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LETTER TO THE EDITOR

Host immunity dictates influenza A(H1N1)pdm09 infection outcome in hematology–oncology patients

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Hematology–oncology patients can develop remarkably prolonged influenza virus excretion with an enigmatic wide clinical spectrum.[1–6] These patients often develop mild virus-associated symptoms and occasional bacterial or fungal pneumonic co-infections, but significant numbers of cases develop severe influenza virus-associated lower respiratory tract infection (LRTI) and acute respiratory distress syndrome (ARDS). Risk factors for influenza LRTI include profound lymphopenia, lack of early antiviral treatment and old age.[5,6] Pathogenesis of severe viral LRTI and ARDS is unclear and may include virus-induced pathology or excessive immunopathology.[7] Impaired influenza-specific host immune responses are well-established in hematology–oncology patients but surprisingly little is known about the interactions with virus-associated clinical manifestations and outcomes. The recent introduction of influenza A (H1N1)pdm09 virus in the human population with limited preexistent immunity provided the opportunity to evaluate the role of innate and adaptive host immune findings in determining virus-specific symptoms and viral clearance among hematology–oncology patients with prolonged viral excretion.

In this observational study, adult hematology–oncology patients hospitalized with $\geqslant 14$ days prolonged A(H1N1)pdm09 virus excretion between November 2009 and April 2013 were eligible for inclusion. The institutional review board approved the pre-established study protocol and informed consent forms that were obtained from all subjects. Patients or legal representatives signed informed consent for voluntary study participation and confidentiality. Permission was granted for clinical data collection, blood draw of a research specimen and immunologic studies. Exclusion criteria were patients aged < 18 years, patients deemed unfit by the treating physician (for example, owing to severe underlying bleeding disorders, religious background including Jehova's witnesses, altered mental or emotional status) and patients or legal representatives not wishing to enter the study. Medical records were reviewed for relevant clinical findings, virus-associated symptoms and outcomes. Respiratory specimens were assessed for A(H1N1)pdm09, neuraminidase gene H275Y mutation encoding oseltamivir resistance and other respiratory viruses using real-time PCR and viral culture.[8] Pulmonary imaging (chest radiography or computed tomography), bronchoalvolar lavage microbiology results and broad-spectrum antimicrobial treatment regimens were evaluated to confirm severe A(H1N1)pdm09-associated LRTI and ARDS requiring invasive mechanical ventilation and to exclude concomitant infections (viral, bacterial or fungal) and non-infectious cardiopulmonary causes (lung embolism, pneumothorax or congestive cardiac failure).

Influenza A (H1N1)pdm09 virus isolates were routinely cultured in established lines of monkey kidney cells (LLCMK2) and Madin-Darby canine kidney cells and further characterized using duplicate hemagglutinin inhibition (HI) tests to confirm antigenic similarity with the corresponding vaccine strain. Humoral responses were determined against homologous virus and reference A/California/007/09 vaccine strain using serum duplicate HI tests, turkey erythrocytes and four hemagglutinin units of virus. Seroconversion was defined as a fourfold rise of HI titers and HI titers $\geqslant 80$ were considered seroprotective. White blood cell differential counts were evaluated. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and lymphocyte subsets were quantified by flow cytometry using CD3, CD4, CD8, CD16, CD19 and CD56 fluorochrome-labeled antibodies.[9] Minimum absolute cell count references were defined for granulocytes (500/mm^3), monocytes (100/mm^3), lymphocytes (1000/mm^3), $CD3^+CD4^+$ (560/mm^3) T cells, $CD3^+CD8^+$ (260/mm^3) T cells, $CD3^-CD16^+$ and/or $CD56^+$ nature killer (NK) cells (40/mm^3) and $CD19^+$ B cells (60/mm^3). T-cell counts $\leqslant 20\%$ of minimum reference were defined as profound T-cell lymphopenia.[10] Defined surrogate antibody-dependent cell-mediated cytotoxicity (ADCC) markers included the combined presence of seroprotective HI titers with $CD16^+$ FcIgG receptor-bearing cytotoxic NK cells and monocytes in blood. The presence of virus-specific T cells was evaluated by flow cytometry. In brief, thawed PBMCs were stimulated with conserved peptides derived from the nucleoprotein and M1 protein, live influenza A/Netherlands/602/09 (H1N1)pdm09 virus or reference strain Resvir-9 (H3N2), 1 μg/ml *Staphylococcus* enterotoxin B (SEB; Sigma-Aldrich, Zwijndrecht, the Netherlands), or left untreated. The cells were permeabilized and incubated with antibodies directed against differentiation (CD3, αCD3 PerCP BD Biosciences (Breda, The Netherlands) # 345766; CD4, αCD4 Pacific blue BD Biosciences # 558116; CD8, αCD8 PECy7 eBioscience # 25-0088-42), activation (CD69, αCD69 APC BD Biosciences # 340560) and intracellular cytokine expression (IFNγ, αIFN-γ FITC eBioscience (Vienna, Austria) # 11-7319-82) markers and analyzed by flow cytometry ($\sim 1\times10^6$ cells per sample). Dead cell staining excluded cells with non-specific results. SEB was used as a positive control and to monitor functional integrity of T cells. Functional virus-specific T cells were confirmed by duplicate detection of $CD3^+CD4^+CD69^+IFN\gamma^+$ or $CD3^+CD8^+CD69^+IFN\gamma^+$ cells,[9,11] excluding profound T-cell lymphopenia. Virus- and SEB-specific T cells were calculated by subtracting $CD4^+IFN\gamma^+$ and $IFN\gamma^+CD8^+$ cell percentages observed after incubation with medium only.

Six adult hematology–oncology patients (age, range 39–67 years) hospitalized with prolonged A(H1N1)pdm09 excretion (duration, 29 to >90 days) were enrolled (Figure 1a). Patient 1 (cutaneous T-cell lymphoma) and patient 2 (acute undifferentiated leukemia) received immunosuppressive agents for the prophylaxis or treatment of GvHD following allogeneic hematopoietic stem cell transplantation (allo-HSCT) 7 and 22 months earlier. Patient 3 (chronic lymphocytic leukemia) received high-dose steroids for leukemic hyperleukocytosis. Pre-allo-HSCT conditioning regimens were provided to patient 4 (progressive multiple myeloma), patient 5 (refractory T-cell non-Hodgkin lymphoma) and patient 6 (refractory B-cell non-Hodgkin lymphoma). Never was the decision taken to proceed with allo-HSCT during any knowledge of active A(H1N1)pdm09 infection. Four patients received a well-matched influenza vaccine (patients 1, 3, 4 and 5) during the corresponding season but had no seroprotective HI Ab titers during onset (Figure 1b). Oseltamivir or zanamivir antiviral treatment was provided to all patients ($n=2$, $\leqslant 48$ h; $n=4$, >48 h). Four patients developed H275Y resistant virus (Figure 1a) and variable clinical outcomes (2 mild, 2 severe).

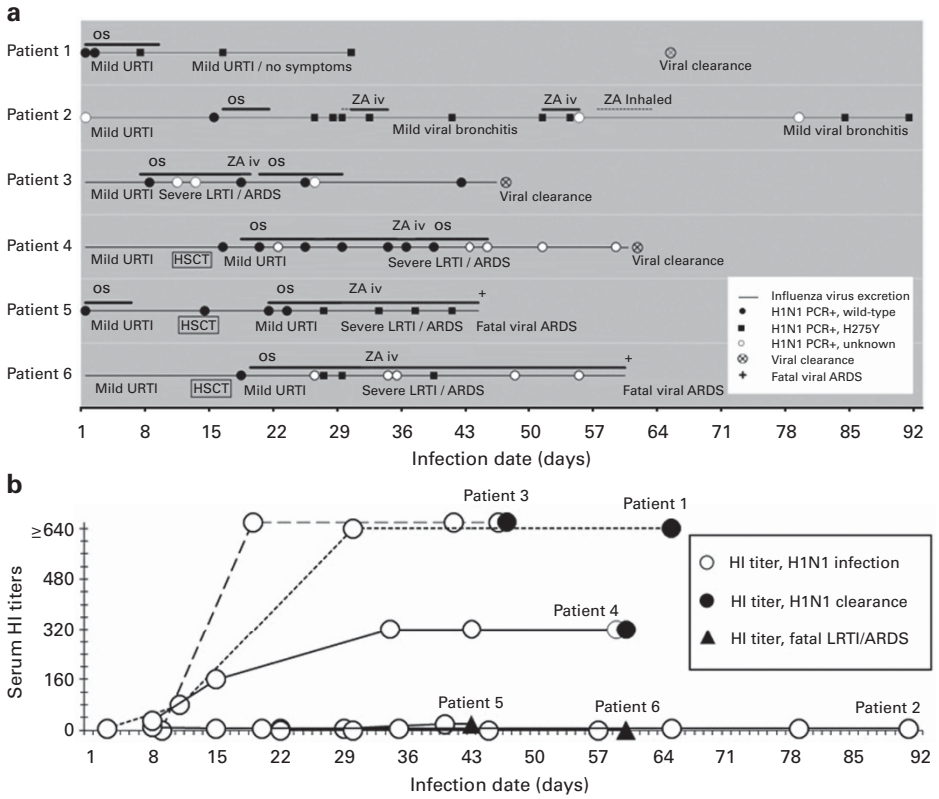

Figure 1. (**a**) Timeline of prolonged A(H1N1)pdm09 virus infection and antiviral treatment. (**b**) Humoral responses during prolonged A(H1N1) pdm09 virus infection. HI titers are expressed as the reciprocals of the highest serum dilution that inhibited homologous virus hemagglutination.

All six patients displayed prolonged viral excretion during $CD4^+$ and $CD8^+$ T-cell lymphopenia (Figure 2a). Virus excretion duration was not influenced by antiviral treatment, seroprotective HI titers (Figures 1a,b) or ADCC markers (data not shown). The six patients developed a wide spectrum of virus-associated symptoms ranging from mild URTI to severe LRTI and ARDS (Figure 1a). Intercurrent pulmonary co-infections and non-infectious cardiopulmonary diagnoses (congestive cardiac failure, pneumothorax) were excluded or effectively treated and did not seem to confound virus-associated measures. Two cases (patients 1 and 2) displayed mild symptoms during the presence of $CD8^+$ T cells (Figure 2a). Mild symptoms of patient 1 completely alleviated in the presence of ADCC markers whereas patient 2 manifested sustained mild bronchitis and recurrent *Haemophilus influenzae* bronchopneumonia during absence of seroprotective antibodies. Four cases (patients 3, 4, 5 and 6) developed severe viral LRTI during profound $CD4^+$ and $CD8^+$ T-cell lymphopenia (Figures 1a and 2a) and intercurrent combined absence of ADCC markers. The onset of severe viral LRTI and ARDS during profound $CD4^+$ and $CD8^+$ T-cell lymphopenia coincided with innate cell-mediated immune reconstitution in all four patients (Figure 2b). Patients 4, 5 and 6 manifested granulocyte, monocyte and NK cell reconstitution after recent allo-HSCT and patient 3 displayed granulocyte reconstitution during leukemic hyperleukocytosis.

Complete viral clearance occurred strictly during $CD4^+$+ and $CD8^+$+ T-cell reconstitution and functional virus-specific T-cell responses (Figure 2a) in patient 1 ($CD4^+CD69^+IFN\gamma^+$ 0.02%; $CD8^+$ $CD69^+IFN\gamma^+$ 0.12%), patient 3 ($CD4^+CD69^+IFN\gamma^+$ 0.47%; $CD8^+$ $CD69^+IFN\gamma^+$ 0.20%) and patient 4 ($CD4^+$ T-cell reconstitution with homologous virus seroconversion and emergence of $CD8^+$ T cells).

The findings from this study confirm that influenza virus-infected hematology–oncology patients develop a wide clinical spectrum ranging from mild[1] to severe respiratory symptoms[2–4] during prolonged viral excretion. Innate and adaptive host immune responses appear to be major determinants of virus-associated outcome and viral clearance.[3,6,7] Earlier studies report that undefined lymphopenia increases the risk for prolonged influenza virus excretion[5,6] and that lymphocyte reconstitution is associated with viral clearance.[3] Our findings show that prolonged viral excretion more specifically correlated with T-cell lymphopenia. It is generally assumed that T cells induce viral clearance,[7] but this has not clearly been demonstrated in human influenza cases. The role of $CD8^+$ T cells has frequently been characterized in influenza animal models but the role of $CD4^+$ T cells remains unclear.[12] We confirm that viral clearance occurs during $CD4^+$ and $CD8^+$ T-cell reconstitution in the presence of functional virus-specific T cells (Figure 2a). Viral clearance did not occur during profound $CD4^+$ T-cell and $CD8^+$ T-cell lymphopenia (patients 5 and 6) or during profound $CD4^+$ T-cell lymphopenia with a low-level $CD8^+$ T-cell count (patient 2). This observation supports the assumption that both $CD4^+$ and $CD8^+$ T-cell responses determine complete viral clearance.[12]

The wide clinical spectrum among six hematology–oncology patients with prolonged influenza virus excretion prompted an investigation into the protective role of host immune responses and pathogenesis. Mild symptoms (patients 1 and 2) correlated with a low-level presence of $CD8^+$ T cells even during absence of seroprotective HI titers (patient 2). In contrast, severe viral LRTI (patients 3, 4, 5 and 6) manifested during profound $CD4^+$ and $CD8^+$ T-cell lymphopenia and intercurrent absence of ADCC markers even during the presence of seroprotective HI titers (patients 3 and 4). Patients 3 and 4 developed seroprotective HI titers despite transient profound $CD4^+$ T-cell lymphopenia. High virus-specific Ab titers did not prevent the development of severe

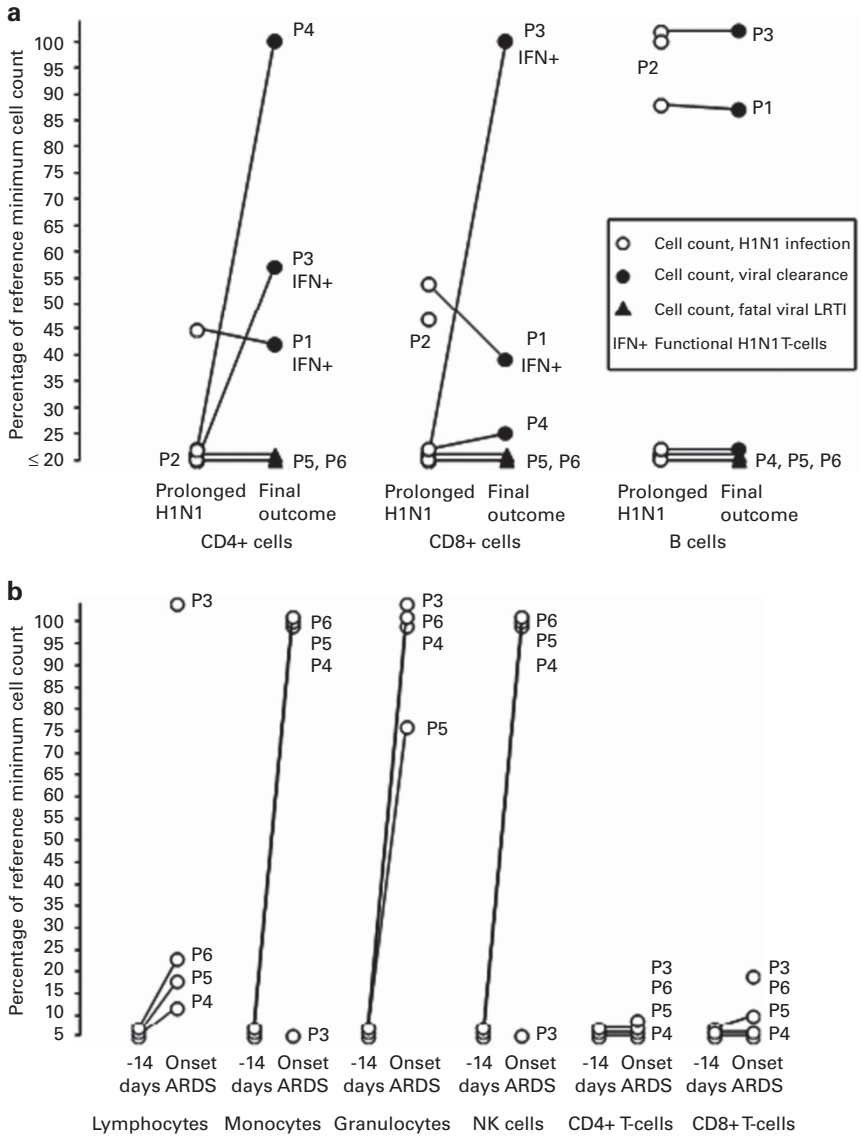

Figure 2. (**a**) T- and B-cell counts during prolonged A(H1N1)pdm09 virus infection and during final outcome. (**b**) Cell-mediated responses during a 2-week development period of severe viral LRTI and ARDS.

viral LRTI and ARDS probably due to intercurrent absence of ADCC effector cells. Altogether, these results support the hypothesis that $CD8^+$ T cells independently mediate clinical protection[7,11] and that ADCC provides additional clinical protection.[13]

The pathogenesis of severe influenza LRTI and ARDS remains unclear and is likely multifactorial. Four cases (patients 3, 4, 5 and 6) with (transient) profound T-cell lymphopenia and absence of ADCC markers developed severe virus-associated LRTI and ARDS during innate cell reconstitution. Our findings suggest that profound $CD4^+$ and $CD8^+$ T-cell lymphopenia and (transient) absence of ADCC markers may have provided a window of opportunity for the virus to reach lower alveolar compartments and trigger severe immunopathology by the excessive influx of neutrophils and macrophages.[14] Timely antiviral treatment is therefore important when protective immune responses are still lacking and early IV zanamivir therapy may benefit patients who are most at risk.[15]

Study limitations include a small sample size due to the rare occurrence of prolonged influenza virus excretion, which does not permit statistical analysis. Additional limitations include technical difficulties of measuring T-cell and ADCC responses, unblinded clinical and outcome assessments by the clinical investigator and incomplete detection of existing antiviral resistance mutations. Despite these limitations, the study provides new insights into the role of host immune responses in determining influenza infection outcomes. Our findings underline the importance of influenza prevention strategies in hematology–oncology patients and show that vaccine improvements are needed to raise immunogenicity in this vulnerable patient group.

In conclusion, prolonged influenza virus excretion is associated with T-cell lymphopenia in hematology–oncology patients. $CD8^+$ T cells and ADCC markers afford clinical protection and combined $CD4^+$ and $CD8^+$ T-cell responses mediate viral clearance. Pathogenesis of severe viral LRTI and ARDS is likely the result of virus reaching lower compartments of the lung during a lack of combined T-cell- and ADCC-mediated immunological protection followed by excessive immunopathology triggered by innate cell-mediated responses. More insight into the role of influenza host immune responses can improve the clinical management of infected hematology–oncology patients and may limit the emergence of antiviral-resistant viruses.

CONFLICT OF INTEREST

GFR reported his part-time employment as a consultant of Viroclinics Biosciences BV. The remaining authors declare no conflict of interest.

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