Analysis of Serum Th1/Th2 Cytokine Levels in Patients with Acute Mumps Infection

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ABSTRACT

Background: The mumps virus is frequently the causative agent of parotitis. There has been no study on serum cytokine levels of acute mumps parotitis except for a few which document cytokine levels in cerebrospinal fluid of mumps meningitis. It is with this notion, our study aimed to find Th1/Th2 cytokine levels from patients with acute mumps parotitis. **Materials and Methods:** Concentrations of mumps-specific IgM, mumps, measles, rubella-specific IgG antibody, and Th1/Th2 cytokines, namely interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4, and IL-10 were measured simultaneously in serum from 74 patients (42 pediatric and 32 adult cases), 40 healthy subjects (20 pediatric and 20 adults) and in the supernatant of peripheral blood mononuclear cells stimulated with mumps virus genotype C which served as the positive control. Statistical significance was analyzed between each group by means of Mann–Whitney U-test, Kruskal–Wallis test, and Spearman's rank correlation coefficient test. **Results:** IgM positivity confirmed acute infection in all 74 patients and of these 67 were vaccinated cases; however, very few of them (10/67) were positive for mumps IgG. We found that IFN-γ, IL-2, and IL-10 showed a statistically significant increase in both pediatric and adult patients with acute mumps infection when compared to healthy controls and values were comparable to the positive control. **Conclusion:** The Th1 cells play important roles during the acute phase of mumps parotitis.

Key words: Acute mumps, IgM and IgG antibodies, measles, mumps, rubella vaccine, Th1 and Th2 cytokines

INTRODUCTION

Mumps is acute, highly contagious, systemic, communicable viral infection found throughout the world, characterized by parotitis of one or both salivary glands (90%), aseptic meningitis (−15%), transient deafness (−4%), and encephalitis (−0.1%). Other clinical features include orchitis (20–38% in postpubertal males), oophoritis (0.5–7%), and respiratory symptoms (40–50%). Transmission occurs through inhalation of respiratory droplets or by direct person-to-person contact; reinfection may occur after natural infection.[1-4]

Mumps infection is not rare in childhood and adults, but sometimes severe complications arise in the form of meningitis and deafness in children or orchitis in postpubertal males. Mumps infection still prevails in India because mumps virus vaccine has not been made mandatory for children.[5,6] It is generally believed that pro-inflammatory interferon-γ (IFN-γ) responses prevail at the sites of infection, while the systemic response is anti-inflammatory interleukin-10 (IL-10). There have been no reports on serum/plasma cytokines levels in mumps infection except for a few studies documenting cerebrospinal fluid (CSF) cytokine levels in mumps mediated meningitis in comparison with other viral

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meningitis and a ferret in vivo model.[7-12] The aim of our study was to determine reflection of specific mechanisms of host response to mumps infection by analyzing the serum cytokine levels of Th1 (IFN-γ and IL-2), Th2 (IL-4 and IL-10) in children and adults with acute mumps infection.

MATERIALS AND METHODS

Patient sample collection

For the selection of mumps cases, WHO guidelines were adopted.[3,13] An informed consent, proforma, and human ethical clearance for sample collection were obtained. Blood was collected from patients at the outpatient department of V. K. Nursing Home and Sri Ramachandra Medical College and Research Institute, Chennai, India. The sample was collected 1-7 days after onset (average = 3 days). Following collection, samples were allowed to clot, then centrifuged at 1500 rpm for 5 min in order to remove cells, and serum was separated, divided into aliquots and immediately stored at −86°C until use.

Controls

The negative control groups consist of afebrile, noninfected and nonmeasles, mumps, and rubella (MMR) vaccinated subjects (20 pediatric and 20 adults) aged from 5 years to 20 years. Patients and control subjects having a history of recent infections, receiving prolonged steroid therapy, central nervous system disorders, convulsions or epilepsy, those who received blood, plasma, or immunoglobulin within the last 3 months, those diagnosed with malignancy or immunodeficiency diseases were excluded from the study.

For positive control, human blood was obtained from healthy donors (Blood Bank, Voluntary Health Services, Chennai, India). Peripheral blood mononuclear cell (PBMC) isolation was done by using Histopaque (Sigma Cat No. 1077), and the procedure used was as directed by the manufacturer.[14,15] Isolated PBMCs were plated at a concentration of 2 ×10^6 cells/ml/well in 24 well-tissue culture plates and infected with 50 μl of (5000 tissue culture infectious dose $[TCID]_{50}$) mumps virus genetype C (virus positive control) as described earlier.[4,16] Cells were stimulated with 5 μg/ml of mitogen that is phytohemagglutinin ([PHA] - positive control [assay control]). Plates were incubated in a CO_2 incubator at 37°C for 72 h. After 72 h culture supernatants were collected, aliquoted, and immediately stored at −86°C until use.

Determination of antibody and cytokine concentrations

Serum samples were tested for mumps-specific IgM antibody by ELISA (Labor Diagnostika Nord GmbH and Co., KG, Germany), and MMR-specific quantitative IgG ELISA (Techno Genetics, Italy) was done to quantify MMR-induced specific IgG antibody in all participants, in order to confirm that they had been vaccinated. IgG antibody titers were calculated from the sample absorbance and reference standard curve generated from the reference sera provided with the kit. IgG antibody titers of >115 mU/ml for mumps, >115 mIU/ml for measles, and >15 IU/ml for rubella were defined as seropositivity.

Serum samples and culture supernatants were tested for Th1 cytokines and Th2 cytokines by standard ELISA as described before.[16] Cytokines evaluated in this study were the Th1 cytokines, IFN-γ (BD Pharmingen Cat. No. 555142) and IL-2 (Cat. No. 555190), and Th2 cytokines IL-10 (Cat. No.555157) and IL-4 (Cat. No.555194). Cytokine evaluation was done as per manufacturer's instructions. Briefly, all wells of 96 well-microtiter plates were manually coated with 100 μl of capture antibody/well and kept at 4°C overnight. After overnight incubation, the plates were washed with wash buffer 3 times. Wells were blocked with 200 μl/well-assay diluent and incubated at room temperature for 1 h. After washing, standards, test serum, and culture supernatants were added to appropriate wells. The plates were sealed and incubated for 2 h at room temperature. The plates were washed again for 5 times, and 100 μl/well of working detector (detection antibody + SAv-horseradish peroxidase streptavidin) was added and incubated for 1 h, after which the plate was washed 7 times. Finally, 100 μl/well of 3′,5,5′-tetramethylbenzidine substrate was added; incubated in a dark room for 30 min, and the reaction was stopped by adding 50 μl/well-stop solution. The absorbance was then read using an ELISA reader at 450 nm wavelength. Concentration of each cytokine of the sample was calculated based on the standard curve. All assays were repeated thrice, and the results were represented as the average (mean) of 3 experiments.[17]

Statistical analysis

Values were reported as the median with range, and differences in the results between two groups were analyzed by means of student *t*-test and nonparametric Mann–Whitney U-test. Difference between more than two groups was analyzed by means of One-way ANOVA and nonparametric Kruskal–Wallis test. A $P < 0.05$ was considered to be statistically significant. Correlations were

analyzed with use of Spearman's rank correlation coefficient test. For all the above analyses, a GraphPad Prism 6.

RESULTS

Patient demographic and antibody analysis

A total of 74 mumps parotitis cases were collected over a period of 16 months (July 2011-November 2012). Of the 74 samples collected from various age groups, 42 (57%) were from pediatrics with a median age of 6 years, and 32 (43%) were from adults with a median age of 21 years. Of these, 54% were males and 46% were females. There was no significant difference in patients with regard to age and gender. Of the 74, 67 (91%) had been vaccinated, and 7 of them were nonvaccinated. Tests for mumps IgM in vaccinated cases indicated that an alarming 67/67 (100%) samples tested positive, whereas 57/67 (85%) samples were negative for mumps IgG. These facts inevitably state that MMR vaccine failed to offer protection in vaccinated individuals against mumps infection. Around 67 (100%) samples tested positive for rubella-specific IgG and 65 (97%) samples tested positive for measles-specific IgG, suggesting that the mumps component in the MMR vaccine had low efficacy. Of the 74 acute mumps cases, 7 of them were nonvaccinated; mumps IgM was positive for these samples; however, they were negative for both mumps and rubella IgG. Measles IgG was positive for these samples because a separate measles vaccine is given at 9 months of age, prior to the MMR vaccine [Table 1]. The median value for mumps, measles, and rubella-specific IgG was 422 mU/ml, 1009 mIU/ml, and 298 IU/ml, respectively.

Th1/Th2 cytokine profiles in acute mumps parotitis

The lower detection limit of the IFN-γ, IL-2, IL-4, and IL-10 cytokines evaluated in this study was 2.35 pg/ml, 1.95 pg/ml, 0.9 pg/ml, and 1.95 pg/ml, respectively. The concentrations below the detection limit were taken as 0 pg/ml. The median and concentration range of the pediatric control subjects were 11.2 (9.6-17.61 pg/ml), 2.7 (1.26-6.15 pg/ml), 0 (0–3.01 pg/ml), and 2 (0-3.2 pg/ml), respectively and in adult control were 15.1 (9.74-23.6 pg/ml), 6 (1.98-8.91 pg/ml), 0.9 (0-3.24 pg/ml), and 2.2 (0-3.68 pg/ml), respectively. Genotype C of mumps virus was isolated from our previous study and quantified by $TCID_{50}$.[18] The quantified virus was used for PBMC infection as virus control and the PHA used as assay positive control. The median concentrations of virus control were 217.1 (210-223 pg/ml), 210.5 (192.6-206.4 pg/ml), 2 (1.94-2.12 pg/ml), and 194.2 (192.3-199.4 pg/ml), respectively [Table 2]. IFN-γ, IL-2, and IL-10 showed a statistically significant increase in both pediatric and adult acute mumps patients when compared to healthy controls (pediatrics: $P < 0.0001$, $P < 0.0001$, $P < 0.0001$; and adults: $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, respectively) and values were almost same and significant with virus control ($P = 0.0075$, $P = 0.0302$, $P = 0.0003$, respectively). IL-4 levels were slightly reduced when compared to controls $P = 0.115$, $P = 0.0016$, $P = 0.0003$ [Tables 2 and 3a, Figure 1].

Correlation among immunological variables

Table 3b shows the correlation among the pairs of measures of IFN-γ, IL-2, IL-4, and IL-10 as represented by a Spearman's correlation matrix. The IFN-γ levels correlated with IL-10 and IL-2 in both pediatric and adult patients (pediatric: $r = 0{\cdot}621$, $P < 0.00001$; $r = 0{\cdot}523$, $P < 0.0004$; adult: $r = 0{\cdot}724$, $P < 0.000003$; $r = 0{\cdot}026$, $P = 0.888$, respectively) but did not correlate with IL-4 secretion.

DISCUSSION

The immune system responds to viral infections by using a set of cytokines that are beneficial to the host as they coordinate

Table 1: Details of patients with mumps infection and control subjects

Patients details	Acute mumps — Pediatric cases	Acute mumps — Adult cases	Pediatric control	Adult control
n	42	32	20	20
Age (median age)	<12 years (6)	13-42 years (21)	5-12 years (8)	18-20 years (19)
Sex (male:female)	26:16	14:18	10:10	10:10
Onset of parotitis to sampling (average) (days)	3	3	—	—
Duration of fever (average) (days)	5	6	—	—
MMR vaccination (%)	35/42 (83)	32	0	0
Number positive for mumps IgM antibody	42	32	—	—
Number positive for mumps IgG antibody (%)	2/35 (6)	8/32 (27)	0	0
Number positive for measles IgG antibody	33/35	32/32	19	16
Number positive for rubella IgG antibody	35/35	25/32	0	0

Table shows the case distribution along with controls. A total of 74 patients with acute mumps were recruited in this study. Cases were divided into pediatrics (<12 years) and adults (13–42 years). Of the 74 cases, all were positive for parotitis and mumps IgM. Of them, 67 (90%) patients had a clear MMR vaccine history while the others 7/74 (age of 20, 22, 23, 29, 36, 41, and 42) were nonvaccinated; MMR: Measles, mumps, and rubella

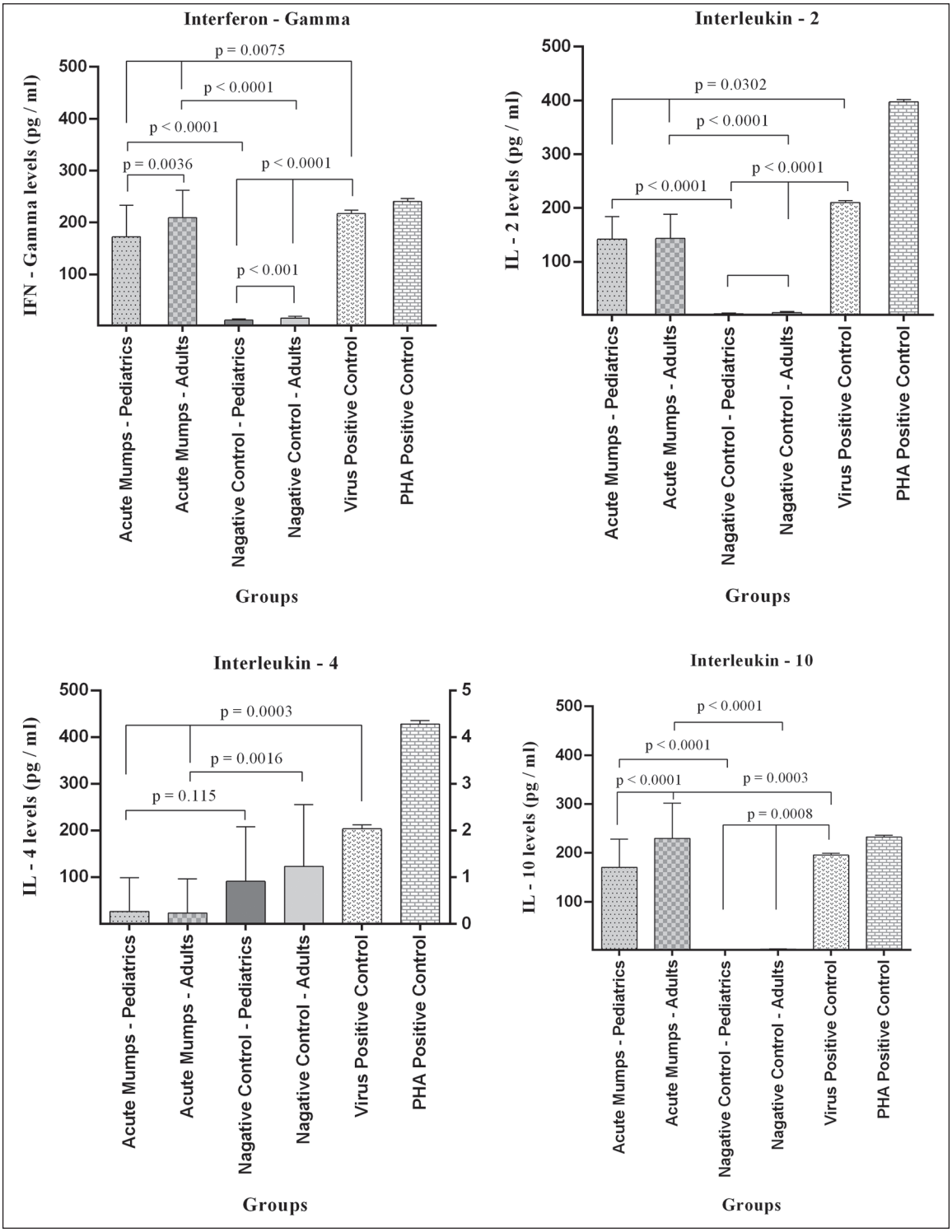

Figure 1: Serum concentrations of interferon-γ, interleukin-2, interleukin-4, and interleukin-10 in patients with mumps parotitis and controls. Data are represented as medians with range. The interferon-γ, interleukin-2, and interleukin-10 levels of cytokine concentrations were higher in adult patients when compared to pediatric patients. In adult and pediatric patients, the cytokine levels were comparable to virus control and higher when compared to control subjects except interleukin-4. Interleukin-4 level was slightly reduced when compared to control ($P = 0.115$, $P = 0.0016$, $P = 0.0003$)

with host immune cells and eliminate viruses efficiently. The Th cell response can be divided into Th1 or Th2 based on the pattern of cytokine secretion. Th1 cells promote cell-mediated immune response and produce gamma IFN-γ, IL-2, and tumor necrosis factor-β, whereas Th2 cells promote humoral immunity and produce IL-4, IL-5, IL-6, IL-10, and IL-13.[19,20] T-cells that produce cytokine pattern distinct from the well-defined Th-1/Th-2 sets have been described as Th-0 and Th-17, etc.[19,21]

Table 2: Levels of cytokine concentrations (pg/mL) observed in this study

Groups	IFN-γ			IL-2		
	Range (pg/mL)	Mean (pg/mL)	Median (pg/mL)	Range (pg/mL)	Mean (pg/mL)	Median (pg/mL)
A. Acute mumps - pediatric (42)	86.2-329.4	172.2	164.4	85.2-248.7	141.6	132.7
B. Acute mumps - adult (32)	110.1-329	209	200.4	89.8-325.5	143.1	138.6
C. Pediatric control (20)	9.6-17.61	11.6	11.2	1.26-6.15	3.2	2.7
D. Adult control (20)	9.74-23.6	15.5	15.1	1.98-8.91	5.5	6
E. Virus positive control	210-223	217.1	217.1	192.6-206.4	210.1	210.5
F. PHA positive control	325.6-246.1	240	240.2	392.6-401.5	397.1	397.2
Groups		IL-4			IL-10	
	Range (pg/mL)	Mean (pg/mL)	Median (pg/mL)	Range (pg/mL)	Mean (pg/mL)	Median (pg/mL)
A. Acute mumps - pediatric (42)	0-2.5	0.26	0	96.1-322.7	170.5	156.5
B. Acute mumps - adult (32)	0-3.2	0.22	0	27.4-365.1	229.7	254
C. Pediatric control (20)	0-3.01	0.91	0	0-3.2	1.3	2
D. Adult control (20)	0-3.24	1.2	0.9	0-3.68	1.8	2.234
E. Virus positive control	1.94-2.12	2.0	2.0	192.3-199.4	195.3	194.2
F. PHA positive control	420.6-435.6	427.6	426.8	229.1-236.4	232.3	231.6

IFN-γ: Interferon-γ; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-10: Interleukin-10

Table 3a: Comparison of significant differences (*P* values) between the groups

Cytokines	*P*								
	B versus A	C versus A	D versus B	A versus B versus E	A versus B versus *F*	D versus C	F versus E	C versus D versus *E*	C versus D versus F
IFN-γ	0.0036	<0.0001	<0.0001	0.0075	0.0042	<0.001	0.1000	<0.0001	<0.0001
IL-2	0.7884	<0.0001	<0.0001	0.0302	0.0135	0.0002	0.1000	<0.0001	<0.0001
IL-4	0.7952	0.0115	0.0016	0.0003	<0.0001	0.5039	0.1000	0.3804	0.0073
IL-10	<0.0001	<0.0001	<0.0001	0.0003	0.0003	0.2870	0.1000	0.0088	0.0088

IFN-γ: Interferon-γ; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-10: Interleukin-10

Table 3b: Correlation between IFN-γ, IL-2, IL-4, and IL-10 responses in study subjects as represented by a Spearman's correlation matrix

Variable	Acute mumps — Pediatrics *P*			
	IFN-γ	IL-2	IL-4	IL-10
IFN-γ	—	<0.0004	0.510	<0.00001
IL-2	0.004	—	0.227	<0.00015
IL-4	0.5104	0.2274	—	0.426
IL-10	1.25	<0.0002	0.426	—
Variable	Acute mumps — Adults *P*			
	IFN-γ	IL-2	IL-4	IL-10
IFN-γ	—	0.888	0.965	<0.000003
IL-2	0.888	—	0.759	0.217468
IL-4	0.965	0.759	—	0.482792
IL-10	2.7	0.217	0.483	—

IFN-γ: Interferon-γ; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-10: Interleukin-10

In this study, we evaluated the ability of patients to respond to mumps virus by measuring cytokine levels. It is generally believed that pro-inflammatory (IFN-γ), responses prevail at the sites of infection while the systemic response is anti-inflammatory (IL-10). Our results showed that the simultaneous activation of pro and anti-inflammatory immune responses in the serum may reflect the specific mechanisms of host response to mumps infection. The previous studies demonstrated that CD8+cytotoxic T lymphocytes (CTL) play an important role in CSF of mumps meningitis.[12,20] Taking these reports into consideration, a higher level of IFN-γ in this study may indicate that IFN-γ is mainly produced by CD8+CTL in serum of mumps infection. On the other hand, levels of IL-2, a Th1 cytokine, elevated. This finding indicates the activation of CD4+ Th1 cells. Therefore, it cannot be regarded that only CD8+CTL produce IFN-γ in mumps infection because activated CD4+ Th1 cells can also produce IFN-γ. With respect to the cytokine levels in patient sera, we suggest that CD8+CTL and CD4+ Th1 cells play an important role in mumps during the acute phase infection. An increased level of IL-10, which is mainly produced by CD4+ Th2 cells, inhibits cytokine production by CD4+ Th1 cells.[22,23] Therefore, we suggest that IL-10 is induced in response to higher production of IFN-γ to modulate the balance of Th1 and Th2. The levels of cytokine concentrations were higher in adult patients when compared to pediatric patients. In both adult and pediatric patients, the cytokine levels were comparable to virus control and higher when compared to control subjects.

There have been several reports on the immunological response to vaccine against the mumps virus. The mumps antigen-specific lymphoproliferative response to the vaccine is comparable to that which occurs in naturally immune individuals and healthy control subjects. In response to

the mumps vaccine, serum IFN-γ, IL-10, and IL-12 are reported to be the central immunoregulatory cytokines.[24-27] The proinflammatory cytokine IFN-γ is a good indicator of antigen-specific cellular immunity while the anti-inflammatory cytokines IL-10 and IL-13 may be important in maintaining sufficient humoral, or Th2 immune responses during antigen stimulation.[26] Low levels of IL-4 production are found in tissues affected by organ-specific autoimmune diseases, but this may simply reflect the relative dominance of inflammatory Th1 cells and few studies using animal model document increased production of IFN-γ and IL-10 with strong absence of IL-4 and IL-5.[23,28] Our data also suggest same kind of results wherein IFN-γ and IL-10 concentrations were significantly increased when compared to control subjects with strong positive correlations and little-reduced level of IL-4 reflected by raised Th-1 cytokines. Furthermore, the stimulation of mumps virus-specific memory cells in vaccinated individuals would have led to the increased Th1 cytokine levels. In conclusion, the Th1 cells play important roles during the acute phase in the pathogenesis of mumps parotitis, and further studies are needed to understand the immune response to mumps virus.

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Conflicts of interest

There are no conflicts of interest.

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