CHEST

Lower Respiratory Tract Myeloid Cells Harbor SARS-Cov-2 and Display an Inflammatory Phenotype

To the Editor:

Severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) pneumonia may induce an aberrant immune response with brisk recruitment of myeloid cells into the airspaces.[1] Although the clinical implications are unclear, others have suggested that infiltrating myeloid cells may contribute to morbidity and mortality rates during SARS-CoV-2 infection.[1-3] However, few reports have characterized myeloid cells from the lower respiratory tract, which appears to be the primary site of viral-induced disease, during severe SARS-CoV-2 pneumonia.

Methods

Endotracheal aspirate (ETA) samples were collected prospectively from seven patients whose condition required mechanical ventilation for severe pneumonia due to SARS-CoV-2 infection, which was documented by reverse transcriptase polymerase chain reaction from April to June 2020. All patients were enrolled in a University of Pittsburgh lung injury registry and biospecimen repository (IRB# PRO10110387). ETA were fixed in 4% (volume/volume) paraformaldehyde overnight then processed for subsequent imaging. Briefly, ETA samples were washed twice then pelleted at 600*g*. Samples for electron microscopy were resuspended in 1% (volume/volume) glutaraldehyde, repelleted at 600*g*, and processed as described in a previous report.[4] Samples for light microscopy and immunofluorescence were resuspended in phosphate-buffered saline solution then prepared as cytospins by spinning at 300 rpm onto a Superfrost plus microscope slide (Fisher Scientific). Manual cell counts of ETA samples were performed after Diff-Quick (Siemens; Healthcare Diagnostics, Inc) staining, and representative images were obtained with the use of an Olympus Provis I microscope (Olympus Corporation). For immunofluorescence, cytospin slides were placed in 70% (volume/volume) ethyl alcohol followed by 90% (volume/volume) ethyl alcohol for 10 minutes each, then allowed to air dry. Antibodies that were used include SARS-CoV-2 nucleocapsid protein (NB100-56576; Novus Biologicals), CD14 (#347490; BD Biosciences), CD16 (MA1-84008; Invitrogen), CD142 (Tissue Factor; BD 550252; BD Biosciences), and IL-6 (Novus NBP2-44953; Novus Biologicals). Subsequent staining was performed, and slides were imaged using a Nikon A1 confocal scanning fluorescence microscope (Nikon Inc). Preembed immune-electron microscopy was performed after cytospin preparation with colloidal gold-conjugated secondary antibodies (CD14, 18 nm; SARS-CoV-2, 6 nm nucleocapsid; Jackson ImmunoResearch) using a JEOL JEM 1400 transmission electron microscope (JEOL USA, Inc) at 80 kV with image capture via an Advanced Microscopy Techniques 2K digital camera. RNAscope was performed per manufacturer instructions. Quantitative imaging analysis was performed with the use of object-based area overlap analysis via Nikon Elements software. Statistical comparisons of co-expression of CD14, CD16, IL-6, and tissue factor in cells with or without SARS-CoV-2 nucleocapsid protein were performed with non-parametric testing in GraphPad Prism version 7.05 (GraphPad Software).

Results

The median age of the seven patients was 58 years (range, 56-77 years), and five patients (71.4%) were men. The median duration of reported symptoms prior to initiation of mechanical ventilation was 7 days (range, 3-11 days). Samples were collected within a median of 5 days (range, 1-14 days) after initiation of mechanical ventilation. Notably, three patients required extracorporeal membrane oxygenation, and two patients were dead by 60 days of follow up from ICU admission. There were no patients with known immune deficits in this cohort as defined by history of immunosuppressive therapy, which included chemotherapy or chronic systemic steroids, or known immune deficiency. ETAs were composed primarily of mononuclear and polymorphonuclear leukocytes (range, 70.6%-97.5% of nucleated cells) (Fig 1A). Electron tomography of ETAs revealed intracellular localization of presumptive SARS-CoV-2 virions in mononuclear leukocytes (Fig 1B) and polymorphonuclear leukocytes (Fig 1C). The identification of SARS-CoV-2 virions by electron tomography was consistent with immune-electron microscopy with an antibody against the nucleocapsid protein of SARS-CoV-2 that confirmed the presence of virus in $CD14^{+}$ cells in the lower airways (Fig 1D). Quantitative imaging of ETA cells revealed SARS-CoV-2 nucleocapsid protein expression (n=6; patient 7 did not have sufficient ETA available for imaging) (Fig 2A), many of which were also positive for CD14, IL-6, and tissue factor immunostaining (Fig 2B).

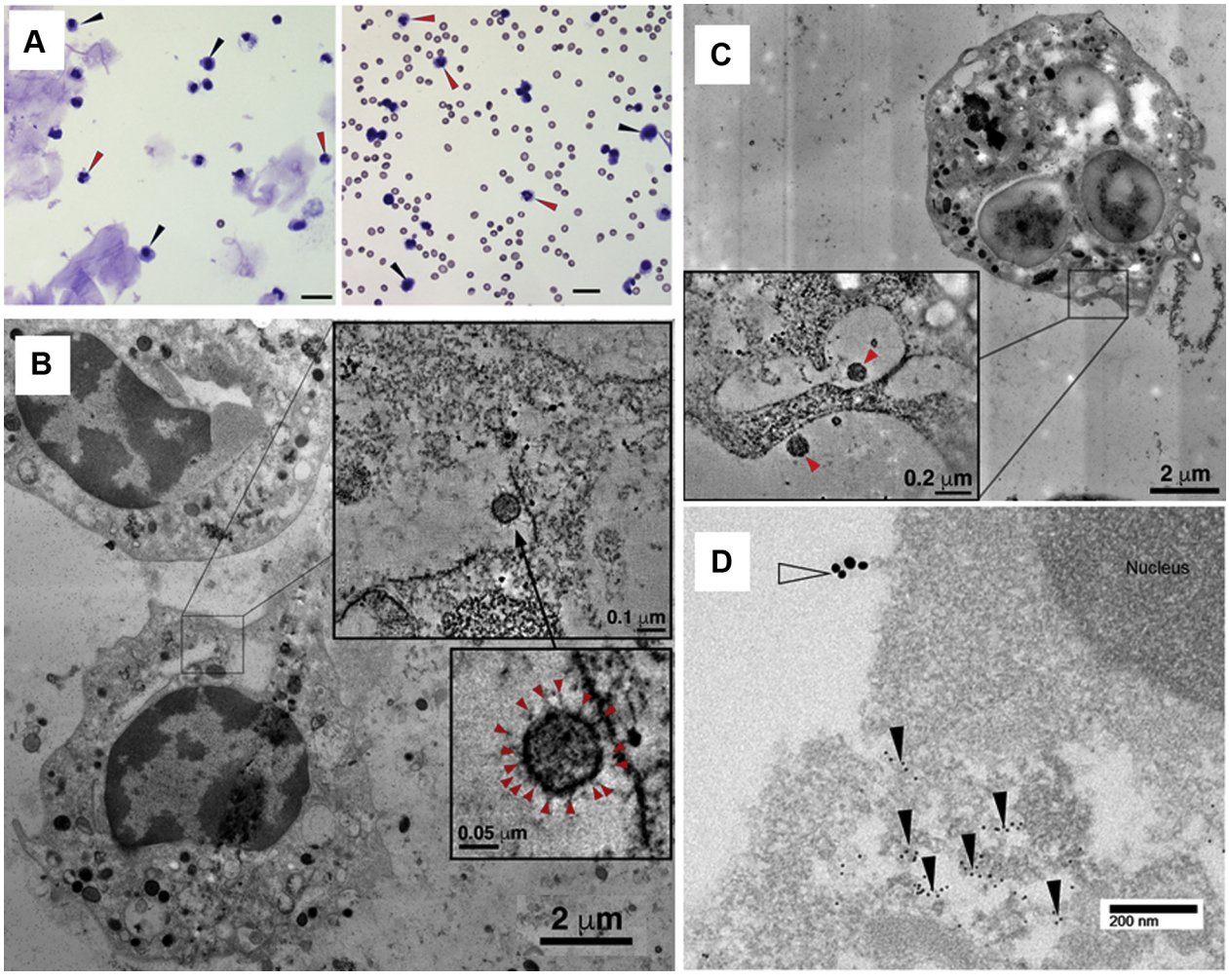

Figure 1 – *A-D, Lower respiratory tract myeloid cells can harbor SARS-CoV-2 virions. A, Representative images of cytospins prepared from endotracheal aspirate samples; patient 3 is on the left and patient 6 is on the right. Black arrowheads denote mononuclear cells, and red arrowheads denote polymorphonuclear cells. Black scale bar in lower right portion of each image indicates 20 microns. B, Electron microscopy overview of lower respiratory tract mononuclear leukocyte (presumptive macrophage) from patient 4: the upper inset shows the region indicated by the square that shows a tomographic slice with presumptive SARS-CoV-2 virion in a smooth-walled compartment or surface invagination; the lower inset shows a higher magnification tomographic view of presumptive virion with apparent spike proteins indicated by red arrowheads. C, Polymorphonuclear leukocyte (presumptive neutrophil) from patient 7; the inset shows the region indicated by the square in the overview that contained presumptive SARS-CoV-2 virions (red arrowheads). D, Immunotransmission electron microscopy of lower respiratory tract mononuclear leukocyte from patient 6 with CD14 (18 nm gold colloid; open arrowhead) surface immunostaining and internal immunostaining of SARS-CoV-2 Nucleocapsid protein (6 nm gold colloid; black arrowheads at clusters of staining). SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.*

Myeloid cells that expressed SARS-CoV-2 nucleocapsid protein were more likely to express the inflammatory markers CD14 and CD16 ($P < .01$ by Mann-Whitney test) (Fig 2C) compared with myeloid cells without viral co-localization within each sample. Similarly, lower respiratory tract cells that expressed SARS-CoV-2 nucleocapsid protein were more likely to express IL-6 ($P < .01$) (Fig 2D) and tissue factor ($P < .05$) (Fig 2D) compared with cells without co-localization of viral protein within each sample. Finally, we noted that ETA myeloid cells showed *IL6, F3,* and *CD14* transcripts (representative image of polymorphonuclear leukocyte) (Fig 2E).

Discussion

Taken together, our findings suggest that lower respiratory tract myeloid cells found in ETA samples harbor SARS-CoV-2 virus and display an inflammatory phenotype marked by expression of CD14, CD16, IL-6, and tissue factor. Although others have shown co-localization of SARS-CoV-1 and H1N1 influenza virus with human monocyte/macrophages in autopsy studies,[5,6] we believe this to be the first description and confirmation of the presence of SARS-CoV-2 virions inside lower respiratory tract myeloid cells, including polymorphonuclear leukocytes, from human samples. Although the clinical implications of our findings are

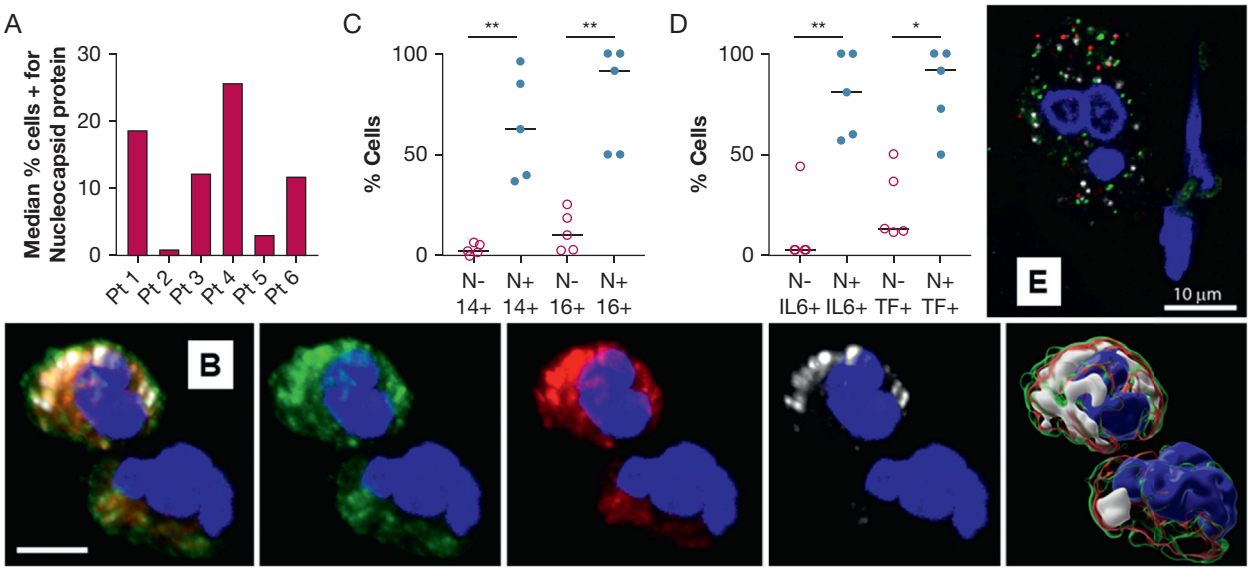

Figure 2 – *A-E, Lower respiratory tract myeloid cells that harbor SARS-CoV-2 virions display an inflammatory phenotype. A, Quantitative immunofluorescence with median percentage (n=3 slides per patient) of total endotracheal aspirate cells that expressed SARS-CoV-2 nucleocapsid protein (n=6 patients; patient 7 did not have sufficient endotracheal aspirate for immunofluorescence staining). B, Representative montage from a single polymorphonuclear cell shows co-localization by immunofluorescence. Panels from left to right show merge, CD14 (green), IL-6 (red), SARS-CoV-2 nucleocapsid protein (white), and Imaris (Bitplane) surface-rendered image of the overlapping areas of labeling. The blue nuclear stain in all panels is DAPI; the white scale bar is 10 microns. C, Comparison of endotracheal aspirate cells that co-expressed CD14 (14) or CD16 (16) with (N+) or without (N-) SARS-CoV-2 nucleocapsid protein in each sample (n=5 patients; patient 2 was removed due to low number of cells with nucleocapsid protein). Statistical comparison by Mann-Whitney test. The double asterisks indicate a probability value of <.01. D, Comparison of endotracheal aspirate cells that co-expressed IL-6 (IL-6+) or tissue factor (TF+) with (N+) or without (N-) SARS-CoV-2 nucleocapsid protein in each sample (n=5 patients; patient 2 was removed due to low number of cells with nucleocapsid protein). Statistical comparison by Mann-Whitney test; the single asterisk indicates a probability value of <.05; the double asterisks indicate a probability value of <.01. E, Representative in situ localization of CD14 (green), IL6 (white), and tissue factor or F3 (red) transcript and DAPI nuclear staining (blue) in an endotracheal aspirate myeloid cell. Pt = patient; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.*

unclear, we speculate that the benefits of dexamethasone in patients with SARS-CoV-2 pneumonia whose condition requires mechanical ventilation[7] potentially result from modulation of inflammatory myeloid cells recruited to lung airspaces, which are deleterious in mouse models of SARS-CoV-1 pneumonia.[8] Notably, we found that lower respiratory tract myeloid cells can harbor virus as long as 14 days after initiation of mechanical ventilation.

Despite the novelty of these findings, the mechanisms by which virions enter lower respiratory tract myeloid cells and survive phagocytic degradation are unclear. Others have demonstrated SARS-CoV-1 virions within monocytes/macrophages without productive replication in vitro, [9,10] and previous reports have shown survival of HIV virions in bone marrow macrophages in a humanized mouse model.[4] Therefore, it remains to be determined whether productive replication of SARS-CoV-2 can occur in these cells. A limitation of our study is that ETAs may not reflect fully the distal airspaces; we suggest that these findings should be validated in BAL samples. Further work in preclinical models of SARS-CoV-2 pneumonia are needed to determine the protective or maladaptive role of viral uptake by myeloid cells and the associated inflammatory phenotype to help guide future clinical research strategies.

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Author contributions: W. Bain takes responsibility for the content of this manuscript. W. Bain enrolled patients, collected samples, performed the experiments, designed, analyzed, and interpreted the data, and wrote the manuscript. H. Peñaloza and M. Ladinsky performed the experiments and designed and interpreted the data. R. van der Geest, M. Sullivan, and M. Ross performed the experiments and revised the work for important intellectual content. G. Kitsios enrolled patients, collected samples, provided critical input to the design of the experiments, and revised the work for important intellectual content. B. A. Methé, B. McVerry, and A. Morris provided critical input to design of the experiments and revised the work for important intellectual content. A. Watson and S. Watkins provided critical input to the design of the experiments, expert imaging input, and revised the work for important intellectual content. C. St Croix and D. Stolz performed the experiments, designed and interpreted the data, and revised the work for important intellectual content. P. Bjorkman conceived, designed, interpreted the data, and revised the work for important intellectual content. J. Lee conceived, designed, analyzed, interpreted the data, and wrote the manuscript.

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