Soluble CD40 ligand and Oxidative Response are Reciprocally Stimulated during Shiga Toxin-associated Hemolytic Uremic Syndrome.

Running title: Role of sCD40L and oxidative response in HUS

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Abstract: Shiga toxin (Stx) produced by *Escherichia coli* is the main pathogenic factor of diarrhea-associated hemolytic uremic syndrome (HUS), which is characterized by obstruction of renal microvasculature by platelet-fibrin thrombi. It is well known that the oxidative imbalance generated by Stx induces platelet activation, contributing to thrombus formation. Moreover, activated platelets release soluble CD40 ligand (sCD40L) which in turn contributes to oxidative imbalance, triggering the release of reactive oxidative species (ROS) on various cellular types. The aim of this work was to determine if the interaction between the oxidative response and platelet-derived sCD40L participates in the pathogenic mechanism during HUS. Activated human glomerular endothelial cells (HGEC) by Stx2 induced platelets to adhere to them. Although platelet adhesion did not contribute to endothelial damage, high levels of sCD40L were released to the medium. The release of sCD40L by activated platelets was inhibited by antioxidant treatment. Furthermore, we found increased levels of sCD40L in plasma from HUS patients, which were also able to trigger the respiratory burst in monocytes, in a sCD40L-dependent manner. Thus, we concluded that platelet-derived sCD40L and the oxidative response are reciprocally stimulated during Stx2-associated HUS. This process may contribute to the evolution of glomerular occlusion and the microangiopathic lesions.

Keywords: hemolytic uremic syndrome; oxidative stress; blood platelets; Shiga toxin 2; CD40L

Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is associated with development of hemolytic uremic syndrome (HUS), which is characterized by hemorrhagic diarrhea followed by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure [1].

Stx is considered the main pathogenic factor and necessary for HUS development [2]. Damaged intestinal epithelium allows Stx to enter the circulation. There, it binds to its specific globotriaosylceramide (Gb3) receptor, which is present on microvascular endothelial cells and epithelial cells of target organs and monocytes. The main target organ is the kidney, but other organs such as the brain are also affected[3]. Endothelial damage plays a crucial role in the sequence of events leading to the microangiopathic process during HUS [4]. Microvascular endothelial cells, mostly of renal glomeruli, become activated in response to Stx and lose their anti-thrombogenic properties. This leads to the upregulation of adhesive molecules, such as vitronectin receptor, PECAM-1, and P-selectin, on endothelial surface that mediate platelet adhesion and

thrombi formation [5].   Thus, platelet adhesion and subsequent aggregation contribute to the formation of platelet-fibrin thrombi characteristic of the thrombotic microangiopathy during Stx-associated HUS[6]. Furthermore, soluble factors such as cytokines and/or chemokines released by Stx-activated microvascular endothelium [7] and monocytes [8] have also been implicated in the stimulation of platelet function. Thereby, thrombosis and inflammation are strictly correlated and constitute the major pathogenic components of Stx-associated HUS.

Studies performed on platelets from patients with HUS showed impaired aggregating responses [9,10] and reduced β-thromboglobulin content [10] indicating that the aggregation process had occurred *in vivo*. However, aggregate formation is not the only function of platelets. It has been recently recognized that platelets modulate immuno-inflammatory reactions through cytokine secretion and subsequent interaction with leukocytes and endothelial cells[11]. In fact, platelets are an important source of potent autocrine and paracrine factors, including several vasoactive and inflammatory mediators, such as P-selectin, CD40 ligand (CD40L), chemokines, growth factors and others [12,13].

CD40-CD40L interaction constitutes one of the main pathways initiated by platelets that leads to inflammation, and is also implicated in the pathogenesis of atherosclerosis and other immuno-inflammatory diseases [14-20]. CD40L, a membrane glycoprotein belonging to the TNF superfamily, is expressed mainly by activated T cells and activated platelets [21,22]. Platelet CD40L is stored in α-granules and translocated to the platelet surface upon activation [23,24]. Surface-expressed CD40L is then cleaved generating a soluble fragment, sCD40L. It retains the ability to activate its widely expressed receptor CD40[21], promoting inflammatory or thrombotic response by causing further platelet activation[23].   More than 95% of circulating CD40L is derived from platelets [25,26].

The biological function of sCD40L has recently been a subject of intense investigation; sCD40L binds to CD40 on target cells triggering an inflammatory response [14,24,27,28]. Furthermore, it is capable to induce oxidative stress and reactive oxidative species (ROS) generation in various cellular types such as endothelial cells[18], platelets[29], and neutrophils [30]. The involvement of the oxidative stress in tissue damage and renal failure processes during Stx-intoxication has also been subject of investigation; oxidative stress is generated by Stx systemically and locally in the kidney and has been shown to enhance platelet activation [31]. Thus, oxidative stress and platelet-derived sCD40L could stimulate each other. In the present study, we investigated the effects of Stx2 and oxidative stress on renal microvasculature, platelet adhesion and sCD40L release in order to identify a novel mechanism contributing to thrombotic microangiopathy. The *in vivo* relevance of sCD40L and its role in oxidative stress was studied in HUS patients.

Results

Platelets did not contribute to Stx2-mediated damage to HGEC

In order to accurately model the pathological renal environment during HUS, confluent HGEC cultures were incubated overnight with different concentrations of purified Stx2. Then, isolated human platelets (1x 10^8) were added or not to HGEC cultures. After 1 h, HGEC were stained with H&E and observed by optical microscopy to register any change in the cellular morphology associated with toxicity (shape and cellular detachment). In parallel, viability of living cells was analyzed by the neutral red assay.

Although no significant changes were observed with 0.1ng/mL Stx2, 1ng/mL Stx2 induced a significant toxicity on HGEC as it was demonstrated by a decreased uptake of the vital dye neutral red (Fig. 1A and B). No additional toxicity signs were evident upon platelet addition. These results confirmed the dose-dependent toxic effect of Stx2 on HGEC, and showed that platelets did not enhance endothelial damage, even when HGEC were incubated with Stx2 at a suboptimal concentration.
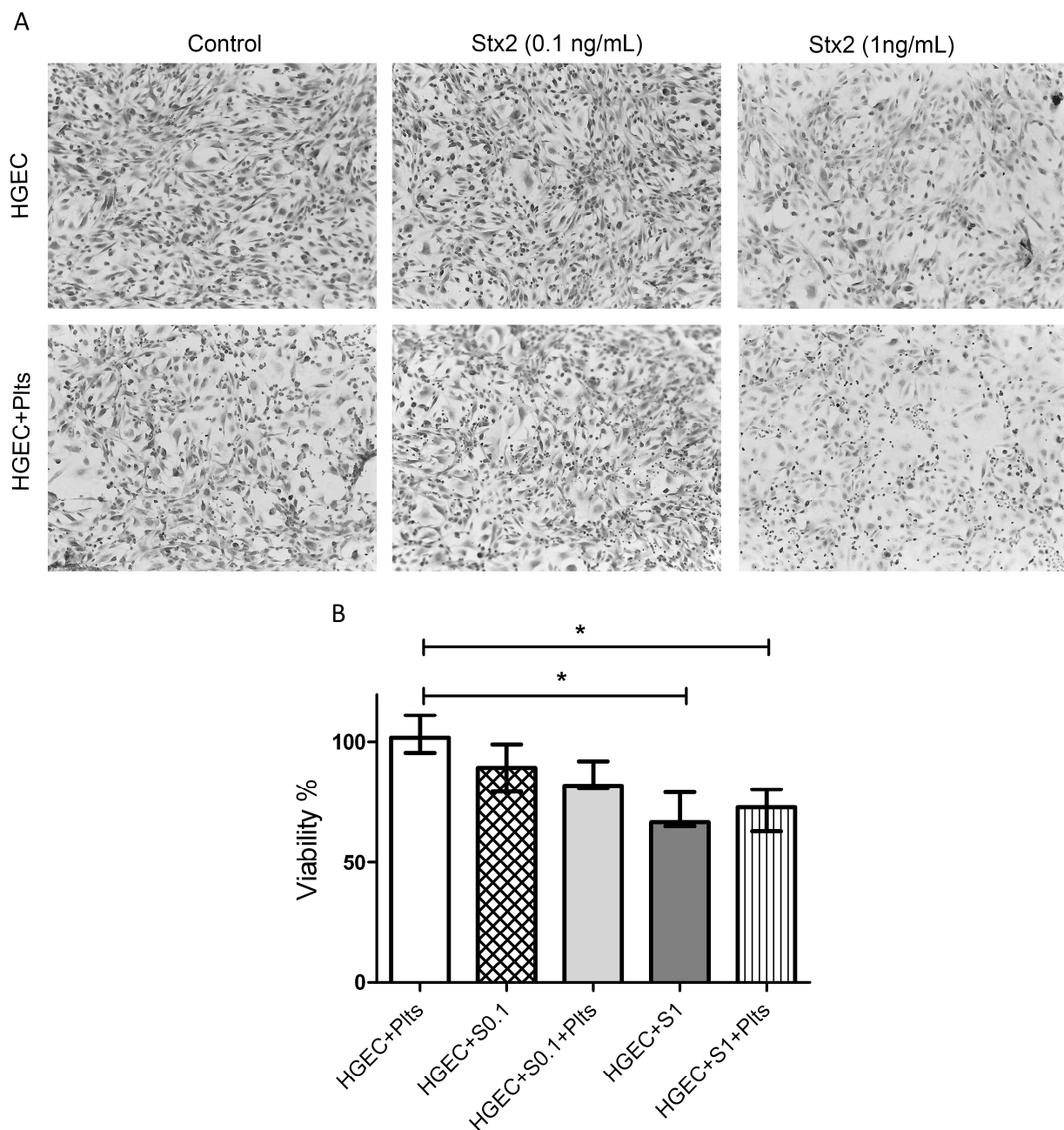
Figure 1. Microvasculature damage by Stx2. HGEC were seeded on glass coverslips (A), or placed in 24-well plates (B) treated or not with 0.1 ng/mL (S0.1) or 1 ng/mL (S1) Stx2.   After 24 h platelets (Plts) (1×10^8/well) were added for 1 h at 37°C in 5% CO_2. (A) Representative images by light microscopy from each experimental condition stained with H&E (×10) are shown. (B) Cells were incubated with neutral red for an additional 1 h at 37°C in 5% CO_2. The percentage of viable cells was calculated considering that absorbance (*A*) obtained for cells incubated without toxin treatment represents 100% viability. Each condition was performed in duplicate in each experiment. Data are expressed as median and interquartile range (*n*=3). *$P < 0.05$.

    To assess whether platelets respond to Stx2-mediated endothelial injury by switching to a pro-thrombotic state, adhesion studies were made. Platelet adhesion was measured by the acid phosphatase assay, reading *A* at 405 nm. Fig. 2B shows a significantly higher *A* values only when platelets were added to HGEC cultures that were pre-treated with 1ng/ml Stx2, indicating that the damaged endothelium stimulated platelet adhesion. This conclusion was confirmed by counting platelets recovered from cultures under different experimental conditions. The percentage of platelets recovered was around 50% from HGEC pre-treated with 0.1ng/mL Stx2 and only 25% from HGEC pre-treated with 1ng/mL Stx2 compared to platelets recovered from non-treated HGEC (Fig. 2C). These results suggest that although Stx2-induced endothelial

injury was not evident, platelet adhesion was triggered even at sub-optimal Stx2 doses. However, maximal platelet adhesion was observed when HGEC were incubated with 1ng/mL Stx2.
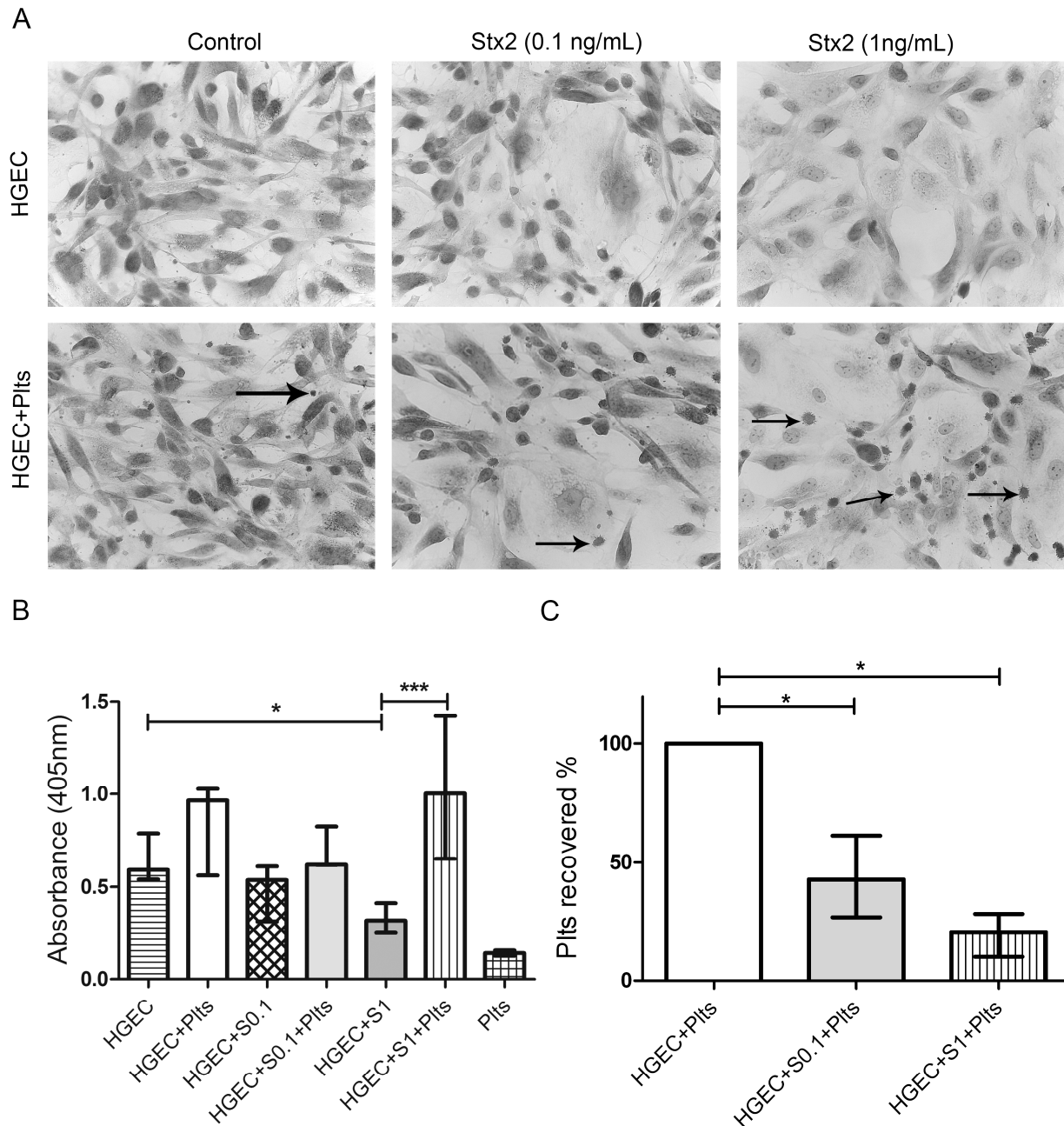

Figure 2. Platelet adhesion to damaged endothelium. HGEC were seeded on glass coverslips (A), or placed in 24-well plates (B and C) treated or not with 0.1 ng/mL (S0.1) or 1 ng/mL (S1) Stx2.   After 24 h Plts (1x10^8/well) were added for 1 h at 37°C in 5% CO_2. (A) Representative images by light microscopy from each experimental condition stained with H&E (×400) are shown. Platelets are indicated by arrows. (B) Cells were incubated with PNP and acid phosphatase activity was measured reading *A* at 405 nm. (C) Supernatants from HGEC-Plts cultures were collected and the number of Plts recovered was determined by hematology analyzer. The number of Plts recovered in the well without Stx2 treatment represented 100%. Each condition was performed in duplicate for each experiment. Data are expressed as median and interquartile range (*n*=5). *$P<0.05$ *** $P<0.001$.

Stx2-mediated HGEC damage induced platelet to release sCD40L

Then, we further analyzed if platelets, besides showing increased pro-thombotic properties, were able to release sCD40L upon incubation with Stx2-damaged HGEC. For this purpose, culture supernatants under the same experimental conditions were recovered and sCD40L was quantified by ELISA with a commercial kit.

As shown in Fig. 3A, supernatants from platelets incubated with 1ng/mL Stx2-treated HGEC, had significant increased levels of sCD40L, suggesting that Stx2-damaged HGEC were able to induce platelets to release this pro-inflammatory mediator. As expected, HGEC pre-treated with 1ng/mL Stx2 *per se*, did not produce sCD40L, confirming that platelets are the major source of sCD40L. Moreover, HGEC incubated with a suboptimal Stx2 dose, stimulated a low, but not significant, release of sCD40L by platelets.

Considering that controversial results regarding Stx direct effect on platelets have been shown [32-35], we determined if Stx2 was able to directly stimulate platelets to release sCD40L. Thus, platelets were incubated with 1ng/mL Stx2 for one hour and thrombin was used as a positive control. Then, supernatants were collected and sCD40L concentration measured by ELISA.

As shown in Fig. 3B, supernatants from Stx2-stimulated platelets have a similar sCD40L concentration than control platelets incubated with medium. In contrast, supernatants from thrombin-stimulated platelets presented a significant increase in sCD40L levels. It is important to highlight that although Stx2 was not removed from the HGEC cultures before adding platelets, supernatants were tested on VERO cells and did not show toxic activity (data not shown).

These results suggest that Stx2 did not directly stimulate platelets to release sCD40L, but instead, indirectly through endothelial damage.
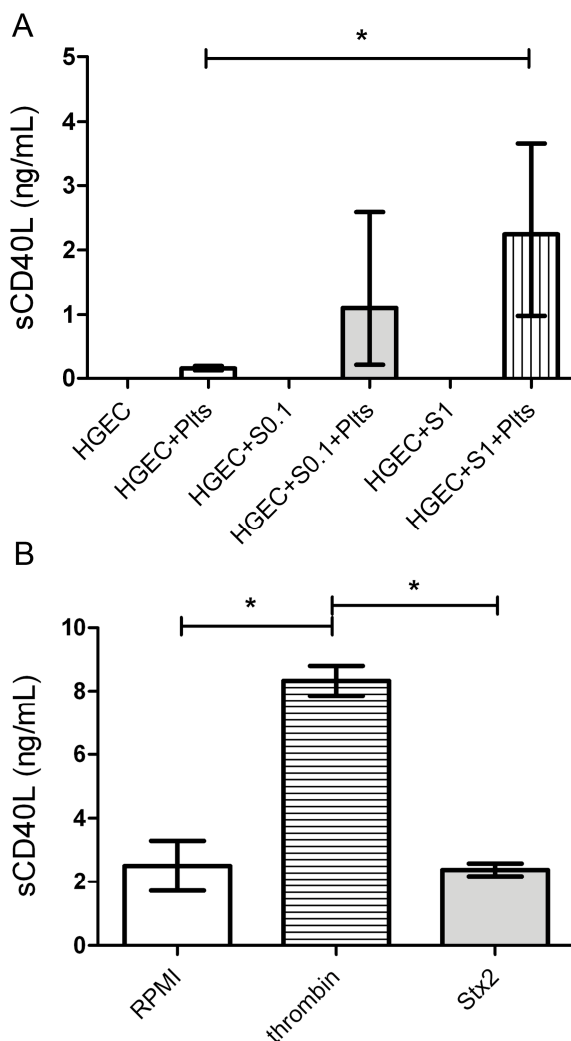

Figure 3. Release of sCD40L by Plts. (A) HGEC were placed in 24-well plates treated or not with 0.1 ng/mL (S0.1) or 1 ng/mL (S1) Stx2. After 24 h Plts (1×10^8/well) were added for 1 h at 37°C in 5% CO_2. Supernatants of HGEC cultures were collected and sCD40L levels were measured by ELISA kit. (B) Isolated Plts were stimulated with 0.2 U/mL thrombin or 1 ng/mL Stx2 during 1 h at 37°C in 5% CO_2. Supernatants were collected and sCD40L levels measured by ELISA kit. Each condition was performed in duplicate for each experiment. Data are expressed as median and interquartile range (*n*=8). * $P < 0.05$.

Oxidative stress did not contribute to Stx2-mediated damage to HGEC or platelet adhesion

Stx2 intoxication induced a marked prothrombotic status and simultaneously a pro-oxidative imbalance demonstrated in both, experimental mouse models [31], and most important in HUS-patients [36,37]. Considering that NAC is a well-known anti-oxidant and glutathione precursor [38], we decided to evaluate if it was able to inhibit Stx2-mediated effects on HGEC cultures.

HGEC incubated with or without 1ng/ml Stx2 were incubated with or without 1mM NAC[30] and toxicity and viability were analyzed by H&E staining and neutral red uptake, respectively.

We did not observe any difference in the viability of HGEC between Stx2-treated and Stx2/NAC-treated cultures, showing that anti-oxidant treatment did not inhibit Stx2-mediated injury to endothelium (Fig. 4A and B). Then, we determined platelet adhesion as previously described. We tested NAC effect in two experimental protocols, incorporating NAC from the beginning of culture (together with 1ng/ml Stx2), and incorporating NAC previous to platelets addition. As shown in Fig. 4C, NAC did not inhibit the increase of platelet adhesion, secondary to Stx2-mediated endothelial damage, in any of the protocols evaluated. These results demonstrate that the oxidative stress does not contribute to the endothelial damage induced by Stx2. Moreover, platelet adhesion is only a consequence of this damage and the oxidative stress is not involved.
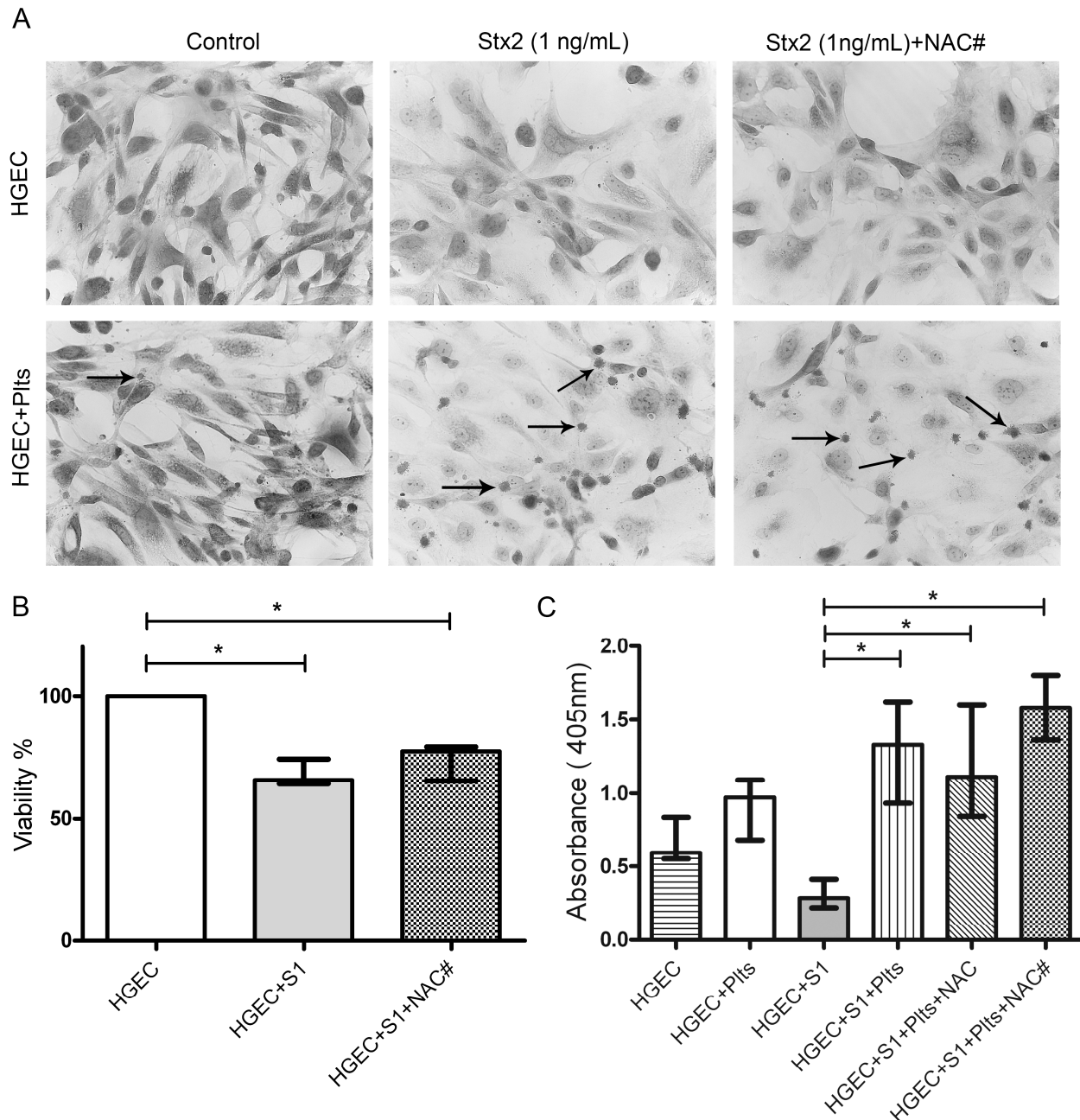
Figure 4. Role of oxidative stress in endothelial damage and platelet adhesion. HGEC were seeded on glass coverslips (A), or placed in 24-well plates (B and C) treated or not with 0.1 ng/mL (S0.1) or 1 ng/mL Stx2 (S1).   After 24 h Plts (1x10^8/well) were added for 1 h at 37°C in 5% CO_2. NAC (1 nM) was incorporated simultaneously with Stx2 (NAC#) or previous to Plts (NAC). (A) Representative images by light microscopy from each experimental condition stained with H&E (×400). Plts are indicated by arrows. Number of viable cells and Plts adhered to HGEC were observed by light microscopy (×400). (B) Cells were incubated with neutral red for an additional 1 h at 37°C in 5% CO_2. The percentage of viable cells was calculated considering that *A* obtained for cells incubated without toxin treatment represents 100% viability. (C) Cells were incubated with PNP and acid phosphatase activity was measured reading *A* at 405 nm. Each condition was performed in duplicate for each experiment. Data are expressed as median and interquartile range (*n*=8). * *P* <0.05

Even though NAC did not modify Stx2-mediated endothelial damage or platelet adhesion, we further analyzed if NAC affected the release of sCD40L by platelets under the same experimental protocol. Supernatants were collected after one hour of platelet addition, and sCD40L was quantified by ELISA as previously described. Fig. 5 shows that supernatants from platelets incubated with Stx2-pretreated HGEC had increased sCD40L levels, and sCD40L release was inhibited only when NAC was added before platelets.

The fact that NAC was effective on blocking sCD40L release but not platelet adhesion, suggests that both processes are modulated by different pathways and oxidative stress is only involved in platelet degranulation.
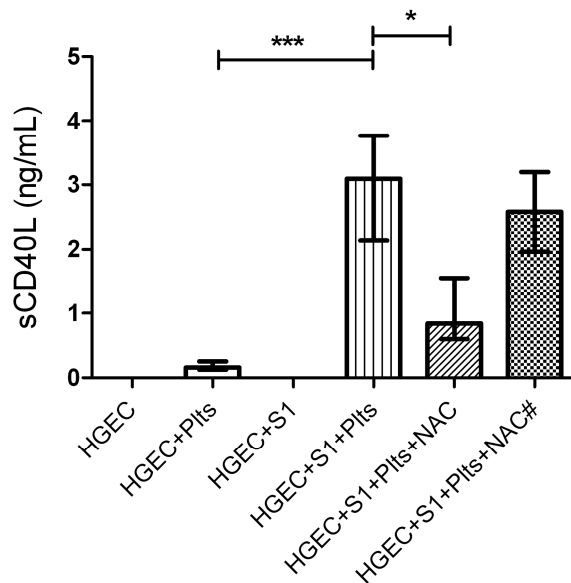

Figure 5. Role of oxidative stress in sCD40L platelet release. HGEC were placed in 24-well plates treated or not with 0.1 ng/mL (S0.1) or 1 ng/mL (S1) Stx2. After 24 h Plts (1×10^8/well) were added for 1 h at 37°C in 5% CO_2. NAC (1 nM) was incorporated simultaneously with Stx2 (NAC#) or previous to Plts (NAC). Supernatants of HGEC cultures were collected and sCD40L levels were measured by ELISA kit. Each condition was performed in duplicate for each experiment. Data are expressed as median and interquartile range (*n*=8). * $P < 0.05$ *** $P < 0.001$.

HUS patients had increased levels of sCD40L in circulation

Because we found that Stx2-mediated damage to HGEC leads to the release of sCD40L by platelets, we assayed the plasmatic levels of sCD40L in HUS patients and HC. With this aim we collected plasma from HUS patients at diagnosis and healthy age-matched controls, and sCD40L concentration was quantified by ELISA. HUS patients were retrospectively classified according their evolution and renal dysfunction. Thus, patients were classified in two groups: those classified as grade 1 and 2 (up to 7 days of dialysis) and grade 3 (more than 7 days of anuria or dyalisis), according to Gianantonio *et al*'s criteria[39]. As depicted in Fig. 6A, only plasmas from grade1-2 patients had significantly increased sCD40L levels, compared to those from HC. It is noteworthy that grade 3 patients presented sCD40L circulating levels similar to controls. Considering that platelets are the major source of sCD40L and HUS patients had different degree of thrombocytopenia, we analyzed the relationship between the number of circulating platelets and the plasmatic levels of sCD40L in HUS patients and HC. Fig. 6B shows that HUS patients (from all severity groups) had higher values of sCD40L produced per platelet than HC, indicating that platelets are activated and releasing sCD40L. In addition, grade1-2 patients had the highest sCD40L plasma levels, suggesting that in this group, platelet activation and consequently sCD40L release are probably ongoing at the moment of hospitalization.

Finally, we analyzed the existence of any correlation between levels of sCD40L and renal dysfunction in HUS patients, assessed as plasma levels of urea and creatinine (Fig. 6C and D). We found a weak negative correlation between sCD40L and urea and creatinine levels. All together these results suggest that the onset of sCD40L release was an early event in the course of STEC infection-HUS and previous to renal dysfunction.
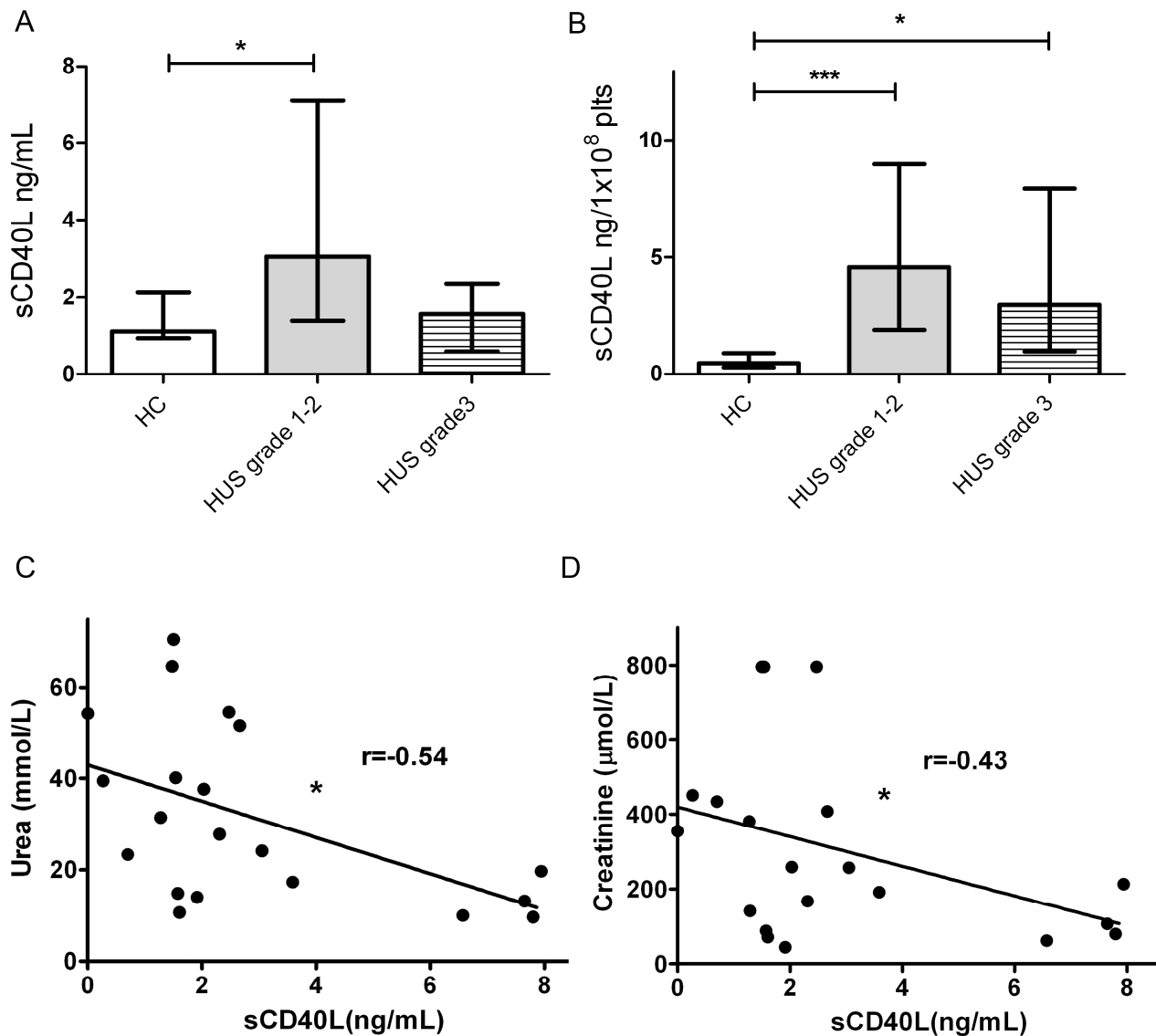
Figure 6. sCD40L levels in HUS plasma. (A) sCD40L levels in HUS patients and healthy controls (HC) plasmas were determined by ELISA kit. HUS patients were retrospectively classified according to Gianantonio et.al´s criteria in grade 1/2 (n=13) and grade 3 (n=10). Data are expressed as median and interquartile range. (B) Quantities of sCD40L (ng) released per $1x10^8$ Plts from HUS patients and HC. Data are expressed as median and interquartile range. (C). Correlation between sCD40L and plamatic creatinine levels in HUS patients. (D). Correlation between sCD40L and plasmatic urea levels in HUS patients. Points represent independent individuals. * P <0.05 *** P <0.001.

Plasmatic sCD40L induced ROS generation by monocytes

It has been reported that CD40-CD40L interaction in endothelial cells[18] or neutrophils[30] leads to ROS generation. Thus, we further examined whether plasmas from patients and controls induced ROS generation by monocytes and if this process was related to the concentration of sCD40L. For this, PBMC were incubated with plasma from HUS patients containing high levels of sCD40L (>3ng/mL) or plasma from HC (≈1.5ng/mL) during one hour and respiratory burst was evaluated by flow cytometry using DHR-123 as substrate. ROS generation in monocytes was evaluated analyzing MFI in monocytes gate. Plasma samples from HUS patients induced a higher ROS generation by monocytes compared to plasma samples from HC (Fig. 7B). In order to determine role of plasmatic sCD40L in triggering ROS generation, it was depleted with an anti-CD40L antibody. Plasma samples depleted or not for sCD40L, were analyzed in parallel. Fig. 7B

shows that sCD40L depletion significantly decreased ROS generation induced by HUS plasmas, but did not modify the ROS generation induced by control plasmas.

These results suggest that sCD40L present in plasma from HUS patients is biologically active inducing monocytes to release ROS.
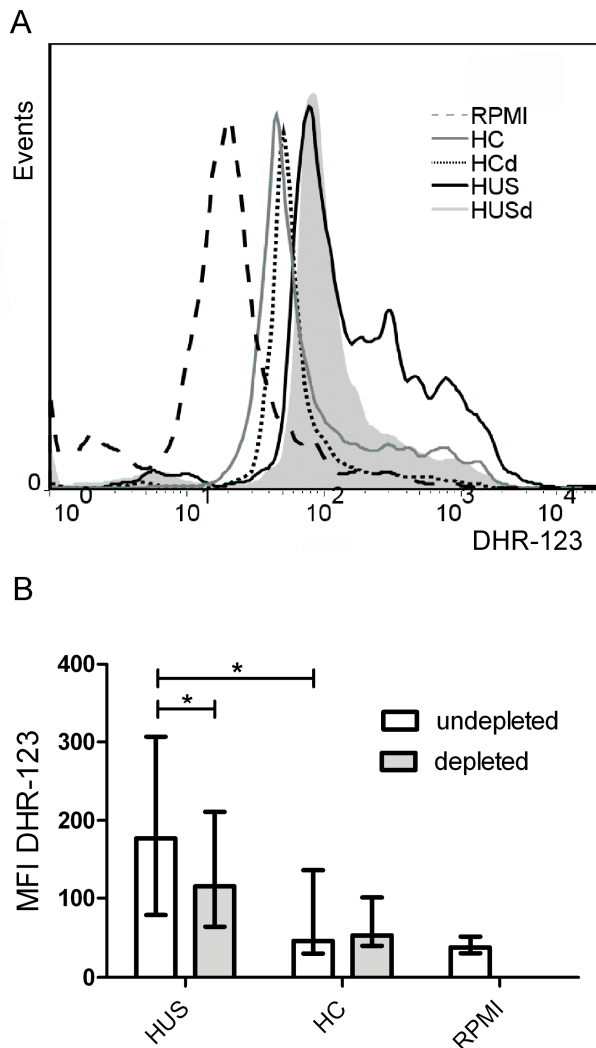

Figure 7. ROS production by monocytes. PBMC were incubated overnight with plasmas from HUS patients or HC. Then, PMBC were washed and incubated with DHR-123 and ROS production by monocytes was measured by flow cytometry. Monocytic population was gated by FSC-H/SSC-H and CD14 staining. (A) Representative histogram of each experimental condition: HC (healthy control plasma), HCd (Healthy control plasma depleted for sCD40L, HUS (HUS patient plasma) and HUSd (HUS patient plasma depleted for sCD40L. (B) Each bar shows the mean fluorescence intensity (MFI) of monocytes under different conditions. Data are expressed as median and interquartile range (*n*=6). * *P* <0.05

Discussion

Platelets play a significant role in the development of thrombosis and inflammation, two effector arms of the HUS pathogenesis. Particularly, it has been reported that platelets interact with monocytes via the CD40–CD40L pathway leading to their adherence to the inflamed endothelial layer [40-42].

Results presented here lead us to propose that the interaction between platelets and monocytes occurs in a pathogenic loop that involves the CD40–CD40L pathway and the oxidative response. The primary pathogenic event during systemic complications secondary to STEC infections is the microvascular injury mediated directly and/or indirectly by Stx. As consequence, platelets adhere to the endothelium and release sCD40L into the circulation, in a redox-sensitive manner. sCD40L binds to CD40 on monocytes, triggering ROS generation, which in turn can stimulate additional platelets. Thus, platelets and monocytes are reciprocally stimulated via sCD40L-CD40 dyad and ROS, secondary to microvascular injury.

In this regard, we demonstrated that release of sCD40L by platelets was induced by Stx2-damaged endothelium and not directly by Stx2. In addition, treatment of endothelium with antioxidant 1h before platelet addition significantly inhibited the release of sCD40L, suggesting that ROS derived from endothelial cells and/or from platelets itself, could stimulate sCD40L release. In contrast, antioxidant treatment added 24 hours before platelets was not able to inhibit sCD40L release, probably as consequence of a rapid loss of antioxidant capacity of NAC and a sustained ROS production by endothelial cells. In this regard, previous studies have shown that platelet expression of CD40L involves activation of the NADPH oxidase subunit, gp91phox [43] and subsequent studies revealed that the release of sCD40L by platelets involves oxidative stress, and is inhibited by antioxidants, such as vitamin C [44]. It has also been reported that NAC is able to decrease sCD40L release by platelets upon thrombin stimulation[30].

In conclusion, the enhancement of sCD40L release following platelet exposure to Stx2-injured HGEC and the NAC blocking effect implies that endothelial-mediated platelet activation is potentiated by ROS. The relevance of the *in vitro* results was confirmed in this study by demonstrating elevated levels of sCD40L in plasma samples from patients with Stx-associated HUS at the moment of diagnostic. It is important to highlight that the highest levels of sCD40L were detected in plasmas from those patients classified as mild/moderate (grade 1-2). However, when the amount of sCD40L released per platelet was evaluated, it was observed that all groups of patients (grade 1-2 and 3) have an increased sCD40L production compared to HC. Moreover, it is important to highlight that the levels of sCD40L locally released in the renal microvascular environment should be higher than those detected in systemic circulation.

Since we found a negative correlation between plasmatic sCD40L concentration and urea and creatinine levels in HUS patients, we propose that sCD40L could represent an early and sensitive marker of platelet activation previous to renal insufficiency.

The sCD40L plasmatic values showed by HUS patients were similar to those reported in other pathological conditions such as cardiovascular diseases, diabetes, HIV infection or smokers [41,45-49] and it can be considered as a marker of thrombotic risk [26]. Therefore, and based on the present results, we propose that quantification of sCD40L in plasma could be used as a surrogate and early marker of microvascular dysfunction and/or platelet activation in Stx-associated diarrheas.

The biological activity of circulating sCD40L in HUS patients was further confirmed by its capacity to induce the oxidative burst in monocytes. The enhancement of ROS generation by monocytes upon incubation with plasmas from HUS patients with elevated levels of sCD40L supports the notion that platelets may stimulate ROS generation via the CD40–CD40L interaction. The specificity of the CD40–CD40L pathway in this reaction was demonstrated using plasmas depleted from sCD40L, which induced a lower ROS production compared to the same plasmas previous to sCD40L depletion. In line with these results, Ha YJ *et al* demonstrated that CD40 ligation on monocytes enhances the production of ROS via activation of NADPH oxidase and PI-3-Kinase [50].

Thus, the sCD40L present in plasmas from HUS patients is able to interact with monocytes and to trigger ROS production. This in turn would contribute to endothelial damage and to further platelet activation leading to a positive feedback loop between platelets and monocytes, in which ROS and sCD40L stimulates each other. This represents a new pathogenic pathway mediated by monocyte-platelet interaction during HUS, that adds to the already described monocyte-platelet aggregate-formation[51].

Oxidative stress is widely considered as a common signaling mechanism of the vascular response to injury. Enhancement of the oxidative stress has been reported in patients with HUS[36,37] and vascular diseases which have also been associated with elevated levels of plasma sCD40L as has been discussed above[52-54]. In this regard, other authors have previously reported that recombinant human CD40L promotes oxidative burst in human neutrophils via a PI3-kinase-dependent signaling pathway [30,55,56] and Vanichakarn *et al* described a positive feedback loop between platelets and neutrophils, similar to that reported in our study between platelets and monocytes[30].

In conclusion, we described a new pathway of platelet-monocyte interaction, mediated by sCD40L and oxidative stress that may contribute to the progression of endothelial dysfunction during Stx2-associated HUS. Moreover we suggest that antioxidant treatments may be useful to reduce platelet activation and thrombus formation improving renal microcirculation and kidney function.

Materials and methods

Reagents

Recombinant purified Stx2 was purchased from Tufts University, Boston, USA. It contained less than 5 pg of LPS (per μg of Stx) quantified by Limulus amebocyte lysate assay. N-acetylcysteine (NAC), dihydrorhodamine-123 (DHR-123), phorbol myristate acetate (PMA), prostaglandin E$_1$ (PGE$_1$) M199, endothelial cell growth supplement (ECGS) and thrombin were obtained from Sigma (St Louis, MO, USA). Fetal calf serum (FCS), L-glutamine, and penicillin/streptomycin was obtained from GIBCO (USA).

HGEC cultures

Human glomerular endothelial cells (HGEC) were isolated from kidneys removed from different pediatric patients undergoing nephrectomies performed at Hospital Nacional "Alejandro Posadas", Buenos Aires, Argentina (written informed consent was obtained from the next of kin, caretakers, or guardians on the behalf of the children participants involved in our study). The Ethics Committee of the University of Buenos Aires approved the use of human renal tissues for research purposes. The method used for HGEC isolation was previously described [57].

Human platelets isolation

Blood samples were obtained from healthy donors. This study was performed according to institutional guidelines (Academia Nacional de Medicina, Buenos Aires, Argentina) and was approved by the Institutional Ethics Committee. Written consent was obtained from all subjects. Four volumes of blood were drawn directly into plastic tubes containing one volume of acid-citrate-dextrose (ACD). Platelet rich plasma (PRP) was obtained by the centrifugation of blood samples at room temperature (RT) at 200xg for 15 min. PRP samples were centrifuged at RT at 800×g for 10 min in presence of PGE$_1$ (4μg/mL). The pellet was resuspended in washing buffer (0.01M Tris, 0.15M NaCl, ph:7,4) and centrifuged at RT at 800×g for another 10 min in presence of PGE$_1$. The final pellet was resuspended in RPMI at 1x10^9/mL.

HGEC-platelets cultures

HGEC were plated (50000 cells/well) in gelatin coated 24-well plates and grown to confluence in complete medium (M199 medium supplemented with 20% FCS, 3.2mM L-glutamine, 100 U/ml penicillin/streptomycin and 25 μg/ml ECGS). Cells were then exposed to Stx2 (0.1 ng/mL or 1 ng/mL) in growth-arrested conditions for 24 h. Then, platelets (1x10^8/well) were incorporated for 1 h at 37°C in 5% CO$_2$. After that, supernatants were collected and stored at -20ºC.

NAC treatment

NAC (1mM) was added to HGEC cultures simultaneously with Stx2 or 1 h before platelet addition.

Hematoxylin-Eosin (H&E) staining

HGEC were seeded in glass coverslips and after treatments cells were fixed for 2 h at room temperature with alcohol 96º, stained with (H&E) hematoxylin-eosin and observed by light microscopy as previously described [57,58].

Neutral red cytotoxicity assay

The neutral red cytotoxicity assay was adapted from previously described protocols [59]. Briefly, HGEC were incubated with five hundred microliters of freshly diluted neutral red in M199 to a final concentration of 10μg/mL during 1 h at 37°C in 5% CO$_2$. Cells were then washed with 500μL 1% CaCl2 + 1% formaldehyde and solubilized in 500μL 1% acetic acid in 50% ethanol. Absorbance (*A*) in each well represents neutral red uptake and was read in an automated plate spectrophotometer at 540nm. The percentage of viable cells was calculated considering that *A* obtained for cells incubated without toxin treatment represents 100% viability.

Acid phosphatase assay

After treatments, HGEC were washed twice with PBS and then were incubated with 500µL of p-nitrophenylphosphate (PNP) diluted in reaction buffer (0.1M sodium citrate, 0.1% Triton X-100, ph:5,4) to a final concentration of 1mg/mL during 1 h at 37°C in 5% CO_2. Reaction was stopped adding 150µL of NaOH 2N. *A* in each well represents acid phosphatase activity and was read in an automated plate spectrophotometer at 405nm. Increased *A* in wells with platelets compared with wells without platelets was considered as a function of the number of adherent platelets.

Patients and samples

The study was approved by the Hospital Ethical Committees: the Comité de Bioética del Hospital Municipal del Niño de San Justo, San Justo, Buenos Aires, Argentina and the Comité de Bioética del Hospital Jose Penna, Bahia Blanca, Buenos Aires, Argentina. All patients were enrolled after informed consent was obtained from their parents. The study included 23 children during the acute period of HUS. All patients developed HUS after a prodrome of gastroenteritis with bloody diarrhea. There were 8 girls and 15 boys in the study. Clinical and biochemical data of patients are presented in Table 1. Blood samples (2 mL) were obtained by venopuncture into EDTA plastic tubes, before dialysis and/or transfusion at different days after the onset of diarrhea (Table 1). Blood samples from healthy controls (HC) were collected and processed identically. Plasma was obtained by blood centrifugation at RT at 800xg for 10 min, aliquoted and stored at -80ºC until analysis.

Table 1. Clinical and biochemical data of patients with HUS. According to Gianantonio et al's criteria, 23 patients were retrospectively classified as mild-moderate cases (grade 1-2: less than 7 days of anuria) or severe cases (grade III: more than 7 days of anuria). Data are presented as median (interquartile range)
[a] The number of days between the the onset of diarrhea and time of blood sample collection.

	Severity of renal dysfunction	
	Grade 1 y 2	**Grade 3**
General parameters	(n=13)	(n=10)
Age (month)	45.0 (22.0-81.2)	21.5 (10.5-60.5)
Time from the onset of diarrhea (days)[a]	4.0 (3.0-5.0)	4.5(1.7-9.7)
Blood and renal parameters		
Platelets (x 10^9/L)	77.5 (39.2-34.5)	45.0 (34.5-57.0)
Leukocytes (x 10^9/L)	14.0 (8.9-22.3)	17.4 (13.8-31.0)
Hematocrit (%)	24.0 (19.3-26.0)	24.2 (23.0-25.7)
Urea (mmol/L)	15.6 (10.5-25.9)	39.7 (26.6-54.3)
Creatinine (µmol/L)	110.5 (61.8-223.2)	419.9 (235.4-795.6)

sCD40L measurement

sCD40L levels in plasma and supernatants were determined by using an ELISA kit following manufacturer's instructions (e-Biosciences, USA).

Plasma depletion of sCD40L

96-well plate was coated with 2µg/well anti-CD40L (Becton Dickinson, USA) overnight at 4ºC. Wells were washed three times with PBS, blocked with PBS-BSA (0.5%) during 1h. Then, washed three times with PBS, and incubated with 100µL of plasma at RT for 2 hours. Then, depleted plasmas were collected and stored at -80ºC until analysis. Non-depleted plasmas were treated in identical conditions but without anti-CD40L coating. Those plasmas with sCD40L levels higher than 3ng/mL were selected for depletion and the effectiveness of procedure was confirmed by ELISA.

PBMC isolation

Blood was diluted 1:2 with saline, layered on a Ficoll-Hypaque cushion (Ficoll Pharmacia, Uppsala, Sweden; Hypaque, Wintthrop Products, Argentina) and centrifuged at $400xg$ for 30 min as previously described [60]. Peripheral blood mononuclear cells (PBMC) were collected, washed twice, and resuspended in RPMI. Viability of PBMC was more than 96% as determined by trypan blue exclusion test.

ROS generation measurement

PBMC ($1x10^6$) were incubated with 10% of HUS and HC plasma, depleted or not for sCD40L, overnight at 37°C in 5% CO_2, then washed in PBS and resuspended in 200μL of RPMI. DHR-123 (5μM) was added for 15 min at 37°C. Afterwards, the cells were washed and suspended in 200μL of Isoflow (International Link, SA, Argentina). Green fluorescence was measured on 10000 events with a Becton Dickinson (Franklin Lakes, USA) fluorescence activated cell sorter (FACScan) and analysed using the Cell-Quest program. Monocytes were identified and gated using forward/side-scatter (FSC/SSC) dot-plot profiles and CD14 staining by using anti-CD14 (Becton Dickinson, USA).

Data Analysis

Data are presented as median and interquartile range. All data were analyzed by a non-parametric Kruskal–Wallis test followed by the Dunnett's multiple comparisons test. The non-parametric Spearman test was used for correlations. A p value lower than 0.05 was considered to be statistically significant.

Author contribution

M.J.A-R performed and designed experiments, analyzed the data and wrote the manuscript. R.S.A performed HGEC isolation and cultures. F.A, M.V.R and R.J.F-B contributed to obtain samples from healthy donors, performed platelets and PBMC isolation and flow cytometric assays. M.P.M and A.C.B performed ELISA assays. R.A.E and L.A contributed to obtain samples from HUS patients and HC. C.I, M.M.A and M.S.P designed and directed the study and wrote and edited the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest

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