ARTICLE

Excessive neutrophil levels in the lung underlie the age-associated increase in influenza mortality

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Neutrophils clear viruses, but excessive neutrophil responses induce tissue injury and worsen disease. Aging increases mortality to influenza infection; however, whether this is due to impaired viral clearance or a pathological host immune response is unknown. Here we show that aged mice have higher levels of lung neutrophils than younger mice after influenza viral infection. Depleting neutrophils after, but not before, infection substantially improves the survival of aged mice without altering viral clearance. Aged alveolar epithelial cells (AECs) have a higher frequency of senescence and secrete higher levels of the neutrophil-attracting chemokines CXCL1 and CXCL2 during influenza infection. These chemokines are required for age-enhanced neutrophil chemotaxis in vitro. Our work suggests that aging increases mortality from influenza in part because senescent AECs secrete more chemokines, leading to excessive neutrophil recruitment. Therapies that mitigate this pathological immune response in the elderly might improve outcomes of influenza and other respiratory infections.

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INTRODUCTION

The elderly exhibit a greater mortality and morbidity to influenza viral infection than younger people, which poses a considerable burden on health-care resources throughout the globe.^{1–4} Aging of the immune system is a likely reason why older people exhibit this increased mortality. Impaired immunity to influenza infection with aging has been examined mostly in murine models and can be broadly categorized by changes in the innate or adaptive immune system.^{5,6} Regarding innate immunity and influenza infection, there is evidence of reduced dendritic cell and natural killer cell activation and impaired in vitro type I interferon responses in human monocytes with aging.^{7,8} With respect to adaptive immunity to influenza, aging impairs CD8⁺ T cell and follicular T cell responses and leads to increased expansion of regulatory T cells.^{9–12} Overall, these findings indicate that declines in both innate and adaptive immunity with aging compromise host defense to influenza.

Neutrophils are circulating innate immune cells that traffic to sites of infection to contain pathogens.¹³ Neutrophils are recruited to infected sites by attaching to the vascular endothelium followed by para and transcellular migration through the endothelium into the tissue.¹⁴ Not only do neutrophils exhibit effector functions, for example, phagocytosis, degranulation, and the production of extracellular traps to contain pathogens but they also guide adaptive immune cells, e.g., CD8⁺ T cells, to sites of infection to promote pathogen clearance.¹⁵ Thus, neutrophils are key innate immune cells that orchestrate innate immunity and regulate adaptive immunity.

In addition to combating bacterial infections, there is evidence that neutrophils control viral infections, including influenza.^{16–18} The local inflammatory environment within the lung promotes neutrophil recruitment.¹⁹ Once neutrophils have arrived within the lung, they assist in viral clearance, as shown in young mice depleted of neutrophils at the time of infection, which exhibited accelerated

mortality and impaired viral clearance.¹⁷ Neutrophils promote clearance of influenza virus in the lung by producing extracellular traps, phagocytosing viral particles, and activating the inflammasome.^{18,20} However, excessive neutrophil functions beyond viral clearance can promote immune pathology.^{21–23} To prevent immune pathology during infection, neutrophils are removed by resident macrophages.²⁴ The dual functions of neutrophils during influenza infection were suggested in a prior study in young mice which found that partial depletion of neutrophils, via low-dose depleting monoclonal antibody administration, improved survival during influenza viral infection, whereas full depletion of neutrophils with high-dose monoclonal antibody accelerated mortality.²¹

People aged >60 years exhibit cell-intrinsic defects within circulating neutrophils, including decreased phagocytosis of bacteria, reduced production of reactive oxygen species, and reduced chemotaxis compared to neutrophils isolated from younger humans.²⁵ Furthermore, with human aging, peripheral blood neutrophils exhibit dysregulated migration^{26,27} and a reduced ability to produce extracellular traps.²⁸ In addition to these cell-intrinsic defects with aging, there is evidence that aging leads to heightened systemic, low-grade inflammation such as increases in tumor necrosis factor (TNF)- α ,²⁹ which could act as cell-extrinsic factors to impact neutrophil response during influenza infection. However, it is not known how aging impacts neutrophil responses to influenza viral infection in vivo.

RESULTS

Aging is associated with increased neutrophil accumulation during influenza infection

To examine whether aging impacts the role of neutrophils during influenza infection, we employed a murine model that exhibits an

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Fig. 1 Aging is associated with increased neutrophils within the lung during influenza infection. Aged (18–22 months of age) and young (2–4 months of age) C57BL/6 mice were intranasally inoculated with PR8 strain influenza virus. Lung tissue was harvested and digested, and cells were obtained and suspended to permit incubation with fluorescent monoclonal antibodies followed by flow cytometric analysis. **a** Representative flow cytometric plot at day 6 p.i. (postinfection) of a young and aged mouse showing increased proportion of neutrophils (CD11b⁺, Ly6G^{hi}) within the lung in the aged mouse than the young mouse. The flow cytometric plots are gated on CD11b⁺Ly6G^{hi} cells. **b** Absolute number of neutrophils before and during influenza infection in young and aged mice in the entire lung (airspace, vasculature, and lung interstitium). **P* < 0.05, (Mann–Whitney test). *n* = 3–6/group/time point. Data are representative of one of two independent experiments. **c–e** Proportion of neutrophils analyzed by flow cytometry in non-infected and infected (day +6 p.i.) young and aged mice in BAL (**c**), blood (**d**), and bone marrow (**e**). **P* < 0.05, ***P* < 0.01 (Mann–Whitney test). Data are representative of one of two independent experiments, which yielded similar results. Error bars indicate mean ± SEM

age-dependent increase in both morbidity and mortality during influenza infection.^{12,30,31} First, we infected young (2–4 months of age) and aged (18–22 months of age) C57BL/6 mice intranasally (i. n.) with influenza virus (PR8 strain) and used flow cytometric analysis to count the numbers of neutrophils (i.e., Ly6G^{hi} CD11b⁺ cells) within whole lungs (Fig. 1a). Neutrophil accumulation peaked 1 day postinfection (p.i.) for both aged and young mice. Infected aged mice showed a three-fold increase in neutrophil levels 6 days p.i. compared to infected young mice (Fig. 1a, b). The age-associated increase in neutrophil levels was prominent throughout the first week p.i. (Fig. 1b). After the first week p.i., neutrophils numbers declined in both groups but still remained higher in aged mice compared to young mice at day 12 p.i.

To examine whether the increase in neutrophils in the infected lungs was in the airspace compartment, we examined bronchoalveolar lavage (BAL). Before infection, we did not detect neutrophils in the BAL of either young or aged mice (Fig. 1c). At day 6 p.i., we detected neutrophils in the BAL of influenza-infected young and aged mice, and the proportion of neutrophils was two-fold higher in aged mice compared to young mice (Fig. 1c). Relative to young mice, aged mice also showed higher frequencies of circulating blood neutrophils after infection but not before (Fig. 1d). Lastly, before infection, aged mice exhibited a 5% increase in neutrophil frequency within the bone marrow, compatible with the known myeloid skewing that occurs within the bone marrow with aging,³² although this difference was not evident at day 6 p.i. (Fig. 1e). Overall, these data demonstrate that neutrophil levels in the lung airspace are higher in aged infected mice than in young infected mice.

Depletion of neutrophils before or after infection has opposing effects on mortality
Antibody-mediated depletion of neutrophils administered at the time of viral inoculation increased the mortality of young mice

from 10% to 70% at 20 days p.i. (monoclonal antibody IA8, Fig. 2a), consistent with prior observations that neutrophils are critical for combatting influenza early during infection.¹⁷ However, depletion of neutrophils on day 0 did not significantly increase lethality among aged mice (Fig. 2b).

We next examined whether depletion of neutrophils after infection of young and aged mice impacted mortality, given our results that aging enhanced the increase in neutrophils after infection. Neutrophil depletion p.i., from day 6 to 12 p.i., did not impact the survival of young mice (Fig. 2c). However, similarly treated infected aged mice showed a substantial and significant increase in survival (Fig. 2d). Indeed, depleting neutrophils p.i. in aged mice improved survival to 80% from 40% in controls (Fig. 2d). Excessive neutrophil extracellular traps, an important neutrophil effector mechanism, have been implicated in lung injury during influenza viral infection in mice.³³ As DNase treatment disrupts these traps,³⁴ we examined whether DNase impacted survival in our model. DNase treatment via i.n. administration from day 6 to 12 p.i. did not significantly enhance survival in either aged or young infected mice (Supplemental Fig. 1a, b). In sum, these data indicate that neutrophils promote survival at the time of viral infection in young mice but substantially promote mortality towards the end of infection in aged infected mice.

Neutrophil depletion reduces lung damage and lung inflammatory cytokine levels in aged mice without impacting viral clearance during influenza infection

Our prior study demonstrated that aged mice exhibit increased mortality with elevated protein levels in the BAL³⁰, a marker of lung damage. Furthermore, proinflammatory cytokines such as interleukin (IL)-1β and TNF-α can activate neutrophils, be produced by neutrophils, or are induced by neutrophils to enhance tissue damage.^{20,35,36} Compared to young mice, aged

Fig. 2 Neutrophil depletion at the end of the first week p.i. (postinfection) enhances survival in aged mice. **a** Young mice were administered a neutrophil-depleting monoclonal antibody just prior to inoculation with influenza virus and following infection (as described in Materials and methods) and survival was monitored. Compared to isotype control treated mice, neutrophil-depleted young mice exhibited a significant increase in mortality. $*P < 0.05$ (Log rank), $n = 8-10$ per group. **b** Aged mice were treated per **a** and survival postinfection was monitored. P value between neutrophil-depleted and control group, $P = 0.23$ (Log-Rank), $n = 8-10$ per group. **c** Young mice were administered a neutrophil-depleting antibody at day +6 p.i., then intermittently until day +12 p.i. (as described in Materials and methods) and compared to isotype control treated, young infected mice. $*P = 0.10$ (Log-rank), $n = 8-10$ per group. **d** Aged mice were treated according to **c** and survival was measured postinfection. Aged neutrophil-depleted mice exhibited a significant increased survival as compared to the control group. $*P < 0.05$ Log-rank, $n = 8-10$ per group

mice exhibited four-fold higher levels of IL-1 β on day 6 p.i. and four-fold higher levels of TNF- α on day 9 p.i. (Supplemental Fig. 2a, b). We also examined lactate dehydrogenase (LDH), a marker of cell death,^{37,38} and found that young and aged mice displayed similar levels of LDH in BAL at day 10 p.i. (Fig. 3a). However, BAL protein levels were higher in aged mice compared to that in young mice at day 10 p.i. (Fig. 3b).

Next, we examined whether depletion of neutrophils during days 6–12 p.i. affected the levels of proinflammatory cytokines or lung damage. P.i. neutrophil depletion reduced the BAL levels of LDH, protein, and IL-1 β in both young and aged mice at day 10 p.i. (Fig. 3a–c) and reduced TNF- α levels in aged mice (Fig. 3d). However, p.i. neutrophil depletion did not significantly impact viral clearance in either aged or young mice (Fig. 3e). These results suggest that, at the end of the first week post-influenza infection, neutrophils promote death by increasing lung damage and lung inflammatory cytokine levels without impacting viral clearance.

Neutrophils from aged mice exhibit chemotactic defects in response to C-X-C chemokine motif ligand 1 (CXCL1). Neutrophils are generated and stored in the bone marrow, where they are poised to traffic to sites of inflammation.³⁹ We considered that neutrophils from aged mice have a higher intrinsic ability to migrate towards a chemo-attractant, resulting in the increased neutrophil levels in the lungs of aged infected mice. To test this, we purified neutrophils from the bone marrow of young and aged non-infected mice and measured their chemotaxis towards CXCL1, a known neutrophil chemo-attractant. However, neutrophils purified from bone marrow of aged mice exhibited reduced chemotaxis towards CXCL1 than neutrophils isolated from young mice (Fig. 4a). We also examined C-X-C chemokine motif receptor 2 (CXCR2), the chemokine receptor for CXCL1 (Fig. 4b), which is critical for

neutrophil recruitment during influenza infection in mice.⁴⁰ We observed reduced surface expression of CXCR2 in aged vs. young bone marrow neutrophils. CXCR4, a chemokine receptor that contributes to neutrophils releasing from and returning to the bone marrow,⁴¹ was expressed at low levels on neutrophils, with no differences between the age groups (Fig. 4b). Fetal calf serum, which contains a variety of neutrophil chemo-attractants, induced a higher degree of chemotaxis than CXCL1 but without any differences between the age groups (Fig. 4a). These data are consistent with human studies demonstrating that aged neutrophils exhibit chemotactic defects to specific chemo-attractants.²⁶ Thus, bone marrow neutrophils from aged mice display impaired chemotactic function, even though aged mice exhibited increased neutrophil levels within the lung during viral infection.

Age-associated changes in chemo-attractants and neutrophil chemoreceptors during infection

We hypothesized that aging alters cell-extrinsic factors that impact neutrophil recruitment towards the lung during influenza infection. To test this, we harvested lung lysate and BAL from young and aged mice before and after infection. We then performed chemotaxis assays with neutrophils isolated from the bone marrow of non-infected young mice. Neutrophils displayed increased chemotaxis towards the lung lysate and BAL obtained from infected compared to that from non-infected lungs (Fig. 5a, b). Importantly, we observed higher neutrophil chemotaxis towards the lung lysate and BAL harvested from aged mice compared to that from young mice, particularly after infection (Fig. 5a, b). Consistent with these findings, aged mice exhibited a two-to-four-fold increase in the BAL levels of the neutrophil chemo-attractants CXCL1 and CXCL2 at day 6 p.i., compared to young mice (Fig. 5c, d). Also, on day 6 p.i., aged mice exhibited a

Fig. 3 Neutrophil depletion in mice from the first week p.i. (postinfection) reduces lung damage and lung inflammatory cytokine levels without impacting viral clearance. Aged and young mice were infected with influenza virus and depleted of neutrophils or treated with isotype control at day 6 p.i. and then intermittently until day 10 p.i. At day 10 p.i., the BAL was obtained and LDH (a), protein (b), IL-1β (c), and TNF-α (d) was measured via ELISA. **P* < 0.05, ***P* < 0.01 (Mann–Whitney test). Data are representative of one of two independent experiments, which yielded similar results. **e** Young and aged mice were infected with influenza and depleted of neutrophils or treated with isotype control at day 6 p.i. and then intermittently until day 10 p.i. At day 10 p.i., the lungs were obtained and viral load was measured by plaque assay. There were no significant differences in viral load between control and neutrophil-depleted groups. *P* = 0.49 (young Rat IgG vs Anti-Ly6G) (Mann–Whitney test) and *P* = 0.47 (aged Rat IgG vs Anti-Ly6G) (Mann–Whitney test). Data are representative of two independent experiments. Error bars indicate mean ± SEM

three-fold increase in the levels of the proinflammatory cytokine IL-17 (Supplemental Fig. 2c), which induces the production of chemokines from lung epithelial cells and promotes immune pathology during influenza infection.^{42–44} However, aged mice did not exhibit an increase in the chemokine CXCL5 during the course of infection (Supplemental Fig. 2d).

Next, we examined the surface expression of CXCR2 and CXCR4 on lung neutrophils. CXCR2 on lung neutrophils was higher on days 6 and 9 p.i. of aged mice compared to young mice, whereas the surface expression of CXCR4 on lung neutrophils was diminished in aged mice at day 6 p.i. compared to that in young mice (Fig. 5e, f, Supplemental Fig. 3). We also examined bone marrow neutrophils from aged and young mice on day 6 p.i. and observed similar expression of CXCR2, whereas those from aged mice displayed reduced expression of CXCR4 (Supplemental Fig. 4). Overall, these data indicate that influenza-infected lungs of aged mice are characterized by increased levels of CXCL1 and CXCL2 and increased CXCR2 but reduced expression of CXCR4 on lung neutrophils.

Fig. 4 Aging impairs bone marrow neutrophil chemotaxis towards CXCL1 and reduces surface expression of CXCR2. Neutrophils were purified via negative magnetic separation from the bone marrow of young and aged non-infected mice. **a** Neutrophil chemotaxis was assessed in response to CXCL1 or fetal calf serum (FCS) at the indicated doses. **b** Surface expression of CXCR2 and CXCR4 via flow cytometry was assessed on purified bone marrow neutrophils from young and aged mice. Experiments are representative of one experiment repeated twice with consistent results. **P* < 0.05 (Mann–Whitney test). Error bars indicate mean ± SEM

CXCL1 and CXCL2 in the aged influenza-infected lung contribute to increased neutrophil chemotaxis

Next, we examined to what extent CXCL1 and CXCL2 contribute to neutrophil chemotaxis in young versus aged infected mice. As above, we purified neutrophils from the bone marrow of young mice and assessed chemotaxis in response to lung lysates isolated from aged or young mice after infection. We found that antibody-mediated blockade of either CXCL1 or CXCL2 significantly reduced neutrophil chemotaxis with the lung lysate from aged infected mice (Fig. 6a). The anti-CXCL1 antibody was significantly more effective at reducing neutrophil chemotaxis induced by the aged infected lung lysate than the anti-CXCL2 antibody (Fig. 6a). Combined blockade of both chemokines further reduced chemotaxis by the aged lung lysate, indicating an additive effect of dual blockade (Fig. 6a). In lung lysate from young infected mice, blockade of either chemokine led to a similar reduction in chemotaxis compared to control (Fig. 6b). Dual blockade also further reduced chemotaxis induced by the lung lysate from young mice (Fig. 6b). Thus, elevated levels of CXCL1 and CXCL2 in the lungs of infected aged mice enhance neutrophil chemotaxis.

Alveolar epithelial cells (AECs) show an age-associated increase in CXCL1 and CXCL2 secretion during influenza infection

As the alveolar surface is protected by epithelial cells that contribute to the first line of defense against viral infection,⁴⁵ we hypothesized that AECs are a source of the age-associated increase in CXCL1 and CXCL2 in the lung during influenza infection. To test this hypothesis, we isolated AECs from the lungs of young and aged mice before and after infection, cultured them ex vivo, and measured CXCL1 and CXCL2 in culture supernatants by enzyme-linked immunosorbent assay (ELISA). AECs from aged

Fig. 5 Aging increases the levels of CXCL1 and CXCL2 in the lung during influenza viral infection and enhances neutrophil chemotaxis in vitro. **a, b** Bone marrow neutrophils were purified from young non-infected mice and added to chemotaxis assays in which the chemo-attractant was either the BAL (**a**) or lung lysate (**b**) of non-infected or infected (day 6 postinfection) young and aged mice. Young neutrophils exhibited a significant increased chemotaxis towards the fluid obtained from the lungs of aged mice than young mice (before or after infection). * $P < 0.05$, ** $P < 0.01$ (Mann–Whitney test). **c, d** BAL was obtained during the course of influenza infection in young and aged mice and CXCL1 (**c**) and CXCL2 (**d**) measured within the BAL via ELISA. $n = 5$ /time point/group. * $P < 0.05$, *** $P < 0.001$ (Mann–Whitney test). **e, f** Surface expression of CXCR2 and CXCR4 on neutrophils in the lung before and during the course of influenza infection in young and aged mice. $n = 4$ /time point/group. * $P < 0.05$ (Mann–Whitney test). Error bars indicate mean $\pm$ SEM

infected mice produced the highest levels of both CXCL1 and CXCL2 (Fig. 7a, b). Importantly, the AECs from aged mice exhibited an over five-fold increase in CXCL1 levels and a two-fold increase in CXCL2 levels upon infection, whereas AECs from young mice showed only a two-fold increase in CXCL1 and a marginal increase in CXCL2 upon infection (Fig. 7a, b). AECs from infected mice produced TNF- α and IL-1 β , although there were no differences between the age groups (Fig. 7c, d). TNF- α and IL-1 β levels were not detectable in the supernatants of cultured AECs from uninfected mice (data not shown). These results indicate that AECs contribute to the increased levels of CXCL1 and CXCL2 upon influenza infection of aged versus young mice.

Aged mice display a higher frequency of senescent AECs with aging. Senescent cells secrete CXCL1 and CXCL2.⁴⁶ To determine whether AECs from aged mice exhibited a higher frequency of senescence than young mice either before or after influenza infection, we measured senescence-associated (SA)- β -galactosidase activity, a well-described marker of senescence.⁴⁷ We found that aged infected mice displayed the highest frequency of AECs with SA- β -galactosidase activity, which was five-fold higher than the frequency of senescent AECs in young infected mice (Fig. 8a, b). Our data indicate that there is a higher frequency of senescent AECs in aged mice both before and after

influenza infection, which might contribute to the elevated levels of CXCL1 and CXCL2.

DISCUSSION

Clinical reports and experimental studies have shown that aging leads to increased mortality during influenza infection, yet the exact cellular mechanisms behind these observations remain unclear.^{1–3} In our study, we focused on neutrophils, as prior work has shown that these cells are not only critical for clearing influenza virus at the time of infection¹⁷ but can also promote lung damage during influenza infection.^{23,33} We found that aged mice exhibit increased levels of neutrophils during the course of influenza infection compared to young mice. The increased neutrophil levels towards the end of the first week of infection were critical for mortality in aged mice, as we found that neutrophil depletion at this time point increased survival to >80% from <40% in controls. Neutrophil depletion was effective at reducing lung damage in young mice but did not extend survival during influenza infection. It is possible that the reduced level of lung damage or an increased tolerance to the level of lung damage in young mice led to a high survival rate during influenza infection without neutrophil depletion. Only a few therapies have been shown to improve outcome in older hosts during active influenza infection, and these therapies have mostly focused on

Fig. 6 CXCL1 and CXCL2 are critical for neutrophil chemotaxis towards the influenza-infected lung with aging. The lung lysate from young and aged infected mice were obtained and employed in chemotaxis assays with neutrophils purified from the bone marrow of young mice. CXCL1 or CXCL2 inhibition reduced neutrophil chemotaxis to aged (a) and young (b) lung lysate. $n = 3-6$. * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney test). Isotype control antibody did not significantly reduce neutrophil chemotaxis. Data are representative of one of two independent experiments, which yielded similar results. Error bars indicate mean $\pm$ SEM

inhibiting viral replication rather than impacting toxic host immune responses to the pathogen.⁴⁸ To our knowledge, our study is the first to demonstrate that reducing an excessive immune response, in this case increased neutrophil accumulation during active influenza infection, substantially and specifically improves survival with aging.

Our study suggests that increased production of CXCL1 and CXCL2 but not CXCL5 contributes to enhanced neutrophil recruitment to the lung during influenza infection with aging. Why aging leads to an impaired increase in CXCL5 levels during influenza infection is not clear and will require future investigation. We previously reported that alveolar macrophages, cells with important functions for host defense and homeostasis,⁴⁹ are compromised in their ability to uptake and phagocytose apoptotic neutrophils with aging.³⁰ Together, our studies suggest that increased neutrophil accumulation in the lungs during influenza infection with aging arises from both AECs producing higher levels of CXCL1 and CXCL2 and declining neutrophil clearance by alveolar macrophages.³⁰

We found that aging impairs chemotaxis of bone marrow-derived neutrophils towards specific chemotactic signals, which is consistent with prior work, predominantly in humans.^{26,50} Human neutrophils isolated from aged individuals also exhibit an impaired ability to migrate due to increased activation of Class 1 phosphoinositide 3-kinase activity.²⁷ Despite the observed age-

dependent defect in neutrophils chemotaxis, we found that neutrophils accumulated within the lung airspace to a greater degree in aged mice than in young mice during influenza infection. The higher production of both CXCL1 and CXCL2 by aged, influenza-infected lungs was essential for enhanced neutrophil chemotaxis. AECs from aged mice were a source of these elevated chemokines, though we cannot exclude other cellular sources of chemokines, including infiltrating and resident macrophages, dendritic cells, recruited neutrophils, and vascular cells, such as endothelial cells. Regardless of the source of CXCL1 and CXCL2, the levels of these chemokines were increased within the lung during influenza with aging. These higher levels of chemokines may have mobilized neutrophils to exit the bone marrow and may explain the increased proportion of circulating neutrophils during infection in aged mice. We also found that bone marrow neutrophils of aged mice downregulated CXCR4 at day 6 p.i. Downregulation of this receptor has been shown to release neutrophils from the bone marrow and to lead to neutrophilia.^{41,51,52} Hence, the reduced expression of this receptor on bone marrow neutrophils also could have contributed to the increase in peripheral blood neutrophil levels noted with aging during influenza infection.

We found that neutrophils in the lungs of aged mice during influenza infection upregulated cell surface expression of CXCR2 but downregulated CXCR4. CXCR2 was downregulated on aged bone marrow neutrophils before infection but was similarly expressed on bone marrow neutrophils between young and aged mice at day 6 p.i. CXCR4, as stated above, was downregulated on both bone marrow and lung neutrophils with aging during influenza infection. It is possible that the increase maturation status (i.e., increased expression of CXCR2 and reduced expression of CXCR4) on lung neutrophils could be due, in part, to the impaired clearance of neutrophils in the lung by alveolar macrophages. Alternatively but not mutually exclusively, the neutrophils that enter the aged lung during infection may already have downregulated their CXCR4 receptor. Overall, chemokine receptor expression may be impacted by both the aged bone marrow niche and the altered inflammatory environment within the aged lung that occurs during influenza infection. Future investigation will be required to dissect the contributions of the bone marrow and the lung environment on the dysregulated chemokine receptor expression on neutrophils with aging.

Recently, there has been increasing interest in the contribution of the host microbiome to the development of the chronic inflammation observed in aging ("inflamm-aging").⁵³ Elderly nursing home residents have elevated inflammatory markers that correlate with distinct fecal microbial signatures compared to community-dwelling elderly subjects.⁵⁴ Similarly, aged mice housed under conventional specific pathogen-free conditions demonstrated elevated circulating inflammatory cytokines (e.g., TNF- α , IL-6) that appeared to be associated with increased gut epithelial permeability, whereas germ-free mice did not exhibit age-associated microbial dysbiosis and inflammation.²⁹ Interestingly, co-housing germ-free mice with aged specific pathogen-free mice resulted in elevated inflammatory markers, whereas germ-free mice co-housed with young specific pathogen-free mice did not.²⁹ Given that the gut microbial community has been shown to play a critical role in immune priming against respiratory pathogens, including influenza and bacteria,⁵⁵⁻⁵⁷ a potential mechanism of the augmented chemokine responses in our aged mice during influenza infection may be aged-induced gut dysbiosis, which will be examined in future studies.

Cellular senescence leads to the production of inflammatory mediators including chemokines and cytokines.^{46,58} Importantly, recent experimental and clinical evidence has revealed that senescence of AECs contributes to idiopathic pulmonary fibrosis,⁵⁹ an age-related disease. Specifically, AECs from patients with idiopathic pulmonary fibrosis or from mice treated with bleomycin

Fig. 7 AECs secrete higher levels of CXCL1 and CXCL2 with aging during influenza infection. AECs were purified from young and aged non-infected and infected mice (6 days postinfection) and cultured ex vivo. After 36 h, the supernatants were harvested and CXCL1 (a), CXCL2 (b) TNF-α (c), and IL-1β (d) were measured via ELISA. * $P < 0.05$ (Mann-Whitney test). Data are representative of two independent experiments, repeated with similar results. Error bars indicate mean $\pm$ SEM

Fig. 8 AECs from aged mice upregulate senescence-associated β -galactosidase activity. AECs were purified from young and aged non-infected and infected mice (6 days postinfection) and cultured ex vivo. After 36 h, cells were incubated for 2 h with C12FDG, a β -galactosidase substrate that produces fluorescent product upon cleavage. β -Galactosidase activity was measured via flow cytometry. Cells incubated with PBS were used as negative control. **a** Representative flow cytometry plots gated on cells expressing the cleaved fluorescent product. **b** Graph indicating the frequency of senescent cells showing the β -galactosidase activity. * $P < 0.05$. Error bars indicate mean $\pm$ SEM

exhibit senescence and exhibit an increased inflammatory, pro-fibrotic phenotype.⁵⁹ In our study, we observed a higher frequency of senescent AECs in aged mice than young mice both prior to and after influenza infection. Hence, senescence could explain why AECs from the aged mice secrete high levels of chemokines both before and after influenza infection.

Neutrophils produce extracellular traps, reactive oxidative species such as superoxide, enzymes (e.g., myeloperoxidase and elastase), and antimicrobial peptides (e.g., defensins and cathelicins) to kill pathogens.⁶⁰ Our data suggest that extracellular traps do not contribute to the age-associated increase in mortality, since DNase treatment did not extend survival during infection. Neutrophils also produce inflammatory cytokines and communicate with other innate immune cells, such as alveolar macrophages, to produce cytokines during influenza infection.²⁰ Our results show that, during influenza infection, aged mice exhibit higher levels of IL-1β, TNF-α, and IL-17 in the BAL compared to young mice. Each of these cytokines can either be produced by neutrophils^{61–63} or can activate neutrophils directly^{20,35,64} or lead to epithelial cells to produce more chemokines to promote neutrophil recruitment.^{42,64} IL-1β, TNF-α, and IL-17 can also be produced by a variety of cells including macrophages, effector, memory, and tissue memory T cells.^{65–67} Future studies will be required to determine the source of the cytokines in aged hosts and whether any of the elevations in these inflammatory cytokines or any of neutrophil's known other effector functions could contribute to inflammatory tissue damage and mortality during influenza infection with aging.

Our study implies that potential therapies that target AECs, and possibly other lung epithelial cells, neutrophils, and the interaction between neutrophils and epithelial cells, may provide a therapeutic benefit for older patients infected with influenza. If AECs from older humans exhibit senescence, one potential therapeutic

approach to target AECs would be with drugs that remove senescent cells, so called senolytics.⁶⁸ Indeed, an experimental study has shown that senolytics reduce AEC senescence and ameliorates pulmonary fibrosis.⁵⁹

In conclusion, our study has revealed that aging is associated with increased neutrophil accumulation in the lungs during influenza infection. An age-associated increase in the production of CXCL1 and CXCL2 by AECs may contribute to enhanced neutrophil chemotaxis. Thus, our study has revealed that controlling excessive neutrophil levels may improve outcomes in older people infected with influenza virus and possibly other respiratory pathogens.

MATERIALS AND METHODS

Mice and in vivo viral infection

Female C57BL/6 mice aged 2–4 months and 18–22 months were obtained from the National Institute of Aging rodent facility. Mice were infected with purified influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) (Advanced Biotechnologies) per our prior study.³⁰ Mice were anesthetized with isoflurane and instilled i.n. with 40 μ l phosphate-buffered saline (PBS) containing 1×10^4 plaque-forming units (PFU) of PR8 virus. Following infection, mice were monitored daily for changes in weight and mortality. Mice were euthanized when 30% of their original weight was lost, which was recorded as death in survival experiments. No animals were used in the study if they displayed evidence of infection or other illnesses prior to PR8 administration.

Treatment in mice

Anti-Ly6G treatment. (i) Treatment at day 6 p.i.: mice were treated intraperitoneally (i.p.) with 0.5 mg in 100 μ l anti-Ly6G (clone: 1A8, BioXCell) antibody and also i.n. with 0.25 mg in 50 μ l on alternate days starting from day 6 p.i. until day 12 p.i.

(ii) Day 0 p.i.: mice were treated i.p. with 0.5 mg in 100 μ l anti-Ly6G antibody and also i.n. with 0.25 mg in 50 μ l on alternate days from day 0 to day 18 p.i., as described before.^{17,33} Control mice were treated with Rat IgG.

DNase I treatment. Mice were treated i.n. with 50 μ g/mouse (1 μ g/ μ l) DNase I (Affymetrix), on alternate days starting from day 6 p.i. until day 12. Control mice were treated with PBS.

Sample collection and preparation

Mice were euthanized with an overdose of isoflurane. To harvest the lungs, the chest cavity was opened and the lungs were removed. Non-perfused lung tissue were harvested from PR8-infected mice at 1, 3, 6 and 9 days p.i. The BAL was obtained by washing the lungs twice with 1 ml cold sterile $1 \times$ PBS. Fresh BAL was centrifuged at 1400 rpm for 5 min and supernatant was stored at -80°C . The cells from BAL were used for flow cytometry.

Assessment of tissue injury

Lung injury was assessed by measuring total protein and LDH levels in the BAL fluid. LDH activity was measured with an LDH Assay Kit (Roche; NY, USA) according to the manufacturer's instructions. Total protein in BAL was measured using the BCA Protein Assay Kit (Thermo Scientific; MA, USA) following the manufacturer's instructions.

Viral load measurement

Tissue preparation. Lung lysates from left lung lobe from mice were prepared in PBS using TissueLyser II (Qiagen). Lung lysate supernatants were collected by centrifugation at $350 \times g$ for 10 min and stored at -80°C . Lung lobe weights were calculated by subtracting weight of the tube without lung lobe from weight of the tube with lung lobe.

Plaque assay. Live lung viral titers were determined by plaque assay using Madin Darby canine kidney (MDCK) cells (provided by Dr. Adam Lauring, University of Michigan). MDCK cells were cultured in 6-well plates overnight until the cell monolayer reached $>70\%$ confluence. Lung supernatants were thawed at room temperature and diluted to different dilutions (i.e., 10^{-1} – 10^{-6}) with 0.1% bovine serum albumin (BSA) in $1 \times$ PBS. Cells were washed twice with $1 \times$ PBS followed by 1 h incubation with 400 μ l lung supernatants at 37°C with shaking every 15 min. Cells were washed twice with $1 \times$ PBS to remove excess virus. The cells were then covered with 2 ml over-layer medium containing a 1:1 mixture of 2% agarose and $2 \times$ Dulbecco's modified Eagle's medium (DMEM) containing 2 μ g/ml (final concentration) acetylated trypsin (Sigma-Aldrich). Plates were incubated at 37°C for 72 h. The agarose was removed from the plates and the cells stained with 0.3% crystal violet for 5 min. The plates were washed and viral plaques were counted. The PFU per gram (PFU/g) were calculated using the formula: (# plaques $\times$ dilution factor)/weight of the lung lobe.

Flow cytometry

To obtain single-cell suspension, the lungs were harvested from animals, minced, and then digested with 1 mg/ml Collagenase D (Roche) and 100 U/ml DNase (DN25, Sigma) in PBS without calcium and magnesium for 45 min at 37°C . After digestion, lung tissue was disrupted into single-cell suspension and passed through a 100- μ m sieve followed by 70- μ m sieve (Fisherbrand). To remove red blood cells, cells were incubated with red blood cell lysis buffer (Biolegend) for 3 min.

Live cells were spun and re-suspended in PBS. Dead cells were stained using the LIVE/DEAD Fixable Aqua Dead Cell Staining Kit (ThermoFisher Scientific) or Zombie Aqua Fixable Viability Kit (Biolegend) for 30 min. Cells were washed by centrifugation at 1400 rpm for 5 min at 4°C and resuspended in PBS containing 0.5% BSA (fluorescence-activated cell sorter (FACS) buffer). Fc receptors were blocked by incubating cells with anti-CD16/32 antibody (clone: 93, Biolegend) for 10 min. Cells were thereafter stained with antibodies for the desired surface markers for 20 min. Cells were washed in FACS buffer and fixed in 2% paraformaldehyde for 20 min. Following another wash, cells were stored in FACS buffer until analysis. Flow cytometric data were acquired on Cytoflex for <5 colors (Beckman-Coulter) or Mo-Flo for >5 colors (Beckman-Coulter) and analyzed using the FlowJo software. For the expression of CXCR2 and CXCR4, median fluorescent intensity is shown in arbitrary units.

Antibodies

APC: anti-Ly6G (clone: 1A8, Biolegend); APCeF780: CD8 (clone: 53-6.7, eBioscience), CD45 (clone: 30-F11, eBioscience), CD24 (clone: M1/69, eBioscience); BV421: CXCR4 (clone: L276F12, Biolegend); eF450: CD11b (clone: M1/70, Invitrogen); FITC: Ly6C (clone: HK1.4, Biolegend); PE: CXCR2 (clone: SA044G4, Biolegend), SiglecF (clone: E50-2440, BD bioscience); PECy5: CD19 (clone: 1D3, eBioscience); PECy7: CD4 (clone: GK1.5, eBioscience); PEeF610: CD11c (clone N418, eBioscience); PEVio770: CD64 (clone: REA286, Miltenyi Biotec); PerCPeF710: MHCII (clone: M5/114.15.2, eBioscience).

Enzyme-linked immunosorbent assay

CXCL1, CXCL2, CXCL5, IL-1 β , TNF- α , and IL-17 levels in BAL and cell culture supernatants were measured with ELISA kits (R&D Systems for CXCL1, 2, 5 and IL-17; Invitrogen for IL-1 β and TNF- α) following the manufacturers' instructions. Briefly, 96-well plates were coated with capture antibodies overnight at room temperature for measurement of CXCL1/2/5 and IL-17 and at 4°C for measurement of IL-1 β and TNF- α . The unbound antibody was removed by washing plates three times with wash buffer. The unspecific binding was removed by incubating plate with blocking buffer for 2 h at room temperature. The test samples and standard were

added and the plate was incubated overnight at 4 °C. The plate was washed as before and incubated with detection antibody overnight at 4 °C or 1 h at room temperature. After washing as before, the bound antibody was detected using streptavidin and TMB substrate. Reaction was stopped with stop solution (2 N H₂SO₄). The absorbance was measured at 450 nm using a plate reader (BioTek). The concentration was determined based on absorbance values of the standard. The ELISA kits had the following limits of detection: CXCL1, 2, 5 and IL-17: 15.6 pg/ml, IL-1β and TNF-α: 8 pg/ml.

Cell migration assay

Neutrophils were isolated from mouse bone marrow using the EasySep Mouse Neutrophil Enrichment Kit (Stem Cell Technologies). Neutrophil migration was analyzed using the CytoSelect Cell Migration Kit (Cell Biolabs Inc) containing 12-well plates with polycarbonate membrane of 3-μm pore size as described in the manufacturer's instructions. Briefly, neutrophils were resuspended in serum-free RPMI 1640 medium and added to the upper chamber of the plates. The lower chamber consisted of BAL or lung lysate from experimental mice or neutrophil-attracting chemokine as positive control or PBS as negative control. CXCL1 and CXCL2 in lung lysates were blocked using anti-CXCL1 (R&D, clone 48415) and anti-CXCL2 (Invitrogen, clone 40605) neutralizing antibodies. The concentration of the antibodies used for neutralizing was as per the vendor's guidelines. The plates were incubated for 1 h at 37 °C. The neutrophils that migrated to the lower chamber were counted under light microscope using a Neubauer chamber and then flow cytometric analysis.

AEC isolation

AECs were isolated by magnetic associated cell sorting (MACS) as previously described.⁶⁹ Briefly, single-cell suspensions from the lungs were first incubated with CD45 microbeads and CD31 beads in PBS containing 2 mM EDTA and 0.5% BSA. The unbound antibody was removed by centrifuging at 300 × g for 5 min at 10 °C. Cells were then passed through a MACS column placed in the magnetic field of MidiMACS separator. The number of cells in flow though containing CD45⁺ CD31⁺ cells were counted and the cells were incubated with anti-mouse biotin-labeled CD326 (EpCAM) antibody (Miltenyi Biotec) according to the manufacturer's instructions. Cells were washed as before and incubated with anti-biotin microbeads (Miltenyi Biotec). After washing, cells were again passed through a MACS column. The flow through was discarded and CD326⁺ AECs attached to the column were removed by firmly applying a plunger. We typically obtained >84% purity of CD326⁺ cells. The number of cells were counted and resuspended at a concentration of 1 million cells/ml in DMEM/F12 medium containing 10% FCS and allowed to adhere to tissue culture plates for 24 h followed by DMEM/F12 medium containing 1% BSA overnight. Cell culture supernatants following the overnight culture were stored at −80 °C until analysis.

SA-β-galactosidase detection by flow cytometry

Flow cytometric-based detection of SA-β-galactosidase was performed as described before.⁴⁷ Briefly, AECs from young and aged non-infected and influenza-infected mice were isolated and cultured as described above. Following 36-h culture, the cells were treated with Bafilomycin A1 (100 nM, Enzo Life Sciences) for 1 h followed by 2-h incubation with C12FDG (33 μM, Thermo-Fischer). Cells were harvested, washed once in ice-cold PBS, and immediately measured on a flow cytometer (Cytoflex, Beckman-Coulter). Data were analyzed using the Flow-Jo software.

Statistics

Mann-Whitney *U* (equivalently Wilcoxon Rank Sum) test was used to compare two groups (e.g., young vs. aged) for continuous outcomes (e.g., neutrophils). Log rank (Mantel-Cox) test was used for

comparing two groups in terms of survival. Statistical analysis was carried out using the Prism 7 (GraphPad) software. All results are based on two-sided tests and we evaluated statistical significance at the level of 0.05. Instead of reporting the exact *P* values, in figures we present the range of *P* values using **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Error bars are presented as means with SEM.

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AUTHOR CONTRIBUTIONS

U.K. and D.R.G. conceived and designed the experiments. U.K. performed the experiments with the help of C.S. and S.W. Data analysis was done by U.K. D.R.G. wrote the first draft of the paper that was then edited by U.K. All authors subsequently contributed in writing and carefully reviewing the experiments and the manuscript.

ADDITIONAL INFORMATION

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