

RESEARCH ARTICLE

Rotavirus intestinal infection induces an oral mucosa cytokine response

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Abstract

Introduction

Salivary glands are known immune effector sites and considered to be part of the whole mucosal immune system. The aim of the present study was to assess the salivary immune response to rotavirus (RV) infection through the analysis of the cytokine immune profile in saliva.

Material and methods

A prospective comparative study of serial saliva samples from 27 RV-infected patients (sampled upon admission to the hospital during acute phase and at convalescence—i.e. at least three months after recovery) and 36 healthy controls was performed. Concentrations of 11 salivary cytokines (IFN- γ , IFN- α_2 , IL-1 β , IL-6, IL-8, IL-10, IL-15, IL12_{p70}, TNF- α , IFN- λ_1 , IL-22) were determined. Cytokine levels were compared between healthy controls acute infection and convalescence. The correlation between clinical data and salivary cytokine profile in infected children was assessed.

Results

The salivary cytokine profile changes significantly in response to acute RV infection. In RV-infected patients, IL-22 levels were increased in the acute phase with respect to convalescence (P -value < 0.001). Comparisons between infected and control group showed significant differences in salivary IFN- α_2 , IL-1 β , IL-6, IL-8, IL-10 and IL-22. Although acute-phase levels of IL-12, IL-10, IL-6 and IFN- γ showed nominal association with Vesikari's severity, this trend did not reach statistical significance after multiple test adjustment.

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Conclusions

RV infection induces a host salivary immune response, indicating that immune mucosal response to RV infection is not confined to the intestinal mucosa. Our data point to a whole mucosal implication in the RV infection as a result of the integrative mucosal immune response, and suggest the salivary gland as effector site for RV infection.

Introduction

Epithelial cells are more than mere physical barriers to infection; they also have a central role in immune responses and are key in tissue remodeling after healing [1]. Oral mucosa epithelial cells, located at the interface between the external and internal environment, are routinely exposed to large amounts of substances, including pathogens. Several studies have revealed that the oral epithelium—like all other epithelia—, is highly dynamic and displays a broad spectrum of activities related to immunity and host defense [2,3]. The major secretion associated with the oral mucosa epithelium is saliva, produced by the salivary glands and secreted to the oral cavity through the oral mucosa. Among the substances produced in saliva are cytokines [4], soluble regulating agents of immune responses, secreted by epithelial cells in response to diverse stimuli such as injury, infection or inflammation [5].

Saliva from the sublingual compartment has demonstrated to be an excellent non-invasive proxy for intestinal immune induction [6,7]. Induction of mucosal immunity in the intestinal Peyer's patches, results in an effector response at distant sites such as the oral mucosa, functionally based on the compartmentalized mucosal immune system [7,8]. This fact has long been demonstrated with IgA and plasma cells [9–12], but, to the best of our knowledge, never before with the induction of an epithelial cytokine response. Until now, studies had only shown an oral epithelial cytokine response to local inflammation or infections of the mouth such as periodontitis [13–15], or in autoimmune diseases affecting oral mucosa such as Sjögren disease [16].

Here, we hypothesized that RV intestinal infection induces in the host a complete cytokine response at the oral mucosa epithelial cells, which can be measured in the sub-lingual compartment. If true, this would constitute supportive evidence to the suggestion that RV infection induces a whole mucosal immune response in locations as distant as the oral mucosa.

Methods and material

Patients and controls

A total of 27 RV infected patients admitted to hospital and 36 healthy children scheduled to receive RV vaccination were enrolled in this study. Written consent from parents was obtained for all subjects involved in this study. Approval for this project was obtained from the Ethics Committee for Clinical Research of Galicia before patients and healthy controls were recruited.

RV-infected patients were prospectively recruited at the Hospital Clínico Universitario of Santiago de Compostela (Spain) during the period 2013–2014, all of them hospitalized with acute gastroenteritis and with a positive RV antigen detected in stool. For the RV-infected group, demographic and clinical data were obtained, including detailed symptoms scores during the course of illness such as temperature, number of vomiting episodes per day and duration of vomiting, the severity of diarrhea (number of stools per day, duration of diarrhea and level of dehydration), and length of stay in hospital, as well as Vesikari's severity score.

In the same period, 36 healthy children who attended to the infectology consultation for scheduled rotavirus vaccination were enrolled in the study.

Samples

Saliva samples were collected at recruitment in acute phase and at convalescence (>90 days after infection) for RV-infected patients, and at recruitment (baseline pre-vaccination) for control group.

A sample from unstimulated sublingual saliva was obtained with oral swabs (*Whatman*) placed under the tongue for 5 min. The swabs were eluted in 0.4 ml of phosphate buffered saline (PBS), and then centrifuged at 800 g for 10 min to remove mucin and epithelial cells. Supernatants were stored at -30°C prior to analysis.

Detection of cytokines in saliva samples

The cytokine detection assay was performed according to the instructions of the Milliplex Map human cytokine detection kit purchased from Millipore (Merck Millipore, Billerica, MA). The assay kit consisted of a “9-plex” panel of several cytokines (IFN- α 2, IL-8, IL-1 β , IL-10, IL-15, IL12_{p70}, TNF- α , IFN- γ and IL-6). Assays were carried out on a Luminex™ 200 platform.

For IFN- λ and IL-22 detection, a DuoSet ELISA kit containing capture/detection antibodies and recombinant protein standard were purchased from R&D Systems (Minneapolis, MN)

Statistical analysis

Data are reported as median and range interquartile, unless otherwise indicated. Statistical analysis was performed using R software v. 3.0.2 [17] (with Mann-Whitney for comparison between patients of different groups); P -value ≤ 0.05 was considered as the nominal threshold for statistical significance. Non-parametric statistics were used for analysis because the data were not normally distributed.

Cytokine levels in patients from the same group were compared by the Wilcoxon rank-sum test. Spearman's rank correlation coefficients were used to quantify the association between cytokine concentration and clinical parameters. Bonferroni correction was employed to correct for multiple test.

Results

Characteristics of patients and clinical data

A total of 63 children were enrolled in the study, classified in two groups: RV-infected group ($n = 27$) and control group ($n = 36$). RV-infected group age ranged from 1 to 40 months (median of 12 months) at recruitment and from 5 to 47 (median of 18 months) at convalescence. Clinical characteristics of the patients are summarized in **Table 1**. Control group age ranged from 1.8 to 2.3 months (median of 2.1 months) at recruitment.

Salivary cytokine levels were measured in all groups, RV-infected (acute phase), RV-convalescence and control group.

Cytokine profiles in saliva of RV patients

RV-infected patients in the acute phase showed differences in median levels of IL-22 compared to the convalescence period (**Tables 2 and 3** and **Fig 1**). Levels of IL-22 were found significantly increased (P -value < 0.001) in acute-phase samples (47.9 pg/ml) compared with the convalescence period (12.5 pg/ml). All other differences found were not significant after Bonferroni adjustment. We could not detect signal for IFN- λ in any sample of both groups.

Table 1. Summary of main clinical data of rotavirus infected patients included in the study. Data are expressed as mean (SD).

	RV-Infected (acute phase)	RV-Convalescence	Control group
	(n = 27)	(n = 27)	(n = 36)
Age (months)	12 (1–40)	18 (5–47)	2.1 (1.8–2.3)
Sex (Male:Female)	16:11	16:11	18:18
Vesikari's score	11.0 (3.4)	–	–
Length of stay in hospital (days)	5.7 (3.0)	–	–
Temperature (°C)	38.7 (0.6)	–	–
Vomiting episodes per day	3.1 (4.7)	–	–
Duration of vomiting (days)	2.0 (1.9)	–	–
Number of stools per day	4.9 (5.0)	–	–
Duration of diarrhea (days)	4.5 (2.1)	–	–
Level of dehydration	1.6 (1.1)	–	–

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Saliva from acute-phase infected patients showed significant differences when compared to those measured in for healthy controls for the following cytokines: IFN- α_2 , IL-1 β , IL-6, IL-8 and IL-22 (Tables 2 and 3, and Fig 1).

Finally, IL-1 β , IL-6, IFN- α_2 , IL8, and TNF- α were differentially expressed in convalescent vs. healthy control children (Tables 2 and 3, and Fig 1).

Statistically significant correlations between cytokine levels were observed between acute and convalescence patients (Fig 2 and S1 Table). As expected, saliva sample from acute-phase patients showed a correlative rise of pro-inflammatory cytokines (e.g. IFN- γ , IL-1 β , IL-6, IL-8 and TNF- α). Convalescence saliva samples showed similar significant correlation and rise of pro-inflammatory cytokines, especially at IL-6, IL-10 and IL-15 rise. IL-22, a cytokine of non-epithelial origin did not show correlation with any other cytokines in both groups of samples.

Salivary cytokine profiles and clinical parameters

We first conducted analyses to examine if salivary cytokine levels in acute-phase infected children were age dependent, to avoid potential interferences in our analysis. There were no

Table 2. Salivary concentrations of selected cytokines in RV-infected patients (acute phase and at convalescence) and in healthy control children. Data are shown as median (pg/ml) and interquartile ranges (25%–75%) for all groups. nd: not detectable.

Cytokines	RV-infected patients		Control Group
	Acute phase	Convalescence	
IFN- α_2	30.1 (12.2–68.5)	71 (31.2–76.8)	216.1 (110.4–337.1)
IFN- γ	4.1 (1.9–6.5)	4.3 (3.9–5.2)	4 (2–7.5)
IL-1 β	3.2 (1.8–4.6)	3 (2.3–3.5)	1.4 (0.9–2)
IL-6	1.4 (0.7–2.9)	1.2 (1.1–2.3)	3.6 (2.1–6.8)
IL-8	387 (168.4–1435.3)	248.6 (178.8–249.7)	62.6 (48.7–143.9)
IL-10	3.8 (1–13)	9 (2.1–10.9)	13 (6.6–17.1)
IL-15	2.6 (1.3–5.1)	2.3 (1.6–2.6)	1.7 (0.8–3.1)
IL12 _{p70}	7.6 (3.2–15.4)	7.6 (4.3–11.4)	3.2 (2.5–9.4)
TNF- α	2 (1–4.4)	2.9 (1.8–3.3)	0.9 (0.5–1.5)
IL-22	47.9 (6.8–58.8)	12.5 (11.7–13.5)	7.9 (5.2–15.5)
IFN- λ_1	nd	nd	nd

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Table 3. P-values of comparisons between the different groups analyzed in the present study.

Cytokines	AC vs. CV	AC vs. HC	CV vs. HC
IFN- α_2	0.0547	<0.0001*	0.0001*
IFN- γ	0.3038	0.7845	0.908
IL-1 β	0.1429	0.0001*	0.0001*
IL-6	0.0502	0.0011*	0.0007*
IL-8	0.0290	<0.0001*	<0.0001*
IL-10	0.1907	0.0014*	0.0053
IL-15	0.2774	0.0479	0.3517
IL12 _{p70}	0.4524	0.0374	0.0245
TNF- α	0.1893	0.0053	0.0017*
IL-22	0.0002*	0.0032*	0.0769
IFN- λ_1	-	-	-

AC: acute patients; CV: convalescence patients; HC: healthy control children;

*: significant after Bonferroni correction.

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significant age-related differences in any cytokine response (Table 4), as determined by Spearman’s rank correlation coefficients.

We next examined possible associations between the levels of cytokines in acute-phase saliva, and several clinical parameters of disease (Table 4). We did not find any cytokine significantly associated to Vesikari’s total severity score, length of hospital stay, or age.

Discussion

To the best of our knowledge, the results from the present study provide the first evidence for a cytokine-mediated response in oral mucosa in children exposed to natural RV infection. The oral epithelial cells recognize pathogen invaders through their specific innate receptors and stimulate epithelial cells to produce cytokines either by constitutive or inductive pathways [18]. This epithelial cytokine response in oral mucosa had been demonstrated for local inflammatory or infectious events but, it had not been investigated previously for an intestinal infection such as RV.

Rotavirus, the leading cause of severe gastroenteritis in infants and young children around the world, infects preferentially terminally differentiated villous enterocytes of the upper small intestine [19]. Although many case reports show that infection is not limited to the gastrointestinal tract [20] [21] with evidences of systemic transcriptional changes caused by rotavirus infection [22], so far there was no evidence for RV infection of oral mucosa epithelium.

Significant differences in the levels of several cytokines were observed in infected RV-patients, suggesting a systemic response to RV also expressed at the oral mucosa. Our data also show that there is a complex pattern of cytokine co-expression, suggesting that a coordinated immune response associated to a rise of pro-inflammatory cytokines exists. This is particularly remarkable in acute-phase saliva samples, suggesting transference of the inflammatory response from intestine to saliva in a well-orchestrated way.

Acute-phase RV-infected patients showed increased levels of several cytokines, and particularly of IL-22, all of them representative of innate immunity. IL-8 and IL-12, both increased in our patients, are potent pro-inflammatory cytokines and chemo-attractant factors for diverse immune cells such as polymorphonuclear cells and lymphocytes. These cytokines are secreted by the epithelium in response to pathogen entry [23,24]. IFN- γ , a cytokine from the adaptive system that is produced mainly by T-lymphocytes, was not found to be differently expressed in

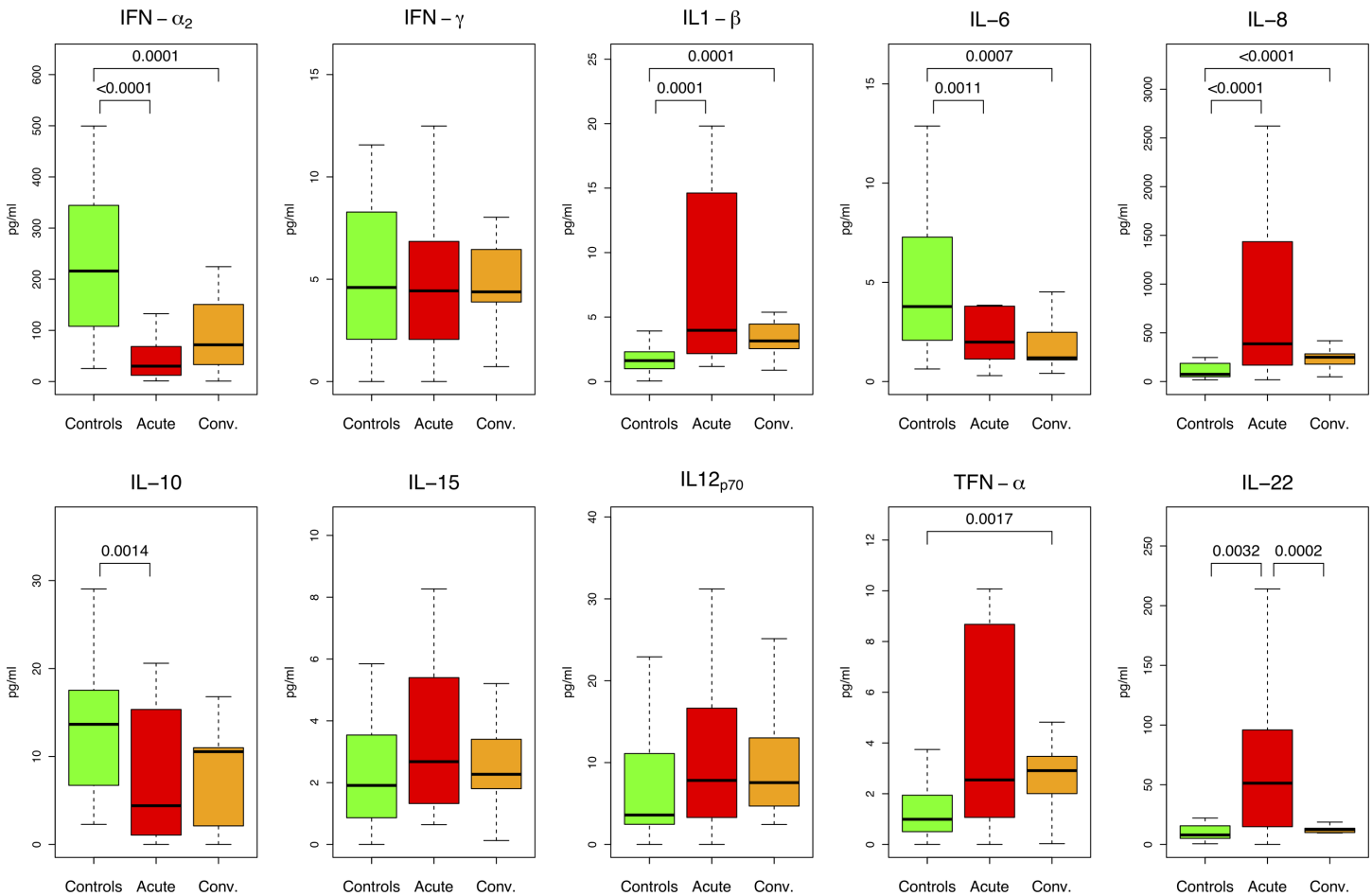


Fig 1. Salivary cytokine concentrations for all groups. Data are represented as median \pm interquartile range. Only P-values surpassing Bonferroni threshold are indicated.

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our subjects. IFN- λ_1 , a recently described cytokine that seems to play an important role in the epithelium defense from viral pathogens, was not even detected in our patients. Although there is no evidence of RV entry into oral mucosa epithelial cells, our data suggest that the oral mucosa responds by secreting these pro-inflammatory mediators, presumably through immune communication from intestinal epithelia, showing a global host mucosal immune response to RV infection.

IFN- α_2 levels were found significantly low in acute-phase infected patients when compared to healthy children. Several studies indicate that the constitutive expression of type I interferon in mucosal surfaces plays an important role in controlling the proliferation and function of the epithelium, and regulating epithelial renewal [25–27]. Rotaviruses have evolved a range of specific mechanisms to evade the type I interferon antiviral response of the host in order to achieve successful replication in the host epithelium [28], for example preventing detection of viral components, or inhibiting the function of transcription factors that initiate interferon response [29]. However, it is unlikely that the low levels of IFN- α observed in oral mucosa of infected patients are due to specific inhibition promoted by RV, since there is no evidence for entry of virus in oral epithelia. It seems more likely that intestinal mucosa injury interferes with the normal homeostasis of interferon production in the whole mucosa.

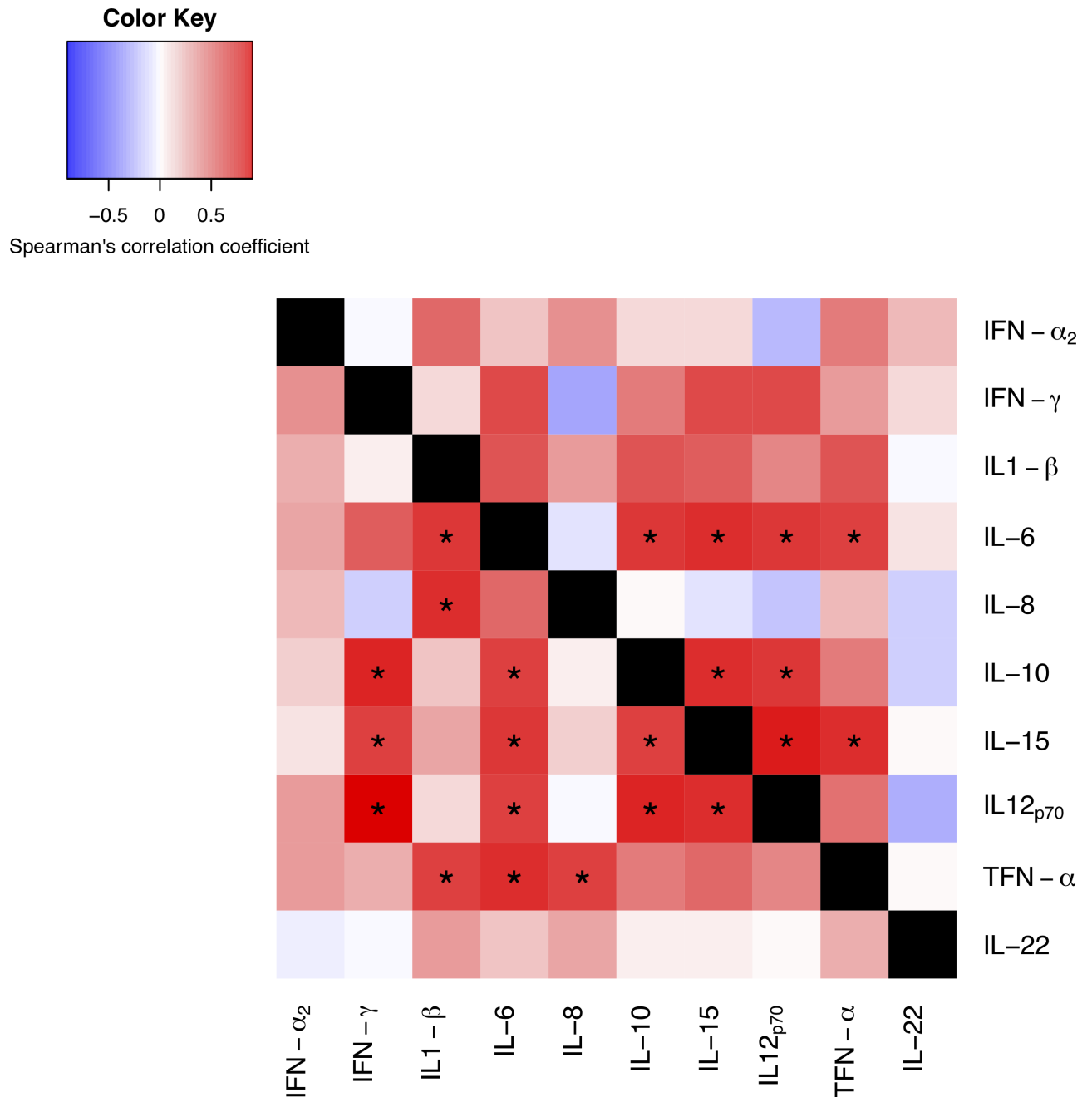


Fig 2. Heatmap of cytokine expression correlations in RV-infected patients (acute-phase vs convalescence). Heatmap of infected patients: below the diagonal are the correlations in acute phase samples, while above the diagonal are the correlations in convalescence samples. Blue and red colors indicate the direction of the correlation as indicated in the legend. Asterisks indicate statistical significant correlations after Bonferroni correction.

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High levels of IL-22, a cytokine not associated with an epithelial origin, were found in oral mucosa of acute-phase RV-infected patients. This cytokine belongs to the superfamily of IL-10 [30] and is mainly synthesized by innate lymphoid cells (ILC3), dendritic cells and natural killer (NK) cells [31]. There is no evidence of IL-22 synthesis by epithelial cells; therefore, the presence of this cytokine in oral mucosa is most likely associated with a lymphoid innate immune response, suggesting presence of activated lymphoid cells in oral mucosa in response

Table 4. Correlation coefficients between saliva cytokine levels in RV-infected patients and age, Vesikari’s severity score and length of hospital stay. *P*-value of Spearman’s rank correlation and Pearson’s correlation coefficients for cytokines values are expressed between parentheses.

Cytokines	Age (months)		Vesikari’s severity score		Length of stay (days)	
	Spearman’s correlation	Pearson’s correlation	Spearman’s correlation	Pearson’s correlation	Spearman’s correlation	Pearson’s correlation
IFN- α_2	-0.09 (0.660)	-0.09 (0.67)	-0.09 (0.648)	-0.1 (0.611)	-0.16 (0.419)	0.03 (0.881)
IFN- γ	-0.3 (0.132)	-0.17 (0.396)	-0.4 (0.039)	-0.23 (0.249)	-0.04 (0.83)	0.02 (0.925)
IL-1 β	0.16 (0.436)	0.12 (0.543)	0.09 (0.670)	-0.01 (0.978)	-0.32 (0.109)	-0.15 (0.449)
IL-6	-0.28 (0.155)	-0.26 (0.184)	-0.43 (0.026)	-0.25 (0.204)	-0.11 (0.582)	0.44 (0.020)
IL-8	0.14 (0.474)	0.18 (0.370)	-0.09 (0.665)	-0.11 (0.571)	-0.1 (0.633)	-0.01 (0.948)
IL-10	-0.24 (0.236)	0.02 (0.918)	-0.41 (0.035)	-0.07 (0.712)	-0.08 (0.690)	-0.26 (0.199)
IL-15	-0.32 (0.099)	-0.28 (0.153)	-0.31 (0.121)	-0.22 (0.271)	-0.09 (0.658)	-0.06 (0.758)
IL12 _{p70}	-0.44 (0.020)	-0.32 (0.101)	-0.42 (0.031)	-0.35 (0.072)	0.03 (0.867)	0.26 (0.192)
TNF- α	0.02 (0.916)	0.07 (0.735)	-0.18 (0.374)	-0.03 (0.884)	-0.25 (0.205)	-0.31 (0.111)
IL-22	0.37 (0.059)	0.27 (0.179)	0.04 (0.837)	-0.07 (0.729)	-0.26 (0.190)	-0.16 (0.417)
IFN- λ_1	-	-	-	-	-	-

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to intestinal RV infection. A major target for this cytokine are epithelial cells (respiratory and gut) where it acts as a potent mediator of cellular inflammatory process against invader pathogens [32]. Administration of IL-22 and IL-18 in intestinal epithelial cells in mice, produced elimination of RV infection acting as a broad-spectrum antiviral agent [33]. Also, IL-22 has been related to protective functions in epithelia through a regenerative action on injured epithelia after infection [32,34,35]. Salivary levels of IL-22 show a significant correlation with clinical parameters of Sjögren’s disease, indicating a critical role for this cytokine in the pathogenesis of this autoimmune disease that targets salivary glands [36]. The finding of elevated levels of IL-22 in oral mucosa shows that not only epithelial cells respond to the intestinal RV infection producing pro-inflammatory signals, but also mucosal lymphoid innate cells are involved in this response.

Several clinical parameters were investigated with regards to cytokine levels, although none of them reached statistical significance. IFN- γ , IL-6, IL-10 and IL12_{p70} were statistically correlated with Vesikari’s severity score, but significance did not surpass multiple test correction (Table 4). Whether this reflects just a lack of association or insufficient statistical power to detect them can only be addressed through more powerful cohort studies.

Besides limited sample size, age differences between children from RV-infected (acute-phase and convalescence) and healthy control groups might constitute a limitation to our study. However, we did not find any link between age and cytokine levels in saliva samples in either our samples or the literature, even for sera cytokine levels. [37]

In conclusion, our data suggest the existence of an oral mucosa cytokine host response to RV infection. Several pro-inflammatory innate cytokines produced by epithelial cells, such as IL-8, IL-1 β , IL-12 and TNF- α , were elevated in saliva after infection. In addition, production of IL-22, presumably from mucosa innate lymphoid circulating cells, was elevated. IFN- α_2 was inhibited probably as consequence of RV homeostasis disruption.

Although the present study needs further validation, our preliminary data indicate that RV infection is not confined to the intestinal mucosa, suggesting a whole mucosal implication as a result of the integrative mucosal immune response.

Supporting information

S1 Table. Correlation coefficients between saliva cytokine levels in RV-infected subjects at baseline and at convalescence. Spearman’s correlations rank coefficient for each pair of

cytokine values (P-value between parentheses) for RV-infected patients. Correlations for cytokines measured in acute-phase are shown below the diagonal; correlations for cytokines measured in convalescent-phase are shown above the diagonal.

(DOCX)

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