

## Expression of triggering receptor on myeloid cell 1 and histocompatibility complex molecules in sepsis and major abdominal surgery

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was  $44.39 \pm 20.25\%$ , with a significant difference between healthy and septic groups ( $P < 0.05$ ) for both molecules. In the surgical patients, TREM-1 and HLA-DR expressions were  $56.8 \pm 20.85$  MFI and  $71 \pm 13.8\%$  before surgery and  $72.65 \pm 29.92$  MFI and  $72.82 \pm 22.55\%$  after surgery. TREM-1 expression was significantly different ( $P = 0.0087$ ) between the samples before and after surgery and svTREM-1 expression was  $0.8590 \pm 0.1451$  MFI,  $0.8820 \pm 0.1460$  MFI, and  $2.210 \pm 0.7873$  MFI in the healthy, surgical (after surgery) and septic groups, respectively. There was a significant difference ( $P = 0.048$ ) in svTREM-1 expression between the healthy and surgical groups and the septic group.

**CONCLUSION:** TREM-1 expression is increased during systemic inflammatory conditions such as sepsis and the postoperative phase. Simultaneous low expression of HLA-DR molecules correlates with the severity of illness and increases susceptibility to infection. Additionally, TREM-1 expression is distinctly different in surgical patients at different stages of the inflammatory response before and after surgery. Thus, surface TREM-1 appears to be an endogenous signal during the course of the inflammatory response. svTREM-1 expression is significantly increased during sepsis, appearing to be an indicator of severity of illness. Together, these data indicate that TREM-1 may play an important role in establishing and amplifying the systemic inflammatory response. TREM-1, HLA-DR, and svTREM-1 expression analysis can provide useful diagnostic and prognostic indicators during SIRS, CARS, and sepsis.

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**Key words:** TREM-1; HLA-DR; SIRS; CARS; Sepsis

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### Abstract

**AIM:** To evaluate the surface expression of triggering receptor on myeloid cell 1 (TREM-1), class II major histocompatibility complex molecules (HLA-DR), and the expression of the splicing variant (svTREM-1) of TREM-1 in septic patients and those subjected to major abdominal surgery.

**METHODS:** Using flow cytometry, we examined the surface expression of TREM-1 and HLA-DR in peripheral blood monocytes from 11 septic patients, 7 elective gastrointestinal surgical patients, and 10 healthy volunteers. svTREM-1 levels were analyzed by RT-PCR.

**RESULTS:** Basal expression of TREM-1 and HLA-DR in healthy volunteers was  $35.91 \pm 14.75$  MFI and  $75.8 \pm 18.3\%$ , respectively. In septic patients, TREM-1 expression was  $59.9 \pm 23.9$  MFI and HLA-DR expression

## INTRODUCTION

Inflammation constitutes the initial and essential response of the host against infection and injury. In the presence of infection, inflammation is triggered through the recognition of microorganism-associated molecular structures by Toll-like receptors (TLRs)<sup>[1,2]</sup>, which are expressed in a nonclonal manner on the surface of innate immune cells such as monocytes, macrophages, and dendritic cells<sup>[3,4]</sup>. This interaction leads to the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and to the release of pro-inflammatory (tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin (IL-12, IL-1, IL-6, IL-8, *etc.*) and anti-inflammatory (IL-10 and TGF- $\beta$ ) cytokines<sup>[3,5-7]</sup>. In the absence of infection, injuries such as trauma, burns, pancreatitis, and major surgery also induce an inflammatory response. In most cases, inflammation is a local response, but in some situations, it becomes a systemic phenomenon and leads to the development of systemic inflammatory response syndrome (SIRS), characterized by the massive release of pro-inflammatory mediators in the absence of infection<sup>[8-10]</sup>. On the other hand, the excessive release of anti-inflammatory mediators leads to the development of compensatory anti-inflammatory response syndrome (CARS)<sup>[8,11]</sup>. During this phase, patients frequently suffer from temporary impairment of immunological functions, which in its most severe form is referred to as immunoparalysis<sup>[8-10]</sup>. This diminished ability to respond to stimuli is paralleled by a strong downregulation of MHC class II (mainly HLA-DR, invariant chain) expression on the surface of monocytes. Such monocytes produce only minor amounts of pro-inflammatory cytokines, as well as NO<sup>[12,13]</sup>. Very similar alterations of monocyte function exist with experimental endotoxin tolerance and in patients after trauma, burns, pancreatitis, and major surgery<sup>[10,14-17]</sup>. These alterations often predispose patients to life-threatening infections and to the development of sepsis. Furthermore, it has been reported that immunoparalysis correlates with the severity of sepsis and injury as well as the postoperative course<sup>[15,17-19]</sup>.

However, progress has been made in sepsis research improving the knowledge of the basic pathophysiological processes of sepsis, in daily intensive care unit (ICU) practice, it remains difficult to adequately identify and treat sepsis and its related conditions<sup>[20]</sup>. In some cases, measurement of serum proteins such as interleukin 6 (IL-6) has been demonstrated to be useful in evaluating the severity and in predicting the outcome for patients with SIRS/sepsis, but not in CARS and susceptibility to infection<sup>[21,22]</sup>. Therefore, it is essential to search for and identify endogenous mediators of inflammatory response suitable for use as early indicators of infection in cases of sepsis and as prognostic indicators during SIRS and CARS<sup>[20,23-27]</sup>.

TREM-1 is an activating receptor of the Ig superfamily exclusively expressed on blood neutrophils and monocytes<sup>[28]</sup>. Expression of TREM-1 on these cells is induced by TLR ligands such as lipopolysaccharide and lipoteichoic acid<sup>[29]</sup>. Though its natural ligand has not been

identified, engagement of TREM-1 on monocytes with agonist monoclonal antibodies results in the production of pro-inflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) as well as chemokines such as IL-8 and monocyte chemoattractant protein-1. When monocytes are stimulated with lipopolysaccharide, leading to a pro-inflammatory response, engagement of TREM-1 dramatically enhances this effect when used as a costimulus, showing that TREM-1 can amplify inflammatory responses initiated by TLRs<sup>[28-30]</sup>.

TREM-1 surface expression is greatly increased on infiltrating neutrophils isolated from the peritoneal cavity of patients with septic shock due to bacterial peritonitis. In contrast, peritoneal lavage cells of patients with SIRS caused by nonmicrobial peritoneal inflammation show normal levels of TREM-1, and it has been proposed that TREM-1 is upregulated in the presence of infection<sup>[30]</sup>. At the mRNA level, an alternative splicing variant of TREM-1 (svTREM-1) has been described, which might encode a soluble form<sup>[31]</sup>, as it has been detected in patients receiving mechanical ventilation. Rapid detection of soluble TREM-1 in bronchoalveolar lavage fluid has been proposed for establishing or excluding a diagnosis of bacterial or fungal pneumonia<sup>[32,33]</sup>, and TREM-1 mRNA upregulation correlates with the severity of acute pancreatitis<sup>[34]</sup>.

In this study, we aimed to explore the expression of TREM-1, svTREM-1, and HLA-DR as early indicators of infection or as prognostic indicators during systemic inflammation, using surgical and septic patients as models of the inflammatory response.

## MATERIALS AND METHODS

### Patients

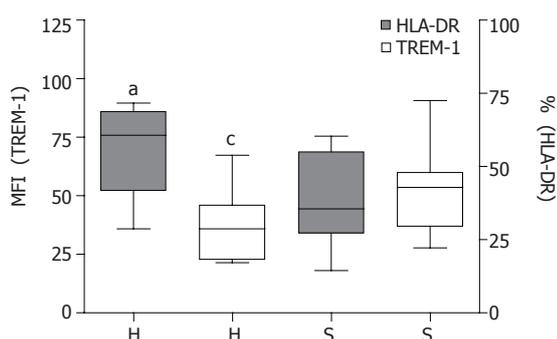
Samples were obtained from 11 septic patients, 7 elective gastrointestinal surgical patients (pre- and post-surgery) and 10 healthy volunteers who gave their informed consent, according to the protocol approved by the Institute Ethics Committee (reference number 034/2004, IMSS 2004-3601-0064). Sepsis diagnosis was determined by the presence of at least two of the following signs: hypothermia or hyperthermia, tachycardia, tachypnea or hyperventilation as defined by PaCO<sub>2</sub> of 4.256 Kpa and leukocytosis, bandemia or leukopenia.

### Blood samples

Blood (15 mL) was collected from healthy volunteers, septic patients (at their admission to gastro surgery service with sepsis diagnosis) and surgical patients (first sample 30 min before surgery and second sample 12-24 h after surgical procedure ended) and heparinized (10 U/mL).

### Flow cytometry

Peripheral blood mononuclear cells were obtained from the heparinized samples by centrifuging at 800 r/min for 30 min over a Ficoll-Hypaque gradient. Cells were washed thrice with isotonic solution at 150 r/min for 10 min, the



**Figure 1** TREM-1 and HLA-DR expression in healthy volunteers and septic patients. Comparison of expression levels of TREM-1 (mean of fluorescence intensity) and HLA-DR (percentage of CD14+HLA-DR+cells) between healthy volunteers (H) and septic patients (S). <sup>a</sup> $P < 0.05$  healthy vs septic for HLA-DR expression, <sup>b</sup> $P < 0.05$  healthy vs septic for TREM-1 expression.

supernatant was discarded, and the pellet was resuspended in an isotonic solution. Trypan blue staining was done on an aliquot of the cell suspension to determine cell numbers and to assess their viability. Cells were transferred to a 96-well conical well plate ( $1 \times 10^6$  cells/well). Cells were blocked with a 20 g/L human gamma globulin solution (Beriglobina<sup>®</sup>, Aventis) for 1 h at 4 °C and washed by centrifuging at 800 r/min for 3 min at 4 °C. The supernatant was discarded and the pellet was resuspended. Cell staining for flow cytometry was done by adding anti-CD14/FITC, anti-HLA-DR/PE (BD Biosciences), anti-TREM-1/PE (R&D Systems) and an isotype control. The contents in each well were transferred to flow cytometry tubes in 400  $\mu$ L of sheath fluid. Finally, samples were analyzed in a BD FACScalibur flow cytometer.

#### Total RNA extraction, RT-PCR and semiquantitative analysis

Total RNA was extracted from peripheral blood mononuclear cells using TRIzol reagent (Invitrogen). The concentrations and purity of the extracted RNA were determined spectrophotometrically at 260 and 280 nm (Beckman DU640, USA). Single-stranded cDNA was synthesized by mixing 1  $\mu$ g RNA with 1  $\mu$ L oligo-dT (0.5 g/L, Promega), 1  $\mu$ L dNTP mix (10 mmol/L, Promega), 4  $\mu$ L reaction buffer (Promega), 2  $\mu$ L DTT (0.1 mol/L, Promega), 1  $\mu$ L SuperScript II RNase H reverse transcriptase (200 U/ $\mu$ L, Promega) and DEPC-treated water to 20  $\mu$ L. The reaction mixture was incubated at 42 °C for 50 min, followed by 70 °C for 15 min to inactivate the reverse transcriptase. cDNA (1  $\mu$ g) was used as the template for PCR amplification of TREM-1. The mixture consisted of 0.5  $\mu$ L dNTP mix (10 mmol/L, Promega), 1.5  $\mu$ L MgCl<sub>2</sub> (25 mmol/L, Promega), 5  $\mu$ L primers (5'-GGACGGAGAGATGCCCAAGACC-3' and 5'-ACCAGCCAGGAGAATGACAATG-3' for TREM-1 or 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3' for  $\beta$ -actin)<sup>[31]</sup>, 0.25  $\mu$ L DMSO, 0.25  $\mu$ L Taq DNA polymerase (5 U/ $\mu$ L, Promega), 2.5  $\mu$ L PCR buffer (Promega) and DEPC-treated water to 25  $\mu$ L. The reaction was performed

using a programmable thermocycler (Techne, Progene). The DNA was predenatured at 94 °C for 15 s, followed by 32 cycles of denaturation (94 °C for 15 s), annealing (60 °C for 15 s), and extension (72 °C for 20 s). The mixture was finally extended for 5 min at 72 °C.  $\beta$ -Actin served as an internal control in each experiment. PCR products (10  $\mu$ L) were electrophoresed using 20 g/L agarose gels in TAE at 70 V for 60 min. The samples were stained with ethidium bromide (0.1 mg/L) and observed by ultraviolet illumination. The images were photographed and analyzed with an IS-1000 digital imaging system (Alpha Innotech Corporation). The pixel densities of TREM-1 and  $\beta$ -actin bands were determined and the ratios of TREM-1/ $\beta$ -actin were calculated. These ratios were represented as the relative expression levels (expression index) of TREM-1 mRNA and were used for semiquantitative analysis.

#### ELISA

Sera levels of IL-6 were measured using a commercial kit (BD-Pharmingen) according to the manufacturer's instructions.

#### Statistical analysis

Due to the sample size, we performed qualitative analysis with the data expressed as medians and quartiles. For comparison between each group, the nonparametric Mann-Whitney *U*-test was used.  $P \leq 0.05$  was considered statistically significant.

## RESULTS

Twenty-eight subjects were included: 11 septic patients, 7 elective gastrointestinal surgical patients (pre- and post-surgery) and 10 healthy volunteers. The age range was 20-83 years and the average age over each group was 52.7, 52.25, and 35.4 years for the surgical, septic and healthy groups, respectively. Demographic data for all the subjects included in this study, as well as diagnostic and disease evolution data, are shown in Table 1.

#### Expression levels of TREM-1 and HLA-DR

Surface expression levels of TREM-1 and HLA-DR were measured by flow cytometry. Results were expressed as mean fluorescence intensity (MFI) related to the entire monocyte population for TREM-1 and as the percentage of HLA-DR-positive monocytes in the total monocyte population<sup>[35]</sup>.

The expression of TREM-1 and HLA-DR in healthy volunteers was  $35.91 \pm 14.75$  MFI and  $75.8 \pm 18.3\%$ , respectively. We considered this group as showing the basal level of expression of both molecules. In septic patients, the TREM-1 expression level was  $59.9 \pm 23.9$  MFI and HLA-DR expression was  $44.39 \pm 20.25\%$ . There was a significant difference between healthy and septic groups ( $P < 0.05$ ) for both molecules (Figure 1).

In the surgical patients, TREM-1 and HLA-DR expressions were  $56.8 \pm 20.85$  MFI and  $71 \pm 13.8\%$  before

**Table 1** Patients' data

Group	Sex	Age	Diagnosis	Procedure	Survival	HLA-DR (%)		TREM-1 (MFI)	
						pre-s	post-s	pre-s	post-s
Surgery	M	52	Diverticulitis	Hemicolectomy	L	N	82	56.8	46.5
Surgery	F	43	Portal hypertension	Gastro-gastro <sup>2</sup>	L	64.18	87.56	50.8	82.9
Surgery	F	76	GRD	ARS <sup>4</sup>	D	49.8	82.19	73.4	102.6
Surgery	F	50	GRD	Nissen funduplication	L	77.9	63.45	39.1	44.1
Surgery	M	52	GRD	Nissen funduplication	L	83.82		94	
Surgery	F	39	Choledocal cyst	H-J <sup>3</sup>	L	59.8	53.07	32.8	62.4
Surgery	M	62	Cholangiocarcinoma	H-J	L	82.38		63.7	
Septic	M	48	AP	NA	D	52.88		36.9	
Septic	M	53	Acute abdominal pain	Splenectomy	D	22		59.9	
Septic	M	73	Gastric cancer	Total Gastrectomy	D	44.3		48.3	
Septic	M	58	AP	Laparotomy	L	33.98		29.1	
Septic	F	33	Pancreatic neoplasm <sup>1</sup>	Laparotomy	L	67.28		50.06	
Septic	F	83	Obstructive jaundice	Cholecystectomy	D	70.97		54.7	
Septic	M	50	AP	Pseudocyst drainage	D	68.82		90.7	
Septic	F	58	Bile duct injury	H-J <sup>3</sup>	L	37.63		27.8	
Septic	M	24	Pancreatic fistulae	NA	L	75.4		54.8	
Septic	M	50	EC <sup>5</sup>	NA	D	18.6		53.6	
Septic	M	46	AP	Laparotomy	D	38.12		63.1	
Healthy	M	67	NA	None	L	81.37		47.6	
Healthy	M	42	NA	None	L	53.37		35.2	
Healthy	M	55	NA	None	L	87.5		83	
Healthy	M	24	NA	None	L	82.4		37.8	
Healthy	M	50	NA	None	L	70.31		44.3	
Healthy	M	29	NA	None	L	89.66		67.3	
Healthy	F	24	NA	None	L	50.92		21.4	
Healthy	M	20	NA	None	L	84.43		35.9	
Healthy	F	23	NA	None	L	35.87		24.1	
Healthy	M	20	NA	None	L	63.16		21.4	

A = years; M = masculine; F = feminine; AP = acute pancreatitis; GRD = gastroesophageal reflux disease; L=live; D=death; <sup>1</sup>mixed papillary neoplasm of the pancreas; <sup>2</sup>gastro-gastro-anastomosis; <sup>3</sup>H-J = hepatico-jejunostomy; <sup>4</sup>ARS = anti-reflux surgery; <sup>5</sup>EC = emphysematous cholecystitis; NA = not applicable; pre-s = pre-operative; post-s = post-operative.

surgery and  $72.65 \pm 29.92$  MFI and  $72.82 \pm 22.55\%$  after surgery, respectively. There was a significant difference ( $P < 0.05$ ) in TREM-1 expression between the samples before and after surgery (Figure 2).

#### svTREM-1 expression levels

svTREM-1 expression was  $0.8590 \pm 0.1451$  in the healthy group,  $0.8820 \pm 0.1460$  in the surgical group (after surgery; pre-surgical value was  $0.377 \pm 3.7$ ) and  $2.210 \pm 0.7873$  in the septic group. There was a significant difference ( $P < 0.05$ ) when svTREM-1 in both the healthy and surgical groups was compared to the septic group (Figure 3).

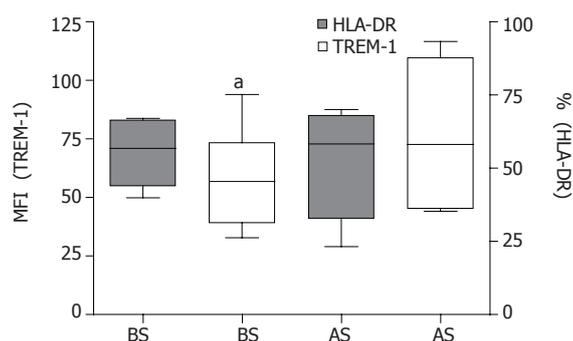
#### Serum interleukin-6 levels

Serum level of IL-6 was  $0.0 \pm 28.49$ ,  $0.1320 \pm 1.547$ ,  $24.53 \pm 51.94$ , and  $155.5 \pm 124.5$  (pg/mL) in the healthy surgical (before and after surgery) and septic groups, respectively. There was a significant difference ( $P < 0.05$ ) when the serum IL-6 levels in the healthy and surgical groups were compared to the septic group. There was also a significant difference between the surgical patients before and after surgery (Figure 4).

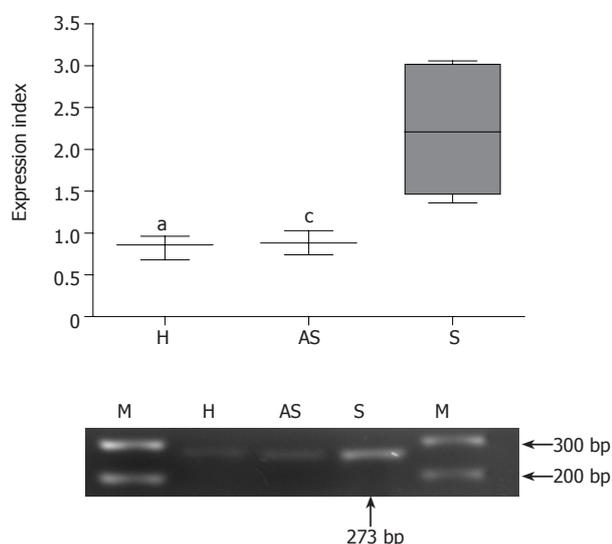
## DISCUSSION

The SIRS concept is valid to the extent that a systemic inflammatory response can be triggered by a variety of infectious and noninfectious conditions. Sepsis is a clinical syndrome defined by the presence of both infection and a systemic inflammatory response. However, these definitions are also considered nonspecific in clinical diagnosis or prognosis<sup>[9,20,36,37]</sup>. Here we have demonstrated that both low levels of HLA-DR and high levels of TREM-1 expression were related with sepsis and its resolution.

Major injuries such as extensive abdominal surgery, which involves stress and tissue necrosis, could lead to immunoparalysis as a consequence of CARS and get infected independent of their surgical and medical treatment, developing sepsis<sup>[15,38]</sup>. In addition to the massive release of pro- and anti-inflammatory mediators such as cytokines, acute phase proteins, heat shock proteins, and intracellular molecules such as HMGB1<sup>[19,24,25,39-43]</sup> there is a modification of expression of surface receptors such as TLRs, TREM-1 and HLA-DR in monocytes during the evolution of inflammatory response (SIRS, CARS, and sepsis)<sup>[15,30,35,38,44,45]</sup>.



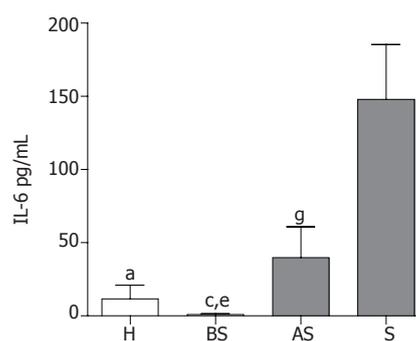
**Figure 2** TREM-1 and HLA-DR expression in surgical patients. Comparison of expression levels of TREM-1 (mean of fluorescence intensity) and HLA-DR (percentage of CD14+HLA-DR+cells) in surgical patients before (BS) and after surgery (AS). <sup>a</sup> $P < 0.05$  before surgery vs after surgery for TREM-1.



**Figure 3** mRNA expression of svTREM-1. **A:** Expression index of svTREM-1/ $\beta$ -actin in each group. <sup>a</sup> $P < 0.05$  healthy vs septic groups, <sup>c</sup> $P < 0.05$  surgical (after surgery) vs septic groups; **B:** Representative svTREM-1 PCR products from healthy volunteers (H), surgical patients after surgery (AS) and septic patients (S).

HLA-DR expression level in septic patients was strongly diminished compared to pre-surgical patients and healthy volunteers. This correlates with studies in which low expression of HLA-DR is considered as a sign of immunoparalysis and is related to the presence of infection in septic patients<sup>[12,13,35]</sup>. The relationship between high levels of TREM-1 and low HLA-DR expression correlates with the severity of illness. On the other hand, healthy volunteers showed low levels of TREM-1 and high levels of HLA-DR, as the surgical patients did before and after surgery, indicating that the immunocompetence of these subjects correlates with their optimal outcome and recovery.

During the onset of an infection, monocytes and neutrophils trigger inflammation in response to the recognition of microorganism-associated molecular structure by pattern recognition receptors, which may lead to the upregulation of surface TREM-1 expression<sup>[29]</sup>. Subsequently, stimuli through TREM-1 may amplify the inflammatory response by the release of TNF $\alpha$ , IL-1 $\beta$ ,



**Figure 4** Serum interleukin-6 levels. Serum levels of IL-6 in healthy volunteers (H), surgical patients before surgery (BS) and after surgery (AS), and septic patients (S). <sup>a</sup> $P < 0.05$  healthy vs septic groups; <sup>c</sup> $P < 0.05$  surgical groups before vs after surgery; <sup>e</sup> $P < 0.05$  before surgery vs septic groups; <sup>g</sup> $P < 0.05$  after surgery vs septic groups.

and IL-8, as well as the upregulation of co-stimulatory molecules<sup>[28,30]</sup>.

In our study, the TREM-1 expression level in septic patients was significantly higher than in healthy volunteers. This correlates with previous reports where TREM-1 surface expression is strongly increased in infiltrating neutrophils isolated from the peritoneal cavity of patients with septic shock due to bacterial peritonitis<sup>[30]</sup>.

Among the surgical patients, TREM-1 expression before surgery increased when compared to healthy volunteers, while after surgery there was a statistically significant upregulation as compared to the pre-surgical and healthy volunteer groups. This increase without clinical signs of infection might be the result of the inflammatory status due to the disease that led to operation and/or to surgical stress, instead of other factors such as age or gender. Thus, TREM-1 appears to be an endogenous signal reflecting the course of the inflammatory response.

We also measured the svTREM-1 mRNA level in mononuclear cells of each group, since this molecule might encode soluble TREM-1, which has been proposed as a marker for establishing the diagnosis of bacterial or fungal pneumonia<sup>[33]</sup>. svTREM-1 could serve as a soluble receptor for a yet unknown ligand, and its upregulation at the transcriptional, translation and secretion level may be a negative regulation mechanism by preventing surface TREM-1 engagement and pro-inflammatory activity during severe inflammatory processes such as SIRS and sepsis. We speculate that the increased svTREM-1 expression observed in septic patients compared to both healthy and surgical groups may correlate with the presence of pathogens that activate the immune response leading to complications such as sepsis.

We measured serum IL-6 in each group as an indicator of disease severity and for prediction of patient outcomes. IL-6 increased as previously described<sup>[21,46]</sup>.

We found a correlation between the upregulation of surface TREM-1 and svTREM-1 and the downregulation of surface HLA-DR in critically ill patients.

In conclusion, TREM-1 may play an important role in establishing and amplifying the systemic inflammatory response. In the future, with the support of epidemiological data, TREM-1, HLA-DR and svTREM-1

expression may be used as diagnostic and prognostic indicators to identify different phases of the inflammatory response, such as in SIRS, CARS and sepsis.

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