Increased Viral Loads and Exacerbated Innate Host Responses in Aged Macaques Infected with the 2009 Pandemic H1N1 Influenza A Virus

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In contrast to seasonal influenza virus infections, which typically cause significant morbidity and mortality in the elderly, the 2009 H1N1 virus caused severe infection in young adults. This phenomenon was attributed to the presence of cross-protective antibodies acquired by older individuals during previous exposures to H1N1 viruses. However, this hypothesis could not be empirically tested. To address this question, we compared viral replication and the development of the immune response in naïve young adult and aged female rhesus macaques infected with A/California/04/2009 H1N1 (CA04) virus. We show higher viral loads in the bronchoalveolar lavage (BAL) fluid and nasal and ocular swabs in aged animals, suggesting increased viral replication in both the lower and upper respiratory tracts. T cell proliferation was higher in the BAL fluid but delayed and reduced in peripheral blood in aged animals. This delay in proliferation correlated with a reduced frequency of effector CD4 T cells in old animals. Aged animals also mobilized inflammatory cytokines to higher levels in the BAL fluid. Finally, we compared changes in gene expression using microarray analysis of BAL fluid samples. Our analyses revealed that the largest difference in host response between aged and young adult animals was detected at day 4 postinfection, with a significantly higher induction of genes associated with inflammation and the innate immune response in aged animals. Overall, our data suggest that, in the absence of preexisting antibodies, CA04 infection in aged macaques is associated with changes in innate and adaptive immune responses that were shown to correlate with increased disease severity in other respiratory disease models.

Influenza virus infections pose a significant health problem and remain among the leading causes of morbidity and mortality in the elderly (19). This is in part due to the lower rate of seroconversion and poor immunogenicity of the seasonal influenza vaccines observed in the elderly (6, 28, 30). The threat of influenza virus infection is amplified by antigenic drift (mutations in the genes encoding hemagglutinin [HA] and neuraminidase [NA]) as well as the potential to create new genomic reassortants (especially during interspecies transmission), which leads to the introduction of new viral strains that the population is largely naïve toward (23).

In April 2009, a new reassortant swine-origin H1N1 influenza virus emerged, and by June 2009, the World Health Organization declared this virus to be the cause of the first influenza pandemic of the 21st century. In contrast to seasonal influenza virus, in which 90% of reported deaths are associated with advanced age, the pandemic H1N1 virus caused more severe morbidity and mortality in children and young adults, with 87% of deaths involving patients between the ages of 5 and 59 years and 70% of the reported hospitalizations in patients <50 years of age. (4a, 12). Several hypotheses were put forth to explain the reduced severity of the disease in the elderly. The most prevalent hypothesis was that older individuals were protected due to the presence of preexisting cross-reactive antibodies acquired by exposure to circulating H1N1 strains during the 1950s (9, 34). However, only 30%

of individuals who were born before 1950 had cross-neutralizing antibodies, and only 50% of individuals who received the 1976 vaccine generated cross-neutralizing antibodies (9). These observations suggest that additional factors may have contributed to the protection of older individuals. Another mechanism put forth to explain the increased severity of the disease in younger adults is the deposition of pulmonary immune complexes created by weakly cross-reactive nonneutralizing antibodies generated by exposure to seasonal influenza virus (21). Although these immune complexes correlated with severe lung injury, whether they are a consequence or the cause remains to be determined. Thus, it is possible that additional factors contributed to this reversal in age-related susceptibility.

To characterize the impact of age on the host response to 2009 H1N1 pandemic influenza virus infection in the absence of pre-

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existing immunity, we compared viral loads and host response between naïve young adult and aged rhesus macaques infected with 2009 H1N1 virus strain CA04. Although influenza virus studies are usually carried out in rodents or ferrets, the close genetic and physiological homology of nonhuman primates to humans makes them a robust model to investigate influenza virus pathogenesis. Indeed, studies in cynomolgus macaques (*Macaca fascicularis*) have provided invaluable insight into the pathophysiology of the highly virulent H5N1 and 1918 viruses (3, 5, 14, 29). A few studies have compared disease severity and host response following infection with pandemic 2009 isolates to those following infection with seasonal H1N1 viruses in cynomolgus macaques (10, 11, 17, 25). These studies revealed that in comparison to seasonal H1N1 virus, CA04 replicated more efficiently in the respiratory tract (11, 17), resulting in increased cellular infiltrates (13, 14) and proinflammatory cytokine production (11) in the lungs. In addition, a recent study in cynomolgus macaques recapitulated the significant clinical differences between two early isolates of the 2009 H1N1 pandemic virus: A/Mexico/4108/2009 (Mex/4108; isolated from a mildly ill child) and A/Mexico/InDRE4487/2009 (Mex/4487; isolated from a 26-year-old individual from a family cluster of severe disease) (25). Therefore, clinical differences in pathogenicity between pandemic and seasonal H1N1 viruses as well as between different pandemic isolates were maintained following experimental inoculation in macaques. Another study demonstrated that T cells generated following seasonal influenza virus infection can play a critical role in early control of CA04 replication (32).

Although these studies were instrumental in understanding the pathogenesis of the 2009 influenza pandemic, to date, no studies have investigated how age affects the host immune response to CA04 from either a functional or transcriptional standpoint. In this study, we investigated the impact of age on viral replication and the adaptive immune response to the pandemic 2009 H1N1 CA04 virus in naïve rhesus macaques. Data presented herein show that aged animals (i) experience higher viral loads than young adult animals, (ii) produce a comparable CD8 T cell response but a lower frequency of effector CD4 T cells, (iii) generate a higher hemagglutination inhibition (HI) titer than young adult animals, and (iv) produce higher levels of inflammatory cytokines. Moreover, gene expression studies revealed higher upregulation of genes associated with inflammation and the innate immune response and a lower upregulation of T cell-specific genes in aged animals.

MATERIALS AND METHODS

Virus. A/California/04/2009 (H1N1) virus was grown in MDCK cells and harvested when >70% of the cells exhibited a cytopathic effect for virus stock generation. The virus was titrated on MDCK cells using a 50% tissue culture infective dose (TCID₅₀) assay as previously described (4).

Animal studies and sample collection. The study was carried out in strict accordance with the recommendations described in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (22a), the Office of Animal Welfare, and the United States Department of Agriculture. All animal work was approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee, which is accredited by the American Association for Accreditation of Laboratory Animal Care (PHS/OLAW Animal Welfare Assurance A3304-01). All procedures were carried out while the animals were under ketamine anesthesia by trained personnel under the supervision of veterinary staff, and all efforts were made to ameliorate the welfare and to

minimize animal suffering, in accordance with the recommendations in *The Weatherall Report on the Use of Non-Human Primates in Research* (24a). Animals were housed in adjoining individual primate cages allowing social interactions under controlled conditions of humidity, temperature, and light (12-hour light/12-hour dark cycles). Food and water were available *ad libitum*. Animals were monitored and fed commercial monkey chow, treats, and fruit twice daily by trained personnel. Environmental enrichment consisted of commercial toys.

Sixteen female rhesus macaques (*Macaca mulatta*) 10 to 12 and 20 to 24 years of age were used in these studies ($n = 8/\text{group}$). These animals had no preexisting immunity against CA04, as evidenced by lack of T cell responses (see Fig. 4) or antibodies (see Fig. 5E and F) on day 0. Animals were infected using a combination of intratracheal (4 ml), intranasal (0.5 ml/nostril), and conjunctival (0.5 ml/eyelid) routes for a total dose of 7×10^6 TCID₅₀s dose. Blood samples were collected preinfection and on days 0, 1, 2, 4, 7, 10, 14, 21, 28, 35, 77, and 99, and bronchoalveolar lavage (BAL) fluid samples were collected preinfection and again on days 0, 4, 7, 10, 14, 21, 28, 35, 77, and 99. Nasal, ocular, and throat swab specimens were collected on days 0, 1, 2, 3, 4, 7, 10, 14, and 21. At the end of the study, the animals were released back to the colony.

Viral loads. Viral RNA was extracted from BAL fluid supernatant and nasal, ocular, and throat swabs. RNA isolation was performed using a ZR viral RNA kit per the manufacturer's instructions (Zymo Research, Irvine, CA). Briefly, 100 μl of supernatant was transferred to a tube containing 300 μl of ZR viral RNA buffer. This mixture was bound to a Zymo-Spin IC column by centrifugation at $16,000 \times g$ for 2 min. The flowthrough was discarded, and the column was washed twice with 300 μl of RNA wash buffer. Residual wash buffer was removed by centrifugation, and the purified RNA was eluted with 12 μl of RNase-free water. Purified RNA was reverse transcribed using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions for 20- μl reaction mixtures. Viral loads were determined using absolute quantitative RT-PCR using primers and probes specific for CA04 HA viral RNA and an amplicon standard. The forward primer sequence is 5' GAT GGT AGA TGG ATG GTA CGG TTA T 3', the reverse primer sequence is 5' TTG TTA GTA ATC TCG TCA ATG GCA TT 3', and the probe sequence is 5' 6-FAM ATA TGC AGC CGA CCT GAA GAG CAC ACA 3' BHQ (where FAM is 6-carboxyfluorescein and BHQ is black hole quencher dye). Primers and probes were purchased from Applied Biosystems. cDNA was subjected to 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Experiments were carried out using a StepOnePlus real-time PCR system from Applied Biosystems.

Lymphocyte proliferation. Peripheral blood mononuclear cells (PBMCs) and BAL fluid cells were surface stained with antibodies against CD8 β (Beckman Coulter, Brea, CA), CD4 (eBioscience, San Diego, CA), and CD28 and CD95 (BioLegend, San Diego, CA) to delineate the naïve (CD28⁺ CD95⁻), central memory (CM; CD28⁺ CD95⁺), and effector memory (EM; CD28⁻ CD95⁺) T cell subsets (20). PBMCs and BAL fluid cells were also surface stained with antibodies against CD20 (Beckman Coulter, Brea, CA), IgD (Southern Biotech), and CD27 (BioLegend) to delineate the naïve (IgD-positive [IgD⁺] CD27⁻), marginal-zone like (MZ-like; IgD⁺ CD27⁺), and memory (IgD-negative CD27⁺) B cell subsets (20). Cells were fixed and permeabilized according to the manufacturer's recommendations (BioLegend), followed by nuclear permeabilization (10% dimethyl sulfoxide [DMSO] in permeabilization buffer), before the addition of Ki67-specific antibody (BD Pharmingen). The samples were acquired using an LSRII instrument (Becton Dickinson, San Jose, CA), and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Intracellular cytokine staining. The peptide libraries were designed using software available on the Sigma website (St. Louis, MO) as 20-mers overlapping by 12 amino acids. The GenBank accession numbers of the CA04 genes are as follows: NS1-NEP, JF915191; PB1(F2), JF915189; M1-M2, JF915185; PB2, JF915190; NP, JF915187; PA, JF915188; NA, JF915186; and HA, JF915184. Peptides were reconstituted in DMSO, and

libraries of peptides were made for HA-NA (128 peptides), PA-NP (148 peptides), PB1-NS1-NEP (133 peptides), and PB2-M1-M2 (136 peptides).

BAL fluid cells and PBMCs were stimulated with the overlapping peptide libraries for 16 h. At the end of the incubation, cells were first stained with anti-CD4 and anti-CD8 β antibodies. The cells were then permeabilized, followed by the addition of anti-gamma interferon (anti-IFN- γ) and anti-tumor necrosis factor alpha (anti-TNF- α). Samples were acquired using an LSRII instrument, and the data were analyzed using FlowJo software.

Assessment of humoral immune response. IgG antibody titers were measured by enzyme-linked immunosorbent assay (ELISA) using plates coated with recombinant CA04 HA protein (Sino Biological Inc., Beijing, China). ELISA plates were coated with 1 μ g/ml solution antigen overnight at 4°C, washed three times with 0.05% Tween-phosphate-buffered saline (PBS), and incubated with heat-inactivated (56°C, 30 min) plasma samples in 3-fold dilutions in triplicate for 1 h. After washing three times with 0.05% Tween-PBS, horseradish peroxidase (HRP)-conjugated anti-rhesus IgG (Open Biosystems, Rockford, IL) was added for 1 h, followed by addition of *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma, St. Louis, MO). The reaction was stopped with the addition of 2 M HCl. IgG endpoint titers were calculated using log-log transformation of the linear portion of the curve and 0.1 optical density (OD) units as the cutoff. IgG titers were standardized using a positive-control sample that was included in every ELISA plate.

Hemagglutination inhibition titer assays were performed using chicken red blood cells as previously described (4). Results are expressed as the reciprocal serum titer at which inhibition of hemagglutination by the CA04 virus was no longer observed.

Plasma cytokine levels. Plasma samples (stored at -80°C) were thawed and diluted 1:2 in a serum matrix for analysis with Milliplex non-human primate magnetic bead panel as per the manufacturer's instructions (Millipore Corporation, Billerica, MA). Concentrations for TNF- α , interleukin-6 (IL-6), IL-12/23p40, IL-8, monocyte chemoattractant protein 1 (MCP-1), IL-1 receptor antagonist (IL-1ra), soluble CD40L (sCD40L), IL-15, IFN- γ , IL-4, and IL-17 were determined for all samples. Values below the limit of detection of the assay were assigned a value half that of the lowest value detectable in that assay.

RNA extraction and microarray analysis. One million BAL fluid cells were resuspended in TRIzol (Applied Biosciences), and RNA was extracted using an RNeasy microkit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was resuspended in RNase-free water, and the RNA concentration was measured using a Thermo Scientific Nano-drop 2000 spectrophotometer (Fischer, Houston, TX). The integrity of each RNA sample was determined using an Agilent 2100 bioanalyzer with Expert software (Agilent Technologies, Santa Clara, CA). Probe labeling and microarray slide hybridization were performed for each biological replicate using a rhesus macaque gene expression microarray kit (Agilent Technologies), according to the manufacturer's instructions. One hundred nanograms of total RNA was used for cRNA synthesis and probe preparation using an Agilent one-color low-input Quick Amp labeling kit (Agilent Technologies). Labeled cRNA (1,500 ng) was hybridized to each slide of the Agilent rhesus macaque 4 $\times$ 44K microarrays (representing over 20,200 transcripts). Dry slides were scanned on an Agilent DNA microarray scanner (model G2505B) using the XDR setting. Raw images were analyzed using Agilent Feature Extraction software (version 9.5.3.1) and the GE1 (version 5_95_Feb07) extraction protocol. For each microarray, raw intensities, probe mappings, and quality control (QC) metrics were output to a custom laboratory information management system (LabKey software).

Microarray data analysis. Extracted raw data were background corrected using the norm-exp method with an offset of 50 and quantile normalized using the limma package in the R environment. Replicated probes were mean summarized, and control probes were filtered out. Transcriptional similarities between samples were visualized using nonmetric mul-

tidimensional scaling (MDS) to map Euclidian distances calculated on transcriptomic data expressed in intensity scale into the 2-dimensional space with minimal loss of information (evaluated by Kruskal's stress). Gene expression data for each animal at each time point were calculated as the ratio of its gene expression at day 0 postinfection (p.i.). These ratios were logarithm base 2 transformed and are referred to as log₂ ratios. A Student *t* test on log₂ ratios was performed to determine the probes that were differentially changed upon infection between aged and young adult animals. Criteria for differential expression were an absolute difference between log₂ ratios of aged and young adult animals of >2 and a Benjamini-Hochberg adjusted *q* value of <0.05.

Functional analysis of statistically significant gene expression changes was performed using the Ingenuity Pathways Knowledge Base (IPA; Ingenuity Systems), with enrichment scores calculated using all probes present in the microarray as the background data set.

Meta-analysis of immune cells and interferon-stimulated genes. A meta-analysis was performed using publicly available microarray data from the immune response *in silico* (IRIS) database of 12 different types of human leukocytes from peripheral blood and bone marrow before and after treatment to induce activation and/or differentiation (1). Raw CEL files were downloaded from the NCBI GEO website (GEO accession number GSE22886), preprocessed in R using the affy package, and normalized using the robust multiarray analysis (RMA) method. For comparison with our data set, the Entrez Gene accession number was chosen as the common gene identifier. A gene was defined to be highly expressed and specific in a cell subset compared to all other immune population genes using the Student *t* test with a Benjamini-Hochberg adjusted *q* value of <0.05 and an absolute log₂ fold change of >2.

In-house data for Calu-3 cells treated with IFN- α , - β , or - γ for 3, 6, and 21 h were used to define genes induced after IFN treatment (interferon-stimulated genes [ISGs]). ISGs were defined as probes differentially expressed after treatment with at least one type of IFN and at one time point (Student *t* test with *q* value of <0.05 and an absolute log₂ fold change of >2).

Fisher's exact test was used to determine whether the proportion of specific immune genes in each cluster was significantly greater than the proportion of immune genes in the background. To look for a correlation between immune cell proportions and gene expression, the Spearman rank correlation was used on transcriptomic data in log₂ intensities, and time-matched cell frequency (in total number of cells) was determined by flow cytometry.

Statistical analysis. Repeated-measures analysis of variance (ANOVA) with a first-order autoregressive covariance structure was used to compare the immune response measures between young adult and aged animals. Preplanned contrasts were used to compare the difference in immune response measures between young adult and aged animals at each day postinfection. Prior to application of the repeated-measures ANOVA, ELISA titers and viral genome copy number data were transformed using the logarithm function with base 10 to hold the normal distribution assumption. Statistical significance was determined at the level of 0.05. The area under the curve (AUC) as an overall response was calculated by trapezoidal integration. A Mann-Whitney-Wilcoxon test was used for assessing differences in AUCs between aged and young adult animals.

Microarray data accession number. Raw microarray data have been deposited in NCBI's Gene Expression Omnibus database under GEO Series accession number GSE38502 and are also accessible through the website of the M. G. Katze lab (<https://viromics.washington.edu>).

RESULTS

Animal characteristics and clinical outcomes. To characterize the impact of age on the immune response to pandemic H1N1 influenza virus infection, 8 young adult and 8 aged female rhesus macaques were inoculated with A/California/04/2009 (CA04) virus. Prior to infection, aged animals had significantly fewer naïve

FIG 1 Aged animals display an increased viral burden compared to young adult animals. Viral loads were measured using quantitative reverse transcriptase PCR using primers and probes specific for CA04 HA in throat (A), nasal (B), and ocular (C) swabs as well as BAL fluid samples (D). Our analysis revealed a slightly longer duration of viral shedding in nasal swabs, a higher ocular viral load at 10 dpi, and higher BAL fluid viral loads at 4 and 7 dpi. Means $\pm$ SEMs are shown. *, $P < 0.05$, **, $P < 0.005$.

T cells and a higher frequency of terminally differentiated (EM) T cells, the cardinal hallmark of immune senescence (see Fig. S1 in the supplemental material). Animals were inoculated via the tracheal, nasal, and ocular routes with a total dose of 7×10^6 TCID₅₀s. Animals were monitored for signs of clinical disease by visual inspection (rhinorrhea and respiratory rate) and by collecting temperatures on a daily basis for the first 4 days and then on days 7, 10, 14, and 21 p.i. Overall, CA04 infection was asymptomatic in both young adult and aged animals, with no notable increase in temperature, respiratory rate, or rhinorrhea (see Fig. S2A in the supplemental material; data not shown).

Pandemic H1N1 infection results in higher viral loads in aged animals. Viral loads were measured in nasal, ocular, and throat swabs as well as in BAL fluid samples using absolute quantitative PCR with primers and a probe specific for HA viral RNA (Fig. 1). Viral genome copy number in throat swabs reached a peak at 2 days p.i. (dpi) before declining uniformly in both young adult and aged animals at 4 dpi, followed by an increase at 7 dpi and a final drop at 10 dpi to undetectable levels at 14 dpi (Fig. 1A). It is possible that CA04 shedding has a biphasic course, as was observed for tracheal viral loads following infection with r1918 (14) or, more recently, H5N1 (26) influenza virus. It is also possible that the drop on day 4 was due to changes in the personnel collecting these samples. Similarly, viral RNA was detectable in nasal swabs at 1 dpi, with levels peaking at 2 dpi in both young adult and aged animals (Fig. 1B). In contrast to young adult animals, where genome copy numbers began to decline at 4 dpi, aged animals experienced a more sustained viral replication in nasal swabs ($P = 0.02$ for days 7 and 10), but viral genomes ceased to be detected at 21 dpi in both young adult and aged animals. To estimate overall viral loads in nasal swabs, we compared the AUCs between young adult and aged animals and found them to be

slightly higher in aged animals ($P = 0.06$). Viral RNA could also be detected in ocular swabs at 1 dpi, albeit at a lower level than in throat or nasal swabs (Fig. 1C). Viral loads were also higher in aged animals at 2 and 10 dpi ($P < 0.001$), with an overall higher level of shedding in aged animals (AUC $P = 0.06$). CA04 genomes were no longer detected at 21 dpi in either group. While ocular viral titers are not usually measured in macaque studies, it is interesting to note that CA04 virus replicated effectively in this compartment, with a biphasic distribution that could suggest a self-reinfection from the throat or the nose. Higher levels of viral RNA were detected in the BAL fluid, where they reached 10^7 to 10^8 genomes/100 μ l BAL fluid at 4 dpi (Fig. 1D). These levels began to decline at 7 dpi, becoming undetectable by 21 dpi in young adult and aged animals. Overall, aged animals experienced higher viral loads in the BAL fluid ($P = 0.04$ for AUC), especially at 7 dpi ($P < 0.001$). Taken together, these data suggest that CA04 infected both the upper and lower respiratory tracts and was able to replicate to higher levels in aged animals, but the infection resolved with similar kinetics in both young adult and aged animals.

Aged animals generate higher T cell proliferation in BAL fluid but delayed T cell proliferation in peripheral blood compared to young adult animals. We first characterized changes in the frequency of CD4 and CD8 T cells as well as changes in CM and EM T cell subset frequencies in peripheral blood and BAL fluid using flow cytometry (FCM). CM and EM T cell subsets were defined on the basis of the expression of CD28 and CD95, as previously described (20). Our analysis revealed a profound remodeling of the T cell compartment in the BAL fluid of infected animals. Prior to CA04 infection, frequencies of CD4 and CD8 T cells in BAL fluid were each at $\sim 20\%$ of circulating lymphocytes in both aged and young adult animals (see Fig. S3 in the supplemental material). Following infection, there was a dramatic increase in

FIG 2 Influenza virus infection induces robust proliferation of CD4 and CD8 T cell subsets in BAL fluid. (A) T cell proliferation was assessed by measuring changes in expression levels of the cell cycle marker Ki67 in CM (CD28⁺CD95⁺) and EM (CD28⁻CD95⁺) T cell subsets using flow cytometry. A representative example of CD4 T cell subsets (left) and frequency of Ki67 cells within EM subsets on 0 dpi (middle) and 7 dpi (right) from one of the animals is shown. (B, C) Frequencies of CD4 CM and EM T cells expressing Ki67; (D, E) frequencies of CD8 CM and EM T cells expressing Ki67⁺. The overall size of the proliferative burst was measured from the AUC. Aged animals generated a larger proliferative burst than young adult animals. Means $\pm$ SEMs are shown. *, $P < 0.05$; **, $P < 0.005$.

the frequency of CD8 T cells in the BAL fluid, which reached $\sim 70\%$ of the circulating lymphocytes, whereas the relative frequency of CD4 T cells decreased to $\sim 10\%$. This change seemed to be relatively stable, since it was still evident at 99 days postinfection. There were also significant changes in the relative frequencies of CM and EM cells within the CD8 subset in the BAL fluid. More specifically, influenza virus infection resulted in redistribution toward EM cells between days 7 and 35 postinfection. No significant changes in CD4 CM and EM frequencies in the BAL fluid were detected. These changes were specific to the BAL fluid, as we did not detect changes in the relative frequencies of CD4 or CD8 T cells in peripheral blood (see Fig. S4 in the supplemental material), despite an increase in the number of lymphocytes in whole blood at 10 to 14 dpi (see Fig. S2B in the supplemental material).

We then characterized the kinetics and magnitude of the T cell proliferative burst that usually takes place after antigenic encounter. These changes can be monitored by FCM by measuring the upregulation of Ki67, a nuclear protein associated with entry into the cell cycle, within CM and EM T cell subsets (8, 32). CA04 infection led to a dramatic increase in the frequency of Ki67-ex-

pressing (Ki67⁺) T cells, with $>50\%$ of the cells in all four T cell subsets expressing Ki67 starting at 4 dpi. T cell proliferation peaked at 10 dpi, returning to baseline at 14 dpi in the BAL fluid (Fig. 2). Interestingly, aged animals experienced a significantly larger T cell proliferative burst, especially within the CD8 CM ($P < 0.001$ for AUC) and CD8 and CD4 EM subsets ($P = 0.007$ for AUC).

In contrast to these findings, young adult animals generated an earlier and more robust proliferative burst in peripheral blood following infection with CA04 (Fig. 3). A dramatic increase in Ki67⁺ T cells was detected in PBMCs from young adult animals at 7 dpi, whereas T cell proliferation was not detected until 10 dpi in aged animals ($P < 0.001$ for CM T cells and $P < 0.01$ for EM T cells at 7 dpi). This proliferative burst was sustained through day 14, after which frequencies of Ki67⁺ T cells slowly returned to baseline levels.

Young adult animals generate a more robust CD4 T cell response than aged animals. To better characterize the T cell response to CA04, we measured the frequency of CA04-specific T cells by intracellular cytokine staining (ICS), where BAL fluid cells

FIG 3 T cell proliferation in peripheral blood of aged animals is delayed. T cell proliferation in PBMCs was determined as described for Fig. 2. Changes in frequencies of Ki67⁺ cells in CD4 CM and EM T cell subsets (A, B) and CD8 CM and EM T cell subsets (C, D). The onset of the T cell proliferative burst was delayed in aged animals compared to young adults. The overall size of the proliferative burst was measured from the AUC. Means $\pm$ SEMs are shown. *, $P < 0.05$; **, $P < 0.005$.

(Fig. 4) or PBMCs (see Fig. S5 in the supplemental material) were stimulated with overlapping peptide libraries covering the entire proteome of CA04 and the frequency of T cells that produced IFN- γ and/or TNF- α was determined by FCM. Due to the paucity of cells collected, the peptide libraries of several genes were pooled together (see Materials and Methods).

The kinetics of the antiviral T cell response in the BAL fluid were comparable between the different peptide pools, peaking at between 14 and 21 dpi for both CD4 and CD8 T cells and then declining before establishing a memory set point at 77 dpi (Fig. 4). In contrast, the magnitude of the T cell response differed between the pools, with a larger response against PA-NP and HA-NA than the other CA04 proteins.

In addition, CA04-specific CD4 T cell responses were either detected earlier in young adult animals or reached higher magnitudes (at 7 or 14 dpi; $P < 0.05$). These differences are best illustrated when considering the total CD4 response against CA04, where responses generated by young adult animals were significantly higher than those generated by aged animals at 7 and 14 dpi ($P = 0.05$; see Fig. S5A in the supplemental material). These data were surprising, considering that aged animals generated a bigger CD4 EM cell proliferative burst than young adult animals (CD4 CM cell proliferation was comparable) in the BAL fluid (Fig. 2). However, these data are in line with the delay in T cell proliferation observed in peripheral blood (Fig. 3), suggesting a delay or a defect in generation of effector CD4 T cell function with age.

In contrast to the CD4 response, CD8 T cell responses were comparable between young adult and aged animals (Fig. 4B, D, F, and H), as were the total CD8 T cell responses (see Fig. S5B in the supplemental material). Exceptions were the response to HA-NA, which was slightly higher in aged animals at 14 and 21 dpi ($P = 0.059$, Fig. 4B), and the response to PA-NP, which was higher in

young adult animals at 28 and 35 dpi (Fig. 4D). These results were also surprising, given that aged animals generated a larger CD8 T cell proliferative response in the BAL fluid (Fig. 2C and D), and suggest that generation of effector T cells might be compromised in the aged.

Despite the robust T cell proliferative response observed in peripheral blood, CA04-specific T cells were rare in this compartment, with responses representing 0.4 to 1% of the total CD4 and CD8 response in the PBMCs (see Fig. 5C and D in the supplemental material). Given the low frequency of responding T cells in PBMCs, we used IFN- γ enzyme-linked immunosorbent spot (ELISPOT) assay to more accurately enumerate CA04-specific T cells at 7 and 14 dpi (see Fig. S5E and F in the supplemental material, respectively). The ELISPOT assay analysis revealed that young adult animals generated a marginally bigger T cell response against HA-NA peptide pools and PA-NP in PBMCs than aged animals at 7 dpi and 14 dpi.

Aged rhesus macaques generate a more robust humoral response than young adult animals following pandemic H1N1 virus infection. We next assessed the humoral response in these animals. As described for T cells, we measured B cell proliferation (via Ki67 expression) within memory and MZ-like B cell subsets in peripheral blood and BAL fluid samples (Fig. 5). CA04 infection resulted in an increase in the number of Ki67⁺ cells within memory and MZ-like B cells in the BAL fluid at as early as 4 dpi. This proliferative burst peaked at 7 dpi, and the number of proliferating B cells returned to baseline at 28 dpi (Fig. 5A and B). As described for T cells in the BAL fluid, aged animals generated a bigger memory B cell proliferative response ($P = 0.001$ at 7 dpi and $P = 0.01$ at 10 dpi), but the magnitude of MZ-like B cell proliferation was comparable. B cell proliferation was delayed in peripheral blood compared to that in the BAL fluid, peaking at 14 dpi (Fig. 5C

FIG 4 Frequency of influenza virus-specific T cells in BAL fluid. Frequency of CA04-specific CD4 and CD8 T cells was determined using intracellular cytokine staining. (A, B) CD4 and CD8 T cell responses to HA-NA; (C, D) CD4 and CD8 T cell responses to PA-NP; (E, F) CD4 and CD8 T cell responses to PB1-pB1F2-NEP-NSI; (G, H) CD4 and CD8 T cell responses to PB2-M1-M2. Means $\pm$ SEMs are shown. *, $P < 0.05$.

and D), and the proliferative peak was comparable in young adult and aged animals.

To characterize the impact of age on B cell function, we measure CA04 HA-specific IgG titers and found them to be comparable between young adult and aged animals (Fig. 5E). In contrast, HI titers were significantly higher in aged animals than young adults ($P < 0.001$ at 21 and 28 dpi, $P = 0.08$ for AUC; Fig. 5F). Thus, aged animals generated a better primary humoral response following CA04 infection.

CA04 infection induces a robust cytokine response in the lungs. To get a global assessment of the local immune response in the lungs, we measured changes in cytokine concentrations in BAL fluid supernatants collected during acute CA04 infection us-

ing a multiplex ELISA that allows the simultaneous detection of 12 cytokines. After infection, we did not detect any changes in the levels of IL-17, TNF- α , or the IL-12/23p40 common chain (data not shown). In contrast, levels of IL-15, IL-1ra, MCP-1, IL-8, IL-6, and IFN- α increased at 4 dpi and, with the exception of those for IL-15, remained high or increased at 7 dpi (Fig. 6A to F). IFN- γ and sCD40L levels increased at 7 dpi and returned to baseline at 10 dpi (Fig. 6G and H). Changes in cytokine levels were comparable between young adult and aged animals, with the exception of those of IL-8 ($P < 0.01$ at 7 dpi, $P = 0.05$ for AUC; Fig. 6D) and IL-6 ($P < 0.01$ at 7 dpi; Fig. 6E), which were induced to a greater extent in the aged animals. IL-15, MCP-1, and IL-1ra levels were initially found to be higher in aged animals ($P < 0.05$). We also

FIG 5 B cell response is comparable in young adult and aged animals. Proliferation of memory and MZ-like B cells in both BAL fluid (A, B) and peripheral blood (C, D). (E, F) Endpoint titers of IgG antibody to HA antigen (E) and HI titers (F) in plasma. Means $\pm$ SEMs are shown. *, $P < 0.05$; **, $P < 0.005$.

measured the IFN- α level in the BAL fluid supernatant and found this cytokine to be induced to a higher level in aged animals (Fig. 6). This higher level of cytokine induction is likely due to a higher viral load in the BAL fluid in aged animals.

Young adult and aged animals had distinct transcriptional responses in BAL fluid at day 4. To uncover the mechanisms underlying increased T cell proliferation in BAL fluid and HI titers in response to CA04 infection in aged animals, we performed gene expression profiling of BAL fluid resident cells collected at days 0, 4, 7, 10, and 14 p.i. BAL fluid samples included immune cells as well as desquamated epithelial cells (see Table S1 in the supplemental material). Viral mRNA was found in these samples, indicating the presence of infected cells in BAL fluid (data not shown). We first used nonparametric MDS to visualize global changes induced after CA04 infection (Fig. 7). This analysis revealed that samples collected at 4 and 7 dpi exhibited the largest Euclidian distance from day 0 and, thus, the greatest gene expression changes after infection, with gene expression profiles gradually returning to baseline at 10 and 14 dpi. A strong clustering according to the age of the animals was observed for day 4 samples, indicating that young adult and aged macaques mounted distinct responses at that time point. This observation was confirmed by supervised statistical analysis, which confirmed that day 4 was the

time when the largest gene expression differences between aged and young adult animals were observed. We found 709 genes differentially expressed between young adult and aged macaques. These genes were organized into three clusters on the basis of their expression levels (Fig. 8; see Data Set S1 in the supplemental material). Cluster 1 consists of 194 genes that were more highly induced in young adult animals, cluster 2 contains 287 genes that were more highly induced in aged animals, and cluster 3 contains 228 genes that were less downregulated in aged animals after infection.

Overall, the 709 genes differentially expressed between young adult and aged macaques were highly enriched for genes associated with various immunological responses (see Data Set S2 in the supplemental material), which is consistent with the large proportion of immune cells observed in BAL fluid samples (43 to 66% of total cells at day 4; see Table S1 in the supplemental material). We annotated these genes using the IRIS database, which is a compendium of microarray expression data from six key immune cell types and their activated and differentiated states (1). Figure 8 and Table S2 in the supplemental material show that the genes in cluster 1 were mostly associated with naïve B cells ($P = 1e-13$) and resting CD8 and CD4 T cells ($P = 2e-21$ and $2e-20$, respectively). A large number of the genes within cluster 2 are ISGs ($P =$

FIG 6 Influenza virus infection induces a robust cytokine response in the BAL fluid. Cytokine levels were determined using Luminex technology. Influenza virus infection was associated with increased levels of IL-15 (A), IL-1ra (B), MCP-1 (C), IL-8 (D), IL-6 (E), IFN- α (F), IFN- γ (G), and sCD40L (H). Means $\pm$ SEMs are shown. *, $P < 0.05$. No changes in IL-17, TNF- α , and IL-12/23p40 with influenza virus infection were detected (data not shown).

$4e-23$); some are associated with activated dendritic cells (DCs; $P = 2e-42$) and differentiating macrophages ($P = 2e-74$). Finally, the genes within cluster 3 were mostly enriched in genes specific to fully differentiated macrophages ($P = 2e-23$).

Genes within cluster 1 were induced to a greater extent in young adult than aged animals and were highly enriched in genes specific to naïve T or B cells (Fig. 2; see Table S2 in the supplemental material). Upregulation of most of these genes was associated with increased trafficking of T and B cells into the BAL fluid at 4 dpi in both young adult and aged animals (Spearman's $\rho > 0.7$, $P < 0.05$). Genes encoding T cell markers (CD3E, CD3D, and CD3G) and B cell markers (CD79B, CD20, and CD19) as well as transcriptional regulators TCF7 (T cells) and POU2AF1 (B cells) were more highly upregulated in young adult than aged macaques

(see Data Sets S1 and S3 in the supplemental material). However, expression of some T cell-specific (*SIGIRR*, *AES*, *APBA2*) and B cell-specific (*CD22*, *TSC22D3*) genes correlated with changes in the frequency of T and B cells in young adult animals only, while their expression remained unchanged between 0 and 4 dpi in aged animals (see Data Sets S1 and S3 in the supplemental material).

Within cluster 2, 62 ISGs, including those for classical proinflammatory cytokines, numerous chemokines, and complement genes, were more highly induced in aged than young adult animals (Fig. 8; see Data Set S1 in the supplemental material). Most of these genes are known targets of the NF- κ B transcription factor, which was predicted to be highly activated in aged animals only, based on the levels of expression of the genes discriminating young adult and aged animals (IPA regulation $z = 4.2$; see Data Set

FIG 7 Global view of dynamic pulmonary transcriptional response across infection, using nonparametric MDS. Each mRNA sample is represented as a single point colored by the age of the animal and with a different shape according to the time p.i. Euclidian distance was calculated using all genes of the array, such that proximity indicates similarity of gene expression profiles, whereas distance indicates dissimilarity of gene expression profiles. Kruskal's stress evaluates the loss of information in the representation.

S2 in the supplemental material). The increased activation of the IFN response could be due to the increased viral loads observed in the BAL fluid of aged animals, leading to a higher activation of NF- κ B (Fig. 1). A higher induction of ISGs could explain the increased levels of inflammatory cytokines and chemokines seen in BAL fluid supernatants of aged animals (Fig. 6; IL-8, IL-6, IL-15, MCP-1, and IL-1ra). The higher interferon response could also explain the enrichment in genes associated with DC activation ($P = 2e-42$).

The downregulation of macrophage-related genes (cluster 3) observed at 4 dpi could be partially explained by the decrease of macrophage frequency between days 0 and 4 in BAL fluid (from 66.9% to 23% in aged animals and 69.2% to 22% in young adults; see Table S1 in the supplemental material). Our results suggest, though, that macrophages present in BAL fluid at day 4 are differentiating macrophages in aged animals, while they are undifferentiated monocytes in young adults ($P = 3e-8$ in monocyte-related genes for cluster 1).

DISCUSSION

Influenza A viruses typically cause a mild self-limiting febrile disease. However, in infants, the elderly, and immunocompromised individuals, seasonal influenza A virus infection can lead to pneumonia and acute respiratory distress syndrome and even death. The outbreak of swine-origin influenza A H1N1 virus in early 2009 highlighted the ability of influenza viruses to recombine, generating new strains that can spread efficiently between humans and for which herd immunity is limited. Overall, human infections with the 2009 H1N1 virus appeared to be mild, but an alarming number of young adult individuals with no underlying comorbidities developed severe symptoms atypical of seasonal influenza. Indeed, it is estimated that only 13% of deaths and 10% of hospitalizations occurred among individuals older than 65 years of age (12, 15). The prevailing hypothesis put forth to explain the difference between the outcome of the 2009 H1N1 infection and that of

seasonal influenza is that older individuals had cross-protective neutralizing antibodies generated during previous encounters with swine-origin H1N1 viruses that were in circulation in the 1950s and 1970s (9, 34). However, other factors could have contributed to increased disease severity in the younger individuals, such as early hypercytokinemia and a more significant innate immune response that could have damaged the lungs.

To investigate age-related disease severity caused by the H1N1 pandemic virus in the absence of preexisting immunity, we compared virological and immunological parameters in young adult and aged macaques infected with A/California/04/2009 H1N1 (CA04), a virus isolated from a pediatric patient with uncomplicated, upper respiratory tract illness. These studies are the first to investigate the impact of age on the host response to influenza virus infection in nonhuman primates. Our analysis revealed sustained viral replication and shedding for 21 days after infection in both the lower and upper respiratory tracts, as previously shown for other 2009 H1N1 strains (10, 11, 25). Viral loads were highest in the BAL fluid, followed by nasal, throat, and finally, ocular swabs. Interestingly, we observed a biphasic pattern of viral replication in throat and ocular swabs which has not been reported in previous studies of CA04 infection in cynomolgus macaques, though in those studies, viral loads were measured over a more limited time course (day 0 to day 4 [10] and day 3 and day 7 in tonsils [11]). However, in cynomolgus macaques infected with r1918 H1N1 virus, a rebound in viral titer was observed in trachea at 6 and 8 dpi (14). Similarly, a recent study in rhesus macaque reported biphasic tracheal viral titers at 12 h and 6 days after H5N1 infection which were associated with a biphasic fever pattern (26). Despite reports of ocular complication following influenza virus infection, including by 2009 H1N1 virus (18), ocular titers are not usually reported in macaque studies. The rebound in ocular titer is delayed (day 10) compared to the second viral peak in throat (day 7), which suggests possible self-reinfection, with the viral source being the throat or the nasal compartment.

In line with robust viral replication in the lungs, CA04 infection led to a significant increase in the frequency of CD8 T cells in the lungs that was sustained for over 2 months after the resolution of infection in both young adult and aged animals. Aged animals exhibited increased peak viral loads in nasal, ocular, and BAL fluid samples, but infection resolved with similar kinetics in both young adult and aged animals, with no viral genomes detected after 21 dpi.

Interestingly, aged animals generated a bigger T cell proliferative burst in the BAL fluid than young adult animals. However, despite this increased proliferation, the frequency of CA04-specific effector CD4 T cells in the BAL fluid was lower in aged animals, whereas the CD8 T cell response was comparable. In contrast to the BAL fluid, T cell proliferative burst in peripheral blood was delayed and smaller in aged animals. This observation suggests that aged T cells have a delay in acquiring effector functions, such as IFN- γ production. Moreover, these data also suggest that CA04-specific CD4 T cells might be recruited primarily from the periphery and T cell proliferation in the BAL fluid is in part due to cytokine-mediated bystander activation. The delayed appearance of effector CD4 T cells could in part explain the increased peak viral loads in aged animals. Indeed, a recent study in humans showed that the frequency of influenza virus-specific CD4 T cells correlated negatively with disease severity (33). Gene expression profiling of BAL fluid cells also supports this notion of impaired T

FIG 8 Acute influenza virus infection induces differential gene expression profiles in young adult and aged animals at 4 dpi. Gene expression for each animal is shown as the $\log_2$ ratio of expression at day 4 relative to that at day 0. Genes were clustered on the basis of their expression values across samples using Euclidian distance and complete linkage function. This revealed three major groups of genes, indicated on the left of the heat map. The association of each molecule within an immune cell subset or interferon response is shown in red on the right. Genes specific to each immune cell population were defined using the IRIS data set as genes highly expressed in one population compared to the other. ISGs were defined using Calu-3 cells treated with IFN- α , - β , or - γ . Mono/Mac, monocytes/macrophages; LPS, lipopolysaccharide.

cell effector function in aged animals. Specifically, we show decreased expression (*CD3*, *TCF7*) or lack of upregulation (*SIGIRR*, *AES*, *APBA2*) of T cell-specific genes at 4 dpi in aged compared to young adult macaques. In particular, the absence of *SIGIRR* in T cells was shown to result in increased Th17 cell proliferation, with proinflammatory consequences (7).

Memory B cell proliferation reached a higher peak in aged animals. Although plasma HA-specific IgG titers were comparable between the two groups, the plasma HI titers observed in aged animals were significantly higher than those observed in young adult animals. However, the HI titers peaked at 21 dpi, after viral replication ceased in the animals. Therefore, it is unlikely that the increased antibody response could have modulated disease severity during primary infection. It is more likely that increased antigen load (due to higher viral replication) enhanced B cell proliferation in aged macaques.

Our study also revealed that aged animals mobilized several inflammatory cytokines to a higher magnitude than young adult animals. In BAL fluid supernatant, we detected higher levels of IL-8, MCP-1, IFN- α , and IL-6 in aged animals. These cytokines play a critical role in the activation of the innate immune response (IFN- α) and recruitment of phagocytic cells, notably, neutrophils and monocytes (IL-8, IL-6, and MCP-1), as well as lymphocytes (MCP-1 and IL-6). We also detected marginally higher increased levels of IL-15 at 4 dpi, which could lead to an increased recruitment of NK cells, which in turn could contribute to the early increased production of IFN- γ observed at 7 dpi. Increased levels of IL-15 and IFN- γ in the BAL fluid could also be due to the higher CD8 and CD4 EM T cell proliferation observed in the BAL fluid of aged animals. Increased cytokine production in the BAL fluid of aged animals is most likely in response to increased viral replication at this site. Early increased cytokine production has been associated with increased disease severity in previous respiratory disease studies and is in line with the higher peak viral loads that we observed in aged animals.

Our gene expression data also show that the early transcriptional response to CA04 infection is highly modulated with age in macaques. Specifically, aged animals generated a more robust interferon and inflammatory response, which was associated with activation of macrophages and DCs. Moreover, genes associated with chemotaxis (*CCL22*, *CCL8*, *CCL2*, *CCL4*), recruitment of innate immune cells (IL-8, IL-6, Toll-like receptor 2) as well as T cells (IFN- γ , IL-12B, IL-23), and complement activation (*C3*) were also more highly upregulated in aged than young adult animals. It is possible that increased inflammation was associated with increased viral replication and lung lesions in aged macaques. Baas et al. showed that the lung area with influenza virus-induced lesions had a higher innate immune response than regions where no viral mRNA was detected (2). Early and sustained innate immune responses have also been associated with severe influenza in previous macaque studies (3, 5, 16, 31). Interestingly, NF- κ B was predicted to be highly activated in aged macaques, based on gene expression levels of its targets. Increased induction of NF- κ B with age was also found in rhesus macaques infected with severe acute respiratory syndrome coronavirus (SARS-CoV), and in that study, this was associated with a greater severity of infection (27). Similarly, in a mouse model of SARS-CoV, infection is lethal only in aged animals, where it is characterized by earlier induction in pulmonary expression of genes related to the inflammatory response, including NF- κ B (24). In line with the gene expression

data, we observed a stronger induction of several proinflammatory cytokines, such as IL-6, IL-1, IL-1 α and - β , IL-8, and IFN types I and II (IFN- α 8, IFN- β 1, IFN- γ), in aged macaques after influenza virus infection at both the gene expression and protein levels (IFN- α).

Overall, data presented in this report demonstrate that in naïve animals with no preexisting immunity, older age results in increased viral replication, hypercytokinemia, and delayed effector CD4 T cell responses following CA04 infection compared to those in young adult animals. At the transcriptional level, aged macaques upregulate several genes associated with inflammation and the innate immune response to a greater extent than young adult animals. Although aged animals generated a more robust humoral response, the kinetics of this response preclude it from playing a major role during primary infection. Taken together, these data suggest that in the absence of preexisting immunity, aged macaques display several hallmarks usually associated with severe respiratory disease following pandemic H1N1 influenza virus infection in comparison with young adult animals. Therefore, these data support the hypothesis that preexisting immunity plays a critical role in modulating disease severity following infection with swine-origin H1N1 influenza virus. In addition, two recent clinical studies have shown that older individuals generate a broader antibody response with higher avidity than young adults following vaccination (14) or infection (20, 28) with the 2009 H1N1 virus. The improved antibody response is most likely mediated by memory B cells specific for swine-origin influenza A viruses generated during previous exposures. Preexisting T cell responses could also have played an important role in dampening disease severity in the elderly following the 2009 H1N1 pandemic. This hypothesis is supported by a recent macaque study where the presence of cross-reactive H1N1 virus-specific T cells reduced viral loads in the absence of cross-protective antibodies (32). In summary, our studies indicate that age results in increased disease severity in naïve animals and provide support for the hypothesis that preexisting immunity acquired during previous exposure to swine-origin H1N1 viruses most likely protected older individuals from severe influenza during the 2009 H1N1 pandemic.

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