Chronic Viral Infections and Immunosenescence, with a Focus on CMV

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Abstract: The term immunosenescence is used to describe the decreased function of the immune system with age, and also to describe the phenotypic alterations in immune cells and cytokines that develop with age. The most dramatic phenotypic change is seen in the T cell compartment, where the percentage of cells with an effector memory phenotype increases, and the number and diversity of naïve cells decrease. In particular, large, often oligoclonal accumulations of CD28-CD8+ T cells develop. This hallmark change is largely attributable to CMV infection; the accumulating cells are the enormous "inflationary" virus-specific T cell population that are responding to this lifelong, smouldering, subclinical infection. In many populations, at least 80% of the elderly carry CMV, so CMV-driven changes in old age were easily ascribed to aging per se. There is broad agreement that CMV drives this characteristic phenotype of the aged immune system. There is also considerable evidence that the size of the CD8+CD28- population can be used to describe an "immune risk phenotype" which correlates with an increased inflammatory milieu ("inflammaging"), which may be a predictor of all cause mortality. However, the evidence that CMV contributes causally to the functional failings of the immune system in old age, rather than innocently providing a convenient biomarker, is much less convincing. This important question needs to be addressed with studies including enough CMV seronegative individuals to provide statistically valid data.

Keywords: Immunosenescence, cytomegalovirus, CD8 T cells.

INTRODUCTION

As life expectancy has increased in the past few decades, so have studies of the affect of age on the immune system. These studies have shown that the body undergoes an age-related decline in immune function. Currently there are 600 million elderly worldwide, and that number is expected to increase to 2 billion by 2050 [1]. The age-related decline in the elderly immune system termed immunosenescence affects a variety of cells in the immune system, including both the innate and adaptive immune systems. These age-related changes have multiple affects on the elderly population, including decreased vaccine responses and higher incidence of infectious disease, both of which result in greater mortality of the aged population [1]. While multiple definitions have been assigned to immunosenescence, the end result of immunosenescence is the dysregulation of the immune system leading to its decline in function. In addition, there is a lot of interest in the idea that chronic viral infections (particularly cytomegalovirus) play a major role in driving immunosenescence [2]. Inflammation may also contribute to, or be a consequence of, the altered immune state in the elderly, in a concept referred to as "inflammaging" [3]. Lastly, a series of studies from Sweden described an immune risk phenotype (IRP) as a predictor of mortality [4, 5]: the IRP is mainly attributable to large CD8+ T cell populations in CMV seropositive individuals. However, the field as a whole is best by problems of definition and the ability to distinguish cause and effect. We will review here the basic alterations in the immune system in the elderly, and consider the contributions of chronic herpesvirus infections to those changes. We will then discuss the evidence that chronic herpesvirus infections contribute to functional immunosenescence.

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FEATURES OF IMMUNOSENESCENCE

Immunosenescence and Innate Immunity

Immunosenescence affects both the innate and adaptive immune systems in the aging population. The innate immune system plays a crucial role in host defense against pathogens and in initiating the adaptive immune response. Multiple cells within the innate immune system including neutrophils, macrophages, dendritic cells and NK cells are affected by immunosenescence. Neutrophils in the elderly have reduced migration, phagocytosis and also don't respond well to survival factors [6]. Extensive studies on the monocyte/macrophage population reveal a decline in expression and function of toll like receptors (TLRs) [7], resulting in decreased production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα. This is an interesting contrast to the observation that there is an overall increase in inflammatory cytokine levels (such as IL-6) in the elderly, and that this correlates with an "immune risk phenotype" [5]. This is one of many apparent paradoxes that are evident when taking a "snapshot' in time of available material (basically, blood). Similarly, the NK cell population in the elderly is increased, but has decreased cytotoxicity [6]. The function of NK cells

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is of interest to our topic, since NK cells are particularly important in herpesvirus immunity [8].

Immunosenescence and Adaptive Immunity

The impact of aging is more readily seen in the composition of the adaptive immune system, particularly T cells, to the extent that the term ”immunosenescence” is sometimes used as a description of those changes. We prefer to think of immunosenescence in terms of immune function, and would like to define it as impaired immune function due to aging. However, although impaired immunity in aging seems self-evident, it is rather difficult to quantify in human populations. Poor response to the annual vaccination with trivalent inactivated flu vaccine is the most common correlate, and even this is difficult to quantify, given that the elderly often have high titers of pre-existing antibodies. Several factors contribute to the decline of the adaptive immune response, including decreased numbers of naïve T cells and increased numbers of antigen-experienced memory T cells, particularly those bearing an “effector memory” phenotype. The decrease in naïve T cells is mainly attributed to the involution of the thymus [9], and this decline is often blamed for poor immunity [10-12]. The T cell compartment is maintained by an increase in homeostatic proliferation in the face of decreased thymic output. TCR repertoire diversity of the naïve population tends to be maintained until old age. Naïve cells that have undergone multiple divisions before seeing antigen for the first time may be expected to have a lower capacity for antigen-driven expansion. The fact that the naïve repertoire becomes distorted in the elderly has led to the suggestion that homeostatic dysregulation contributes to the age-related defects in the immune system [13].

Although both CD4+ and CD8+ T cell compartments are altered in the elderly, the changes in the CD8+ T cell compartment are more dramatic and have been more extensively studied. This fixation with the CD8+ T cell compartment may seem odd, given that the main clear functional correlate of immunosenescence is poor response to killed flu vaccine, a response that involves APCs, CD4+ T cells and B cells, but not CD8+ T cells. Still, it is hard not to be mesmerized by the dramatic alterations in CD8+ T cell populations that occur with aging. Multiple studies have attempted to characterize the CD8+ T cells in the elderly, using a variety of cellular markers and functional capacity. Cellular surface markers commonly used throughout the literature to phenotype T cell subsets include: CD45RA, CD45RO, CD28 and CD27. In addition, other markers such as CCR7, CD57 and CD95 have been used [14]. Expression patterns of these cellular markers can be used to delineate between the differentiation status of T cells. While a variety of these cellular markers are useful in defining CD8+ T cell subsets, CD28 and CD27 have proven to be the most reliable in defining CD8+ T cell subsets in the aging immune system.

CD28 is a co-stimulatory molecule that is expressed on naïve T cells, and is involved in activation, proliferation and survival. Loss of CD28 expression typically occurs following activation, but as cells differentiate into central memory cells, CD28 is re-expressed. Some memory cells remain CD28-; these are effector memory (Tem) cells that traffic through peripheral tissues and not through lymp nodes. As a population in human blood, CD28- cells have reduced TCR diversity as well as decreased proliferation in response to antigen [15]. CD27 also serves as a co-stimulatory molecule, and downregulation of this marker is associated with a fully or terminally differentiated CD8+ T cell [16]. Loss of CD28 and CD27 have been found to occur in a stepwise fashion with naïve T cells expressing both CD28 and CD27, followed by loss of CD28 and finally loss of CD27. Curiously, in mice the reverse is true. Tem cells are CD27- but loss of CD28 is rarely or never seen in memory cells. The immunology community is firmly divided into those who study human and those who study mice, with little cross-over, and in consequence there has been distressingly little attempt to perform well-controlled cross-species comparisons. In humans, multiple studies have found shown that CD28- CD8+ T cells are associated with aging. Fagnoni *et. al.* analyzed circulating lymphocytes of 120 healthy donors and found increased in CD28- T cells with age [11]. Other studies found that loss that CD28-CD27- expression correlated with increased granzyme B and perforin [17]. Another study found that increases in the CD28- population correlated with increased CD8+ T cells, decreased IL-2 production, and increased IFNγ and TNFα [18]. These findings are all consistent with these cells being terminally differentiated virus-specific effector memory cells. Tem don’t typically make IL-2, but have more immediate cytotoxic and inflammatory cytokine function than their proliferating, IL-2-producing central memory counterparts. Microarray analysis of CD28- and CD28+ CD8+ T cells in healthy young and elderly persons revealed that CD28- CD8+ T cells in both groups were similar in their gene expression profiles. Differences were observed in the CD28+ group where the young had expression of proteins involved in cell growth and differentiation, but the elderly had genes involved in inflammation and apoptosis induction. This suggests that the CD28+ CD8+ T cell population were more differentiated compared to the young, similar to the CD28- population [19]; again, this might be attributed to having undergone many more rounds of homeostatic cytokine-driven divison.

Cells that have lost CD28 and CD27 expression have shortened telomere length due to loss of telomerase activity, which has long been associated with replicative senescence in T cells. Telomerase is a telomere-extending enzyme, responsible for maintaining telomere length in replicating cells, such as CD8+ T cells. Initially CD8+ T cells demonstrate robust telomerase activity following activation, but additional rounds to stimulation result in a loss of telomerase activity [20]. Interestingly, the loss of telomerase activity correlates with the loss of CD28 expression on T cells [20]. Continual division of CD8+ T cells eventually results in short telomeres that are associated with senescent T cells that have the same characteristics as CD28- T cells [21]. Additionally, chronic viral infection, such as HIV and CMV, are associated with short telomeres in peripheral CD8+ T cells [22, 23]. While one could question cause and effect, attempts have been made to reverse the “senescent” phenotype in HIV-specific T cells, by transducing these cells with telomerase, or with the costimulatory molecule CD28. Both

manipulations led to some restoration of proliferation and IL-2 production [24, 25].

Consequences of Immunosenescence

The functional significance of these altered T cell populations in the elderly is presumably the relationship to the ability of the elderly to respond to vaccines and defend themselves against pathogens. The elderly are far more likely to develop severe influenza and pneumonia, and account for the vast predominance of influenza associated hospitalizations [26]. Flu vaccination (with inactivated vaccine) is only 30-50% protective in the elderly, compared to 65-80% in young adults [27]. Poor response (antibody production) following immunization has been correlated with the size of the CD8+CD28- T cell population, which accumulate to large levels in the elderly [28,29]. Since CD8+ T cells are not involved in the response to a killed vaccine, the meaning of this correlation is not clear. However, it is reasonable to postulate that these population distortions reflect an immune system with impaired function.

INFLAMMATION AND THE AGING IMMUNE SYSTEM

Inflammaging

One component of the aging immune system that contributes correlates with its dysregualtion is a pro-inflammatory status, termed "inflammaging" [3]. This pro-inflammatory environment has been shown to be detrimental to the elderly population because it increases their risk for cardiovascular disease, Alzheimer's disease and frailty [30]. While a variety of pro-inflammatory cytokines exist, the major players that have been implicated in multiple studies are IL-6, IL-1β, tumor necrosis factor alpha (TNFα), and interferon gamma (IFNγ). In addition, c-reactive protein (CRP) has been used extensively as a marker for inflammation, as its production is induced by IL-6 [30].

In 1993, it was found that peripheral blood mononuclear cells from older people (mean age 80.2 years) produced higher amounts of pro-inflammatory cytokines, including IL-1β, IL-6 and TNFα when stimulated *in vitro* with mitogens, compared to the young group (mean age 26.8 years) [31]. While the innate immune system plays a role by increasing levels of IL-1β and IL-6, CD8+ T cells have also contributed to this phenotype, with increasing production of inflammatory cytokines, IFNγ and TNFα, with age [18]. The increase in pro-inflammatory cytokines was attributed to the larger CD28- CD8+ T cell population in the elderly. However, to make sense of studies such as this, it would be important to know how often these massive T cell populations are actually stimulated *in vivo* to produce inflammatory cytokines.

Immune Risk Phenotype

The immune risk phenotype was originally defined Ferguson and colleagues in a longitudinal study of very old individuals, in which baseline immune parameters of the individuals who were alive at the end of a two-year period were compared with those of the deceased. The initial definition of the IRP included increased CD8 percentages, poor T cell proliferative responses to concanavalin A, low CD4 and CD19 percentages [32]. These studies were continued with the OCTO and NONA longitudinal studies performed in Sweden. Over the years, the IRP underwent a confusing set of changing descriptions, with an inverted CD4/CD8 ratio and CMV seropositivity being considered the crucial predictors associated with IRP in more recent studies [33, 34]. A more recent follow-up of individuals who reached 100 years of age found little evidence of the IRP, suggesting that very altered T cell populations was not compatible with survival into extreme old age [4]. Perhaps the most interesting feature of these studies was the observation that individuals who did not display an IRP at baseline could develop it several years later, reflecting a rather dramatic alteration in their T cell populations. This change seemed to reflect a "tipping point" in what may have previously been a stable equilibrium and was more predictive of incipient mortality than prevalent disease status.

These are very interesting studies that unfortunately involved a rather small number of individuals. As these studies were in progress, it became clear that the large numbers of CD8+CD28- T cells that underlie the IRP were found almost exclusively in CMV seropositive individuals, and may indeed primarily consist of T cells that are specific for CMV. The prevalence of CMV seropositivity is very high in the elderly (80-90%), which makes it very difficult to know whether CMV is a driver of the apparent risk associated with the IRP, or merely acts as a convenient litmus paper reflecting some other underlying disturbance.

ROLE OF CHRONIC VIRAL INFECTION IN IMMUNOSENESCENCE

Cytomegalovirus

The fact that the characteristic T cell populations associated with aging (frequently by themselves described as "immunosenescence") are largely attributable to CMV infection has prompted the view that immunosenescence is infectious. There are multiple infections an individual experiences early in life that establish life-long latent infections these include cytomegalovirus (CMV), Epstein Barr virus (EBV), varicella-zoster virus (VZV), herpes simples virus (HSV), adenovirus, human papilloma viruses and many others. For the purpose of this review, we are going to focus mainly on CMV and EBV, as these are the only two that elicit very large T cell responses in the peripheral blood, with CMV being far more culpable than EBV.

Cytomegalovirus is a β herpesvirus that commonly infects during an individual during early childhood. It is believed that transmission occurs though bodily fluids such as saliva, breast milk and urine: infected infants excrete large amounts of CMV in their urine for several years and provide an excellent mode of transmission of the virus to children (and adults) who may have failed to be infected by their mothers. Seroprevalence of cytomegalovirus varies depending on the socioeconomic status of the population, with anywhere between 50-90% of the population being infected [35]. In highly infected populations, virtually everyone is seropositive by age 10, whereas in some high socioeconomic groups less than 20% of individuals may reach adulthood

carrying CMV. Overall, in the US, the seroprevalence rate is about 50%, much lower than in developing countries, and presumably much lower than 50 years ago in the US. However, in some ethnic groups- African Americans, Mexican Americans and Asian American, the current seroprevalence is about 90%. These changing demographics will become very important if CMV is indeed found to be a "driver" of immunosenescence and perhaps some other chronic diseases.

During primary infection, typically during infancy, there are no signs of disease. Cytomegalovirus then establishes a latent, lifelong infection in cells of the myeloid lineage. It is believed that viral reactivation occurs during differentiation of myeloid cells into macrophages and dendritic cells, but the frequency of CMV reactivation in immunocompetent individuals is not known. What is clear is that it is uncommon to detect infectious CMV in the secretions of most infected adults, so, if reactivation occurs frequently, it is extremely rapidly controlled. While the CMV latency is poorly understood, the immune system plays a significant role in keeping CMV in check. Multiple components of the immune system are involved in the CMV immune response including NK cells, antibodies, and CD4+ and CD8+ T cells. Multiple studies have shown that a large percentage of CD8+ T cells are specific for CMV in healthy adults [36,37]. Since there is no good way to know which CD8+ T cells are specific for CMV without stimulating them with peptide antigen, and CMV is a large virus with over 200 genes, it has been very difficult to accurately quantify the true size of the response. However, one massively heroic and expensive study utilized overlapping 15mer peptides covering the entire CMV genome to stimulate CD8+ T cells for an ICS assay; this found that an average of 7% of CD8+ T cells were CMV specific in healthy adults [38]. The CD8+ T cell response to CMV is necessary to maintain control of the virus, although there is no reason to believe that these huge populations are needed for viral control. Most CMV-specific CD8+ T cells are CD28- and CD27- [39], and this, along with the size of the response, makes the CMV-immune T cell population unique. For example, although EBV-specific T cell populations can be substantial in size, they are smaller than those specific for CMV [40], and no other chronic infection in healthy individuals comes close to these two. In addition, EBV-specific CD8+ T cells typically maintain expression CD27 for the long term [39, 40], perhaps suggesting that they are stimulated by antigen less frequently.

The Impact of Cytomegalovirus on Aging Immune System

The large CMV-specific CD8+ T cell populations that are present in adults can become truly enormous in the elderly [27, 41]. Many studies have been done on the elderly (60 years of age or older) to examine how CMV infection alters their immune function and CD8+ T cell compartment. The combination of CMV infection with other factors have contribute to the immunosenescent nature of the aging immune system. As discussed earlier there are several hallmark features of immunosenescence including decreased numbers of naïve T cells, increased terminally differentiation CD8+ T cells that are senescence, and increased inflammation. Many of these features may be attributed to CMV infection in the elderly. One of the initial studies to define changes CMV infection has on the elderly T cell population was by Looney and colleagues. They found that CMV+ individuals had increased numbers of CD28- CD4 and CD8 T cells, and that these changes were specific to CMV infection, not age [42]. Multiple additional studies have confirmed the dramatic impact that CMV has on T cell populations in the peripheral blood: CMV seropositives have increased percentages of CD28- CD4+ and CD8+ T cells (with the CD8+ population being more dramatic), and fewer naïve T cells [34, 40, 43, 44]. The increased CD8+CD28- population results in a net increase in CD8+ T cells, and decreases the CD4:CD8 ratio. Thus the hallmarks of "immunosenescence", when regarded as altered peripheral blood lymphocyte populations, are largely if not entirely attributable to hosting CMV.

Recent studies examined the telomere length of CMV-specific CD8+ T cells. Since CMV infections results in a large population of terminally differentiated CD4 and CD8 T cells (defined as CD28-CD27-) this results in a lymphocytes pool with reduced telomere length in the CD8+ T cell subset. This is maintained 3 years post-infection. Telomere length also correlated with age, but CMV exacerbated this affect. CD8+ T cells in CMV- individuals lost on average 77bp/year whereas CMV+ individuals lost 94bp/year [23]. Again, CMV seems to accelerate the development of a hallmark of the senescent immune system.

An extensive study by Chidrawar and colleagues analyzed the T cell compartment of a healthy cohort of CMV+ individuals across a broad age range. They found that CMV+ elderly (60+ years of age) had approximately 20% more T cells, compared to their CMV- counterparts. In addition, they had a decreased CD4/CD8 ratio. While all CMV+ individuals had increased numbers of CD8+ T cells, the elderly had a 41% increase compared to the young population, this was associated with increased numbers of CD8+ memory T cells. Although naïve CD8+ T cells decline with age in both the CMV+ and CMV- groups, individuals with CMV had a lower number of naïve T cells [45]. Additionally, CMV infection has been shown to drive the development of oligoclonal expansions in old age, which may be contributing to the results observed by Chidrawar and colleagues. Khan *et. al.* examined TCR repertoires of CMV-specific CD8+ T cells and found clonality increased with age in CMV seropositive individuals. In addition, these clonal expansions could accumulate to approximately 25% of the memory CD8+ T cell compartment (defined as CD28-CD57+) [46]. A similar study found comparable results, finding that clonal expansions of CD8+ T cells increased the CMV seropositive elderly group but were not detectable in the middle age group. Additionally, they found that when looking at IRP status, those without IRP had higher clone numbers compared to individuals with IRP. The authors suggest that this shrinkage was a result of exhaustion of the CD8+ T cell clones [34].

The question of T cell exhaustion in CMV-specific T cell populations is somewhat controversial. In the prototypical mouse viral infection, LCMV, chronic infection leads to progressive loss of function (exhaustion) of CD8+ T cells. This has led to a widespread assumption that T cell exhaustion is a common feature of chronic viral infections, and that it should be expected in CMV. Indeed, lack of function has

been observed in some very large oligoclonal CMV-specific CD8+ T cell populations in the elderly [47, 48]. However, the vast majority of CMV-specific T cells, even in the elderly, are fully functional, secreting IFN-γ and TNF α in response to antigen, and are capable of cytotoxicity. In fact, the total IFN-γ and TNF α response to CMV antigens was higher in the elderly than in a middle aged group [49].

BUT WHAT DOES IT SIGNIFY?

Some things have become clear through all this work. CMV has a dramatic impact on the peripheral blood T cell populations, resulting in particular in the accumulation of CD8+CD28- T cells, but also of CD4+CD28- T cells. In the elderly the size of these populations can become truly huge. CMV is responsible for, or at least accelerates the development of, the characteristic profile of the T cell compartment in the elderly. Since most old people are CMV seropositive, the CMV-driven changes were originally ascribed to aging per se, and referred to as "immunosenescence". However, the relationship between these changes and what we would like to call functional immunosenescence is much less clear.

For example, CMV is clearly responsible for the T cell changes that comprise the IRP. But does this mean that CMV is a bad actor? Those who propose that CMV is the cause of immunosenescence and its corollaries- poor vaccine responsiveness, increased mortality- suggest that some people handle CMV better than others, and hence that CMV only causes serious immune compromise in those who handle it poorly- i.e. those who develop the IRP. However, an equally plausible explanation would be that some peoples immune systems become less effective earlier than others, for some completely different reasons (genes, environment…). If these "immunoelderly" are CMV seropositive, CMV will become more active, and this immune dysfunction will be manifest by increasing distortions in the T cell compartment- the IRP. If the immunoelderly are CMV seronegative, no such distortions will develop, but the underlying problem will still be there. In both cases, the immunoelderly would have poorer response to vaccines, earlier mortality, etc. If CMV is really participating in the problem, then CMV seropositives should have worse outcomes than CMV seronegatives. The problem with most analyses is that the high prevalence of CMV seropositivity in this age group makes it difficult to make this comparison. For what it is worth, the very small number of CMV seronegatives in the OCTO and NONA studies that defined the IRP died at the same rate as the CMV seropositives (although without developing the IRP): however, the numbers are too small to be significant. With regard to flu vaccination, studies are inconsistent as to whether CMV seropositivity per se contributes to a poorer outcome. Prospective studies that specifically recruit larger numbers of CMV seronegatives are needed to properly address these questions. Furthermore, the demographics of CMV infection mean that all such studies need to be very carefully controlled to take account of socio-economic differences.

It is very easy to imagine how CMV could contribute to many diseases of the elderly. In particular, the idea that these enormous T cell responses contribute to the inflammatory environment of "inflammaging" is highly appealing. Regrettably, the evidence is not yet there. However, even if CMV is found "not guilty", the T cells responding to it may at least provide a window to the underlying problem, and in so doing, help us to understand it.

CONFLICT OF INTEREST

None declared.

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