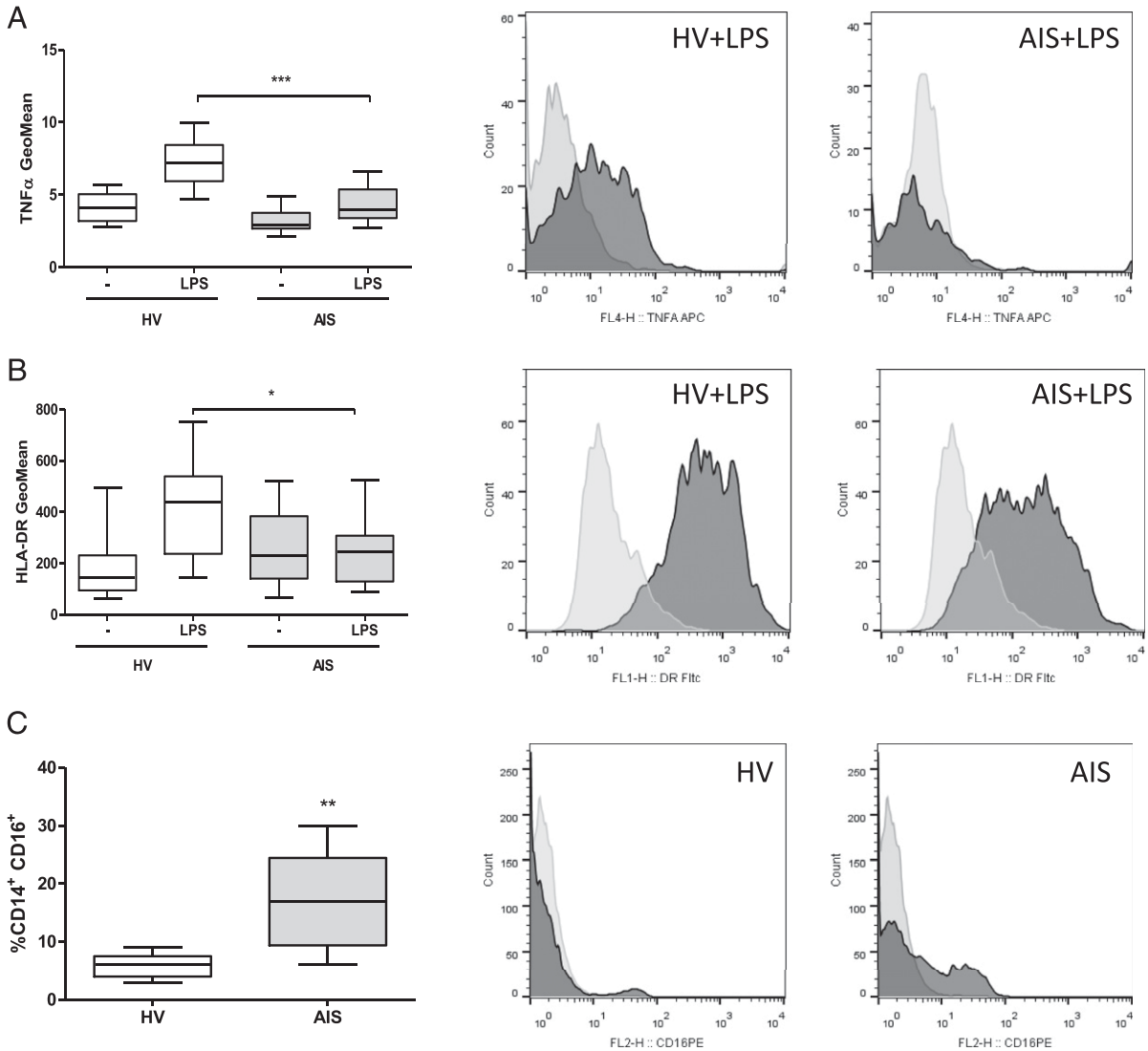

FIGURE 3. Phenotype of human monocytes from patients with AIS after LPS challenge. Monocytes were isolated from HV (n = 10, open boxes) or patients with AIS (n = 14, filled boxes) with or without LPS (16 h, 10 ng/ml). Next, cells were stained with anti–TNF-α, anti–HLA-DR, anti-CD16, and anti-CD14 and were analyzed by flow cytometry. (**A**) The geometric mean of intracellular TNF-α on the $CD14^+$ monocyte is shown. Standard histograms of TNF-α production with respect to isotype control are shown (right panel). (**B**) The geometric mean of HLA-DR in the $CD14^+$ monocyte is shown. Standard histograms of HLA-DR expression with respect to isotype control are shown (right panel). (**C**) Percentage of $CD14^+CD16^+$ is shown. Standard dot plots of CD14 and CD16 expression are shown (right panel).*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ AIS versus HV using the Mann–Whitney U test.

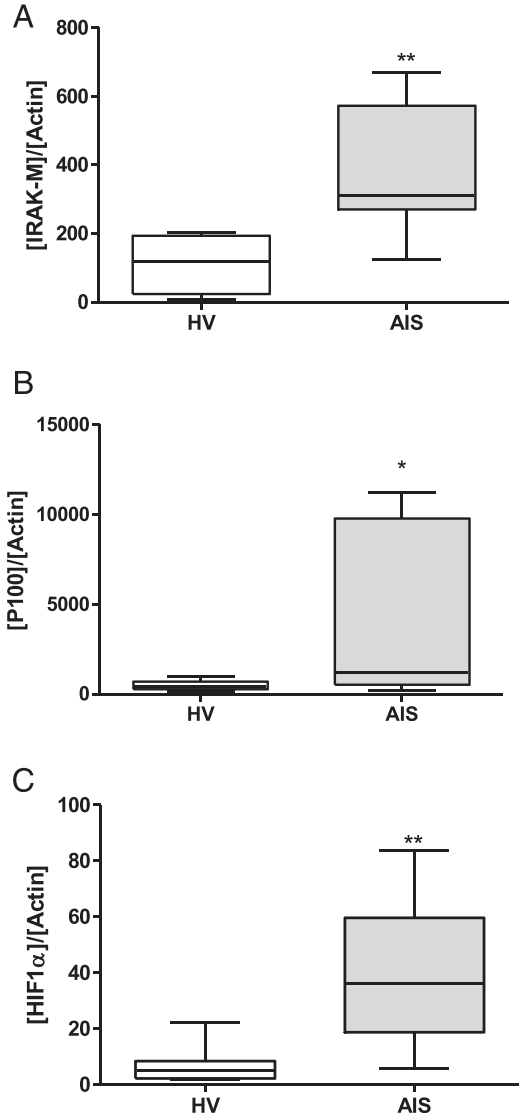

FIGURE 4. ET markers of human monocytes from patients with AIS. Isolated monocytes from HV (n = 10, open boxes) or patients with AIS (n = 14, filled boxes) were analyzed. Total mRNA from these cells was isolated and cDNA was synthesized. Real-time qPCR analyses of (**A**) IRAK-M, (**B**) p100, and (**C**) HIF1α were performed. *$p < 0.05$, **$p < 0.01$ AIS versus HV using the Mann–Whitney U test.

Intracellular TNF-α analysis

For intracellular cytokine staining, the cells were incubated for 16 h at 37°C in the presence of brefeldin A (0.1 μg/ml). The staining was performed after fixation using FACS permeabilizing solution 2 (BD Biosciences), according to the manufacturer's instructions. The cells were washed with BD Perm/Wash solution (BD Pharmingen) and stained with an allophycocyanin-conjugated anti-human TNF-α mAb from Miltenyi Biotec at 4°C for 20 min. The cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Cytometric bead array and flow cytometry analysis

The cytokine levels in the culture supernatants from the human samples were determined using the cytometric bead array (CBA) Flex Set (BD Biosciences), following the manufacturer's protocol. Supernatants from rat sera were evaluated using the CBA rat TNF-α kit (BD Biosciences). The data collected were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences).

RNA isolation and quantification

The cells were washed once with PBS, and the RNA was isolated using the High Pure RNA isolation kit (Roche Diagnostics). The cDNA was obtained by reverse transcription of 1 mg of RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). The mRNA quantitation gene expression levels were analyzed by real-time quantitative PCR using the LightCycler system (Roche Diagnostics), and the cDNA was obtained as described above. Real-time quantitative PCRs were performed using the QuantiMix Easy SYG kit from Biotools and specific primers. The results were normalized to the expression of β-actin (actin), and the cDNA copy number of each gene of interest was determined using a 7-point standard curve, as described previously (16–18, 23–25). The products were amplified using primers for TNF-α, 5′-GCC TCT TCT CCT TCC TGATCG T-3′ (forward) and 5′-CTC GGC AAA GTC GAG ATA GTC G-3′ (reverse); IL-6, 5′-CAA AGA ATT AGA TGC AAT AA-3′ (forward) and 5′-GCC CAT TAA CAA CAA CAA TCT G-3′; and CCL2, 5′-GAT CTC AGT GCA GAG GCT CG-3′ (forward) and 5′-ATT CTT GGG TTG TGG AGT GAG TGT TCA-3′ (reverse). All the primers were synthesized, desalted, and purified by Bonsai Biotech.

Statistical analysis

The statistical significance was calculated using a Mann–Whitney U test and paired t test where appropriate. The differences were considered significant at $p < 0.05$, using Prism 5.0 software (GraphPad Software).

Results

Isolated monocytes from patients 24 h after AIS revealed an ET status

Monocytes from HV and patients with AIS were isolated and exposed to LPS ex vivo for 1 h, and cytokine expression was analyzed by real-time qPCR. The proinflammatory cytokines TNF-α and IL-6 were downregulated in patients with AIS, whereas IL-10 and CCL2 were upregulated after LPS challenge (Fig. 1). On basal levels, TNF-α but not IL-6 showed lower levels

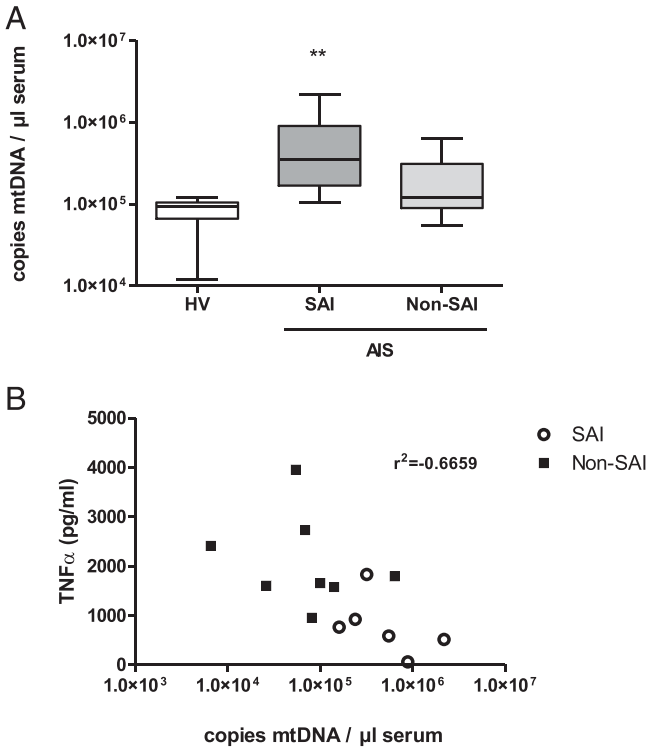

FIGURE 5. mtDNA levels in sera and mtDNA correlation with TNF-α protein levels in monocytes isolated from patients after 72 h from AIS. (**A**) Sera mtDNA levels of HV (n = 10, white box), SAI (n = 6, dark gray box), and non-SAI (n = 8, light gray box) patients after 72 h of AIS were quantified by qPCR. **$p < 0.01$ for infected patients with AIS versus HV using the Mann–Whitney U test. (**B**) Correlation between mtDNA levels and TNF-α after LPS challenge (16 h, 10 ng/ml) of isolated monocytes from patients after 72 h of AIS (n = 14). Spearman correlation coefficient (r) is shown. SAI patients (n = 6, ○) and non-SAI (n = 8, ■) are shown.

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in AIS than in HV monocytes (Fig. 1A, 1B). Both IL-10 and CCL2 also exhibited a patent increase in untreated AIS monocytes (Fig. 1C, 1D, Table I).

An analysis of the soluble protein levels of TNF-α, IL-6, and CCL2 after 16 h of LPS treatment corroborated the ET status in isolated monocytes from patients with AIS (Fig. 2A–C). There was no statistically significant difference, however, in IL-10 levels between AIS and HV (data not shown). Moreover, we performed an in vivo animal model of stroke (pMCAO, $n = 11$) to study the implications of a vascular event inducing ET status. We found a significant decrease in circulating levels of TNF-α after LPS challenge in the pMCAO group compared with the sham group (Fig. 2D).

The cytometry analysis supported the ET status in the patients with AIS. TNF-α intracellular expression after LPS challenge was impaired in $CD14^+$ cells from patients with AIS compared with HV (Fig. 3A). Moreover, HLA-DR expression in the $CD14^+$ cells was not increased after LPS challenge in patients with AIS (Fig. 3B). After AIS, monocytes exhibited a significant increase in the nonclassical population subset ($CD14^+/CD16^+$, Fig. 3C). Collectively, these results suggest that the AIS monocytes were locked into a state of ET.

We also analyzed the expression of three reported factors that control the evolution of human monocytes into a refractory state: IRAK-M, p100, and HIF1α. Monocytes from patients with AIS at basal levels showed increased expression of these three factors compared with HV monocytes (Fig. 4).

mtDNA correlated with SAI and impaired the inflammatory response in patients after 72 h of AIS

Monocyte exposure to mitochondrial DAMPs, including mtDNA, induces a transient state in which these cells are refractory to further endotoxin stimulation. In this context, we explored the potential role of mtDNA levels after 72 h of AIS in response to infections. As Fig. 5A shows, circulating levels of mtDNA after 72 h of AIS were significantly elevated in patients with SAI compared with HV. The levels of TNF-α after LPS challenge in monocytes isolated from SAI and non-SAI patients after 72 h of AIS were correlated with circulating levels of mtDNA after 72 h of AIS (Fig. 5B). These results confirm that mtDNA in sera correlated not only with a marked ET, but also with infections.

Ultimately, to validate the potential role of mtDNA as a biomarker of secondary infections after AIS, we evaluated the sera mtDNA levels from an independent cohort of patients with AIS ($n = 23$), and a significant increase in mtDNA levels from SAI sera compared with non-SAI sera was found (Table II).

Sera from patients with AIS induce an ET state depending on mtDNA

To study the modulation of mtDNA on the inflammatory response in monocytes, we performed mtDNA in vitro assays with TLR9 antagonist (ODN 5′-TTAGGG-3′) or DNase pretreatment, followed by an LPS challenge (see the schematic design in Fig. 6A). We found a significant reduction in TNF-α and IL-6 in the mtDNA pretreatment monocytes compared with controls. In contrast, CCL2 was upregulated in mtDNA pretreatment monocytes. This ET status induced by mtDNA was negated using a TLR9 antagonist (ODN 5′-TTAGGG-3′) or DNase pretreatment (Fig. 6).

Taking into account the data shown in Fig. 5, we studied the effect of sera from AIS patients with SAI on monocytes isolated from HV (see the schematic design in Fig. 7A). AIS sera induced a deregulation of the inflammatory response against LPS compared with HV sera, decreasing TNF-α and IL-6 cytokine levels and increasing CCL2 production. In contrast, TLR9 antagonist (ODN 5′-TTAGGG-3′) pretreatment negated this ET status effect, increasing TNF-α and diminishing CCL2; IL-6 levels, however, showed no significant differences. In line with these findings, DNase pretreatment reduced the ability to induce refractoriness of

Table II. Baseline, demographic data, risk factors, stroke etiology, disease severity, 90-d outcome, and mtDNA in AIS patients

	SAI ($n = 10$)	Non-SAI ($n = 13$)	p Value
Men, *n* (%)	6 (60)	6 (46.15)	ns
Median age, y (SD)	81 (6.8)	77 (12.4)	ns
Systolic blood pressure on admission; median (SD)	149.5 (29.3)	155 (21.2)	ns
Blood glucose on admission, mmol/l; median (SD)	112 (24.4)	124.5 (33.7)	ns
Leukocytes, $\times 10^9$ cells/ml; median (SD)	8.2 (4.5)	8.6 (1.8)	0.004
Hypertension, *n* (%)	7 (70)	8 (61.5)	ns
Diabetes mellitus, *n* (%)	3 (30)	3 (23.1)	ns
Hyperlipidemia, *n* (%)	4 (40)	7 (53.8)	ns
Atrial fibrillation, *n* (%)	3 (30)	5 (38.5)	ns
Current smoking, *n* (%)	3 (30)	3 (23.1)	ns
Alcohol abuse, *n* (%)	2 (20)	1 (7.7)	ns
Coronary heart disease, *n* (%)	2 (20)	1 (7.7)	ns
Peripheral arterial disease, *n* (%)	1 (10)	0 (0)	ns
Stroke etiology			
Large artery atherosclerosis, *n* (%)	4 (40)	3 (23.1)	ns
Cardioaortic embolism, *n* (%)	4 (40)	6 (46.1)	ns
Small artery occlusion, *n* (%)	0 (0)	0 (0)	ns
Other causes, *n* (%)	0 (0)	0 (0)	ns
Undetermined causes, *n* (%)	2 (20)	4 (30.8)	ns
NIHSS score on admission; median (IQR)	15.5 (17)	10 (21)	ns
90-d outcome			
mRS 0–3, *n* (%)	5 (50)	13 (100)	0.054
mRS 4–6, *n* (%)	5 (50)	0 (0)	0.002
Sera mtDNA levels after 72 h of AIS, copies mtDNA/μl serum; median (SD)	752,489; 115 (±790,194; 2,651)	174,951; 7 (±89,473; 42,231)	0.0169

Comparisons between groups were performed by ANOVA or a χ^2 test.
IQR, interquartile range; mRS, modified Rankin Scale. NIHSS, National Institutes of Health Stroke Scale; ns, not significant.

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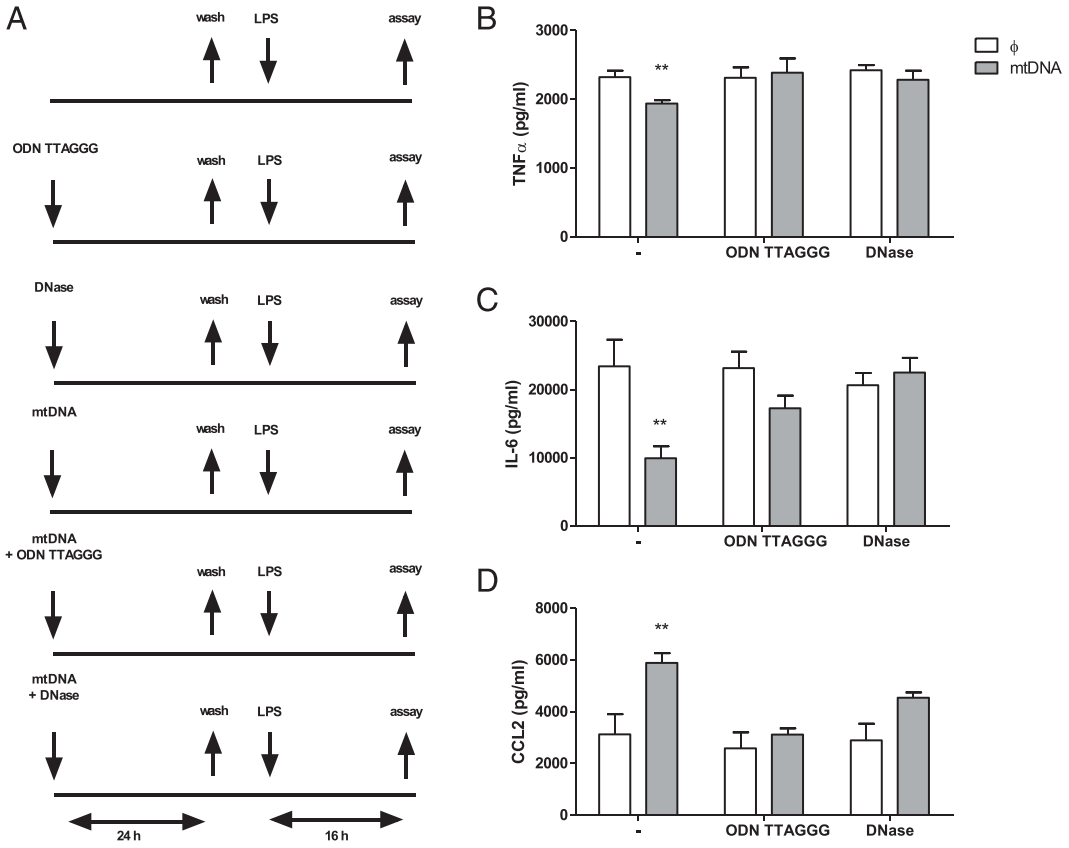

FIGURE 6. Modulation of human monocytes by exposure to mtDNA. (**A**) Workflow diagram. Monocytes from HV ($n = 5$) were pre-exposed to human mtDNA (0.3 μg/ml, filled bar) and without mtDNA (ϕ, open bar), and in some conditions the TLR9 antagonist (ODN 5′-TTAGGG-3′, 10 μM) was added or the sera were treated with DNase I (DNase, 180 U) for 20 min at 37°C before use. Next, cultures were washed and challenged with LPS (10 ng/ml) for 16 h. Finally, levels of cytokines in the supernatants were evaluated by CBA. Protein levels of TNF-α (**B**), IL-6 (**C**) and CCL2 (**D**) are shown. $^{**}p < 0.01$ mtDNA versus ϕ using the *t* test.

AIS sera in the TNF-α, IL-6, and CCL2 cytokines analyzed (Fig. 7B–D).

Discussion

The immune response after AIS is receiving increased attention, although it could be closely related to the occurrence of comorbidities. Several studies have indicated that patients with AIS are colonized by pathogens after ischemia, suggesting a brain-induced immunosuppression (4, 6–8). More specifically, our data showed that monocytes isolated from AIS patients exhibit an ET status, with a patent upregulation of the three primary ET controllers (IRAK-M, p100, and HIF1α). Besides, an in vivo animal model of stroke (pMCAO) corroborated the main ET hallmark, that is, a downregulation of TNF-α after LPS challenge.

In most cases, ET has been associated with previous endotoxin contact, which induces a refractory state in a second endotoxin challenge (11, 12, 16, 17); this ET signature also helps to predict sepsis and organ dysfunction (26). However, we previously reported that mitochondrial DAMPs, including mtDNA, induced ET in human monocytes under "sterile" conditions (10). Our cohort of patients with AIS exhibited high levels of circulating mtDNA after 72 h from the vascular event, and these levels were significantly higher in those patients who suffered from bacterial infections. After ischemic brain injury, disruption of the blood–brain barrier (BBB) occurs early (27). In humans, opening of the BBB has been documented within 2–6 h (median, 3.8 h) of stroke onset (27). We hypothesize that this early disruption of the BBB might spread the mitochondrial DAMPs resulting from the vascular event. A previous report has shown that, in a large cohort of patients with AIS, mtDNA levels increase after AIS (28).

The in vitro assays corroborated our hypothesis, which indicated that mtDNA was an important factor in the ET observed in patients with AIS. Both sera from patients with AIS and mtDNA induced an ET status in monocytes isolated from HV, and this effect was primarily negated in the presence of a blocking TLR9 antagonist. Note that published data suggest that mtDNA carries bacterial motifs due to its endosymbiotic origin (i.e., its DNA is rich in CpG motifs and molecules such as cardiolipin in its membrane) (29). Moreover, our findings suggested that the well-described DNA bacterial sensor TLR9 (30) recognized the mtDNA. Additionally, when DNAse degraded the mtDNA in the sera samples, the ET effect was completely negated. Neither bacterial DNA nor endotoxin traces were found in the sera samples (data not shown).

We have shown that monocytes from patients with AIS exhibit a manifest ET state that correlates with circulating mtDNA levels after 72 h from AIS. These patients could be classified into two groups: those who were infected and those who were not, according to circulating mtDNA levels. A putative role of mtDNA as a new biomarker of SAIs, and thus a clinical target for preventing poststroke infection, has also been identified. This finding was validated in an independent cohort of patients in whom levels of mtDNA in circulation allowed their classification into SAI and non-SAI. Understanding the mechanisms of ET after AIS is necessary to establishing its role in stroke outcome and to aid the further development of therapeutic targets that allow improvements in stroke recovery.

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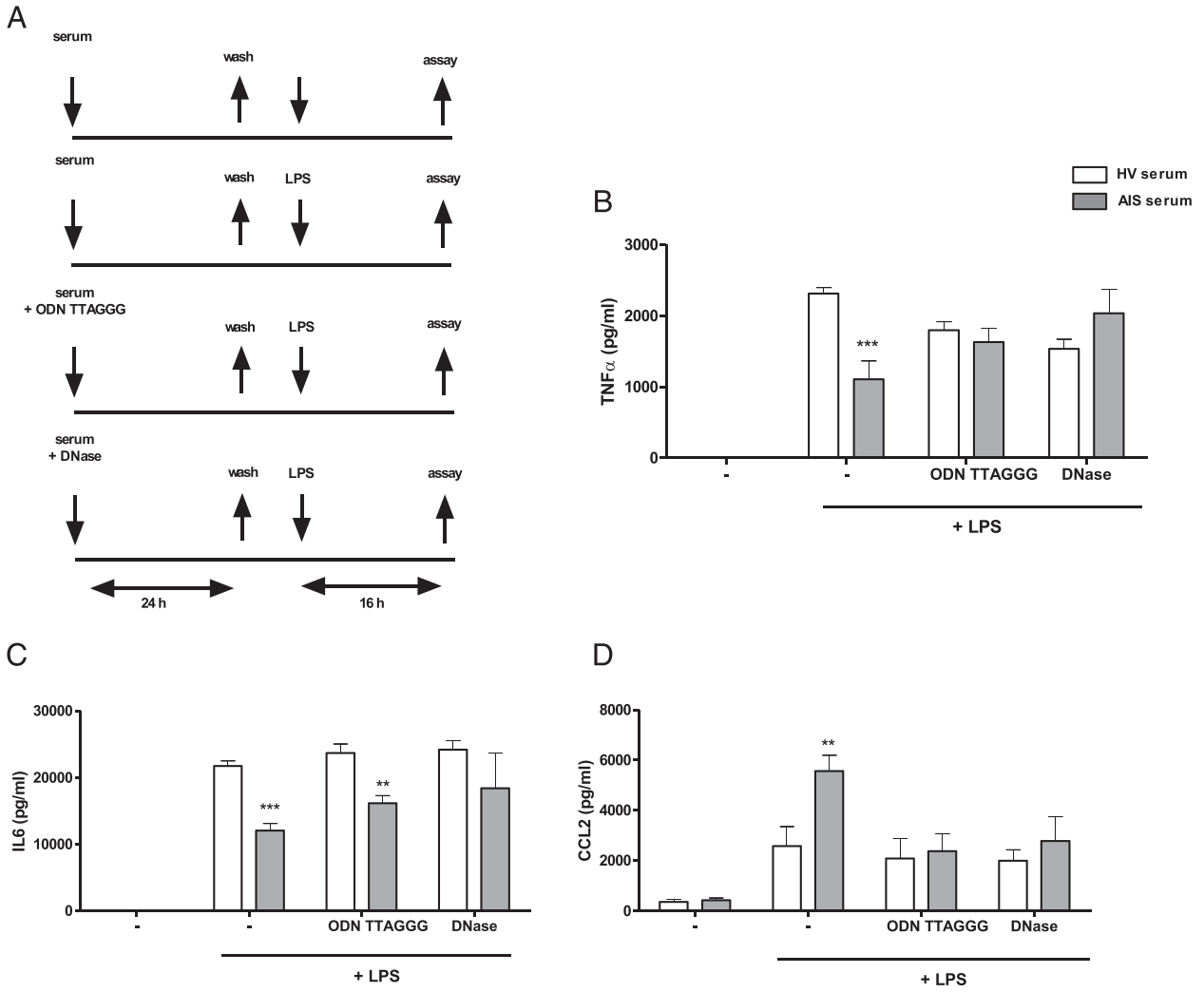

FIGURE 7. Modulation of human monocytes by exposure to sera from patients with AIS. (**A**) Workflow diagram. Monocytes from HV ($n = 7$) were pre-exposed to sera from HV ($n = 4$, open bars) or patients with SAI after 72 h of AIS ($n = 4$, filled bars); in some conditions the TLR9 antagonist (ODN 5′-TTAGGG-3′, 10 μM) was added or the sera were treated with DNase I (DNase, 180 U) for 20 min at 37°C before use. Next, cultures were washed and challenged with LPS (10 ng/ml, filled bars) or without for 16 h. Finally, levels of cytokines in the supernatants were evaluated by CBA. Protein levels of TNF-α (**B**), IL-6 (**C**), and CCL2 (**D**) are shown. $^{**}p < 0.01$, $^{***}p < 0.001$ AIS serum versus HV serum using the *t* test.

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Disclosures

The authors have no financial conflicts of interest.

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