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T cell responses in symptomatic moderate patients with pandemic 2009 H1N1 influenza A virus infection

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Summary. – The response of the host immune system should be appropriate to fight against pandemic 2009 H1N1 (pH1N1) influenza A virus without causing damage to its self. T cells play an indispensable role in the fight against the virus, but have the potential to cause host immunopathological changes. A better understanding of the immunoregulation that occurs during pH1N1 infection is necessary for preventing severity of the disease. In this study, we found that a significantly higher percentage of Vδ1$^+$ T cells and increased expression of activation markers in total T cells in patients with moderate pH1N1 infection could lead to its efficient fight against the virus. On the other hand, the percentages of total and CD4$^+$ T cells were decreased along with an increased expression of exhaustion marker-Tim-3 on T cells that might suppress excessive T cell responses in the host. This tuning of T cell responses might be necessary in efficient combat against pH1N1 virus, without aggravating T cell mediated immunopathology in patients with moderate pH1N1-infection.

Keywords: pH1N1; T cells; activation; exhaustion; Tim-3

Introduction

Pandemic 2009 H1N1 (pH1N1) influenza A virus that appeared in 2009 has rapidly spread throughout the world, contributing to morbidity and between 105,700–395,600 deaths during the first year of the viral circulation (Dawood *et al*., 2012). The most common symptoms in patients with mild or moderate pH1N1 infection include fever, cough and sore throat (Dawood *et al*., 2009). Recent study showed a higher risk for serious complications and deaths in patients with pH1N1 compared to patients infected with H3N2 or influenza B (Delgado-Sanz *et al*., 2020). The main features of severe pH1N1 infection were high fever, lower respiratory tract infection, dyspnea, pneumonia, restlessness, deterioration of underlying diseases and in the critical cases, the clinical signs included respiratory failure, viral encephalitis and coma (Wu *et al*., 2013).

Both CD4$^+$ and CD8$^+$ T cells play a pivotal role in protecting the host against influenza A virus (Brown *et al*., 2004; Peiris *et al*., 2010; Topham *et al*., 1997). Upon exposure to influenza A viral infection, CD4$^+$ T cells augment CD8$^+$ T cell and B cell responses by secreting a number of cytokines, including IL2, IL4, IL5 and IFN-γ (Graham *et al*., 1994; La Gruta *et al*., 2007; Sarawar and Doherty, 1994). The CD8$^+$ T cells in turn act against influenza A virus either via direct lysis of infected cells or by the production of cytokines such as IFN-γ and TNF-α (Doherty *et al*., 1997). Besides CD4$^+$ T cells and CD8$^+$ T cells, NK cells and γδ T cells expressing Vδ2$^+$ receptor have been shown to fight against pH1N1 virus by its cytolytic activity as well as by means of soluble factors (Jegaskanda *et al*., 2019; Qin *et al*., 2011). Although the role of γδ T cells expressing Vδ1$^+$ receptor in respiratory viral diseases is lacking, anti-viral

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Abbreviations: CTLA-4 = T-lymphocyte-associated protein 4; HI = healthy individual; ILI = influenza-like illness; Lag-3 = lymphocyte activation gene 3; PBMC = peripheral blood mononuclear cells; PD-1 = programmed death 1; pH1N1 = pandemic 2009 H1N1 influenza A virus; Tim-3 = T-cell immunoglobulin and mucin domain 3

properties of Vδ1$^+$ T cells against cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human immuno-deficiency virus (HIV) have been previously described (Fausther-Bovendo *et al*., 2008; Knight *et al*., 2010; Orsini *et al*., 1994; Siegers and Lamb, 2014). Upon pathogenic challenge, Vδ1$^+$ T cells release copious amounts of cytokines such as IFN-γ and IL17, and show potent cytotoxic effector function (Fenoglio *et al*., 2009; Halary *et al*., 2005). Paradoxically, the potential immunosuppressive role of Vδ1$^+$ T cells with increased expression of regulatory molecules, such as FoxP3, CD25 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and corresponding production of anti-inflammatory cytokine-TGF-β have been reported (Hua *et al*., 2013; Kuhl *et al*., 2009). Thus, Vδ1$^+$ T cells can play the dual role of combating infectious diseases and immunoregulatory functions.

An excessive and dysregulated immune reaction to viral infection could be detrimental to the host if not appropriately controlled. The pro-inflammatory cytokines such as interferons, IL6 and TNF-α produced as a result of infection with influenza virus contributes to the systemic effect of fever, and have been shown to be correlated with severity of illness in the patients (Guo *et al*., 2017; Peiris *et al*., 2009, 2010). Additionally, these cytokines promote secretion of chemokines resulting in recruitment of immune cells and subsequent amplified inflammatory response in the internal organs, such as lungs and central nervous system. For instance, pulmonary immunopathology has been shown to be mediated by CD8$^+$ T cells in response to influenza HA antigen (Xu *et al*., 2004). A significantly higher expression of several cytokines, including IL17, IL8, IL15, IL12 and IL6 has also been observed in critical patients severely infected with the pH1N1 virus (Bermejo-Martin *et al*., 2009; Keshavarz *et al*., 2019). Intranasal administration of mice with mouse-adapted strain of pH1N1 has been shown to induce acute lung injury due to Th1 polarization with an increased IFN-γ production accompanied by infiltration of macrophages to the lungs (Liu *et al*., 2019). Other studies have highlighted a strong correlation between TNF-α neutralizing antibodies and decreased pathological severity associated with prolonged survival in influenza infected mice (Hussell *et al*., 2001; Peper and Van Campen, 1995). Additionally, the release of damage associated molecular patterns (DAMPs), including mitochondrial DNA, formyl peptides and cardiolipin from injured tissue of the lungs impairs lung structure and functions (Ray *et al*., 2010; Zhang *et al*., 2010). This series of events which leads to hyperinflammation and immune dysregulation in pH1N1 infection causes development of acute lung injury (ALI) or other pathological conditions such as sepsis or multiple organ failure and even death.

Immune response to pH1N1 virus is therefore a highly complex phenomenon that is known to result in efficient virus clearance, but excessive immune responses can be detrimental to the host. A proper immunoregulation is necessary for the host, and persistent antigenic challenge is linked to the phenomenon of 'immune exhaustion' that has been associated as one of the possible mechanisms to suppress the immune response (Gogoi *et al*., 2015; Wherry, 2011). Immune exhaustion is characterized by upregulation of inhibitory receptors such as T-cell immunoglobulin and mucin domain 3 (Tim-3), lymphocyte activation gene 3 (Lag-3), CTLA-4 and programmed death 1 (PD-1) on T cells, resulting in its decreased effector functions (Gogoi *et al*., 2015). Although T cell exhaustion is a major hurdle for the treatment of chronic viral diseases and cancer, it also averts collateral tissue damages that can occur due to excessive immune reaction (Cornberg *et al*., 2013; Speiser *et al*., 2014; Waggoner *et al*., 2012).

In the present study, T cell response in patients with moderate pH1N1-infection has been addressed. We characterized T cells in pH1N1-infected patients and found that the frequencies of total circulating T cells as well as CD4$^+$ T cells were significantly lower whereas the percentage of circulating Vδ1$^+$ T cells was significantly higher in these patients. Activation of T cells is marked by higher expression of activation markers such as CD69 and CD25 with the commencement of their effector functions (Gogoi *et al*., 2014). We found that pH1N1 infection is associated with significant increase in both early (CD69) and late (CD25) activation markers. Interestingly, we also observed a remarkable increase in the expression of Tim-3 in pH1N1-infected patients. The increased expression of exhaustion marker-Tim-3 might be playing a role in suppressing immunopathological changes associated with excessive activated T cells.

Materials and Methods

Study group. Peripheral blood samples of patients and controls were collected from hospitals and residents of Dibrugarh district of Northeast India. Outpatients having mild/moderate influenza-like illness (ILI) aged 25–36 years without any history of major preexisting diseases and no or modest respiratory insufficiency were considered for the study. The symptoms include fever, sore throat with or without cough, nasal discharge, headache/body ache and fatigue. The samples were collected within three days after the onset of ILI. All patients were confirmed for pH1N1 infection by testing throat/nasal swabs using RT-PCR. Age and sex-matched healthy individuals with no history of fever in the previous three months of recruitment and without any history of major illness were considered as controls. The study was approved by the institutional Ethics Committee, and written informed consent was obtained from the participants before collection of the samples.

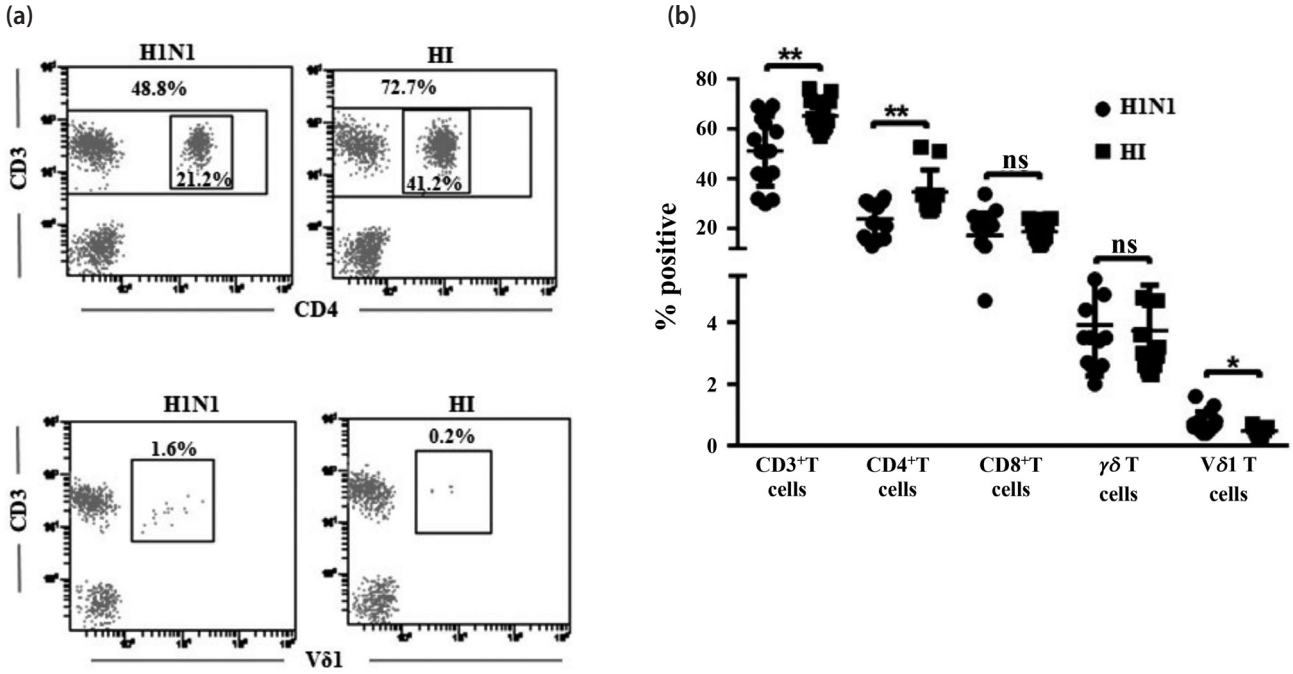

Fig. 1

Characterization of T cells and its subsets in PBMCs from pH1N1 infected patients and healthy individuals (HI)
(a) Representative density plot showing expression of $CD3^+$, $CD4^+$ T cells (top) and $V\delta1^+$ T cells (bottom) in pH1N1 patients and HI. **(b)** Expression of $CD3^+$ T cells and $CD4^+$ T cells were significantly decreased and $V\delta1^+$ was significantly increased without any significant changes in $CD8^+$ and total γδ T cells in pH1N1 positive cases compared to HI. The Mann-Whitney test was used as the test of significance (*p <0.05, **p <0.005).

RNA extraction and real time PCR. Nasal and throat swab specimens were collected in HiViral transport medium (Himedia, Mumbai, India) from ILI cases. The swab specimens were transferred to the laboratory under cold chain conditions. In the laboratory, the samples were vortexed and centrifuged at 1000 rpm for 10 min following which 140 µl of supernatant was used for the extraction of viral RNA using commercially available QIAamp Viral RNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Next, the samples were tested for pH1N1 using real-time reverse transcription polymerase chain reaction (RT-qPCR). Samples were analyzed using AgPath-ID™ One-Step RT-PCR reagents (Applied Biosystems, Foster City, USA). The sequences of the forward and reverse primers used for PCR amplification of the pH1N1 influenza virus are: F: GTGCTATAAACACCAGCCTYCCA, R: CGGGATATTCCTTAATCCTGTRGC and for probe: 6-FAM-CAGAATATACA"T" CCRGTCACAATTGGARAA-BHQ1. RT qPCR was carried out by Step One Plus system (Applied Biosystems).

Human PBMC separation. Peripheral blood mononuclear cells (PBMCs) from pH1N1-positive patients and pH1N1-negative healthy individuals as confirmed by RT-qPCR were isolated by Ficoll-Hypaque (Sigma-Aldrich, USA) density gradient centrifugation. Cells were suspended in FACS buffer (0.01 M PBS pH-7.4, 1% FCS, 0.01% sodium azide) for subsequent use.

Flow cytometry. PBMCs were stained for surface markers with the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8, anti-γδ TCR, anti-CD69, anti-CD25, anti-CTL-A4, anti-PD-1 (BD Bioscience, USA), anti-Vδ1, anti-Tim-3 and anti-Lag-3 antibodies (Miltenyi Biotec, Germany). After staining the PBMCs with required antibodies for 45 min at 4°C, cells were washed and intensity of fluorescence was measured using flow cytometer (FC500, Beckton Dickinson). The cells were gated on the basis of their forward and side scatter characteristics and the fluorescence intensity was measured. Cells were analyzed using FC500 software.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5.0. The Mann-Whitney test was used as the test of significance (p-value = 0.05 or less).

Results

Characterization of peripheral T cell subsets in pH1N1 positive patients and healthy individuals

The expression of T lymphocytes and its subsets were determined in PBMCs from 15 pH1N1-positive patients and 15 healthy individuals. The percentage of T lympho-

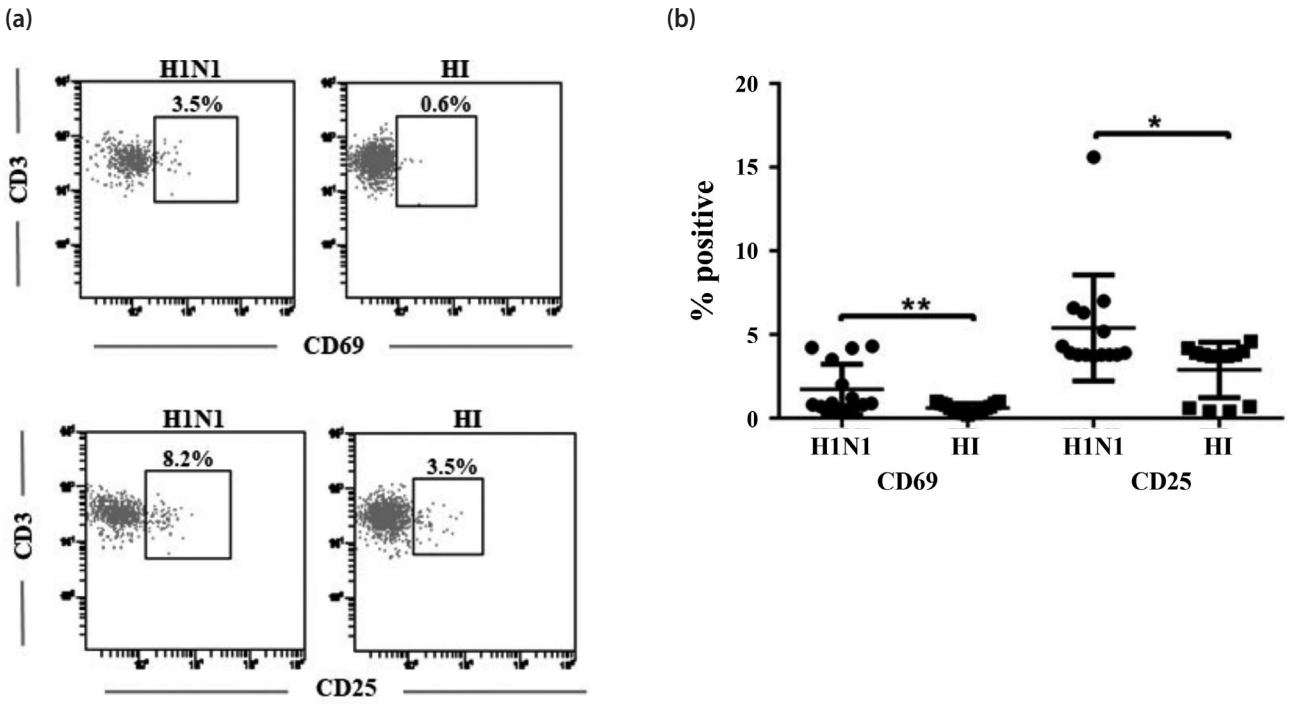

Fig. 2

Expression of early (CD69) and late (CD25) markers on CD3⁺ T cells

(a) Representative figure shows expression of CD69 and CD25 on CD3⁺ T cells from pH1N1-positive patients and HI. **(b)** The expression of both early (CD69) and late (CD25) activation markers was significantly higher in CD3⁺ T cells from pH1N1 positive cases. *p <0.05, **p <0.005 by the Mann-Whitney test of significance.

cytes and its subsets were measured using specific markers for total T cells ($CD3^+$), T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), γδ T cells (γδ TCR) and Vδ1 T ($V\delta1^+$ TCR) cells by flow cytometry. Representative flow cytometry data showed lower percentages of $CD3^+$ T cells in pH1N1-infected patients (48.8%; Fig. 1a) compared to that of healthy individuals (72.7%; Fig. 1a). Likewise, we found decreased percentage of $CD3^+CD4^+$ T cells (21.2%, Fig. 1a) in pH1N1-infected patients in contrast to $CD3^+CD4^+$ T cells (41.2%, Fig. 1a) of healthy individuals. $V\delta1^+$ T cells comprise a minor population of peripheral blood lymphocytes in healthy individuals (0.2%, Fig. 1a), but the percentage of $V\delta1^+$ was found to be increased in pH1N1-infected individuals (1.6%, Fig. 1a). Overall, the frequency of $CD3^+$ T and $CD3^+CD4^+$ T cells were significantly lower in pH1N1 positive patients ($p < 0.005$ and $p < 0.05$ respectively; Fig. 1b). In contrast, a significantly higher $CD3^+V\delta1^+$ population in patients with pH1N1 compared to healthy individuals was observed ($p < 0.05$; Fig. 1b). There were no significant differences between the frequencies of $CD3^+CD8^+$ T cells and γδ T cells in healthy individuals and pH1N1 positive patients (Fig. 1b). The data thus suggested that mild/moderately infected pH1N1 patients had lower levels of $CD3^+$ and $CD4^+$ T cells but higher $V\delta1^+$ T cells.

Activation status of T cells in pH1N1-infection

To determine the effect of pH1N1 infection on the activation status of $CD3^+$ T cells, we assessed the expression of early and late activation markers, CD69 and CD25, respectively. As shown by the representative flow cytometry dot plots (Fig. 2a), expression of CD69 and CD25 on $CD3^+$ T cells from one of the pH1N1-infected patients were 3.5% and 8.2% respectively. Whereas, in case of healthy individuals, the expression of early and late activation markers on $CD3^+$ T cells were 0.6% and 3.5%, respectively. The overall frequency of both CD69 and CD25 were significantly higher in patients with pH1N1 compared to healthy controls ($p < 0.005$ and $p < 0.05$ respectively, Fig. 2b). Thus, pH1N1 infection results in activation of peripheral blood T lymphocytes that are required for mounting an effective immune response to the virus.

Higher expression of Tim-3 on T cells from pH1N1-infected patients

T cell exhaustion occurs to minimize tissue damage while still reconciling a critical level of pathogen clearance (Speiser *et al*., 2014). We have looked for the expres-

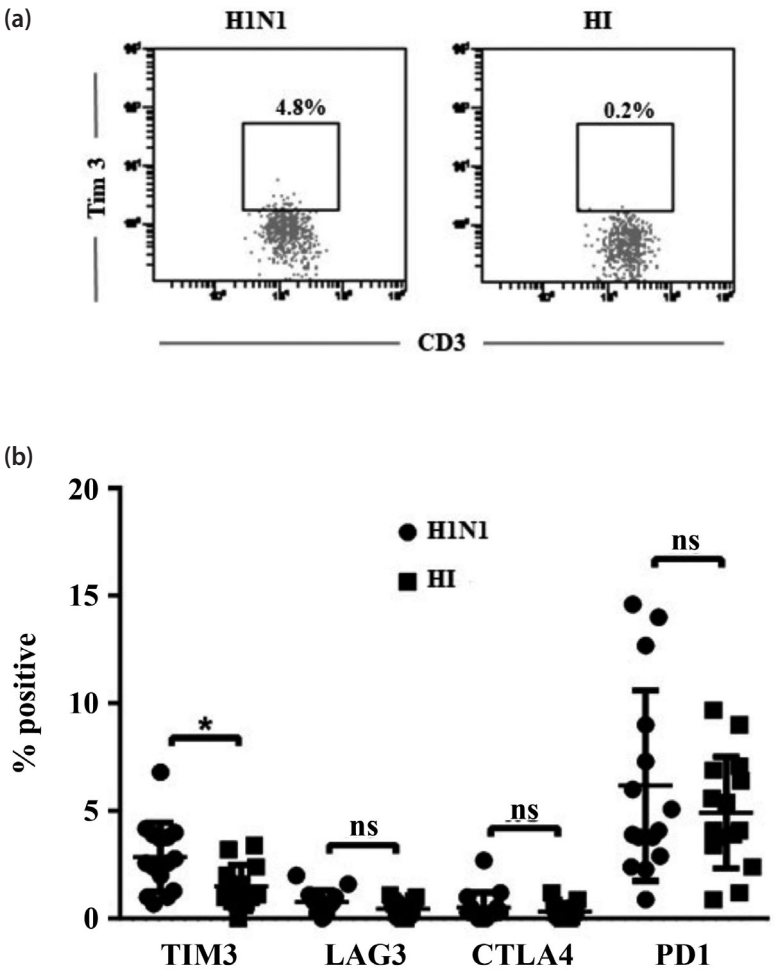

Fig. 3

Analysis of exhaustion markers on CD3⁺ T cells
(a) Representative figure showing expression of Tim-3 on CD3+ T cells from pH1N1 infected patient and HI. **(b)** Comparison of various exhaustion markers on CD3+ T cells from pH1N1 positive cases and HI. Tim-3 expression was significantly upregulated in pH1N1 infected patients; the Mann-Whitney test was used as the test of significance (*p <0.05).

sion of four different types of exhaustion markers, Tim-3, Lag-3, CTLA-4 and PD-1 on CD3⁺ T cells from mild/moderate pH1N1-positive cases. As shown in the representative Fig. 3a, the expression of Tim-3 was upregulated in CD3⁺ T cells of pH1N1-infected patients (4.8%) in contrast to that of healthy individuals (0.2%). In general, the expression of Tim-3 was significantly increased in T lymphocytes from pH1N1-positive patients compared to healthy individuals ($p < 0.05$, Fig. 3b). With respect to other markers for T cell exhaustion (Lag-3, CTLA-4 and PD-1), there were no significant changes in their expression pattern (Fig. 3b). Accordingly, the increased expression of Tim-3 on CD3⁺ T cells during pH1N1 infection might prevent immunopathological changes associated with aggressive T cell responses.

Discussion

The pathogenesis of pH1N1 influenza A virus remains poorly understood. An efficient immune response is required to clear pH1N1 viral load, on the contrary, an excessive immune reaction is harmful to the host. The cellular branch of immune response is involved in the clearance of influenza A virus, but it may also contribute to immunopathology and self-injury (Peiris *et al.*, 2010). We therefore found it relevant to characterize different subsets of T cells, its activation status and also the expression pattern of exhaustion markers in pH1N1-infected patients.

In the present study, we have analyzed the frequency of total T (CD3⁺), CD4⁺ and CD8⁺ T cells as well as γδ⁺ and Vδ1⁺ T cells in patients with pH1N1 moderate infection. The percentage of CD4⁺ T cells have been previously reported to be significantly lower in severe pH1N1 patients in comparison to mild cases without any noteworthy difference in the frequency of CD8⁺ T cells (Guo *et al.*,

2011). We found that the percentage of circulating total T cells and $CD4^+$ T cells were significantly lower in pH1N1 patients in comparison to the healthy individuals. Although both $CD8^+$ T and γδ T cells expressing $V\delta2^+$ receptor is known to act against influenza A virus, these cells were not altered in the patients. The γδ T cells expressing $V\delta1^+$ T cells are predominantly found at mucosal sites and respond to virally-infected cells (Gogoi and Chiplunkar, 2013; Halary *et al*., 2005; Shin *et al*., 2005). $V\delta1^+$ T cells constitute a minor subset of peripheral blood lymphocytes that have been shown to be expanded and act against viruses such as HIV, CMV and EMV (Autran *et al*., 1989; Fujishima *et al*., 2007; Halary *et al*., 2005). On the contrary, $V\delta1^+$ T cells can exhibit potent immunoregulatory role by secreting anti-inflammatory cytokines (Hua *et al*., 2013; Kuhl *et al*., 2009). An important finding in this report is that the frequency of circulating $V\delta1^+$ T cells was significantly higher in the patients and therefore, these cells might be playing one of the possible roles of conferring protection against pH1N1 virus mediated pathogenesis. The physiological role of $V\delta1^+$ in host defense and immunoregulatory function during pH1N1 infection needs further in-depth investigation.

Multiple proteins are known to be upregulated on the surface of lymphocytes during the immune activation process. Among these, the early (CD69) and late activation (CD25) markers are known to be expressed on activated T cells (Gogoi *et al*., 2014). The existence of activated T cells is crucial to control the virus infection. The expression of these markers was analyzed on $CD3^+$ T cells from pH1N1-infected patients and healthy individuals. Our data revealed that during pH1N1 infection, the patients exhibit a marked increase in both early (CD69) and late (CD25) activation markers. Although, we observed an increased expression of both early and late activation markers of T cells, but the frequency of total T cells was significantly decreased. This could be due to activation induced cell death (AICD) of T cells, akin to the cytokine mediated AICD of T cells during Ebola or classical swine fever virus diseases (Renson *et al*., 2010; Younan *et al*., 2017).

Elevated systemic levels of cytokines in response to influenza A virus has been linked to severity and high-mortality rates (Bermejo-Martin *et al*., 2009; Wang *et al*., 2014). A properly balanced immune response is indispensable to the host protection; wherein immune cells react adequately to the threat in a controlled way to avoid immunopathological changes. The pathogenicity of pH1N1 virus is associated with dysregulated and excessive immune responses (McAuley *et al*., 2015). In order to decreased excessive immune response, T cells can progress to 'immune exhaustion' wherein their effector function is adjusted to minimize tissue damage (Speiser *et al*., 2014). The observation that pH1N1 infection results in activation of T lymphocytes prompted us to look at the expression of exhaustion markers such as Tim-3, Lag-3, CTLA-4 and PD-1 on $CD3^+$ T cells from pH1N1-positive cases with mild/moderate symptoms. Interestingly, we found that the expression of exhaustion marker-Tim-3 was significantly increased in T lymphocytes from pH1N1-positive mild/moderate patients in contrast to healthy individuals. We have also found higher expression of other exhaustion markers, Lag-3, CTLA-4 and PD-1 in some of the patients compared to healthy controls, albeit statistically non-significant. The severity of illness in influenza-infected patients is linked to increased secretion of Th1 cytokines, but increased expression of Tim-3 on T cells diminishes the secretion of Th1 cytokines (Anderson *et al*., 2016; Bermejo-Martin *et al*., 2009; Hastings *et al*., 2009; Monney *et al*., 2002; Peiris *et al*., 2010). Additionally, Tim-3 expression represents an immunoregulatory mechanism to protect vital tissues of lungs (Isshiki *et al*., 2017). Thus, the increased expression of Tim-3 on T cells during pH1N1 infection might be one of the possible mechanisms of preventing immunopathological changes associated with aggressive T cell responses in patients with moderate infection. Future in-depth studies might be useful in the design of novel therapeutic Tim-3 agonist in preventing pH1N1 virus associated severe complications.

However, few limitations of this study, such as small group-level sample size, non-consideration of pH1N1 severe cases and restricting to cross-sectional study warrants further investigations to better understand the relationship between pH1N1 infection and the host immunomodulation.

In summary, the study demonstrates higher percentage of $V\delta1^+$ T cells and increased expression of activation markers on total T cells to act against the virus; contrarily, decrease in the percentages of total and $CD4^+$ T cells along with an increased expression of exhaustion marker-Tim-3 on T cells might prevent immunopathology associated with activated T cells in patients with moderate pH1N1-infection.

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