Cytokine 121 (2019) 154732

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Rab7b participation on the TLR4 (Toll-like receptor) endocytic pathway in Shiga toxin-associated Hemolytic Uremic Syndrome (HUS)

Andrea Florencia Lafalla Manzano[a], Andrea Fernanda Gil Lorenzo[b], Victoria Bocanegra[c], Valeria Victoria Costantino[c], Valeria Cacciamani[c], María Eugenia Benardon[b], Patricia G. Vallés[b,c,d,*]

[a] Laboratorio de Citometría de Flujo, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina
[b] Área de Fisiopatología, Departamento de Patología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina
[c] IMBECU-CONICET (Instituto de Medicina y Biología Experimental de Cuyo – Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina
[d] Hospital Pediátrico Humberto J. Notti, Servicio de Nefrología, Ministerio de Salud, Mendoza, Argentina

ARTICLE INFO

Keywords:
Toll-like receptor 4
Rab7b
Intracellular cytokines
Monocytes
Shiga toxin-associated Hemolytic Uremic Syndrome

ABSTRACT

Background: The inflammatory response of the host to Shiga toxin and/or lipopolysaccharide (LPS) of *Escherichia coli* (*E. coli*) is included in (HUS). The TLR4-LPS complex is internalized and TLR4 induced inflammatory signaling is stopped by targeting the complex for degradation. Rab7b, a small guanosine triphosphatase (GTPase) expressed in monocytes, regulates the later stages of the endocytic pathway. Objective: we studied the Rab7b participation on the TLR4 endocytic pathway and its effect on monocyte cytokine production along the acute course of pediatric Shiga toxin-associated HUS.

Methods and results: Monocytes were identified according to their positivity in CD14 expression. Surface TLR4 expression in monocytes from 18 HUS patients significantly increased by day 1 to 6, showing the highest increase on day 4 compared to monocytes of 10 healthy children. Significant higher surface TLR4 expression was accompanied by increased proinflammatory intracellular cytokines, tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). In contrast, after these time points, surface TLR4 expression and intracellular TNF-α levels, returned to near control levels after 10 days. Furthermore, confocal immunofluorescence microscopy proved colocalization of increased intracellular TLR4/Rab7b determined by Pearson's coefficient in monocytes from HUS patients from day 1 on the highest colocalization of both proteins by day 4. Decreased TLR4/Rab7b colocalization was shown 10 days after HUS onset.

Conclusion: The colocalization of TLR4 and Rab7b allows us to suggest Rab7b participation in the control of the TLR4 endocytic pathway in HUS patient monocytes. A consequential fall in cytokine production throughout the early follow up of HUS is demonstrated.

1. Introduction

The Shiga toxin (Stx)-producing *E. coli*-associated Hemolytic Uremic Syndrome (HUS) is the most frequent cause of acute renal failure in children [1]. The primary clinical features are thrombocytopenia, hemolytic anemia and renal failure [2,3]. Endothelial injury has been recognized as the trigger event in the microangiopathic process [4]. Host endothelial cell inflammatory response to *E. coli* Stx and/or LPS contributes to the ongoing vascular damage from the infection, which results in HUS [5]. Evidence demonstrated that LPS augments Stx toxicity through the upregulation of kidney Stx globotriaosylceramide 3 receptor (GB3) in an experimental model of HUS [6] and also both Stx and LPS were necessary for developing an HUS-like response in mice [7,8]. The recognition of LPS by innate immune cells is crucial for the host defense against Gram-negative bacteria. Upon LPS recognition TLR4 undergoes oligomerization that leads to the recruitment of downstream adaptors, critical for MyD88-dependent and MyD88-independent transduction signal pathways [9,10]. Innate inflammatory response to such infection regulated by way of Toll-like receptors (TLRs), triggers tightly regulated signaling cascades through transcription factors, including nuclear factor-κB (NF-κB) [11,12].

Recently we showed an increase in surface receptor TLR4 protein

* Corresponding author at: Area de Fisiología Patológica, Departamento de Patología, Facultad de Ciencias Médicas. Universidad Nacional de Cuyo. Mendoza, Argentina.
E-mail address: pvalles@fcm.uncu.edu.ar (P.G. Vallés).

https://doi.org/10.1016/j.cyto.2019.05.019
Received 19 February 2019; Received in revised form 14 May 2019; Accepted 19 May 2019
Available online 30 May 2019

expression on neutrophils of HUS patients within 24 h after the disease diagnosis, TLR4 activation driven proinflammatory cytokines release, resulting in a negative regulation after 10 days of HUS onset [13]. The receptor-ligand intracellular membrane trafficking and routing is crucial to reach a controlled inflammatory response and a timely and regulated termination of inflammatory signaling. The TLR4-LPS complex is rapidly internalized and TLR4-induced inflammatory signaling is stopped by targeting the complex for degradation [14]. Endosomes play an essential role in this process, serving as sorting facilities for the biosynthetic and endocytic pathways. Endosomes acidification leads to the receptor oligomerization disruption and ligand-receptor interaction, thereby initiating the first step in shutting off proinflammatory signaling [15]. The LPS/TLR4 complex internalized by endocytosis in early endosomes is then delivered to late endosomal/lysosomal for degradation or toward the Golgi apparatus for recycling [16].

Rab7, a small GTPase localized to late endosomes, regulates the later stages of the endocytic pathway and it is involved in trafficking and lysosomal degradation of several kinds of receptors [17]. Rab7b, homologous to Rab7, is localized to lysosome-associated compartments and is selectively expressed in monocyte, monocyte-derived dendritic cells and promyeloid or monocytic leukaemia cell lines [18].

Herein we studied the Rab7b participation on the TLR4 endocytic pathway and its effect on monocyte intracellular cytokines production along the acute course of HUS.

2. Patients

The study was approved by the Ethical Committees of Humberto J. Notti Pediatric hospital, Mendoza, Argentina. All patients were enrolled in this study after informed consent was obtained from their parents. In total, 18 children (aged 24 ± 5.8 months), in the acute period of HUS were studied. All patients developed HUS after a prodrome of gastroenteritis with bloody diarrhea that lasted for 3.8 ± 0.9 days. 11 children were positive to Stx-producing *E. coli* O157 diagnosed by stool culture. The presence of Stx antibodies in serum samples was demonstrated in seven children. Microangiopathic hemolytic anemia with schizocytes and thrombocytopenia and increased number of circulating leukocytes were shown in the studied group at onset. 11 patients required peritoneal dialysis for 9 ± 4 days because of acute kidney injury.

Blood samples (2 mL) were obtained for biochemical and immunological studies by venopuncture into EDTA plastic tubes before dialysis and/or transfusion at the first day of hospitalization. A second, third and fourth sample was taken at 4, 6 and 10 days after disease onset. 10 healthy children (aged 23 ± 10 months), showed peripheral WBC counts ranging between 5.000 and 9.000 cells/mm^3. Platelet count, urea and creatinine serum levels, and hematocrit were within the normal range for healthy children (controls) (Table 1).

3. Methods

3.1. Microbiologically confirmed Shiga toxin Escherichia coli (STEC) infection

Stool samples were inoculated directly and after an enrichment step (37 °C, 4–6 h) in trypticase soy broth with cefixime and potassium tellurite (CT-TSB) into sorbitol-MacConkey agar (SMAC). A multiplex polymerase chain reaction (PCR) was performed to detect Stx1 and Stx2 sequences (Stx-1A: 5′GAAGAGTCCGTGGGATTACG3′, Stx-1B: 5′AGCG ATGCAGCTATTAATAA3′, Stx-2A: 5′TTAACCACACCCCACCGGGC AGT3′, Stx-2B: 5′GCTCTGGATGCATCTCTGGT3′), in the confluent growth zone and in the isolated colonies. Colonies that did not ferment sorbitol were tested for agglutination with O157 antiserum. If Stx was detected, colonies were forwarded to the National Reference Laboratory (Instituto Nacional de Enfermedades Infecciosas – ANLIS Dr. Carlos G. Malbrán, Buenos Aires) for serotyping, biotyping, and virulence profiling.

3.2. Flow cytometry

First, 500 μL EDTA whole blood was transferred to a polypropylene tube, diluted with 100 μL phosphate buffered saline (PBS), and incubated with 1 μg/mL of LPS (O111:B4, Sigma-Aldrich Corp., Argentina) to evaluate intracellular cytokines; and Brefeldin A, a protein transport inhibitor (1 μg/mL, BD Biosciences) for 4 h at 37 °C in 5% CO_2. Afterwards, the cells were incubated with antibodies mouse anti human CD14/PerCP (MφP9, BD Biosciences, Argentina) and mouse anti human TLR4/biotin (HTA 125, BD Biosciences, Argentina) for 30 min at 4 °C. Then, the cell suspension was washed with PBS and incubated with streptavidin/FITC for 30 min at 4 °C. The red blood cells were lysed using BD FACS™ Lysing Solution (BD Biosciences, Argentina).

Subsequently, the cells were incubated with permeabilization/fixation solution (BD Bioscience, Argentina) for 20 min at 4 °C and washed with perm/wash buffer (BD, Bioscience, Argentina). The intracellular monoclonal antibodies mouse anti human TNF-α/APC (6401.1111, BD Biosciences, Argentina), and rat anti human IL6/PE (MQ2-6A3, BD Biosciences, Argentina) were added to the cell suspension and kept in the dark for 30 min at 4 °C. Two washes were performed with commercial wash solution 1:10 (BD Perm/ Wash buffer, BD Biosciences, Argentina), to eliminate traces of fixation solution and keep cell membranes permeable. Finally, the cells were washed in 1 mL of PBS supplemented with 0.5% of bovine albumin (Sigma-Aldrich Corp., Argentina), and cells were resuspended in 200 μL of PBS for subsequent acquisition. In all cases, isotype-matched antibodies were assayed in parallel. At least 50,000 events were counted per analysis. All samples were evaluated in BD FACSAria™ III cytometer (BD Biosciences, Argentina) and analyzed using the FACSDiva™ Software v6.1.3 and FlowJo software (Tree Star, Inc.).

Monocyte population was identified and gated according to their positivity for CD14 expression (Fig. 1A). No differences were found in the surface expression of TLR4 in CD14+ monocytes from control children and HUS in LPS stimulated and unstimulated samples (Fig. 1C). A lack of cytokine TNF-α and IL-6 expression was shown in CD14+ monocytes from peripheral blood samples taken from control and HUS children incubated without LPS (Fig. 1D).

3.3. Confocal immunofluorescence microscopy

Indirect immunofluorescence was performed in primary culture of monocytes obtained from peripheral blood of patients with HUS and healthy controls by Ficoll Histopaque gradient. Monocytes obtained by density gradient from mononuclear cells were cultured in D-MEM/F12 medium supplemented with 10% FCS and antibiotic/antimycotic mixture. Cells were plated on sterile coverslips and cultured at 37 °C, 5% CO_2 and humidified environment for 48 h. Then, the cells were fixed in 4% paraformaldehyde, PBS washed and blocked with 50 mM NH_4Cl and permeabilized with PBS 0.05% / saponin 0.5% BSA. For the identification of CD14+ monocytes, the cells were incubated with the primary antibody mouse anti human CD14/PerCP overnight at 4 °C (Fig. 1B). For the determination of TLR4 and Rab7b colocalization in monocytes, the cells were incubated overnight with primary antibodies rabbit anti human TLR4 (Abcam) and mouse anti human Rab7b (Santa Cruz), washed and incubated for 1 h with anti rabbit/FITC secondary antibody and anti mouse/Cy3 secondary antibody. Whole nuclei were visualized using Hoechst. Images were obtained with an Olympus FV1000, (Olympus, Tokyo, Japan). The specificity of the immunostaining was evaluated by omission of the primary antibody. Colocalization coefficients were determined with JACoP Plugin Mc Biophothonics, Image J, using the Pearson overlap coefficient. The resulting coefficient value was the average of select image areas containing predominantly colocalization in 20 cells from 3 independent

Table 1
Clinical and biochemical values. Data from 18 HUS patients at onset (day 1) and control group.

Patients	Age	Diarrhea	HCT	Platelets	WBCs	Leukocyte formula (%)				LDH	Serum Creatinine	Uremia
(n)	(months)	(days)	(%)	(n°/mm^3)	(n°/mm^3)	Neutrophils	Monocytes	Lymphocytes	E/B	(UI/L)	(mg/dL)	(g/L)
1	43	4	30	58000	12000	58	6	33	3	3029	0.86	0.79
2	10	4	30	65000	20500	54	22	20	4	6143	2.35	0.68
3	18	2	27	39000	14830	61	10	27	2	2740	0.46	0.57
4	46	4	33	27000	24900	54	16	26	4	6300	2.43	1.75
5	24	7	19	80000	14900	34	12	52	2	3117	0.36	0.49
6	41	2	22	52000	15800	54	16	30	0	5146	3.43	1.73
7	44	4	33	78000	9950	46	12	40	2	1096	2.51	2.30
8	24	3	33	96000	19900	69	3	22	6	7776	1.36	1.32
9	27	3	29	25000	20200	81	6	13	0	5709	1.65	1.32
10	24	3	35	20000	26000	60	14	26	0	6132	3.87	1.10
11	10	4	21	62000	14000	41	15	41	3	1900	3.00	1.50
12	19	7	28	63000	7600	40	10	40	10	5705	4.54	0.93
13	36	3	22	108000	8300	60	15	25	0	2706	1.81	1.00
14	13	5	22	232000	15200	39	12	49	0	2698	0.94	0.93
15	9	5	32	130000	11000	38	6	53	3	1270	0.46	0.41
16	12	3	36	136000	12900	67	13	20	0	3010	1.48	0.81
17	24	3	24	35000	14500	53	12	34	1	4954	2.33	1.8
18	19	4	25	52000	22000	52	12	35	1	4208	2.8	1.9
Mean	24.61	3.89	27.83	75444.44	15804.44	53.39	11.78	32.56	2.28	4091.06	2.04	1.19
SEM	5.80	0.92	6.56	17782.43	3725.14	12.58	2.78	7.67	0.54	964.27	0.48	0.28
Controls (n = 10)												
Mean	23.00	0.00	38.79	297400.00	7469.00	41.31	8.23	46.42	4.04	400.00	0.50	0.20
SEM	10.00		1.22	21480.00	670.00	3.53	0.81	2.67	0.67	60.00	0.06	0.03

Abbreviations: **HCT:** hematocrit; **WBCs:** white blood cells; **LDH:** lactate dehydrogenase; **E/B:** eosinophils/basophils.

cultures for each day of HUS and in 20 cells from 5 independent cultures in the control group.

3.4. Statistical analysis

Results were assessed by one-way analysis of variance (ANOVA) to compare groups. Significance of differences between groups was estimated by the Bonferroni post test. A p value < 0.05 was considered statistically significant. Values are expressed as mean ± standard error of mean (SEM). Statistical tests were performed with GraphPad Prism statistical software (GraphPad Prism 6.0).

4. Results

4.1. Monocyte cells display sustained expression of TLR4

Surface expression of TLR4 was determined by flow cytometry. Surface TLR4 expression in CD14+ monocytes increased by days 1, 4 and 6 compared to 10 healthy children monocytes. The highest increase of TLR4 protein expression was demonstrated on day 4 in HUS children vs controls, $p < 0.01$. In the early follow up, monocytes from HUS patients displayed reduction in surface TLR4 expression from day 6 onward, with TLR4 expression similar to control after 10 days (Fig. 2).

4.2. Intracellular cytokines production in HUS monocyte cells

Production of intracellular cytokines TNF-α and IL-6 in LPS stimulated CD14+ monocytes from peripheral blood samples from control children and HUS patients at days 1, 4, 6 and 10 was determined by flow cytometry using anti IL-6/PE, anti TNF-α/APC and the corresponding isotype control (Fig. 3A).

Increased percentage of CD14+ monocytes producing both intracellular cytokines TNF-α and IL-6 was shown on day 1 to day 6 when related to control. The highest percentage of TNF-α and IL-6 producing CD14+ monocytes was shown in HUS patients on day 4 compared to control, $p < 0.001$. The reduction in the percentage of CD14+ monocytes expressing intracellular cytokines in HUS began on day 6 onward. Decreased CD14+ monocytes producing intracellular TNF-α near control values was shown on day 10 (Fig. 3 B). In HUS CD14+ monocytes, a significant decrease of intracellular IL-6 cytokine was shown on day 10 related to day 4, $p < 0.001$ (Fig. 3C).

4.3. Rab7b participation in the TLR4 endocytic pathway in early follow up of HUS

TLR4 is internalized and transported to Rab7b positive compartments after LPS ligation. Rab7b, a small GTPase localized to lysosome-associated subcellular compartments, is predicted to regulate the membrane transport of proteins [19].

We further examine the effects of subcellular Rab7b on TLR4 distribution. As expected, dynamic changes of TLR4 on monocyte cells from HUS patients was shown (Fig. 4A). Confocal microscopy analysis revealed that TLR4 and Rab7b colocalized into vesicular and perinuclear membrane organelles at day one of the disease with significantly higher colocalization related to control (Pearsońs Coefficient: 0.77 ± 0.06 vs 0.54 ± 0.2 ($p < 0.01$). On HUS monocytes day 4, a clear overlap between increased cytoplasmatic TLR4 expression and Rab7b was demonstrated exhibiting a discrete vesicular pattern in perinuclear and cytoplasmatic distribution. Increased TLR4 and Rab7b colocalization vs control was shown, Pearsońs Coefficient: 0.87 ± 0.06 vs 0.54 ± 0.2 ($p < 0.01$), (Fig. 4B). Lower staining of both proteins was observed in HUS monocytes on days 6, showing few and small vesicles and low punctuate staining pattern in perinuclear and cytoplasmatic distribution. Absence of cytoplasmatic vesicles was shown on day 10. Colocalization of TLR4 and Rab7b showed no differences to control on day 6 and 10 at the disease follow up (Fig. 4B).

5. Discussion

The internalization and targeted degradation of activated TLRs is a critical component in ending inflammatory signaling, which result in the production of proinflammatory cytokines [11]. Although Stx is the

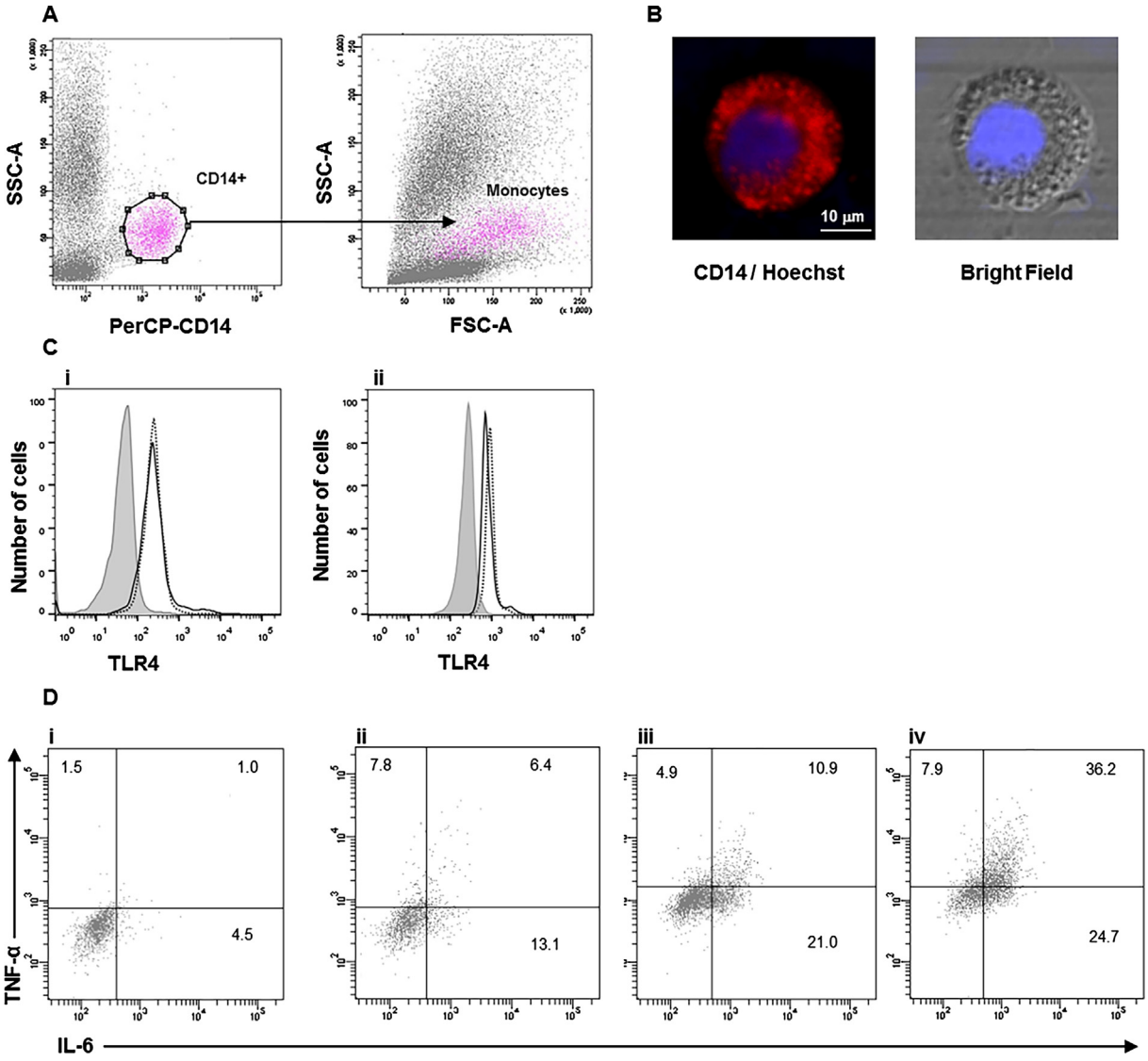

Fig. 1. Identification of monocytes CD14+ in HUS patients. A. Forward scatter (FSC) and side scatter (SSC) biparametric dot plot showing the monocyte population (pink area). The CD14+ population was defined in monocyte gate as it is shown in representative flow cytometry dot plots of SSC vs PerCP-CD14. Backgating strategy: the arrow shows the biparametric position in relation to SSC vs FSC where the events are shown in the monocyte gate. **B**. Indirect immunofluorescence of monocyte culture from a single HUS patient stained with specific antibody against CD14 (red) and Hoechst (blue) and bright field. Scale bar 10 µm. Magnification 600X. **C**. Representative flow cytometry histograms corresponding to the surface TLR4 expression in peripheral monocytes from a control child (i) and a HUS patient (ii), at basal condition (dotted line) and after LPS (1 µg/mL) stimulation for 4 h (solid line). The cells were labeled with anti human CD14/PerCP, anti human TLR4/biotin with streptavidin/FITC. All samples were compared to isotype control (gray shaded histograms). **D**. Representative flow cytometry dot plots of intracellular TNF-α and IL-6 from one control child without LPS stimulation (i) and in the presence of LPS (1 µg/mL) (ii); and one HUS patient without LPS stimulation (iii) and after LPS stimulation (1 µg/mL) of the sample (iv). Cut off quadrant markers based on the negative control were set individually for each measurement. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

main pathogenic factor and is necessary for epidemic HUS development, evidence has shown that the inflammatory response potentiates Stx toxicity. Indeed, both bacterial LPS and polymorphonuclear neutrophils play crucial roles in the full development of HUS [20,21]. Upon recognition of LPS binding with CD14, TLR4 recruits a specific set of adaptor molecules and initiates downstream signaling events that lead to the secretion of inflammatory cytokines. The LPS/TLR4/MD-2 complex is internalized by endocytosis in early endosomes, but it then targeted to other intracellular destinations such as the recycling endosomes and the Golgi apparatus/trans-Golgi Network (TGN) for recycling or late endosomes which fuse with lysosomes for degradation [14,22]. Retrograde transport of transmembrane cargo from endosomes to the TGN is mediated by the retromer complex composed of a sorting nexin (SNX) dimer that binds to endosomal membranes and a vacuolar protein sorting (Vps) 26/29/35 trimer, the latter specifically binds to Rab7 [23].

Rab7b localized on late endosomes and lysosomes, and similarly to Rab7, it is involved in the control of the transport to late endosomes and lysosomes in the endocytic pathway [18].

Our study revealed that LPS from *E. coli* triggers an early surface TLR4 signaling in monocytes from HUS patients. In this way, increased surface TLR4 expression was demonstrated from day 1 to day 6 with the highest surface TLR4 expression on day 4. Moreover, TLR4 activation drives an inflammatory response characterized by an increased generation of intracellular cytokines TNF-α and IL-6 in CD14+ monocytes from patients on day 1 to day 4. After an early disease follow up, on day 10, in contrast, monocytes displayed decreased surface TLR4 expression near control values and a significant reduction of intracellular cytokines. The intracellular expression of TNF-α levels was shown in a time dependent manner from day 4 to day 10.

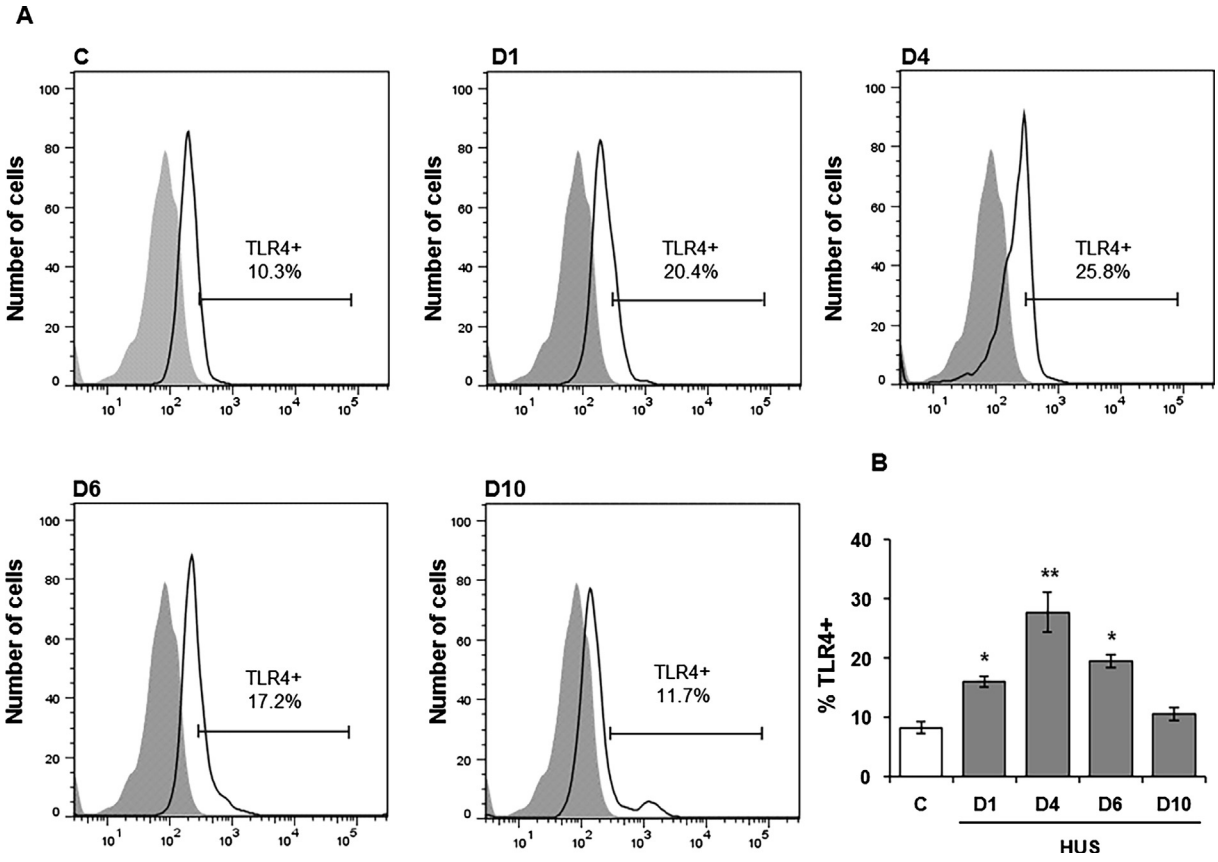

Fig. 2. Analysis of surface Toll-like receptor 4 (TLR4) expression on peripheral blood monocytes from patients within 1, 4, 6 and 10 days of HUS onset and from controls. A. Representative flow cytometry histogram of the TLR4 expression for the cell population defined in the monocyte gate in control children (C) and HUS patients within 1 (D1), 4 (D4), 6 (D6) and 10 days (D10). **B.** Each bar represents the quantification of TLR4 on the surface of CD14 positive monocytes for each subpopulation among patients at HUS (n = 18) within 1, 4, 6 and 10 days of onset and controls (n = 10). Data are presented as mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$ vs control).

We previously reported higher surface TLR4 expression on circulating leucocytes from HUS patients at onset. Neutrophil TLR4 protein expression determined by flow cytometry was upregulated. We found increased dependent proinflammatory plasma cytokines, TNF-α and IL-8 along with decreased anti-inflammatory IL-10 release within one day after HUS when compared with EHEC diarrhea patients and controls [13]. In this study, the monocyte population was identified and selected through the use of specific monoclonal antibodies against its cell line marker, CD14. This selection allows a very accurate analysis of TLR4 expression for this cell type, much more than when the selection was made by forward and side scatter like in our previous study, where TLR4 expression showed no changes in circulating monocytes [13].

Brigotti et al. described TLR4 as the receptor that specifically recognizes not only LPS but also Stx1 and Stx2 in human neutrophils. Upon stimulation with Stx1 or Stx2, neutrophils induced the same pattern of cytokine expression in culture supernatants as in response to LPS [24]. Due to the fact that monocytes have both receptors the Stx receptor Gb3Cer and TLR4, we cannot dismiss the possible contribution of Stx binding to TLR4 nor the exclusion of Stx binding to Gb3Cer for inducing enhanced intracellular cytokine generation at the earliest stage of HUS. This issue should be studied in future investigations.

Fernandez et al. demonstrated at basal conditions that the highest TNF-α and IL-10 producing monocytes was found in moderate/severe HUS when related to healthy children (HC); whereas LPS produced a similar increase in TNF-α and IL-10-producing monocytes for HUS groups. However, decreased secretion of TNF-α and IL-10 plasma levels was demonstrated in the HUS group when compared to healthy controls at basal and LPS conditions [25].

The recognition of Gram-negative bacteria LPS bound to CD14 by TLR4 leads to TLR4 ligation and induction of proinflammatory cytokines. Therefore, TLR4 expression levels may be required in regulating cytokine synthesis by immune cells following LPS stimulation, these events leading to an initial response for the elimination of Gram-negative bacterial infections [26,27]. Excessive or uncontrolled TLR4 activation are partly responsible for the initiation of inflammatory and infectious diseases. Prolonged or exaggerated production of LPS-induced mediators might result in a severe disease, septic shock. This was shown in a paper that we published concerning clinical data about the amplified acute inflammatory response in a limited number of patients during the beginning phase of HUS with high leukocyte count at admission. We showed that an extreme compromise of hemodynamic parameters similar to septic shock brings about multisystemic organ injury in these patients [28].

TLR4 signaling is tighly regulated at several levels. A number of diverse inhibitors of TLR4 signaling have been reported. Some of these negative regulators interfere with the initial stage of TLR4 signaling as ST2L (also known as IL1Rl) and SIGIRR (single immunoglobulin IL-1R-related molecule) [29]. Although others, like Triad3A (triad domain-containing protein 3 variant A) and SOCS1 (suppressor of cytokine signaling), two E3 ubiquitin protein ligases, are involved in the ubiquitin-dependent degradation of TLRs or signalling components [30,31].

In the last years, the endocytic trafficking of the LPS/TLR4 complex as well as the functional consequences of the lysosomal degradation pathway have been analyzed for the signalling ending.

Rab7b, specifically expressed in CD14 positive cells in peripheral blood, is involved in the transport regulation to degradative compartments in the endocytic pathway. Notably, the receptor complex has

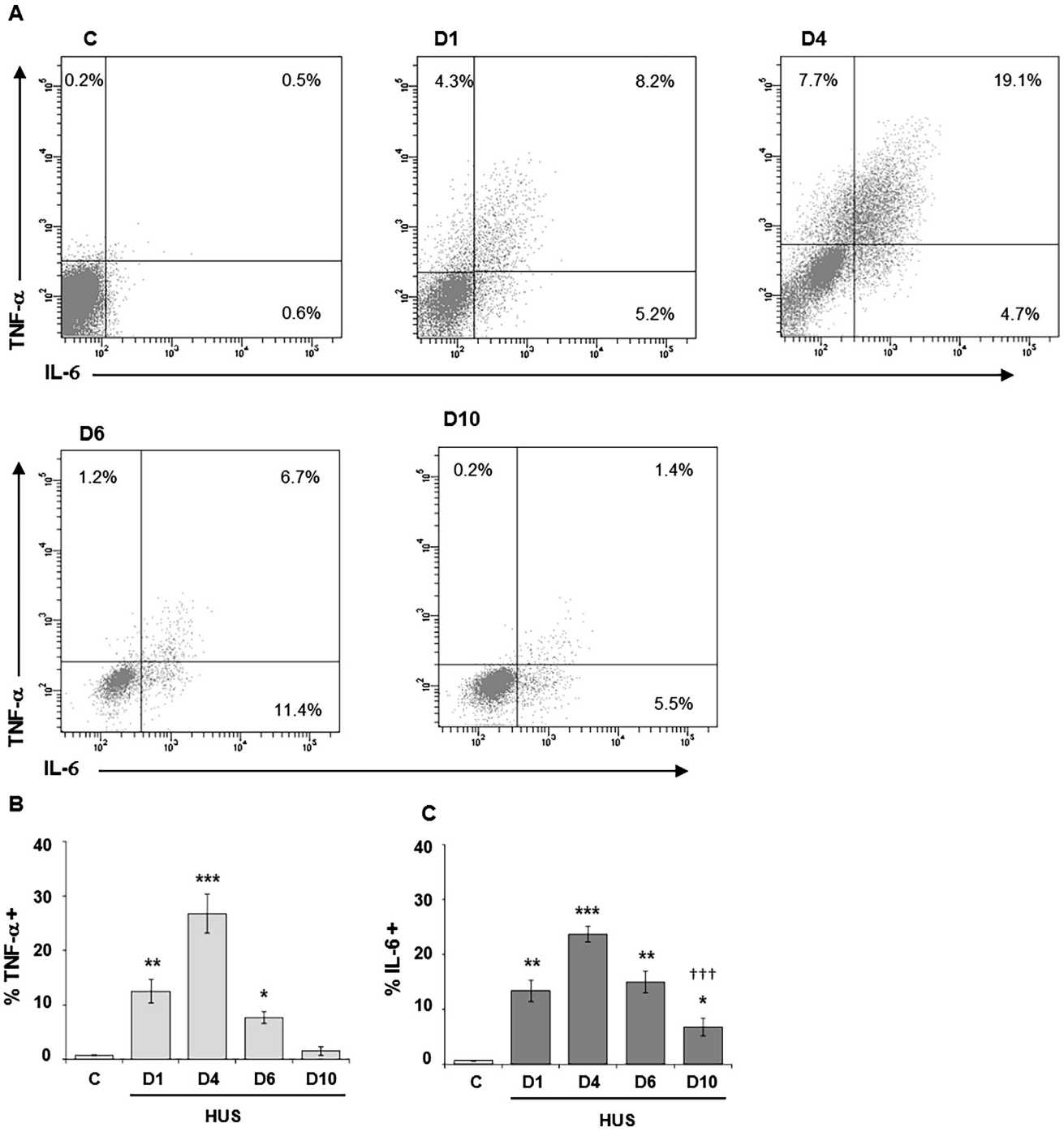

Fig. 3. Analysis of the intracellular cytokines TNF-α and IL-6 expression in CD14+ monocytes during the acute course of HUS. A. Representative flow cytometry dot plot quadrant analysis of monocytes CD14+ using anti-TNF-α and anti-IL-6 antibodies in samples from control children (C) and HUS patients, within 1 (D1), 4 (D4), 6 (D6) and 10 days (D10). Numbers in each plot denote the percentage of the corresponding intracellular cytokine from the total CD14+ population. **B.** Quantification of monocyte TNF-α/CD14+. A significant increase in the percentage of CD14+ monocytes expressing intracellular TNF-α was observed on days 1 (D1), 4 (D4) and 6 (D6) of HUS vs control ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{*}p < 0.05$) respectively. Control, n = 10; HUS n = 18. **C.** Quantification of monocyte IL-6/CD14+. Higher percentage of monocytes CD14+ expressing intracellular IL6 was observed on HUS samples from D1, D4 and D6 vs control, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{**}p < 0.01$ respectively. Decreased percentage of CD14+ monocytes expressing intracellular IL-6 was shown in HUS on day 10 vs day 4. ($^{\dagger\dagger\dagger}p < 0.001$). Data are presented as mean ± SEM.

been detected in Rab7b positive compartments [19].

Preliminary investigations demonstrated that Rab7b can regulate LPS-induced cytokine production in macrophages, suggesting that Rab7b may be involved in the control of TLR4 signaling. In Rab7b silenced cells after LPS treatment, it has been observed that a prolonged persistence of the receptor in early endocytic compartments delays the presence of TLR4 in late endosomes and lysosomes. This suggests that Rab7b may negatively regulate TLR4 signaling, which promotes TLR4 targeting to lysosomes for degradation [32]. Therefore, we investigated the role of Rab7b in LPS-initiated TLR4 signaling in monocytes during the acute course of HUS. We found that at the very beginning of the disease, days 1 and 4, Rab7b colocalizes with TLR4 in intracellular vesicles with maximal colocalization at day 4. These results suggest a localization of TLR4 within Rab7b-coated vesicles.

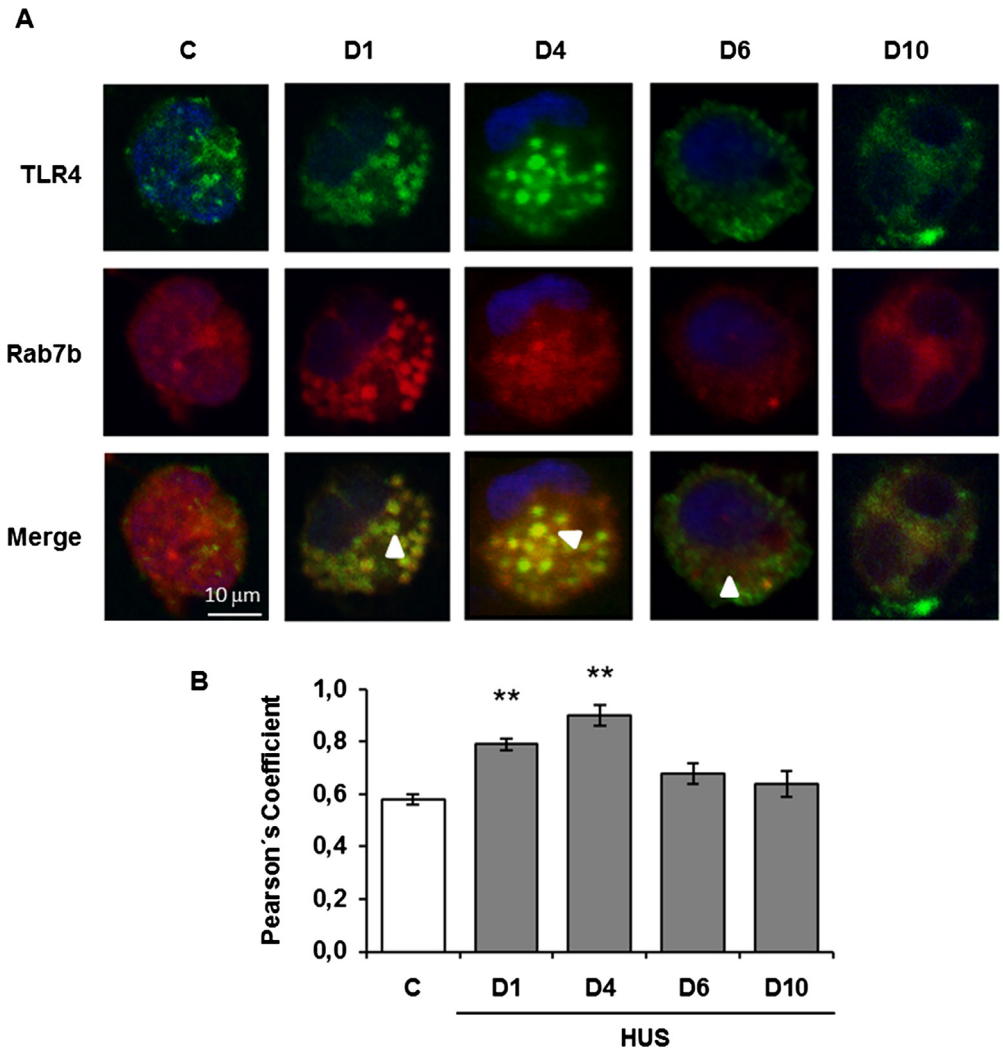

Fig. 4. Colocalization analysis of Rab7b and TLR4 in monocytes during the acute course of HUS. TLR4 and Rab7b colocalization in monocytes was determined throughout the progression of HUS and in controls by indirect immunofluorescence and confocal microscopy. Specific rabbit anti TLR4/anti rabbit FITC (green), mouse anti Rab7b/anti mouse Cy3 (red), and Hoechst to visualize the nuclei (blue) were used. **A.** Representative immunofluorescence of both proteins in monocytes from one HUS patient and one control. The arrows indicate the colocalization areas. Scale bar 10 µm. Magnification 600X. **B.** Graphic bar shows the TLR4 and Rab7b colocalization, which was determined by using the Pearsońs Coefficient. $^{**}p < 0.01$ vs control. 20 cells from 3 independent cultures were analyzed for each day of the HUS follow-up. In addition, 20 cells from 5 independent cultures in the control group were evaluated. Data are expressed as the mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Both proteins run together in intracellular trafficking directed towards degradation. It is likely that the higher TLR4 observed at the surface level by flow cytometry could demonstrate the recycling of the receptor in a step prior to the proteolytic degradation; and/or, it may be possible that the sustained stimulus of the pathogen could generate the *in novo* synthesis, which also would contribute to the increased receptor expression in the cell membrane on day 4.

As the disease progresses, surface TLR4 decreases as well as the monocyte intracellular cytokine inflammatory response. Monocyte cell analysis on day 10 showed surface TLR4 expression and intracellular proinflammatory cytokines expression near controls. Lower expression of Rab7b and TLR4 and minimal and punctual colocalization of both proteins was observed with no differences related to control. In this way, the early colocalization of Rab7b/TLR4 may account for an adequate/successful trafficking of TLR4 to the degradation pathway leading to the downregulation of proinflammatory state or inflammation during the early follow up (10 days) of the disease.

In a previous report, subsequently to LPS stimulation, Brucia et al. demonstrated that Cystic Fibrosis transmembrane conductance regulator $CFTR^{-/-}$ macrophages have prolonged TLR4 retention in the early endosome and reduced translocation into the lysosomal compartment. The abnormal TLR4 trafficking led to an increased LPS-induced activation of NF-κB and decreased TLR4 degradation. This may cause a persistent inflammation of the chronic infection that characterizes the Cystic Fibrosis disease [33].

Our results demonstrate a synchronic movement of TLR4 through various levels of expression that are within the clinical follow up of the disease. Increased surface TLR4 expression and enhanced monocyte intracellular cytokine generation lead to a robust inflammatory response to LPS. This is shown at the very beginning of the disease. The colocalization of TLR4 with Rab7b on days 1 and 4 demonstrates how TLR4 moves to late endosomes promoting TLR4 targeting to lysosomes for degradation (Fig. 5).

In the early follow up of HUS, downregulation of surface TLR4 expression and decreased Rab7b/TLR4 colocalization led us to assume a controlled TLR4 signaling and degradation.

In conclusion, our findings suggest that Rab7b participates as a negative regulator in the control of the TLR4 endocytic pathway in pediatric HUS patient monocytes. The resulting decline in monocyte cell cytokine generation is demonstrated by the induction of the TLR4 receptor endocytosis during the early follow up of HUS.

Knowledge of the daily expressions of TLR4 and the regulation pathways in the early progression of the disease allow for potential therapeutic strategies for controlling the host response against inflammation, which may avoid the severe consequences of the disease.

Compliance with ethical standards

Funding: The work was performed with financial support from CONICET, PICT N°:2086 and from SECTyP-UNCUYO 06/J474, Argentina, to P.G. Vallés.

Ethical approval: All procedures performed in studies involving

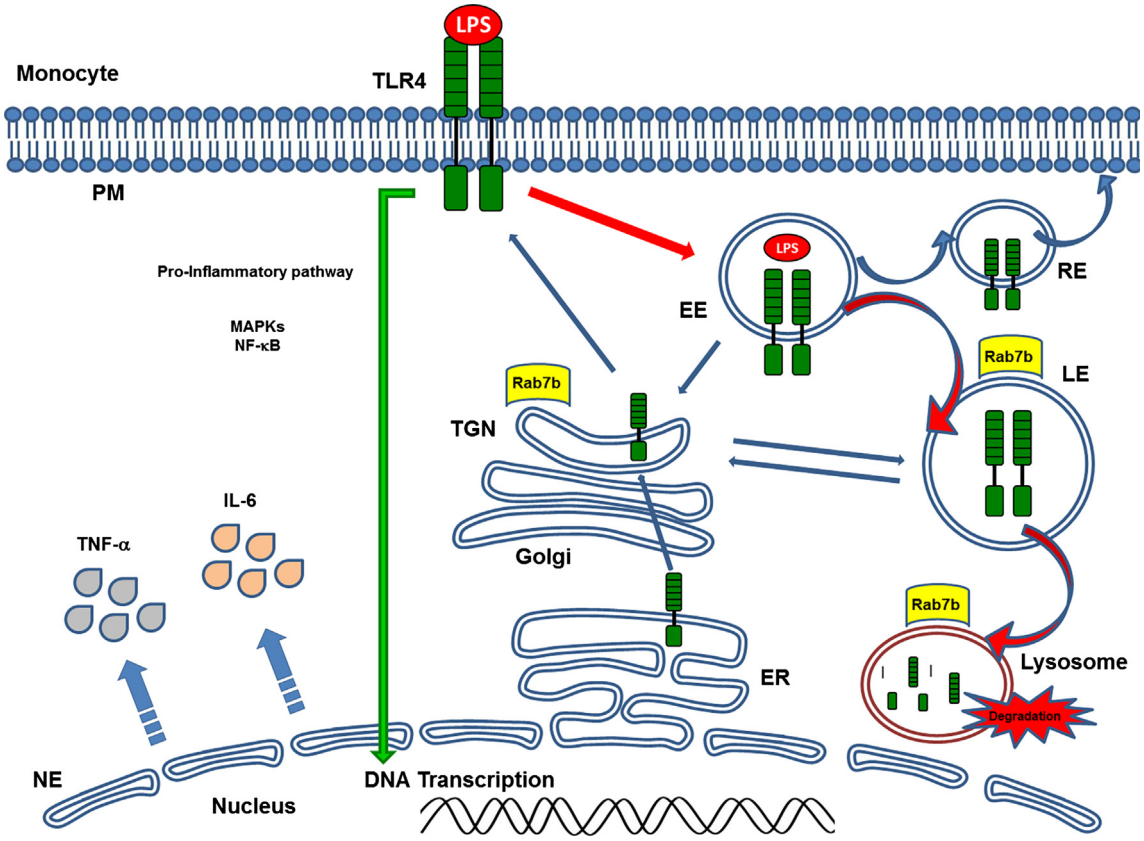

Fig. 5. Involvement of Rab7b on the TLR4 (Toll-like receptor) endocytic pathway degradation. On the left, the inflammatory response to LPS/TLR4 internalization and TLR4 activation inducing enhanced intracellular cytokine generation on the very beginning of HUS (day 1 to 4), is presented. On the right, the figure displays the involvement of Rab7b on TLR4 degradation pathway leading to the downregulation of proinflammatory state in the early follow up of the disease (10 days). **LPS**: lipopolysaccharide, **PM**: plasma membrane, **NE**: nuclear envelope, **EE**: early endosome, **RE**: recycling endosome, **LE**: late endosome, **ER**: endoplasmic reticulus, **TGN**: trans-Golgi network, **DNA**: desoxyribonucleic acid.

human participants were in accordance with the ethical standards of the institutional and/or national research committee at which the studies were conducted (IRB approval number 0030992/2011) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

References

[1] C. Mele, G. Remuzzi, M. Noris, Hemolytic uremic syndrome, Semin. Immunopathol. 36 (4) (2014) 399–420.
[2] H.A. Repetto, Haemolytic-uraemic syndrome–the experience in Argentina, Nephrol. Dial. Transplant. 14 (3) (1999) 548–550.
[3] C.J. Cobeñas, L.F. Alconcher, A.P. Spizzirri, R.C. Rahman, Long-term follow-up of Argentinean patients with hemolytic uremic syndrome who had not undergone dialysis, Pediatr. Nephrol. 22 (9) (2007) 1343–1347.
[4] T.N. Petruzziello, I.A. Mawji, M. Khan, P.A. Marsden, Verotoxin biology: molecular events in vascular endothelial injury, Kidney Int. Suppl. 75 (2009) S17–S19.
[5] C. Zoja, S. Buelli, M. Morigi, Shiga toxin-associated hemolytic uremic syndrome: pathophysiology of endothelial dysfunction, Pediatr. Nephrol. 25 (2010) 2231–2240.
[6] F. Clayton, T.J. Pysher, R. Lou, et al., Lipopolysaccharide upregulates renal Shiga toxin receptors in a primate model of hemolytic uremic syndrome, Am. J. Nephrol. 25 (2005) 536–540.
[7] M. Ikeda, S. Ito, M. Honda, Hemolytic uremic syndrome induced by lipopolysaccharide and Shiga-like toxin, Pediatr. Nephrol. 19 (2004) 485–489.
[8] T.R. Keepers, M.A. Psotka, L.K. Gross, T.G. Obrig, A murine model of HUS: Shiga toxin with lipopolysaccharide mimics the renal damage and physiologic response of human disease, J. Am. Soc. Nephrol. 17 (2006) 3404–3414.
[9] M. Triantafilou, K. Miyake, D.T. Golenbock, K. Triantafilou, Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation, J. Cell Sci. 115 (2002) 2603–2611.
[10] P.G. Motshwene, M.C. Moncrieffe, J.G. Grossmann, C. Kao, M. Ayaluru, A.M. Sandercock, V. Carol, C. Robinson, E. Latz, N.J. Gay, An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4, J. Biol. Chem. 284 (37) (2009) 25404–25411.
[11] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (2004) 499–511.
[12] T. Nishiya, A.L. DeFranco, Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors, J. Biol. Chem. 279 (2004) 19008–19017.
[13] P.G. Vallés, S. Melechuck, A. González, W. Manucha, V. Bocanegra, R. Vallés, Toll-like receptor 4 expression on circulating leucocytes in hemolytic uremic syndrome, Pediatr. Nephrol. 27 (3) (2012) 407–415.
[14] H. Husebye, O. Halaas, H. Stenmark, et al., Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity, EMBO J. 25 (2006) 683–692.
[15] M. Kobayashi, S. Saitoh, N. Tanimura, Regulatory roles for MD-2 and TLR4 in ligand-induced receptor clustering, J. Immunol. 176 (2006) 6211–6218.
[16] LuY. Yong-Chen, W.C.H. Wen-Chen Yeh, P.S. Ohashi, LPS/TLR4 signal transduction pathway, Cytokine 42 (2008) 145–151.
[17] H. Stenmark, Rab GTPases as coordinators of vesicle traffic, Nat. Rev. Mol. Cell Biol. 10 (2009) 513–525.
[18] M. Yang, T. Chen, C. Han, N. Li, T. Wan, X. Cao, Rab7b, a novel lysosome-associated small GTPase is involved in monocytic differentiation of human acute promyelocytic leukemia cells, Biochem. Biophys. Res. Commun. 318 (2004) 792–799.
[19] Y. Wang, T. Chen, C. Han, et al., Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4, Blood 110 (2007) 962–971.
[20] R.A. Exeni, G.C. Fernandez, M.S. Palermo, Role of polymorphonuclear leukocytes in the pathophysiology of typical haemolytic uremic syndrome, Sci. World J. 7 (2007) 1155–1164.
[21] V. Landoni, M. Campos-Nebel, P. Schierloh, et al., Shiga toxin 1-induced inflammatory response in lipopolysaccharide- sensitized astrocytes is mediated by endogenous tumor necrosis factor alpha, Infect. Immun. 78 (2010) 1193–1201.
[22] N. Thieblemont, S.D. Wright, Transport of bacterial lipopolysaccharide to the golgi apparatus, J. Exp. Med. 190 (1999) 523–534.

[23] R. Rojas, T. van Vlijmen, G.S. Mardones, Y. Prabhu, A.L. Rojas, S. Mohammed, A.J. Heck, G. Rapodo, P. van der Sluijs, J.S. Bonifacino, Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab 7, J. Cell Biol. 183 (3) (2008) 513–526.
[24] M. Brigotti, D. Carnicelli, V. Arfilli, N. Tamassia, F. Borsetti, E. Fabbri, P.L. Tazzari, F. Ricci, P. Pagliaro, E. Spisni, M.A. Cassatella, Identification of TLR4 as the receptor that recognizes Shiga toxins in human neutrophils, J. Immunol. 191 (2013) 4748–4758.
[25] G.C. Fernández, M.V. Ramos, V.L. Landoni, et al., Cytokine production is altered in monocytes from children with hemolytic uremic syndrome, J. Clin. Immunol. 32 (2012) 622–631.
[26] F. Proulx, E.G. Seidman, Karpman, Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome, Pediatr. Res. 50 (2001) 163–171.
[27] C. Zoja, S. Angioletti, R. Donadelli, et al., Shiga toxin-2 triggers endothelial leukocyte adhesion and transmigration via NF-kappaB dependent up-regulation of IL-8 and MCP-1, Kidney Int. 62 (2002) 846–856.
[28] P.G. Vallés, S. Pesle, L. Piovano, et al., Postdiarrheal Shiga toxin-mediated hemolytic uremic syndrome similar to septic shock, Med. (B Aires) 65 (5) (2005) 395–401.
[29] E.K. Brint, D. Xu, H. Liu, A. Dunne, A.N. McKenzie, L.A. O'Neill, et al., ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance, Nat. Immunol. 5 (2004) 373–379.
[30] T.H. Chuang, R.J. Ulevitch, Triad3A and E3 ubiquitinprotein ligase regulating Toll-like receptors, Nat. Immunol. 5 (2004) 495–502.
[31] A. Mansell, R. Smith, S.L. Doyle, P. Gray, J.E. Fenner, P.J. Crack, et al., Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation, Nat. Immunol. 7 (2006) 148–155.
[32] C. Bucci, O. Bakke, C. Progida, Rab7b and receptors trafficking, Commun. Integr. Biol. 3 (5) (2010) 401–404.
[33] E.M. Bruscia, P.X. Zhang, A. Satoh, et al., Abnormal trafficking and degradation of TLR4 underlie the elevated inflammatory response in cystic fibrosis, J. Immunol. 186 (2011) 6990–6998.