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B Cell–Specific Biomarkers for Optimal Antibody Responses to Influenza Vaccination and Molecular Pathways That Reduce B Cell Function with Aging

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Abstract

This review highlights recent findings on the effects of aging on influenza vaccine responses, with major emphasis on T and B cells, which are significantly impaired by aging. We discuss changes in T cell production and thymic output; T cell subsets; and TCR repertoire, function, and response to latent persistent infection. We also discuss changes in B cell subsets, repertoire, and function, and how function is impaired by increased intrinsic B cell inflammation and reduced signal transduction. This review presents age-related effects on antigen-presenting cells, summarizes recent studies, including our own, aimed at the identification of biomarkers of protective vaccine responses, and provides examples of recent technical advances and insights into human vaccine responses that are helping to define the features associated with successful vaccination and that may enable a more predictive vaccinology in the future.

Keywords

aging; B cells; inflammation; vaccine responses

I. INTRODUCTION

The impaired humoral immune responses observed with age lead to increased frequency and severity of infectious diseases and are responsible for the reduced vaccine responses observed in elderly individuals.^{1–8} The world population is aging rapidly; as a consequence, the prevention of infectious diseases in the elderly represents an important public health issue.

Vaccination against influenza is strongly recommended for individuals over 65 years of age to protect them from infection, and it has been shown to significantly decrease disease burden and influenza transmission in the elderly community. For this reason, annual vaccination is recommended. In the case of seasonal influenza vaccination, for example, there is evidence that elderly individuals who have routinely received the vaccine can still contract the infection with secondary complications leading to hospitalization, physical

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debilitation, exacerbation of underlying medical conditions, and ultimately death.^{1,3,7} The population of elderly individuals accounts for more than 90% of seasonal influenza–related deaths and hospitalizations.

It has been shown that repeated vaccinations with a vaccine containing the same viral strains result in a significant increase in protective antibodies and titers in both young and elderly individuals.^{9,10} In general, the composition of the influenza vaccine changes each year, with one or more viral strains replaced annually to provide protection against strains that are anticipated to circulate. Since 2009, influenza vaccines have included the pandemic (p)2009 H1N1 strain. A recently published study conducted on 136 young and 122 elderly individuals vaccinated for 4 consecutive influenza vaccine seasons showed the effect of prior vaccination history on vaccine response. Results demonstrated that young individuals respond better than elderly individuals to the first vaccination, but the difference in response between young and elderly individuals declines rapidly after subsequent vaccinations, suggesting that prior vaccination history and/or infection history must be taken into consideration when influenza vaccination is examined.¹¹

Challenges for the field are to better understand the mechanisms of induction, development, and maintenance of protective immunity in the elderly in response to vaccination against emerging and reemerging diseases, and to better characterize vaccine responders and nonresponders.

II. IMMUNOSENESCENCE AND INFLUENZA VACCINE RESPONSES

Decreased T cell function has for many years been considered the most significant feature of immunosenescence, sufficient on its own to explain the age-related decrease in antibody response to anti- gens and vaccines observed in the elderly. Much work has been carried out in recent years showing that defects in other components of the innate and adaptive immune system in the elderly also occur with age and contribute to the increased frequency and severity of infectious diseases.

Age-related defects in immune cells (T, B, antigen-presenting) induce inefficient primary and recall responses to influenza vaccination in elderly individuals. These defects are summarized next.

A. Age-Related Changes in T Cells

Although the mechanisms underlying T cell immunosenescence are multifactorial, thymic involution plays a crucial role.¹² Naïve T cells are reduced and CD4 T cell receptor (TCR) excision circles, which are markers of thymic output, significantly decrease between 25 and 60 years of age.¹³ TCR diversity is also compromised by decreased thymic output of naïve T cells. Concomitant with decreased frequency of circulating naïve T cells, the frequency of memory/effector T cells increases.¹⁴ A study published in 2014 showed that aging induces a significant decrease in naïve CD8+, but not CD4+, T cells.¹⁵ The decrease in naïve CD8+ T cells was not altered by cytomegalovirus (CMV) seropositivity, whereas the decrease in naïve CD4+ T cells showed significance in CMV-seropositive individuals, who also

exhibited an absolute increase in effector/effector- memory CD4+ and CD8+ T cells with age.

With age, T cells downregulate expression of the CD28 molecule, and subsets of CD4+CD28– and CD8+CD28– T cells emerge.¹⁶ The downregulation of CD28 expression due to chronic immune activation of human T cells is one of the signatures of replicative senescence¹⁷ and has been associated with impaired influenza vaccine responses.^{18,19}

Aging has also been associated with increased frequency of a subset of CD8+ T cells that are phenotypically naïve but capable of secreting multiple factors (TNF- α , IFN- γ , Granzyme B) in response to persistent viral antigens. These cells, known as memory T cells with naïve phenotype (T_{MNPs}), are transcriptionally different from memory and effector T cells. T_{MNP} frequencies and numbers significantly increase months and years after symptomatic infection with the West Nile virus and are positively correlated with the severity of acute viral infection.²⁰ It is not known if T_{MNPs} also increase in individuals infected with the influenza virus.

The cytotoxicity of CD8+ T cells decreases with age.²¹ Virus-specific killing to clear influenza virus infection from the lungs is mediated by granzymes—primarily Granzyme B—contained in granules in cytotoxic T cells. The granules migrate to the immunological synapse between the activated cytotoxic T cells and the virus-infected target cell and are transported through the cell membrane into the cytoplasm of the infected host cell. This process is facilitated by perforin. Granzyme B is a key element in the enzymatic cascade that leads to apoptotic cell death²² and clearance of the influenza virus from the lungs.^{23,24} In elderly individuals, Granzyme B activity in virus-stimulated lymphocytes correlates with protection against influenza.^{25,26} It has been reported that there is a significant reduction in the frequency of cells that coexpress Granzyme B and perforin and that this is associated with a decline in Granzyme B and perforin cytotoxic activity.²⁷ This decline has been shown to predict not only increased risk of influenza illness^{25,26} but also illness severity²⁸ in elderly individuals.

Although aging is generally associated with increased inflammation, increases in anti-inflammatory response can also occur which are characterized by IL-10 production. A decreased IFN- γ /IL-10 ratio in influenza-stimulated lymphocytes has also been shown to be associated with reduced cytolytic capacity of the CD8+ T cells that clear the influenza virus from infected lungs.²¹

The effects of aging on the specific response of CD4+ T cells to the influenza vaccine have been evaluated in the context of CMV infection. CD4+ T cells provide help for antiviral B cell responses, antibody class switch, affinity maturation, and the generation of long-lived plasma cells; moreover, preexisting CD4 memory T cell levels are associated with disease protection in humans.²⁹

CMV is a β -herpes virus that latently infects a large proportion of the human population; this proportion increases with age.³⁰ 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.³¹ The effect of CMV infection on influenza vaccine response is controversial, with most studies showing a negative effect^{32–35} and others showing no effect at all.³⁶ The negative response in CMV-seropositive individuals, both young and elderly,^{32,35,37} has been associated with the presence of either CD27–CD28–CCR7–CD45RA+ or CD28–CD57+CD4+ T cells, both types identified as late differentiated/exhausted T cells that produce proinflammatory cytokines and therefore have a significant role in age-related immune pathologies. Moreover, it has been reported that specific memory CD4 T cell responses are reduced in elderly individuals but only if they are CMV-seropositive,³² suggesting that advanced age plays a role in reducing CD4 response to influenza although only in concert with CMV infection. In contrast, CMV seropositivity has been reported to induce the expansion of polyfunctional CD8+ T cells (CD8+CD57+), which produce multiple cytokines (IFN- γ , TNF- α), degranulate in response to stimulation,³⁸ and correlate with better response to vaccination.³⁹ Moreover, the late differentiated/exhausted-memory CD4+ T cells that are expanded in elderly individuals are specific for previously encountered CMV antigens; their presence correlates with the ability to mount robust proinflammatory responses against major CMV antigens and is therefore positively associated with longer survival in elderly individuals, suggesting that these cells are at least partially functional and necessary for further protection against subsequent infection.⁴⁰

Studies on the contribution of age-associated defects to T cell response to the trivalent inactivated influenza vaccine (TIV), which is the most common influenza vaccine and is delivered intramuscularly (only rarely subcutaneously), have been exclusively focused on peripheral blood, as the immune cells recruited to the site of vaccine injection are not T cells but innate cells. When the live attenuated influenza vaccine (LAIV) was used, mucosal T cells were evaluated, but to our knowledge no such studies focused on different age groups. As to other vaccines—the HIV vaccine for example—age-related decreases in mucosa-associated T cells are more relevant but not a topic of this review.

B. Age-Related Changes in B Cells

Antibody responses represent the gold standard of influenza vaccine efficacy, which is reduced in the elderly in part because of decreased generation of specific serum antibodies,^{41–46} switched-memory B cells,^{42,43,47–49} and long-lived plasma cells.^{50,51}

We previously showed that intrinsic B cell defects that develop with age contribute to suboptimal vaccine responses in humans.^{37,42,43,48,52,53} These defects include (1) reduced activation-induced cytidine deaminase (AID), the enzyme for class switch recombination (CSR), and somatic hypermutation (SHM); (2) reduced E47, encoded by the *E2A* gene, a key transcription factor regulating AID^{54,55}; and (3) reduced ability to generate higher affinity antibodies to a new antigen. Table 1 summarizes our major results obtained during consecutive influenza vaccine seasons (2011–2014).

We measured antibody response to the influenza vaccine in serum and associated this response with B cell response to the vaccine *in vitro* after vaccination. *In vivo* and *in vitro* B cell responses were measured, respectively, by hemagglutination inhibition assay (HAI) and

AID mRNA expression by qPCR after B cell restimulation with the vaccine. AID is a measure of CSR and B cell function. Our published results show that the specific *in vivo* and *in vitro* responses of B cells to vaccination decrease with age and are significantly correlated.^{42,43,56} These results support our initial hypothesis that the *in vitro* AID response recapitulates what occurs *in vivo* in the germinal center in the generation of memory B cells. Since 2009, the (p)H1N1 has been repeated each year in vaccine preparation, leading to higher levels of seroprotection in vaccinated individuals. Therefore, possible defects seen in AID in B cells from elderly individuals may have been underestimated.

In a recently published study, we evaluated how aging affects the generation of memory B cells. We expected to see reduced memory B cell responses in elderly individuals because AID is decreased in this population. To our surprise, memory B cell levels were maintained in the elderly probably because of further amplification in response to repeated vaccination; however, the fold-increase in serum titers after vaccination was lower although most subjects were seroprotected. This finding suggests that in this case low seroconversion in the elderly is mostly due to cell intrinsic defects in the differentiation of plasma cells.⁵⁷ One explanation for similar memory B cell responses in young and elderly individuals, despite lower AID in the elderly, is that IgG+ cells in the latter can be positively selected and can proliferate in response to repeated vaccines, as has been seen in mice.⁵⁸ Similar to our results, memory B cells but not antibody responses have been shown to be maintained in both immunodeficient HIV-infected children and in controls vaccinated yearly with the influenza vaccine.⁵⁹ These data suggest that, at least in the case of the influenza vaccine, because of intrinsic age-related impairment in plasma cell differentiation, regular booster vaccinations should be recommended to support seroprotective titers and protect vulnerable populations from infectious diseases.

Analysis of the quality of antibodies secreted from single plasmablasts 7 days after vaccination has shown an age-related decrease not only in the number of vaccine-specific plasmablasts but also in the number of antibodies made by these cells.⁵¹ However, the avidity of these vaccine-specific antibodies and the affinity of recombinant monoclonal antibodies obtained from single-cell plasmablasts were similar in the two age groups. In line with these findings, analyses of the clonal structure and mutation distribution of B cell repertoires have shown that elderly individuals exhibit increased mutations in their repertoires before vaccination, suggesting that priming by previous infections or vaccinations may have occurred.⁶⁰ Moreover, most of these elderly individuals show reduced B cell clonal diversity when compared to young individuals.

Using spectratype analysis and high-throughput sequencing, another study showed that the B cell repertoire of elderly individuals evinces nonspecific clonal expansion in the absence of challenge, and that this loss of specific B cell diversity correlates with poor health.⁶¹ No major differences in repertoire between different cell subsets or between different classes and subclasses of antibodies were found.

C. Age-Related Changes in Antigen- Presenting Cells

Defects in cytokine production by antigen-presenting cells (APCs) from elderly individuals have been associated with poor influenza vaccine response. Dendritic cells (DCs) are

professional APCs. Human DCs, classified as myeloid (mDCs) or plasmacytoid (pDCs), have distinct functions: mDCs produce IL-12 and induce Th1 and CTL response, whereas pDCs produce IFN- α/β in response to bacteria and viruses.^{62,63} Both mDCs and pDCs from elderly individuals are significantly impaired in their capacity to secrete TNF- α /IL-6/IL-12 (p40) in response to Toll-like receptor (TLR) stimulation,⁶⁴ and these impairments have been associated with poor antibody response to the influenza vaccine.

Monocytes from elderly individuals have also been shown to be impaired in their capacity to secrete the proinflammatory cytokines TNF- α and IL-6 but not the anti-inflammatory cytokine IL-10 in response to influenza vaccination.⁶⁵

III. PREDICTORS OF OPTIMAL VACCINE RESPONSES

A major challenge in vaccinology is to build models that can accurately predict vaccine efficacy using baseline evaluations of immune cell populations. These models may be applied to predict response to many vaccines and infectious diseases, but they are particularly important in influenza vaccination, in which a partial immunity is present at baseline that leads to significant differences across individuals.⁶⁶

Recent technological advances in multiple research areas (genetics, immunology, structural biology, bioinformatics, computational biology, nanotechnology, systems biology) offer tools for the discovery of biomarkers of protective immunity. Among these, systems vaccinology employs systems biology to identify early molecular signatures that may predict immune responses to vaccination and possibly lead to discovery of new correlates of protection.^{67,68} Systems vaccinology has provided a global picture of the immune response to vaccination and has been successfully used to study the immune response to vaccines against influenza,^{69–73} pneumococcus,⁷⁴ yellow fever,^{75,76} and meningococcus.⁷⁷ However, most studies so far have provided data obtained after vaccination rather than at baseline, making their use problematic as predictive models of vaccine response given that the system is already perturbed by the vaccine. Baseline predictive models also have limitations because they are highly dependent on cell population frequencies. Indeed, transcriptomic measurements of PBMCs have been shown to be insufficiently sensitive to differences across individuals in contrast to the highly transcriptionally active profile of cells evaluated after vaccination.⁶⁶

Unlike the studies just cited, a study by Kennedy et al.⁷⁸ found that composition of the immune cell pool and level of activation of immune cells measured by expression of CD86 and HLA-DR are good predictors of vaccine-specific antibody response as quantified by HAI, virus neutralization assay, and memory B cell ELISpot. In this study, baseline (t0), early (t3), and late (t28) B cell frequencies were calculated using flow cytometry. Changes from t0 to t28 and from t3 to t28 were found to be significantly associated with good response. Variations in individual responses to influenza vaccination were explained by variations in the composition of the B cell pool.

In another recent study from the same group,⁷⁹ transcriptional signatures associated with the generation of vaccine-specific memory B cells were identified. In this study, mRNA-Seq

transcriptional profiling of PBMCs was performed before (t0) and after vaccination (t3, t28) to analyze cell signaling, cytokine signaling, cell adhesion/migration, and transcriptional regulation of metabolic pathways. Results revealed significant associations of metabolism and immune response genes with specific memory B cell responses. This was the first study, to our knowledge, to use system vaccinology, unbiased high-throughput transcriptional analysis, and modeling to identify signatures of memory B cell responses.

A. Cell Predictive Biomarkers of Vaccine Responses

In the case of the influenza vaccine (and vaccines against frequently encountered pathogens), antibodies produced during a previous pathogen exposure influence new responses. It has been shown that serum antibody titers at baseline are important predictive markers of good response. This suggests that in-depth serological characterization of human vaccines should be performed in every human vaccine study, particularly for viruses such as influenza, for which extensive data on circulating strains in multiple years are available.

Our work has mainly focused on the identification of B cell predictive biomarkers of good influenza vaccine responses. As stated previously, the focus of most studies published so far has not been to predict vaccine responses from gene expression in samples taken prior to vaccination but to dissect distinct sets of immunological events in vaccine recipients that correlate with the longer-term outcomes of vaccination.

We identified baseline B cell biomarkers of response and characterized the inflammatory phenotype of our cohort of young and elderly individuals, measuring multiple inflammatory markers in blood (TNF- α , IL-6, CRP, CMV, leptin, adiponectin, lipopolysaccharide). We measured the influenza vaccine response by HAI before (t0) and after (t7, t28, t120, t180) vaccination. We isolated B cells from the peripheral blood using magnetic beads and measured AID, a marker of optimal B cell function, in CpG-stimulated B cells isolated from young and elderly individuals at t0. We found CpG-induced AID before vaccination to be a good B cell biomarker that is reduced in B cells from elderly individuals when compared to young individuals, and to be significantly correlated with *in vivo* antibody response to the vaccine.^{42,43,55} For this reason, we proposed AID as good predictive biomarkers for response to vaccines and infectious agents in humans.^{42,43} Levels of the transcription factor E47 in CpG-stimulated B cells were also correlated with *in vivo* response to the vaccine.⁵⁵

More recently, we showed that AID correlates with high-affinity antibodies specific for the pandemic (p)H1N1 influenza vaccine.⁵⁶ The role of AID in CSR has been well characterized,^{80,81} but its role in SHM and polyclonal antibody affinity maturation has been associated only with genetic deficiency in mice⁸⁰ and humans⁸² or weakly associated.^{83,84} Antibody affinity maturation is a key aspect of an effective response to vaccination and has a significant impact on clinical outcomes following exposure to infectious agents. Our results showed that AID mRNA expression before vaccination in stimulated B cells, as well as the fold-increase of AID mRNA expression after vaccination, directly correlates with the increase in polyclonal antibody affinity to the HA1 globular domain of pH1N1, which is the domain most associated with protection against infection.⁵⁶ In young individuals, significant maturation of affinity to the HA1 globular domain has been observed and has been associated with initial levels of and a fold-increase in AID after vaccination. In most of the

elderly individuals studied, high affinity to the HA1 domain was observed before vaccination, resulting in minimal change in antibody affinity and correlating with low AID induction. This likely reflected previous vaccination or infection with highly conserved pH1N1 determinants that generated an optimal memory response. These findings demonstrated for the first time a strong correlation between AID induction and *in vivo* antibody affinity maturation in humans.

Switched memory B cells (CD19+IgD–CD27+), which are also good B cell biomarkers, are reduced in elderly individuals^{48,55,85,86} when compared to young individuals and are significantly correlated with *in vivo* antibody response to the vaccine.^{42,43} Switched memory B cells are generated from naïve and/or IgM memory B cells that have undergone CSR.^{86–88} We showed that, in response to a new vaccine, the percentage of these cells increases after influenza vaccination in young subjects whereas there is little increase in elderly subjects. These results⁴² are of great importance because memory B cells can carry the history of the individual in terms of specific immune responses.⁴⁷ We know that some elderly subjects have higher t0 antibody titers, when compared to younger controls, because they have undergone prior immunizations with related influenza virus antigens; however, they do not show increased switch-memory B cells after vaccination as younger people show. Therefore, a target for future-generation vaccines is to potentiate this B cell subset in the elderly.

We also showed that the ability to generate a vaccine-specific antibody response is negatively correlated with levels of serum TNF- α .⁵² Human unstimulated B cells from elderly individuals produce higher levels of TNF- α than those from young individuals, and these higher levels positively correlate with serum TNF- α levels. B cell and serum TNF- α levels negatively correlate with B cell function as measured by AID. Although it has been shown that human B cells from young individuals can be directly activated via Toll-like receptors or anti-CD40 and BCR cross-linking to secrete TNF- α ,⁸⁹ our results were the first to show that aging increases TNF- α production by B cells before exogenous stimulation. In the elderly when compared to young individuals, only memory B cells—especially the terminally differentiated B cell subset CD19+IgD–CD27– (referred to as late memory/exhausted memory, tissuelike, or double-negative), which is increased in this population^{85,90} and is the most pro-inflammatory B cell subset—produce TNF- α and more.⁸⁵ These findings identify serum and cytoplasmic B cell TNF- α as other B cell-specific biomarkers, which can help to predict the quality of *in vivo* and *in vitro* B cell responses. Not only TNF- α but other markers of intrinsic B cell inflammation (i.e., microRNAs) have been negatively correlated with AID,⁹¹ suggesting that AID can be downregulated in B cells from elderly individuals by inflammation through distinct mediators. Moreover, the possibility of controlling inflammation represents a powerful tool for modulating and counteracting major age-related pathologies in order to target therapeutic interventions and improve the health status of the elderly population.

B. Age Effects on Signal Transduction Pathways in B Cells

Defects in cell activation and signal transduction occur with aging in B cells. However, most relevant studies that arrived at this finding were carried out using mouse, not human, B cells. Those from the 1990s showed that signal transduction pathways in B cells, leading to

phosphorylation of either tyrosine or serine/threonine residues, are modified during aging. In particular, reduced proliferation in response to BCR engagement was shown to induce decreased phosphorylation of both tyrosines and serines/threonines in young and aged B cells, but no aging effect on stimulation with phorbol esters or anti-CD40 was observed.⁹² Age-related impairments in mobilization of cytosolic protein kinase C enzymatic activity and in cytosolic Ca⁺⁺ concentration were also observed in stimulated B cells.⁹²

It was recently shown that the calcium/calmodulin-dependent protein kinase type IV (CaMKIV) is rapidly phosphorylated after *in vitro* stimulation of PBMCs from young individuals given the influenza vaccine.⁷³ Expression and phosphorylation levels at day 3 after vaccination were inversely correlated with antibody titers, suggesting a possible role for CaMKIV in the regulation of antibody responses to the influenza vaccine. CaMKIV belongs to the family of calcium/calmodulin-dependent protein kinases, which are integral to immune response, mediating Ca⁺⁺-dependent cell function and regulating inflammation through TNF- α production.^{93,94} Interestingly, CAMKIV is activated by AMP-activated protein kinase (AMPK), the energy-sensing enzyme and key metabolic regulator ubiquitously expressed in mammalian cells⁹⁵ that is spontaneously activated in proinflammatory B cells from elderly individuals and is responsible for the secretion of inflammatory mediators by aged B cells.⁸⁵

In our recently published study,⁸⁵ we sorted the major peripheral B cell subsets (naïve, IgM memory, switched memory, and late/exhausted memory) and determined their percentages in the peripheral blood as well as their level of immune activation by measuring basal levels of expression of multiple senescence-associated secretory phenotype (SASP) markers, such as proinflammatory cytokines (TNF- α /IL-6/IL-8), inflammatory micro-RNAs (miRs, miR-155/16/93), and cell cycle regulators (p16^{INK4}). We found that only memory B cells express SASP markers, especially the terminally differentiated B cell subset (CD19+IgD-CD27-), called late-memory/exhausted-memory, tissuelike, or double negative, which of all B cell subsets is the most pro-inflammatory. This subset has been reported to be increased in the blood of healthy elderly individuals^{85,90} and in patients with autoimmune⁹⁶⁻¹⁰¹ and infectious diseases,¹⁰²⁻¹⁰⁴ suggesting that, in a favorable inflammatory microenvironment, these cells may expand *in vivo* in the presence of autoantigens or pathogen-derived antigens, leading to the production of pathogenic (autoimmune) or protective antibodies, respectively.

Although late-memory (LM) B cells do not proliferate, they are metabolically and transcriptionally active and affect the microenvironment by secreting proinflammatory mediators that in turn sustain and propagate the inflammatory response and negatively regulate the function of other immune cells. The expression of SASP markers in LM B cells is associated with activation of NF- κ B due to spontaneous activation of AMPK, which leads to (spontaneous) p38MAPK activation by phosphorylation of Thr180/Tyr182 residues, whereas IgM memory B cells activate the p38MAPK (Y323) pathway upon 30 min of stimulation with CpG. This pathway leads to class switch and antibody production measured by levels of AID mRNA after stimulation with CpG. The spontaneous activation of AMPK, together with the expression of RNA for proinflammatory cytokines and miRs, as well as NF- κ B activation, makes LM cells refractory to further stimulation—for example, with CpG. Altogether our results suggest that senescence and signaling pathways sensing nutrients (i.e.

glucose) converge to regulate functional responses in these cells,⁸⁵ similar to terminally differentiated T^{105,106} and NK¹⁰⁷ cells. Changes in glucose levels in the extracellular milieu occur with age¹⁰⁸ and may be responsible for decreased function because B cells use glucose for proliferation and differentiation.¹⁰⁹ This has also been shown for T cells^{110–112} and macrophages.¹¹³ We believe that the signaling pathways leading to the spontaneous activation of AMPK in terminally differentiated immune cell subsets should be interrogated given that the detrimental effects of chronic AMPK activation may overshadow the beneficial systemic effects of treatments like metformin, which is now used in clinical trials to target aging and age-related diseases (e.g., the Treating Aging with Metformin (TAME) clinical trial).

The mammalian target of rapamycin (mTOR) pathway integrates multiple signals from nutrients, growth factors, and oxygen to regulate cell growth, proliferation, and survival.¹¹⁴ Inhibition of mTOR signaling has been shown to increase the longevity of yeast,¹¹⁵ *C. elegans*,¹¹⁶ *Drosophila*,¹¹⁷ and mice,¹¹⁸ and has been suggested to also have a role in humans. The effects of mTOR inhibition on influenza vaccine responses were recently evaluated using the mTOR inhibitor RAD001 (an analog of rapamycin) with the purpose of increasing influenza vaccination response.¹¹⁹ Results showed enhanced response and broadened serologic response in elderly volunteers to influenza vaccination through increased antibody titers to heterologous strains of influenza not contained in the seasonal influenza vaccine. Although the underlying mechanisms require further elucidation, the results from this study convincingly indicate an increased serologic response to heterologous influenza strains that may confer enhanced protection against influenza illness.

IV. CONCLUDING REMARKS

Aging increases susceptibility to infectious diseases. For this reason, the prevention of infections is one of the necessary steps toward healthy aging. Vaccines are more effective in fighting influenza infection in the young than in elderly individuals, who represent most seasonal influenza-related deaths. A thorough analysis of the effects of aging on immune reactivity is necessary in order to design tailored vaccines and therapeutic strategies that can be maximally effective. Recent advances in technology have made possible a broad approach to study simultaneously multiple immune cells responding to vaccination.

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ABBREVIATIONS

AID	activation-induced cytidine deaminase
AMPK	AMP-activated protein kinase
APC	antigen-presenting cell
BCR	B cell receptor

CaMKIV	calcium/calmodulin-dependent protein kinase type IV
CMV	cytomegalovirus
CSR	class switch recombination
DC	dendritic cell
DN	double negative
HAI	hemagglutination inhibition assay
LM	late memory/exhausted memory
miR	micro-RNA
mDC	myeloid DC
mTOR	mammalian target of rapamycin
PBMC	peripheral blood mononuclear cell
pH1N1	pandemic (p)H1N1
pDC	plasmacytoid DC
qPCR	quantitative PCR
SASP	senescence-associated secretory phenotype
SHM	somatic hypermutation
TCR	T cell receptor
TLR	Toll-like receptor
T_{MNP}	memory T cell with naïve phenotype

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TABLE 1

Aging significantly reduces the number of individuals with a responding phenotype in different measures.

Markers [*]	Number of individuals with positive responses (%)	
	Young	Elderly
HAI	21/33 (64)	6/26 (23)
CpG-induced AID [†]	27/33 (82)	2/26 (8)
Vaccine-induced AID [†]	27/33 (82)	5/26 (19)
E47 [†]	31/33 (94)	2/26 (8)
swIg percentages [‡]	17/33 (52)	0/26 (0)
LM percentages [‡]	31/33 (94)	3/26 (12)
TNF- α [†]	20/33 (61)	5/26 (19)
miR-155 [†]	24/33 (73)	0/26 (0)
miR-16 [†]	24/33 (73)	0/26 (0)

Note: Results are from 3 consecutive influenza vaccine seasons (2011–2014). Young: 33 individuals; elderly: 26 individuals, all healthy.

^{*} The 9 markers identifying a responding phenotype are the following: fold-increase in the reciprocal of the HAI titers ≥ 4 ; qPCR of CpG-induced AID mRNA expression at t0 ≥ 0.1 ; fold-increase in qPCR values of vaccine-induced AID mRNA expression at t0 ≥ 2 ; qPCR of CpG-induced E47 mRNA expression at t0 ≥ 0.05 ; percentage of switched memory B cells (swIg, CD19+IgD–CD27+) at t0 ≥ 2 ; percentages of late-memory B cells (LM, CD19+IgD–CD27–) at t0 ≥ 3 ; qPCR of RNA expression of TNF- α ≥ 4 , miR-155 ≥ 0.05 , miR-16 ≥ 0.1 at t0.

[†] qPCR results are $2^{-\text{Ct}}$ Ct values of sample RNAs (AID, E47, TNF- α , miR-155, miR-16) normalized to GAPDH.

[‡] Results are percentages of peripheral blood CD19+ cells.