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Impaired heterologous immunity in aged ferrets during sequential influenza A H1N1 infection

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ABSTRACT

The major burden of influenza morbidity resides within the elderly population. The challenge managing influenza-associated illness in the elderly is the decline of immune function, where mechanisms leading to immunological senescence have not been elucidated. To better represent the immune environment, we investigated clinical morbidity and immune function during sequential homologous and heterologous H1N1 influenza infection in an aged ferret model. Our findings demonstrated experimentally that aged ferrets had significant morbidity during monosubtypic heterologous 2° challenge with significant weight loss and respiratory symptoms. Furthermore, increased clinical morbidity was associated with slower and shorter hemagglutinin antibody generation and attenuated type 1 T-cell gene responses in peripheral blood. These results revealed dampened immune activation during sequential influenza infection in aged ferrets. With the presence of an aged model, dissecting clinical morbidity, viral dynamics and immune response during influenza infection will aid the development of future prophylactics such as age specific influenza vaccines.

Introduction

The elderly influenza disease rate in humans carries a heavy burden on healthcare systems and perpetuates virus circulation in the general population. During the 2012–2013 season, persons aged ≥ 65 years accounted for ~50% of all influenza-related hospitalizations in the US (Centers for Disease Control and Prevention (CDC), 2013). Since the host immune system deteriorates significantly during aging, the inability to generate effective, broad-spectrum immune memory following infection or vaccination contributes to increased influenza burden among the elderly (Bridges et al., 2000; Castle, 2000; Dao et al., 2010; Thompson et al., 2003). Aging influences various facets of the immune response, but T-cell populations are prominently affected due to thymus involution limiting naïve T-cell production (Aw and Palmer, 2011; Buchholz et al., 2011; Castle, 2000). Post-thymic homeostatic T-cell proliferation compensates for production deficit (Aw and Palmer, 2011; Buchholz et al., 2011), but long-term T-cell replication leads to cell-intrinsic dysfunction highlighted by progressive loss of repertoire diversity and weakened responses (Buchholz et al., 2011). This directly influences cell-mediated T-cell responses, most evident during viral infection (Buchholz et al., 2011; Deng et al., 2004; Effros et al., 2003), while indirectly affecting humoral responses (Eaton et al., 2004). Together with high rates of antigenic change of circulating virus population, this process puts the elderly at greater risk of recurring influenza infection (Bridges et al., 2000).

Age-related immune dysregulation modestly impacts disease severity in animal models of primary influenza infection (Guo et al., 2012; Josset et al., 2012; Muto et al., 2012; Pica et al., 2012). Heterosubtypic influenza A immune memory is severely impaired in aged animals (Bender and Small, 1993; Decman et al., 2010), although the elderly's sensitivity to monosubtypic antigenic change is unclear. To address this question, we investigated clinical morbidity, viral dynamics, and subsequent immune responses to sequential influenza A H1N1 infection for the first time in an aged ferret model. Here we report dampened immunity in aged ferrets

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upon monosubtypic heterologous 2° challenge associated with diminished antibody production and altered T-cell responses in peripheral blood. These findings help elucidate the immune dynamics which contribute to elderly influenza susceptibility and put forth the aged ferret model for further study of aging immunity and influenza.

Results

Aged ferrets develop more severe disease than adults during heterologous monosubtypic 2° challenge

Ferrets closely mimic the clinical manifestations of influenza infection in humans as shown previously (Banner and Kelvin, 2012; Belser et al., 2011; Huang et al., 2011, 2013; Rowe et al., 2010). Naïve adult (4–6 months old) and aged ($\geq$ 4 years old) male ferrets were placed into groups for either homologous or heterologous H1N1 sequential infection studies. The homologous sequentially infected group was first infected intranasally with pandemic 2009 H1N1 strain A/Mexico/4108/2009 (Mex/4108) as the 1° infection after 46 days the animals were then infected with pandemic 2009 H1N1 A/California/07/2009 (Cal/07) as 2° challenge. The heterologous 1° infection–2° challenge group was infected with seasonal H1N1 A/Brisbane/59/2007 (Bris/59) and then subsequently infected with Mex/4108 Day 39 post 1° infection. Animals were intranasally infected with the indicated virus strain at 10^6 EID_{50} and clinical signs were monitored for 14 days post-infection/challenge (temperature, weight change, nasal discharge (clear discharge or color dry mucus/exudate), sneezing, and lethargy).

During both 1° strain infections aged animals exhibited clinical morbidity that was modestly more pronounced than in adults, with greater weight loss (7–8% peak loss) and more frequent production of mucus/exudate. Sneezing was also observed more frequently in our aged cohorts, but sneezing incidence rates in aged ferrets (50%) were consistent with previously reported rates in adults during sH1N1 or H1N1pdm infection (Huang et al., 2011). Upon homologous 2° challenge with Cal/07, neither age group exhibited clinical symptoms except for sporadic detection of clear nasal discharge and sneezing (Fig. 1A). In contrast, during heterologous 2° challenge with Mex/4108, adult ferrets showed mild clinical morbidity whereas the aged animals developed greater illness with $>$6% peak weight loss, sneezing, prominent nasal discharge (yellow–brown color exudate or dry mucus), and lethargy.

Having detected disease differences between aged and adult ferrets during sequential influenza infection, we next examined if morbidity was associated with viral burden in nasal washes. Live virus titers were measured at Days 3 and 7. No significant differences in viral burden/clearance were detected between the age groups at any time-point tested. Virus was undetectable by Day 3 following homologous 2° challenge in both age groups. Mex/4108 virus titers were dramatically reduced at Day 3 post-heterologous 2° challenge when compared to 1° infection ($\sim$ 100-fold), consistent with previous reports (Fang et al., 2012) (Fig. 1C). Interestingly, aged animals still developed severe clinical morbidity during heterologous 2° challenge (Fig. 1B) despite reduced viral titers.

Antibody production in aged ferrets is delayed and not sustained at levels equivalent to adults

The increased disease severity in aged ferrets during heterologous 2° challenge prompted us to investigate possible causes of the disease disparity between aged and adult animals. Roles for humoral (Fang et al., 2012) and cell-mediated immunity (Guo et al., 2011; Tu et al., 2010) have been identified in heterologous influenza A H1N1 rechallenge models. First we investigated humoral responses in our cohort by assaying sera taken from the ferrets at designated time points for haemagglutination inhibition (HI) against Bris/59 or Mex/4108 viruses. Aged ferrets failed to maintain antibody titers at the same levels as adults following either 1° Bris/59 or Mex/4108 infection. By Day 28 post Mex/4108 1° infection, aged ferrets had significantly reduced haemagglutinin antibody levels compared to adults which remained significantly lower through the 2° infection with Cal/07 (Fig. 2A). Aged ferrets were also slower to mount an initial humoral response to the Bris/59 virus during 1° infection, as adults had generated antibodies toward Bris/59 by Day 7 post-infection whereas aged animals had undetectable levels (Fig. 2B). Similar antibody responses were seen between adult and aged ferrets during the 2° challenge with the Mex/4108 virus. Together, these results show that aged ferrets respond differently (slower and less) compared to adult ferrets in respect to protective antibody generation which may be dependent on the virus strain and the sequence of insult.

Peripheral type 1 T-cell gene responses to heterologous monosubtypic 2° challenge are attenuated in aged ferrets

We next investigated other peripheral immune responses to complement the humoral immunity evaluation. Host gene expression analysis for the molecular dissection of circulating immune cell regulation (Fig. 3) was performed on in-life peripheral blood samples taken from animals throughout the infection time course. In our analysis we included gene sets for cell mediated immunity, inflammatory cytokines and T cell regulation. Strikingly, we detected rapid increases in CD4 and CD8 mRNA expression during heterologous 2° challenge in adult but not aged ferrets, with peak differences in CD4 (2-fold) and CD8 (3-fold) expression detected at Day 1 post-2° challenge (p2°) (Fig. 3B (i and ii)). A similar expression profile was detected for CD28 (T-cell activation cell surface marker) with significantly higher expression in adults. (Fig. 3B (iii)). Together, these profiles suggested rapid mobilization of adult T-cells to peripheral blood during heterologous challenge which was diminished in the aged. Moreover, we also detected a trend of reduced CD19 (B-lymphocyte maker) expression in aged ferrets (Fig. 3B (iv)), consistent with impaired humoral responses as suggested by our HI data. In contrast, similar innate response gene profiles (CXCL8, CXCL10, and TLR3) were detected in both groups with mostly no changes in gene regulation throughout the time course except for acute upregulation of CXCL8 and CXCL10 during heterologous 2° challenge at Day 1 p2° (Fig. 3 (v–vii)) (2-fold and 10–20-fold increase, respectively).

Given the role of type 1 T-cell responses in heterologous immunity (Guo et al., 2011; Tu et al., 2010), we further investigated circulating T-cell population effector status by measuring TBX21 (transcription factor expressed in Th1-commited CD4+ T-cells and CD8+ T-cells) (Sullivan et al., 2003; Szabo et al., 2000), GZMA (cytotoxic T-cell effector molecule) (Anthony et al., 2010), as well as IFNγ and TNFα (type 1 cytokines) (Grivennikov et al., 2005; Xu et al., 2004) levels (Fig. 3(viii–xi)). As above, TBX21 and TNFα mRNA expression declined early (Day 1 p2°) during heterologous 2° challenge in aged ferrets while remaining stable in adult ferrets (TBX21: 2-fold) (TNFα: 2-fold) (Fig. 3B (viii and x)). Furthermore, peak GZMA mRNA expression was reduced in aged ferrets (Fig. 3 (ix)). Together, these findings suggest a potential age-related decrease in type 1 T-cell responses specific to heterologous 2° challenge which may have contributed to disease (Bender and Small, 1993; Decman et al., 2010).