Original Article

Higher Viral Load and Prolonged Viral Shedding Period is Associated with Impaired Th17 Cell Response in Patients with H1N1 Influenza A

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Objective To explore whether age, disease severity, cytokines and lymphocytes in H1N1 influenza A patients correlate with viral load and clearance.

Methods Total of 70 mild and 16 severe patients infected with H1N1 influenza A virus were enrolled in this study.

Results It was found that the patients under 14 years old and severe patients displayed significantly higher viral loads and prolonged viral shedding periods compared with the patients over 14 years old and mild patients, respectively ($P < 0.05$). Moreover, the patients under 14 years old and severe patients displayed significantly lower Th17 cell frequency than the patients over 14 years old and mild patients ($P < 0.01$). The viral shedding period inversely correlated with the frequency of IL-17⁺IFN- γ CD4⁺ T cells. Additionally, the decreased concentration of serum TGF- β correlated with the decreased frequency of IL-17⁺IFN- γ CD4⁺ T cells.

Conclusions Both younger and severe patients are associated with higher viral loads and longer viral shedding periods, which may partially be attributed to the impaired Th17 cell response.

Key words: Viral load; Viral shedding period; H1N1 influenza A; Th17 cells; TGF- β

In March 2009, an outbreak of H1N1 influenza A occurred in Mexico and the United States of America. Subsequently, it spread quickly around the world.¹ A vast majority of patients displayed mild symptoms and a self-limited disease. However, a small percentage of patients may develop into pneumonia, respiratory failure or multiple organ failure, or even death.² High-risk populations include pregnant women and patients with underlying diseases such as obesity, cardiac and lung diseases, diabetes, and specifically children < 5 years old and adults over 65 years old.³⁻⁶ In seasonal flu patients, severe patients with pneumonia exhibited higher viral loads and longer viral shedding periods compared with mild flu patients without pneumonia.⁷ However, we don't know whether there is any difference and which lymphocytes or cytokines are involved in viral load and clearance between mild and severely infected patients. Recently, a new subset

of helper T cells was identified. They are characterized by secretion of IL-17, IL-1 β , IL-6, IL-21, IL-23 and TNF- α , called Th17 cells, which are generally considered pro-inflammatory cells and contribute to the development of autoimmune diseases.⁸ Th17 cells can play a critical immune protective role in bacterial and fungal infections, and tumor growth. However, it is unknown whether Th17 cells can play a role in viral clearance and acute lung injury in patients with H1N1 influenza A. In this study, we investigated clinical features, duration of viral shedding, kinetic viral load, and immunological features. We followed 70 mild cases without pneumonia and 16 severe H1N1 influenza A patients with pneumonia. Thirty patients with seasonal flu and 68 healthy subjects were also enrolled as controls. We explored the relationship among viral load, viral shedding period and Th17 cells response in mild patients without pneumonia and severe patients with pneumonia infected with H1N1 influenza A virus.

PATIENTS AND METHODS

Patients

Mild cases are defined as laboratory confirmed patients with H1N1 influenza A but without pneumonia ($n = 70$), severe cases are defined as patients with

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Table 1. Characteristics and clinical manifestations of mild and severe patients with H1N1 influenza A

Characteristics	Values		<i>P</i> values
	Mild	Severe	
Sex (male/female)	41/29	10/6	> 0.05
Age (years)			
median	18	15	-
range	0.6-51	1.5-57	-
Clinical symptoms			
Fever, n (%)	67 (95.7%)	16 (100%)	> 0.05
The highest temperature ($\bar{x} \pm s$, °C)	38.2 ± 0.8	39.2 ± 0.4	< 0.01
Cough, n (%)	36 (51.4%)	16 (100%)	< 0.01
Dyspnea, n (%)	0	4 (25%)	< 0.01
Rhinorrhea, n (%)	16 (22.9%)	2 (12.5%)	> 0.05
Sore throat, n (%)	22 (31.4%)	4 (25%)	> 0.05
Myalgia, n (%)	6 (8.6%)	3 (18.8%)	> 0.05
Diarrhea, n (%)	5 (7.1%)	3 (18.8%)	> 0.05
Tonsil enlargement, n (%)	37 (52.9%)	9 (56.3%)	> 0.05
Hospitalization period ($\bar{x} \pm s$, d)	6.0 ± 1.7	9.6 ± 3.5	< 0.01

pneumonia identified by chest X-ray or chest CT (computerized tomograph) scanning (n = 16). Thirty cases of seasonal influenza and 68 healthy donors were also recruited. Written informed consent was obtained from the patients and volunteers, and the Ethics Committee Board of Shenzhen Third People's Hospital reviewed and approved the study. The confirmed cases are in accordance with the diagnostic criteria for 2009 H1N1 influenza A published online by WHO and the Chinese Ministry of Health. The clinical characteristics of the patients studied were shown in Table 1. Routine blood, liver function and cardiac biochemistry tests were performed according to the manufacturer's instructions.

Collection of specimen

Nasopharyngeal-swab specimens of the patients were consecutively collected daily during their hospitalization from admission through release with viral clearance. Specimens were put in transport medium and kept at 2-4°C until tested. Peripheral blood and serum were collected as well.

Quantitative real-time PCR for detection of A/H1N1 virus

Primers and probes specific for 2009 human H1N1 influenza A virus (H1 and H3 subtypes) were developed and quantitative real-time PCR tests were performed for detection of the virus according to published guidelines by World Health Organization (WHO). These developed reagents allowed for a rapid detection of H1N1 influenza A virus. Technical details on this assay had been published on the WHO Global Influenza Program Website. In order to establish

copy number as a unit of viral load, the primers were designed to establish PCR assay to amplify a part of the M gene of 2009 H1N1 influenza A virus according to the published sequences online by WHO, then the amplified PCR products were cloned into vector pMD18-T and subsequently produced a new plasmid containing M gene. Concentration of the resultant new plasmid was serially diluted from 10^9 to 10^3 copies/ml and quantitative real-time PCR assay was performed to detect the copy number of viral load in nasopharyngeal-swab specimens of the patients using the resultant new plasmid containing M gene as a standard curve. The unit of mol can be transformed into copy number according to the following equation: $(6.02 \times 10^{23} \text{ copies/mol}) \times (\text{concentration of plasmid g/ml}) / (\text{MW g/mol}) = \text{copies/ml}$.

Flow cytometry analysis

IL-17, IFN- γ , IL-4 and Foxp3 production using intracellular staining by flow cytometry was determined after 6 hours' stimulation of PBMCs with 20 ng/ml (Sigma) PMA and 1 $\mu\text{g/ml}$ ionomycin (Sigma), in the presence of 3 $\mu\text{g/ml}$ Brefeldin A (e-Bioscience). First, CD3 and CD8 surface stainings were carried out, subsequently cells were fixed with Fixation/Permeabilization solution (BD, Biosciences) for 10 min. This was followed by washing with 1 $\times$ Perm/Wash buffer (BD Biosciences) and permeabilized with Cytofix/Cytoperm reagent for 10 min. PBMCs were incubated with phycoerythrin-labeled monoclonal anti-human IL-17, IFN- γ , IL-4 and Foxp3 or control isotype following manufacturer's instructions (BD Biosciences).

Detection of cytokine production by ELISA

IL-6, IFN- γ and TGF- β concentrations in sera were measured by ELISA according to the manufacturers' instructions (R & D System).

Statistical analysis

Data were expressed as means (M) $\pm$ standard deviation (SD) and analyzed with the SPSS15.0 software. Unpaired *t* tests or non-parametric tests (Newman-Keuls tests) were performed before ANOVA analysis of variation was conducted to analyze continuous data of multiple groups. A *P* value < 0.05 was considered statistically significant.

RESULTS

Characteristics of clinical presentation

In the patients, the most common symptoms and signs were fever and cough, and enlargement of tonsils. However, severe patients suffered from higher temperatures and more dyspnea compared with mild patients (Table 1). Among the mild patients, 44 patients (62%) received oral oseltamivir treatment, with 26 patients (38%) not receiving oseltamivir. Among the severe patients, 4 patients (25%) suffered from dyspnea, 2 patients were pregnant women, 1 patient with an underlying condition of diabetes, 1 patient with chronic hepatitis B, 2 patients with obesity, 2 patients receiving mechanical ventilation support, and all the severe patients received oseltamivir treatment. All the patients recovered from their diseases.

Laboratory investigations

In mild cases, 26% showed a decrease in white blood cell count (WBC), and 34% presenting with a decrease in neutrophil, 31% presenting with lymphopenia. In the severe patients, 5 patients showed an increase in WBC and neutrophil counts, and 2 patients presenting with decreased WBC and lymphopenia. Moreover, the severe patients exhibited significantly higher values of WBC, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and C reaction protein (CRP) in comparison to the mild patients (*P* < 0.05) as shown in Table 2.

Patients under 14 years old and severe patients display higher viral load and longer viral shedding than the patients over 14 years old and mild patients respectively

Viral load was quantified in nasopharyngeal-swab specimens daily by quantitative real-time PCR assay until viral load was undetectable. Duration of viral shedding was calculated from admission until viral load was at an undetectable level. It was found that the peak viral load and viral shedding period of the patients under 14 years old was significantly higher than that of patients aged or elder than 14 years old ($4.86 \pm 1.23 \log_{10}$ copies/ml vs. $4.17 \pm 0.89 \log_{10}$ copies/ml, *P* < 0.05 ; 5.33 ± 0.49 d vs. 3.63 ± 0.28 d, *P* < 0.01) (Figure 1A and Figure 1B). Moreover, the severe patients also displayed significantly higher viral load and longer viral shedding period compared with the mild patients ($6.36 \pm 1.44 \log_{10}$ copies/ml vs. $4.35 \pm 0.99 \log_{10}$ copies/ml, *P* < 0.001 ; 5.75 ± 1.77 d versus 4.24 ± 1.96

Table 2. Laboratory findings of mild and severe patients with H1N1 influenza A

Variables	Mean $\pm$ Standard		Normal reference values	<i>P</i> values
	Mild	Severe		
WBC (/ml)	5.8 ± 2.0	7.8 ± 4.9	3.5-9.5	< 0.01
Granulocyte (/ml)	3.7 ± 1.6	5.4 ± 4.7	2.0-7.0	> 0.05
Lymphocyte (/ml)	1.4 ± 0.9	1.8 ± 1.4	1.5-4.0	> 0.05
ALT (U/L)	19.4 ± 14.6	30.6 ± 22.2	5-40	< 0.05
AST (U/L)	25.0 ± 8.9	38.7 ± 30.8	5-40	< 0.01
CK (U/L)	149.5 ± 168.4	223.4 ± 366.5	24-195	> 0.05
CK-MB (U/L)	8.1 ± 3.7	9.7 ± 4.9	3-25	> 0.05
Cr (μ mol/L)	82.9 ± 24.8	73.1 ± 22.8	2.9-7.0	> 0.05
BUN (mmol/L)	3.7 ± 1.6	3.0 ± 1.2	30-120	> 0.05
Blood glucose (mmol/L)	6.0 ± 2.5	6.2 ± 3.1	3.9-6.1	> 0.05
CRP (mg/L)	13.8 ± 16.4	30.5 ± 38.7	< 8	< 0.05
CD4 (%)	34.1 ± 8.3	32.5 ± 7.6	35-50	> 0.05
CD8 (%)	25.4 ± 6.1	27.1 ± 7.7	21-39	> 0.05
CD4/CD8	1.5 ± 0.6	1.4 ± 0.8	1.5-2.5	> 0.05

Notes: ALT: alanine aminotransferase; AST: aspartate aminotransferase; CK: creatine kinase; CRP: C reactive protein.

Figure 1. Viral load and viral shedding of patients of or over and under 14 years old.

Notes: The patients were divided into age of or over 14 years old and under 14 years old, as well as mild and severe patients. Viral load in the nasopharyngeal-swab specimens was determined by quantitative real time PCR. The $\Delta\Delta Ct$ value of viral load was transformed into copy number as described in Materials and Methods above, with copy number of viral load converted into $\log_{10}$. Viral load of 50 copies ($1.7 \log_{10}$) is defined as a cut-off value. Comparison of the peak viral loads (A) and viral shedding periods (B) between the mild patients under 14 years old vs. over 14 years old. Comparison of the peak viral loads (C) and viral shedding periods (D) between the mild patients and severe patients.

d, $P < 0.01$) (Figure 1C and Figure 1D).

Oseltamivir can significantly decrease the viral shedding period

Before oseltamivir treatment, in the mild patients, the viral loads of the patients who received oseltamivir were compared with those patients without oseltamivir treatment (Figure 2A). In order to avoid a bias caused by an age factor, patients under 14 years old were excluded from this comparison. The duration of viral shedding of the patients receiving oseltamivir within 4 days after symptoms onset was significantly reduced as compared to that of patients without oseltamivir (Figure 2B). These data indicate that oseltamivir can significantly reduced the viral shedding period. Furthermore, early oseltamivir treatment (especially within 48 h after symptoms onset) can more effectively suppress viral load than late oseltamivir treatment more than 48h after symptom onset ($P < 0.01$, data not shown). In mild patients, 85.7% patients cleared the virus on day 5 after oseltamivir treatment and the viral loads of all the patients became undetectable on

day 8 after oseltamivir treatment. However, in severe patients, only 50% patients cleared the virus on the 5th day after oseltamivir treatment, and 1 patient aged 7 years who suffered from severe pneumonia with acute respiratory failure didn't clear the virus until up to 20 days after oseltamivir treatment. The percentage of viral load at undetectable level in mild patients on day 5 after oseltamivir treatment was significantly higher than that in severe patients ($P < 0.01$) (Figure 2C).

IL-17⁺ T cells production in H1N1 influenza A patients by flow cytometry analysis is significantly decreased

In order to investigate which T cell subset or cytokines result in the differences in viral load and the viral shedding period among these patients, Th1, Th2, Th17 and Treg cells of the patients were determined by flow cytometry through intracellular staining (Figure 3). It was found that the percentage of IL-17⁺IFN- γ CD4⁺ T cells from the patients with H1N1 influenza A was significantly decreased, with severe patients more extensive decreased, compared with seasonal

Figure 2. Effect of oseltamivir on viral load and viral shedding of the patients.

Notes: Before oseltamivir treatment, the initial viral load of patients with oseltamivir treatment in mild patients was compared with that of patients without oseltamivir treatment (A). Oseltamivir treatment within 4 days after symptoms onset can significantly reduce the duration of viral shedding of patients compared with the patients not receiving oseltamivir (B). Because age can affect the viral load and viral shedding of the patients, those patients under 14 years old were excluded from these two groups before statistical analysis was performed by a Student *t* test. Viral clearance percentage on the 5th day after oseltamivir treatment between mild patients and severe patients (C).

flu patients and healthy subjects ($P < 0.05$) (Figure 4A). Furthermore, IFN- γ (Th1), IL-4 (Th2) and Foxp3 (Treg) productions were also measured, however, no significant differences in the IFN- γ , IL-4 and Foxp3 proportions were found among these groups (data not shown, $P > 0.05$, respectively). After the patients recovered (defined as virus elimination and disappearance of clinical symptoms), the percentage of IL-17⁺IFN- γ CD4⁺ T cells in these patients was not back to normal (Figure 4C). On the other hand, we also compared the percentage of the IL-17⁺IFN- γ CD4⁺ T cells of patients under 14 years old with that of the patients of or above 14 years old, and a statistical difference was found ($P < 0.01$) (Figure 4B). More importantly, the frequency of IL-17⁺IFN- γ CD4⁺ T cells was significantly correlated with the viral shedding period ($r = 0.42$, $P < 0.05$) (Figure 4D). This demonstrates that lower proportion of Th17 cells may partially be responsible for delayed viral clearance and higher viral load in children and severe patients.

TGF- β production detected by ELISA is significantly decreased

To determine which cytokines may result in a decrease in Th17 cells in the patients, IL-6, IFN- γ and TGF- β productions in the serum were measured by ELISA, which can promote or suppress Th17 differentiation

and expansion. It disclosed that serum TGF- β concentration from patients with H1N1 influenza A was significantly decreased, and the severe patients with a more extensive decrease in TGF- β content, compared with those patients with seasonal flu and healthy donors (Figure 5C). However, serum IFN- γ (Figure 5A) and IL-6 levels (Figure 5B) among these groups showed no significant differences ($P > 0.05$). Furthermore, the decreased serum TGF- β level of the patients correlated with the decreased frequency of IL-17⁺IFN- γ CD4⁺ T cells of the patients ($r = 0.55$, $P < 0.01$) (Figure 5D).

DISCUSSION

2009 H1N1 influenza A virus which consists of molecular features of North America and Eurasian swine, avian and human influenza viruses was recently identified.^{2,9} Antigenically the virus is homogeneous and similar to North American swine A (H1N1) viruses but distinct from seasonal human A (H1N1),¹⁰ with symptoms like seasonal influenza. In this study, we found that the duration of viral shedding and the peak viral load of children were significantly longer or higher than that of adolescent and adult patients, and severe patients with pneumonia also exhibited significantly higher peak viral load and prolonged viral shedding period compared with mild patients

Figure 3. Representative plots of IL-17 frequency by flow cytometry.

Notes: IL-17 production was assessed on CD4⁺-gated T cells (CD8⁺ T cells) by flow cytometry analysis, Th1 (IFN- γ), Th2 (IL-4) and Th17 (IL-17) as well as regulatory T cells (Foxp3) were detected by flow cytometry through intracellular staining among healthy donors, seasonal flu patients, mild and severe H1N1 influenza A patients. These were representative of 4 typical plots from 4 subjects of 4 different groups, respectively, the numbers shown in the plots represented the percentages of IL-17, IFN- γ , IL-4 and Foxp3 cells.

Figure 4. IL-17 production among the different groups by flow cytometry analysis and association of IL-17 production and the duration of viral shedding.

Notes: IL-17⁺IFN- γ ⁻CD4⁺ cells proportion among these groups was determined by flow cytometry (A). The frequency of IL-17⁺IFN- γ ⁻CD4⁺ cells among the patients under 14 years old and over or of 14 years old (B) as well as patients at acute stage and recovered stage (C). The relationship between viral shedding period and IL-17⁺IFN- γ ⁻CD4⁺ T cells frequency from mild patients (D). HD represents healthy donors and SF represents seasonal flu patients.

Figure 5. Cytokine productions in sera and association of TGF- β and IL-17.

Notes: Concentrations of IFN- γ (A), IL-6 (B) and serum TGF- β content (C) among these groups were quantified by ELISA. The relationship between TGF- β and IL-17⁺IFN- γ ⁻CD4⁺ T cells in the patients (D).

without pneumonia. This indicates higher viral loads and longer viral shedding periods can be associated with condition severity of this disease. In a ferret pathogenesis and transmission model, it was found that the 2009 A (H1N1) influenza virus was found to be more pathogenic than a seasonal A (H1N1) virus, with more extensive virus replication occurring in the respiratory tract. Replication of seasonal A (H1N1) virus was confined to the nasal cavity of ferrets, but the 2009 A (H1N1) influenza virus also replicated in the trachea, bronchi, and bronchioles.¹¹ However, we can't exclude that immune-mediated responses play a critical role in pneumonia or acute lung injury and is required to be elucidated in the future.

This study also indicates early oseltamivir treatment can significantly reduce the viral shedding period of patients consistent with previous reports on patients with highly pathogenic H5N1 avian influenza or seasonal influenza early treated with oseltamivir, in particular within 48 h after onset of the symptoms.¹²⁻¹⁶ Previous studies reported early treatment with oral oseltamivir can reduce the duration of viral shedding and viral titer of human influenza A, and also reduce symptom scores, nasal pro-inflammatory cytokine levels and secondary complications such as bronchitis and sinusitis.^{12,13,17} However, some seasonal flu patients with NA gene H274Y mutation could develop seasonal oseltamivir-resistant influenza A (H1N1) viruses.¹⁸

The frequency of Th17 cells in patients with H1N1 influenza A was significantly diminished compared

with patients with seasonal flu and healthy donors, with patients under 14 years old and severe patients more extensively decreased. However, no significant differences in the frequencies of Th1, Th2 and Treg cells were found among the patients. It suggested that H1N1 influenza A virus may cause a decrease in Th17 cells frequency of the patients. However, it's still unknown which antigens or proteins of the virus can give rise to the impaired Th17 cells response, which needs to be furtherly investigated. In addition, the frequency of Th17 cells in the patients correlates to the viral shedding period. This demonstrates that Th17 cells may play a protective role in reducing viral shedding period and viral titer. As we know, TGF- β in combination with IL-6 or IL-21, or with IL-1 and IL-23, can induce Th17 cell differentiation.¹⁹⁻²² In humans, IL-1 β as well as IL-6 or IL-23 is thought to drive the generation of Th17 cells, while IL-1, IL-23^{8,23} and IL-21²⁴ promote the production of IL-17 by memory T cells.⁷ However, IFN- γ can inhibit Th17 differentiation.²⁵⁻²⁶ Recently, it has been demonstrated that TGF- β plays a critical role in driving Th17 cell development and differentiation.²⁷⁻³¹ In this study, it was found that TGF- β content in the serum of patients with H1N1 influenza A was significantly decreased, with severe patients more extensively decreased, compared with that of seasonal influenza patients and healthy subjects. Moreover, the TGF- β level in serum of the patients correlated with the frequency of IL-17⁺IFN- γ ⁻CD4⁺ T cells of the patients. This suggests that the

impaired TGF- β production may result in the decreased Th17 cells frequency. On the other hand, whether Th17 cells can play a protective or a detrimental role in acute lung injury is required to be explored in the future, which can recruit neutrophils into lung tissue through CXCL1, CXCL6 and CXCL8 chemokines and result in lung injury.³²

Taken together, in this study, higher viral loads and prolonged viral shedding periods may be associated with younger ages or disease severity. Th17 cells-mediated immune response seems to play a part role in control of the spread of novel H1N1 influenza A.

Competing interests

The authors have no financial conflict of interests.

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Abbreviations used in this paper

WBC, white blood cell count; PBMCs, peripheral blood mononuclear cells; TGF- β , transforming growth factor-beta; ELISA, enzyme-linked immunosorbent assay; IFN- γ , interferon-gamma; Treg, T regulatory T cells; RT-PCR, reverse-transcription-polymerase-chain-reaction; CRP, C reaction protein; CK, creatine kinase; BUN, blood urine nitrogen; Cr, creatine; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

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