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Original Paper

Association of haemolytic uraemic syndrome with dysregulation of chemokine receptor expression in circulating monocytes

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Abstract

Haemolytic uraemic syndrome (HUS) is the major complication of *Escherichia coli* gastrointestinal infections that are Shiga toxin (Stx) producing. Monocytes contribute to HUS evolution by producing cytokines that sensitize endothelial cells to Stx action and migration to the injured kidney. As CC chemokine receptors (CCRs) are involved in monocyte recruitment to injured tissue, we analysed the contribution of these receptors to the pathogenesis of HUS. We analysed CCR1, CCR2 and CCR5 expression in peripheral monocytes from HUS patients during the acute period, with healthy children as controls. We observed an increased expression of CCRs per cell in monocytes from HUS patients, accompanied by an increase in the absolute number of monocytes CCR1$^+$, CCR2$^+$ and CCR5$^+$. It is interesting that prospective analysis confirmed that CCR1 expression positively correlated with HUS severity. The evaluation of chemokine levels in plasma showed that regulated on activation of normal T-cell-expressed and -secreted (RANTES) protein was reduced in plasma from patients with severe HUS, and this decrease correlated with thrombocytopenia. Finally, the expression of the higher CCRs was accompanied by a loss of functionality which could be due to a mechanism for desensitization to compensate for altered receptor expression. The increase in CCR expression correlates with HUS severity, suggesting that the dysregulation of these receptors might contribute to an increased risk of renal damage. Activated monocytes could be recruited by chemokines and then receptors could be dysregulated. The dysregulation of CCRs and their ligands observed during the acute period suggests that a chemokine pathway would participate in HUS development.

Key words: chemokine receptors, functionality, HUS, monocytes, Shiga toxin, up-regulation.

INTRODUCTION

The postdiarrhoeal form of the haemolytic uraemic syndrome (HUS) has been associated with enterohaemorrhagic infections caused by Shiga toxin (Stx)-producing *Escherichia coli* (STEC) [1]. HUS mainly affects children and is characterized by microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure [2]. Endothelial dysfunction induced by Stx is the main event in the development of this thrombotic microangiopathy. However, clinical and experimental data support the host's inflammatory response playing a pivotal role in the pathogenesis of the disease and renal damage [3]. In this regard, inflammatory cytokines such as tumour necrosis factor α (TNF-α) can be released from monocytes/macrophages in response to Stx [4,5], further increasing Gb3 expression (a specific receptor for Stx) and Stx toxicity in human endothelial cells [6]. In addition, the interaction of leukocytes with blood vessels involves the synthesis of cytokines and chemokines by injured cells [7]. The interaction between these chemokines and their receptors drives the migration of specific subsets of inflammatory cells to different tissues [7–9]. The temporal and spatial recruitment of immune cells in the kidney during renal damage are orchestrated

Abbreviations: CCR, CC chemokine receptor; DARC, Duffy antigen receptor for chemokines; HUS, haemolytic uraemic syndrome; IL, interleukin; IQR, interquartile range; LPS, lipopolysaccharide; mAb, monoclonal antibody; MCP, monocyte chemotactic protein; MFI, mean fluorescence intensity; MIP, macrophage inflammatory protein; PBMC, peripheral blood mononuclear cell; RANTES, regulated on activation of normal T-cell-expressed and -secreted; STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin; TNF-α, tumour necrosis factor α.

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Table 1 Clinical and biochemical data from HUS patients
According to Gianantonio's criteria [19], patients were classified as mild cases (grade I: no anuria), moderate cases (grade II: <7 days of anuria) or severe cases (grade III: >7 days of anuria). Data are presented as means ± S.D.s.

	Severity of renal dysfunction		
	Grade I ($n=7$)	**Grade II ($n=3$)**	**Grade III ($n=11$)**
General parameters			
Age (months)	52.4 ± 5.3	73.0 ± 38.1	35.5 ± 26.2
Duration of diarrhoea (days)	5.3 ± 2.0	4.1 ± 1.0	4.8 ± 1.9
Blood and renal parameters			
Platelets ($\times 10^9$/l)	112.6 ± 62.3	56.0 ± 16.0	64.8 ± 69.9
Haematocrit (%)	26.1 ± 3.7	24.5 ± 7.7	22.8 ± 4.7
Urea (mmol/l)	67.1 ± 11.4	253.3 ± 117.5	261.3 ± 32.0
Creatinine (mmol/l)	0.10 ± 0.04	0.24 ± 0.11	0.60 ± 0.31
Days from the onset of diarrhoea[a]	5 ± 2	3 ± 0	5 ± 3

[a]Refers to the number of days from the time of blood sample collection to the onset of diarrhoea.

by an array of host cytokines, chemokines and their cognate receptors.

Chemokines are low-molecular-mass proteins classified according to the presence or absence of an amino acid between the first two cysteine residues, yielding CXCL and CCL chemokines according to the more recent nomenclature. CXC chemokines act predominantly on neutrophils and T-lymphocytes, whereas CC chemokines are able to chemoattract monocytes, T-lymphocytes, eosinophils, basophils, dendritic cells and natural killer (NK) cells, depending on the specific protein [10]. Among the various chemokines studied, monocyte chemotactic protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP-1β (/CCL4) and regulated on activation of normal T-cell-expressed and -secreted (RANTES/CCL5) protein have been shown to have an important role in the resolution or progression of renal diseases [11]. In addition, renal tubular cells have been shown to be a primary target of Stx in animal models and cell culture, inducing both apoptosis and cytokine secretion in these cells [12,13]. MCP-1, RANTES and MIP-1α are the most abundant renal chemokines induced by Stx plus lipopolysaccharide (LPS) in mice, and their neutralization significantly reduced renal macrophage recruitment [14]. Moreover, we have reported that CC chemokine receptor (CCR)-1 blockage increases mice survival, reduces leukocyte recruitment in the kidney and reduces renal damage after Stx intoxication [15]. These data suggest that chemokine interaction with their receptors in monocytes is the primary mechanism that regulates renal macrophage infiltration.

Other chemokines such as fractalkine/CX_3CL_1 and its receptor CX_3CR_1 have also been implicated in HUS. We previously reported that CX_3CR_1 is down-regulated on peripheral leukocytes from HUS patients [16] and Zanchi et al. [17] showed that Stx induces the synthesis of fractalkine by human endothelial cells. Thus, locally secreted chemokines participate in the accumulation of inflammatory cells at the kidney level [18] and amplify the inflammatory processes fundamental to the activation of renal microvascular endothelial cells.

Therefore, in the present, prospective, non-therapeutic study in children with HUS, we examined CCR1, CCR2 and CCR5 expression and function in circulating monocytes, as well as their corresponding plasma chemokine levels. In addition, we determined whether changes in chemokine receptor expression were associated with the most severe forms of the disease.

MATERIALS AND METHODS

Patients and samples

The study was approved by the hospital ethical committee, Comité de Bioética del Hospital Municipal del Niño de San Justo, and has been performed in accordance with the 1964 Declaration of Helsinki and its later amendments. All patients and healthy children as the control group, admitted for routine surgical procedures and matched for age and sex, were enrolled after informed consent was obtained from their parents. The diagnostic criteria for HUS were microangiopathic haemolytic anaemia, thrombocytopenia (platelet count $<150\times10^9$/l) and acute renal failure (serum creatinine level >0.100 mmol/l). All patients developed HUS after a prodrome of gastroenteritis with bloody diarrhoea. Half the children showed evidence of STEC O157 by stool culture and/or the presence of Stx antibody in serum. There were 11 girls and 15 boys in the study. Severe illness was defined by Gianantonio et al. [19] according to days of anuria. Clinical and biochemical data of patients are presented in Table 1, and the absolute counts of peripheral leukocytes are summarized in Table 2. Blood samples (2 ml) were obtained by venepuncture into EDTA plastic tubes during the acute period, before dialysis and/or transfusion at different days after the onset of diarrhoea (Table 1). In some patients, a small amount of blood sample precluded measurement of all studied parameters. For evaluation of parameters in recovered patients, blood samples were obtained at least 6 months after resolution of the signs and symptoms of HUS. To perform experiments in which a monocyte subpopulation was isolated, blood samples from normal adult volunteers were obtained. The present study has been reviewed and approved by the local ethical committee.

Table 2 Absolute number of circulating leukocytes in different clinical groups
All data are expressed as the median and IQR values. The Mann–Whitney U-test was used to determine significant differences between the two groups.

	HC ($n = 20$) Median (IQR)	HUS ($n = 22$) Median (IQR)
WBC count ($\times 10^9$/l)	7.70 (6.25–9.07)	11.10*** (9–15.93)
Monocytes (%)	6.15 (4.35–7.38)	7.70** (5.85–11.38)
Monocytes ($\times 10^8$/l)	0.35 (0.22–0.65)	0.96* (0.59–1.64)

WBC, white blood cell.
*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$, HUS compared with healthy children.

Antibodies and studies

Monoclonal antibodies (mAbs) for characterization of monocyte subpopulations by flow cytometry were applied: CD14-PECy5 (mouse IgG2a, Immunotech), CD16-FITC (mouse IgG1, BD Biosciences), CCR1-PE (mouse IgG, R&D Systems), CCR2-PE (mouse IgG, R&D Systems), CCR5-PE (mouse IgG1, eBioscience) and CD11b-FITC (mouse IgG1, Caltag Laboratories). Whole-blood samples (100 μl) were incubated with the specific conjugated mAb for 30 min at room temperature; then they were treated with FACS Lysing Solution (BD Biosciences), washed and resuspended in 0.2 μl of ISOFLOW (International Link, SA). In all cases, isotype-matched antibodies were assayed in parallel, and the fluorescence was measured on 100 000 events using the CellQuest Pro software on a BD FACScan (Becton Dickinson). Monocytes were identified and gated according to their forward- and light-scattering (FSC/SSC) dot–plot profiles and positive staining for CD14.

Monocyte purification

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy children or HUS blood samples by centrifugation over a Ficoll (Pharmacia)/Hypaque (Winthrop Laboratories) gradient.

Isolated monocytes were obtained from blood samples from normal adult volunteers. For this purpose, PBMCs were obtained as described, and then cells were centrifuged on a Percoll gradient (Amersham Pharmacia Biotech) [20]. The viability and purity of monocytes were >96% and >90%, respectively.

Chemotaxis assays

Monocyte migration towards plasma from healthy or HUS children

Purified monocytes (5.3×10^6/ml) were added to the (upper) top compartment of a chemotaxis chamber (MultiScreen 96-well filtration plate, 5.0 μm polycarbonate sterile, Millipore), and allowed to transmigrate at 37 °C for 3 h against RPMI 1640 medium (Hyclone Laboratories Inc.) or plasma from healthy or HUS children (diluted at 50%) in the lower compartment.

Monocyte migration towards chemokines

Purified PBMCs (6×10^5) from HUS or healthy children were added to the top compartment and allowed to transmigrate at 37 °C for 3 h against 200 μl of RPMI 1640 medium, or MIP-1α or RANTES placed in the bottom compartment of the chemotaxis chamber.

In both experimental designs for monocyte migration, input cells and cells recovered from the bottom chamber were stained and analysed by CD14/CD16. The total number of monocytes in 1 ml was calculated using a predetermined number of beads, carboxylate microspheres (Spherotech), added to each tube.

Up-regulation of CD11b by MCP-1 and MIP-1α on monocytes

Whole-blood samples (50 μl) were stimulated with MCP-1 or MIP-1α at 100 ng/ml for 10 or 45 min at 37 °C. Then CD11b expression was analysed on monocytes identified according to their FSC/SSC dot–plot profiles and positive staining for CD14.

Cytokine measurement

Plasma MIP-1α, MCP-1 and RANTES were measured by ELISA using commercial kits (eBioscience), according to the manufacturer's instructions.

Correlations

Retrospectively, the existence of a correlation between the immunological alterations found and the severity of disease in HUS patients, according to Gianantonio's criteria, based on the number of days of anuria [19] was analysed. In addition, immunological alterations were correlated with biochemical parameters.

Statistical analysis

Results are expressed as median and interquartile range (IQR) values. Comparative analyses were performed using a non-parametric, unpaired, two-tailed Mann–Whitney U-test. A multisample Kruskall–Wallis test followed by Dunn's comparison test was performed for comparison between more than two treatments. $P < 0.05$ was considered significant. Correlations between immunological and clinical data were done using the non-parametric Spearman's rank–correlation test.

RESULTS

Enhanced expression of chemokine receptors on monocytes from HUS patients

Circulating monocytes were identified by their FSC/SSC profile and CD14 membrane expression after whole-blood analysis by flow cytometry. Therefore, comparative analysis of CCR expression on leukocytes was performed by multiparameter flow cytometry using the anti-CCR1, anti-CCR2 and anti-CCR5 mAbs on whole blood to avoid variations in its cellular expression during isolation of leukocyte subpopulations (Figure 1A). As shown in Figures 1(A) and 1(B), the predominant chemokine receptor expressed on the cell surface of circulating monocytes from healthy children is CCR2, which binds MCP-1 [21]. Monocytes also express CCR1 (which binds MIP-1α and RANTES), and very low levels of CCR5 (which binds MIP-1α, MIP-1β and RANTES) [22]. The whole monocytic population from HUS patients showed a significant increase in the expression of the receptors CCR1, CCR2 and CCR5 compared with healthy children, giving rise to higher percentages of positive cells for CCR1, CCR2 or CCR5

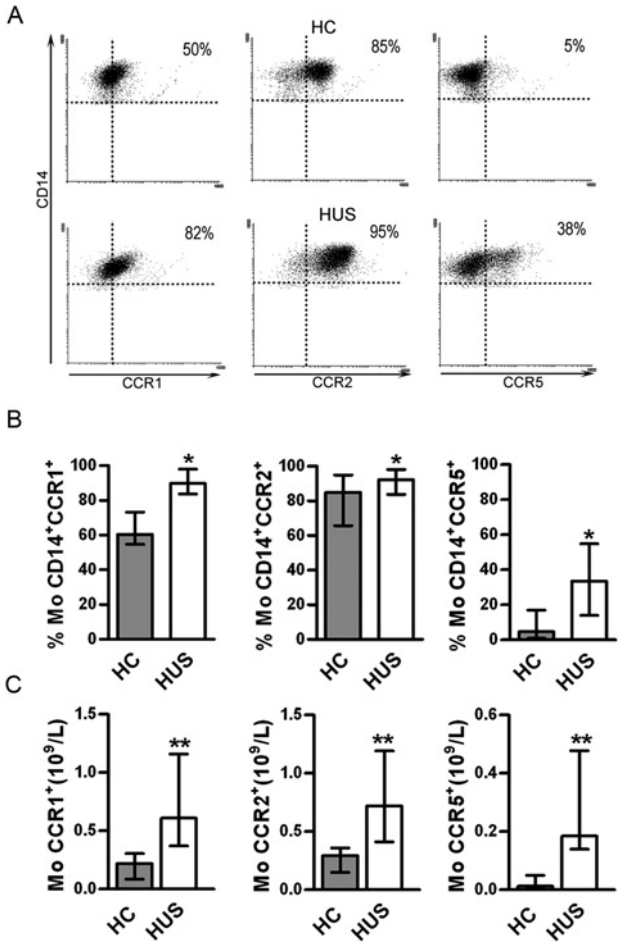


Figure 1 CCR1, CCR2 and CCR5 expression in monocytes
(**A**) Representative dot plot of CD14 versus CCR1, CCR2, or CCR5 expression of one healthy child (HC; $n = 20$) and one HUS patient ($n = 20$). (**B**) Each bar represents the median with the IQR percentage of $CD14^+$ monocytes that express CCR1, CCR2 or CCR5 in each clinical group. (**C**) Each bar represents the median with IQR of the absolute number of cells for each monocyte subset within the different clinical groups. $^*P < 0.05$, $^{**}P < 0.1$ vs healthy children. Statistical analyses were performed using the Mann–Whitney U-test.

(Figures 1A and 1B), as well as higher absolute numbers of monocytes positive for each CCR (Figure 1C).

Monocyte heterogeneity has largely been demonstrated by flow cytometry according to the expression of CD14 and CD16 molecules [23]. These markers define mainly two distinct subsets: classic monocytes ($CD14^+CD16^-$) and inflammatory monocytes ($CD14^+CD16^+$). Both subsets differ in the surface expression of chemokine markers and major histocompatibility complex (MHC) class II, and also in their capacity to produce cytokines and phagocytosis of microbial particles. Inflammatory monocytes produce proinflammatory cytokines such as TNF-α and are expanded in certain inflammatory conditions including sepsis, HIV infection [24,25] and HUS (Figure 2A) as we previously described [16,26]. Thus, we analysed CCR1, CCR2 and CCR5 expression per cell in both subsets of $CD14^+$ monocytes: classic and inflammatory ($CD16^-$ and $CD16^+$, respectively). As shown in Figures 2(B) and 2(C), HUS children presented an enhanced expression of CCR1 and CCR2 in inflammatory monocytes whereas CCR5 expression is increased in both subsets. These results suggest that monocyte differentiation into the inflammatory phenotype is accompanied by alterations in chemokine receptor expression in both subpopulations of HUS children during the acute period.

Decreased functionality of chemokine receptors in monocytes from HUS patients

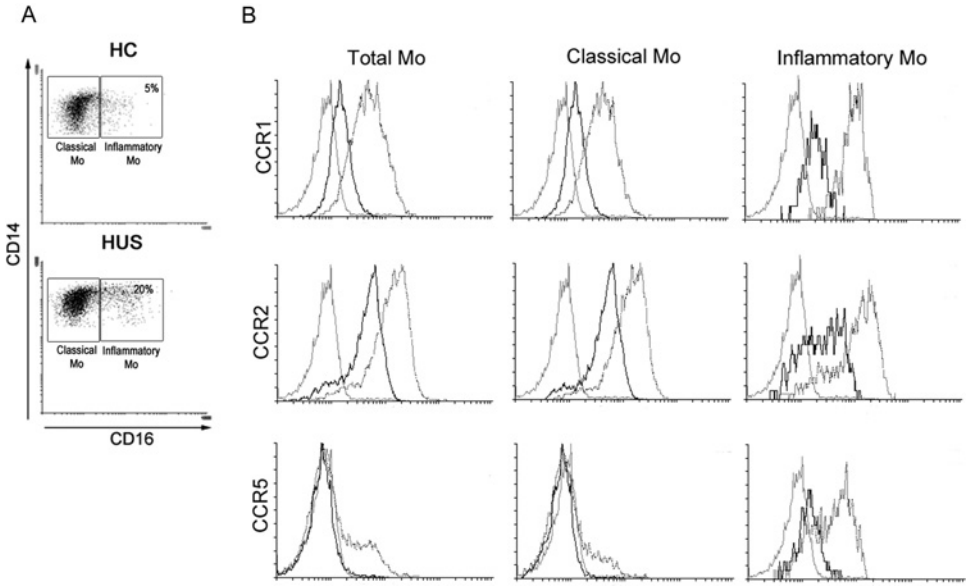
To assess whether the higher CCR expression on monocytes from HUS patients correlates with a higher CCR-dependent functionality, CD11b regulation was investigated after challenge with the chemokines. It has been reported that CD11b, an adhesion molecule essential during chemotaxis and infiltration of monocytes, can be modulated after interaction between chemokines and their receptors [27]. As shown in Figure 3(A), MCP-1 (which targets CCR1/CCR5) and MIP-1α (which targets CCR2) clearly increased the expression of CD11b on monocytes from healthy children, at 10 and 45 min, respectively. In contrast, they had no effect on monocytes from HUS patients. We used PMA as a positive control, and it is interesting that cells from healthy children and HUS patients responded similarly to the PMA challenge (Figure 3). With the consideration that CCR1 and CCR5 were the more altered chemokine receptors, the functional state of these CCRs was analysed by a direct chemotactic assay. For this purpose PBMCs were purified using blood samples from HUS or healthy children, and the capacity of these cells to migrate to CCR1 and CCR5 ligands, MIP-1α and RANTES was analysed. As shown in Figure 3(B), chemotaxis was diminished in HUS patients. Collectively, these data indicate that the increase in the expression of CCR1, CCR2 and CCR5 was associated with a loss of the receptor functionality on chemokine challenge.

Differential chemotactic responsiveness of control monocytes to plasma from healthy or HUS children

We tested the chemotactic activity of plasma from healthy and HUS children towards control monocytes isolated from normal adult volunteers, in transwell migration assays. As shown in Figure 4(A), HUS plasma samples induced increased monocyte chemotaxis compared with plasma from healthy children. When patients were retrospectively segregated according to degree of renal dysfunction, we found that only plasma from severe cases presented greater chemotactic activity. Flow cytometric analysis of migrated monocytes revealed that plasma from HUS patients preferentially induced the migration of classic $CD16^-$ monocytes (Figure 4B).

Plasma chemokine concentration in HUS patients

With consideration of the higher monocyte chemotactic activity induced by plasma from HUS patients, we measured the concentration of the main chemokines for monocytes, MCP-1, MIP-1α and RANTES, in plasma samples from healthy and HUS children. We did not observe significant differences in plasma concentrations of MIP-1α, MCP-1 or RANTES between healthy and HUS patients. However, when patients were retrospectively stratified by severity, the RANTES levels were significantly lower



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Chemokine Receptor		Whole Monocytes		Classical Monocytes		Inflammatory Monocytes	
		HC	HUS	HC	HUS	HC	HUS
CCR1	Median	25	28*	21.9	25.44	18	34.5*#
	IQR	(16.4-31)	(21.7-38.9)	(16-27.2)	(18.4-37.6)	(13-26)	(27.4-48.8)
CCR2	Median	42.5	52.28	41	46.8	27.54#	47*
	IQR	(30.8-68.5)	(38.8-72.6)	(27.2-69.4)	(39.1-67.3)	(18.8-37)	(28.4-65.7)
CCR5	Median	8.4	10.47*	5.5	8.07*	13.1#	16.61*#
	IQR	(6-11.6)	(8-17.6)	(4.2-9.2)	(6.8-13.6)	(10.6-17.7)	(14.4-36.2)

Figure 2 CCR1, CCR2 and CCR5 expression on classic and inflammatory monocyte subsets
(**A**) Representative dot plot of CD14 versus CD16 to identify classic and inflammatory monocytes of one healthy child (HC; $n = 20$) and one HUS patient ($n = 20$). (**B**) Representative histograms of CCR1, CCR2 and CCR5 expression on total monocytes (Mo) (left panel), classic monocytes (middle panel) or inflammatory monocytes (right panel). Healthy children (black line) or HUS (dotted line) expression is shown for each chemokine receptor in each monocyte subpopulation. Grey histograms represent the isotype controls. (**C**) Level of expression per cell of each receptor (CCR1, CCR2 and CCR5) in both subpopulations. Results are expressed as the median with IQR of the mean fluorescence intensity (MFI) values for each chemokine receptor. $^*P < 0.05$ vs same subset from healthy children, $\#P < 0.05$ vs classic monocytes inside healthy children the HUS group. Statistical analyses were performed using the Mann–Whitney U-test.

in patients with severe HUS compared with healthy children or patients with mild HUS (Figure 5).

Correlation of chemokines, chemokine receptors and HUS severity

For further understanding of the co-ordinated interplay between chemokine receptors and chemokines during HUS, Spearman's correlation analysis was performed. As shown in Table 3, CCR1 expression on whole monocyte populations, and also on each monocyte subpopulation, showed a significant positive correlation with the severity of renal dysfunction in HUS patients. In addition, we observed a negative correlation between RANTES concentration and severity of HUS, and a positive correlation with platelet numbers.

Recovered patients

To evaluate the possibility that differences in CCR expression on monocytes between healthy and HUS children were attributable to inherent genetic differences, such as polymorphism variability in chemokine expression, five HUS patients were evaluated after recovery and their CCR expression was compared with that during the HUS acute period. At least 6 months post-HUS, recovered patients did not show increased levels of CCR expression, in contrast to those observed during the HUS acute phase (Figure 6). The absence of significant differences in CCR2 and CCR5 levels between the acute period and after recovery is probably related to the relatively modest number of patients evaluated. Anyway, these results indicate that alterations observed during the acute period did not pre-exist, but were rather the consequence of the inflammatory alterations associated with HUS.

DISCUSSION

Cellular responses to chemokines, which are essential for mounting an effective host defence against pathogens, depend critically

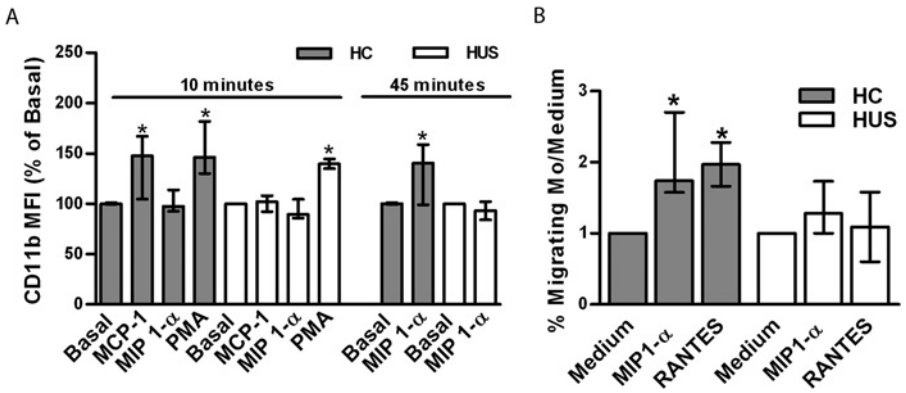

Figure 3 Diminished functionality of chemokine receptors in monocytes from HUS patients
(**A**) Whole blood from healthy children (HC; $n=5$) and HUS patients ($n=3$) were incubated with MCP-1 (100 ng/ml) or MIP-1α (100 ng/ml) for 10 or 45 min at 37 °C, as indicated. PMA (50 ng/ml) was used as positive control of CD11b modulation. CD11b expression per cell (measured as the MFI) is expressed as the median with IQR of the percentage above baseline (i.e. incubation without a chemokine). $^*P<0.05$ vs CD11b basal expression in each clinical group. (**B**) PBMCs (6×10^5) from HUS or healthy children were incubated in the upper compartment of transwell cell culture chambers towards RPMI medium, MIP-1α (100 ng/ml) or RANTES (100 ng/ml) in the lower compartment. After 3 h, migrating monocytes were recovered from the lower compartment, stained with anti-CD14, and counted. Results are expressed as a percentage of migrating monocytes to chemokines/migrating monocytes to medium (median with IQR from three experiments). $^*P<0.05$ vs medium. Statistical analyses were performed using the Kruskal–Wallis and Dunn's tests.

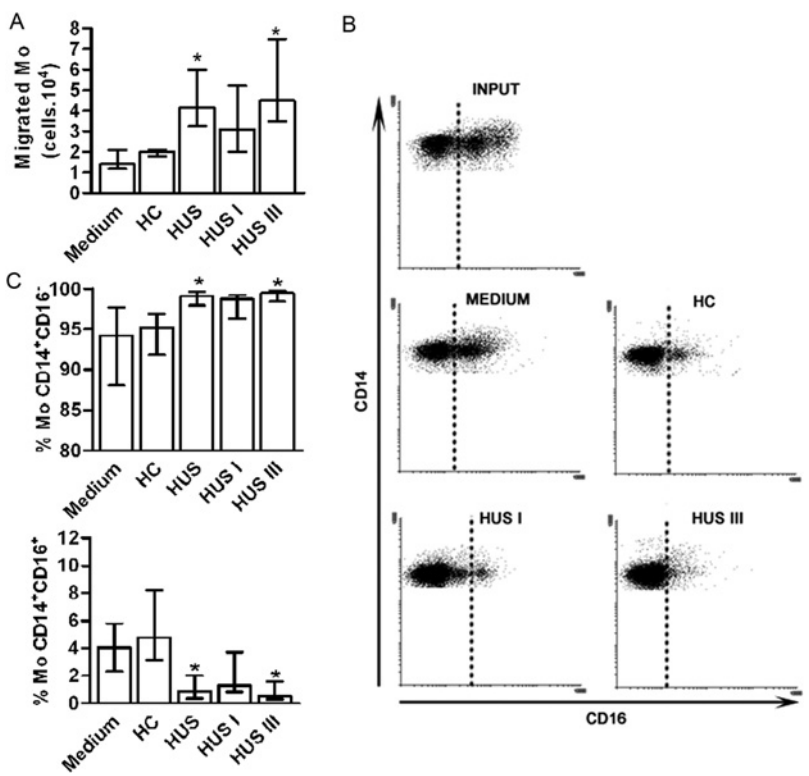

Figure 4 Differential chemotactic responsiveness of control monocytes to plasma from healthy or HUS children
Control monocytes (4×10^5) isolated from normal adult volunteers were incubated in the upper compartment of transwell cell culture chambers. RPMI medium, or plasma from healthy (HC) or HUS children, was placed in the lower compartment. After 3 h, migrating monocytes were recovered from the lower compartment and stained with anti-CD14/anti-CD16 mAbs. (**A**) The absolute number of migrating monocytes was calculated as described in Materials and methods (the median with IQR of three independent experiments). (**B**) A representative dot plot of CD14 versus CD16 to identify both monocyte subsets before (input) and after chemotaxis (lower chamber) induced by medium or plasma. (**C**) The percentage of each subset was calculated inside migrating monocytes. The results are expressed as the median with the IQR. $^*P<0.05$ vs healthy children. Statistical analyses were performed using the Kruskal–Wallis and Dunn's tests.

Table 3 Correlation between plasmatic chemokine concentrations or the expression of CCRs by peripheral Monocytes and clinical parameters of HUS patients
Data were analyzed with nonparametric Spearman rank-correlation test and Pearson's coefficients (r) are shown.

	Chemokine Concentrations				Chemokine Receptors		
	RANTES	MCP-1	MIP 1α		CCR1	CCR2	CCR5
Severity	−0.731*	0.024	−0.241	Whole Monocytes	0.520*	0.340	−0.055
				$CD16^-$	0.514*	0.365	−0.175
				$CD16^+$	0.465*	0.306	−0.011
Platelet numbers	0.518*	−0.075	0.212	Whole Monocytes	−0.172	−0.323	0.212
				$CD16^-$	−0.138	−0.294	0.374
				$CD16^+$	−0.141	−0.220	0.338

*$P < 0.05$ was considered significant.

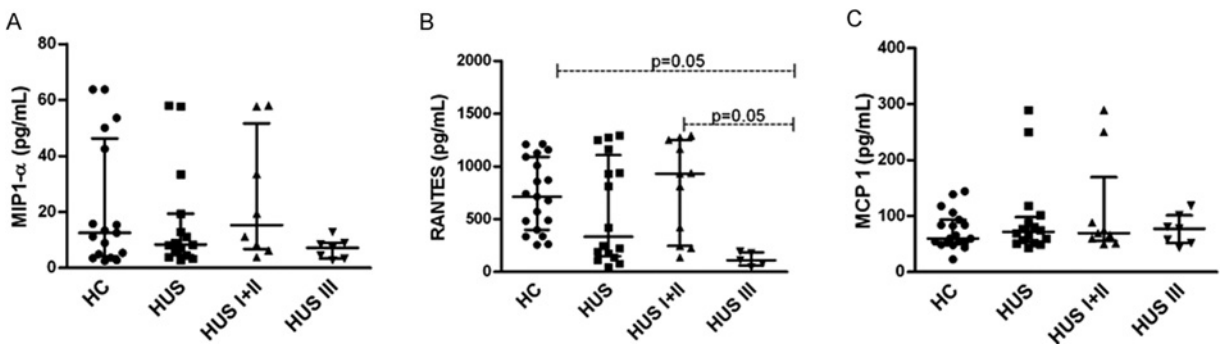

Figure 5 Chemokine concentration in plasma
Levels of (**A**) MIP-1α, (**B**) RANTES and (**C**) MCP-1 (expressed as picograms per millilitre, median with IQR) were measured in plasma from healthy (HC; $n = 16$) and HUS ($n = 16$) children, as indicated in the section Materials and methods. Statistical analyses were performed using the Kruskal–Wallis and Dunn's tests.

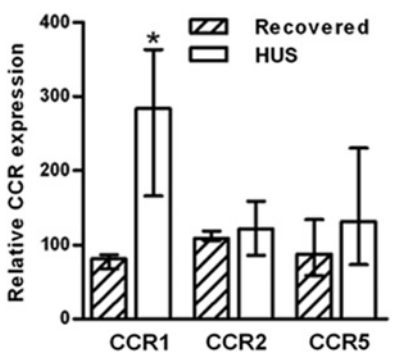

Figure 6 CCR expression in recovered HUS patients
Chemokine receptors were evaluated in peripheral monocytes from the same children ($n = 5$) during the HUS acute period (white bars) and after at least 6 months (hatched bars). Results are expressed as the median with IQR of the MFI of CCR1, CCR2 and CCR5 relative to the corresponding CCR expression in the healthy children assayed in parallel on the same day. *$P < 0.05$ compared with CCR1 expression after recovery. Statistical analyses were performed using the Mann–Whitney U-test.

on the expression of functional chemokine receptors on the surface of inflammatory cells. Our results show a marked dysregulation in the expression of chemokine receptors in monocytes and chemokine responsiveness in children with HUS, which is more pronounced in the subgroup of patients with a poor outcome. The correlation between the increase in CCR expression on monocytes and the severity of the HUS suggests that dysregulation of chemokine receptors might contribute to the risk of renal damage.

Previous reports have shown an increase in monocyte- and/or neutrophil-activating factors [28], and in the levels of the inflammatory cytokines interleukin (IL)-6 and IL-8, and the anti-inflammatory cytokines IL-10 and IL-1Ra [29–31] preceding the expansion of the inflammatory monocyte subset in HUS patients [16,26]. These results suggest an autocrine feedback loop that leads to the emergent expansion and activation of this subset under inflammatory conditions. Thus, the enhanced expression of all CCRs in the inflammatory monocyte subset, and CCR5 in the classic one, may be consistent with features of cytokine-preactivated or more differentiated monocytes. In this regard, it has been reported that CCR5 is up-regulated during maturation of monocytes in adherent culture [32], further emphasizing the growing complexity of the regulation of the chemokine receptor in monocytes.

Our results underscore the disparity between chemokine receptor expression and function. Although the levels of inflammatory CCRs are significantly higher on monocytes from patients with HUS, the specific biological response seems to be impaired. Only when receptor function is evaluated does the alteration of chemokine receptor become apparent, with monocytes being almost completely unresponsive to chemokine ligands, but not to a non-receptor-dependent stimulus such as PMA. This may be a consequence of immunoreactive but biologically inactive

cell-surface receptors. Alternatively, it could be caused by a general hyporesponsiveness of circulating monocytes from HUS patients rather than a specific impairment of chemokine receptor functionality. The high levels of circulating cytokines and chemokines described in HUS patients during the acute phase [28–31] may lead to the loss of chemokine receptor function in circulating monocytes via cross-desensitization. In this regard, it has been reported that the expression of CCR1, CCR2 and CCR5 in monocytes exposed to microbial agents or proinflammatory cytokines can be up-regulated by IL-10. However, these receptors, which are retained on monocyte cell surfaces, are uncoupled from the signal transduction pathway and unable to elicit migration. In contrast they would act as scavengers for inflammatory chemokines [33]. Thus, within an inflammatory microenvironment, IL-10 generates functional decoy receptors that act as molecular sinks and scavengers for inflammatory chemokines. With the consideration that IL-10 is also elevated in HUS patients, such a scenario could be possible.

Besides, we found a positive correlation between the level of expression of CCR1 in monocytes and severity of HUS. It is worth noting that, in the mouse model of HUS by Stx2 intoxication, knocking out CCR1 significantly improves survival, attenuates neutrophilia and monocytosis, and reduces renal damage and renal monocyte infiltration [15]. In addition, the increase of the inflammatory cytokines TNF-α and IL-6 in plasma was delayed in CCR1$^{-/-}$ mice compared with control mice, suggesting that CCR1 participates in cell recruitment to the kidney and in the amplification of the inflammatory response that contributes to HUS development [15]. These findings, together with the present data in HUS patients, point to a critical role for CCR1 and its ligands in renal damage during HUS. However, the initial stimulus that triggers the up-regulation of chemokine receptors is not easily identifiable. The regulation of both chemokine and receptor expression is modulated by a wide range of stimuli, including growth factors [31], cytokines [34], cellular stressors [35], cellular activation by apoptotic bodies [36] and release of cellular debris from necrotic cells [37]. Chemokines function as paracrine signals and in autocrine loops, with both positive and negative feedback elements.

In the present study, we observed that plasma samples from HUS patients have a higher chemotactic activity for monocytes from healthy donors, particularly to the classic subset. However, none of the chemokines evaluated was increased. The promiscuous nature of many chemokines and their receptors suggest the redundancy of these molecules in pathophysiological conditions. In this regard, and because we evaluated the level of only three chemokines, we cannot discard the notion that other chemokines are responsible for the higher chemotactic activity induced by HUS plasma. In addition, several factors could be responsible for our finding of normal values for MIP-1α, MCP-1 and RANTES concentrations in plasma from HUS patients, among them being the time elapsed from the start of STEC infection to the re-collection of blood samples. In fact, MIP-1α has been reported to be transiently enhanced during the acute phase (maximal within the first week) of infection, whereas MCP-1 increases later during the repair phase (from day 21 onwards) [38].

Another possible alternative factor is that circulating chemokines do not always reflect local tissue production. Previous studies on a murine model of HUS have shown increased levels of MCP-1, RANTES and MIP-1α in the kidney [14]. Similarly, it has also been shown that MCP-1 is over-expressed in the kidneys from diabetic animals, and it has become evident that MCP-1 contributes to kidney damage not only by inducing mononuclear cell recruitment, but also by direct activation of resident renal cells [39]. Finally, the absence of an increase in the levels of MCP-1, MIP1-α and RANTES in plasma from HUS patients may be a consequence of the effects of scavenger receptors, as discussed above. In addition, a specific subfamily of silent chemokine receptors has been described, which includes Duffy antigen receptor for chemokines (DARC) [40] and D6 [41]. DARC is expressed by erythrocytes, but also by endothelial cells [42] of the skin, kidneys, lungs, brain, thyroid and spleen, as well as in inflamed tissues and during various kidney diseases. DARC has a promiscuous chemokine-binding profile, interacting with chemokines such as MCP-1 and RANTES [43].

Once chemokines bind to DARC, at least on the erythrocyte surface, they are no longer accessible to other chemokine receptors, this indicates that DARC effectively sequesters chemokines from the tissue microcirculation. The other scavenger receptor, D6, also recognizes and scavenges chemokines usually considered as inflammatory mediators, including agonists for the receptors CCR1 and CCR5 [44]. D6 is weakly expressed in haematopoietic cells, although it is strongly expressed by placenta and endothelial cells lining the afferent lymphatics in certain anatomical sites, such as the skin [45]. Future experiments should be necessary to evaluate the involvement of DARC and D6, as responsible for removing inflammatory chemokines (i.e. MIP1-α, MCP-1 and RANTES) from circulation during HUS.

Another remarkable finding of the present study is the negative correlation between plasma concentrations of CCL5/RANTES, the ligand of CCR1 and CCR5, and the severity of the disease in HUS patients. When considering that platelets are the major reservoir of RANTES [46], we suggest that marked thrombocytopenia in severe HUS patients could lead to a reduction of this chemokine in plasma. Moreover, low levels of RANTES correlate with disease severity and mortality in individuals with severe malaria, Chikungunya fever infection or septic shock, who were also correspondingly thrombocytopenic [47–49].

In conclusion, we have shown a transitory dysregulation of the chemokines/chemokine receptor network in HUS patients, which is associated with disease severity. Although the present study does not define the cellular basis or underlying mechanism for the observed alterations, it supports the immense complexity of these networks during acute renal injury. Our results show that chemokine receptors CCR1, CCR2 and CCR5 are over-expressed on peripheral monocytes, accompanied by loss of functionality. In addition, HUS plasma showed increased monocyte chemotactic activity, although MIP-1α, MCP-1 and RANTES concentrations were conserved. The dysregulation of CCRs and their ligands observed during the HUS acute period suggests that the chemokine pathway is involved in HUS development.

CLINICAL PERSPECTIVES

- Endothelial dysfunction induced by Stx is the main event in the development of HUS. However, the host's inflammatory response is inextricably linked to this phenomenon and plays a pivotal role in the pathogenesis of the disease. During inflammation, endothelial cells produce chemokines that drive the migration of monocytes to injured tissue.
- In the present study, we analysed whether the expression and functionality of the chemokine receptors CCR1, CCR2 and CCR5 on circulating monocytes was altered in HUS patients during the acute period. These receptors were over-expressed on monocytes but were unable to elicit migration, possibly due to cross-desensitization of CCRs induced by circulating cytokines.
- The increase in CCR expression correlates with HUS severity, suggesting that CCR dysregulation might contribute to an increased risk of renal damage. These data could be useful for further therapeutic strategies to block CCR function on activated monocytes in order to modulate the inflammatory response during HUS.

AUTHOR CONTRIBUTION

Maria Victoria Ramos, Matias Ruggieri and Analia Cecilia Panek performed phenotypic studies on circulating monocytes from patients and control groups; Andrea Exeni, Catalina Barilari and Ramon Exeni selected patients and control children, obtained the informed consent from their parents and approval from the ethics committee, collected blood samples, and followed the clinical and renal evolution; Maria Pilar Mejias, Maria Jimena Abrey-Recalde and Romina Jimena Fernandez-Brando collaborated in the evaluation of chemokine receptor functions. Maria Victoria Ramos and Matias Ruggieri performed ELISA for measurements of soluble chemokines; Maria Victoria Ramos co-designed this study with Marina Sandra Palermo and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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