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Review

Immunosenescence and Challenges of Vaccination against Influenza in the Aging Population

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ABSTRACT: Influenza is an important contributor to morbidity and mortality worldwide. Accumulation of genetic mutations termed antigenic drift, allows influenza viruses to inflict yearly epidemics that may result in 250,000 to 500,000 deaths annually. Over 90% of influenza-related deaths occur in the older adult population. This is at least in part a result of increasing dysregulation of the immune system with age, termed immunosenescence. This dysregulation results in reduced capacity to cope with infections and decreased responsiveness to vaccination. The older adult population is in dire need of improved vaccines capable of eliciting protective responses in the face of a waning immune system. This review focuses on the status of immunity, responses to influenza vaccination, and strategies that are currently being explored to elicit enhanced immune responses in this high risk population.

Key words: Aging; Influenza; Vaccination; Immune Response; Immunosenescence

Annual influenza epidemics are the leading cause of severe virus-induced respiratory disease in the older adult population [1,2]. Influenza epidemics cause three to five million cases of severe illness, and may lead to 250,000 to 500,000 deaths annually [3]. Influenza-associated morbidity and mortality disproportionately affects older adults; defined here as adults age 65 and older. Even in a population with a high rate of influenza vaccination, influenza infects 2–5% of older adults, resulting in 10- to 30-times more hospitalizations annually compared to younger individuals [1,2,4,5]. The average length of a hospital stay due to pneumonia and influenza increases from 5.8 days for those between the ages of 5 and 49, to over 8 days for those older than 65 [4]. The influenza-associated mortality rate for individuals 5–49 years of age is 0.2 deaths per 100,000 person-years, compared with 1.3 deaths for those aged 50–64 years [1]. The influenza-associated mortality rate for individuals 65 years and older is 22.1 deaths per 100,000 [1]. Influenza impacts the frail older adult population even more severely. Individuals older than 85 years of age are 16-times more likely to die of influenza-related illness and 32-times more likely to die of influenza-associated pneumonia than those between 65 and 69 years of age [1].

Effectiveness of Influenza Vaccination in Older Adults

Vaccine Efficacy in Older Adults

Vaccination remains the most cost-effective method currently available for reducing the morbidity and mortality associated with influenza infection. Several studies have been conducted which demonstrated the ability of influenza vaccines to stimulate significant immune responses in older adults compared to placebo [6,7] However, these studies only evaluated immune parameters, while protection from disease was not evaluated. During the 1991-1992 influenza season, Govaert et al. conducted the only large, published, randomized, placebo-controlled trial of influenza

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vaccines in older adults, examining vaccine effects on clinical outcomes [8]. This study estimated influenza vaccine efficacy to be 58% for the prevention of clinical influenza with serologic evidence of infection in relatively healthy, adults aged 60 years and older. This study also suggested that subjects who had received the influenza vaccine in previous years were better protected than first-time vaccinees [9]. However, placebo-controlled influenza vaccine trials in the aged population are no longer considered ethical, because the United States and other countries have recommended that all persons aged 65 years and older receive annual influenza vaccination. These recommendations have limited current evaluations of influenza vaccine effectiveness in older adults to observational studies that compare the relative reduction in influenza disease outcomes in those who elect to be vaccinated compared to those who are not vaccinated. This reliance on observational studies, all of which are subject to various forms of bias, has led to considerable controversy over the effectiveness of influenza vaccination in older adults. Differences in the analysis methodology, a limited number of subjects, and variability in influenza attack rates from season to season have resulted in variable and often conflicting conclusions [10-12]. While some studies claim a definite benefit from influenza vaccination of older adults, others question the benefit of vaccination, and point to a 'healthy vaccine bias' that may confound the results of many large analyses [13-15].

In an effort to overcome these potential problems, several studies have been performed with large numbers of subjects across multiple influenza seasons, aimed at assessing the effectiveness of influenza vaccination in older adults. Nichol et al. evaluated data from subjects aged 65 or over, across 10 influenza seasons from 1990 to 2000 [16]. This study pooled data from community-dwelling individuals from 18 cohorts across the United States. Influenza vaccination, in this study, was associated with a 27% reduction in the risk of hospitalization due to influenza, and a 48% reduction in the risk of death [16]. A similar study by the same group examined three large managed care organizations across two influenza seasons (1998-2000) resulting in similar estimates [17]. However, concern has been raised about the likelihood of a healthy vaccinee bias resulting in an overestimation of influenza vaccine effectiveness in older adults in large cohort studies of this type [13,14]. Recent analyses designed to control for unmeasured health factors potentially associated with both vaccination status and risk of mortality have recently been developed [18,19]. More studies of this type are needed to better differentiate vaccine effects for confounding, especially the healthy vaccinee bias, particularly in studies with a mortality outcome.

While considerable controversy exists over the benefit to influenza vaccination in older adults compared to unvaccinated, it is generally agreed that vaccine responses in older adults are significantly reduced compared to young, healthy adults. In young, healthy adults, influenza vaccine effectiveness have ranged from 47% to as high as 86% effective in reducing laboratory-confirmed illness, depending upon antigenic similarity between the vaccine and the circulating influenza strains [20-23]. In older adults, influenza vaccines have been estimated to be 17% to 53% effective at reducing pneumonia and influenza hospitalizations; however, these observational studies did not use laboratory-confirmed influenza illness as a criterion [16,24,25].

A quantitative review of studies by Goodwin et al. evaluated the antibody responses to influenza vaccination in older adults [24]. Of 4,492 vaccinated subjects, 42%, 51% and 35% of subjects seroconverted (defined as a 4-fold increase in antibody titers) to H1N1, H3N2 and influenza B, respectively, compared with 60%, 62% and 58%, of younger subjects (913 subjects), respectively. Responses in subjects ≥75 years of age were especially impaired with seroconversion (a 4-fold or greater increase in serum HI antibody titer) rates of 32%, 46% and 29%, respectively. Seroprotection (defined as hemagglutination inhibition (HI) titers ≥ 40) in older adults (4,643 subjects) occurred in 69%, 74%, and 67% to H1N1, H3N2 and influenza B, respectively, compared to 83%, 84%, and 78% of younger subjects (1,151 subjects). Seroprotection in subjects age 75 or older was demonstrated in 65%, 68%, and 71% of subjects to H1N1, H3N2 and influenza B, respectively [24]. It should be noted; however, that while the so-called seroprotective titer (HI titer ≥ 40) is a widely accepted immune correlate of protection, this standard was established in younger adults [26-29]. While both the HI titer of ≥ 40 and seroconversion parameters are commonly applied to influenza studies performed in older adults to evaluate immunogenicity, there is little evidence to suggest that achievements of HI titers ≥ 40 correlate with protection in this age group. On the contrary, there is evidence to suggest that antibody titers are poor correlates of protection in the aged; T cell responses appear to correlate better with immune protection to influenza in older adults [30,31]. Further studies examining immune correlates in the older population are greatly needed for influenza research in this age group.

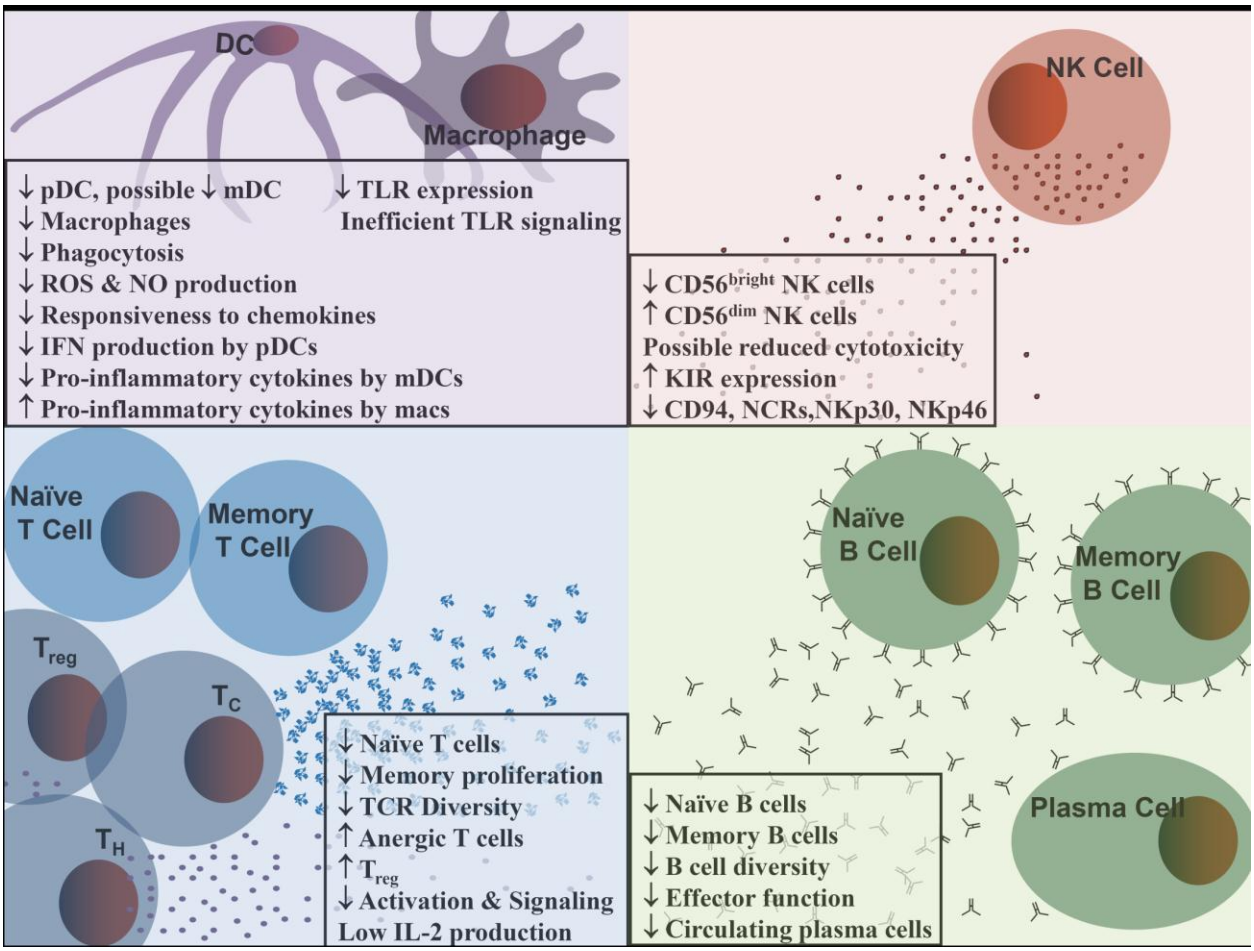

Figure 1. Immunosenesence of the aging immune system. The adult immune system becomes less responsive to vaccination, and less able to cope with infectious disease with age. Multiple arms of the immune system have been shown to develop age-related defects resulting in the loss or decreased effectiveness of key protective functions. Development of improved influenza vaccines for older adults must overcome these age related defects to improve protection of this high risk population.

Immunity in Aging

The impaired ability of older adults to develop effective immunity in response to influenza vaccines has been attributed to immunosenescence, defined as a decline in the body's ability to fight infection, mount adequate protective immune responses, and develop immunological memory for future protection [32,33]. This age-related immunological dysfunction is summarized in Figure 1, and discussed in detail below.

Immunosenescence in Innate Immunity

The innate immune system, the first line of defense against microbial pathogens, is mediated by a diverse group of cell types composed primarily of neutrophils, natural killer (NK), natural killer T cells (NKT), monocytes/macrophages and dendritic cells (DCs). DCs are the most potent, professional antigen presenting cells and play a central, unique role in bridging innate and adaptive immunity in response to pathogen invasion [34,35]. DC progenitors in the bone marrow give rise to circulating precursors that home to the periphery where they reside as immature DCs. Upon stimulation with microbial products, DCs mature and migrate to the draining lymph nodes to stimulate antigen-specific T cells [35,36].

Defects in Dendritic Cell Populations

DCs in humans are heterogeneous and are subdivided into two major categories, conventional or myeloid DCs

(mDC) and plasmacytoid DCs (pDC). mDCs play a crucial role in antigen presentation but show rather limited capacities in type I interferon (IFN) production. pDCs can produce large amounts of type I IFNs and are thought to be the major source of these cytokines *in vivo* [37,38]. Although the number and phenotype of DCs in aged mice seems preserved [39,40], studies in humans are not conclusive. DC number & phenotype were shown to be comparable between the young and the aged in some studies [41,42]. However, Della Bella et al. and Panda et al. demonstrated that the number of mDCs in human blood progressively declines with age, and mDCs appeared to have a more mature phenotype and impaired ability to produce pro-inflammatory cytokines (TNF-α, IL-6 and IL-12) upon Toll-like receptor (TLR) engagement [43,44]. In addition, mDCs were severely depleted in frail older subjects [41]. Most studies showed that overall pDC numbers in peripheral blood were lower in healthy older adults and the amount of IFN generated by pDCs was decreased in response to virus infection [41,45]. Because of the importance of pDCs for antiviral responses, the age-related changes in pDCs likely contribute to the impaired immune response to viral infections in older persons, especially when combined with the mDC dysfunction occurring in those with compromised health.

In terms of antigen uptake and migration, DCs from older individuals were shown to display significantly reduced capacity to phagocytose antigens via macropinocytosis and endocytosis in contrast to DCs from the young. In the same study, impaired capacity to migrate *in vitro* in response to chemokines was observed in DCs from older subjects [42]. DCs differ from other APCs in that they have the unique capacity to prime naïve T cells, which is critical for mounting an effective response against novel antigens. Earlier studies in aged mice demonstrated a decreased ability to prime a robust T cell response against an infectious agent [39,46]. In humans, DCs generated from peripheral blood of older adults were equally effective as those from younger adults in inducing the proliferation of T cell clones after antigenic stimulation [47,48]. However, the DCs were generated *in vitro* with cytokines that may have overcome age-related defects that are observed *in vivo* in older adults. Although the role of DCs in response to foreign antigens seems controversial, DCs from aged subjects display impaired capacity to phagocytose self antigens in the form of apoptotic cells [49,50]. This would result in the accumulation of apoptotic cells leading to secondary necrosis and release of self-antigens such as DNA. DCs from older adults were shown to display increased reactivity to intracellular human DNA. These DNA-primed DCs from older adults enhanced T cell proliferation compared to younger adults [51,52]. An impaired uptake of apoptotic cells and increased reactivity to intracellular human DNA by DCs from older adults may result in both increased inflammation and autoimmunity commonly associated with aging.

Defects in Macrophages

Macrophages function as 'pathogen sensors' and play an important role in the phagocytosis of antigen, microorganisms and cellular debris, and elimination of invading microorganisms and tumors [53]. A significant decrease in the number of macrophages in older adults has been described [54]. While macrophage precursors, monocytes, are found in the peripheral blood. Macrophages are found primarily in tissues. This has made studies of human macrophages difficult, and restricted studies primarily to human alveolar macrophages which can be isolated more readily than those from other sites. As a result, many of the studies examining the effect of aging on macrophage function have been done in animal models, such as mice.

Phagocytosis constitutes the first line of immune defense against pathogenic bacteria that have penetrated the epithelial barrier. An age-related decline in particle internalization, reactive oxygen species (ROS) and nitric oxide (NO) production were observed in murine macrophages from aged animals which weaken defense mechanisms to clear infectious agents [55-59].

Activated macrophages secrete pro-inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6 and metalloproteinases to initiate inflammatory responses that recruit neutrophils and natural killer cells. *In vitro*, macrophages from the alveoli and spleen of older mice generally produced more cytokines compared to younger counterparts. Conversely, macrophages from the peritonea of older mice generally produced less cytokine *in vitro* compared to younger counterparts [60,61]. Macrophages play an important role during the inflammatory phase of wound healing. They keep the wound free from infection and promote angiogenesis [62]. The alterations in chemokine secretion, and a concurrent decline in wound macrophage phagocytic function may contribute to the delayed repair response of aging [63]. In addition, defects in secretion of vascular endothelial growth factor and expression of cell adhesion molecules are also thought to contribute to the delay in wound healing in the aged [64]. However, not all inflammatory mediators are produced in reduced amounts with aging, some of which may actually increase with age. Macrophages from old mice have significantly higher levels of PGE2 production compared with those from young mice [65,66]. PGE2 plays a critical role in the age-associated dysregulation of the

immune and inflammatory responses. In particular, several studies have shown that increased PGE2 production in macrophages from old mice contributes to the suppression of T cell function with aging [66]. In summary, the effects of advancing age have been reported on the broad range of functional capabilities of macrophages. However, the reported impact of aging on macrophage function varies from study to study due to differences in experimental conditions assessing their function and the underlying medical conditions of the population evaluated.

Defects in Pattern Recognition Receptors with Age

As main cellular components of innate immunity, DCs and macrophages recognize molecules shared by groups of related microbes that are essential for the survival of those organisms and are not found associated with mammalian cells. These unique microbial molecules are called pathogen-associated molecular patterns or PAMPs. These PAMPs are sensed by the host's germline encoded pattern recognition receptors (PRRs) expressed on DCs and macrophages. Toll-Like Receptors are the most widely studied PRRs and are considered to be the primary sensors of pathogens. Eleven human TLRs and 13 murine TLRs have been described to recognize specific molecular patterns present on the surface of pathogens [67]. Toll-Like Receptors are evolutionarily conserved molecules and the expression of TLRs varies in different cell subsets: the expression of TLR3 on primary human macrophages from older individuals was lower than macrophages from young individuals [68]. Decreased expression of TLR3 and TLR8 in mDC and decreased expression of TLR7 in pDC was observed [44].

After recognition of microbial pathogens, TLRs trigger intracellular signaling pathways that result in the induction of inflammatory cytokines, type I IFN and chemokines. Panda et al. found substantial decreases in older compared with young individuals in TNF-α, IL-6, and/or IL-12 (p40) production in mDCs, and in TNF-α and IFN-α production in pDCs in response to TLR engagement [44,69]. These results support the concept that increased susceptibility to infections and poor adaptive immune responses in aging may be due to the decline in TLR expression and function [70-72]. Upon ligation, TLR signaling activates a cascade of intermediates including myeloid differentiation factor-88 (MyD88), IL-1 receptor-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF6) leading to activation of nuclear factor (NF)-κB and activating protein-1 (AP-1) [73-75]. Both basal and downstream signaling components, such as the adaptor molecule MyD88, TRAF6 and several members of the NF-κB pathway, such as c-Rel, p65, NF-κB p50 and p52 were reduced in the aged suggesting that the TLR-dependent pathway is working at a significantly reduced efficiency [76]. Qian et al. observed no significant age-related difference in expression or nuclear translocation of signaling molecules in initial antiviral responses. But they showed that DCs from older donors have diminished induction of late-phase responses (eg, STAT1, IRF7, and IRF1), suggesting defective regulation of type I IFN [77].

In specific TLR 4 signaling, LPS stimulation results in endothelial activation through a receptor complex consisting of TLR4, CD14 and MD2. Serum levels of LPS Binding Protein are unaltered in aged mice or humans. However, the expression of CD14 as well as MD2 on macrophages is reduced in mice [78]. Excessive immune responses are detrimental to the host and negative feedback regulation is crucial for the maintenance of immune-system integrity. TLR signaling during subsequent or continuous exposure induces the expression of IL-1 receptor-associated kinase-M and suppressor of cytokine signaling as negative regulators. Ageing increases SOCS-3 expression in rat hypothalamus and human muscle tissue, and SOCS1 and SOCS3 in human neutrophils [79-81]. In addition, increased levels of IRAK-M mRNA as well as protein have been observed with increased age in mice after LPS stimulation [82]. Therefore, in older adults, dysfunction of TLR signaling could arise from either decreased activation of TLR signaling adaptor molecules and/or increased activity of negative regulators of TLR function.

Defects in NK Cells

NK cells play an essential role in the innate immune defense against tumors and viral infections. They mediate MHC-independent cytotoxicity through perforin and granzyme B, and regulate adaptive immune responses by the production of chemokines and cytokines for recruitment and activation of T and B cells. Two distinct populations of NK cells can be identified by the density of surface CD56 molecules. While $CD56^{dim}$ NK cells mediate cytotoxicity of virus-infected cells, the low density $CD56^{bright}$ subpopulation appears to be the primary source of NK cell-derived immunoregulatory cytokines such as IFN-γ, TNF-α, GM-CSF, IL-10 and IL-13 [83,84]. Although there is a reduction in $CD56^{bright}$ NK cells with aging [85,86], an expansion of $CD56^{dim}$ NK cells results in an increase in the absolute number [85-87].

Age-related changes in NK cell functionality are controversial. Several studies have demonstrated diminished, unaffected or even enhanced activity of NK

cells from older adults [86-90]. Reduced cytotoxicity of NK cells based on diminished perforin expression was observed in older adults [87,88], although a more recent study revealed a preserved cytotoxic ability of NK cells from childhood through old age [86]. Interferon-γ production upon NK cell activation was previously considered to be reduced in older adults [90], but was recently shown to be significantly higher in the aged, particularly in subjects older than 85 years of age compared to younger older adults [89]. Altered chemokine (RANTES, MIP-1a, IL-8) production [87] and suppressed trafficking of matured NK cells into draining lymph nodes [91] have also been associated with ageing.

Several potential mechanisms have been observed to account for the functional decline of NK cells in older adults. Reduced cytotoxicity may be a consequence of impaired zinc [87,92] or calcium [93] homeostasis in NK cells from older adults. An increased expression of Ly49-receptors downregulates NK activation [94], and diminishes sensitivity of aged NK cells to stimulatory effects of IL-2 [90] and IFN-α [95]. Moreover, several studies [86,96,97] demonstrated significant age-related changes in expression of NK cell receptors that inhibit or activate NK cells to lyse target cells, including killer cell immunoglobulin-like receptors (KIRs), natural cytotoxicity receptors (NCRs) and C-type lectins. The increased expression of KIR [96] particularly on the $CD56^{bright}$ subset [86], along with the decreased expression of C-type lectin CD94 [97] and activating NCRs, NKp30 and NKp46 in both CD56 subsets [86] were observed in older adults compared to younger adults.

Defects in T cells with Aging

Aging affects all stages of T cell responses including generation, maturation, differentiation, activation and functionality of both CD4 and CD8 T cells. Progressive accumulation of these defects leads to several key changes in T cell immunity: decreased production of naïve T cells, increased proportion of memory cells, decreased activation and T cell signaling, and decreased proliferation and antigen-specific response. Beginning with the most crucial event – depletion of naïve T cells due to thymic involution – all subsequent changes are strongly interconnected revealing imbalanced T cell responses with altered survival and activity of immature and mature T cells.

Thymic Involution and T cell Repertoire

The size of the naïve T cell pool is significantly decreased in aging. Naïve T cells are generated from bone marrow precursors and develop into mature T cells upon migration to the thymus. The age-associated involution of the thymus begins in the initial years of life and continues into almost complete adipose tissue replacement by age 70 [98,99]. Thymic atrophy involves considerable contraction of thymic epithelial space and substantial increase in perivascular space that contains low levels of recruited peripheral T and B cells and is not directly involved in thymopoiesis [100]. Another factor contributing to naïve T cell pool depletion in older adults is a lack of IL-7 production. IL-7 plays a key role in survival of both preselected and matured T cells, including memory cells that express the IL-7 receptor. Treatment with IL-7 reversed thymic atrophy and increased thymic output in aged mice [101,102], and increased the number of naïve CD4 and CD8 T cells in aged rhesus macaques [103]. In a small human study involving volunteers under 60 years old, recombinant IL-7 slightly enhanced CD45RA+ and diminished CD45RO+ levels in CD4 and CD8 T cells, indicating expansion of the naïve T cell pool [104]. Decreased production of IL-7 was associated with observed thymic involution both in animal studies [105], and aged humans [106].

In addition to limited development and survival, naïve T cells in older adults appear to be more susceptible to apoptosis. Decreased expression of bcl-2 molecules in naïve CD4 and CD8 T cells along with increased activation of caspase 8 and 3 was observed in aged humans indicating that aged naive T cells maintain both apoptotic pathways [107-109]. The age-related decrease in the ability of naïve T cells to survive and renew, results in a significant loss in the ability to mount primary responses to novel antigens [110,111].

The age-associated reduction in naïve T cells in older adults is partially compensated by an increased memory T cell pool that provides cross reactive responses to new antigens. This age associated increased memory T cell pool is not simply a proportional increase as the naïve T cell pool wanes due to reduced thymic output. Several animal studies reported an increased memory phenotype even in newly developed CD4 T cells [112,113]. Bone marrow cells from young mice were transferred to both young and aged animals. Peripheral CD4 T cells reconstituted in aged mice revealed phenotype and lymphokine profiles similar to those from the control aged mice. However, these are distinctly different from those in the young mice. These data suggest that the aged microenvironment is defective and influences the maturation of newly produced CD4 T cells. Besides antigen-independent accumulation of memory T cells, clinical data indicate that chronic viral stimulation in the older adults leads to expansion of CD4 and CD8 T cells

with a typical memory phenotype [114,115]. However, memory T cells from older adults have decreased differentiation and proliferative ability [116] and demonstrate low CD28 and CD27 expression reflecting their poor activation [116].

Poor adaptive immune response in aging is also related to narrowing of the T cell repertoire by decreased TCR diversity and increased levels of anergic CD28- cells. Reduced TCR diversity reveals the inability of naïve T cells to differentiate efficiently into new effector T cells with high specificity for antigen. Animal studies and *in vitro* analysis of isolated human CD4 and CD8 T cells indicate significantly decreased TCR beta-chain diversity [117-119]. In individuals over 70 years old, CD4 T cell repertoire decreased 100 times compared with young adult controls [117]. Another factor that narrows T cell repertoire is progressive accumulation of cells lacking the costimulatory molecule, CD28, which occupy available immunological space. CD28-negative T cells are anergic to antigen stimulation [120] and resistant to apoptosis [121], allowing them to expand considerably *in vivo* [120,122,123]. Significant expansion of CD28null T cells was observed upon chronic stimulation with viral [115,122-124], auto- [125] and tumor-associated antigens [126,127]. In addition CD8+CD28- cells seem to retain cytokine production that disrupt APC function, suppress T cell responses, and disturb the Th1:Th2 balance [128,129].

Regulatory T Cells

Regulatory T cells (Treg) expressing a CD4+CD25+ phenotype along with effector/memory markers are believed to suppress redundant T cell immune responses, preventing autoimmune diseases and immune pathology following excessive immune reactivity [130]. Although the data for Treg functional activity in older adults is controversial [131-133], a significant increase in the number of Treg cells with age is well established [131,133,134]. Resistant to apoptosis, and independent of IL-7 signaling [135,136], Tregs seem to suppress CD4 and CD8 T cell functionality with aging [136,137] by impairment of costimulatory CD40 and CD86 molecule expression on DCs, thereby diminishing the ability of APCs to form stable contacts with responding T cells [138]. Several studies show a correlation between increased levels of Tregs in aged mice and older adults, and low levels of effector/memory T cells to viral antigens [139-142], suggesting that age-associated defects in the regulatory T cell compartment affects the immune control of acute and persistent viral infections.

Decreased T Cell Activation and Signaling

Although the T cell compartment appears to accumulate effector/memory CD4 and CD8 T cells with age, the ability of those cells to respond to antigenic stimulation, achieve activation and eventually respond is significantly decreased with aging. Several factors are considered to be involved in this process: (i) the lack of costimulatory molecules; (ii) low efficiency of receptor synapse formation between the T cell receptor and antigen-MHC complex on the surface of antigen-presenting cells; (iii) weak activation of intracellular signaling; and (iv) low expression of surface activation/differentiation markers. T cells from older individuals lose the costimulatory molecule CD28, becoming anergic to antigen stimulation and thus resulting in a weak immune response. A significant proportion of CD4 and CD8 T cells expressing a CD28null phenotype was observed in subjects over 65 years old vaccinated with influenza trivalent vaccine, and only 17% of vaccine recipients demonstrated increased antibody titers to all three vaccine components, while almost half of the vaccinees failed to respond at all [143]. The whole synaptic complex of TCR and antigen-MHC presented by APCs becomes less efficient with aging [144-146]. Subsequent intracellular signal transduction is weak due to defects in signaling pathway gene expression [147], calcium and tyrosine kinase metabolism and alterations in plasma membrane lipids [144,145,148,149]. All these factors together provide poor T cell activation that is reflected in low expression of activation/differentiation markers.

Decreased T Cell Functionality

Defects in the functionality of aged naïve and memory T cells reveal suboptimal effector responses. The decreased proliferation of peripheral CD4 and CD8 T cells isolated from older adults to mitogens and antigens is well established [150,151]. Altered functionality of the effector/memory compartment has been associated with low IL-2 production [148,151]. While there has been an overall cytokine imbalance demonstrated in mice with polarization towards Th2, it was not very clear in humans [129,152-159]. Furthermore, antigen-experienced T cells from aged mice exhibit decreased trafficking into sites of inflammation [160,161], and reduced helper or cytolytic activity [118,162-164]. Furthermore, young mice that received naïve CD4 cells from aged donors have dramatically decreased numbers of antigen-specific B cells after immunization, suggesting reduced help from CD4 T cells from the older animals [162]. Clinical trials of influenza vaccines show substantially lower Granzyme B expression by effector CD8 T cells in vaccine recipients over 65 years old [163,164]. Besides altered functionality, memory T cells in older adults also seem to be more susceptible to

apoptosis, presenting decreased expression of bcl-2 and increased Fas-mediated activation of caspase 8 and 3 [165,166]. In addition, diminished T cell responses with aging are partially a result of age-associated changes in antigen presenting cells. Aged mice demonstrated limited expansion of influenza-specific CD8 T cells transferred from young mice [167], but the ability of CD8 cells to proliferate and produce IFN-γ significantly increased after co-transfer of DCs from young donors [168].

Immunosenescence in NK T Cells

NK T cells represent a heterogeneous population expressing T cell markers such as CD3, CD4, CD8 and CD1d restricted TCR (Vα24/Vβ11 in humans) as well as NK-cell markers such as CD56, CD94 and KIR [169]. While an age-associated decrease in NK T cell numbers was observed in mice [170,171], ageing in humans does not seem to affect NK T cell levels [86,172], although a significant decrease in expression of CD94 in aged human NKT cells was noticed [86,96]. In contrast to T lymphocytes maturating in the thymus, NK T cells may migrate directly from the bone marrow to the liver. The ability to maintain extrathymic development is considered to be a compensatory mechanism for thymus involution during ageing [173]. However, studies with viral infections in aged mice identified the NK T cells as producers of high levels of IL-17 which was associated with increased inflammation and hepatic injury [174,175] suggesting that NK T cells have the potential to account for adverse infection-related outcomes in aging.

Aging in Humoral Immunity

The effect of aging on humoral immunity is characterized by quantitative and qualitative alterations in antibody responses. These render older adults increasingly susceptible to infectious diseases and prone to suboptimal responses to vaccination. Since most current vaccines are designed to generate a neutralizing antibody response against infectious pathogens such as influenza viruses, the senescence of humoral immunity directly influences vaccine immunogenicity and efficacy in the aged. While several players of the immune system intricately affect each other's function, defects in antigen presenting cell function, T cell number and function, namely diminished CD4 T cell help and increased T cell-mediated suppression play significant roles in reduced humoral immunity [99,160]. However, a number of studies point to intrinsic changes in B cells associated with aging, indicating senescence of humoral immunity itself. These changes include reductions in cellular composition, in B cell diversity, and in B cell effector function. In this section, we discuss findings of intrinsic alterations of B cells associated with aging. Although much of our knowledge on immunosenescence of humoral immunity derives from animal studies, there are some discrepancies between animal vs. human data [176]. Therefore, in this review, we focus on findings from human B cells.

Cellular Composition

Both the total number and percentage of mature B cells and antigen-specific B cells in the periphery alter with aging [177]. A number of studies have found that the absolute number and percentage of mature B cells including naïve and memory B cells in the periphery decrease compared to younger counterparts [178]. Especially, the reduction in overall human memory B cells as well as compromised recall memory responses is noted. Human memory B cells are composed of IgM memory and class-switched memory B cells, which include IgG, IgA and IgE memory B cells. IgM memory B cells play an important role in bacterial infection, such as pneumococcal infection [179]. In older adults, the frequency of antigen-specific IgM memory B cells was shown to be dramatically reduced, i.e. the pneumococcal bacteria-specific IgM response was reduced following vaccination [180]. A reduction in IgG responses and alteration in IgG subclass composition in response to influenza vaccination has also been reported [181]. In contrast to a dramatic reduction in mature B cells in the periphery, B lymphopoiesis in bone marrow stays relatively active and the percentage and numbers of B cell precursors decline only moderately with aging [182,183]. This pattern may reflect age-associated changes in the microenvironment that supports the survival of mature B cells in the periphery. B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are TNF ligand superfamily proteins that are critical survival factors for mature B cells in the periphery [184,185]. It has been reported that in healthy older adults, the blood plasma levels of BAFF and APRIL are reduced [186]. Primary sources of BAFF and APRIL are monocytes, macrophages and dendritic cells [187,188], and aging is associated with the decline in the number and function of dendritic cells [41] as outlined above. Thus, these studies point out a global alteration in innate and adaptive immunity associated with aging.

B Cell Diversity

Changes in the B cell repertoire associated with aging involve an increase in the reactivity against autologous antigens and impaired responses against foreign antigens, expansion of oligoclonal populations, reduced

size and diversity of the B cell repertoire, and reduced antibody specificity and affinity [189-191]. A shift in the B cell repertoire from foreign to self-antigens accompanies selectively decreased activity of conventional B2 (CD5-) B cell subsets compared to autoreactive B1 (CD5+) B cell subsets, resulting in production of autoantibodies [192]. Reduced B cell diversity could be in part due to impaired B cell lymphopoiesis in the bone marrow and due to diminished diversification in the germinal center [191]. The diversity of the B cell repertoire can be measured by the diversity of mRNA coding for the 3rd complementarity-determining region (CDR3) of the immunoglobulin molecule [193]. This region is highly diverse both in sequence and length of the sequence and important for antigen binding. The diversity of the Ig heavy chain CDR3 region is due to the addition and deletion of nucleotides when variable (V_H), diversity (D), and joining (J_H) genes rearrange [194,195]. Mutations in this region are reduced in older adults compared to young adults [196] and the preference for certain V_H family genes in older individuals has been reported [197]. In healthy, young individuals, the mRNA CDR3 size diversity is represented by a bell-shaped profile of peaks. However, frail older individuals exhibit a distorted profile in their CDR3 spectratype from the normal distribution, indicating a collapse in B cell diversity [189]. Interestingly, the collapse in B cell diversity is associated with poor health status (frail vs. healthy) in this study cohort. Reduced B cell diversity also means oligoclonal expansions in the B cell repertoire [189]. Most of these oligoclonal B cells are derived from the CD5+ subset (B1) of B cells, which accounts for increased autoreactive antibodies in the aged [190,198].

B Cell Activation and Effector Function

Immunosenescence is also evident at the individual cell level in biochemical and signaling pathways. In older adults, the activity of protein tyrosine kinase (PTK) and the expression of protein kinase C (PKC) in response to B cell receptor engagement is reduced, thus perturbing downstream signaling events, such as antigen-induced proliferation [199]. Detailed molecular mechanisms behind the reduced antibody response associated with immunosenescence is mostly derived from murine studies [176]. In aged mice, the number and duration of germinal center formation is reduced [200]. Within the germinal center, molecular events including class switch recombination (CSR) and somatic hypermutation (SHM) in the IgH and IgV genes are critical in generating high affinity antibodies against antigen. In aged mice, reduced germinal center function accompanies reduced activity of activation-induced cytidine deaminase (AID), an enzyme necessary for CSR and SHM events, and reduced expression of E47 protein, a transcription factor regulating the gene encoding AID, and reduced stability of E47 mRNA [201-204]. However, corresponding, direct evidence concerning human germinal centers has been limited for obvious reasons. Recently, studies employing human peripheral blood B cells (from subjects 18-86 years of age) showed that expression of E47, AID and Igγ1 circle transcripts progressively decrease with age [205]. This, together with the reduction in the magnitude and duration of antibody responses to specific antigen, such as tetanus toxin, encephalitis viruses, Salmonella, or pneumonococcus indirectly indicates the diminished germinal center reaction of aged human B cells in lymphoid organs [206].

B Cell Differentiation

Progressive defects in mounting high-affinity antibody responses in older people is in part due to a decrease in the number and percentage of circulating plasma cells in the peripheral blood [207]. This decrease could be due to reduced survival niches that support plasma cells in the bone marrow, due to progressive replacement of hematopoietic bone marrow with fat cells, or reduced differentiation processes from mature B cells to plasma cells [208]. These changes could lead to a reduction in the number of antigen-specific plasma cells in peripheral blood as well as in bone marrow. The reduction in antigen-specific plasma cells, together with the oligoclonal expansion of plasma cells with cross-reactivity and low affinity to specific antigen, may account for reduction in the antibody responses to influenza vaccination in older adults [24].

Mechanisms for Improving Influenza Vaccine Efficacy in Older Adults

Older adults are at high risk for severe complications resulting from influenza infection, and are increasing rapidly as a proportion of the world population [209]. Adults over the age of 65 currently compose 8% of the world's population, but projections by the U.S. Census Bureau predict that proportion will more than double by 2050 [209]. In some regions of the world, the older adults are expected to grow to nearly 30% of the population. Growth of this high-risk population, as projected by the U.S. Census Bureau, is depicted in Figure 2. In the near future, there will be an increasing need for protection of this age group from influenza.

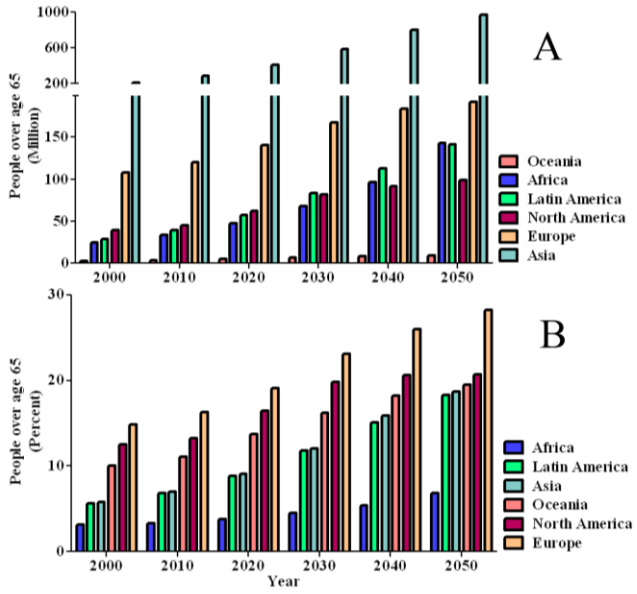

Figure 2. Projected increase in the aged population. Adults over the age of 65 are projected to increase worldwide over the next 40 years. The summary above, generated from population projections by the U.S. Census Bureau [209], predict that the older population within every region of the world will drastically increase in (A) number, and (B) will make up a larger proportion of the overall population. This could have a significant impact on influenza spread and control as the proportion of high risk individuals dramatically increases.

Despite controversies that exist about the effectiveness of influenza vaccines in older adults, it is clear that improved vaccines need to be developed that enhance the immunogenicity of influenza vaccines for this age group [210,211]. These improved vaccines need to overcome the age-related decline in immune function and stimulate both humoral and cellular immunity. Several strategies in various stages of development have been proposed and tested, including the use of adjuvants, cytokines, immunostimulatory complexes, increased antigen doses, intradermal vaccine delivery, live-attenuated vaccines, and virosomal vaccines [210,212-216]. However, very few of these proposed strategies have demonstrated the level of safety and efficacy required for licensure (Fig. 3). Two adjuvanted influenza vaccines containing MF59 and AS03 marketed by Novartis Vaccines and GlaxoSmithKline (GSK), respectively, are licensed for use primarily in Europe for older adults [217,218]. Canada recently granted licensure for the Arepanrix™ pandemic H1N1 vaccine and Fluad® seasonal influenza vaccine containing AS03 and MF59, respectively [219,220]. These adjuvants are based on an oil-in-water formulation utilizing the biodegradable oil, squalene [217,218].

MF59, licensed in 1997, is the first adjuvant approved for the use in humans since alum [221]. Although the MF59-adjuvanted TIV vaccine has been marketed in Europe for older adults for over 13 years and has been shown to be safe and immunogenic [221-223], it is not yet approved for use in the US [224]. MF59 has been shown to significantly enhance the immunogenicity of influenza vaccines in older adults, resulting in higher antibody titers than conventional unadjuvanted vaccines [225-227]. Additionally, the antibody responses induced by MF59-adjuvanted vaccine demonstrate broader cross-reactivity with influenza strains not included in the vaccine, potentially offering improved protection in the event of vaccine mismatch with currently circulating strains [228,229]. In studies performed in mice and humans, MF59 has been shown to enhance the number of influenza specific CD4 T cells and memory B cells [230-232]. In clinical trials evaluating MF59 as an adjuvant for a H5N1 pre-pandemic vaccine, MF59 induced significant broadly reactive, H5-specific, CD4 T cells with a Th1 prone effector/memory phenotype after 1 injection [230]. A second vaccine dose stimulated H5N1-specific, IgG+ memory B cells, and microneutralization titers of ≥ 80. It should be noted that MF59's enhancement of cellular immunity has only been studied in subjects aged 18 to 64, and has yet to be evaluated in older subjects [233]. Fluad®, the MF59 adjuvanted seasonal influenza vaccine, as well as the H5N1 pre-pandemic mentioned above are subunit vaccines containing influenza hemagglutinin (HA) and neuraminidase (NA), but lacking influenza nucleoproteins, matrix proteins, and polymerases. As it has been shown that the majority of influenza T cell epitopes are derived from the nucleoproteins, matrix proteins, and polymerases, with minimal epitopes derived from HA and NA [234,235], the capacity of these vaccines to expand a broader repertoire of cross-reactive T cells may be narrower than that of a comparable split-virion vaccine. This may be especially true from the perspective of older adults,

whose T cell repertoire has been shown to be narrower than that of younger adults due to immunosenescence [117-119]. An evaluation of this vaccine's ability to expand cross-reactive T cells in older adults would give a more complete understanding of the breadth of this vaccine's effectiveness from the perspective of cellular as well as humoral immunity in the older population.

From studies primarily performed in mice, MF59 has been shown to enhance vaccine responses by increasing antigen uptake at the site of injection, stimulating the release of chemokines to attract additional immune cells, and stimulating localized inflammation at the injection site, enhancing responses [236-239]. MF59 has also been shown to induce the differentiation of monocytes into dendritic cells [237].

More recently, GSK Biologicals has developed its proprietary adjuvant system, AS03, to enhance responses to pre-pandemic vaccines. AS03 was initially used with an H5N1 split-virion pre-pandemic vaccine called Prepandrix™ [218]. In test subjects aged 18 to 60, subjects received 2 doses of a split virion vaccine, 21 days apart [240]. Subjects received 1 of 4 concentrations of antigen. All adjuvanted vaccines in this trial were significantly more immunogenic than their unadjuvanted counterparts as determined by HI and microneutralization titers, including the lowest antigen concentration of 3.8μg (25% the amount commonly used in conventional seasonal vaccines). Furthermore, the AS03 adjuvant was shown to stimulate cross-neutralizing antibody to related drift strains, as well as cross-reactive B and T cells [240-242]. Studies in subjects over the age of 61 showed significantly higher geometric mean HI titers than unadjuvanted vaccine, and antigen specific CD4 T cells [243]. Prepandrix™ is currently licensed to market in all members of the European Union [218]. With the emergence of H1N1 pandemic influenza in 2009, the monovalent H1N1 pandemic vaccine, Pandemrix™ was adjuvanted with AS03. In clinical trials evaluating the immunogenicity of the H1N1 pandemic vaccine, 85.7% of subjects older than 60 years of age demonstrated seroprotection (defined as an HI titer ≥ 1:40) after the first dose, and 87.8%, after a booster vaccination at 21 days [244]. An AS03-adjuvanted seasonal influenza vaccine for older adults is currently in development [224].

Vaccine Type	Adjuvant	Licensed Vaccine	Mechanism
Adjuvanted	MF59	Fluad® (seasonal) Focetria® (H1N1 pandemic) Celtura ® (H1N1 pandemic)	↑ Ag uptake and presentation [225-228] ↑Chemokines ↑Cellular recruitment Localized inflammation Promote differentiation of DCs & Macs
Adjuvanted	AS03	Prepandrix™ (H5N1 pre-pandemic) Pandemrix™ (H1N1 pandemic) Arepanrix ™ (H1N1 pandemic)	↑ Cytokines and chemokines [236] Granulocyte recruitment Localized inflammation ↑ Ag presentation (APC recruitment) ↑APC activation Promote recruitment to draining lymph nodes
High-dose	None	Fluzone® (seasonal)	Mechanism not known: Probable ↑ Ag uptake & presentation Possible localized inflammation
Virosomal	None	Inflexal® (seasonal)	Mechanism not known: Probable ↑ Ag uptake & presentation
Intradermal	None	Intanza® (seasonal) Fluzone Intradermal® (seasonal)	Mechanism not known: Probable ↑ Ag uptake & presentation

Figure 3. Licensed influenza vaccines eliciting improved immune responses in older adults. Conventional vaccines have been shown to elicit poor immune responses in older adults. Several novel mechanisms have resulted in influenza vaccines which elicit improved immune responses in this population, yet are considered safe enough by regulatory agencies to warrant licensure.

In studies performed in mice and humans, the AS03 adjuvant system has been shown to enhance immune responses by inducing localized inflammation at the injection site, increasing production of cytokines and chemokines, and recruiting granulocytes, macrophages and dendritic cells to the injection site and draining lymph nodes [245]. Antigen presenting cells displayed increased activation and increased antigen presentation [245].

Several studies have shown that increasing the vaccine dose from the standard 15 μg of HA per virus strain can enhance the level of protective antibodies [246-249]. In studies in older adults, vaccines were generated matching that year's seasonal vaccine strains (2001-2002, and 2004-2005 seasons), but containing 60 μg of HA for each viral strain rather than the conventional 15 μg. These high-dose vaccines generated significantly higher HI and neutralizing antibody titers than the conventional-dose vaccines [244,247]. While each of the vaccine doses was well tolerated, a dose related increase in injection site reactions was observed in the studies. A recent study by Chen et al., demonstrated similar findings using a 60 μg dose influenza vaccine [249]. While the high-dose vaccine did not increase responses to the level observed in younger volunteers, the number of complete non-responders in the older adult group was significantly reduced.

In December of 2009, the US Food and Drug Administration (FDA) granted a license to Sanofi Pasteur for Fluzone® High-Dose, a trivalent, inactivated influenza vaccine containing 60 μg of HA for each of the viral strains [250,251]. Approval for this vaccine was granted under an accelerated process based on favorable safety and efficacy data [250]. Under this accelerated process, the manufacturer is required to provide additional data evaluating vaccine effectiveness in preventing seasonal influenza [250]. The Advisory Committee on Immunization Practices (ACIP) reviewed data generated during the first year after licensure, acknowledging the vaccine's favorable immunogenicity and safety data. However, the committee has expressed no preference for Fluzone® High-Dose, nor for any other influenza vaccine, for preferred use in older adults [250,252].

The use of virus-like particles, called virosomes, as an alternative delivery vehicle has been proposed for some time. Virosomes are liposomes with the viral surface antigens on their surface, but with relevant T cell antigens inside rather than the viral genetic material. This creates a delivery system which mimics viral infection, without the pathology associated with live virus. Virosomes are able to present intact surface antigen to B cells, and are readily taken up by APCs for presentation to T cells. Upon uptake by APCs, the virosomes fuse with the endosomal membrane [253,254]. This fusion releases the internal antigens into the cytosol where they can be loaded onto MHC class I for presentation to T cytotoxic cells, while the surface antigens are processed within the endosome for presentation on MHC class II to T helper cells [253,255]. The first virosomal influenza vaccine, Inflexal® V marketed by Crucell N.V., was introduced in Switzerland in 1997, in Italy in 1998, and throughout the rest of Europe in 2001 [256]. Safety studies have demonstrated that Inflexal® V is safe and well tolerated by all age groups, including children, older adults, and immunocompromised patients [256-260]. However, studies in older adults examining the immunogenicity of virosomal vaccines have varied widely in their conclusions, ranging from greater responses to inferior responses compared with conventional vaccines [257,261-265].

The route of delivery has long been known to affect the immune response elicited by a vaccine. The most common route of influenza vaccine delivery is by intramuscular injection. This method does not require significant skill for delivery thereby making mass vaccination during influenza season possible. However, the muscle environment is not considered to be an efficient site for vaccination due to the low numbers of APCs [266]. In contrast, intradermal vaccination is considered a significantly more efficient route of delivery due to the high number of resident macrophages and dendritic cells [266-269]. Intradermal vaccine delivery; however, requires more skill, making intradermal vaccines less popular in the past. Recent advances in intradermal vaccine delivery systems, including patches, microneedle injection systems, and needle free systems have made this route of vaccine delivery more attractive and feasible for influenza vaccines [270-273]. Clinical trials in the elderly evaluating intradermal delivery of influenza vaccines using these new delivery systems compared to conventional intramuscular vaccines has shown equivalent or enhanced immunogenicity by the intradermal route [270,274-276]. The intradermal influenza vaccine, Intanza® produced by Sanofi Pasteur, was granted licensure by the European Medicines Agency for marketing throughout the European Union in February 2009, and was approved for marketing in Australia by the Therapeutic Goods Agency in March of 2009 [277]. Intanza® became available in Canada in 2010, and was recently licensed in May 2011 by the FDA for marketing in the U.S. under the name Fluzone Intradermal® [278,279]. However, this vaccine is only

approved for ages 18 to 59 years in Canada and Australia, and ages 18 to 64 years in the U.S. The European Union has approved a 15µg Intanza® vaccine for use in adults over age 60 years (compared to the 9µg dose approved for ages 18 to 59 years). In a recent phase III, multi-center, randomized, controlled study performed in adults aged 65 years or more, Intanza® was shown to have immunogenicity and safety comparable to the MF59 adjuvanted Fluad® vaccine [280].

Many strategies to enhance the immunogenicity of influenza vaccines for older adults are still in the early stages of development and these include the use of toll-like receptor (TLR) ligands as adjuvants to enhance immune responses to vaccines. These ligands act by binding TLRs on the surface of APCs resulting in APC activation, release of pro-inflammatory cytokines and chemokines, thus enhancing adaptive immune responses. However, as discussed earlier, older adults demonstrate a reduced capacity to respond to many TLR ligands which may reduce the effectiveness of this mechanism. A recent study performed in an aging mouse model has shown that Poly I:C, a TLR-3 ligand, retains its ability to stimulate APCs resulting in release of pro-inflammatory cytokines, leading to enhanced T helper function [281]. In a brief report, McElhaney suggested that a TLR4 ligand may also be effective in improving the IFN-γ:IL-10 ratio and Granzyme B activity in aged mice [282]; functions that she has previously demonstrated correlate with protection in the elderly [30,31].

Conclusions

Influenza causes considerable morbidity and mortality worldwide, but disproportionately affects older adults. Conventional TIV vaccines provide adequate protection in young, healthy adults against seasonal epidemics. However, older adults do not develop effective protective immunity in response to these vaccines due to immunosenescence which impacts the cellular components of both innate and adaptive immune systems. While the dysregulation of the immune system with aging is becoming better understood, strategies for overcoming these deficiencies are being explored, which include high-dose vaccines, adjuvanted-vaccines, and alternate routes of immunization.

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