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Pneumonia Severity and Phase Linked to Virus-Specific T Cell Responses with Distinct Immune Checkpoints during pH1N1 Infection

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The detailed features and the longitudinal variation of influenza-specific T cell responses within naturally infected patients and the relationship with disease severity remain uncertain. In this study, we characterized the longitudinal influenza-specific $CD4^+$ and $CD8^+$ T cell responses, T cell activation, and migration-related cytokine/chemokine secretion in pH1N1-infected patients with or without viral pneumonia with human PBMCs. Both the influenza-specific $CD4^+$ and $CD8^+$ T cells presented higher responses in patients with severe infection than in mild ones, but with distinct longitudinal variations, phenotypes of memory markers, and immune checkpoints. At 7 ± 3 d after onset of illness, effector $CD8^+$ T cells ($CD45RA^+CCR7^-$) with high expression of inhibitory immune receptor CD200R dominated the specific T cell responses. However, at 21 ± 3 d after onset of illness, effector memory $CD4^+$ T cells ($CD45RA^-CCR7^-$) with high expression of PD1, CTLA4, and LAG3 were higher among the patients with severe disease. The specific T cell magnitude, T cell activation, and migration-related cytokines/chemokines possessed a strong connection with disease severity. Our findings illuminate the distinct characteristics of immune system activation during dynamic disease phases and its correlation with lung injury of pH1N1 patients. *The Journal of Immunology*, **2022, 208: 2154–2162.**

The prevalence of the 2009 pandemic influenza A (H1N1) virus (pH1N1) has been continuously sustained for several seasons worldwide since arising in April 2009 (1). This virus has resulted in thousands of confirmed cases with >18,500 confirmed deaths in >200 countries from April 2009 to August 2010 (2). The most common cause of death is viral pneumonia (3, 4). Additionally, the most prominent histopathological feature observed during autopsy was diffuse alveolar damage in the lung (5, 6). However, the lung injury–associated ingredients and causations, which could guide the diagnosis and treatment of severe influenza virus infections, are still largely unknown.

Both $CD4^+$ and $CD8^+$ T cells play a critical protective role in the host defense against influenza viruses (7, 8). Wilkinson et al. (9) found that pre-existing $CD4^+$ T cells responding to influenza internal proteins were associated with lower virus shedding and less severe illness after influenza challenge in humans. In a prospective cohort study involving natural infections by pH1N1 (10), individuals who developed mild or no symptoms after pH1N1 influenza infection were found to have higher circulating levels of pre-existing influenza-specific $CD8^+$ effector memory T cells.

However, an impaired or exaggerated host immune response could be detrimental. There is evidence that dysregulation of immune cells and early inflammatory mediators can contribute to lung immunopathology during pH1N1 infections (11). The extravasation of T cells and uncommon release of inflammatory mediators have been observed in the lungs and peripheral blood of pH1N1-infected patients (12). In humans, Ag-specific $CD4^+$ T cell counts in blood correlated with disease severity during pH1N1 virus infection (13). Meanwhile, an increased proportion of activated effector $CD8^+$ T cells in the circulating blood is observed as expected in cases of severe and lethal pH1N1 (14). In mouse models, disease severity directly correlates with the frequency of $CD8^+$ or $CD4^+$ T cell responses, which can be attenuated by intervention with TNF-α (15), IFN-γ (16), or CD28 antagonist (17). However, the detailed features

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B.C., G.F.G., and W.J.L. conceived and designed the study. H.L. contributed to patient enrollment and sample collection. M.Z., S.T., W.J.L., D.Z., and Y.G. contributed to ELISPOT assays, flow cytometry, and cytokine measurements. Y.L. was responsible for pH1N1 virus load detection. H.L., M.L., and C.X. contributed to clinical information collection and disease severity assessments. Data analysis was performed by W.J.L., H.L., H.Z., Y.Z., C.Q., and M.Z. B.C., G.F.G., G.W., W.J.L., H.L., and M.Z. interpreted the data and wrote the paper. B.C., G.F.G., and W.J.L. acted as overall supervisors of the study.

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Abbreviations used in this article: Ct, cycle threshold; LIS, lung injury score; MOI, multiplicity of infection; PaO_2/FiO_2, ratio of arterial partial pressure of oxygen to fraction of inspired oxygen.

and the longitudinal variation of influenza-specific T cell responses within naturally infected patients and the relationship with disease severity remain uncertain.

We recruited influenza-infected patients with different disease severity. We sought to characterize the detailed features of the Ag-specific T cells, including the phenotypes of activation/memory markers and immune checkpoints, in the patients from the early phase to late phase and the associations with the severity of viral pneumonia.

Materials and Methods

Patient enrollment

Patients were prospectively recruited from a tertiary teaching hospital in Beijing during two consecutive influenza seasons. Recruiting criteria included PCR-confirmed pH1N1 infection and age >18 y. Those who came to the hospital later than 10 d after disease onset, who refused to provide written informed consent, and those without enough blood samples were also excluded. Within the first week (day 7 ± 3) and the third week (day 21 ± 3) after symptom onset (18, 19), K_3EDTA anti-coagulated blood samples and pharyngeal swabs/sputum were collected. Simultaneously, the indices representing lung injury (e.g., ratio of arterial partial pressure of oxygen to fraction of inspired oxygen [PaO_2/FiO_2], lung injury score ([LIS, Ref. 10]) (20) were evaluated for severe patients with influenza viral pneumonia. Briefly, the LIS system comprises four different parameters reflecting the condition of the lung, including chest roentgenogram, hypoxemia, positive end-expiratory pressure, and pulmonary compliance. The final value of LIS was obtained by dividing the summary of the above scores by the number of parameters used.

Healthy adult staff and students (>18 y of age) of the University of Chinese Academy of Sciences were invited to participate as the healthy control group, within whom individuals already vaccinated for influenza or with higher Ab titers to pH1N1 (>1:40) measured by the hemagglutination inhibition assay were excluded. PBMCs were isolated from whole blood as previously described (21).

Laboratory confirmation of pH1N1 infection and virus load

RNA was extracted from pharyngeal swabs/sputum using an RNeasy mini kit (Qiagen, Hilden, Germany) within 24 h after sampling. Real-time RT-PCR experiments were performed as described previously (22).

Phenotypic staining of PBMCs and flow cytometry

PBMCs from patients were stimulated with live virus A/California/07/2009 at a multiplicity of infection (MOI) of 1.0 for 1 h and then added FBS for 3 h. Subsequently, PBMCs were incubated for an additional 18 h with GolgiPlug (BD Biosciences, 1:2000) at 37°C in 5% CO_2. Cells stimulated with medium alone or PMA were used as negative and positive controls, respectively. After stimulation, the PBMCs were stained for 30 min on ice with different surface fluorescence-labeled mAbs in FACS buffer, including CD4, CD8, CD45RA, CCR7, CD49a, CCR5, TIM3, PD1, LAG3, and CD200R. Subsequently, cells were fixed and permeabilized with BD fixation/permeabilization buffer on ice for 30 min and stained in a BD wash buffer for 30 min on ice with corresponding intracellular markers, including IL-2, IFN-γ, TNF-α, and CTLA-4. After washing, PBMCs were resuspended in FACS buffer for flow cytometry analysis (BD LSRFortessa). All of the fluorescence-labeled Abs were obtained from BD Biosciences. Data were analyzed with FlowJo software version 10.

Design and synthesis of influenza virus–derived peptide pools and ELISPOT assay

Previously identified HLA-I–restricted influenza virus epitopes (23) were retrieved from published reports as described in our previous study (24). All peptides were synthesized and purified to ~90% purity.

The Ag-specific T cell responses were detected through an IFN-γ–secreting ELISPOT assay (BD China, Beijing, China) as described previously (21). Briefly, flat-bottom, 96-well plate membranes were precoated with anti–IFN-γ mAb and incubated overnight at 4°C. Subsequently, plates were blocked with culture medium with 10% FBS for 1 h at 37°C. A total of 2.5×10^5 PBMCs from patients in 100 μl of RPMI 1640 supplemented with 10% FBS were seeded in each well. To stimulate the effector cells, peptide pools or the HLA-A2–restricted peptide GL9 were diluted in 100 μl of RPMI 1640 supplemented with 10% FBS, added to each well, and incubated at 37°C in 5% CO_2 for 18 h. PHA was added as a positive control for nonspecific stimulation. Cells incubated with medium alone were employed as a negative control. Finally, the cells were removed and the plates were processed according to the manufacturer's instructions. Spot-forming cells were counted and analyzed using an automatic ELISPOT reader.

Cytokine/chemokine measurements

Concentrations of cytokines/chemokines related to T cell activation and migration in plasma were measured with a human cytokine multiplex assay (Bio-Rad, Beijing, China).

Statistical analyses

A Student *t* test was used for comparison between two groups. For comparisons between multiple groups, a one-way ANOVA analysis with a Bonferroni posttest was performed. The Pearson correlation coefficient test was used for correlation analyses. All tests were two-tailed with a significance level of 0.05. All data analyses were performed with the use of SPSS 25.0 Mac version and the graphs were plotted with GraphPad Prism (GraphPad Software, San Diego, CA).

Ethics statement

The Research Ethics Committee of Beijing Chao-Yang Hospital approved this study (project approval number 10-KE-17), and written informed consent was obtained from all patients.

Table I. Baseline characteristics of influenza virus–infected patients and healthy controls

Characteristics	Healthy (n = 22)	Mild (n = 22)	Severe (n = 57)
Male	7 (31.8)[a]	8 (36.4)	18 (31.6)
Age, y (mean ± SD)	27.6 ± 5.2	34.4 ± 10.5	54.1 ± 18.0
Underlying diseases	0	2 (9.1)	34 (59.6)
Hypertension	0	0	14 (24.6)
Coronary heart disease	0	0	5 (8.8)
Chronic renal insufficiency	0	0	3 (5.3)
Diabetes mellitus	0	0	5 (8.8)
Bronchiectasis	0	1 (4.5)	2 (3.5)
Asthma	0	0	2 (3.5)
Chronic obstructive pulmonary disease	0	0	7 (12.3)
Main laboratory finding on admission			
WBCs ($\times 10^9$/ml)	3.5–9.5[b]	6.3 ± 1.7	6.5 ± 3.6
Neutrophils ($\times 10^9$/ml)	1.8–6.3[b]	4.8 ± 1.6	5.0 ± 3.3
Lymphocytes ($\times 10^9$/ml)	1.1–3.2[b]	1.0 ± 0.6	0.9 ± 0.6
Aspartate aminotransferase (U/L)	15–40[b]	22.6 ± 5.5	55.6 ± 58.1
Creatine (U/L)	57–97[b]	75.1 ± 14.8	94.9 ± 91.9
Secondary infection	—[c]	0	7 (12.3)
Treatment during the whole course of disease			
Oxygen requirement	—	0	51 (89.5)
Invasive mechanical ventilation	—	0	15 (26.3)
ICU admission	—	0	15 (26.3)

[a]Percentages are shown in brackets.
[b]The normal range of healthy adults.
[c]A dash indicates not applicable.

Results

Patient enrollment and characteristics

In total, 79 patients with laboratory-confirmed pH1N1 infection were enrolled in the study. Patients with influenza viral pneumonia (25) were classified as the severe group; otherwise, patients were classified as the mild group. All 44 patients enrolled in the first influenza season were severe cases with viral pneumonia. Among the 35 cases enrolled in the second influenza season, 13 were diagnosed as severe cases, whereas the other 22 were mild cases without pneumonia and only presented influenza-like illness, such as fever and a sore throat. Meanwhile, during the two influenza seasons, we recruited 6 and 16 healthy adults as controls, respectively.

Characteristics of all patients and healthy controls enrolled are summarized in Table I. The mean age of the severe patients was 54.1 y, older than the mild patients (34.4 ± 10.5 y) and the controls. Compared to the mild patients (9.1%, 2/22), 59.6% (34/57) of the severe patients had underlying diseases. Furthermore, in the severe group, >80% (51/57) of the patients needed oxygen support, 26.3% (15/57) of the patients had to receive mechanical ventilation to maintain oxygenation, 26.3% (15/57) of the patients were admitted to the intensive care unit, and 10.5% (6/57) of the patients died during the study. No significant clinical difference was observed between the severe patients enrolled during the two influenza seasons (Supplemental Table I).

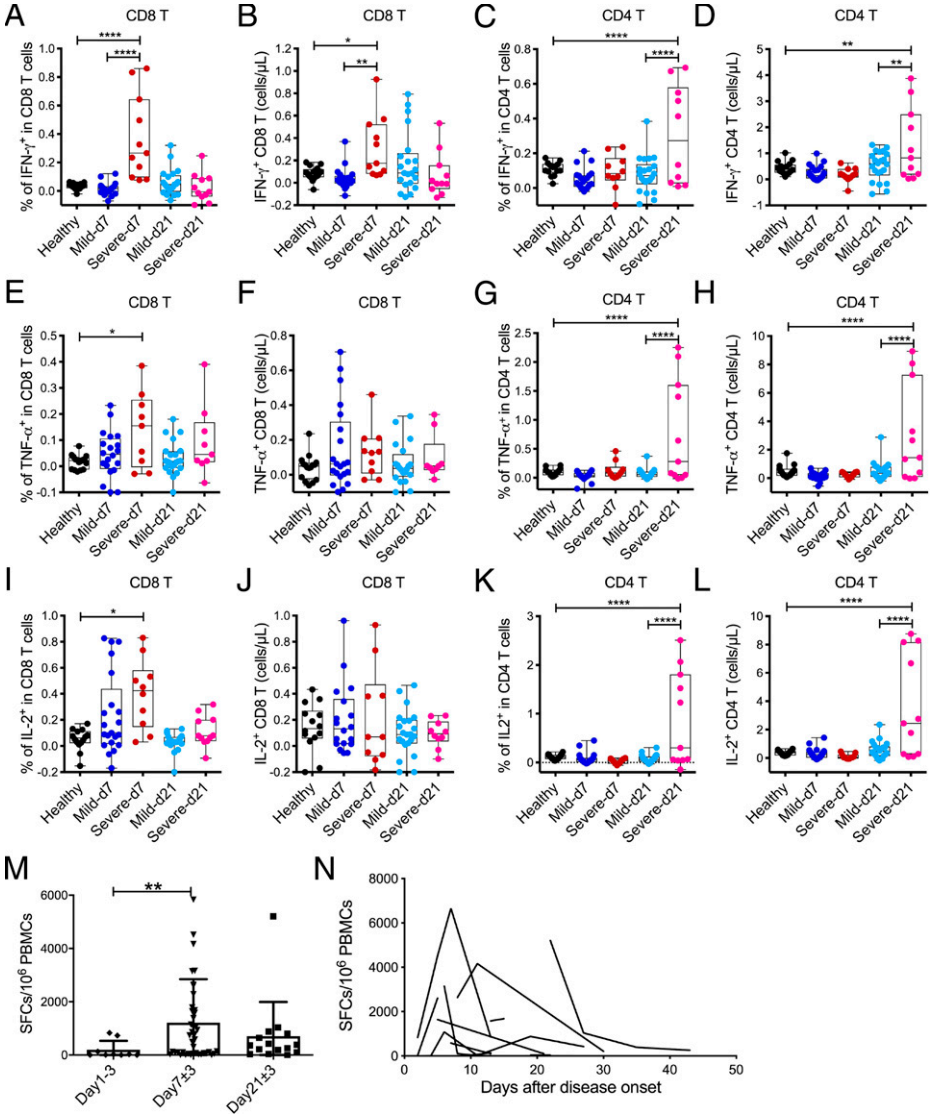


FIGURE 1. $CD8^+$ and $CD4^+$ T cell responses in pH1N1 virus–infected patients. (**A**–**L**) The frequencies of Ag-specific $CD8^+$ and $CD4^+$ T cells of the 35 patients (13 severe cases, 22 mild cases) and 16 healthy adults enrolled during the second influenza season were tested through ex vivo intracellular cytokine staining of PBMCs, under the stimulation of live pH1N1 virus at an MOI of 1.0, or no virus as the background. The numbers of these virus-specific cells were calculated according to the lymphocyte counts in the peripheral blood test, which was performed the same day that the sample was collected. The "no virus" background was subtracted for each sample in the data analysis and healthy adults were recruited contemporaneously as controls. (A, B, E, F, I, and J) Percentage (A, E, and I) and number (B, F, and J) of Ag-specific $CD8^+$ T cells that secreted IFN-γ, IL-2, and TNF-α, respectively. (C, D, G, H, K, and L) Percentage (C, G, and K) and number (D, H, and L) of Ag-specific $CD4^+$ T cells that secreted IFN-γ, IL-2, and TNF-α, respectively. (**M**) pH1N1-specific T cell responses were detected in patients ($n = 44$) enrolled in the first influenza season using IFN-γ ELIPOST assays. The $CD8^+$ T cell response levels were divided into three stages by the sampling time of the patients after disease onset (day 1–3, day 7 ± 3, and day 21 ± 3), and the error bars represent the SD. (**N**) The individual analyses of repeated sampling from similar patients showed peaks of $CD8^+$ T cell responses appearing between day 7 and day 10. Each line represents the detection of T cell responses of one patient with at least two sampling points, denoted as terminal vertexes and inflections. Statistical significance of differences between healthy controls and pH1N1 infected patients at different disease phase and with different disease severity, and between mild and severe patients at the same disease phase, was determined by a one-way ANOVA analysis with Bonferroni posttests. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Ag-specific T cell response characteristics of the pH1N1 patients with different disease severities during different disease stages

To illuminate the characteristics of the influenza-specific T cells, blood of the 35 patients and 16 healthy adults enrolled during the second influenza season was assessed via flow cytometry. The frequency of Ag-specific $CD8^+$ and $CD4^+$ T cells was tested through ex vivo intracellular cytokines IFN-γ, IL-2, and TNF-α staining of PBMCs, under the stimulation of live pH1N1 virus at an MOI of 1.0, or no virus as the background. The proportion and numbers (Figs. 1A, 1B, 1E, 1F, 1I, 1J, 2A–F) of Ag-specific $CD8^+$ T cells that secreted the three different cytokines of the severe patients had an early elevation at day 7 ± 3 after onset of illness, as compared with that of the mild patients. Detailed analysis indicated that the difference was mainly due to singly IFN-γ–secreting $CD8^+$ T subpopulation (Fig. 3A, 3B). In contrast, a late increase of IFN-γ–, IL-2–, and TNF-α–secreting $CD4^+$ T cells was observed in the severe patients at day 21 ± 3 after disease onset compared with the mild patients (Figs. 1C, 1D, 1G, 1H, 1K, 1L, 2G–L). During this phase in the severe patients, the cytokine secretion of the Ag-specific $CD4^+$ T cells presented diversified patterns with large proportions of single, double, and triple cytokine–secreting cells (Fig. 3C, 3D).

With the PBMCs harvested from the A(H1N1)pdm09 patients during the first influenza season, we determined the IFN-γ production by $CD8^+$ T cells utilizing previously well-defined HLA-I peptides (8–11 aa) spanning the entire A(H1N1)pdm09 proteome and similar results were observed. The magnitude of the overall $CD8^+$ T cell responses rapidly peaked at day 7 ± 3 after onset of illness and dropped to a lower level from the beginning of the third week after disease onset (Fig. 1G). Accordingly, based on the individual analyses of the repeated PBMC sampling from the same patients, the peaks of $CD8^+$ T cell responses appeared between day 7 and day 10, followed by a decreasing trend in the responses after day 10 (Fig. 1H).

In addition, we analyzed the memory phenotype of the IFN-γ–secreting virus-specific T cells by CD45RA and CCR7. We found that $CD45RA^+CCR7^-$ T cells represented most of the $CD8^+$IFN-γ^+ T cells in the pH1N1 patients, especially in severe patients during the acute phase (Supplemental Fig. 1A, 1B), indicating a dominant role for effector $CD8^+$ T cells in these patients. In contrast, Ag-specific $CD4^+$IFN-γ^+ T cells with the effector memory phenotype $CD45RA^-CCR7^-$ were dominant in the pH1N1 patients in both the acute and recovery phases (Supplemental Fig. 1C, 1D).

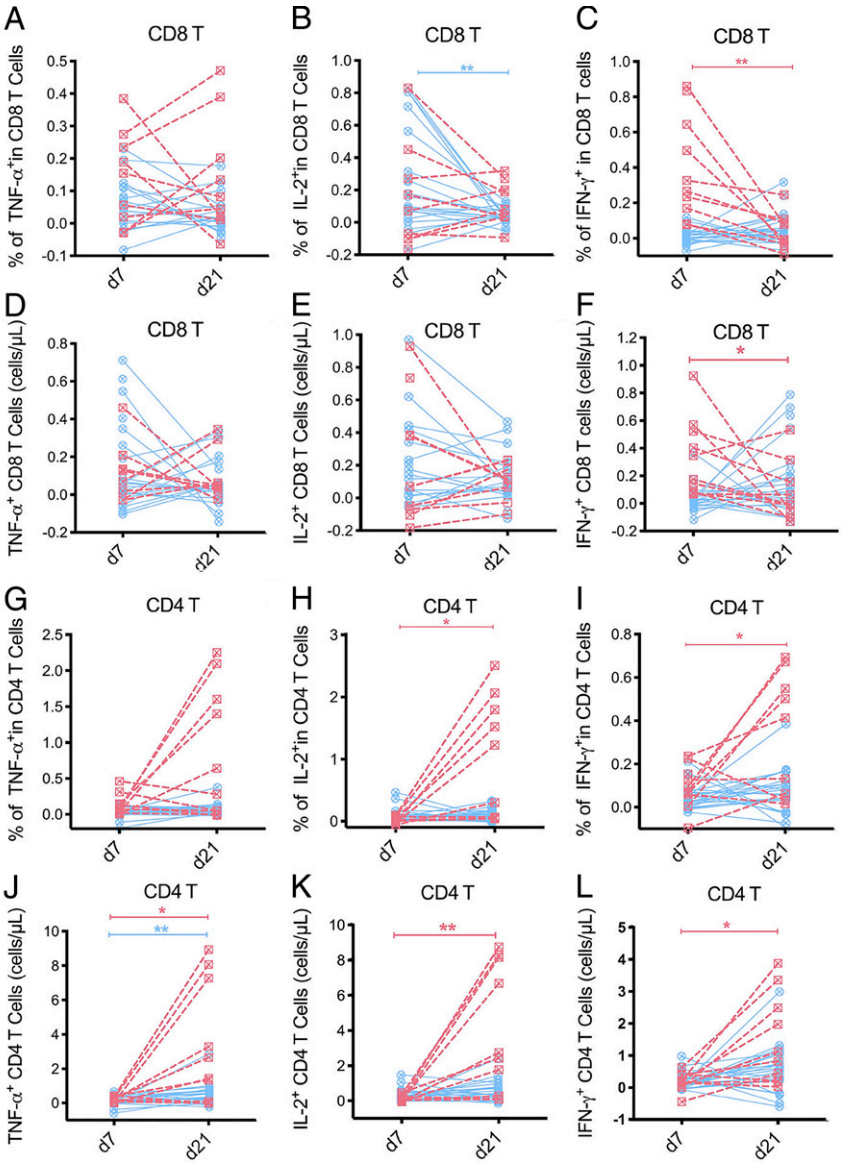


FIGURE 2. Longitudinal $CD4^+$ and $CD8^+$ T cell responses in pH1N1 virus–infected patients. The frequency and number of Ag-specific $CD8^+$ and $CD4^+$ T cells was tested as mentioned in Fig. 1. (**A–C**) The frequency of longitudinal $CD8^+$ T cell responses in pH1N1 virus–infected patients. (**D–F**) The number of longitudinal $CD8^+$ T cell responses in pH1N1 virus–infected patients. (**G–I**) The frequency of longitudinal $CD4^+$ T cell responses in pH1N1 virus–infected patients. (**J–L**) The number of longitudinal $CD4^+$ T cell responses in pH1N1 virus–infected patients. Each line indicates data of one individual patient from day 7 ± 3 after disease onset to day 21 ± 3 after disease onset, with the pink dashed line representing the severe cases and the blue line showing the mild case. A paired *t* test was performed to test the difference between day 7 and day 21 of mild cases and the severe cases group, respectively. $*p < 0.05$, $**p < 0.01$.

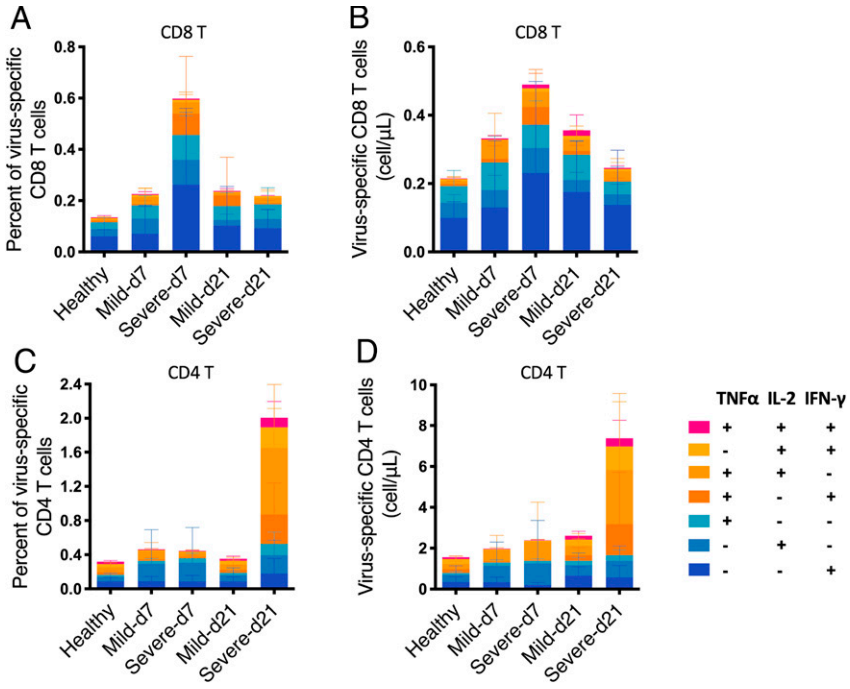


FIGURE 3. Polyfunctional populations of virus-specific $CD4^+$ and $CD8^+$ T cells. The intracellular cytokines IFN-γ, IL-2, and TNF-α were tested by flow cytometry after staining of PBMCs, under the stimulation of live pH1N1 virus at an MOI of 1.0. (**A**–**D**) The frequencies of single-positive, double-positive, and triple-positive intracellular cytokines producing $CD8^+$ (A and B) and $CD4^+$ T (C and D) cells of all patients in the mildly ill group (n = 22), severely ill group (n = 13), and healthy control group (n = 16) are plotted as stacked bars. The bar charts show mean ± SD of different groups.

Magnitude of T cell responses correlated with lung injury in severe pH1N1 patients

Considering the strong T cell responses in severe pH1N1 patients, the next step we took was to further determine the relationship between influenza-specific T cell responses and the lung injury degree of the patients with pneumonia, in combination with the influence of viral load. We collected the viral cycle threshold (26) value from real-time PCR assays of pharyngeal swabs or sputum during the acute phase (day 7 ± 3) of the 44 patients enrolled in the first influenza season, which inversely reflected the virus load in the patients (13). With PBMCs harvested contemporarily, we determined their magnitude of influenza-specific $CD8^+$ T cells by ELISPOT. Simultaneously, the indices representing the severity of lung injury (e.g., LIS, Ref. 10; PaO_2/FiO_2) (20) were evaluated for the severe patients with influenza viral pneumonia. LIS and PaO_2/FiO_2 have a trend to be correlated with virus shedding in the patients (Fig. 4A, 4B), which illuminated that the lung injury was partially induced by the influenza virus infection. The frequency of total influenza-specific $CD8^+$ T cells correlated with LIS and PaO_2/FiO_2 (Fig. 4C, 4D).

Further flow cytometry data analysis with the 13 patients with pneumonia collected during the second influenza revealed similar correlation trends between an influenza-specific $CD8^+$ T cell response at day 7 ± 3 after disease onset and disease severity indexes (Fig. 4E, 4F). Also, severe patients presented a higher influenza-specific $CD4^+$ T cell response at day 21 ± 3 after disease onset (Fig. 4G, 4H). Nevertheless, due to the small sample size, the correlation was not statistically significant.

Functional status of $CD4^+$ and $CD8^+$ T cells during pH1N1 infection

To further investigate the functional status of the $CD4^+$ and $CD8^+$ T cells, we detected the expression of inhibitory immune receptors, including PD1, CTLA4, LAG3, TIM3, and CD200R in the T cell populations from the 35 patients enrolled during the second influenza season, as well as the contemporarily collected 16 healthy controls, respectively. For the general population of $CD8^+$ T cells in the patients, consistently elevated expression of CD200R at both day 7 ± 3 and day 21 ± 3 after disease onset was observed in the severe patients. The expression of CTLA4, LAG3, and PD1 on $CD8^+$ T cells remained normal in both mild and severe patients, whereas the TIM3 expression had a minor debasement, especially in the severe patients at day 21 ± 3 after disease onset (Fig. 5A–E).

In contrast, the inhibitory immune receptor expression profile was totally distinct on the general $CD4^+$ T cells from the patients. The CD200R had no significant variation in either mild and or severe patients compared with that in the healthy controls. Notably, the expression levels of CTLA4, LAG3, and PD1 were all highly expressed on $CD4^+$ T cells in severe patients at day 21 ± 3 after disease onset compared with those in the mild patients. Similar to the TIM3 on $CD8^+$ T cells, the TIM3 expression on $CD4^+$ T cells also had a decreased trend in both mild and severe patients (Fig. 5F–J).

T cell–associated cytokines/chemokines

Cytokines/chemokines relating to the generation and migration of activated T cells of severe pH1N1-infected patients enrolled during the first influenza season were measured and we found that multiple plasma cytokines/chemokines of pH1N1-infected patients significantly increased compared with those of healthy controls (Fig. 6A–H). Among these inflammatory mediators, IP-10 (CXCL10), MIG (CXCL9), IL-8 (CXCL8), and eotaxin, which act as the chemoattractants, were associated with short peptide-specific $CD8^+$ T cell responses (Fig. 6I). Similarly, the T cell activation-related cytokines IL-9, IL-15, IL-18, and IFN-γ also displayed a linear relationship with the Ag-specific T cell responses in the patients. Further analysis indicated that most of these cytokines/chemokines were correlated with the LIS. The plasma levels of IP-10, MIG, eotaxin, IL-9, and IL-15 were inversely related to the cycle threshold (Ct) value of the real-time PCR for influenza virus in the patients. Our data suggested that the elevation of some cytokines/chemokines in plasma of pH1N1-infected patients were correlated with the virus. Additionally, the association of T cell function-related mediators and lung injury provides additional evidence for the correlation between the high virus-specific T cell responses and lung injury in the patients.

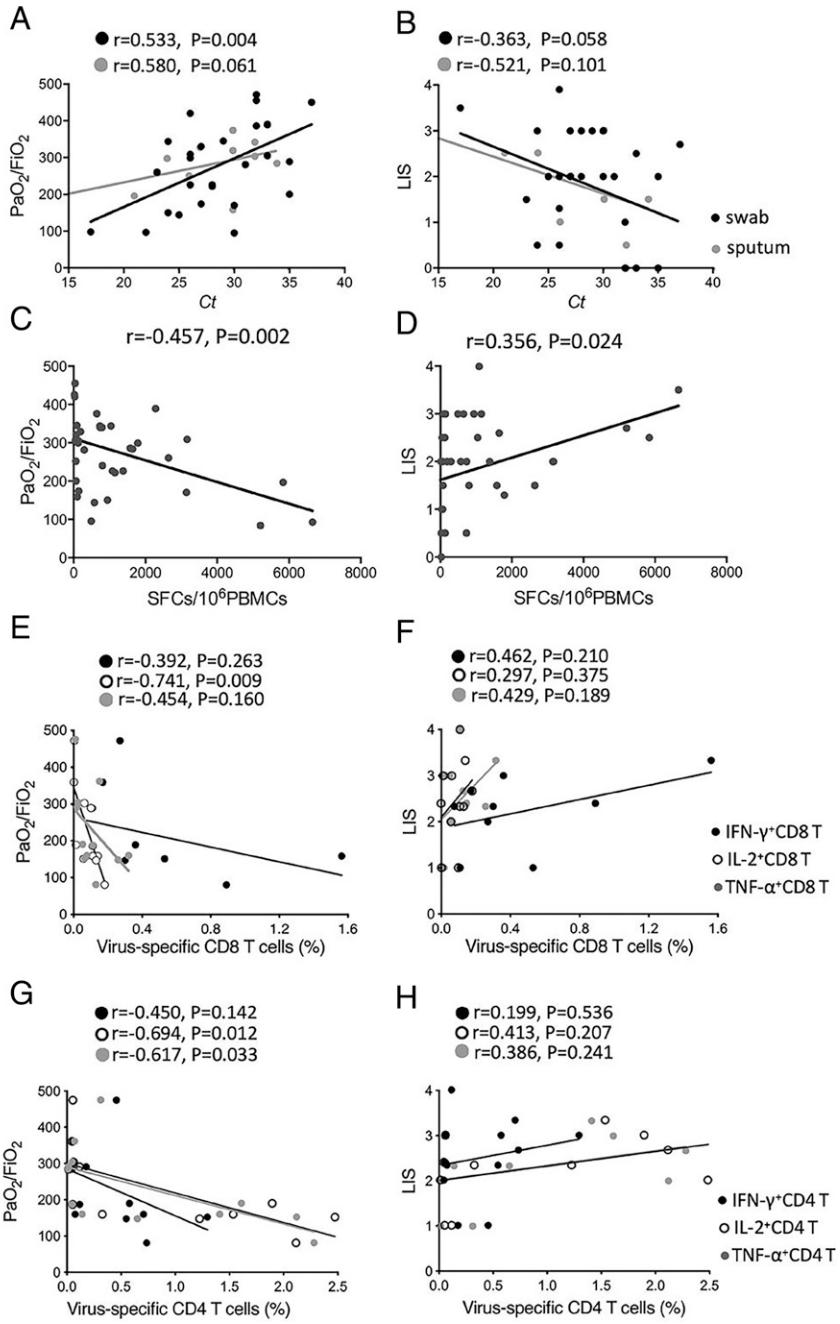


FIGURE 4. Correlation between viral loads, Ag-specific T cell responses, and lung injury. At day 7 ± 3 after disease onset of the influenza virus infection, all Ct values of influenza virus shedding in patients were collected based on RT-PCR tests using throat swabs (n = 32) or sputum (n = 10). (**A** and **B**) Patients' Ct values of throat swabs or sputum were analyzed, respectively, for the correlation with the indices reflecting lung injury, that is, LIS (A) and PaO_2/FiO_2 (B). pH1N1-specific $CD8^+$ T cell responses during the acute phase were detected in all 44 patients enrolled in the first influenza season using IFN-γ ELISPOT assays. (**C** and **D**) Correlations between influenza virus-specific $CD8^+$ T cell responses and the indices of lung injury were analyzed with Pearson correlation tests. (**E** and **F**) IFN-γ–, TNF-α–, and IL-2–secreting influenza-specific $CD8^+$ T cells at day 7 ± 3 after disease onset were assessed with flow cytometry in the 13 severe influenza patients enrolled during the second influenza season. Specific T cell magnitudes were analyzed with the lung injury–related indexes (LIS and PaO_2/FiO_2). (**G** and **H**) TNF-α–, IFN-γ–, and IL-2–secreting influenza-specific $CD4^+$ T cells at day 21 ± 3 after disease onset were assessed as correlated to lung injury indexes.

Discussion

We studied the nature of influenza-specific T cell–mediated immunity in pH1N1 patients and found that both the influenza-specific $CD4^+$ and $CD8^+$ T cells presented higher responses in severe patients, but with distinct longitudinal variations, phenotypes of memory markers, and immune checkpoints. We investigated the T cell response profile of the pH1N1 patients, which may benefit a better understanding of immune responses and the immunopathogenesis of influenza viral pneumonia.

Given the correlation of the immune characteristics with parameters representing disease severity of the pH1N1-infected patients (22, 27), the robust $CD8^+$ T cell responses in acute phase, the late $CD4^+$ T cell responses, and overwhelmed cytokine levels in the patients may relate to the immunopathogenesis and disease severity. Although sustained innate immunity contributed to the pathology in high pathogenic influenza virus infections, adaptive immunity played a vital role in the pathogenic and recovery period (24, 28, 29). However, different key orchestrators of immunopathogenesis, including underlying comorbidities, pre-existing anti-influenza immunity, innate immunity, and host genetic background, which have been identified previously, may help to explain the disease severity and lung injury (30, 31). In this study, the underlying medical conditions of the patients were analyzed, including lung, heart, liver, renal, cerebrovascular, and other neoplastic diseases. Patients with chronic respiratory diseases (including chronic obstructive pulmonary disease, asthma, bronchiectasis, and pulmonary fibrosis) had relatively higher viral loads and influenza-specific $CD8^+$ T cell responses (Supplemental Fig. 2), whereas other comorbidities had no effect on these parameters (data not shown). In our study, we found that IFITM3 single-nucleotide polymorphism rs12252 may contribute to susceptibility for rapid replication of the virus in the lung, although with no significant difference, which might be due to the limitation of sample size. It is reasonable to deduce that underlying medical conditions, for example, chronic lung diseases and genetic background of

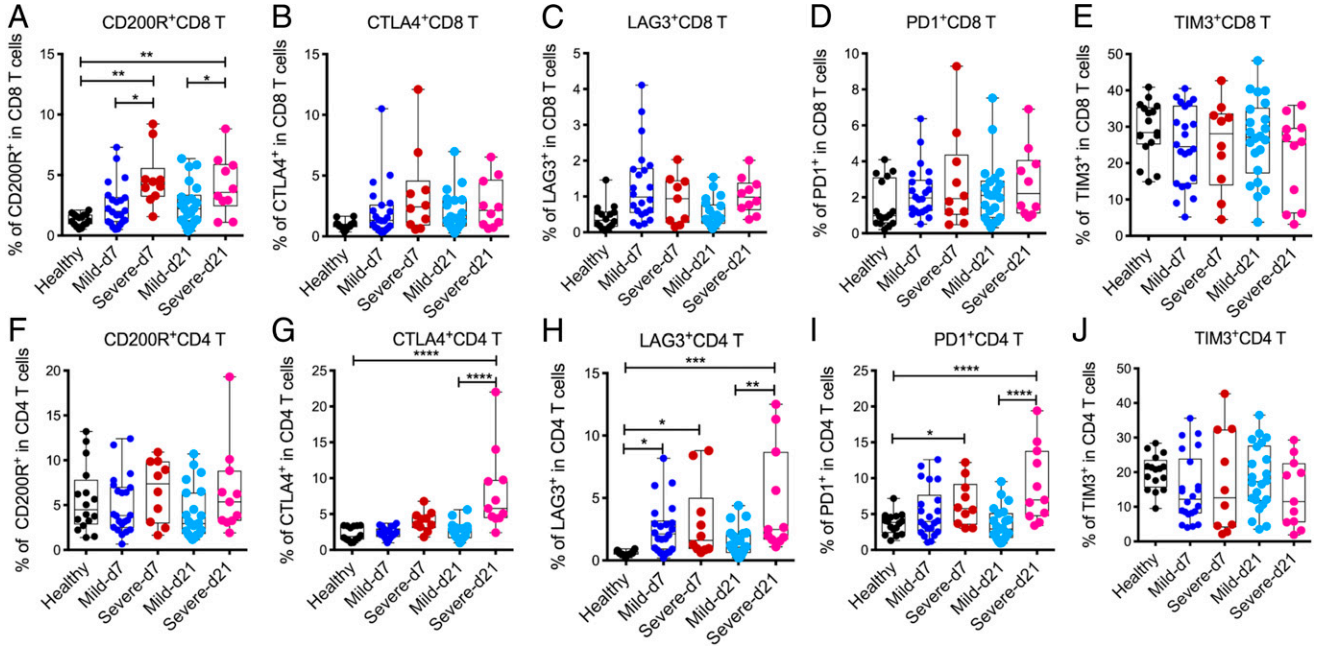


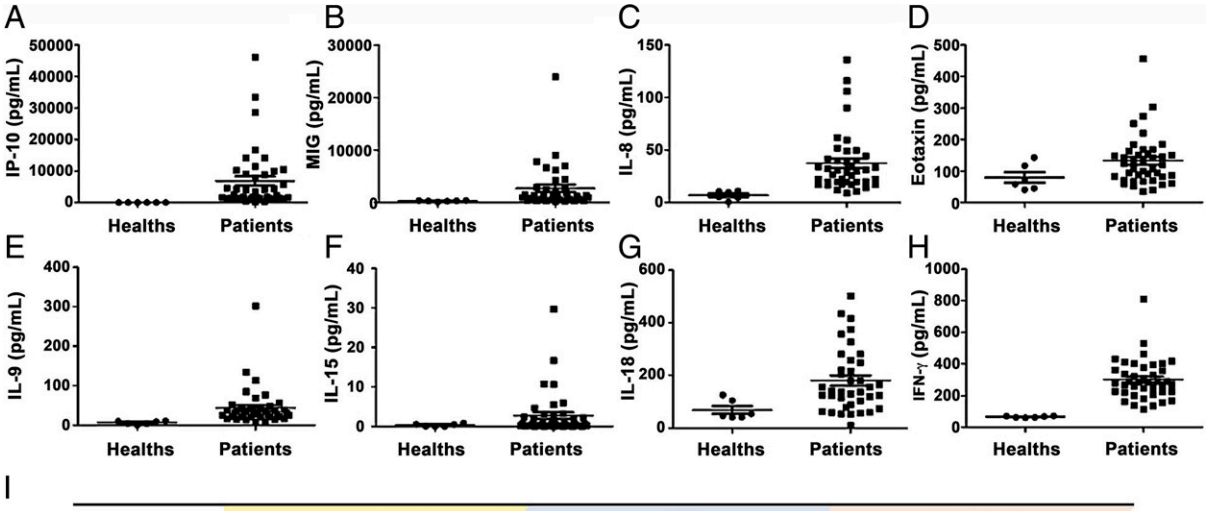
FIGURE 5. The expression of inhibitory immune receptors on general $CD8^+$ and $CD4^+$ T cell populations in influenza patients. Quantification of the expression level of these inhibitory immune receptors is presented as standard box-and-whisker diagrams (whiskers were minimum to maximum) with individual values represented by dots; patients in the mild group ($n = 22$), severe group ($n = 13$), and healthy controls ($n = 16$) in different disease phases are shown. (**A**–**E**) Expression of inhibitory immune receptors (CD200R, CTLA4, LAG3, PD1, TIM3) on general $CD8^+$ T cell populations in influenza patients. (**F**–**J**) Expression of inhibitory immune receptors on general $CD4^+$ T cell populations in influenza patients. Statistical significance of differences between healthy controls and pH1N1-infected patients at different disease phase and with different disease severity, and between mild and severe patients at the same disease phase, was determined by one-way ANOVA analysis with a Bonferroni posttest. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

the influenza patients, may contribute to susceptibility for the rapid replication of the virus in the lung and thus induce robust influenza-specific T cell responses. In our previous studies on patients of H7N9 avian influenza virus infection and patients of SARS-CoV infection, we found that elevated plasma levels of selective cytokines in the patients reflect viral load, lung injury, and even fatal outcome (32, 33). Meanwhile, the pre-existing anti-influenza immunity may also play a pivotal role in the immune response profile during the acute infection phase. It is indicated that pre-existing influenza-specific $CD4^+$ and $CD8^+$ T cells correlate with disease protection against influenza challenge in humans (9, 10). However, the causal relationships between these elements during the influenza virus infections are more complicated and need further exploration.

It is well known that many of the effector functions of $CD4^+$ T cells essential for host antiviral defenses also have the potential to cause serious immunopathology (34). However, little is known about the involvement of $CD8^+$ T cells in lung injury of patients with influenza pneumonia. A recent study involving pH1N1-infected patients reports an unexpectedly higher level of Ag-specific IFN-γ–secreting $CD4^+$, but not $CD8^+$, T cell immunity in severely sick patients compared with those with mild illness (13). In our study, we found that influenza-specific $CD8^+$ T cells tested either via an ELISPOT assay with well-defined HLA-I peptides or with flow cytometry during the acute phase were higher in the severe patients than in the mild patients and correlated with lung injury severity of influenza patients. The T cell response plays an important role in adaptive immunity to accelerate viral clearance through the Fas/Fas ligand pathway and perforin-mediated cytolysis and cytokine secretion. However, the same antiviral mechanisms may also contribute to immunopathology. A previous study of H7N9 patients found that patients with a prominent early $CD8^+$ T response recovered faster (35). There are no significant differences in the $CD8^+$ T cell responses between the survivors and fatal cases in our A(H1N1)pdm09 patients (data not shown). This may correlate with the low mortality rate of A(H1N1)pdm09 compared with H7N9 patients, or it may reflect different immunopathogenic processes of the infections by the two influenza viruses.

Several studies suggest that effector $CD8^+$ T cells play a critical role during influenza virus infection (36), whereas the contribution of effector $CD4^+$ T cells to virus clearance is modest (37). It is indicated that previous infection-installed $CD4^+$ memory T cells are capable of producing multiple cytokines and differentiating into secondary effector T cells rapidly (34, 38). The strong responses of $CD4^+$ effector and effector memory T cells during the late phase may contribute to the delayed recovery in severe patients. A study of H7N9 patients found that the delayed recovery patients shown a more prominent $CD4^+$ T response, which is concordant with our findings in the pH1N1 patients (35). Therefore, it will be crucial to determine the optimal magnitude of T cell response for protection with the lowest cost of immunopathology for the host following influenza virus infection.

Although inhibitory immune receptors are responsible for a negative regulatory role during chronic viral infection, it is indicated that these receptors also play an important role in shaping the immune responses to acute viral infections (39–41). As the CD200/CD200R axis could negatively regulate immune responses during severe influenza infection, a high level of CD200R on $CD8^+$ T cells in severe patients during the acute phase found in our study may be the result of immune-repressing progress (33). Our results showed that not only CTLA4, but also PD1 and LAG-3, in $CD4^+$ T cells were higher in patients with pneumonia than that in mild patients or healthy controls. Block of Gal-9/Tim-3 generally triggered superior CD8 T cell and humoral immune responses in influenza A virus infection (39). Interestingly, a slight decline of TIM-3 was observed for both $CD4^+$ and $CD8^+$ T cells in the patients compared with the



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	Ct		LIS		CD8 T-cell	
	r	P	r	P	r	P
IP-10	**-0.415**	**0.028**	0.140	0.382	**0.541**	**<0.001**
MIG	**-0.476**	**0.012**	0.229	0.069	**0.400**	**0.013**
IL-8	-0.237	0.224	**0.526**	**<0.001**	**0.422**	**0.006**
Eotaxin	**-0.370**	**0.052**	**0.293**	**0.063**	**0.365**	**0.019**
IL-9	**-0.407**	**0.031**	**0.375**	**0.016**	**0.479**	**0.002**
IL-15	-0.349	0.069	**0.455**	**0.003**	**0.512**	**0.001**
IL-18	-0.209	0.295	0.087	0.604	**0.484**	**0.002**
IFN-γ	-0.160	0.416	**0.333**	**0.033**	**0.288**	**0.068**

FIGURE 6. Plasma cytokine and chemokine levels in pH1N1 patients. Plasmas at day 7 ± 3 after disease onset in patients with severe infection enrolled in the first influenza wave (n = 44) were measured for the levels of chemokines and cytokines that are related with T cell activation and migration (IL-9, IL-15, IL-18 and IFN-γ, IP-10 [CXCL10], MIG [CXCL9], IL-8 [CXCL8], and exotoxin). Six healthy controls were recruited in the same period. (**A**–**H**) The levels of chemokines and cytokines were analyzed by utilizing a human cytokine multiplex assay (Bio-Rad, Beijing, China). (**I**) Correlation of plasma cytokine/chemokine with T cell responses and disease severity by Pearson r analysis. The error bars represent the SD. $CD8^+$ T cell indicates the T cell responses determined by an ELISPOT assay.

healthy controls, indicating a distinct checkpoint role of TIM-3 in influenza virus infection. These results indicated that inhibitory receptors might act synergistically to regulate T cell responses during the influenza virus infection to dampen the immunopathology. However, the molecular mechanisms by which the inhibitory receptors control the T cell response remain unclear. Future studies are needed to develop the immune-based interventions.

In conclusion, our results shed more light on the detailed characteristics of T cell immunity during influenza virus infection and also encourage future work on the possibility of T cell immune intervention in clinical practice of severe viral infections.

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Disclosures

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