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REVIEW

# Aging, cytomegalovirus (CMV) and influenza vaccine responses

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**ABSTRACT**

Influenza vaccination is less effective in elderly as compared to young individuals. Several studies have identified immune biomarkers able to predict a protective humoral immune response to the vaccine. In this review, we summarize current knowledge on the effects of aging on influenza vaccine responses and on biomarkers so far identified, and we discuss the relevance of latent cytomegalovirus (CMV) infection on these vaccine responses.

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## Influenza infection in the elderly

Influenza infection is associated with morbidity and mortality in children ≤2 years of age, in individuals ≥65 years of age and in those at risk for complications due to other co-morbidities (immunodeficiency diseases, ischemic heart disease, cerebrovascular disease, and diabetes).[1,2] It has been reported that ≥90% of annual influenza-related deaths occur in individuals ≥65 years of age.[2] The complications of influenza infection include secondary bacterial infections and exacerbations of pre-existing medical conditions.[3,4] Hospitalization and consequent decline in physical activities has been described as a major contributor to the development of disability in elderly individuals[5] and represent a significant economic burden due to both direct (medical) and indirect costs (inability to work, reduction in productivity).[6]

Infection with the influenza virus is initially controlled by an antibody response which allows time for the CD8+ and CD4+ T cell-mediated immune responses to develop. Available evidence indicates that CD8+ cells are more effective than CD4+ cells.[7-10] Infection results in a localized pulmonary infection and inflammation and elicits an influenza-specific CD8+ T cell immune response which is necessary for virus clearance.[11-14] These CD8+ T cells have been shown to be able to control the infection by killing infected pulmonary cells. Clearance by the virus-immune CD8+ population has generally been considered to require cognate interaction between cytotoxic T lymphocytes and virus-infected target cells and occurs through different mechanisms, which are perforin-[14], Fas-[14], and/or TRAIL-mediated.[15]

Although antiviral drugs against influenza are available, vaccination is the most effective method to prevent influenza infection.[16] The influenza vaccine induces an antiviral response in B and T cells, resulting in humoral and cellular immunity, respectively.[17] The antibody response to the vaccine is the first line of protection from subsequent infection. An essential step in the generation of vaccine-induced antibody-secreting cells is the interaction of vaccine-specific B cells and T follicular helper cells to generate B cell proliferation, class switch recombination (CSR) and somatic hypermutation (SHM).[18,19] Secretory IgA and IgM provide protection against the establishment of initial infection, whereas IgG antibodies neutralize newly replicating virus once infection has been established.[20] Annual influenza vaccinations help individuals to make protective levels of antibodies against the currently circulating strains.[21,22] Although for long time a general consensus has existed that there is little or no pre-existing immunity to newly emerging influenza variants in humans,[21,22] it has recently been demonstrated that seasonal influenza vaccination can induce polyclonal heterosubtypic neutralizing antibodies which are cross-reactive with both the swine-origin pandemic H1N1 virus and the H5N1 avian virus.[23]

## Aging decreases influenza vaccine responses

The effects of influenza vaccination are different in individuals of different ages[24-28] and successive annual vaccinations increase protection against influenza.[29-31] In the case of seasonal influenza vaccination there is evidence that elderly individuals who have routinely received the vaccine can still contract the infection,[32-34] likely due to decreased immunocompetence of the elderly, generally referred to as 'immunosenescence,' as well as to a reduced 'match' of the vaccine with seasonal viruses. Moreover, a mismatch between the virus strain in the vaccine and the circulating virus strain, can also cause reduced vaccine effectiveness.[35] The fact that the influenza vaccines prevent complications from influenza (e.g. pneumonia) strongly supports vaccination campaigns targeted to improve immune functions in elderly individuals. Current influenza vaccination campaigns are able to reduce hospitalization rates,[36] but hospitalizations due to influenza-related disease are still high [http://www.cdc.gov/flu/weekly/overview.htm and[31]].

For many years, functional alterations in T cells have been considered to be the most significant for immunosenescence

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and sufficient *per se* to explain the age-related decrease in antibody responses to vaccines observed in elderly individuals. However, a large amount of work has been done more recently showing that defects in other components of the innate and adaptive immune systems also occur with age and contribute to the increased frequency and severity of infectious diseases in the elderly. Systems vaccinology approaches have recently been successfully employed to investigate innate and adaptive immune responses to influenza as well as other vaccines.[37,38] Several studies have clearly demonstrated that the age-dependent differences in the response to influenza vaccination may be due to age-related differences in the innate and adaptive immune systems. These include decreased T cell function[39-42] and loss of CD28 expression,[39] decreased memory B cells[43-48] and reduced specificity and class of antibody produced,[49,50] reduced natural killer cell cytotoxicity on a per cell basis,[51] and reduced number and/or function of dendritic cells in blood.[42,52,53] In addition cytomegalovirus (CMV) positivity is increased with age.[54,55]

A recent study has shown that the length of telomeres is an indicator of the robustness of B and T cell responses of elderly individuals to the influenza vaccine.[56] In particular, B cells from individuals with protective titers to the influenza vaccine had significantly longer telomeres than those with a poor antibody response, whereas monocyte-derived antigen-presenting cells of both short and long telomere groups induced similar expansions of influenza-specific CD8+ T cells. Vaccination-specific CD8+ T cells that underwent more expansions had significantly longer telomeres than cells with fewer divisions.

We will summarize below published results on the effects that the age-related changes in T cells, B cells, dendritic cells and monocytes may have on influenza vaccine responses.

## T cells

The reduced response of the elderly to influenza vaccination has been correlated with a reduction in naïve T cells and,[57] an accumulation of late-differentiated memory CD4 and CD8 T cells with a loss in CD28 expression,[39,58-60] increased CMV seropositivity (see below).[55] It has recently been shown that aging is significantly correlated with a significant loss of naive CD8, more than naïve CD4 T cells, and this loss is not associated with an increase in memory T cells and is not affected by CMV seropositivity.[61] Conversely, the loss of naïve CD4+ T cells is associated with an increase in effector/effector memory CD4 and CD8 T cells and is observed only in CMV seropositive individuals. These findings demonstrate that aging and CMV have both distinct and joint influence on peripheral T cell homeostasis in humans but the mechanisms for these are still not determined.

CD28 is a costimulatory molecule required for the productive activation, proliferation, and differentiation of effector function in T cells.[62] The irreversible loss of CD28 expression due to chronic immune activation of human T lymphocytes in long term culture is one of the signatures of replicative senescence[60] and even in young individuals this has been associated with persistent infections, autoimmunity and inflammatory conditions.[63-66] CD28-mediated costimulation is crucial for the formation of germinal centers (GCs) and the generation of effective B cell responses and antigen-specific high-affinity antibodies. In response to immunization, defective T helper-cell function has been indicated in contributing to antibodies not being hypermutated and without SHM.[67,68] It has been shown that the lack of antibody production following influenza vaccination is associated with increased frequency of CD8+CD28- T cell clones, which express effector cell markers and are mostly CD45RA+. When isolated and stimulated with anti-CD3 or autologous cells, these clones do not proliferate, but produce IFN-$\gamma$, suggesting that in elderly individuals a change in the polarization of the immune system occurs and this may be responsible for the development of age-related immune deficiencies.[59] Others have confirmed and extended these results showing that the frequencies of CD8+CD28- T cells can be useful biological markers of compromised immune competence, identifying individuals at risk for insufficient antibody responses, whereas the size of the CD4+CD28- T cell subsets has been shown to have no effect on the ability to mount effective antibody responses.[39]

It has recently been shown that terminally differentiated (CD27-CD28-) CD4+ T cells utilize an intracellular signaling pathway for the activation of the p38 MAPK that senses changes in intracellular levels of glucose as well as genotoxic stress and spontaneously engages the metabolic master regulator AMPK to trigger autophosphorylation of p38. Signaling through this pathway inhibits telomerase activity, T cell proliferation and the expression of key components of the TCR signaling machinery, and has been proposed to drive the senescence of human T cells.[69] These results are in line with the hypothesis that aging is powerfully influenced by alterations in nutrient sensing and metabolism.[70]

Although aging is associated with increased inflammation,[71-74] increases in the anti-inflammatory response can also occur, and the increased production of IL-10 and the decreased IFN-$\gamma$:IL-10 ratio in influenza-stimulated lymphocyte cultures has been shown to be associated with reduced cytolytic capacity of CD8+ T cells which clear influenza virus from infected lungs.[8] Moreover, IL-10 suppresses CTL responses and down-regulates the expression of costimulatory molecules on antigen-presenting cells,[75] and together with the down-regulation of IFN-$\gamma$ production leads to reduced stimulation of T cell memory and poorer responses to influenza vaccination in the elderly.[8,76]

## B cells

Our laboratory has shown that age-related intrinsic B cell defects also occur, generating sub-optimal antibody responses to vaccines.[44,46,77,78,79] Some of the B cell defects we have identified include a reduction in activation-induced cytidine deaminase (AID), the enzyme necessary for class switch recombination (CSR) and SHM; E47, a key transcription factor regulating AID[80]; and the ability to generate higher affinity antibodies to a new antigen. In the last 5 influenza vaccine seasons (2009–2013), we have measured the antibody response to the seasonal and pandemic influenza vaccines in serum and we have associated this response with the B cell response after vaccination to the vaccine *in vitro*. *In vivo* and *in vitro* B cell responses have been measured respectively by the hemagglutination inhibition (HAI) assay and by AID mRNA expression

by qPCR after B cell re-stimuation with the vaccine. AID is a measure of CSR and of B cell function which we have previously established to reflect the generation of specific IgG and to associate with other mechanistic markers such as E47,[81] encoded by the *E2A* gene, which transcriptionally activates AID,[80] which is crucial for all processes generating antibody diversity, such as V(D)J recombination, CSR and SHM.[82-86] Our published results have shown that the specific response of B cells to vaccination *in vivo* and *in vitro* are both decreased with age and are significantly correlated.[44,46, 87], Moreover, the percentages of switched memory B cells and CpG-induced AID before vaccination are both good B cell biomarkers that are reduced in elderly as compared to young individuals and are significantly correlated with the *in vivo* antibody response to the vaccine.[44,46] Therefore, we have proposed these as predictive biomarkers of optimal vaccine-induced antibody responses.[44,46]

Polymerase chain reaction (PCR)-based spectratyping analyses of the lengths of the complementarity determining region (CDR)3, used to assess repertoire diversity, have shown age-related changes in the relative proportions of large clonal populations of both T cells and B cells (reviewed in[88]). In particular, it was shown that a significant proportion of elderly individuals have a dramatic collapse in their B cell repertoire diversity and that the extent of loss in B cell diversity correlates with their health status.[89] Additionally, a study in which influenza and pneumococcal vaccines were administered to 6 young and 6 elderly individuals showed that the vaccine-induced expansion of B cells with short and hydrophilic IgH CDR3 regions was lower in older individuals.[90] Moreover, elderly individuals had impaired IgM and IgA anti-pneumococcal antibody responses, which correlated with features of the spectratypes for their IgM and IgA expressing B cells (baseline repertoire with larger CDR3 regions than in the younger group). Another study has used high-throughput long read sequencing to perform immunogenomic characterization of human antibody repertoires in the context of influenza vaccination.[91] This analysis of the clonal structure and mutational distribution of individuals' repertoires has shown that elderly individuals have decreased numbers of lineages but increased pre-vaccination mutation load in their repertoire and that some of these individuals have an oligoclonal repertoire in which the diversity of the lineages is greatly reduced as compared to young individuals, consistent with earlier reports on contraction of B cell repertoires in the elderly.[92] These findings could help to explain the impaired vaccine responses observed in the elderly.

B cells are significantly affected by inflammation. B cells themselves express innate immune receptors which recognize exogenous pathogens or the adjuvants used to induce an immune response. B cells can either promote immune responses by acting as antigen-presenting cells or they can regulate immune responses by secreting immunoregulatory cytokines. Published data have shown that B cells from mice infected with *T. gondii*, *H. polygyrus* or *P. carinii* can secrete pro-inflammatory cytokines such as TNF-$\alpha$.[93-95] Data from our laboratory have shown that unstimulated B cells from elderly individuals make significantly higher levels of TNF-$\alpha$ than those from young subjects, and these are positively correlated with serum TNF-$\alpha$. Importantly, levels of TNF-$\alpha$ before stimulation are negatively correlated with the response of the same B cells after *in vitro* stimulation which is measured by AID.[77] In line with these results, an anti-TNF-$\alpha$ antibody was found to significantly increase the response in cultured B cells from elderly individuals, providing a proof of principle that it is possible to improve class switch in elderly individuals by counteracting autocrine TNF-$\alpha$.[77] These findings may help to explain the reduced antibody response of elderly individuals to vaccines and also provide biomarkers for good responsiveness and crucial targets for development of more effective vaccines. Results from this study indeed identify TNF-$\alpha$ as another B cell-specific biomarker, which can help to predict the quality of *in vivo* and *in vitro* B cell responses.

### Dendritic cells (DCs)

Defects in cytokine production by dendritic cells (DCs) from elderly individuals have also been associated with poor influenza vaccine responses. DCs are professional antigen-presenting cells that play a key role in the linkage between innate and adaptive immunity. Human DCs, classified as myeloid DCs (mDCs) or plasmacytoid DCs (pDCs), have distinct functional activities: mDCs produce IL-12 and induce Th1 and CTL responses, whereas pDCs produce IFN-$\alpha/\beta$ in response to bacteria and viruses.[96,97] The mDCs from elderly individuals are significantly impaired in their capacity to secrete TNF-$\alpha$/IL-6/IL-12 (p40) in response to TLR1/2, TLR2/6, TLR3, TLR5, TLR8 stimulation. The pDCs from elderly individuals are also functionally impaired and produce less TNF-$\alpha$/IFN-$\alpha$ in response to TLR7 and TLR9 stimulation.[53] These defects have been associated with poor antibody response to the influenza vaccine. It shoud be noted here that the induced inflammatory response to a pathogen is positive for the host and only the chronic inflammatory response previously presented is deleterious for the elderly population.

### Monocytes

Monocytes from elderly individuals have also been shown to be impaired in their capacity to secrete the pro-inflammatory cytokines TNF-$\alpha$ and IL-6, but not the anti-inflammatory cytokine IL-10 in response to influenza vaccination.[98] These results are the first to show that dysregulation of IL-10 production by monocytes is associated with impaired influenza vaccine responses in elderly individuals.

## CMV and influenza vaccine responses

CMV is a $\beta$-herpes virus which latently infects a large proportion of the human population and this proportion increases with age.[99] Once infected with CMV, the immune system is not able to eliminate the virus resulting in latent CMV infection.[99,100] The infection is asymptomatic in immunocompetent individuals, but may cause severe diseases in immunocompromised hosts. CMV has been postulated to be one of the driving forces of immunosenescence. CMV infection is associated with premature mortality and is a component of the immune risk phenotype, which predicts remaining longevity in the very elderly.[101] CMV infects fibroblasts, epithelial, endothelial,

stromal, smooth muscle cells,[102] which present CMV antigens with MHC class I. CMV induces the production of a variety of pro-inflammatory mediators which in turn induce CMV reactivation.[103] In particular, *in vitro* studies have shown that CMV induces rapid translocation of NF-kB in HeLa cells from the cytoplasm to the nucleus, promoting the production of TNF-$\alpha$ which leads to further activation of latent CMV and up-regulation of the inflammatory response,[104] as TNF-$\alpha$ is a powerful stimulator of the promoter/enhancer of the human CMV virus leading to further upregulation and exacerbation of the systemic inflammatory response.[104] This positive feedback loop drives inflammaging more effectively in CMV+ elderly than in young individuals, causing deleterious effects in the immune system of the individual.

CMV seropositivity has been shown to have a negative effect on influenza vaccine-specific antibody responses. CMV has been associated with poor humoral response to influenza vaccination in the elderly[58,105] as well as the young[78] and with the presence of CD27-CD28-CCR7-CD45RA+ or with CD28-CD57+ T cells, both identified as late differentiated/exhausted T cells which produce pro-inflammatory cytokines and have therefore a significant role in age-related immune pathologies.[58,105]

Also in young individuals, CMV has been associated with the induction of CD27-CD28-CD45RA+ T cells and consequent suboptimal influenza vaccine responses, suggesting that this virus may underlie rudimentary aspects of immunosenescence even in chronologically young individuals.[106]

CD4 T cell responses specific for influenza core proteins are absent in half of the CMV seropositive elderly, but present in those not infected with CMV, which respond as well as young individuals, suggesting that advanced chronological age plays a role in reducing the CD4 responses to influenza but only in concert with CMV infection.[107]

The effect of CMV infection on influenza vaccine responses has been controversial with many studies showing a negative effect of CMV[58,78,105,107], and others showing no effect at all.[108] It has recently been shown that CMV infection enhances the response to the influenza vaccine in young but not aged mice and humans.[109] In particular it has been shown that CMV-seropositive young individuals exhibited enhanced *in vivo* antibody responses, increase in the circulating levels of Th1 and Th2 cytokines and stronger CD8+ responses as compared with CMV-seronegative individuals. In parallel experiments, mice infected with murine CMV (MCMV) showed improved T cell responses to influenza virus challenge and significantly lower influenza virus titers and this effect was IFN-$\gamma$-dependent, demonstrating that CMV can boost the immune response of young individuals and therefore shows features of a mutualistic agent confering benefits to the host.

CMV seropositivity also induces the expansion of polyfunctional CD8+ T cells (CD8+CD57+). These cells produce multiple cytokines (IFN-$\gamma$, TNF-$\alpha$) and degranulate in response to stimulation, and are therefore crucial for the generation of optimal responses to infections and vaccines.[110] Polyfunctional T cells also make more cytokine per cell than monofunctional cells.[111,112] Published results have indeed shown that higher numbers of polyfunctional T cells are correlated with better prognosis during HIV infection[111] and better responses to vaccination,[112] suggesting that the efficiency of the response is associated with the capacity to produce several cytokines as a marker of quality. Moreover, the late differentiated/exhausted memory T cells which are expanded in elderly individuals are specific for previously encountered CMV antigens and their presence correlates with the ability to mount robust pro-inflammatory responses against major CMV antigens and are therefore positively associated with longer survival in elderly individuals, suggesting that these cells are at least partially functional and necessary for further protection to subsequent infection.[113]

Our group has recently demonstrated for the first time a negative association between CMV seropositivity and the B cell predictive biomarkers of optimal vaccine responses previously characterized in our laboratory.[78] These biomarkers are switched memory B cells and AID in CpG-stimulated B cell cultures, which are positively correlated with the serum response to the vaccine. We think that this CpG response reflects the inability of B cells from elderly individuals to stimulate AID to an external stimulus and this response in PBMC is consistent with that in the elderly GCs in which B cells are generating a vaccine response, i.e. it accurately reflects the decrement of the aged in vivo B cell response. Moreover, we found CMV seropositivity associated with increased levels of systemic and B cell-intrinsic inflammation and this may be one of the mechanisms through which CMV down-regulates the B cell antibody response. We have proposed that one mechanism through which CMV decreases B cell function may be an increase in systemic TNF-$\alpha$ which induces B cell-derived TNF-$\alpha$ which in turn activates the promoter/enhancer of CMV and pro-inflammatory cytokine production.[104] In addition to this mechanism directly acting on B cells, CMV may also down-regulate the antibody response to the influenza vaccine indirectly through the induction of terminally differentiated T cells and accumulation of senescent T cells,[58,105] which lead to reduced generation of memory T cells.[8,76]

Using high-throughput DNA sequencing of *IGH* gene rearrangements to study the BCR repertoires over 2 successive influenza vaccine seasons, it has been shown that V,D,J usage is comparable in young and elderly individuals, V mutation levels are associated with CMV-seropositivity and frequencies of highly mutated IgM and IgG sequences are increased in B cells from elderly as compared to young individuals and are also associated with CMV seropositivity whereas persistent clonal B cell expansion is associated with EBV.[114] The presence of these persistent clones in the blood of elderly individuals suggests that progressive antigenic exposure has induced both B cell proliferation and Ig gene mutation, leading to the accumulation of highly mutated IGHV genes over the course of human lifespan. The specificity of these clones however is unknown. These studies would support (repeated) vaccination in adults before depletion of their capacity in old age.

CMV seropositivity induces significant changes in NK cells. In particular, the $CD56^{dim}$CD57+NKG2C+ NK subset expands in CMV seropositive individuals[115] and this subset is responsible for degranulation, IFN-$\gamma$ and TNF-$\alpha$ secretion in response to cross-linking of CD16 or natural cytotoxicity receptors, but responds poorly to pro-inflammatory cytokines, suggesting that these cells may be less sensitive to IL-2 produced

by antigen-specific CD4 T cells and to IL-12/IL-18 produced by DCs and macrophages.[116] To our knowledge, there is only one published study on the effect of CMV seropositivity on NK cell responses to the influenza vaccine.[117] This study has shown impaired *in vitro* NK responses to the H1N1 influenza vaccine antigens, such as reduced IFN-$\gamma$ production and degranulation, decreased cytokine responsiveness and decreased cytokine receptor expression.

## Concluding remarks

Yearly influenza epidemics can seriously affect the human population, with high risks of complications occurring in young children ($\leq$2 years) and in individuals over 65 y of age). Annual influenza vaccinations help the population to make protective antibodies against the currently circulating viral strains, but the effects of vaccination decrease with age, mainly due to decreased immunocompetence. Evidence exists that infection with CMV leads to accelerated aging of the immune system and contributes to poor responsiveness to influenza vaccination in the elderly. However, studies summarized in this review have shown that aging and CMV have both independent and joint effects on immune cells. The results presented here may also apply to other routine vaccination programs (e.g., hepatitis B) as well as to vaccines delivered to worldwide travelers (e.g. yellow fever).

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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