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The generation of memory B cells is maintained, but the antibody response is not, in the elderly after repeated influenza immunizations

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Abstract

The success of a vaccine in inducing a protective antibody response depends on the longevity of both long-lived plasma cells (PC) and memory B cells. We have previously shown that the *in vivo* antibody response to a new influenza vaccine, the *ex vivo* plasmablast response, the *in vitro* B cell function, measured by AID (activation-induced cytidine deaminase), and the transcription factor E47, are significantly associated and decreased in elderly individuals. We hypothesized that because AID is decreased in the elderly, the ability to generate memory B cells would also be decreased, but our findings here show that memory B cells are maintained in the elderly probably due to further amplification in response to repeated vaccination. We recruited young and elderly individuals immunized in at least two consecutive influenza vaccine seasons in which the influenza A viral strains H1N1 and H3N2 in the vaccine were the same as in the previous year. PBMC were cultured with CpG/IL2 to measure the frequency of IgG vaccine-specific memory B cells. Serum antibody response was measured by hemagglutination inhibition assay. Blood plasmablasts were measured by flow cytometry. Surprisingly, the frequencies of influenza vaccine-specific memory B cells and plasmablasts were similar in young and elderly individuals, but the fold-increase in serum titers after vaccination was lower in the elderly although most of the elderly were seroprotected. We then measured the transcription factor Blimp-1, considered the master regulator of PC differentiation, and found it significantly reduced in cultures of B cells from elderly *versus* young individuals, as well as E47/AID and IgG secretion. Taken together, these results suggest an impaired memory B cell to PC differentiation in the elderly.

Keywords

Aging; memory B cells; antibody responses; influenza vaccine

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1. Introduction

Vaccination is the most effective method of preventing infections, but the effectiveness of vaccination is different in individuals of different ages [1-6]. Vaccination against Influenza is strongly recommended for individuals over 65 years of age to protect them from infection. According to the CDC (<http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6421a5.htm>), there is evidence that elderly individuals although they have received the vaccine in consecutive years can still be infected with influenza virus and this may lead to secondary complications, hospitalization, and ultimately death [1, 7, 8]. In general, the composition of the influenza vaccine changes each year, with one or more vaccine strains replaced annually to provide protection against circulating viral strains. After 2009, influenza vaccines repeatedly included Influenza A(H1N1)pdm09 virus. Although we [9-11] and others [12-14] have shown that aging significantly impairs the antibody response to Influenza A(H1N1)pdm09 virus [9, 10], elderly individuals respond with protective titers with repeated immunization [9, 11], as we also show in this paper.

The duration and quality of humoral immunity and generation of immunological memory to vaccines is critical for protective immunity. However, these are still poorly defined even in young individuals. Immunological memory in the B cell lineage consists of long-lived plasma cells (PC) and memory B cells. The antibodies made during a memory response significantly differ from those generated during a primary response, as the class/isotype of the secreted antibody is usually switched. Moreover, antibodies appear in serum faster and the affinity of the antibodies generated is higher than in the primary response. Therefore, immunological memory resides in the antibodies which are continuously made by long-lived PC as well as in the expanded B and T memory cells which can cooperate to generate additional antibodies, with higher affinity, improving the immune response.

Influenza vaccination is less effective in elderly as compared to young individuals, in part due to decreased generation of specific serum antibodies [10, 15-19], switched memory B cells [10, 16, 20-22], and long-lived PC [13, 23]. Moreover, antibody titers generated in response to a booster vaccination depend on B cell stimulation, differentiation to B memory cells generated during the primary response, and stimulation and differentiation of these to make new plasmablasts and PC [24].

We have previously shown that intrinsic B cell defects with age contribute to sub-optimal vaccine responses in humans [9, 10, 16, 21, 25, 26]. These defects include a reduction in activation-induced cytidine deaminase (AID), the enzyme for class switch recombination (CSR) and somatic hypermutation; E47, encoded by the *E2A* gene, a key transcription factor regulating AID [27, 28], and the ability to generate higher affinity antibodies to a new antigen. We hypothesize that individuals with good E47/AID/CSR will have good primary and secondary antibody responses and those with reduced CSR will have defects in the generation of memory B cells and therefore their response to a new vaccine would be severely impacted, but the number of existing memory B cells might be maintained/amplified by repeated immunization. Therefore, if serum antibodies were dependent predominantly on memory B cells, then booster vaccination would increase both measures

in the elderly, and the association between the two would be conserved. But if there were further defects, such as the stimulation of memory B cells to make PC, then the antibody response could be lower in the elderly.

In the present study we evaluated whether consecutive vaccinations with an influenza vaccine containing repeated antigens would cause an improvement in the generation of specific memory B cells and antibody responses in elderly individuals. We recruited young and elderly participants, immunized for at least two consecutive seasons with a repeated influenza vaccine. Interestingly, we found that although the frequency of vaccine-specific memory B cells and circulating plasmablasts was not different in young and elderly individuals, the fold-increase in serum titers after vaccination was still significantly reduced in the elderly, suggesting an additional possible defect with age in the generation of PC from memory B cells. We present data that not only are E47/AID decreased in *in vitro* response to the mitogen CpG, but that also the transcription factor Blimp-1, necessary for optimal generation of PC, is significantly reduced in cultures of B cells from elderly as compared to young individuals.

2. Materials and methods

2.1. Subjects

Experiments were conducted using blood from healthy volunteers of different ages (11 young 20-40 years and 11 elderly 60 years) who participated in two consecutive seasons. Participants signed informed consent (IRB protocol #200770481). We designate elderly as 60 because starting at 60 all B cell biomarkers we measure statistically decline. Each participant was asked questions regarding demographics, health behaviors, presence of symptoms associated with inflammatory conditions or respiratory infections at the time of enrollment. No one reported subclinical inflammatory conditions and/or had respiratory tract infections at the time of enrollment, nor was on any anti-inflammatory treatment or on medications known to alter the immune response. Participants were excluded if they had diseases known to alter the immune response.

2.2. Influenza vaccination

The experiments were performed during the 2012-2013 and 2013-2014 Influenza vaccine seasons in which the vaccines had the same Influenza A(H1N1)pdm09 and H3N2 viral antigens. The composition of the vaccines were: 2012-2013 (A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010-like (Yamagata lineage)) and 2013-2014 (A/California/7/2009 (H1N1), A/Victoria/361/2011, B/Massachusetts/2/2012). Blood samples were collected immediately before vaccination (t0), and one week (t7), 4-6 weeks (t28), 4 months (t120) and 6 months (t180) post-vaccination. All elderly participants and 9 out of 11 young participants were vaccinated in the 3 previous seasons characterized by the same Influenza A(H1N1)pdm09.

2.3. Hemagglutination Inhibition (HAI) assay

HAI was performed as previously described [16]. Antibody titers were determined at every time point using the corresponding seasonal vaccine. HAI was performed for all time points

at the end of each season. The HAI test is useful for the measurement of antibody titers in serum and is the most established correlate of vaccine protectiveness. HAI titer 40 is considered to be a seroprotective titer for influenza, whereas a fold-increase of 4 in titers after vaccination is considered as seroconversion.

2.4. Generation of B cell memory in PBMC cell cultures

PBMC were collected using Vacutainer CPT tubes (BD 362761). Cells were washed and cryopreserved at every time point. In order to generate memory B cells, PBMCs were thawed and placed in culture at $3\text{--}6 \times 10^6$ cells/well, for 5 days in recombinant human IL-2 (10^3 U/ml) and CpG (1 $\mu\text{g/ml}$), according to the protocol previously described [29]. Then cells were counted and used for ELISpot.

2.5. Enzyme-linked immunosorbent spot (ELISpot)

Ninety-six-well plates (Millipore S2EM004M99) were coated with the corresponding seasonal vaccine at 1 $\mu\text{g/ml}$ and with goat anti-human IgG Fc specific (Jackson 109-005-098), and incubated overnight at 4°C. Plates were washed, blocked with 1% BSA and then incubated at 37°C for 12-18 hrs with culture-generated memory B cells with a 2-fold serial dilution starting at 2×10^5 cells (for vaccine-specific responses) or 5×10^3 cells (for total IgG responses). Plates were washed and incubated with biotin-goat anti-human IgG (Jackson 109-065-098), 1 hr rt. Plates were then washed, streptavidin-HRP (Jackson 016-030-084) added and reaction developed with AEC substrate (BD 551951). Plates were scanned and analyzed with a CTL ELISpot Scanner. Antibody secreting cells (ASC) were calculated as described [13, 29].

2.6. Flow cytometry

To detect plasmablasts ($\text{CD3-CD19+CD20}^{\text{low}}\text{CD27}^{\text{bright}}\text{CD38}^{\text{bright}}$), we stained fresh PBMC with anti-CD3 (BD 555339), anti-CD19 (BD 555415), anti-CD20 (BD 641396), anti-CD27 (BD 555441), anti-CD38 (BD 551400). The peak of the response was 7 days after vaccination, as measured by both flow cytometry [13, 21, 30] and ELISpot [31]. Up to 2×10^6 events in the lymphocyte gate were acquired on an LSR-Fortessa (BD) and analyzed using FlowJo software. Single color controls were included in every experiment for compensation.

2.7. B cell culture, mRNA extraction and quantitative (q)PCR

B cells were isolated from fresh PBMC using anti-CD19 Microbeads (Miltenyi Biotech), according to the MiniMacs protocol (Miltenyi Biotech). Cells were purified using magnetic columns. At the end of the purification procedure, cells were found to be almost exclusively (>97%) CD19+ by cytofluorimetric analysis. B cells were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, 10 $\mu\text{g/ml}$ Pen-Strep, 2×10^{-5} M 2-ME and 2 mM L-glutamine). B cells ($1 \times 10^6/\text{ml}$ complete medium) were stimulated for 1-14 days in 24-well culture plates with 1 $\mu\text{g}/10^6$ cells of CpG (ODN 2006 In Vivogen). Cells were harvested after 1, 5 and 8 days of stimulation, and mRNA extracted for quantitative (q)PCR to evaluate the peak for E47, AID and Blimp-1 mRNA expression, respectively. Supernatants were

harvested after 14 days of stimulation to measure Ig production by ELISA. The μ MACS mRNA isolation kit (Miltenyi Biotec) was used according to the manufacturer's protocol.

qPCR reactions were conducted in MicroAmp 96-well plates (Life Technologies, ABI N8010560) and run in an ABI 7300 machine. Reagents and primers (Taqman) are from Life Technologies. Primers were: E47 (TCF3) Hs00413032_m1, AID Hs00221068_m1, Blimp-1 (PRDM1) Hs00153357_m1, GAPDH Hs99999905_m1.

2.8. Enzyme-linked immunoabsorbent assay (ELISA)

Serum TNF- α /IL-6/CRP were measured by Life Technologies ELISA kits KHC3013/KHC0062/KHA0032, respectively.

Ig secretion in culture supernatants was measured by a sandwich ELISA as previously described [32].

2.9. Statistical analyses

Data were examined for distributions that violated the assumptions required for parametric analyses and missingness. To examine differences between groups, Student's t tests (two-tailed) were used. To examine the relationships between variables, bivariate Pearson's correlation analyses were performed, using GraphPad Prism 5. A formal power analysis was not computed, however, effect sizes from previous work guided the acquisition of the sample for the analyses presented herein.

3. Results

3.1. Characterization of the participants

Participants were screened for markers of systemic inflammation (plasma TNF- α /IL-6/CRP). Demographic characteristics of the participants, as well as their serum pro-inflammatory profiles, are in Table 1. Serum levels of pro-inflammatory cytokines TNF- α /IL-6/CRP were significantly higher in elderly as compared to young individuals.

3.2. The frequency of influenza-specific antibody secreting cells up to six months after vaccination is not affected by age after consecutive seasonal influenza vaccination

To evaluate influenza-specific ASC generated with previous immunizations we performed ELISpot. Briefly, PBMC from 11 young and 11 elderly healthy individuals recruited during 2012-2013 and 2013-2014 vaccine seasons, were cultured with CpG and IL-2 to measure IgG antigen-specific memory B cells. Results in Fig. 1 show that the frequencies of vaccine-specific memory B cells are not significantly different in young and elderly individuals in both seasons, although limited number of subjects, and will be confirmed in future studies. This similarity in memory between young and elderly individuals may reflect the effect of repeated vaccination given to the elderly which may compensate for their lower levels of E47/AID. One explanation for similar memory B cell responses in young and elderly individuals, despite lower E47/AID in the elderly, is that IgG+ cells in the elderly could be positively selected and proliferate in response to repeated vaccination, similar to what has been shown in mice [33].

Although influenza vaccine-specific antibodies in serum are predominantly IgG, circulating ASCs secrete IgM, IgG and IgA antibodies [13]. In the present study we measured IgG ASCs because circulating IgG antibodies represent the best correlates of protection and it has been reported that the ASC response after influenza vaccination is predominantly IgG-mediated rather than IgA-mediated in both young and elderly individuals [13].

3.3. The frequency of circulating plasmablasts is not significantly affected by age after consecutive seasonal influenza vaccination

We also measured by flow cytometry the plasmablast response at t7 after influenza vaccination in the same subjects above. Others have determined that t7 is the peak for the plasmablast response, which is completely gone at t14 [30], and this response at t7 is vaccine-specific [13, 30]. Results in Fig. 2 show no significant differences between young and elderly subjects in both seasons, at least in response to a repeated influenza vaccine, again with the caveat of low participant numbers. In contrast, in previous years, in response to a new influenza vaccine, the percentages of plasmablasts at t7 were significantly different between young and elderly individuals as we [21] and others [13] have previously published.

3.4. Antibody response to influenza vaccine is decreased by age after consecutive seasonal influenza vaccination

We next measured the serum antibody response to the vaccine by HAI. We [9, 16, 21, 25, 26] and others [18, 34] have previously shown that the HAI test is the most established correlate with vaccine protectiveness. Although we found that the frequency of memory B cells is comparable in young and elderly, their serum responses, measured as fold-increase in titers after vaccination, were significantly different at t7 and t28 in both seasons (Fig. 3A). However, the reciprocal of the titers after vaccination were not different between young and elderly individuals subjects in both seasons, and the repeated immunizations of the elderly have increased their initial response to the vaccine with the majority of elderly individuals having a seroprotective titer at t0 (Fig. 3B). The same happened for young individuals, their t0 titers increased with repeated immunizations and therefore the fold-increase in the reciprocal of the titers was lower than that we have previously seen with a new vaccine [10, 16]. The finding that the fold-increase in titers after vaccination is significantly different between young and elderly individuals suggests that the reduced PC differentiation in the elderly is at least partially due to intrinsic defects such as reduced levels of specific transcription factors.

3.5. Age effects and kinetics on Blimp-1 expression in cultures of B cells

We next investigated whether the expression of Blimp-1, the transcription factor necessary for PC differentiation, was decreased in cultures of B cells from elderly individuals. We stimulated B cells from young and elderly individuals for 1-14 days in culture with CpG. We also measured the transcription factor E47, crucial for CSR, at day 1; AID at day 5; Blimp-1 the transcription factor essential for PC differentiation at day 8; and Ig secretion at day 14. The peak for Blimp-1 is day 7/8, and after the CSR machinery (E47 and AID) is down-regulated, as previously reported in mice [35]. Results show significant age-related decreases in all these measures (Fig. 4). This is to our knowledge the first evidence of an effect of age on the expression of Blimp-1 in cultured B cells.

4. Discussion

In the present paper we show that after consecutive seasonal influenza vaccinations, memory B cell generation in response to the vaccine is maintained in elderly healthy subjects, but the capacity to seroconvert is impaired, although everyone had a protective titer, defined as 40. These findings suggest a possible intrinsic defect in PC generation in the elderly and we show an effect of age on the expression of the transcription factor Blimp-1, a crucial regulator of PC differentiation, in cultured B cells.

Many studies conducted in young individuals have investigated the effects of repeated vaccinations on humoral immune responses to the influenza vaccine showing that annual vaccination with an influenza vaccine containing the same viral strains results in general in an overall increase in protective antibodies and titers. This increase in the initial anti-vaccine titer, however, has been shown to reduce the overall vaccine-induced B cell response and correlate with the generation of plasmablast and memory B cells [36]. This suppressive effect of pre-vaccination antibodies to vaccination-induced antibody response can be explained, at least in part, by immune complex formation [37] and reduction of the effective antigen concentration to stimulate B/T cells. Alternatively, higher levels of pre-vaccination antibodies might have blocked the general activation of B cells upon vaccination [22], or they might have masked viral epitopes in the vaccine, preventing binding and activation of memory B cells recognizing that particular epitope [36]. This negative correlation is broken if a new variant strain is introduced in the vaccine. In this case, the immune response is dominated by B cells that recognize the divergent vaccine strain, leading to a diversification of the antibody response.

In elderly individuals repeated vaccinations also have been shown to generate an increase in protective antibodies and titers [38]. Our knowledge to date about the effects of repeated vaccinations on the generation of memory B cells in the elderly is understudied. Here we show that memory B cells and plasmablasts are generated similarly in young and elderly individuals in response to repeated vaccination, but fold-increase in antibodies is not, suggesting that low seroconversion in the elderly is primarily due to cell intrinsic defects in differentiation of PC rather than to lack of initial memory or naïve cells which might have responded. Although we have not measured PC differentiation directly *in vitro*, we have shown that B cells from elderly individuals express significantly lower levels of Blimp-1 and also secrete IgG, a measure of PC, after *in vitro* stimulation with CpG, as compared to younger individuals. In addition to intrinsic low levels of Blimp-1 in cultured B cells from elderly *versus* young individuals, the reduced capacity of elderly individuals to mount good antibody responses may also be due to a decrease in the capacity of bone marrow niches to support PC survival. The reason for decreased Blimp-1 mRNA expression in cultured B cells from elderly as compared to young individuals may be due to multiple mechanisms, including up-regulation of the repressors of Blimp-1 (Bach2/MITF) and/or down-regulation of the mRNA stability of Blimp-1 with age, and micro-RNAs may be involved in these processes, which are currently under investigation in our laboratory.

One explanation for similar memory B cell responses in young and elderly individuals, despite lower E47/AID in the elderly, is that IgG+ cells in the elderly could be positively

selected and proliferate in response to repeated vaccines, as in mice [33]. Similar to our results, memory B cells but not antibody responses have been shown to be maintained in immunodeficient HIV-infected children, as well as in controls, vaccinated yearly with the influenza vaccine [39]. These data altogether suggest that at least in the case of influenza vaccine, due to intrinsic age-related impairment in PC differentiation, regular booster vaccinations are needed to support seroprotective titers and protect vulnerable populations from infectious diseases.

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Highlights

1. After repeated immunizations, the frequencies of influenza vaccine-specific memory B cells and plasmablasts are similar in young and elderly individuals
2. The fold-increase in serum titers after vaccination is lower in elderly than in young individuals suggesting impaired differentiation from memory B cells to plasma cells in the elderly. However, due to repeated immunizations, most of the elderly individuals have seroprotective titers before vaccination
3. Blimp-1, the master regulator of plasma cell differentiation, is significantly decreased in cultures of B cells from elderly *versus* young individuals

Fig. 1. The frequency of influenza-specific antibody secreting cells up to 6 months after vaccination is not affected by age after consecutive seasonal immunizations

Eleven young (open circles) and 11 elderly (black circles) individuals recruited through the 2012-2013 and 2013-2014 consecutive seasons were evaluated at different time points for the frequency of vaccine-specific antibody-secreting cells, measured by ELISpot. PBMC were cultured with CpG + IL2 to generate memory B cells, and then plated on filter plates coated with influenza vaccine, or an anti-human IgG antibody. Results are expressed as % of vaccine-specific ASC divided by the total IgG ASC. Asterisks refer to the differences between post-vaccination and t0 measures in young and elderly individuals, calculated by paired Student's t test (two tailed), using GraphPad Prism. $p < 0.01$: **; $p < 0.05$: *. No significant differences were detected between young and elderly subjects at any time point.

Fig. 2. The frequency of circulating plasmablasts is not significantly affected by age after consecutive seasonal influenza immunizations

The frequency of blood plasmablasts 7 days (t7) after vaccination in 11 young (open circles) and 11 elderly (black circles) individuals recruited through the 2012-2013 and 2013-2014 consecutive seasons was evaluated by flow cytometry (same individuals as above). A representative staining with gates is shown for t7 on the left (from a young individual recruited in the 2013-2014 season).

Fig. 3. Fold-increase in antibody titers after the influenza vaccine is decreased by age after consecutive seasonal immunizations, whereas titers are comparable

A. Eleven young (open circles) and 11 elderly (black circles) individuals, same as above, were evaluated for serum antibody production to the vaccine at different time points, measured by HAI and shown as fold-increase in titers after vaccination. Removal of the highest young value at t7 does not affect significance of the difference. **B.** Serum antibody production to the vaccine shown as reciprocal of the titers after vaccination. p values are shown for significant differences between t7/t28/t180 and t0, calculated by paired Student's t test (two tailed), using GraphPad Prism. $p < 0.01$: **; $p < 0.05$: *.

Fig. 4. Age effects and kinetics on E47, AID, Blimp-1 expression in cultures of B cells
 B cells from 5 pairs of young (open circles) and elderly (black circles) individuals (included in the ones above, only selected based on availability of samples) were sorted using magnetic beads and stimulated in culture at the concentration of $1 \times 10^6/\text{ml}$ with $1 \mu\text{g}/\text{ml}$ of CpG for 1-14 days. The mRNA was extracted after 1,5,8 days of culture to measure mRNA expression of E47 (A), AID (B), Blimp-1 (C), respectively. Total IgG secretion in culture supernatants is also shown (D). p values are shown for significant differences between young and elderly groups that were calculated by paired Student's t test (two tailed), using GraphPad Prism. $p < 0.001$: ***; $p < 0.01$: **; $p < 0.05$: *.

Table 1
Demographic and serological characteristics of the participants

	Young	Elderly
Participants (n)	11	11
Age (mean years $\pm$ SE)	31 $\pm$ 3	69 $\pm$ 5 **
Gender (M/F)	5/6	6/5
Race (W/B)	7/4	8/3
Ethnic Categories (Hispanic/Non Hispanic) ^a	3/8	4/7
TNF- α (pg/ml)	6 $\pm$ 1	13 $\pm$ 2 *
IL-6 (pg/ml)	50 $\pm$ 9	125 $\pm$ 30 **
CRP (pg/ml)	505 $\pm$ 60	1032 $\pm$ 17 *

Young: 20-40 years, Elderly: 60 years.

^a All races. Hispanic are individuals from Mexico, Puerto Rico, Cuba, Central/South America and from other countries with Spanish culture or origin. All plasma inflammatory cytokines were measured by ELISA and results are means $\pm$ SE. Normal plasma levels of TNF- α (Tumor Necrosis Factor- α), IL-6 (Interleukin-6), CRP (C-reactive protein) are, respectively, 3-10 pg/ml, 30-60 pg/ml, 800 pg/ml.

**
p<0.01,

*
p<0.05 (for differences between young and elderly individuals)