Persistent Pathology in Influenza-Infected Mouse Lungs

To the Editor:

An important emerging concept in the field of regeneration is that the nature and severity of an injurious stimulus can dictate the mechanism of the reparative response. This concept certainly opens up opportunities for new therapeutic approaches. However, it is important to standardize different injuries, and in each case to carefully define the reparative process. This challenge has recently come to the fore in the controversy over the response of the mouse lung to influenza infection, a topic of considerable clinical interest because the virus causes extensive damage to airway and alveolar epithelium. A recent paper claimed that in response to infection with a murinized version of a pandemic H1N1 strain of influenza A (PR8), a preexisting population of keratin 5 (Krt5⁺) stem cells proliferated to form epithelial “pods” that subsequently regenerated alveolar epithelium and fully repaired the lung (1). We also reported the rapid expansion of a Krt5⁺ population of cells within alveoli after PR8 infection but showed that these cells failed to transdifferentiate into alveolar cells unless Notch signaling was inhibited (2).

Here, we confirm that there are extensive regions of Krt5⁺ pods after infection with a sublethal dose of PR8. These persist for up to 200 days post-infection (dpi) in areas largely devoid of unambiguous markers of normal alveolar lineages. In contrast, widespread pods were not observed after infection with the less virulent X31 influenza strain. Therefore, we conclude that Krt5⁺ cells are not an invariant response to influenza infection and are not an essential source of alveolar cells for repair.

Influenza infection was recently repopularized as a lung injury/repair model by a report that used a murinized version of the pandemic PR8 influenza strain (3). The authors reference a previous study showing “histologically complete recovery” from infection, but this was performed with a less virulent H3N2 influenza strain (3, 4). Therefore, we sought to revisit the issue of whether Krt5⁺ regions eventually resolved in the lungs of mice infected with PR8. We infected mice with a single sublethal dose of PR8 and analyzed mice by histology at early (4, 7, 11, 22, 38 dpi) and late (49, 72, 90, 192, 200 dpi) times. At early stages, there was a histological appearance of collapsed alveoli and alveolitis (data not shown). Consistent with previous reports, there was loss of surfactant-associated protein C (SPC)⁺-expressing type 2 alveolar epithelial cells (AEC2) and extensive parenchymal patches of epithelial cells that expressed keratin 5 (Figures 1A and 1B and Ref. 3). Similarly, SPC protein was decreased in total lung lysates of mice 11 dpi, whereas protein was increased compared with vehicle-treated controls (Figure 1C).

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Figure 1. A murinized version of a pandemic H1N1 strain of influenza A (PR8) infection causes loss of alveolar epithelium and the expansion of keratin 5 (Krt5) pods. (A) Lineage-labeled type 2 alveolar epithelial cells (AEC2) and their progeny (red) are lost from large regions of the lung parenchyma of mice 7 days post-infection (dpi) with PR8. (B) Abundant Krt5⁺ “pods” (green) are observed in these regions devoid of normal

In contrast to reports of complete alveolar repair after PR8 infection in mice, we observed that Krt5 pods had not resolved at late stages. Rather, consistent with our previous report, the pods had formed larger cysts lined with Krt5⁺ cells (Figures 1F–1J and ref. 2). Because it has been reported that Krt5⁺ cells give rise to AEC1 and AEC2 after PR8 infection (1, 3), we assessed differentiation in the persistent Krt5⁺ cysts at these later times. Although cells in these cysts expressed some inconclusive markers of type I alveolar epithelial cells (Pdpn), as previously described, the Krt5⁺ regions of the lungs did not express definitive markers of alveolar lineages as far out as 200 dpi (Figures 1H and 1I). Instead, we occasionally observed ciliated and secretory cells in these cysts (data not shown), consistent with bronchiolization of the distal lung parenchyma. Notably, distal expression of Krt5 has been reported in other models of lung injury, including bleomycin (2). We observed a significant decrease in SPC expression 17 d after bleomycin, suggesting this might commonly precede the distal expansion of Krt5 in severe lung injury (see Figure E1 in the online supplement).

Zuo and colleagues used a transgenic construct derived from the bovine Krt5 promoter to lineage trace the putative Krt5⁺ distal airway stem cells after infection with PR8 (1). They reported that this strategy resulted in the labeling of AEC1 and AEC2 after influenza, suggesting that Krt5⁺ cells give rise to AEC1 and AEC2 during injury/repair. The frequency of differentiation was not reported, and low-power images were not provided to assess the efficiency of this process. To more precisely assess the differentiation potential of Krt5⁺ cells after PR8 infection, we used a Krt5-CreER knock-in allele (5). We very rarely observed lineage-labeled cells that expressed markers of alveolar epithelial cells at any stage after injury (Figure 1J). These data are consistent with our previous work in which Krt5⁺ lineage-labeled alveolar cells were only detected in reasonable numbers after pharmacological inhibition of the Notch signaling pathway (2).

Because Kumar and colleagues referenced a manuscript that used an H3N2 influenza A strain as an injury model, we asked whether an H3N2 virus model induced similar Krt5⁺ pods. There was significant alveolar damage in mice infected with X31, a hybrid H3N2 virus containing the PR8 backbone, as assessed by weight loss and lung water (see Figure E1 in the online supplement), some patches of AEC2 loss, and few regions that expressed Krt5 at 7–14 dpi (Figures 1D and 1E). There was an increase in Krt5 protein in whole-lung lysate 11 dpi (Figure 1C), but our immunofluorescence suggests that this increase occurs in the small airways, rather than widespread parenchymal Krt5⁺ pods. Unlike in the lungs of mice infected with PR8, there was not a significant decrease in SPC protein in the lungs of mice infected with X31 (Figure 1C). Therefore, the differences in epithelial response to the two strains are likely attributable to severity of lung injury resulting from viral tropism and host response (6).

In closing, we provide further evidence that the severity of lung injury dictates the epithelial response to restore barrier function and gas exchange. These data have important implications for

researchers choosing experimental models to study regenerative responses in the lung and for clinicians diagnosing lung pathology in humans. Our data are consistent with a model in which Krt5⁺ cells expand to cover basement membrane in response to extreme lung injury, but without further manipulation, these cells are not a significant source of normal alveolar epithelium.

Methods

Mice. PDGFra-GFP (7), SPC-CreERT2 (8), K5-CreERT2 (5), ROSA-mTmG (9), ROSA-Tomato (8) and Rosa-fGFP alleles have been previously described. To lineage trace AEC2, 8-week-old mice heterozygous for both SPC-CreERT2 and a ROSA reporter were given tamoxifen (0.25 mg/g body weight) dissolved in corn oil via intraperitoneal injection every other day for three doses. A chase period of 30 days was used between tamoxifen injection and subsequent influenza infection. To lineage trace cells expressing Krt5, adult mice heterozygous for both K5-CreERT2 and ROSA-mTmG were given a single intraperitoneal dose of tamoxifen (0.123 mg/g body weight) 10 days after infection with PR8, when Krt5⁺ cells are abundant in the distal mouse lung. All studies were performed according to protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

Influenza infections. Mice were anesthetized with isoflurane and infected intranasally with D-PBS (vehicle), 3×10^5 focus forming units (FFU) of influenza A/H3N2 A/Aichi/02/68 $\times$ A/Puerto Rico/8/34 (X31) or 200–280 FFU of influenza A/H1N1/Puerto Rico/8/34 (PR8) dissolved in 30 μ l D-PBS at 8–12 weeks of age.

BLEOMYCIN. Six- to 8-week-old female C57BL/6 mice were intratracheally instilled with saline or 2.0–2.5 units/kg bleomycin (Sigma-Aldrich). Mice were killed on Day 17. The lungs were lavaged, followed by snap freezing in liquid nitrogen for protein extraction.

ASSESSMENT OF LUNG INJURY. C57BL/6 mice were given high-dose ketamine and xylazine and were subsequently killed by bilateral thoracotomy. Blood hemoglobin concentration was assessed, and lung homogenate and homogenate supernatant were collected, weighed, and desiccated for wet-to-dry ratio and excess lung water measurements, as described (10).

MOUSE TISSUE PREPARATION AND IMMUNOFLOUORESCENCE. Mice were killed by CO₂ inhalation, and pulmonary perfusion was performed with 20 ml cold PBS through the right ventricle. Lungs and trachea were removed and inflated to 20 cm H₂O pressure with 4% paraformaldehyde. Lungs were fixed for 30 minutes at 4°C in 4% paraformaldehyde and washed 3 $\times$ 15 minutes in PBS. Cryosections (10 μ m) were stained by standard protocols. Rabbit anti-proSPC (catalog #ab3786, 1:500; Millipore, Billerica, MA), Rabbit anti-RFP (catalog #600-4013791:250; Rockland Immunochemicals Inc., Limeric, PA), rabbit anti-Keratin 5 (catalog

Figure 1. (Continued). alveolar epithelial lineages at 11 dpi. (C) The abundance of pro-surfactant-associated protein C (pro-SPC) is decreased concomitant with an increase in the abundance of KRT5 in the lungs of mice 11 dpi with PR8 compared with mice treated with saline or infected with X31. (D) Lineage-labeled AEC2 and their progeny are lost to a lesser extent 7 dpi X31 infection compared with PR8. (E) Krt5⁺ cells (green) are seen in the airways of mice 14 dpi with X31, but widespread pods are not seen in the distal lung. (F) 49 dpi with PR8, lineage-traced AEC2 (green) have given rise to RAGE (advanced glycosylation end product-specific receptor)⁺ AEC1 (white). In contrast, large adjacent regions of the lung are still devoid of unambiguous markers of AEC1 and AEC2, but still abnormally express K5 (red). (G) Magnified view of boxed region in F. (H, I) Section of wild-type lung 200 dpi with PR8 showing RAGE (white, AEC1) and K5 (red) in discrete regions. (J) Lineage traced K5⁺ cells (red) rarely give rise to RAGE⁺ AEC1 (white) 192 dpi with PR8. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bars: 200 μ m for panels A, B, D, and E. Scale bars: 100 μ m for panels F, I, and J.

PRB-160P; Biolegend, San Diego, CA), rat anti-RAGE (advanced glycosylation end product-specific receptor) (catalog MAB1179 1:250; R&D systems, Minneapolis, MN), mouse anti-acetylated tubulin (catalog T7451; Sigma-Aldrich, St. Louis, MO), and chicken anti-GFP (catalog GFP-1010; Aves Labs, Tigard, OR). Alexa-Fluor-coupled secondary antibodies (Invitrogen, Carlsbad, CA) were used at 1:500. Z stacks of optical sections were captured on a Leica Sp5 laser-scanning confocal microscope. Sections from at least three mice were analyzed for early and late endpoints.

WESTERN BLOT. Snap-frozen mouse lungs were ground and lysed in RIPA buffer (50 mM Tris·HCl at pH 7.5, 150 mM NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100) supplemented with Protease Inhibitor Cocktail, 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM sodium vanadate, 10 mM sodium fluoride, and Phosphatase Inhibitor Cocktail. The lysates were quantified using Pierce BCA protein assay kit (Thermo, #23225), normalized, and blotted for pro-SPC (Millipore, #AB3786), Krt5 (Covance, #PRB-160P), and β -actin (Sigma-Aldrich, #A5441). ■

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