Hematology–oncology patients can develop remarkably prolonged influenza virus excretion with an enigmatic wide clinical spectrum. These patients often develop mild virus-associated symptoms and occasional bacterial or fungal pneumonia 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. Pathogenesis of severe viral LRTI and ARDS is unclear and may include virus-induced pathology or excessive immunopathology. 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 ≥ 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 Jehovah’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. Pulmonary imaging (chest radiography or computed tomography), bronchoalveolar 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 ≥ 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. Minimum absolute cell count references were defined for granulocytes (500/mm³), mononocytes (100/mm³), lymphocytes (1000/mm³), CD3⁺CD4⁺ (560/mm³) T cells, CD3⁺CD8⁺ (260/mm³) T cells, CD3⁺CD16⁺ and/or CD56⁺ nature killer (NK) cells (40/mm³) and CD19⁺ B cells (60/mm³). T-cell counts ≤ 20% of minimum reference were defined as profound T-cell lymphopenia. Defined surrogate antibody-dependent cell-mediated cytotoxicity (ADCC) markers included the combined presence of seroprotective HI titers with CD16⁺ FcγG 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 Reserv-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, cCD3 PerCP BD Biosciences (Breda, The Netherlands) # 345766; CD4, cCD4 Pacific blue BD Biosciences # 558116; CD8, cCD8 PECy7 eBioscience # 25-0088-42; activation (CD69, cCD69 APC BD Biosciences # 340560) and intracellular cytokine expression (IFNγ, antiIFNγ FITC eBioscience (Vienna, Austria) # 11-7319-82) markers and analyzed by flow cytometry (~ 1 × 10⁶ 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⁺IFNγ⁺ or CD3⁺CD8⁺CD69⁺IFNγ⁺ cells, excluding profound T-cell lymphopenia. Virus- and SEB-specific T cells were calculated by subtracting CD4⁺IFNγ⁺ and IFNγ⁺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, < 48 h; n = 4, > 48 h). Four patients developed H275Y resistant virus (Figure 1a) and variable clinical outcomes (2 mild, 2 severe).
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 symptoms of patient 1 completely alleviated in the presence of ADCC markers whereas patient 2 manifested sustained mild symptoms during the presence of CD8+ T cells (Figure 2a). Viral clearance did not occur during CD4+ T-cell and CD8+ T-cell lymphopenia with a low-level presence of CD8+ T cells even during 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+IFNy+ 0.02%; CD8+CD69+IFNy+ 0.12%), patient 3 (CD4+CD69+IFNy+ 0.47%; CD8+CD69+IFNy+ 0.20%) and patient 4 (CD4+CD8+ T-cell reconstitution with homologous virus seroconversion and emergence of CD8+ T cells).

The findings from this study confirm that influenza virus-infected hematologic–oncology patients develop a wide clinical spectrum ranging from mild1 to severe respiratory symptoms2-4 during prolonged viral excretion. Innate and adaptive host immune responses appear to be major determinants of virus-associated outcome and viral clearance.6,7 Earlier studies report that undefined lymphopenia increases the risk for prolonged influenza virus excretion5,6 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,5 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 hematologic–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

© 2016 Macmillan Publishers Limited Bone Marrow Transplantation (2016) 138 – 141
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 and that ADCC provides additional clinical protection.

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. 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.

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.

ACKNOWLEDGEMENTS

We thank Eric Claas (Department of Medical Microbiology) and Geeske Brouwer-Mandema (Department of Hematology) for valuable assistance and advice. We are indebted to Martina Geelhoed-Mieras, Carel van Baalen and Tamara Roelofse Mandema (Department of Viroscience) for excellent technical support. None of these persons were financially compensated for providing assistance and technical support.

J Gooskens1, WAF Marijt2, EHR van Essen3, GF Rimmelzwaan4 and ACM Kros1

1Departments of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands;
2Departments of Hematology, Leiden University Medical Center, Leiden, The Netherlands;
3Departments of Intensive Care, Leiden University Medical Center, Leiden, The Netherlands and
4Department of Viroscience, Erasmus Medical Center, Rotterdam, The Netherlands

E-mail: j.gooskens@lumc.nl

International meeting at which all or part of the information has been presented: 17th International Symposium on Infections in the Immunocompromised Host, Genova, Italy, June 2012. 23rd European Congress of Clinical Microbiology and Infectious Diseases, Berlin, Germany, April 2013. 55th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, USA, September 2015.

REFERENCES

1 Redelman-Sidi G, Sepkowitz KA, Huang CK, Park S, Stiles J, Eagan J et al. 2009 H1N1 influenza infection in cancer patients and hematopoietic stem cell transplant recipients. J Infect 2010; 60: 257–263.
2 Nichols WG, Guthrie KA, Corey L, Boeckh M. Influenza infections after hematopoietic stem cell transplantation: risk factors, mortality, and the effect of antiviral therapy. Clin Infect Dis 2004; 39: 1300–1306.

3 Gooskens J, Jonges M, Claas EC, Meijer A, Kros AC. Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses. J Infect Dis 2009; 199: 1435–1441.
4 Tramontana AR, George B, Hurt AC, Doyle JS, Langan K, Reid AB et al. Oseltamivir resistance in adult oncology and hematology patients infected with pandemic (H1N1) 2009 virus, Australia. Emerg Infect Dis 2010; 16: 1068–1075.
5 Ljungman P, de la Camara R, Perez-Bercoff L, Abecasis M, Nieto Campuzano JB, Cannata-Ortiz MJ et al. Outcome of pandemic H1N1 infections in hematopoietic stem cell transplant recipients. Haematologica 2011; 96: 1231–1235.
6 Choi SM, Boudreault AA, Xie H, Englund JA, Corey L, Boeckh M. Differences in clinical outcomes after 2009 influenza A/H1N1 and seasonal influenza among hematopoietic cell transplant recipients. Blood 2011; 117: 5050–5056.
7 Hillaire ML, Rimmelzwaan G, Kreijtz J. Clearance of influenza virus infections by T cells: risk of collateral damage? Curr Opin Virol 2013; 3: 430–437.
8 van der Vries E, Jonges M, Herfst S, Maaskant J, Van der Linden A, Guldemeneer J et al. Evaluation of a rapid molecular algorithm for detection of pandemic influenza A(H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. J Clin Virol 2010; 47: 34–37.
9 Bodewes R, Fraaij PL, Geelhoed-Mieras MM, van Baalen CA, Tiddens HA, van Rossum AM et al. Annual vaccination against influenza virus hampers development of virus-specific CDB” T cell immunity in children. J Virol 2011; 85: 11995–12000.
10 Hakki M, Riddell SR, Storek J, Carter RA, Stevens-Ayers T, Sudour P et al. Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. Blood 2003; 102: 3060–3067.
11 Hillaire ML, van Trierum SE, Bodewes R, van Baalen CA, van Binnendijk RS, Koopmans MP et al. Characterization of the human CDB” T cell response following infection with 2009 pandemic influenza H1N1 virus. J Virol 2011; 85: 12057–12061.
12 Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. Emerg Infect Dis 2006; 12: 48–54.
13 Jegakandana S, Weinfurter JT, Friedrich TC, Kent SJ. Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques. J Virol 2013; 87: 5512–5522.
14 Narasaraju T, Yang E, Samy RP, Ng HH, Poh WP, Liew AA et al. Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. Am J Pathol 2011; 179: 199–210.
15 Fraaij PL, van der Vries E, Beersma MF, Riezebos-Brilman A, Niesters HG, van der Eijk AA et al. Evaluation of the antiviral response to zanamivir administered intravenously for treatment of critically ill patients with pandemic influenza A (H1N1) infection. J Infect Dis 2011; 204: 777–782.