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Original article

Assessing antigen specific HLA-DR+ antibody secreting cell (DR+ASC) responses in whole blood in enteric infections using an ELISPOT technique

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Abstract

Antibody secreting cells (ASCs) generate antibodies in an antigen-specific manner as part of the adaptive immune response to infections, and these cells increase their surface expression of HLA-DR. We have studied this parameter (HLA-DR+ ASC) in patients with recent diarrheal infection using immuno-magnetic cell sorting and an enzyme linked immunospot (ELISPOT) technique that requires only one milliliter of blood. We validated this approach in adult patients with cholera (n = 15) or ETEC diarrhea (n = 30) on days 2, 7 and 30 after showing clinical symptom at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) hospital in Dhaka, and we compared responses to age-matched healthy controls (n = 7). We found that HLA-DR+ ASC (DR+ASC) responses specific both for T cell-dependent (cholera toxin B subunit), and T cell-independent (lipopolysaccharide) antigens were elevated at day 7 after showing clinical cholera symptom. Similarly, DR+ASCs were elevated against both heat-labile toxin and colonization factors following ETEC infection. We observed significant correlations between antigen-specific DR+ASC responses and antigen-specific, gut homing ASC and plasma antibody responses. This study demonstrates that a simple ELISPOT procedure allows determination of antigen-specific ASC responses using a small volume of whole blood following diarrhea. This technique may be particularly useful in studying DR+ASC responses in young children and infants, either following infection or vaccination.

Keywords: HLA-DR+; ASCs; Immune response; Cholera; ETEC; ELISPOT

1. Introduction

Two major causes of dehydrating diarrheal illness, particularly in young children, are *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) infection. Diarrhea kills approximately 2.2 million people globally each year and causes significant economic loss [1]. To prevent this mortality as well as high morbidity, and to address the economic burden imposed by these illnesses, effective vaccines are needed. To improve vaccine efficacy, a broader understanding of immunological protection following natural disease is needed.

Two major virulence factors of *V. cholerae* O1 are cholera toxin (CT), a T cell-dependent antigen and lipopolysaccharide (LPS), a T cell-independent antigen [2]. The major virulence factors for ETEC are the heat-labile (LT) and heat-stable (ST) enterotoxins [3]. Along with the enterotoxins, ETEC express more than 25 different colonization factors (CFs), which can be subdivided into different families, i.e., the colonization factor I (CFA-I)-like group (including CFA/I, CS1, CS2, CS4,

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CS14, and CS17) [4] and the coli surface 5-like group (with CS5, CS7, CS18, and CS20) [5], and those that are unique (CS3, CS6, and CS10 to CS12) [4]. Within each of these families, there are cross-reactive epitopes that have been considered as candidates for vaccine development [6]. CFA/I and CS6 have been recognized to be important CFs of ETEC, and are frequently detected in ETEC from patients with diarrhea [4,7].

Antibody secreting cells (ASCs) seem to play a crucial role following initial exposure to a pathogen at the mucosal surface, and the antibodies produced by these cells correlate with the protective effects of vaccination against mucosal pathogens [8]. Antigen-specific ASCs have been documented in blood after cholera or ETEC infection [2,9], and after oral vaccination [10]. ASCs have been shown to target two key *V. cholerae* antigens after cholera, lipopolysaccharide (LPS) and cholera toxin B subunit (CTB) [2]. In a previous study, we showed that antigen-specific ASC responses could be enriched in the blood of patients following either *V. cholerae* or ETEC infection, using expression of a gut-homing marker (β7) on the cell surface of circulating ASCs [11]. In our current study, we hypothesized that HLA-DR+ ASC (DR+ASC) responses would also reflect mucosal immune responses, but might be measurable using a smaller quantity of blood, and that these DR+ASC would be antigen-specific.

For evaluation of vaccine efficacy targeting enteric pathogens, mucosal immune responses are most pertinent; however, direct sampling of intestinal tissue is not practical, especially in young children. Assessing mucosal responses using mucosally induced ASC responses is now standardly used; however, blood volume issues can also limit the utility of this approach.

HLA-DR expression on the B cell surface is an indication of B cell activation, and almost all of the antigen-specific ASCs in cholera vaccinated individuals are HLA-DR positive [12]. HLA-DR is also involved in activation of T cells, signal transduction, and regulation of antibody production in response to pathogens [13,14]. Identification of DR+ASCs would help to distinguish recently stimulated, antigen-specific ASCs from other antibody secreting cells in either patients or vaccinees [13,14].

2. Materials and methods

2.1. Study groups and recruitment

The study was approved by the Research Review and the Ethical Review Committees of the icddr,b (PR-13013). We enrolled adult patients hospitalized at the Dhaka Hospital of the icddr,b who presented with severe acute watery diarrhea and gave informed consent. All the day points mentioned throughout are after the appearance of clinical symptoms. Patients from whom stool specimens were positive for *V. cholerae* O1 and negative for other enteric pathogens (e.g.: *Shigella*, *Salmonella* and ETEC) were enrolled as cholera patients, while patients with stools positive for ETEC but negative for *V. cholerae* O1 and other enteric pathogens were recruited as ETEC patients [2,9]. Screening of stools for other common enteric pathogens to exclude patients with coinfections was done as previously described [2,9]. Strains isolated from ETEC-infected patients recruited in the study were analyzed for expression of specific colonization factors, including the CFA/I group or CS6, as well as LT, LT/ST or ST alone by dot blot and PCR, respectively [15,16]. Healthy adult individuals, who had no history of diarrhea, fever, or antibiotic use in the two weeks prior to enrollment, were recruited as control participants and were matched by age and socio-economic status to the *V. cholerae*/ETEC infected participants.

2.2. Subjects and sample collection

Heparinized venous blood was collected by venipuncture on days 2 (acute stage), 7 (early convalescence) and 30 (late convalescence) following presentation for care after hospitalization from *V. cholerae* O1 (n = 15; median age 30) and ETEC infected adults (n = 30; median age 35) and at one time point from healthy controls (n = 7; median age 30).

2.3. Plasma separation

One milliliter of blood was collected in sodium heparin tubes. After centrifugation at 600 × *g* for 10 min, plasma was separated, collected in eppendorf tubes, and stored at −20 °C for plasma antibody analysis.

2.4. Preparation of beads coated with anti-human HLA-DR

Sheep anti-mouse IgG coated magnetic beads (Dynabeads; Invitrogen, Norway) were placed into a 9 ml culture tube (Pyrex, USA) containing 3 ml of cold fluorescence activated cell sorter (FACS) buffer. We prepared 25 μl of beads (10^7 beads) to be used for separation of cells from each 1 ml blood sample (see below). The tube was placed in a magnet (DYNAL MPC-6; Norway) for 2 min. The supernatant was aspirated, without moving or detaching the tube from the magnet. The beads were then washed with 3 ml of cold FACS buffer for 2 min and this procedure was repeated twice. After removing the tube from the magnet, beads were re-suspended in FACS buffer in an initial volume of 25 μl for each blood sample to be analyzed. Mouse anti-human HLA-DR (BD Pharmingen, USA) was added and incubated for 30 min at 2–8 °C. Finally, the beads coated with anti-human HLA-DR were added to mononuclear cells prepared as below.

2.5. Cell preparation

After separation of plasma from each 1 ml blood sample, the remaining fraction containing the cells was transferred into a Falcon tube (BD, USA) and mixed with 30 ml lysing solution (1 M NH_4Cl solution) for lysis of red blood cells. The mixture was incubated for 7 min at room temperature and then centrifuged. The supernatant was removed and the pellet was re-suspended immediately to the initial blood volume (1 ml) in

FACS buffer. The cells were washed twice and were re-suspended in 1 ml FACS buffer and transferred to freshly prepared beads coated with anti-human HLA-DR in a glass tube as above. The cells and beads were incubated at 4° C for 45 min on a horizontal shaker. Tubes were then placed into the magnet and the supernatant was aspirated. Cells were re-suspended in RPMI complete medium (Gibco, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). After counting, bead-separated mononuclear cells were plated onto an ELISPOT plate for determination of total immunoglobulin and antigen-specific spots to ETEC and *V. cholerae* antigens (see below). Depending on the retrieval of cells, 2500–5000 cells were used for determining total immunoglobulin spots and 15,000–20,000 cells were used for detecting antigen-specific spots; duplicate wells were used for total immunoglobulin spots while a single well was used for antigen specific spots.

2.6. ELISPOT assays

For antigen-specific ASC enumerations, ELISPOT wells previously coated with specific antigens (i.e. *V. cholerae* O1 lipopolysaccharide (LPS); 25 μg/ml, cholera toxin B subunit (CTB); 2.5 μg/ml and CFA/I (10 μg/ml) or CS6 (10 μg/ml) colonization factor antigens) were prepared. Similarly, total immunoglobulin-secreting cells (ISCs) irrespective of antigen specificity were enumerated in parallel wells coated with a mixture of affinity-purified goat antibodies to human Ig k and λ light chains [9]. The use of an HRP dependent two color (AEC, BCIP/NBT substrate) ELISPOT procedure for detection has been previously described [17]. Spots were counted manually under a stereomicroscope. Antigen-specific immune responses of patients were calculated as mean + 3SD immune responses of healthy control participants.

2.7. Detection of CTB specific antibodies in plasma

Recombinant cholera toxin B subunit (rCTB)-specific IgA and IgG antibodies in patients infected with ETEC or *V. cholerae* were measured in plasma using a standardized enzyme-linked immunosorbent assay (ELISA) technique, as previously described [18]. As LTB has 80% nucleotide sequence homology and cross-reacts immunologically with CTB [9], plasma responses to LT in ETEC-infected patients were assessed using CTB as the antigen.

2.8. Data calculation

The antigen-specific response was expressed as a spot forming unit (SFU) defined as antigen specific spot found in ELISPOT for 1 ml of whole blood. For correlation, the percentage response was calculated as the number of antigen-specific DR+ASCs divided by the total HLA-DR+ immunoglobulin secreting cells (ISC) of that same antibody isotype; both total and antigen-specific ISC/ASC numbers were first expressed as per 10^6 bead separated PBMCs prior to percentage calculation.

2.9. Statistical analysis

For analyses of ASCs at different time points following infection, multiple comparisons have been performed using the Kruskal–Wallis test with Dunn's post hoc test to analyze differences between individual study groups. The association between ASC and plasma antibody responses was assessed using the Spearman correlation coefficient. We used Graph Pad Prism 5.0 for statistical analyses and generating figures, and considered $p < 0.05$ as significant.

3. Results

3.1. Demographic characteristics of patients

In this study, we enrolled 15 adult patients with cholera and 30 adults infected with ETEC. In addition, we enrolled 7 age-matched healthy controls. All cholera patients recruited for the study were infected with *V. cholerae* O1 Ogawa serotype. Of the patients infected with ETEC, 36.7% (n = 11), 26.6% (n = 8) and 36.7% (n = 11) were infected with LT-, LT/ST-, or ST-expressing ETEC (Table 1), respectively. The majority of the patients were infected with ETEC expressing either CFA/I group CFs (n = 11, 36.7%) or CS6 with or without CS5 (n = 7, 23.3%). Most of the ETEC strains expressing CFA/I group CFs, or CS6 with or without CS5, produced either LT or both LT and ST (81.9% and 85.7%). The demographic, clinical and microbiological characteristics of patients are presented in Table 1.

3.2. Total immunoglobulin responses

Total immunoglobulin responses were reported as total spot forming units (TSFU) defined as isotype-specific HLA-DR+ ASC spot per ml of whole blood at different days after showing the clinical symptoms of diarrheal illness. In patients with cholera, we found a significant increase in total HLA-DR+ IgA immunoglobulin secreting cells (ISCs) at day 7 compared to day 2 ($p < 0.01$) and in healthy controls ($p < 0.05$), and the number of total HLA-DR+ IgA ISCs subsequently decreased from day 7 to day 30 ($p < 0.05$) (Table 2). A similar increase was noted for HLA-DR+ IgG TSFU at day 7 compared to day 2 ($p < 0.01$) and to healthy controls ($p < 0.05$) and a decline from day 7 to day 30 ($p < 0.05$). Similar to cholera patients, patients infected with ETEC also had a significant increase in HLA-DR+ IgA TSFU at day 7 after showing clinical symptom compared to day 2 ($p < 0.05$) and healthy controls ($p < 0.05$), and a decline from day 7 to day 30 ($p < 0.05$) (Table 2). HLA-DR+ IgG TSFU in patients infected with ETEC followed a similar pattern to that of total DR+ IgA ISCs (Table 2). The mean values of total HLA-DR+ IgG were higher compared to total HLA-DR+ IgA in both patient groups (Table 2).

3.3. Antigen specific responses

Antigen specific responses were measured as spot forming units (SFU) defined as antigen-specific spots found in

Table 1
Demographic characteristics of healthy controls (HC), and *Vibrio cholerae* and ETEC-infected patients.

Characteristics	HC	*V. cholerae*-infected	ETEC-infected
No. of study participants	7	15	30
Age (median [25th, 75th percentile]), yr	30 (22,35)	30 (24, 37)	35 (26, 40)
Female gender (no. [%])	5 (71.4)	4 (27)	15 (50)
Duration of diarrhea (mean [SD]), h	N/A	24.1 (18.6)	26.4 (28.2)
Blood group (n [%])			
A	1 (14.3)	3 (20.0)	9 (30.0)
B	5 (71.4)	7 (46.7)	13 (43.4)
O	1 (14.3)	4 (26.7)	4 (13.3)
AB	0 (0)	1 (6.6)	4 (13.3)
Toxin types of ETEC strains (no [%])	N/A	N/A	30
LT/ST	N/A	N/A	8 (26.6)
LT	N/A	N/A	11 (36.7)
ST			11 (36.7)
Colonization factors of ETEC strains (no [%])			30
CFA/I group CS1 + CS3 + CS21, CS2 + CS3, CFA/I + CS21, CS14	N/A	N/A	11 (3,3,1, 4)
CS6 (CS6 only, CS5 + CS6)	N/A	N/A	7 (3,4)
CS7	N/A	N/A	1
CF negative	N/A	N/A	11

Table 2
Spot Forming Unit for total HLA-DR+ immunoglobulin secreting cells (ISCs) and antigen-specific HLA-DR+ ASCs after appearance of clinical symptom following cholera and ETEC infection.

Patients	Mean spot forming[a] IgA (SEM)				Mean spot forming[a] IgG (SEM)			
Antigen	HC[b]	Day 2	Day 7	Day 30	HC	Day 2	Day 7	Day 30
Cholera								
Total[c]	831.9 (435.5)	653.5 (260.9)	1700 (480.6)*#$	562.9 (119.8)	1580 (1093)	916.1 (382.6)	2116 (427.7)*#$	793.5 (89)
CTB[d]	2.3 (1.6)	2 (1.2)	95.9 (37.8)*#$	0.0 (0)	5 (8.3)	2 (1.4)	397.7 (131.9)*#$	4.1 (1.9)
LPS[d]	0.7 (0.7)	1.6 (1.3)	619.6 (162.9)*#$	0.3 (0.3)	2.7 (1.8)	3.6 (2)	199.5 (73.6)*#$	5.6 (3.6)
ETEC								
Total[c]	471 (99.7)	753.9 (753.9)	1506 (316.3)*#$	750.5 (750.5)	482.4 (163.3)	876.7 (266.5, 26, 6826)	1907 (430.2)*	1096 (226.6)
CTB[d]	2.3 (1.6)	0.9 (0.5)	40.7 (23.7)*$	0 (0)	4.7 (2.9)	1 (0.6, 0, 8.5)	93.9 (46.7)*#$	2.3 (1.7)
CFA/I[d]	0.43 (0.43)	2.7 (2.3)	44.95 (19.3)*$	0.9 (0.9)	2.1 (2.1)	1.2 (0.7, 0, 8.5)	21 (9.6)	2.8 (1.3)
CS6[d]	0.4 (0.4)	ND[e]	391.5 (203.9)*$	0 (0)	3.6 (2.4)	ND[e]	268.1 (150)	6 (4.9)

Statistically significant differences ($p < 0.05$) between patients at day 7 with other time points of patients (day 2# and day 30$) and with healthy controls*. Statistical analysis was performed using the Kruskal–Wallis test and the main p values for all individual antigen groups were <0.05. Dunn's post hoc test was used to analyze differences between individual study groups.

[a] Spot forming unit defined as isotype specific spot detected on ELISPOT per ml of blood.
[b] HC, healthy controls.
[c] Total HLA-DR+ isotope specific spot per ml of blood.
[d] Antigen specific HLA-DR+ spot count per ml of blood.
[e] ND, the assay was not done on that day point.

ELISPOT using 1 ml of whole blood. The majority of the patients showed an antigen-specific immune response above the cut off value (defined as mean + 3SD the response of healthy controls) (Table 3). For *V. cholerae* infection, 86.7% and 93.3% of patients developed an IgA DR+ASC response to CTB and LPS, while 86.7% and 80% developed an IgG DR+ASC response for CTB and LPS by day 7, respectively (Table 3). For ETEC infection, 71.4% of patients developed both an IgA and IgG isotype DR+ASC response against CS6, and more than 50% developed a response against CTB (Table 3). 66.6% of patients developed an IgG DR+ASC response to CFA/I, although only 54.5% developed an IgA DR+ASC response above the cutoff.

Table 3
Percentage of DR−ASC responders to different antigens and of different isotypes on day 7 after appearance of clinical symptom in patients infected with *V. cholerae* and ETEC.

Antigen	*V. cholerae*		ETEC		
	CTB (n = 15)	LPS (n = 15)	LT (n = 19)	CFA/I (n = 11)	CS6 (n = 7)
IgA	86.7	93.3	45.5	54.5	71.4
IgG	86.7	80	59.0	66.6	71.4

Healthy control value (mean + 3SD) was used as cut off.

3.3.1. Cholera antigen-specific responses

Antigen-specific ELISPOTs were also performed both from unfractionated blood and from HLA-DR enriched samples,

showing similar results but the requirement for a much smaller volume of blood using enriched samples, so we only present results for that analysis. We assessed quantitative responses to both *V. cholerae* CTB and LPS. We detected antigen-specific HLA-DR+ antibody secreting cell (DR+ASC) IgA and IgG responses to both antigens in cholera patients. Analysis of CTB-specific ELISPOT responses showed that the spot forming unit (SFU) of CTB-specific IgA DR+ASCs was higher at day 7 compared to day 2 ($p < 0.001$) and in healthy controls ($p < 0.01$), and the SFU of CTB-specific IgA DR+ASCs were lower in day 30 compared to day 7 ($p < 0.001$) (Table 2). For IgG responses, a similar pattern was observed; the SFU of CTB specific ASCs was higher at day 7 compared to day 2 ($p < 0.001$) and day 30 ($p < 0.001$), and higher than in healthy controls ($p < 0.01$) (Table 2). The mean SFU of CTB specific IgG DR+ASCs (397.7) of total IgG DR+ASCs was four time higher for IgA DR+ASCs (95.9) (Table 2).

LPS-specific DR+ASC responses followed a similar pattern. Specifically, *V. cholerae*-infected patients had higher levels of IgA DR+ASCs on day 7 after showing clinical symptom compared to day 2 ($p < 0.001$) or day 30 ($p < 0.001$) and to the levels in healthy controls ($p < 0.001$) (Table 2). Similarly, LPS-specific IgG DR+ASCs were significantly elevated on day 7 compared to day 2 ($p < 0.001$), and day 30 ($p < 0.001$), and in healthy controls ($p < 0.01$) (Table 2). Unlike CTB-specific DR+ASCs, the mean SFU of LPS-specific IgA DR+ASCs (619.6) was more than threefold higher than the IgG isotype (199.5) at day 7 after appearance of clinical symptom (Table 2).

3.3.2. ETEC antigen-specific responses

To study specific responses in ETEC patients, we used three different antigens: CTB as a homolog of LTB, CFA/I, and CS6 antigen. Although the mean SFU of CTB-specific DR+ASCs (of total DR+ASCs) was lower than for *V. cholerae*-infected patients, a similar response pattern was observed for the ETEC-infected patients. The frequency of CTB-specific IgA DR+ASCs was higher at day 7 compared to day 2 ($p < 0.05$) and day 30 ($p < 0.001$), and in healthy controls ($p < 0.05$) (Table 2). For the IgG isotype, a similar pattern was observed; the SFU of CTB-specific DR+ASCs was elevated at day 7 compared to day 2 ($p < 0.001$) and day 30 ($p < 0.001$) and to that in healthy controls ($p < 0.05$) (Table 2). The mean SFU of IgG isotype DR+ASCs was more than two-fold higher than of IgA DR+ASCs against CTB at day 7 (Table 2).

We also assessed immune responses using a CFA/I-specific ELISPOT assay for patients infected with ETEC expressing the CFA/I group CFs, and a CS6-specific ELISPOT assay for patients infected with ETEC expressing CS6 alone or together with CS5. We found significantly higher CFA/I-specific responses on day 7 compared to day 2 and day 30 in patients infected with CFA/I positive ETEC and compared to in healthy controls, both for IgA and IgG isotype HLA-DR+ASCs (Table 2). We also detected significant DR+ASC immune responses to CS6 in patients infected with CS6-expressing ETEC. The frequencies of CS6-specific DR+ASCs, both for IgA and IgG isotypes, were higher at day 7 compared to day 30 and to healthy controls (Table 2). We did not analyze day 2 specimens by the CS6 specific ELISPOT assay as we had limited CS6 antigen. No CFA/I-specific DR+ASCs responses were observed in patients infected with CF-negative ETEC strains (n = 11) (not shown).

Table 4
Association of frequencies of DR−ASCs with plasma antibodies in cholera and ETEC patients.

	Cholera patients, CTB (n = 15)				ETEC patients, CTB (LTB) (n = 19)			
	Day 7		Day 30		Day 7		Day 30	
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
Spearman, r	0.63	0.63	0.65	0.86	0.46	0.56	0.39	0.37
p value	0.01	0.01	0.01	<0.0001	0.03	0.007	0.07	0.09

3.4. Association of DR+ASCs with antibody responses in plasma

To determine a potential association between DR+ASC and antibody responses in plasma, we compared CTB-specific IgA and IgG DR+ASC responses at day 7 with corresponding antibody responses in plasma at day 7 and day 30 in patients infected with *V. cholerae* O1 (Table 4) or ETEC expressing LT (Table 4) or LT/ST. We found strong positive correlations between CTB-specific IgA and IgG DR+ASC responses on day 7 with CTB-specific IgA and IgG antibody responses, both on day 7 and on day 30 in cholera patients (Spearman, r = 0.63 to 0.86, $p = <0.0001$ to 0.01) (Table 4). Similarly, in ETEC-infected patients, the frequencies of CTB-specific DR+ASCs, both in IgA and IgG, on day 7 correlated significantly with CTB-specific IgA and IgG ELISA titers in plasma at day 7 (Spearman, r = 0.46 to 0.56, $p = 0.007$ to 0.03) (Table 4; Supplementary Fig. 1); although DR+ASCs at day 7 and ELISA titers on day 30 were related (Spearman, r = 0.37 to 0.39), the association did not reach significance ($p = 0.07$ to 0.09) (Table 4).

3.5. Association of DR+ASCs with gut homing (β7+) ASCs responses following infection

We also assessed the association between DR+ASCs with β7+ gut homing ASCs [11] for pathogen-specific antigens for both *V. cholerae* O1 and ETEC-infected patients at 7 days after hospitalization. In patients infected with *V. cholerae*, CTB-specific DR+ASCs showed a strong positive correlation with the gut homing ASCs specific for CTB, for both IgA (Spearman, r = 0.65; $p = 0.009$) and IgG isotypes (Spearman, r = 0.67; $p = 0.007$) (Table 5, Supplementary Fig. 2A and B). Similarly, LPS-specific DR+ASCs showed significant positive correlations with gut homing ASCs to *V. cholerae* LPS (Spearman, r = 0.51 to 0.73 and $p = 0.05$ to 0.002) (Table 5, Supplementary Fig. 2C and D).

In ETEC-infected patients, CTB-specific DR+ASCs correlated significantly with CTB-specific gut homing IgA (Spearman, r = 0.56; $p = 0.01$) as well as IgG ASCs (Spearman, r = 0.67; $p = 0.002$) (Table 5, Supplementary

Table 5
Association of frequencies of DR−ASCs with frequencies of β7+ ASCs in cholera and ETEC patients at 7 days after hospitalization.

	Cholera patients				ETEC patients					
	CTB (n = 15)		LPS (n = 15)		CTB (LTB) (n = 19)		CFA-I (n = 11)		CS6 (n = 7)	
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
Spearman, r	0.65	0.67	0.73	0.51	0.56	0.67	0.9	0.71	0.6	0.26
p value	0.009	0.007	0.002	0.05	0.01	0.002	0.0002	0.01	0.2	0.6

Fig. 3A and B). When analyzing 11 patients for a possible association between CFA/I group specific DR+ASCs and gut homing ASCs, we found significant positive correlations between these two groups of ASCs, both for IgA (Spearman, r = 0.9 and $p = 0.0002$) and IgG ASCs (Spearman, r = 0.71 and $p = 0.01$) (Table 5, Supplementary Fig. 3C and D). For patients infected with CS6-expressing ETEC, perhaps because of the small sample size (n = 6), we did not confirm a correlation for IgA (Spearman, r = 0.6 and $p = 0.2$) and IgG ASCs (Spearman, r = 0.26 and $p = 0.6$) (Table 5, Supplementary Fig. 3E and F).

4. Discussion

Despite the availability of simple and widely accessible oral rehydration treatment, young children and adults remain vulnerable to the extreme dehydration of severe diarrhea in low and middle income countries. Although the establishment of adequate personal hygiene, food and water safety and good sanitation can help to control these diarrheal episodes, poor socio-economic conditions present in developing countries still leads to acute infectious diarrhea that causes approximately 94,000 deaths annually from cholera [1,19], with all-cause infectious diarrhea accounting for approximately 20% of all childhood deaths [20]. Two major bacterial causes of severe acute watery diarrhea in developing countries are *V. cholerae* O1 and ETEC [4]. Individuals in developed countries are also at risk of these infections when they travel to endemic areas, and the organisms can be readily imported to the industrialized world [21,22].

The host immune system develops adaptive immune responses, particularly via the ASC response [2], against CT and LPS of *V. cholerae,* which make these antigens major targets for effective vaccine design [2]. LTB-specific immune responses, measured here with CTB, also occur in ETEC-infected patients with strains expressing LT toxin because of the homology between LTB and CTB [9,10]. ETEC infection stimulates immune responses to both homologous and cross-reacting colonization factors [9], through different arms of the immune system such as antibody production, ASCs, memory B cells and highly avid antibody responses [9,23,24]. Substantial mucosal and systemic immune responses also develop against CS6 in humans infected with ETEC expressing CS6, with or without CS4 or CS5 [25].

In this study, we confirmed that total DR+ASCs of both IgA and IgG isotypes are significantly elevated in both cholera and ETEC-infected patients on day 7 following presentation for medical care. Antibody levels in the serum correlate with the number of antigen-specific ASCs of that specificity and isotype [26]. Significant correlation exists between circulating LPS-specific IgA ASCs on day 7 and IgA antibody levels to LPS in mucosal extracts [27]. We have recently reported that CTB-specific gut homing ASCs of both IgA and IgG isotype significantly correlate with serum levels of antibodies of the same specificity and isotype in patients with cholera and ETEC infection [11]. To similarly understand the association of DR+ASCs with plasma antibodies, we measured CTB- specific antibody titers in plasma of cholera and ETEC patient, and here showed significant correlations between DR+ASCs in circulation and plasma antibody titers of the same isotype. Therefore, circulating DR+ASCs could be used as a reasonable marker of the early humoral immune response to infection or vaccination following intestinal activation. Induction of HLA-DR+ B lymphocytes targeting LPS would also be consistent with involvement of T cells in B cell maturation during cholera, including to normally T cell independent antigens. Whether HLA-DR+ B cells targeting purified O-specific polysaccharide are induced during cholera is currently unclear.

Mucosal immune responses are essential for mediating protection against cholera and ETEC infection [12,28]. We have previously reported that circulating β7+ASCs are abundant following infection with enteric pathogens like *V. cholerae* and ETEC [11]. In this study, we analyzed the association of gut homing ASCs with DR+ASCs and found significant correlations between them. One of the limitation of this study was to determine the β7+ASC and DR+ASC cells present in the gut directly that would help directly to draw a correlation between β7+ASC and DR+ASC cells present in the gut and in blood and ultimately give an understanding as measureable marker for gut immunity.

In resource-limited settings, the volume of blood available for immunological analyses, particularly in younger children, is often a crucial limitation [29]. Current approaches to measuring circulating ASCs require a relatively large volume of blood (3–5 ml), and this volume may be even larger depending on the number of antigen-specific responses to be measured [27]. The volume of blood required for ASC measurement can be lowered by enriching for specific ASCs using flow cytometry-based technique, but this is more expensive and requires more technology [30]. The findings of the current study suggest that enriching for cells using the HLA-DR surface marker could facilitate assessment of antigen-specific ASCs using a much smaller blood volume, and permits use of a marker of recent stimulation to help discern active or recent infection from background circulating lymphocytes, an advantage over the β7+ enrichment approach.

We did not have sufficient clinical follow up data on these patients to know if clinical outcomes correlate with HLA-DR+ ASC responses on day 7. Because of the number of antigens examined using both IgA and IgG for antigen-specific responses from such a small volume of blood, we were not able to do those assays in duplicate, which will be important in future studies to assess variability.

We could not able to confirm about colonization factors for ETEC patients by day 2 after hospitalization, therefore, we have not carried out ETEC specific antigen immune responses at day 2 except CFA/I. However, we have overcome this situation by including day 0 specimens from healthy participants and compared their immune responses with other day points. Antibody responses has also been measured at acute stage of infection and compared with early convalescence and decreased antibody responses has been noticed at day 2 in compared to day 7 after showing clinical symptom of ETEC infected patients (Supplementary Fig. 1). Moreover, RBC lysis should be optimum to get comparable results like traditional ASC assay. We have already shown that the antibodies in culture supernatants from regular separation of PBMCs and RBC lysis gave comparable results [31]. Therefore, this critical step should be carefully handled during the time of assay.

In summary, this study demonstrates that total as well as antigen-specific circulating HLA-DR+ ASCs can be detected using a small volume of blood after cholera and ETEC infection, and confirms that antigen-specific cells detected in this manner are elevated on day 7 following infection. Measurement of DR+ASC is a good parameter for assessing the early humoral immune responses after infection or vaccination, and correlates with β7+ASCs homing to mucosal surfaces. Utilizing measurement of DR+ASCs in oral vaccine studies may allow determination of antigen specific immune responses following vaccination, without requiring a substantial blood volume.

Conflicts of interest statement

There were no conflicts of interest of any of the authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.micinf.2017.10.005.

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