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Influenza-Induced Production of Interferon-Alpha is Defective in Geriatric Individuals

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Abstract

Background—The majority of deaths (90%) attributed to influenza are in person's age 65 or older. Little is known about whether defects in innate immune responses in geriatric individuals contribute to their susceptibility to influenza.

Objective—Our aim was to analyze interferon-alpha (IFN-alpha) production in peripheral blood mononuclear cells (PBMCs) isolated from young and geriatric adult donors, stimulated with influenza A or Toll-like receptor (TLR) ligands. IFN-alpha is a signature anti-viral cytokine that also shapes humoral and cell-mediated immune responses.

Results—Geriatric PBMCs produced significantly less IFN-alpha in response to live or inactivated influenza (a TLR7 ligand) but responded normally to CpG ODN (TLR9 ligand) and Guardiquimod (TLR7 ligand). All three ligands activate plasmacytoid dendritic cells (pDCs). While there was a modest decline in pDC frequency in older individuals, there was no defect in uptake of influenza by geriatric pDCs.

Discussion and Conclusion—Influenza-induced production of IFN-alpha was defective in geriatric PBMCs by a mechanism that was independent of reduced pDC frequency or viability, defects in uptake of influenza, inability to secrete IFN-alpha, or defects in TLR7 signaling.

Keywords

Aging; influenza; interferon-alpha; plasmacytoid dendritic cells

Introduction

Over 32,000 deaths per year occur in a typical influenza season with over 90% of deaths in persons over 65 years of age [1]. With the current H1N1 global pandemic, the number of influenza cases is expected to dramatically increase resulting in greater numbers of deaths than usual even in individuals over age 65. They are less susceptible than younger individuals to 2009 H1N1 but continue to have greater risk of complications if they develop active influenza infection. Unfortunately, the seasonal influenza vaccine has poor efficacy in this older group. The rates of protection against hospitalization for influenza and pneumonia are estimated to be only 33% in individuals over age 65 [2]. A better understanding of the pathogenesis of influenza infection and disease in older persons is required to develop more effective vaccines or immunomodulatory strategies to reduce morbidity and mortality in this group.

There are likely multiple mechanisms for this increased morbidity and mortality with aging. While decline in T cell function has been extensively documented in the elderly [3–11], potential changes in immune function that impact innate anti-viral responses have been largely unexplored. Type I interferons (IFNs) were first identified for their antiviral properties against influenza [12,13] and have been subsequently shown to induce the transcription of several genes that help degrade viral RNA and block viral replication [14–16]. In addition to their anti-viral properties, type I IFNs have multiple effects on human mononuclear populations including T cells and B cells, and reduced type I IFN production could result in decreased induction of cell-mediated immunity [17].

Plasmacytoid dendritic cells (pDCs) are the main producers of type I IFNs after influenza activation [18]. Influenza virus ssRNA induces expression of type I IFNs in pDCs via activation of Toll-like receptor 7 (TLR7) [19–21]. IFNs induce expression of MxA, a protein with anti-viral activity [22–24] that renders pDCs resistant to virus-induced apoptosis [25]. pDCs have been found in all of the relevant respiratory mucosal sites in humans including nasal mucosa, lung, and bronchialveolar lavage fluid [26–29]. In the influenza challenge mouse model, pDCs in the respiratory tract were found to make two-thirds of the IFN-alpha generated supporting the idea that pDCs in spite of their low numbers in respiratory mucosal sites produce the majority of IFN-alpha [30].

Several animal models suggest a clear role for IFN-alpha in protection against influenza. Ferrets and Guinea pigs have been found to be representative models of human disease as they are susceptible to human influenza strains, are able to transmit infection [31–33], and express the MxA gene [34]. Recent studies in ferrets and Guinea pigs show that exogenous IFN-alpha reduces viral shedding and morbidity to influenza A virus [35,36]. The mouse model has shown variable results regarding the importance of IFN-alpha as many of the studies use BALB and B6 mice that lack the MxA gene [34].

In the current study, we have assessed response of peripheral blood mononuclear cells (PBMCs) isolated from control (<35 years) and geriatric (>65 years) individuals to live influenza. IFN-alpha production was significantly reduced in pDCs from geriatric individuals in response to influenza but not to other TLR ligands that also activate pDCs.

Methods

Donors

Subjects were recruited from the Cleveland, VA, Case Western Reserve University and University Hospitals of Cleveland according to IRB approved protocols.

Media

Unless otherwise stated, cells were cultured in 96-well round-bottom plates at 37°C in 5% CO₂ in X-Vivo 15 serum-free media (Lonza-BioWhittaker, Walkersville, MD, USA) supplemented with Penicillin and Streptomycin (Lonza-BioWhittaker).

Cell Isolation

Blood was obtained in heparin containing green top Vacutainer tubes (BD Biosciences) and processed within 4 h. PBMCs were isolated from blood samples (geriatric and control) by Ficoll Plaque Plus (GE Healthcare/Amersham Biosciences) density gradient centrifugation according to the manufacturer's instructions. PBMCs were resuspended in X-Vivo-15 serum-free media and counted. In some experiments, pDCs were depleted from PBMCs using a CD304 (BDCA-4/Neuropilin-1) Microbead Kit from Miltenyi Biotec (Germany) according to the manufacturer's instructions. Multiple cell types were also purified from PBMCs using positive selection Microbead Kits from Miltenyi Biotec. The cell types isolated included: monocytes (CD14 beads), pDCs (BDCA-4/Neuropilin-1 beads), B cells (CD19 beads), myeloid DCs (mDCs, BDCA-1 beads), and T/NK cells (CD2 beads).

To assess pDC frequency and effectiveness of pDC depletion, 5×10^5 PBMCs were stained in X-Vivo-15 media using the following antibodies: anti-HLA-DR-Pacific Blue (Biolegend, San Diego, CA, USA), Lineage Cocktail 2 (Lin2)-fluorescein isothiocyanate (FITC, Becton Dickinson, San Jose, CA, USA), and anti-CD123-PE (eBioscience, San Diego, CA, USA). Cells were incubated in the dark on ice for 30 min, washed with PBS, and resuspended in 2% paraformaldehyde (Electron Microscopy Sciences). pDC frequency was determined by gating on HLA-DR⁺, Lin⁻, and CD123⁺ cells on a clinically certified LSRII flow cytometer (Becton Dickinson).

Viruses

Influenza A/PR/8/34 (H1N1, Charles River, Wilmington, MA, USA), influenza A/Hong Kong/8/68 (H3N2, BEI Resources, Manassas, VA, USA), and influenza A/Denver/1/57 (H1N1, BEI Resources) in allantoic fluid were stored as aliquots at -80°C.

A/PR/8/34 was UV-inactivated with 18,000 J using a Stratagene UV Crosslinker 1800. UV inactivation of virus was assessed by infecting Vero E6 cells (ATCC, Manassas, VA, USA). Vero E6 cells (3×10^4 /well) were plated overnight in 24-well plates in DMEM (Hyclone, Thermo Fisher Scientific, Waltham, VA, USA) containing 10% fetal calf serum (Hyclone). Media was aspirated and replaced with fresh media containing trypsin (20 µg/ml, Thermo Fisher Scientific). Cells were infected with either live or UV-inactivated A/PR/8/34 at 1.6×10^5 EID₅₀/well and 0.32×10^5 EID₅₀/ml for 48 h at 37°C in 5% CO₂. Cells were washed with warm PBS, fixed with freshly made 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 min at room temperature, washed again, and permeabilized with ice-cold methanol for 20 min at -20°C. Cells were incubated for 1 h at room temperature with blocking buffer (PBS containing 10% goat serum (Invitrogen, Carlsbad, CA, USA) and 5% BSA (Sigma-Aldrich, St. Louis, MO, USA)) and then stained with anti-influenza A monoclonal-FITC antibody (Chemicon-Millipore, Billerica, MA, USA) or isotype control mouse IgG1 (BD Pharmingen, San Diego, CA, USA). Cells were incubated overnight at 4°C, washed three times with PBS, and observed with a Leica DMI 6000 B inverted microscope.

To generate FITC-labeled virus, purified A/PR/8/34 (200 μ l, Charles River) was dialyzed with PBS pH 8.2 for 2 h, incubated with 10 μ l FITC (FLUOS 20 mg/ml stock in DMSO, Roche, Mannheim, Germany) for 25 min at room temperature and dialyzed twice in 200 ml PBS pH 7.3 for 1 h for each exchange. FITC-A/PR/8/34 was frozen at -80°C in aliquots.

Incubation of Cells with Influenza and Ligands

PBMCs (1×10^6 cells/well) in 200 μ l of X-Vivo media were incubated with or without A/PR/8/34 (low = 0.32×10^5 EID₅₀/ml, high = 1.6×10^5 EID₅₀/ml), A/Hong Kong/8/68 (1.8×10^5 EID₅₀/ml), A/Denver/1/57 (1.1×10^6 EID₅₀/ml), type “A” CpG ODN 2216 (3 μ g/ml, InvivoGen, San Diego, CA, USA), ODN 2216 control that lacks CpG motif (3 μ g/ml, InvivoGen), Guardiquimod (1 μ g/ml, InvivoGen), or Poly(I:C)-LMW/LyoVec (4 μ g/ml, InvivoGen) for 20 h at 37°C . Concentration of reagents was determined in initial experiments by incubating control PBMCs with various dilutions of reagents (virus, TLR ligand) for 20 h, analyzing IFN- α in supernatants and selecting concentrations for the subsequent studies that were on the linear part of the IFN- α response (data not shown). For inhibitor studies using chloroquine (10 μ M, Sigma-Aldrich) or control and IRS 661 ODNs (1.4 μ M, manufactured as phosphorothioate ODNs by Invitrogen [19]), inhibitors were added 15 min prior to the addition of A/PR/8/34 or ligands and remained in the culture throughout. Supernatants were stored in microfuge tubes or 96-well plates at -80°C for further analyses.

For experiments that compared A/PR/8/34-induced IFN- α production by various cell types, the following number of cells were added per well to reflect their anticipated proportion in 5×10^5 PBMCs: pDCs (2×10^4 cells/well), B cells (5×10^4 cells/well), mDCs (2×10^4 cells/well), monocytes (1×10^5 cells/well), and T cell/NK cells (2×10^5 cells/well).

ELISAs

IFN- α and IFN- β levels in supernatants were assessed with the Verikine™ Human IFN- α Multi-subtype ELISA kit (detection limit 12.5 pg/ml), and Verikine™ Human IFN- β ELISA kit (detection limit 25 pg/ml, PBL Interferon Source, Piscataway, NJ, USA), respectively. The human IFN- α kit from eBioscience was used for the pDC depletion, cell type specific, and inhibitor experiments. Kits were used according to the manufacturer's instructions. All samples and standards were inactivated with a final concentration of 0.5% Triton X-100 (Bio-Rad, Richmond, CA, USA).

Analysis of Uptake of Influenza by pDCs

PBMCs (1×10^6) were pulsed with or without FITC-A/PR/8/34 for 1 h at 37°C . Cells were washed once with media and chased for 30 min at 37°C . Cells were then washed twice with media, resuspended in MACS buffer (Miltenyi Biotec), and stained with the following antibodies: anti-HLA-DR-Pacific Blue (Biolegend), BDCA-4-APC (Miltenyi Biotec), and CD123-PE-Cy7 (eBioscience). Cells were incubated in the dark on ice for 30 min, washed with PBS, resuspended in 2% paraformaldehyde (Electron Microscopy Sciences), and analyzed by flow cytometry.

Annexin Assay

PBMC (2×10^6) were placed in 400 μ l X-Vivo medium $\pm 1.6 \times 10^5$ EID₅₀/ml A/PR/8/34 at 37°C for 8 h in microfuge tubes. Cells were stained with LIVE/DEAD Violet Fixable Dead Cell Stain Kit (Molecular Probes, Invitrogen), Lin-FITC, CD123-PE, HLA-DR-PECY5 (BD), and Annexin V-APC (eBioscience) in high calcium concentration annexin buffer according to the manufacturer's instructions. Cells were fixed in 2% paraformaldehyde (Electron Microscopy Sciences) in annexin buffer and pDCs analyzed for Annexin V staining by flow cytometry.

Statistical Analysis

The magnitude of IFN- α production and pDC frequency was compared between geriatric and control patients. The distribution of these data were evaluated using scatter plots and were shown to be non-normally distributed. Therefore, Mann–Whitney, nonparametric two-tailed *t* tests were used for statistical comparisons and graphics. Results in the text present both median and mean. All analyses were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, CA, USA, (www.graphpad.com).

Results

PBMCs from Geriatric Individuals Produce Less IFN- α in Response to Influenza than PBMCs from Younger Controls

Subjects are described in Table 1. The control population included 38 individuals in the age range of 21–35 with an average age of 27. The geriatric population consisted of 43 individuals in the age range of 66–96 with an average age of 84. The younger individuals were healthy and not on medications.

PBMCs isolated from control or geriatric individuals were incubated with or without low (0.32×10^5 EID₅₀/ml) or high (1.6×10^5 EID₅₀/ml) concentrations of A/PR/8/34 (H1N1) for 6 or 20 h and supernatants were assayed for IFN- α by ELISA (Fig. 1a,b). IFN- α was barely detectable in the 6 h supernatants (data not shown). In 20 h supernatants, at low-dose A/PR/8/34, geriatric PBMCs ($n=22$, mean=76 pg/ml, median=4.8 pg/ml) produced 78.2% ($p=0.012$) less IFN- α than control PBMCs ($n=21$, mean=348 pg/ml, median=156 pg/ml). At high-dose A/PR/8/34, geriatric PBMCs ($n=24$, mean=2115 pg/ml, median=1,326 pg/ml) produced 66.5% ($p=0.0006$) less IFN- α than control PBMCs ($n=20$, mean=6,203 pg/ml, median=4,325 pg/ml). No response was seen with media or allantoic fluid alone. No IFN- β was detected in the supernatants.

In the geriatric subjects there were several subgroups based on medications and comorbidities. For several of the subgroups we had enough subjects to do a subgroup analysis. These included subjects on statins or aspirin (ASA). We found that their influenza-induced IFN- α levels were not different than those not on these medications ($p=0.53$ statins, $p=0.28$ ASA). Also, diabetes mellitus was the most common comorbid condition in the geriatric population, and those geriatric subjects with diabetes mellitus were not different in their induction of IFN- α ($p=0.45$). Based on these subgroup analyses, we feel fairly confident that the comparisons between the young control and older geriatric populations are yielding valid results across a general geriatric group.

Reduced response to live influenza was not due to increased apoptosis of geriatric cells making IFN- α , since geriatric PBMCs stimulated with UV-inactivated A/PR/8/34 (high dose) for 20 h also made significantly less IFN- α ($p=0.0001$, $n=12$, mean=391 pg/ml, median=312 pg/ml) than control PBMCs ($n=15$, mean=2,566 pg/ml, median=2,114 pg/ml; Fig. 1c). This also suggests that IFN- α production to A/PR/8/34 is at least partially replication dependent and is consistent with that seen by others to influenza and other ssRNA viruses [37,38]. A lack of differences in apoptosis was confirmed by flow cytometric analysis of Annexin V and LIVE/DEAD violet staining of PBMCs after live A/PR/8/34 infection and gating on pDCs. We found no difference ($p=0.83$) in Annexin V and LIVE/DEAD staining in pDC from nine controls (15.9% SD 10.7) and seven geriatric individuals (15.5% SD 11.4). This further supports that apoptosis of pDC is not the cause of the reduction in IFN- α observed in older individuals.

To determine if the reduced response was seen with other strains of influenza A, control and geriatric PBMCs were incubated with A/Hong Kong/8/68 (H3N2, 1.8×10^5 EID₅₀/ml) and A/Denver/1/57 (H1N1, 1.1×10^6 EID₅₀/ml) for 20 h and IFN- α concentrations was assayed

in supernatants (Fig. 2). Geriatric PBMCs ($n=14$, mean=12,904 pg/ml, median=10,408 pg/ml) made 54% less IFN- α ($p=0.0095$) than control PBMCs ($n=11$, mean=28,105 pg/ml, median=22,241 pg/ml) in response to A/Denver/1/57. Geriatric PBMCs ($n=13$, mean=5,365 pg/ml, median=3,976 pg/ml) also made 58% less IFN- α ($p=0.0065$) than control PBMCs ($n=11$, mean 12,842 pg/ml, 9,034 pg/ml) in response to A/Hong Kong/8/68.

In conclusion, geriatric PBMCs made significantly less IFN- α in response to influenza A. This reduced response was not dependent on the viability of the virus, suggesting that viral-mediated apoptosis of geriatric PBMCs was not responsible for the diminished response.

Influenza Activates pDCs Via TLR7

Influenza has been shown to activate pDCs via TLR7 [19–21]. While influenza may additionally signal via the retinoic acid-inducible gene (RIG-I) [39], a cytoplasmic RNA helicase that recognizes 5' triphosphate RNA [40,41], studies have also shown that NS1 protein of influenza inhibits RIG-I [41,42]. Additionally, pDCs, which produce large amounts of IFN- α , use the TLR system rather than RIG-I for viral detection [43]. Since our studies used total PBMC cultures, experiments were performed to confirm that pDCs were the primary producers of IFN- α in response to influenza and to determine whether influenza signaled via TLR7 or RIG-I. PBMCs and PBMCs lacking pDCs were activated with A/PR/8/34 and with other TLR ligands (TLR9 ligand, CpG ODN and TLR7 ligand, and Guardiquimod) known to activate pDCs. Depletion of pDCs from PBMCs completely abrogated the response to all three ligands (Fig. 3a). We further confirmed that pDCs were the primary source of IFN- α production in PBMCs by examining responses of purified cell types found in PBMCs, including purified pDCs, mDCs, monocytes, B cells, and mixed T cell/NK populations, to influenza. Only purified pDCs made IFN- α in response to A/PR/8/34 stimulation (Fig. 3b).

All endosomal TLRs (TLR7, TLR9, and TLR3) signal within the acidic environment of the endosome and can be inhibited by reagents (e.g., chloroquine) that prevent endosomal acidification [44–46]. RIG-I is a cytosolic RNA helicase that cannot be inhibited by chloroquine. To determine whether A/PR/8/34 signaled via a TLR or RIG-I, PBMCs were incubated with A/PR/8/34, Guardiquimod, CpG ODN, and a known RIG-I ligand, poly (I:C):LyoVec, in the presence or absence of chloroquine (Fig. 4a). While chloroquine inhibited IFN- α production in response to A/PR/8/34, Guardiquimod, and CpG ODN, it had little effect on IFN- α production in response to Poly(I:C): LMW LyoVec supporting observations made by others, that influenza signals via a TLR. TLR7 signaling can be inhibited in cells incubated with the inhibitory ODN IRS 661 [19]. ODN IRS 661 inhibited IFN- α production in response to both A/PR/8/34 (80% inhibition) as well as Guardiquimod (82% inhibition) but had no effect on responses to CpG ODN (Fig. 4b) that signals via TLR9. Together, these results support the observation that influenza activates pDCs in a TLR7-dependent manner.

Geriatric PBMCs Have Reduced Frequency of pDCs

Since influenza activates pDCs via TLR7, reduced pDC numbers in the elderly may account for the decrease in response to influenza. It has been reported that the number of circulating pDCs declines during aging [47–49]. Shodell and Siegal reported a decline in pDC numbers as a percentage of all mononuclear cells gated. pDC were 0.29% in individuals 18–29 years of age to 0.17% in individuals 60–91 years of age [49]. We analyzed pDC frequency in control ($n=22$) and geriatric ($n=25$) PBMCs by flow cytometry (Fig. 5). pDCs were identified as HLA-DR+, Lin–, and CD123+. We have similarly observed that pDC frequency significantly declined 38% ($p=0.038$) from a median of 0.27 (mean=0.27) in young controls to a median of 0.14 (mean=0.17) in geriatric PBMCs. Reduced pDC frequency in geriatric PBMCs may partially account for the reduced response to influenza A.

Geriatric PBMCs are not Defective in Production of IFN-Alpha in Response to TLR9 Ligand CpG ODN or Other TLR7 Ligand Guardiquimod

In addition to a decrease in pDC frequency, reduced IFN-alpha responses of geriatric pDCs to influenza maybe due to defects in production of IFN-alpha or in expression and/or signaling via TLR7. Therefore, response of control and geriatric pDCs to type "A" CpG ODN (a TLR9 ligand, [50,51]) and Guardiquimod (a TLR7 ligand, [52]) were analyzed. Control and geriatric PBMCs were stimulated with or without CpG ODN 2216 (3 µg/ml) or Guardiquimod (1 µg/ml) for 20 h and supernatants were assayed for IFN-alpha by ELISA (Fig. 6a,b). No IFN-alpha was detected in medium controls or when ODN 2216 control was used (data not shown). While geriatric pDCs ($n=24$) made slightly less IFN-alpha (mean=21,392 pg/ml, median=15,286 pg/ml) than control pDCs ($n=22$, mean=25,316 pg/ml, median=21,192 pg/ml) in response to CpG ODN 2216 the 15.5% decrease was not statistically significant ($p=0.22$). Response of geriatric PBMCs ($n=24$, mean=411 pg/ml, median=263 pg/ml) to Guardiquimod was equivalent ($p=0.91$) to that of control PBMCs (mean=390 pg/ml, median=275 pg/ml).

Geriatric pDCs were therefore not defective in production of IFN-alpha following activation with TLR9 or TLR7. Since pDC frequency is lower in geriatric individuals, geriatric pDCs should produce more IFN-alpha on a per cell basis than control pDCs, to account for the levels of IFN-alpha made in response to Guardiquimod as well as CpG ODN. Alternatively, a higher percentage of geriatric pDCs responded to both these ligands to produce IFN-alpha.

Geriatric pDCs are Not Defective in Uptake of Influenza A

Since geriatric pDCs were not defective in IFN-alpha production or TLR signaling, reduced uptake of influenza by geriatric pDCs could additionally contribute to the defective response. To assay uptake of influenza, control and geriatric PBMCs were pulsed with FITC-A/PR/8/34 for 1 h, washed, and then chased an additional 30 min at 37°C to maximize uptake. pDCs were identified by staining with anti-HLA-DR, anti-BDCA-4, and anti-CD123, and the mean fluorescence intensity (MFI) of FITC label in pDCs was analyzed by flow cytometry (Table 2). Mean MFI for control pDCs was 13,109 while the mean MFI for geriatric pDCs was slightly higher at 13,523 ($p=0.56$). Although little difference in uptake of FITC-A/PR/8/34 was observed between controls and geriatrics, differences in IFN-alpha production in response to A/PR/8/34 between controls and geriatrics persisted (Table 2).

In conclusion, geriatric pDCs were defective in the production of IFN-alpha in response to influenza A by a mechanism that was independent of reduced pDC viability, defects in uptake of influenza, inability to secrete IFN-alpha, or defects in TLR7 signaling.

Discussion

Susceptibility of older individuals to influenza has often been ascribed to defects in T cell function. The contribution of the innate immune response to this defect remains unclear. Innate immune responses to influenza consist importantly of vigorous production of type I IFNs. They are potent anti-viral cytokines that also regulate other aspects of innate and adaptive immunity. We observed a significant decrease in the levels of IFN-alpha in supernatants from geriatric PBMCs activated with influenza. Surprisingly, no defect in IFN-alpha production was observed in geriatric PBMCs activated with CpG ODN 2216 or Guardiquimod. While influenza activates pDCs via TLR7, CpG ODN 2216 and Guardiquimod activate pDCs via TLR9 and TLR7, respectively. Our observations clearly indicate no defect in TLR7 or TLR9 signaling or IFN-alpha production in the pDC population in the elderly. Consistent with our findings, Jing et al. recently reported that the frequency of IFN-alpha secreting pDCs was reduced in PBMCs from healthy elderly subjects activated with influenza but not CpG 2216 [47]. We also observed a decline in pDC frequency in the elderly similar to that reported by other groups [47–49]. Less

IFN- α secreting pDCs coupled with decline in pDC frequency in geriatric PBMCs may account for the decreased response to influenza in geriatric PBMCs. But geriatric pDCs would have to make more IFN- α on a per pDC basis in response to Guardiquimod and CpG ODN 2216 (Fig. 6) to account for their normal response to these ligands despite lower pDC frequency.

Defect in IFN- α production in geriatric pDCs was only observed with influenza but not with other ligands that also target intracellular TLRs in pDCs. Interaction of ligands with TLR7 and TLR9 occurs in acidic endocytic vesicles, is pH dependent and is abrogated by agents like chloroquine and bafilomycin A that increase endosomal pH [44–46]. Interaction of influenza viral ssRNA with TLR7 requires virus fusion and uncoating from endocytic vacuoles by a process that is pH dependent and occurs in late endosomes through a type I fusion process [53,54]. Wang et al. demonstrated that increasing intraendosomal pH from approximately 4.5 to 5.2 with chloroquine significantly decreased IFN- α production in human pDCs in response to influenza virus but not to TLR7 ligand R848 that is an imidazoquinoline compound like Guardiquimod [55]. Increasing intraendosomal pH to 5.8 abrogated IFN- α production in response to both R848 and influenza. Therefore, subtle variations in late endosomal pH may impact IFN- α production in response to influenza but not other TLR7 ligands. We speculate that a slight increase in pH in late endosomes (and maybe all endosomal compartments) in the elderly may impact influenza virus fusion and uncoating and lead to inhibition of IFN- α production in response to influenza (which is very pH sensitive) but have little impact on IFN- α production to other TLR7 and TLR9 ligands (which may not be as pH sensitive).

The intracellular location of a TLR ligand may also determine the resulting biological response [56]. In human pDCs localization of CpG ODNs to transferrin-receptor-positive early endosomes led exclusively to IFN- α production while localization of CpG ODNs to LAMP-1 positive late endosomes promoted maturation of pDCs [56]. Similarly, in murine pDCs retention of Type “A” CpG ODN in endosomal compartments promoted IFN- α induction [57] while rapid transfer to lysosomal vesicles, as seen with conventional DCs, led to little IFN- α production. When Type “A” CpG ODN was manipulated for endosomal retention, robust production of IFN- α was observed [57]. In geriatric pDCs, viral ssRNA may be rapidly transferred to lysosomes, resulting in reduced IFN- α production.

Entry of influenza virus into cells has been studied extensively and involves binding of the virus to sialic acid-containing receptors on the cell surface followed by internalization by receptor-mediated endocytosis [58]. Since decreased uptake of influenza virus by geriatric pDCs may also lead to decreased IFN- α production, we compared uptake of FITC-labeled virus in geriatric and control pDC by flow cytometry. No defect in uptake of influenza by geriatric pDCs was observed. Although the experiment was designed with an incubation period to allow virus to bind cells, followed by a chase period to maximize uptake of virus, the possibility that virus remained on the cell surface cannot be ruled out.

In addition to their anti-viral properties, type I IFNs mediate both innate and adaptive immune responses. Therefore, reduced IFN- α levels in geriatric individuals after influenza stimulation could have a number of deleterious effects on both innate and adaptive immunity in older adults. Type I IFNs can induce the production of multiple cytokines, chemokines, and other molecules like IL-15 [59], CCXCL10, CCL4, CCL2, and IL-1RA [60]. Type I IFNs have been shown to play a modulatory role in differentiation of human T cells to Th1 development [61,62] and in the development of CD8+ T central memory cells [63,64]. Presence of IFN- α was shown to have mixed effects on proliferation in human memory CD4+ T cells depending on the antigen stimulation [65]. In murine systems, type I IFNs act directly on both CD4+ and CD8+ T cells to allow clonal expansion in response to viral infection [66,67]. An in vitro study by Jego et al. [68] using human DCs and B cells showed that IFN induces activation and IL-6 secretion by DCs, which was required for differentiation of B cells into

antibody-secreting cells. Isotype switching of B cells is also enhanced by type I IFNs [69]. In murine systems, following influenza virus infection, type I IFN receptor signals directly activated local B cells [70] and type I IFN directly modulated respiratory tract B cell responses [71]. Type I IFN has also been shown to enhance B cell receptor-dependent B cell responses [72]. A number of DC functions are enhanced by type I IFNs including MHC-I cross priming and DC maturation [73–75]. Therefore, innate immune defects in IFN- α production by older individuals could lead to multiple defects in their adaptive immune responses.

Conclusions

Geriatric PBMCs made significantly less IFN- α in response to influenza than PBMCs from younger controls. This could have a deleterious effect on anti-influenza responses in geriatric individuals and contribute significantly to the susceptibility of older individuals to influenza.

Abbreviations

ASA	Aspirin
CAD	Coronary artery disease
COPD	Chronic obstructive pulmonary disease
CVA	Cerebrovascular accident
DC	Dendritic cell
IFN	Interferon
PBMCs	Peripheral blood mononuclear cells
pDC	Plasmacytoid DC
TLR	Toll-like receptor

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Fig. 1.

PBMCs from geriatric individuals produce less IFN-alpha in response to A/PR/8/24 than PBMCs from younger controls. Control and geriatric PBMCs (1×10^6 cells/cellswell.well) in 200 μ l of X-Vivo media were incubated with or without **a** A/PR/8/34 (low = 0.32×10^5 EID₅₀/ml), **b** A/PR/8/34 (high = 1.6×10^5 EID₅₀/ml), or **c** UV-inactivated A/PR/8/34 (high = 1.6×10^5 EID₅₀/ml) for 20 h at 37°C. Supernatants were harvested and assayed for IFN-alpha by ELISA. No IFN-alpha was detected in supernatants from media controls. Horizontal bars represent median values. $p < 0.05$ was considered statistically significant

Fig. 2. PBMCs from geriatric individuals make less IFN-alpha in response to clinical isolates A/Hong Kong/8/68 and A/Denver/1/57. Control and geriatric PBMCs (1×10^6 cells/well) in 200 μ l of X-Vivo media were incubated with or without **a** A/Hong Kong/8/68 (H3N2, 1.8×10^5 EID₅₀/ml) or **b** A/Denver/1/57 (H1N1, 1.1×10^6 EID₅₀/ml) for 20 h at 37°C. Supernatants were harvested and assayed for IFN-alpha by ELISA. No IFN-alpha was detected in supernatants from media controls. Horizontal bars represent median values. $p < 0.05$ was considered statistically significant

Fig. 3.

pDC are the source of IFN-alpha in PBMC stimulated with influenza. PBMCs, PBMCs lacking pDCs, or purified cell types were incubated in 200 μ l of X-Vivo media with or without A/PR/8/34 (high = 1.6×10^5 EID₅₀/ml), CpG ODN 2216 (3 μ g/ml) or Guardiquimod (1 μ g/ml) for 20 h at 37°C. Supernatants were harvested and assayed for IFN-alpha by ELISA. **a** IFN-alpha production by control PBMCs or control PBMCs lacking pDCs incubated with A/PR/8/34, CpG ODN 2216, or Guardiquimod. Results are representative of four independent experiments. **b** IFN-alpha production by purified monocytes, T and NK cells, mDCs, B cells, and pDCs incubated with A/PR/8/34. Results are representative of three independent experiments

Fig. 4.

Influenza-induced IFN- α is acidification and TLR7 dependent. **a** PBMCs were stimulated with RIG-I ligand Poly(I:C) LyoVec (4 μ g/ml), CpG ODN 2216 (3 μ g/ml), Guardiquimod (1 μ g/ml), or A/PR/8/34 (high = 1.6×10^5 EID₅₀/ml) with or without 10 μ M chloroquine for 20 h at 37°C. Results are representative of three independent experiments. **b** PBMCs incubated with A/PR/8/34 with or without the TLR7 inhibitory ODN IRS661 or Control ODN. Results are representative of three independent experiments

Fig. 5. pDC frequency is lower in geriatric individuals. Control and geriatric PBMCs (5×10^5) were stained using anti-HLA-DR, anti-Lin2, and anti-CD123 and pDC frequency was quantified by gating on HLA-DR⁺, Lin2⁻, and CD123⁺ cells on a clinically certified flow cytometer. pDC frequency is stated as a percentage of total PBMCs. *Horizontal bars* represent median values. $p < 0.05$ was considered statistically significant

Fig. 6. Geriatric PBMCs are not defective in production of IFN-alpha in response to TLR9 ligand CpG ODN or other TLR7 ligand Guardiquimod. PBMCs (1×10^6 cells/well) were incubated in 200 μ l of X-Vivo media with **a** CpG ODN 2216 (3 μ g/ml) or **b** Guardiquimod (1 μ g/ml) for 20 h at 37°C. Supernatants were harvested and assayed for IFN-alpha by ELISA. No IFN-alpha was detected in supernatants from media controls. Horizontal bars represent median values. $p < 0.05$ was considered statistically significant

Table 1

Clinical Information of Study Subjects

	Control <i>n</i> =38	Geriatric <i>n</i> =49
Age (range)	27 (21–35)	84 (66–96)
Gender, male	37 (97%)	43 (88%)
Comorbidities		
CAD	0	12 (24%)
Diabetes	0	20 (41%)
COPD	0	8 (16%)
CVA	0	2 (4%)
Medications		
Statins	0	24 (49%)
ASA	0	18 (37%)
Insulin	0	4 (8%)
Oral hypoglycemics	0	9 (18%)

CAD coronary artery disease, *COPD* chronic obstructive pulmonary disease, *CVA* cerebrovascular accident, *ASA* aspirin

Table 2

Analysis of Uptake of FITC-Labeled A/PR/8/34 by Control and Geriatric pDCs

Donor	Uptake of FITC-A/PR/8/34 by pDCs mean fluorescent intensity	IFN-alpha (pg/ml; made in response to A/PR/8/34-high)
Control 1	11,658	2,091
Control 2	12,810	12,875
Control 3	12,385	24,595
Control 4	15,584	9,534
Mean	13,109	12,274
Geriatric 1	12,078	1,748
Geriatric 2	15,579	1,764
Geriatric 3	12,014	58
Geriatric 4	14,421	993
Mean	13,523 ($p=0.56$)	1,141