IMMUNE PREDICTORS OF CLINICAL OUTCOMES IN NURSING HOME RESIDENTS

IMMUNE PREDICTORS OF CLINICAL OUTCOMES IN ELDERLY NURSING HOME RESIDENTS

By

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ABSTRACT

Elderly residents of nursing homes are at high risk of respiratory viral infection, mortality and frailty. It is a widely held view that the dysfunctional changes to the immune system that arise from ageing, known as immunosenescence are responsible for the increased risk of infection, mortality and frailty; however only sparse data exist to substantiate this. Furthermore, the majority of studies investigating these associations have excluded elderly nursing home residents, thus little is known about immune phenotypes in this group.

In this thesis, I first characterized immune phenotypes in elderly nursing home residents by comparing immune phenotypes in an elderly nursing home cohort to a group of younger healthy adults. I then explored how age, sex, frailty and nutrition influence immune phenotypes in the elderly group. I subsequently used different statistical analyses, including Cox proportional hazards modeling, hierarchical cluster analysis and multi-level modelling to identifying immune biomarkers predictive of clinical outcomes in elderly nursing home residents including respiratory viral infection, mortality and frailty.

We found that high cytomegalovirus (CMV)-reactive CD4+ T-cells were associated with an increased risk of respiratory viral infection and high T-regulatory cells (T-regs) were associated with a reduced risk of respiratory viral infection. High CMV-reactive CD4+ T-cells were also associated with an increased risk of mortality within the subsequent 1-year in those aged 65-84 years but had no differential effect in those aged 85-104 years. Other immune phenotypes were not predictive of mortality. Higher naïve

CD4+ T-cells and effector memory CD8+ T-cells predicted lower levels of frailty and higher central memory CD8+ T-cells predicted higher levels of frailty.

These findings may help provide more focused care through targeted prevention. Furthermore, knowledge of these immune biomarkers provides insight into how immunosenescence may contribute to these clinical outcomes and will help guide future research into novel prevention strategies.

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LIST OF ABBREVIATIONS USED IN THIS THESIS

ADL	Activities of daily living
ANOVA	Analysis of variance
CI	Confidence intervals
CIHR	Canadian Institutes of Health Research
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
FACS	Fluorescence activated cell sorters
HR	Hazard ratio
IQR	Interquartile range
NK	Natural killer cell
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PCIRN	Public Health Agency of Canada / CIHR Influenza Research Network
PCR	Polymerase chain reaction
RERI	Relative excess risk due to interaction
RSV	Respiratory syncytial virus
SD	Standard deviation
SE	Standard error
T-reg	The regulatory CD4+ T-cell
VIF	Variance inflation factor

PREFACE

This PhD thesis is structured as a sandwich thesis. It begins with an introductory chapter, followed by four manuscript chapters written using data generated from a Canadian Institutes of Health Research (CIHR) funded observational study investigating immune biomarkers associated with immunosenescence that are predictive of clinical outcomes in elderly nursing home residents and ends with a concluding chapter. A summary of my contributions to the observational study and manuscript preparation follow below.

Myself, Dr. Mark Loeb and Dr. Jonathan Bramson, jointly conceived the idea for the observational study in July 2008. I was responsible for drafting the initial project protocol and CIHR grant proposal, which was submitted to the spring 2009 CIHR grant competition. The protocol and grant proposal were critically reviewed by Drs. Loeb and Bramson and had additional input from Dr. James Mahony.

This initial grant application was ultimately unsuccessful, however the study was still initiated in the summer of 2009 and planned as a single center (Hamilton, Ontario) study for the first year. I was responsible for completing the Research Ethics Board application, developing case report forms and engaging and recruiting nursing homes to participate in the study. I was involved in hiring the local research nurse who enrolled participants, abstracted study data, obtained study samples and study outcomes.

I drafted a second CIHR grant application, which was submitted to the spring 2010 CIHR grant competition. This grant application was critically reviewed by Drs.

Loeb and Bramson with additional review by the co-applicants including Drs. Tamas Fulop, Janet McElhaney, Shelly McNeil and Stephen Walter. This grant was successful in receiving $700,000 from CIHR. We were therefore able to expand the study for two additional years (fall 2010 – spring 2012) and recruit participants from nursing homes in four cities across Canada: Halifax, Nova Scotia; Sherbrooke, Quebec; Hamilton, Ontario and Vancouver, British Columbia. The final year of the project (spring 2012 – spring 2013) was spent processing the laboratory specimens obtained during the study. I applied for and obtained additional funds from Public Health Agency of Canada / CIHR Influenza Research Network (PCIRN) for the laboratory to complete the processing of specimens from spring 2012 – fall 2013.

Although I was involved in overseeing the entire project, each city had a local principal investigator who was responsible for hiring local research personnel, recruiting local nursing homes and participants and obtaining and storing study samples until the end of the study. I reviewed the research protocol and case report forms with each local principle investigator and research personnel at the beginning of the study and was available in real-time to answer questions that arose throughout the study. I was responsible for balancing the budget, although Dr. Loeb's Financial Administrator and McMaster University administered the funds. I designed the database used for data entry; however an undergraduate student was hired to input all data and obtain or clarify missing data. I worked with the student to ensure the database was comprehensive, and I audited approximately 10% of all case report forms to ensure the data entry process was valid. No other graduate student was involved in this study.

I was not responsible for any processing of laboratory specimens. Each local site was responsible for organizing the isolation and freezing of peripheral blood mononuclear cells (PBMCs). Dr. Jonathan Bramson's laboratory coordinated the shipment of specimens from each site to his laboratory and generated all immune phenotype data outputs. Dr. Ryan Brinkman's laboratory was responsible for the classification and interpretation of the immune phenotypes. Dr. James Mahony's laboratory processed the nasopharyngeal swabs for respiratory viruses.

Once data were received from Drs. Bramson, Brinkman and Mahony's laboratories, I was responsible for merging these data with the existing database and cleaning the data. I performed all analyses in this thesis. Dr. Walter was available as a resource to help answer statistical questions when they arose.

I am first author of each thesis manuscript. Each manuscript was first reviewed by Drs. Loeb and Bramson, and then critically reviewed by Drs. Walter and Devereaux. The remaining co-authors then provided valuable additional commentary. The manuscripts were all derived from the same study cohort. Thus, the reader should expect overlap in the description of the study design and laboratory methodology for each of the thesis manuscripts. I drafted the introduction and conclusion, and they were critically reviewed by Drs. Loeb, Walter and Devereaux.

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.0 SPECIFIC AIMS OF THE THESIS

Elderly residents of nursing homes are at particularly high risk of respiratory viral infection, mortality and advancing frailty. It is a widely held view that immunosenescence, i.e. waning of the immune system with age, is responsible for the increased risk of infection, mortality and frailty [1, 2]; however only sparse data exist to substantiate this. Immune biomarkers associated with immunosenescence have previously been shown to correlate with outcomes such as mortality [3, 4], but they have not been investigated as predictors of mortality within the elderly nursing home population, and have not been clearly associated with respiratory viral infection or frailty. Identifying immune biomarkers that could identify nursing home residents at highest risk of contracting respiratory viral infection, mortality or worsening frailty could provide more focused care through targeted prevention. Furthermore, knowledge of immune biomarkers may provide insights into how immunosenescence contributes to these clinical outcomes and may help guide future research into novel prevention strategies.

The objective of this thesis was to assess whether immune biomarkers associated with immunosenescence can predict clinical outcomes in an elderly nursing home population. Our specific aims were to:

1. Characterize immune phenotypes associated with immunosenescence in elderly nursing home residents;

2. Identify immune biomarkers predictive of respiratory viral infection in elderly nursing home residents;
3. Identify immune biomarkers predictive of mortality in elderly nursing home residents;
4. Identify immune biomarkers predictive of frailty in elderly nursing home residents.

We hypothesized that immune biomarkers associated with immunosenescence would identify nursing home residents at highest risk of symptomatic respiratory viral infection, mortality within one year and frailty.

2.0 BACKGROUND AND SIGNIFICANCE

2.1 The Burden of Respiratory Viral Infection, Mortality and Frailty in Elderly Nursing Home Residents

Nursing homes are residential facilities for people that require continual nursing care [5]. There are currently over 200,000 Canadians residing in nursing homes [5]. The average age of nursing home residents is 85 years and 70% are women; most have co-morbid illness and require nursing care for activities of daily living (ADL) including toileting and eating [5]. The proportion of adults over 65 years is expected to double in the next 25 years in Canada, and the population of nursing home residents is expected to rise dramatically [6, 7].

Respiratory Viral Infection in Elderly Nursing Home Residents

The burden of respiratory viral infection in elderly nursing home residents is high [8]. Respiratory viral infections are common; with active surveillance the incidence of respiratory viral infection is estimated to range from 1.4 – 2.8 per 1000 resident days [9]. Nosocomial spread within nursing homes is facilitated as residents live in close quarters [10]. Once infected, elderly nursing home residents are more likely to develop complications including lower respiratory tract illness, reduced mobility and death [10-12].

Respiratory viral infection outbreaks due to influenza and respiratory syncytial virus (RSV) are well known to cause morbidity and mortality in elderly nursing home residents [8]. However in recent years, with the advent of newer, more sensitive and comprehensive molecular viral testing using nasopharyngeal specimens [13], it has become clear that other respiratory viruses including parainfluenza, human metapneumovirus, coronavirus and rhinovirus are able to cause outbreaks and severe disease, including pneumonia and death in this population [8, 14-18].

There are few strategies available to prevent respiratory viral infections in nursing homes. General infection control measures include good hand hygiene adherence, having a surveillance system in place to detect respiratory viral infection and isolation of symptomatic individuals [1]. The only targeted prevention strategy currently available is influenza vaccination. However, despite >80% uptake of vaccination against influenza in long-term care facilities [19], outbreaks and their related morbidity and mortality persist. This is likely due to the fact that the vaccine's effectiveness in people over 65 years has

been substantially overestimated [20-23]. Studies that better adjust for functional status or other measures of frailty demonstrate a non-significant benefit of influenza immunization for reducing hospitalization and mortality [20, 22, 23]. Better strategies for the prevention of respiratory viral infection, including influenza in elderly nursing home residents are clearly needed.

Mortality in Elderly Nursing Home Residents

Residents of nursing homes are elderly with a mean age of 85 years, and typically have multiple comorbidities [5]. Two thirds have a mobility impairment or diagnosis of dementia or incontinence, half have an underlying musculoskeletal disease, a third have signs of depression, and between 10-25% have diabetes, chronic obstructive pulmonary disease (COPD), congestive heart failure or malignancy [5, 24]. Given this burden of illness, it is not surprising that mortality is high; between 40% - 65% of residents will die within 5 years [24]. In elderly people over the age of 85 years, the most common causes of death include cardiac disease (26%), cancer (16%), stroke (9%), dementia (5%), chronic lower respiratory disease (5%), influenza/pneumonia (4%) and unintentional injuries (4%)[25]. The precise causes of death in elderly nursing home residents can be difficult to capture when the resident dies in the home, as extensive investigations may not be performed before or after the death. However, based on the fact that the most common reasons for transfer to hospital from nursing homes include cardiac disease, neurological event, injury (typically fracture), and infection [26], the causes of death in elderly nursing home residents are likely similar to those seen in elderly people [25].

Frailty in Elderly Nursing Home Residents

It is expected that the majority of nursing home residents are frail, given their need for additional care [24]. Frailty is defined as a syndrome that arises due to accumulating comorbidity across domains leading to loss of an individual's reserve [27]. Increasing frailty in elderly nursing home residents is associated with increased risk of death [24]. Historically, frailty has been a difficult construct to measure, however there are now three measures of frailty validated in the nursing home setting including Fried Frailty, the Clinical Frailty Scale and the Frailty Index [24, 27-29]. Fried Frailty uses 5 variables to classify whether an individual is robust, pre-frail or frail (Table 1)[29]. The Clinical Frailty Scale uses clinical data to categorize the frailty status on an 8-point scale ranging from 1-8 as follows: (1) very fit, (2) well, (3) well with treated comorbid illness, (4) apparently vulnerable, (5) mildly frail defined as dependence in instrumental ADLs, (6) moderately frail defined as required assistance with basic ADL, (7) severely frail defined as completely dependent on others for ADL and (8) very severely frail (Table 2)[27]. Last, the Frailty Index is a ratio that is derived simply by counting the accumulation of deficits and dividing the deficits by the number of items considered; the more deficits a person has, the higher the level of frailty [28]. The originally described Frailty Index can be found in Table 3[28]. The deficits chosen for inclusion are not necessarily the same in each Frailty Index [30]. The Frailty Index can be customized for each user by following a standard procedure; each Frailty Index requires a minimum of thirty items that must be measured, all related to health status and must cover different

components of health [30]. The ability of the Frailty Index to predict mortality is reproducible across studies, despite the differing deficits [31].

Each of the three frailty measures described above was deemed to be valid for use in elderly nursing home residents [24]. Fried Frailty, Clinical Frailty Scale and Frailty Index were each able to predict mortality within 5 years, decline in cognition, and incident disability in elderly nursing home residents [24]. There are advantages and disadvantages to each measure. The Fried Frailty definition is the easiest to operationalize, however for those who are frail, it does not capture varying degrees of frailty [24]. Both the Clinical Frailty Scale and the Frailty Index can grade frailty, but are more labor intensive to perform than the Fried Frailty [24]. The Frailty Index is the most precise measure of frailty in the elderly [24].

2.2 Using Immune Phenotypes to Summarize Immune System Remodeling Seen in Immunosenescence

Immune phenotype is a term that refers to the proportion of immune cell subsets present within each individual. Immune phenotypes are identified using fluorescence activated cell sorters (FACS) and flow cytometry to analyze either whole blood or peripheral blood mononuclear cells (PBMCs) from study subjects [32]. This approach leads to a descriptive summary of the immune cells present, but it is not a functional assay [33]. However, the characterization of immune phenotypes in aging is an important first step in understanding immunosenescence, as it provides insights into the remodeling of the immune system that occurs with age. Furthermore, immune phenotypes associated

with aging could be used as immune biomarkers predictive of clinical outcomes. Immune phenotype terminology is inconsistent in the literature. The terminology used to describe immune phenotypes in this thesis is summarized in Table 4.

2.3 Immunosenescence as a Possible Explanation for the Increased Risk of Respiratory Viral Infection, Mortality and Frailty in Elderly Nursing Home Residents

As a first step towards discovery of strategies to prevent respiratory viral infection, mortality and declining frailty, fundamental knowledge about the immune mechanisms associated with this morbidity and mortality are needed. It is a widely held belief that the waning immune function associated with advanced age, termed immunosenescence, plays an important role in determining the susceptibility of elderly individuals to infection, mortality and frailty [1, 2]. However, only sparse data exist to support this position.

There are a number of age-related immune phenotype changes that have been observed in the innate and adaptive immune system which are considered indicators of poor immunological function [34]. For example, neutrophil, macrophage and dendritic cell function are impaired and natural killer (NK) cell phenotypes change due to remodeling of NK cell subsets resulting in lower NK cell cytotoxicity [35]. Humoral immune responsiveness and antibody-mediated defense mechanisms are reduced with aging [36]. Although all components of the immune system appear affected by aging, changes in T-cell immune phenotypes are by far the most pronounced [34]. Changes

include reduction in the number of naïve T-cells due to thymic involution and a corresponding increase in memory T-cells subsets due to exposure to pathogens throughout life [37]. In the CD8+ T-cell compartment, aging is associated with higher numbers of terminally differentiated memory T-cells and senescent T-cells [38-41] whereas in the CD4+ T-cell compartment aging is associated with increased numbers of central memory and effector memory T-cells [38].

One theory proposed to explain the increase in memory T-cells in the elderly is the existence of a chronic, low-grade, pro-inflammatory state in the elderly that has been called 'inflammaging' [42]. This imbalance occurs as a result of the relatively preserved innate immune response and the substantially altered adaptive immune response [42]. Cytomegalovirus (CMV) has been proposed as the chronic antigenic stimulus responsible for the pro-inflammatory state [43, 44]. It is thought that CMV drives clonal expansion of terminally differentiated CD8+ T-cells and senescent CD8+ T-cells [43, 44]. This is supported by reports that many of the increased number of senescent T-cells are CMV specific [45]. This leads to higher numbers of CD8+ T-cells relative to CD4+ T-cells [44]. Other viruses in the Herpesvirus group, such as Epstein-Barr virus, contribute minimally to the clonal expansion of senescent T-cells when compared to CMV [43].

The higher numbers of a separate class of T-cell, the regulatory CD4+ T-cell (T-regs) has also been consistently observed in the elderly [46]. T-regs exert suppressive effect on other immune cells following the immune response to infections and other antigenic stimuli [47]. Whether increased T-regs lead to adverse outcomes associated with immunosenescence has yet to be fully elucidated [47].

The majority of immunosenescence studies involve exclusively community dwelling elderly and most have excluded elderly nursing home residents, thus although it is hypothesized that immune phenotypes in elderly nursing home residents are similar to those seen in community dwelling elderly, immune phenotypes in this group have not been well characterized [37]. Describing the immune phenotypes seen in elderly nursing home residents is therefore an important Aim of this thesis (Thesis Aim 1).

Immunosenescence and Respiratory Viral Infection

It is theorized that immunosenescence leads to increased risk of respiratory viral infection because the lack of naïve T-cells is thought to impair the ability of the host to respond to novel pathogens [37, 48] and terminally differentiated memory T-cells are considered to have poor functionality resulting in impaired responses to recall antigens [37]. However, to our knowledge the relationship between immune phenotypes associated with immunosenescence and risk of respiratory viral infection has not been studied. The second Aim of this thesis is to determine immune biomarkers predictive of respiratory viral infection in elderly nursing home residents (Thesis Aim 2).

Immunosenescence and Mortality

Immune biomarkers related to immunosenescence have been successfully validated as predictors of mortality in two aging Swedish cohorts [3, 4, 49]. Building on the theory that immunosenescence leads to an expansion of the CD8+ T-cell compartment relative to the CD4+ T-cell compartment, the CD4+/CD8+ T-cell ratio of <1.0, termed

the immune risk profile, was investigated as an immune biomarker [3, 4]. The immune risk profile was initially found to be predictive of mortality in the OCTO study, a longitudinal study enrolling octogenarians [3] and subsequently in the NONA study, a longitudinal study enrolling nonagenarians [4, 49]. The CD4+/CD8+ T-cell ratio <1.0 also correlates with CMV serostatus and a disproportionate number of CMV reactive senescent T-cells, further supporting the role of CMV as a driver of immunosenescence [4, 45, 49].

However, the immune risk profile is not consistently predictive of mortality in other ageing cohorts. Both a Spanish cohort study and an English cohort study of community dwelling elderly people found the immune risk profile to be associated with time to death in unadjusted analysis, but not after adjusting for potential confounders including age [50, 51]. A nested case control study of elderly community dwelling women in Baltimore found no association between the CD4+/CD8+ T-cell ratio and mortality [52]. Thus, it remains uncertain whether the association between the immune risk profile and death is generalizable to ageing cohorts outside Sweden, and it is unknown whether the immune risk profile can predict mortality in elderly nursing home residents.

It is also unknown whether other immune phenotypes associated with immunosenescence are predictive of mortality. The few studies investigating these associations have used older immune phenotyping unable to effectively delineate between naïve and memory T-cells pools [51-56]. Indeed, these older studies may have misclassified up to 20% of the naïve T-cell compartment [56, 57]. Additionally, the

association between mortality and T-regs has not been investigated. The third Aim of this thesis is therefore to determine whether immune biomarkers including the immune risk profile are predictive of mortality in elderly nursing home residents (Thesis Aim 3).

Immunosenescence and Frailty

The pathophysiological mechanisms that lead to frailty are not yet well understood, but it is hypothesized that frailty is associated with immunosenescence [2]. The results of the few studies addressing the association between immunosenescence and frailty have been mixed. Two studies using data from the Women's Health and Aging Studies cohort found that women with the highest levels of CMV antibody had a greater incidence of frailty [58] and frail women had significantly higher CD8+ T-cells, senescent CD8+ T-cells, and lower CD4+ T-cells [52]. In contrast, a cross-sectional study of community dwelling individuals aged 80 years and older, found that those who had CMV antibodies were less frail than those without CMV antibodies [59] and no difference between physical functioning in those with and without the immune risk profile [60]. A cross-sectional study of elderly people 85 years and older found no association between frailty and CMV serostatus, CD4+/CD8+ ratio less than 1 or memory/naïve CD8+ T-cell ratio [61]. Thus, the fourth Aim of this thesis is to determine whether immune biomarkers are predictive of frailty in elderly nursing home residents, as a first step towards identifying potential candidate immune biomarkers that could be investigated in future studies as predictors for change in frailty status over time (Thesis Aim 4).

2.4 Need for Immune Biomarkers Predictive of Respiratory Viral Infection, Mortality and Frailty in Elderly Nursing Home Residents

If immune biomarkers predictive of respiratory viral infection, mortality and declining frailty are discovered, it will help identify those at risk for these adverse outcomes. This could lead to improved care by providing an opportunity for prevention. For example, identification of those at highest risk of respiratory viral infection could allow care providers of these individuals to become better educated about the importance of hand hygiene adherence. Active surveillance for respiratory viral infection in these residents could also be performed to help prevent spread to other residents and healthcare workers. High-risk residents could also be moved to a single room where feasible as having a room-mate with a respiratory virus is a risk factor for acquiring a respiratory viral infection [10]. Identifying those at risk for mortality could provide an opportunity for prevention through a comprehensive review of medical illnesses to ensure medical care is optimized. In the event that no reversible measures could be implemented, knowledge of risk of mortality could allow the resident and family members to make appropriate preparations prior to death. Last, preventing the decline of frailty is critically important to maintenance of health status [62] as declining health is associated with increased risk of respiratory infection [63, 64], increased burden of care [65], and increased healthcare cost [65]. Identification of those at highest risk of declining frailty could signify the need for a comprehensive multi-disciplinary intervention potentially including medical and nutritional review and rehabilitation.

Immune biomarkers predictive of respiratory viral infection, mortality and frailty will also provide fundamental understanding of how immunosenescence contributes to these clinical outcomes and may help guide research into novel prevention strategies including vaccines or pharmacotherapy for CMV if proven relevant, or targeted immune therapy [66].

3.0 METHODOLOGICAL CONSIDERATIONS

3.1 Clustering Effect of Nursing Homes

Multiple participants were recruited from each nursing home, and we expected that individuals within nursing homes would be more similar to each other than individuals from different nursing homes because of potential differences between homes in levels of support provided (i.e. basic care versus care for residents with end stage dementia) and variation between populations that the nursing homes draw from (i.e. differences in income, race). This clustering effect violated the assumption of independence required in the statistical modeling approaches in both Cox proportional hazards models [67] and linear regression [68]. Ignoring clustering can lead to an increased risk of Type 1 error (finding a statistically significant association when there is no association), incorrect confidence intervals and biased estimates if there is confounding by study center [68].

To appropriately account for the potential clustering effect of nursing homes when using Cox proportional hazards for the analysis of time to respiratory viral infection

(Thesis Aim 2) and time to death (Thesis Aim 3), a sandwich variance estimator was used [67]. The sandwich variance estimator provides calculation of a robust covariance matrix that can be used to derive parameter estimates and confidence intervals when the underlying assumptions of the Cox proportional hazards model are incorrect [67].

When determining immune biomarkers predictive of frailty using the Frailty Index, a continuous variable, a number of potential statistical methods were available that could compensate for the clustering effect of nursing homes including multi-level modeling and generalized estimating equation [69]. The use of multi-level modeling, including the study centers as a random effect (in this case nursing homes) leads to the most efficient effect estimates, thus the multi-level modeling approach was selected as the preferred statistical approach [69].

3.2 Measurement of Frailty

In this thesis, the ability to measure frailty was critically important as frailty was used as a dependent (outcome) variable (Thesis Aim 4) as well as an independent variable to adjust for frailty as a potential confounding variable (Thesis Aim 2 and 3) and frailty was also used to explore its influence on immune phenotypes (Thesis Aim 1). As previously outlined (Section 2.1) frailty can be validly measured in the nursing home setting in three ways; Fried Frailty, Clinical Frailty Scale and the Frailty Index [24]. Fried Frailty was not felt to be a useful measure for our purposes as the majority of nursing home residents are expected to be frail [24]. Both the Clinical Frailty Scale and the Frailty Index are able to grade different levels of frailty [27, 28]. When the studies

presented in this thesis were originally designed in 2008, the Clinical Frailty Scale was chosen over the Frailty Index as the frailty measure of choice because the original Frailty Index required physician assessment of each participant [28], a task that was not logistically feasible. The Clinical Frailty Scale was used to adjust for potential confounding when time to respiratory viral infection was used as an outcome as outlined in our study design *a priori* (Thesis Aim 2) and when time to mortality was used as an outcome (Thesis Aim 3). The influence of frailty on immune phenotypes was also explored using the Clinical Frailty Scale (Thesis Aim 1).

Subsequent to the design of the studies, a standard operating procedure for developing a Frailty Index was published [30], allowing for the creation of a Frailty Index using items collected with the Clinical Frailty Scale as well as other variables prospectively collected during enrollment. The Frailty Index was selected over the Clinical Frailty Scale for use as an outcome variable (Thesis Aim 4) as the Frailty Index is a continuous outcome and therefore allowed for a more powerful analysis than the Clinical Frailty Scale, a categorical variable.

4.0 THESIS OUTLINE

The remainder of this thesis includes four original research manuscripts followed by a concluding chapter. Each original research manuscript seeks to address one of the four Aims outlined in the Introduction. In the first manuscript, I first characterized the immune phenotypes found in elderly nursing home residents by comparing the immune

phenotypes in elderly nursing home residents to a healthy adult population, and examined how age, sex frailty and nutrition influenced the immune phenotypes in the elderly nursing home residents. In the second manuscript, I determined whether immune biomarkers associated with immunosenescence were associated with time to symptomatic respiratory viral infection in elderly nursing home residents using a Cox proportional hazards model, adjusted for age, sex and frailty. In the third manuscript, I determined whether immune biomarkers were associated with time to mortality (within 1-year) using a Cox proportional hazards model adjusted for age, sex and frailty and hierarchical cluster analysis. Last, I investigated immune biomarkers predictive of frailty using a multi-level modeling approach.

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Table 1: Fried Frailty definition [29].

Characteristic	Definition	Score
Weight loss	Loss of 10 pounds or 5% of body weight in past year	1=Weight loss 0=No weight loss
Exhaustion	Self reported feeling of tired all of the time	1=Exhaustion 0=No exhaustion
Low physical activity	Needing assistance with walking or being unable to walk	1=Low physical activity 0=No physical activity
Slowness	19 seconds or more on the timed up and go.	1=Slowness present 0=Slowness absent
Weakness	Abnormal strength on exam	1=Weakness present 0=Weakness absent
Classification		
Frail	Score=3 or more	
Pre-frail	Score=1 or 2	
Robust	Score=0	

Table 2: Clinical Frailty Scale definition [27].

Domain	Characteristic	Additional details
Cognition	Presence of dementia?	Obtain cognition score from minimum data set If dementia, record type
Behavior	Presence of abnormal behavior?	If abnormal, record dominant symptom
Mood	Presence of abnormal mood?	If abnormal, describe
Sensory	Hearing impaired? Vision impaired? Speech impaired?	Comments
Mobility	Transfers independent, assisted or dependent? Ambulates independently, assisted or not at all? Requires Aid? Balance impaired? History of falls?	Record duration of aid Record type of aid Record frequency of falls
Nutrition	Weight stable, loss or gain? Tube feed?	Comments
Function	Bathing independent, assisted or dependent? Dressing independent, assisted or dependent? Toileting independent, assisted or dependent? Eating independent, assisted or dependent? Medication independent, assisted or dependent? Finances independent, assisted or dependent?	Comments
Skin	Ulcers present? Edema present?	Describe type/stage/location
Continence	Bladder continent or incontinent Bowel continent or incontinent	Comments
Social	Married, separated, divorced or single/widowed? Family involved, uninvolved or none	
Planning	Enduring power of attorney?	Record which domains (financial/personal care) Record person Record whether enacted
FRAILTY LEVEL (circle one) 1 2 3 4 5 6 7 8		
1 (Very Fit): Robust, active, energetic and motivated, exercise regularly, fittest for their age.		
2 (Well): No active disease symptoms but are less fit than category 1; exercise or are very active occasionally.		
3 (Managing Well): Medical problems are well controlled, but are not regularly active beyond routine walking.		
4 (Vulnerable): Not dependent on others, but symptoms limit activities; are “slowed up”, and/or tired.		
5 (Mildly Frail): More evident slowing, need help in high order instrumental activities of daily living.		
6 (Moderately Frail): Require help with all outside activities and keeping house, problems with stairs, need help with bathing and might need minimal assistance (cuing, standby) with dressing.		
7 (Severely Frail): Completely dependent for personal care, but are stable, not at high risk of dying within 6 months.		
8 (Very Severely Frail): Completely dependent, approaching the end of life, could not recover from a minor illness.		

Table 3: Originally described 70-item Frailty Index* [28].

Changes in everyday Activities	Mood Problems	Seizures, partial complex
Head and neck problems	Feeling sad, blue, depressed	Seizures, generalized
Poor muscle tone in neck	History of depressed mood	Syncope or blackouts
Bradykinesia, facial	Tired all the time	Headache
Problems getting dressed	Depression (clinical impression)	Cerebrovascular problems
Problems with bathing	Sleeping changes	History of stroke
Problems carrying out personal grooming	Restlessness	History of diabetes mellitus
Urinary incontinence	Memory changes	Arterial hypertension
Toileting problems	Short-term memory impairment	Peripheral pulses
Poor muscle bulk	Long-term memory impairment	Cardiac problems
Rectal problems	Changes in general mental functioning	Myocardial infarction
Gastrointestinal problems	Onset of cognitive symptoms	Arrhythmia
Problems cooking	Clouding or delerium	Congestive heart failure
Suck reflex	Paranoid features	Lung problems
Problems going out alone	History relevant to cognitive impairment or loss	Respiratory problems
Impaired mobility	Family history relevant to cognitive impairment or loss	History of thyroid disease
Musculoskeletal problems	Impaired vibration	Thyroid problems
Bradykinesia, limbs	Tremor at rest	Sin problems
Poor muscle tone in limbs	Postural tremor	Malignant disease
Poor limb coordination	Intention tremor	Breast problems
Poor coordination, trunk	History of Parkinson's disease	Abdominal problems
Poor standing posture	Family history of neurodegenerative disease	Presence of snout reflex
Irregular gait pattern	Other medical history	Presence of palmomental reflex
Falls		

*Items listed above were either dichotomized (0 or 1) or trichotomized (0, 0.5 or 1) with 0 meaning no deficit and 1 meaning presence of deficit. The number of deficits were then added together and the Frailty Index score was calculated by dividing the number of deficits by the total number of possible deficits (n=70).

Table 4: Immune phenotype terminology definitions.

T-cell Phenotype	Name
CD4+	
CD4+CCR7+CD45RA+	Naïve CD4+ T-cell
CD4+CCR7+CD45RA-	Central memory CD4+ T-cell
CD4+CCR7-CD45RA-	Effector memory CD4+ T-cell
CD4+CCR7-CD45RA+	Terminally differentiated CD4+ T-cell
CD4+CD28-CD57+	Senescent CD4+ T-cell
CD8+	
CD8+CCR7+CD45RA+	Naïve CD8+ T-cell
CD8+CCR7+CD45RA-	Central memory CD8+ T-cell
CD8+CCR7-CD45RA-	Effector memory CD8+ T-cell
CD8+CCR7-CD45RA+	Terminally differentiated CD8+ T-cell
CD8+CD28- CD57+	Senescent CD8+ T-cell
T-regs	
CD4+CD25hiCD127loFOXP3+	T-reg

CHAPTER 2

Thesis manuscript 1: *Immunosenescence in the Nursing Home Elderly*

PREFACE TO CHAPTER 2

This chapter characterizes T-cell and natural killer (NK) cell immune phenotypes in elderly nursing home residents by first comparing their immune phenotypes to those of healthy adults and then exploring how age, sex, frailty and nutritional status influenced immune phenotypes in the elderly nursing home residents.

The student contribution to this study included conceiving the study, data cleaning, data preparation, statistical analysis and drafting the manuscript. Co-authors include Jamie Millar, Alina Lelic and Chris Verschoor who analyzed the laboratory whole blood specimens included in this manuscript under the supervision of Dr. Bramson; Dr. Walter provided statistical expertise and provided critical review of the manuscript; Dr. Devereaux critically reviewed the manuscript and Drs. Loeb and Bramson were involved in conception of the study, aided in data interpretation, and provided critical review of the manuscripts. Dr. Loeb provided funding support for the study.

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Immunosenescence in the Nursing Home Elderly

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Abstract

Objective: To describe T-cell and natural killer (NK) cell phenotypes within nursing home elderly.

Materials and Methods: Nursing home elderly were recruited from four nursing homes in Hamilton, Ontario between September 2010 and December 2011. Healthy adults were recruited from McMaster University between September 2011 and December 2011. Nursing home elderly ≥65 years were eligible; those on immunosuppressive medications were excluded. Healthy adults ≥18-64 years were eligible. CD8+ and CD4+ T-cells% and their subsets, T-regs% and NK cell subset% were compared between the nursing home elderly and healthy adults.

Results: 262 nursing home elderly were enrolled; median age 87 years and 81% were female. 16 healthy adults were enrolled; median age 31 and 50% were female. There was no significant difference between CD8+ T-cell% in nursing home and healthy adults (median 17.1 versus 18.0, p=0.56), however there were fewer naïve CD8+T-cell% (median 0.9 versus 5.2, p<0.001), more terminally differentiated CD8+T-cell% (median 7.3 versus 4.1, p=0.004) and more senescent T-cell% (median 5.3 versus 3.1, p=0.04) in the nursing home elderly. There were more CD4+ T-cell% in the nursing home elderly compared to healthy adults (median 45.5 versus 37.1, p=0.001). Nursing home elderly had a higher CD4+/CD8+ ratio than healthy adults (2.6 versus 1.9, p=0.048), higher T-reg% (median 1.8 versus 0.8, p<0.001) and increased mature NK cell% (median 12.1 versus 5.4, p=0.001) compared to healthy adults.

Conclusion: Differences in naïve CD8+ T-cells, terminally differentiated and senescent CD8+ T-cells, T-regs and NK cell subsets were similar to studies involving community dwelling elderly. In contrast, the CD4+/CD8+ ratio was higher in nursing home elderly.

Key Words: Immunosenescence, aging, immune phenotypes, nursing home elderly.

Introduction

Dysfunctional changes to the immune system that arise with age are termed immunosenescence [1]. Although all components of the immune system are affected by aging, changes to T-cells are by far the most pronounced [1]. Changes include a reduction in the number of naïve T-cells due to thymic involution, and an increase in CD8+ memory T-cells subsets including an accumulation of terminally differentiated memory T-cells (CD8+CD45+CCR7-) [2] and senescent cells (CD8+CD28-) [3, 4]. These changes have led to the identification of immune biomarkers; senescent T-cells were predictive of influenza vaccine failure in two community dwelling elderly cohorts [5, 6] and an immune risk profile characterized by a CD4+/CD8+ ratio less than 1 (due to the expansion of the CD8+ T-cell pool relative to CD4+ T-cell pool) was predictive of both mortality and chronic infection with cytomegalovirus (CMV) in aging Swedish cohorts [4, 7-9].

The nursing home elderly are a group that are at high risk of infection, vaccine failure and mortality, and it is postulated that immunosenescence is associated with this increased risk [10]. However, immune phenotypes in this group have not been well characterized, as most immunosenescence studies have excluded the nursing home elderly [10]. Three studies have investigated the immune phenotype of peripheral blood mononuclear cells (PBMCs) in the nursing home elderly [3, 11, 12]; two of these reports contained an elderly population composed of community dwelling and nursing home elderly and did not report specifically on the latter group [11, 12], whereas the other study enrolled exclusively nursing home elderly [3]. The overall observations from these

studies concluded that the nursing home elderly displayed immunosenescent phenotypes similar to the community dwelling elderly. However, it remains unclear whether immune phenotypes in the nursing home elderly are influenced by frailty and malnutrition; two conditions that are present in the nursing home elderly [13, 14] and thought to influence immunosenescence [15-17]. Furthermore, the changes to a separate class of T-cell, the regulatory CD4+ T-cell (T-reg) and to the innate immune natural killer (NK) cell subsets have not yet been described in the nursing home elderly. The goal of this study therefore was to characterize T-cell and NK cell subsets within the nursing home elderly as a precursor to the identification of candidate immune biomarkers.

To this end, we examined circulating CD4+ and CD8+ T-cells and T-cell subsets (naïve, central memory, effector memory, terminally differentiated and senescent T-cells), T-regs and NK cell subset (immature, mature and senescent NK cells) in the nursing home elderly compared to healthy adults, and explored how individual immune phenotypes were influenced by age, sex, frailty and nutritional status in the nursing home elderly.

Methods

Subjects and Setting

Elderly participants were recruited from four nursing homes in Hamilton, Ontario between September 2010 and December 2011. Nursing home residents ≥65 years were eligible. Residents on immunosuppressive medications (including cancer chemotherapy, oral corticosteroid use >21 days, methotrexate, post-transplant medications and/or anti-

cytokine or B-lymphocyte depletion therapies) were excluded, as were participants affected by serious diseases with very poor short-term prognosis (as determined by the supervising physician). Written informed consent was obtained for all participants. A convenience sample of healthy adult participants (≥18 years and <65 years) were recruited from McMaster University in Hamilton, Ontario between September and December 2011. Healthy adults were laboratory students and staff who responded to an advertised request by the study investigators for participants. The study had Research Ethics Board approval from Hamilton Health Sciences and McMaster University Health Sciences ethics boards.

A research nurse abstracted baseline demographics from the nursing home elderly based on participant interview, examination and chart review. Frailty was rated according to the Clinical Frailty Scale, an 8-point scale ranging from very fit (1) to very severely frail (8), which has been validated in the nursing home population [13]. Nutritional status was assessed using the Mini Nutritional Assessment which categorized residents as having normal nutritional status, being at risk of malnutrition or malnourished [14].

Whole Blood Analysis and Flow Cytometry

Whole blood was obtained from participants between 7am and 10am and hand delivered to the research laboratory for immediate processing. An aliquot of whole blood was used for phenotyping. T-cell phenotypes were determined by staining PBMCs in round-bottom 96-well plates with anti-CD3-Qdot605, anti-CD8-Alexa Flour 700, anti-CD4-Pacific Blue, anti-CD45RA-PE Texas Red, anti-CD28-PE, anti-CD57-FITC, anti-

CCR7-PE Cy7. T-regs were identified using anti-CD3-FITC, anti-CD4-Pacific Blue, anti-CD127-PerCP-Cy5.5, anti-CD25-PE, and anti-FoxP3-AlexaFluor700. NK phenotypes were determined by staining PBMCs with anti-CD3-FITC, anti-CD56-PECy7 and anti-CD16-AlexaFluor700. The following antibodies were purchased from BD Bioscience: anti-CD4-Pacific Blue, anti-CD28-PE, anti-CCR7-PE-Cy7, anti-CD25-PE, anti-CD56-PE-Cy7, and anti-CD16-AlexaFluor700. The following antibodies were purchased from eBioscience: anti-CD3-FITC, anti-CD127-PerCP-Cy5.5, anti-FoxP3-AlexaFluor700. The anti-CD3-Qdot605 was purchased from Invitrogen. The anti-CD57-FITC and anti-CD45RA-PE-TexasRed antibodies were purchased from Beckman Coulter. We defined the T-cell subsets as follows: naïve (CD45+CCR7+), central memory (CD45-CCR7+), effector memory (CD45-CCR7-), terminally differentiated (CD45+CCR7-) and senescent (CD28-CD57+). T-regs were defined as CD4+CD25hiCD127loFoxp3+. NK cells were determined based on CD16 and CD56 expression. $CD56^{bright}$CD16- were classified as immature NK cells, $CD56^{dim}$CD16+ were classified as mature NK cells, and a recently described NK cell subset, $CD56^{dim}$CD16-, was classified as senescent NK cells [18]. CD4+ and CD8+ immune phenotypes, T-regs and NK cell subsets were expressed as a percentage of total lymphocytes. Antibody staining was performed using a Beckman Coulter Biomek NX^{p} Laboratory Automation Workstation (Beckman Coulter, Ontario) as described in our recent publication [19], followed by analysis using an LSR II flow cytometer with a high-throughput sampler (BD Biosciences, NJ, USA), and data was analyzed using FlowJo 9.6 (Treestar Inc, Ashland, OR).

PBMCs were isolated and frozen using a validated common standard operating procedure [20]. PBMCs were thawed and CMV-reactive T-cells were identified by stimulating PBMCs with a pool of overlapping peptides spanning the immunodominant pp65 protein of CMV (PepTivator pp65, Miltenyi Biotec) according to our published protocols [21]. Briefly, thawed PBMCs were cultured overnight at 37°C and stimulated with CMV peptides (2 ug/ml) for 1 hr at 37°C. A matched set of PBMCs were stimulated with DMSO as a negative control. Brefeldin A (BD Biosciences) was then added according to the manufacturer's instructions and the cells were incubated for an additional 4 hours. The cells were stained with anti-CD4-PacificBlue and anti-CD8-AlexaFluor700, permeabilized and finally stained with anti-IFN-γ-APC, anti-TNF-α-FITC and anti-CD3-QDot605. CMV-reactive T-cells were identified as CD3+ (CD4+ or CD8+) IFN-γ+ TNF-α+.

Data analysis

Many of the immune phenotype distributions were skewed thus the results were summarized as medians and interquartile ranges (IQR). Non-parametric tests of significance were used including Mann-Whitney U, Kruskal-Wallis and Spearman's rank correlation as appropriate. All statistics were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois).

Results

Demographics

In total, 262 nursing home elderly were enrolled; ages ranged from 65 – 98 years, median age was 87 years (IQR 82-91) and 81% were female. The majority (94%) had at least one co-morbidity, and over half (52%) had a diagnosis of dementia (Table 1). Sixty percent of the participants scored 5 or 6 on the Clinical Frailty Scale, categorizing them as either mildly frail or moderately frail [13](Table 1) and the median frailty according to the Clinical Frailty Scale was 6 (IQR 5-6), [13]. Sixteen healthy adults were enrolled; median age was 31 (IQR 27-36) and 50% were female (Table 1).

Description of Immune Phenotypes

There was no significant difference between the medians of CD8+ T-cell% in the nursing home elderly and healthy adults (median 17.1 versus 18.0, p=0.56), however there was a significantly lower naïve CD8+ T-cell% median in the nursing home elderly compared to healthy adults (median 0.9 versus 5.2, p<0.001), significantly higher terminally differentiated CD8+ T-cell% median in the nursing home elderly compared to healthy adults (median 7.3 versus 4.1, p=0.004) and significantly higher CD8+ senescent T-cell% median in the nursing home elderly group when compared to the healthy adults (median 5.3 versus 3.1, p=0.04)(Table 2).

There was a significantly higher CD4+ T-cell% median in the nursing home elderly compared to healthy adults (median 45.5 versus 37.1, p=0.001)(Table 2). The overall increase in CD4+% appeared due to higher central memory T-cell% and effector

memory T-cell% in the nursing home elderly when compared to healthy adults (central memory% median 12.6 versus 8.4, $p=0.003$ and effector memory% median 18.6 versus 12.3, $p<0.001$) but not the terminally differentiated memory CD4+ T-cell% in the nursing home elderly when compared to healthy adults (median 1.8 versus 1.2, $p=0.09$)(Table 2). In the nursing home elderly, females had higher median CD4+% than males (median 46.1 versus 42.1, $p=0.02$), higher median naïve CD4 T-cell% (median 10.1 versus 7.1, $p=0.04$) and higher median terminally differentiated CD4+ T-cell% (median 1.9 versus 1.4, $p=0.03$)(Table 3)

There was significantly higher median T-reg% in the nursing home elderly group when compared to the healthy adults (median 1.8 versus 0.8, $p<0.001$)(Table 2). In the nursing home elderly, there were significant differences in median T-regs% across frailty ($p<0.001$) and nutritional status ($p<0.001$)(Table 3).

When the CD4+/CD8+ T-cell ratios were compared, the nursing home elderly had a higher CD4+/CD8+ T-cell ratio than the healthy adults (median CD4+/CD8+ ratio 2.6 versus 1.9, $p=0.048$)(Table 2). In the nursing home elderly, the CD4+/CD8+ ratio ranged from 0.42 – 22 and only 17 (6.5%) had a CD4+/CD8+ ratio <1.0. This was in contrast to a far narrower range of values in the healthy adults (CD4+/CD8+ ratio range 1.1 – 3.5). In the nursing home elderly, age, sex, frailty and nutritional status were not associated with changes to the CD4+/CD8+ ratio (Table 3).

There was an increase in the mature NK cell% in the nursing home elderly compared to the healthy adults (median 12.1 versus 5.4, $p=0.001$) and no significant difference between senescent NK cell% (median 1.4 versus 1.7, $p=0.27$) or immature NK

cell% (median 0.2 versus 0.3, p=0.57) (Table 2). In the nursing home elderly, higher senescent NK cell% was associated with increasing frailty (p<0.001) and worsening nutrition (p=0.01)(Table 3). Age, sex, frailty and nutrition did not appear to have a significant association with mature or immature NK cells (Table 3).

Of the 262 nursing home elderly enrolled in the study, 242 had PBMCs available for assessment of T-cell immunity to CMV. In total, 217/242 (90%) of individuals had evidence of prior CMV infection.

Discussion

In this crosssectional study in the nursing home elderly, we observed 1) lower naïve CD8+ T-cells and higher terminally differentiated and senescent CD8+ T-cells; 2) higher CD4+ T-cells driven by central and effector memory CD4+ T-cells; 3) higher CD4+/CD8+ T-cell ratio; 4) higher T-regs; and 5) higher mature NK cells and when compared to healthy adults.

The lower naïve CD8+ T-cells and higher terminally differentiated CD8+ T-cells seen in this study in the nursing home elderly has been well described in community dwelling elderly [2, 3, 22]. These differences are consistent with existing dogma, that the remodelling of the T-cell compartment seen with advanced age is due to a decrease in naïve T-cells due to thymic involution and an increase in terminally differentiated T-cells, possibly due to chronic antigenic stimulation from CMV [1]. Indeed, 90% of the elderly nursing home residents in our study had evidence of CMV immunity, consistent with

prior studies where infection with CMV is estimated to be prevalent in >85% of those aged over 80 years [23]. The reduction of naïve T-cells is thought to impair the ability of the host to respond against novel pathogens [10, 24] and the terminally differentiated memory T-cells are considered to have poor functionality resulting in impaired responses to recall antigens [24], supporting the hypothesis that these immune changes increase the risk of infection and mortality in the elderly.

Higher senescent CD8+CD28- T-cells have been associated with advancing age [3, 4], influenza vaccine failure [5, 6] and CMV infection [4]. In this study, there was a higher median senescent CD8+ T-cells in the nursing home elderly when compared to healthy adults. The one prior immunosenescence study performed in the nursing home elderly also reported higher senescent T-cells when compared to healthy adults [3].

The described differences in CD4+ T-cells in the elderly have been far more inconsistent than for CD8+ T-cells. Studies have described both higher CD4+ T-cells [25, 26] and lower CD4+ T-cells [2, 4, 9, 27, 28]. Two studies found lower CD4+ T-cells in malnourished elderly [16, 17] and the one study that enrolled exclusively nursing home residents showed no change at all [3]. It is possible, that the higher CD4+% seen in our study is partially explained by the predominance of females in the nursing home group (81%), as females had higher CD4% than men, a finding consistent with the literature [25]. The high proportion of females enrolled in our study in the nursing home elderly group was expected given that nursing homes in Canada are comprised of approximately 70% females [29]. Our CD4+T-cell subset results, that the higher circulating CD4+ T-cells in the nursing home elderly was due to higher central memory and effector memory

T-cells, are consistent with another immunosenescence study that enrolled community elderly and used newer immune phenotyping better able to delineate the naïve and memory T-cells pools [2].

The expanded CD4+ T-cell compartment in the nursing home elderly in our study is likely why the CD4+/CD8+ T-cell ratio was higher in the nursing home elderly group than the healthy elderly and only 6% of the nursing home elderly had a CD4+/CD8+ ratio <1.0. This is in contrast to the Swedish OCTO (n=102) and NONA (n=138) cohorts where 14% and 20% of the cohorts had CD4+/CD8+ ratios <1.0 [4, 7]. Our results are very similar to another study that included exclusively nursing home elderly (n=116); the CD4+/CD8+ ratio was 2.0 in the elderly and 1.7 in the young cohort (the difference was not significant, p=0.64)[3]. In a Spanish study that enrolled 151 elderly people ≥65 years only 7.9% of the cohort had a CD4+/CD8+ T-cell ratio <1.0 [30]. The reason for the discrepancy in results among the studies is not clear, but could relate to differences in population. First, only 34% in the NONA cohort were in institutional care [4] versus 100% in our study. Second, the proportion of females enrolled could influence the CD4+/CD8+ ratio given the noted association between female sex and CD4+%. Both our study and the other study that included exclusively nursing home elderly were comprised of approximately 80% females [3] whereas the OCTO and NONA studies consisted of 65% and 70% females, respectively [31]. Last, the OCTO and NONA studies were conducted in Sweden whereas this and the other nursing home study are North American [3]. Indeed, 8% of younger adults in Sweden (ages 20-59) have a CD4+/CD8+ T-cell ratio <1.0 [31]. The NONA cohort has also demonstrated that at very advanced ages (>90

years), it is possible to move from having a CD4+/CD8+ T-cell ratio <1.0 to above 1.0, which is another possible reason why our study had so few people with CD4+/CD8+ T-cell ratios <1.0 [8]. Our findings raise the possibility that CD4+/CD8+ ratios <1.0 may not be predictive of increased mortality in the nursing home elderly, particularly given that this ratio tended to increase in residents with severe frailty.

The accumulation of T-regs has been consistently observed in studies of aging involving the community elderly [32]. How higher T-regs could lead to impairment of host control of infection is not clear, but may be secondary to a decreased ability of aged T-regs to proliferate and produce cytokines [33]. It is interesting to note that the median T-regs% was lower in healthy adults when compared to nursing home elderly, but also in the very frail and the malnourished nursing home elderly. To our knowledge, no other study has reported on the relationship between T-regs and frailty or nutrition. Further study into the relationship between T-regs and human immunosenescence is required.

The higher mature NK subset seen in the nursing home elderly in our study is consistent with immunosenescence studies including community dwelling elderly [34, 35]. These results support the observation that the increase in NK cells seen in the elderly is due to accumulation of mature NK cells. We speculate that the higher numbers of mature NK cells could be due to the same mechanism leading to higher numbers of memory T-cells in the elderly; chronic antigenic stimulation [1]. Although the medians of the recently described senescent NK cells [18] did not differ between the nursing home elderly and the healthy adults, there was higher senescent NK cell medians in those with advancing frailty and worsening nutrition.

Although our study has many strengths including a large sample size of nursing home residents and detailed clinical information including frailty and nutritional status as well as comprehensive immune phenotyping, limitations of our study include the small size of the comparator group, which may have increased our risk of not finding a difference between the two groups when one exists (Type II error). However our control group size was comparable to at least four other similar studies where the control group size ranged from 15 to 21 adults [3, 36-38] and our findings were similar to studies involving community dwelling elderly, increasing the confidence in our results. In addition, due to our study design, we are unable to conclude that the observed differences in immune phenotypes between the nursing home elderly and healthy adults are necessarily associated with aging. However, the goal of this study was not to determine associations, but to characterize differences in T-cell and NK cell subsets in the nursing home elderly, to aid in the identification of immune biomarkers in future studies involving the nursing home elderly.

Conclusion

Differences in naïve CD8+ T-cells, terminally differentiated CD8+ T-cells, T-regs and NK cells subsets in the nursing home elderly were similar to studies involving community dwelling elderly. However, the CD4+/CD8+ T-cell ratio was higher in our study population. These results will aid future studies designed to investigate immune biomarkers predictive of infection, vaccine failure and mortality in the nursing home elderly.

Acknowledgements

Conflict of Interest

All authors report no conflict of interest.

Author Contributions

JJ, ML and JB were responsible for the conception and design of the study, acquisition of data, analysis and interpretation of data. JM, AL, CV, SW and PD aided in data analysis and interpretation of data. JJ drafted the initial manuscript and JM, AL, CV, SW, PD, JB and ML critically revised the manuscript for intellectual content. All authors approved the final version of the manuscript.

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Sponsor's Role

The sponsor had no role in the design, methods, subject recruitment, data collections, analysis and preparation of paper.

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Table 1: Baseline characteristics of the nursing home elderly and healthy adults.

Baseline Characteristics	Nursing home elderly n=262 n (%)	Healthy adults n=16 n (%)
Age (years)		
18-24	-	2 (13)
25-34	-	9 (56)
35-44	-	4 (25)
45-54	-	1 (6)
55-64	-	0 (0)
65-74	21 (8)	-
75-84	73 (28)	-
85-94	147 (56)	-
≥95	21 (8)	-
Sex (F)	213 (81)	8 (50)
Any co-morbidity	245 (94)	0 (0)
Diabetes	75 (29)	-
Stroke	56 (21)	-
Heart failure	36 (14)	-
Coronary artery disease	81 (31)	-
Cancer	21 (8)	-
COPD	30 (11)	-
Dementia	137 (52)	-
≥5 medications	229 (87)	0 (0)
Nutritional status		
At risk of malnutrition	121 (46)	0 (0)
Malnourished	13 (5)	0 (0)
Frailty		
4	64 (24)	0 (0
5	62 (24)	0 (0)
6	94 (36)	0 (0)
7	38 (15)	0 (0)
8	4 (1.5)	0 (0)

Table 2: Immune phenotypes in the nursing home elderly compared to healthy adults.

Immune phenotype	Nursing home elderly Median (IQR) n=262	Healthy adults Median (IQR) n=16	p-value*
CD8+			
CD8%	17.1 (11.9 – 23.9)	18.0 (15.1 – 21.0)	0.56
Naïve CD8+%	0.90 (0.5 – 1.4)	5.2 (3.8 – 7.9)	<0.001
Central memory CD8+%	0.6 (0.3 – 1.0)	0.5 (0.4 – 0.8)	0.86
Effector memory CD8+%	6.3 (3.9 – 9.4)	8.1 (6.6 – 10.1)	0.14
Terminally differentiated CD8+%	7.3 (3.5 – 12.5)	4.1 (2.0 – 6.0)	0.004
Senescent CD8+%	5.3 (2.2 – 9.4)	3.1 (0.7 – 6.2)	0.04
CD4+			
CD4%	45.5 (38.0 - 52.7)	37.1 (29.7 - 43.1)	0.001
Naïve CD4+%	9.2 (5.1 – 13.7)	11.6 (7.1 - 15.4)	0.19
Central memory CD4+%	12.6 (9.1 - 16.2)	8.4 (6.5 - 13.2)	0.003
Effector memory CD4+%	18.6 (14.1 - 22.6)	12.3 (11.8 - 15.8)	<0.001
Terminally differentiated CD4+%	1.8 (1.1 – 2.8)	1.2 (0.6 - 2.1)	0.09
Senescent CD4+%	1.4 (0.2 - 3.4)	0.8 (0.1-2.0)	0.21
Treg			
T-reg%	1.8 (1.1 – 2.6)	0.8 (0.6 – 0.9)	<0.001
Ratio			
CD4:CD8 ratio	2.6 (1.7 – 4.1)	1.9 (1.6 – 2.6)	0.048
NK			
Mature NK cell%	12.1 (7.9 – 16.6)	5.4 (2.7 – 10.0)	0.001
Senescent NK cell%	1.4 (0.89 – 2.2)	1.7 (1.5 – 2.6)	0.27
Immature NK cell%	0.2 (0.1 – 0.4)	0.3 (0.04 – 0.7)	0.57

*Mann-Whitney U test of significance used

Table 3: Immune phenotypes as a function of age, sex, frailty and nutritional status.

Immune phenotype	Age Correlation+ (r) n=262	Sex Median (IQR)) n=262++		Frail Scale Median (IQR) n=262+++				Nutritional Status Median (IQR) n=261a+++		
		Female n=213	Male n=49	4 n=64	5 n=62	6 n=94	7 or 8 n=42	Normal n=127	At risk n=121	Mal-nourished n=13
CD8+										
CD8%	0.05	17.1 (11.8-24.2)	17.4 (12.8-22.2)	18.6 (13.7-23.8)	17.6 (12.3-21.9)	16.3 (11.7-24.1)	16.3 (9.7-26.8)	17.4 (12.2-23.8)	17.1 (11.7-24.2)	17.1 (11.2-20.9)
Naïve CD8+%	-0.13*	0.9 (0.5-1.5)	0.8 (0.4-1.2)	0.9 (0.5-1.4)	0.8 (0.5-1.4)	1.0 (0.5-1.4)	1.2 (0.5-2.1)	0.9 (0.5-1.4)	1.0 (0.58-1.6)	0.7 (0.3-1.2)
Central memory CD8+%	-0.03	0.6 (0.3-1.0)	0.5 (0.3-0.8)	0.5 (0.3-0.9)	0.5 (0.3-0.9)	0.6 (0.3-1.0)	0.7 (0.3-1.5)	0.5 (0.3-0.9)	0.7 (0.3-1.1)	0.5 (0.4-0.9)
Effector memory CD8+%	-0.04	6.1 (3.8-9.5)	7.1 (4.5-9.7)	6.7 (4.1-9.9)	6.3 (4.1-8.7)	6.3 (3.7-10.0)	5.7 (4.0-8.6)	6.5 (3.8-9.9)	6.0 (3.9-8.8)	6.0 (4.0-10.9)
Terminally differentiated CD8+%	0.08	7.8 (3.6-12.6)	5.9 (3.3-12.5)	8.9 (4.2-13.3)	7.6 (3.7-12.8)	6.9 (3.4-11.0)	6.1 (2.4-12.9)	8.1 (3.5-12.7)	7.2 (3.6-12.5)	5.8 (3.1-10.9)
Senescent CD8+%	0.04	5.1 (2.3-8.9)	5.7 (2.0-11.3)	6.2 (2.8-11.4)	5.0 (2.3-9.3)	5.1 (2.2-7.9)	4.9 (0.8-10.0)	5.1 (2.2-10.0)	5.2 (2.3-8.6)	5.6 (1.2-9.5)
CD4+										
CD4%	-0.06	46.1* (39.1-53.8)	42.1* (34.7-49.1)	44.9 (38.6-53.6)	45.8 (37.1-52.6)	46.4 (40.4-53.1)	43.1 (32.4-52.7)	46.1 (38.7-52.6)	45.8 (36.1-53.5)	42.0 (35.9-44.9)
Naïve CD4+%	0.05	10.1* (5.4-14.6)	7.1* (4.6-11.5)	10.5 (5.4-18.0)	9.2 (5.3-14.7)	9.3 (5.4-13.1)	7.2 (4.0-12.2)	10.4* (5.4-16.1)	8.7* (4.8-12.5)	5.9* (3.4-9.9)
Central memory CD4+%	-0.08	12.8 (9.6-16.4)	11.7 (8.7-14.6)	12.5 (9.0-15.4)	12.0 (8.4-16.2)	13.3 (10.7-18.2)	12.8 (8.7-15.8)	12.5 (9.2-16.2)	13.1 (9.0-17.2)	10.7 (9.3-13.3)
Effector memory CD4+%	0.13*	18.9 (14.6-23.0)	18.2 (12.9-21.4)	19.3 (13.6-21.8)	18.1 (12.7-21.9)	19.2 (15.2-24.2)	17.0 (13.5-22.4)	18.6 (13.5-21.8)	19.0 (14.6-22.8)	18.5 (12.8-26.6)
Terminally differentiated CD4+%	0.05	1.9* (1.1-2.9)	1.4* (0.6-2.5)	1.9 (1.1-3.3)	1.8 (1.1-2.6)	1.6 (1.1-2.7)	1.8 (0.8-2.7)	1.9 (1.1-2.9)	1.6 (1.0-2.8)	1.4 (0.9-2.0)
Senescent CD4+%	0.11	1.4 (0.3-3.5)	1.3 (0.2-2.9)	1.5 (0.3-4.2)	1.4 (0.1-3.1)	1.4 (0.3-3.6)	1.2 (0.2-2.9)	1.4 (0.16-3.4)	1.3 (0.27-3.2)	2.0 (0.34-4.4)
T-reg										
T-reg%	0.08	1.8 (1.2-2.6)	1.6 (0.9-2.6)	2.5 ** (1.8-2.9)	2.0** (1.3-2.6)	1.6** (1.1-2.3)	1.0** (0.8-1.5)	2.2** (1.5-2.9)	1.6** (1.1-2.3)	1.0** (0.8-1.2)

Ratio										
CD4:CD8 ratio	-0.06	2.6 (1.7-4.5)	2.5 (1.6-3.5)	2.5 (1.6-3.8)	2.4 (1.8-4.1)	2.7 (1.9-4.5)	2.6 (1.4-5.0)	2.6 (1.7-4.1)	2.6 (1.6-4.5)	2.3 (1.8-3.3)
NK										
Mature NK cell%	-0.05	12 (8.0-16.6)	12.6 (7.1-17)	12.0 (8.3-6.3)	11.3 (7.1-15.2)	12.3 (7.3-16.7)	12.7 (8.5-17.2)	12.3 (8.7-16.0)	11.6 (6.8-16.5)	14.7 (7.9-18.4)
Senescent NK cell%	-0.10	1.4 (0.9-2.2)	1.5 (1.0-2.4)	1.0** (0.7-1.9)	1.1** (0.7-1.9)	1.7** (1.0-2.7)	2.0** (1.3-2.7)	1.3* (0.74-2.1)	1.6* (1.0-2.7)	1.4* (1.1-2.3)
Immature NK cell%	0.03	0.2 (0.1-0.4)	0.3 (0.1-0.5)	0.2 (0.1-0.4)	0.21 (0.1-0.3)	0.3 (0.2-0.4)	0.3 (0.2-0.4)	0.2 (0.2-0.4)	0.2 (0.1-0.4)	0.2 (0.13-0.45)

*p≤0.05
**p≤0.001
[a]One patient excluded as nutritional status unknown
[+]Spearman's rank test of significance used
[++]Mann-Whitney U test of significance used
[+++]Kruskal-Wallis test of significance used

CHAPTER 3

Thesis manuscript 2: *Immune Biomarkers Predictive of Respiratory Viral Infection in Elderly Nursing Home Residents*

PREFACE TO CHAPTER 3

In this chapter, we identified immune phenotypes predictive of risk of respiratory viral infection during the ensuing winter respiratory viral season using a Cox proportional hazards model adjusted for age, sex, frailty status and clustering at the level of the nursing home. In multivariable analysis, high CMV-reactive CD4+ T-cells were associated with an increased risk of respiratory viral infection and high T-regs were associated with a reduced risk of respiratory viral infection.

The student contribution to this study included study conception, data cleaning, data preparation, statistical analysis and drafting the manuscript. Co-authors include Robin Parsons, Fern Botelho and Jamie Millar who analyzed the laboratory peripheral blood mononuclear cell (PBMC) specimens under the supervision of Dr. Bramson; Drs. McNeil, Fulop and McElhaney were local site investigators and responsible for recruiting participants and coordinating laboratory specimens and provided critical review of the manuscript; Dr. Andrew provided the Clinical Frailty Scale and its interpretation and use throughout the study and critically reviewed the manuscript; Dr. Walter provided statistical expertise and provided critical review of the manuscript; Dr. Devereaux criticalled reviewed the manuscript; Mehrnoush Malekesmaeili provided interpretation of the PBMC flow cytometry and created Supporting Information Figure 1, and was supervised by Dr. Brinkman; Dr. Mahony's laboratory performed all respiratory virus testing and critically reviewed the manuscript; and Drs. Loeb and Bramson were involved in conception of the study, aided in data interpretation, and provided critical review of the manuscript. Dr. Bramson created Figures 1, 2 and 3. Dr. Loeb provided funding support

for the study.

This manuscript was submitted to PLOS one on May 22, 2014 and re-submitted with revisions on July 25, 2014. We are currently awaiting a decision regarding acceptance. The citation is:

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I presented the results, in part, as a poster session at the Association of Medical Microbiology and Infectious Disease Canada annual conference, April 3-5, 2014 in Victoria, British Columbia. The published abstract citation is:

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Immune Biomarkers Predictive of Respiratory Viral Infection in Elderly Nursing Home Residents

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Abstract

Objective: To determine if immune phenotypes associated with immunosenescence predict risk of respiratory viral infection in elderly nursing home residents.

Methods: Residents ≥65 years from 32 nursing homes in 4 Canadian cities were enrolled in Fall 2009, 2010 and 2011, and followed for one influenza season. Following influenza vaccination, peripheral blood mononuclear cells (PBMCs) were obtained and analysed by flow cytometry for T-regs, CD4+ and CD8+ T-cell subsets (CCR7+CD45RA+, CCR7-CD45RA+ and CD28-CD57+) and CMV-reactive CD4+ and CD8+ T-cells. Nasopharyngeal swabs were obtained and tested for viruses in symptomatic residents. A Cox proportional hazards model adjusted for age, sex and frailty, determined the relationship between immune phenotypes and time to viral infection.

Results: 1072 residents were enrolled; median age 86 years and 72% female. 269 swabs were obtained, 87 were positive for virus: influenza (24%), RSV (14%), coronavirus (32%), rhinovirus (17%), human metapneumovirus (9%) and parainfluenza (5%). In multivariable analysis, high T-reg% (HR 0.41, 95% CI 0.20–0.81) and high CMV-reactive CD4+ T-cell% (HR 1.69, 95% CI 1.03–2.78) were predictive of respiratory viral infection.

Conclusions: In elderly nursing home residents, high CMV-reactive CD4+ T-cells were associated with an increased risk and high T-regs were associated with a reduced risk of respiratory viral infection.

Key words: Immunosenescence; immune biomarker; respiratory viral infection; nursing home; frailty.

Introduction

The burden of respiratory viral infection in elderly nursing home residents is high [1]. With active surveillance the incidence of respiratory viral infection is estimated to range from 1.4 – 2.8 per 1000 resident days [2]. Influenza and respiratory syncytial virus (RSV) are the viruses commonly responsible for morbidity and mortality associated with infection, but other respiratory viruses including parainfluenza, human metapneumovirus, coronavirus and rhinovirus can also cause severe disease in this population [1, 3-7]. It is a widely held belief that immunosenescence, the waning of immune function associated with old age, is responsible for this increased risk and severity of infection [8]; however, only sparse data exist to substantiate this position [9].

As a first step towards the identification of immune biomarkers predictive of respiratory viral infection in elderly nursing home residents, we characterized immune phenotypes in elderly nursing home residents [10]. Whole blood analysis of circulating CD4+ and CD8+ T-cell subsets was performed in a cross-sectional study involving 262 nursing home elderly participants and immune phenotypes were compared to immune phenotypes from healthy adults. In addition, we explored how individual immune phenotypes were influenced by age, sex, frailty and nutritional status in the nursing home elderly [10]. We observed lower naïve CD8+ T-cells (CD8+CD45RA+CCR7+) and higher terminally differentiated memory T-cells (CD8+CD45RA+CCR7-) and senescent T-cells (CD8+CD28-) when compared to healthy adults [10], consistent with prior findings in elderly people [11-14]. It is hypothesized that the reduced numbers of naïve CD8+ T-cells observed in the elderly due to thymic involution, coupled with an

accumulation of poorly functioning terminally differentiated memory T-cells and senescent cells possibly arising from chronic antigenic stimulation by cytomegalovirus (CMV) [15, 16], predisposes elderly people to infection [17].

Supporting this hypothesis, senescent CD8+ T-cells and high titres of CMV antibody have been found to be associated with influenza vaccine failure in older people [18-20]. Whether these same CD4+ T-cell subsets are associated with infection is less clear. The accumulation of a separate class of T-cell, the regulatory CD4+ T-cell (T-regs) in elderly people has also been observed in elderly nursing home residents [10] and community dwelling elderly people [21]. While T-regs are known to be responsible for controlling the magnitude of CD4+ and CD8+ T-cell responses to viral infections [22], whether the accumulated T-regs in the elderly lead to impairment of host control of infection is not known.

To our knowledge, the relationship between immune phenotypes associated with immunosenescence and risk of respiratory viral infection has not been studied. If a relationship is established, this could help identify elderly nursing home residents at highest risk of become ill and could provide more focused care through targeted prevention. To this end, we sought to identify immune biomarkers predictive of respiratory viral infection during the ensuing respiratory viral season, in an elderly nursing home cohort.

Methods

Subjects and Setting

In this prospective cohort study, elderly participants were recruited from 32 nursing homes in 4 Canadian cities (Halifax, Nova Scotia, Sherbrooke, Quebec, Hamilton, Ontario and Vancouver, British Columbia) in September and October 2009, 2010 and 2011. Residents recruited for a separate study [10] were also eligible for inclusion in this study. Residents ≥65 years of age were eligible for the study. Exclusion criteria included individuals: not planning to be vaccinated against influenza, receiving immunosuppressive medications (including cancer chemotherapy, oral corticosteroid use >21 days, methotrexate, post-transplant medications and/or anti-cytokine or B-lymphocyte depletion therapies), or expected to die within 30 days, as determined by the supervising physician. Written informed consent was obtained from all participants or their legally appointed guardian in the event they were not competent to provide consent themselves. The study protocol was approved by the Research Ethics Board at each participating institution and nursing home.

Trained research personnel abstracted baseline demographics from the participants based on an interview, examination and chart review. Frailty was rated according to the Clinical Frailty Scale, an 8-point scale ranging from 1-8 as follows: (1) very fit, (2) well, (3) well with treated comorbid illness, (4) apparently vulnerable, (5) mildly frail defined as dependence in instrumental activities of daily living (ADL), (6) moderately frail defined as required assistance with basic ADL, (7) severely frail defined as completely dependent on others for ADL and (8) very severely frail [23]. The Clinical Frailty Scale

has been validated in the nursing home population [23]. Participants received the seasonal influenza vaccine, typically in October or November, by public health nurses in accordance with guideline recommendations for the given year [24-26]. Peripheral blood mononuclear cells (PBMCs) were drawn from participants 21 days post vaccination.

Residents were actively followed by research staff for the influenza season immediately following the PBMC draw. The influenza season was defined as spanning from the first week ≥5% of specimens submitted to the local public health laboratory for viral testing were positive for influenza and ending when <5% were positive for influenza for 2 consecutive weeks. The influenza season was chosen as the period of follow-up as the rate of respiratory viral infection is highest during the winter months [2]. Trained research personnel reviewed each participant for the presence of symptoms or signs of respiratory illness twice weekly or more often if notified of symptoms by nursing home staff. Nasopharyngeal swabs (Copan ESwab™, Copan Diagnostics Inc., Murrieta, California) were obtained by the research staff when a resident had one or more of the following new symptoms or signs: fever (≥ 38° Celsius), worsening cough, nasal congestion, sore throat, headache, sinus problems, muscle aches, fatigue, ear ache or infection, chills, not otherwise explained by an alternative diagnosis.

Peripheral Blood Mononuclear Cell Analysis and Flow Cytometry

Blood was obtained from participants between 0700 and 1000 hours and hand delivered to the research laboratory for immediate processing. PBMCs were isolated and frozen using a validated common standard operating procedure [27].

T-cell immune phenotypes were determined by thawing patient PBMCs as previously described [28]. Viability of the PBMCs was found to range between 87% and 98% and the average viability was 94.6%. An aliquot (0.5–16x10^6 cells/stain) was placed in round-bottom 96-well plates with anti-CD3-Qdot605, anti-CD8-Alexa Flour 700, anti-CD4-Pacific Blue, anti-CD45RA-PE Texas Red, anti-CD28-PE, anti-CD57-FITC, anti-CCR7-PE Cy7. T-regs were identified using anti-CD3-FITC, anti-CD4-Pacific Blue, anti-CD127-PerCP-Cy5.5, anti-CD25-PE, and anti-FoxP3-AlexaFluor700. The following antibodies were purchased from BD Bioscience: anti-CD4-Pacific Blue, anti-CD28-PE, anti-CCR7-PE-Cy7 and anti-CD25-PE. The following antibodies were purchased from eBioscience: anti-CD3-FITC, anti-CD127-PerCP-Cy5.5, anti-FoxP3-AlexaFluor700. The anti-CD3-Qdot605 was purchased from Invitrogen. The anti-CD57-FITC and anti-CD45RA-PE-TexasRed antibodies were purchased from Beckman Coulter. We defined the T-cell subsets as follows: naïve (CD45RA+CCR7+), terminally differentiated (CD45RA+CCR7-) and senescent (CD28-CD57+). T-regs were defined as CD4+CD25^{hi}CD127^{lo}Foxp3+. CD4+ and CD8+ immune phenotypes and T-regs were expressed as a percentage of CD3+. Antibody staining was performed using a Beckman Coulter Biomek NX^{p} Laboratory Automation Workstation (Beckman Coulter, Ontario) as previously described [29], followed by analysis using an LSR II flow cytometer with a high-throughput sampler (BD Biosciences, NJ, USA). T-regs were analyzed using FlowJo 9.6 (Treestar Inc, Ashland, OR). T-cell subset analysis employed an automated gating pipeline using the flowDensity algorithm [30]. This approach uses customized threshold calculations for the different cell subsets to mimic a manual gating scheme

based on expert knowledge of hierarchical gating order and 1D density information. Population identification is individually tuned to each cell population in a data driven manner. T-cell subpopulations were identified using characteristics of their density distribution such as the number of peaks, height and width of each peak, change of the slope in the distribution curve, standard deviation, and median density (Supporting Information). CD45RA thresholds were estimated based on control samples, which where then applied automatically to stimulated samples. CCR7 thresholds were estimated based on CD57+ populations given the explicit instruction that CD57+ cells are CCR7- (Supporting Information). A total of 17 populations identified by this approach using high performance computing resources at the Michael Smith Genome Sciences Centre in order to reduce computational time.

CMV-reactive T-cells were identified by stimulating PBMCs with a pool of overlapping peptides spanning the immunodominant pp65 protein of CMV (PepTivator pp65, Miltenyi Biotec) according to our published protocols [28]. Briefly, thawed PBMCs were cultured overnight at 37°C and stimulated with CMV peptides (2 ug/ml) for 1 hr at 37°C. A matched set of PBMCs were stimulated with DMSO as a negative control. Brefeldin A (BD Biosciences) was then added according to the manufacturer's instructions and the cells were incubated for an additional 4 hours. The cells were stained with anti-CD4-PacificBlue and anti-CD8-AlexaFluor700, permeabilized and finally stained with anti-IFN-γ-APC, anti-TNF-α-FITC and anti-CD3-QDot605. CMV-reactive T-cells were identified as CD3+ (CD4+ or CD8+) IFN-γ+ TNF-α+.

Respiratory Virus Detection

Using 200ul of nasopharyngeal swab material, nucleic acid was extracted by the bioMerieux easyMAG automated extractor. Specimens were tested using the xTAG® Respiratory Virus Panel (RVP) assay for influenza A (subtype H1 and H3), influenza B, RSV (subtype A and B), parainfluenza (1-4), coronavirus (NL63, OC43, HKU1 and 229E), human metapneumovirus, entero-rhinovirus, and adenovirus as per the manufacturer's protocol (Luminex Molecular Diagnostics, Inc., Toronto, Ontario).

Statistical analysis

The immune phenotype distributions as well as the age distribution were skewed, and so the distributions of these continuous variables were summarized as medians and interquartile ranges (IQR). The age and sex for those who had PBMCs obtained and those who did not were compared using Mann-Whitney U and chi-square test as appropriate. Complete case analysis of immune phenotypes was planned if there was <10% missing data for each parameter [31].

Unadjusted hazard ratios (HR) and 95% confidence intervals (CIs) using Cox proportional hazards model were first constructed to explore the relationship between immune phenotypes and time to symptomatic respiratory viral infection. In the event a resident had multiple respiratory viral infections, only the first infection was included as an outcome. If a participant died prior to a respiratory viral infection, their time was censored on the date of death. We hypothesized that the following immune phenotypes associated with immunosenescence would be associated with increased risk of infection

[12-17, 21]: low CD4+ and CD8+ naïve T-cells and high CD4+ and CD8+ terminally differentiated and senescent T-cells as well as high CMV-reactive CD4+ and CD8+ T-cells and high T-regs. Low was defined as immune phenotypes in the first quartile of the distribution and high was defined as immune phenotypes in the fourth quartile of the distribution. *A priori*, it was decided that age, sex and frailty would be included in the final model, given their potential for confounding with the effects of primary interest in this population [32]. Immune phenotypes with a p-value <0.20 in univariable analysis were included in the final multivariable model. The final model was determined using backwards elimination. A sandwich variance estimator was used to account for the clustering effect at the level of the nursing homes [33]. The proportional hazards assumption for continuous variables was explored graphically by plotting partial residuals against time to event and tested by regressing the partial residuals against time. The proportional hazards assumption for categorical variables was examined by a log-minus-log graph to ensure the plotted lines remained parallel. The presence of multicollinearity was examined using the variance inflation factor (VIF); presence of multicollinearity was defined as VIF >5.

P-values and 95% CIs were constructed using 2-tailed tests. P-values <0.05 were considered statistically significant. Statistical analyses were performed using R, version 3.0.2 [34].

Results

Nursing Home Cohort

In total, 1165 residents were enrolled in the study and of these, and PBMC were obtained from 1087 (93%). Reasons for not obtaining PBMCs were either refusal of influenza vaccine or refusal of blood draw. There was no statistically significant difference in age (median, 86 (IQR 80 – 90) versus 85 (IQR 79-89) without PBMCs, p=0.42) or sex (%female, 72% versus 64% without PBMCs, p=0.12) between those that did and did not have PBMCs obtained. Fifteen participants (1%) withdrew before the end of the study, leaving 1072 as our final sample size. Seventy-three participants died before a respiratory viral infection could be identified.

Baseline Characteristics

Baseline characteristics of the final cohort are summarized in Table 1. The median age was 86 years (IQR 80 – 90 years) and the ages ranged from 65 – 102 years. Eight persons were ≥100 years. Most (93%) had at least one co-morbidity, and almost half (44%) scored either 7 or 8 on the Clinical Frailty Scale, which is defined as severely and very severely frail respectively. Figures 1, 2 and 3 describe the gating strategies used to define the immune phenotypes. The medians and corresponding IQRs for each immune cell type tested in this study can be found in Table 1.

Respiratory Virus Infection

In total, 269 swabs were obtained from 233 symptomatic people. Nasopharyngeal swabs were positive for viruses in 87 symptomatic residents (Table 2). Coronavirus (32%), influenza (24%), rhinovirus (17%) and RSV (14%) were the most common

viruses found. One nasopharyngeal swab positive for influenza A also had rhinovirus present.

Predictors of Respiratory Virus Infection

We subsequently investigated whether a relationship existed between specific immune cell populations and respiratory virus infection. The proportional hazards assumption was satisfied for all covariates included in the model and there was no concerning evidence of multicollinearity (all VIFs were <3). In univariable analyses, low naïve CD8+ T-cell% (HR 0.69, 95% CI 0.51 – 0.95) and high T-reg% (HR 0.47, 95% CI 0.26 – 0.85) were associated with a reduced risk of respiratory viral infection and high terminally differentiated CD8+ T-cell% (HR 1.57, 95% CI 1.10 – 2.24), high senescent CD8+ T-cell% (HR 1.55, 95% CI 1.11 – 2.17) and high CMV-reactive CD4+ T-cell% (HR 1.82, 95% CI 1.13 – 2.94), were associated with an increased risk of respiratory viral infection (Table 3). In multivariable analysis adjusted for age, sex and frailty, only high T-reg% (HR 0.41, 95% CI 0.20 – 0.81) and high CMV-reactive CD4+ T-cell% (HR 1.69, 95% CI 1.03 – 2.78) remained predictive of respiratory viral infection in the final model (Table 3 and Figure 4).

Discussion

In this prospective cohort study of elderly nursing home residents, CD4+ T cells, in particular T-regs and CMV-reactive CD4+ T-cells were predictive of respiratory viral

infection during the ensuing respiratory viral season in multivariable analysis. In contrast, CD8+ T-cells were not found to be predictive in multivariable analysis. To our knowledge, this is the first study to identify immune biomarkers predictive of respiratory viral infection in elderly people.

T-regs are responsible for creating the balance between the immune response to pathogens and the harmful sequelae of inflammation that arises with this response [35]. High T-regs have been consistently observed in elderly people when compared to healthy adults [10, 21], and it has been hypothesized that this shift may be associated with increased risk of infection seen in the elderly [21, 36]. It is intriguing that in our study higher levels of circulating T-regs were associated with reduced risk of symptomatic respiratory virus infection. We did not systematically test all residents in our study for respiratory virus throughout the study, so we cannot determine whether the association between high T-regs and reduced risk of infection was due to absence of infection or whether there was a higher incidence of asymptomatic infections in the high T-reg group. Little is known about the role of T-regs in preventing acute respiratory viral infection in humans [35]. In mice, a robust T-reg response has been observed during influenza [37] and RSV infection [38] and depletion of T-regs delays RSV viral clearance from lungs [38] suggesting that T-regs play an important role in controlling the immune response to respiratory viral infection. In aged mice, higher percentages of T-regs are observed at baseline and during acute influenza infection when compared to younger mice, and their presence is thought to contribute to a decrease and delay of CD8+ T-cell response during acute influenza infection [39]. In consideration of the murine data, we speculate that

elevated levels of T-regs may suppress immune pathology associated with anti-viral immunity.

CMV has been proposed as the chronic antigenic stimulus responsible for accelerated immunosenescence, including the accumulation of senescent CD8+ T-cells [40, 41]. There have been at least three studies looking at the association between CMV infection and influenza vaccine response in the elderly [20, 42, 43]. In one, CMV was associated with influenza vaccine non-response [20], however two other studies found no association [42, 43]. In contrast to the reports on seropositivity, we focused on the T-cell response to CMV and observed that elevated frequencies of CMV-reactive CD4+ T-cells, but not CMV-reactive CD8+ T-cells, were associated with an increased risk of respiratory viral infection. We are unaware of any other study linking CMV-reactive CD4+ T-cells to increased risk of respiratory viral infection. It is difficult to speculate on the possible biological relationship between the CMV-reactive CD4+ T cells and susceptibility to infection. Given the observation that CD4+ T-regs also correlate with susceptibility, we interpret these collective data as an indication that the distribution of functional cells (i.e. effector, suppressor, Th1, Th2, etc…) within the CD4+ T cell compartment has a strong influence on host resistance in the elderly. Most research to date has focused on CD8+ T-cells in the elderly and these observation strongly support a new line of research in the elderly to understand how and why skewing of the CD4+ T-cell compartment contributes significantly to the outcome of respiratory infection.

Low naïve CD8+ T-cells, high terminally differentiated CD8+ T-cells and senescent CD8+ T-cells have been described in elderly populations [10-14] and have been

hypothesized to predict risk of infection [9, 17]. Although there were associations between these immune phenotypes and risk of respiratory viral infection in univariable analyses, after adjustment for known confounders such as age, sex and frailty, CD8+ T cells were not predictive of respiratory viral infection once all immune phenotypes with suspected association with respiratory viral infection were included in the model. This illustrates the need for a robust statistical approach, including adequate sample size allowing for adjustment for known confounders and other immune phenotypes when exploring associations between immune biomarkers and outcomes.

Frailty is a "state variable" which aims to capture a person's vulnerability to adverse health outcomes. The Clinical Frailty Scale used here has been previously validated in nursing home residents and has been shown to robustly predict outcomes including mortality, disability and cognitive decline [23]. Frailty influences health outcomes through a number of mechanisms, including overall burden of disease/comorbidity and reduced reserve to tolerate further insults. Frailty has also been associated with immunosenescence [44]. Because of its importance as a measure of overall health and its relevance to immune function, it was a relevant measure to include in this study.

Our analysis was greatly facilitated by an automated analysis approach which eliminated what would have otherwise been an extremely time-consuming process of manual gating over 1,000 FCS files using an approach that was unbiased relative to manual gating with variability as low or even lower than manual gating. This approach should help facilitate the efficiency of future large studies of immune biomarkers.

This study provides insights into the role of immunosenescence and the risk of respiratory viral infection in elderly nursing home residents. Although our study was designed to identify associations and not causation, our findings suggest the possibility that strategies to boost circulating T-regs [45] or vaccines to prevent infection with CMV [46] or prophylactic anti-viral therapy to prevent re-activation of CMV may reduce respiratory viral infections in this high-risk population. In addition, those identified at increased risk of respiratory viral infection could be offered alternative prevention strategies such as heightened surveillance during the highest risk periods, which could help prevent nursing home outbreaks and transmission to healthcare workers and their families.

Limitations of this study include lower than expected influenza viral infection. Although influenza is not necessarily the most common virus isolated in nursing homes, [1], we expected to see more than 21 cases in 1072 residents during the influenza season based on prior respiratory viral infection surveillance studies conducted in nursing homes [1, 2]. We do not believe that cases were missed. Indeed, we performed prospective active surveillance for symptomatic respiratory viral infection, and approximately one third of the nasopharyngeal swabs were positive for virus, comparable to another study, which performed active surveillance for respiratory infections in nursing homes in Canada [2]. Instead, we believe the lower numbers were due to circulation of pandemic H1N1, an influenza strain that had less impact on older people during the 2009-2010 influenza season than pre-pandemic years and the relatively low incidence of influenza during 2010-2011 influenza season [47]. In addition, the lower numbers could have been

due to the fact that we only included residents who had been vaccinated against influenza. We felt that influenza vaccination status was too important a confounder to manage statistically, both because of its potential ability to prevent influenza infection and because of its association with the healthy user bias [48, 49]. It remains possible that there are different immune predictors for each of the different respiratory viruses in this vaccinated cohort and combining the respiratory viruses together in one combined endpoint limited its generalizability; however we chose *a priori* to combine the respiratory viruses together in a combined endpoint based on the fact they result in similar outcomes in this population [1, 3-7]. The low number of participants with influenza precluded our ability to perform a sensitivity analysis, looking at immune phenotypes predictive of influenza infection.

An additional limitation was that we were unable to include immune phenotypes as continuous variables in the analysis. In general, maintaining data as continuous is preferred over categorizing data [50]; however an analysis including immune phenotype data as continuous was not feasible as it led to estimates with wide confidence intervals. Thus, the analysis was performed using categorized variables, similar to other aging studies [51-55]. Although seventy-three participants died prior to developing a respiratory viral infection, we do not believe this competing risk introduced significant bias. Competing risks are unlikely to bias the result when the follow-up is short, or the proportion of participants experiencing a competing risk is less than the proportion of participants experiencing the outcome [56]. In this study, the follow-up was short (only one influenza season) and the proportion of participants who died was less than those who

developed a respiratory viral infection. Last, these results were obtained in a frail elderly population and may not be generalizable to community dwelling elderly.

In conclusion, in elderly nursing home residents, high CMV-reactive CD4+ T-cells were predictive of increased risk of respiratory viral infection and high T-regs were predictive of reduced risk during the ensuing respiratory viral season. These findings provide insights into immunosenescence and risk of infection and may help guide future prevention strategies.

Conflict of Interest Statement

All authors report no conflict of interest.

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Table 1: Baseline characteristics of the nursing home elderly.

	Total n=1072
Demographics [n(%)]	
Age (years)	
65-74	131 (12)
75-84	337 (31)
85-94	508 (47)
≥95	96 (9)
Sex (F)	776 (72)
Prior co-morbidity	
COPD	186 (17)
Coronary artery disease	346 (32)
Diabetes	290 (27)
Heart failure	148 (14)
Stroke	273 (25)
Dementia	511 (48)
≥5 medications	966 (90)
Frailty	
4	76 (7)
5	174 (16)
6	354 (33)
7	460 (43)
8	8 (1)
Immune Phenotypes [median (IQR)]	
CD8+ T-cell	
Naïve CD8+ T-cell%	1.10 (0.60 – 1.82)
Terminally differentiated CD8+ T-cell%	8.95 (4.72 – 14.80)
Senescent CD8+ T-cell%	5.87 (2.40 – 11.58)
CD8+ CMV T-cell%	0.32 (.03 – 1.53)
CD4+ T-cell	
Naïve CD4+ T-cell	13.2 (6.90 – 22.85)
Terminally differentiated CD4+ T-cell%	8.46 (4.93 – 14.04)
Senescent CD4+ T-cell%	1.66 (0.28 – 4.54)
CD4+ CMV T-cell%	0.06 (0.006 – 0.40)
T-reg	
T-reg%	2.73 (2.12 – 3.45)

Table 2: Respiratory viruses present in symptomatic elderly nursing home residents.

	Nasopharyngeal swabs positive for respiratory virus* n=87 n(%)
Influenza	21 (24)
Influenza A	16 (18)
Influenza B	5 (6)
RSV	12 (14)
RSV A	10 (11)
RSV B	2 (2)
Coronavirus	28 (32)
Coronavirus OC43	15 (17)
Coronavirus NL63/229E	9 (10)
Coronavirus HKU1	4 (5)
Rhinovirus	15 (17)
Human metapneumovirus	8 (9)
Parainfluenza	4 (5)
Parainfluenza 1	3 (3)
Parainfluenza 2	1 (1)

*One patient had mixed influenza A and rhinovirus

Table 3: Immune phenotype predictors of respiratory viral infection in univariable and multivariable analysis.

	HR (95% CI) Unadjusted	P-value	HR (95% CI) Final Model*	P-value
Age	0.99 (0.97 – 1.01)	0.35	0.99 (0.98 – 1.01)	0.30
Sex				
Male	Reference		Reference	
Female	1.13 (0.65 – 1.98)	0.66	1.03 (0.58 – 1.84)	0.92
Clinical Frailty Scale				
4	Reference		Reference	
5	1.44 (0.34 – 6.17)	0.62	2.68 (0.58 – 12.47)	0.21
6	1.99 (0.59 – 6.70)	0.27	3.67 (1.06 – 12.67)	0.04
7 or 8	1.41 (0.39 – 5.07)	0.60	2.45 (0.55 – 10.86)	0.24
CD8+ T-cell				
Low naïve CD8+ T-cell%	0.69 (0.51 – 0.95)	0.02		
High terminally differentiated CD8+ T-cell%	1.57 (1.10 – 2.24)	0.01		
High senescent CD8+ T-cell%	1.55 (1.11 – 2.17)	0.01		
High CMV-reactive CD8+ T-cell%	1.15 (0.65 – 2.03)	0.64		
CD4+ T-cell				
Low naïve CD4+ T-cell%	0.85 (0.61 – 1.18)	0.33		
High terminally differentiated CD4+ T-cell%	0.96 (0.60 – 1.55)	0.88		
High senescent CD4+ T-cell%	1.08 (0.71 – 1.64)	0.73		
High CMV-reactive CD4+ T-cell%	1.82 (1.13 – 2.94)	0.01	1.69 (1.03 - 2.78)	0.04
T-reg				
High T-reg%	0.47 (0.26 – 0.85)	0.01	0.41 (0.20 – 0.81)	0.01

*Final model adjusted for age, sex, frailty, high T-reg% and high CMV-reactive CD4+ T-cell%

Figure Legends:

Figure 1: Gating strategy for T-cell phenotypes. T cell phenotypes were defined using the flowDensity software package. Lymphocytes were first gated from non-margin events, and then singlets were gated. CD45RA thresholds were calculated based on singlet lymphocytes FMO. CD3+ cells were gated and then separated into CD4+CD8- and CD4-CD8+. Expression of CD57, CD28, CD45RA and CCR7 was analyzed on either CD4+CD8- or CD8-CD4+.

Figure 2: Gating strategy of T-reg. Lymphocytes and singlets were selected. Gates were then set up for CD3+ cells and CD4+. To identify the T-regs, a gate was set up to select CD25+ CD127 $^{lo/-}$ and T-regs were defined as CD25+ CD127 $^{lo/-}$ FOXP3+.

Figure 3: Gating strategy for CMV-reactive T cells. PBMC were stimulated with pp65 peptides to identify CMV-reactive T-cells. As a negative control, PBMC were stimulated with DMSO. Subsequently, the T-cells were stained for surface markers and intracellular cytokines. To define the CMV-reactive T-cells, the flow data was gated on singlet lymphocytes (as shown in Figures 1 and 2) and subsequently gated on CD3+CD8+ cells and CD3+CD4+. The plots show intracellular cytokine staining results for a single patient. CMV-reactive T-cells were defined as IFN-γ+ TNF-α+.

Figure 4: Time to respiratory viral infection stratified by a) T-reg%, adjusted for age, sex, frailty and CMV-reactive CD4+ T-cell%) and b) CMV-reactive CD4+ T-cell%, adjusted for age, sex, frailty and T-reg%.

Supporting Information Figure 1: Comparison of automated versus manual gating for T-cell phenotypes. The same gating hierarchy was used for manual (top row) and automated (bottom row) approaches for the T-cell panel as described in the text. Similar results were obtained for both methods.

Figure 1: Gating strategy for T-cell phenotypes. T cell phenotypes were defined using the flowDensity software package. Lymphocytes were first gated from non-margin events, and then singlets were gated. CD45RA thresholds were calculated based on singlet lymphocytes FMO. CD3+ cells were gated and then separated into CD4+CD8- and CD4-CD8+. Expression of CD57, CD28, CD45RA and CCR7 was analyzed on either CD4+CD8- or CD8-CD4+.

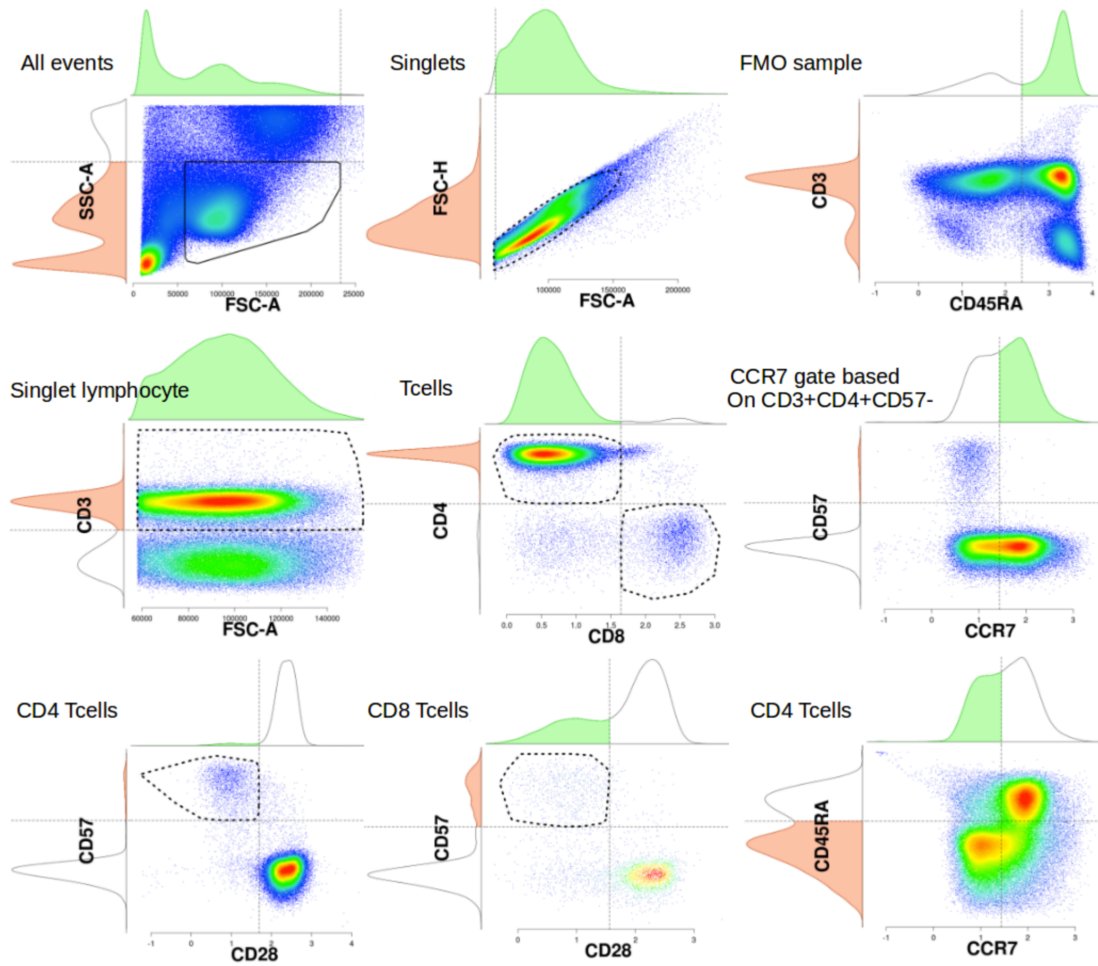


Figure 2: Gating strategy of T-reg. Lymphocytes and singlets were selected. Gates were then set up for CD3+ cells and CD4+. To identify the T-regs, a gate was set up to select CD25+ CD127 $^{lo/-}$ and T-regs were defined as CD25+ CD127 $^{lo/-}$ FOXP3+.

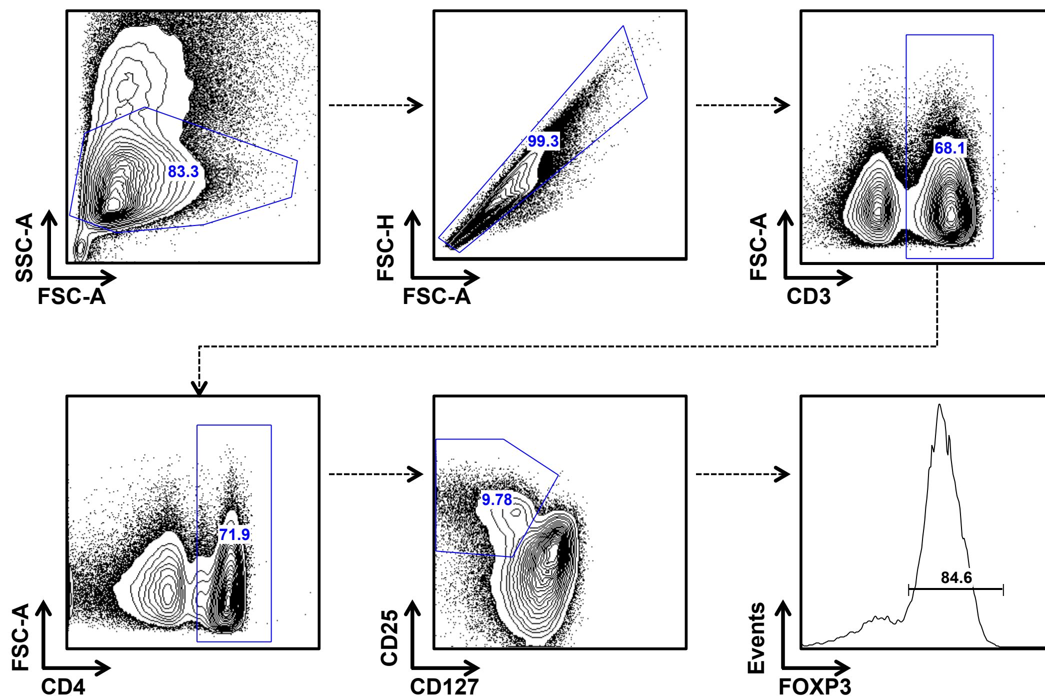


Figure 3: Gating strategy for CMV-reactive T cells. PBMC were stimulated with pp65 peptides to identify CMV-reactive T-cells. As a negative control, PBMC were stimulated with DMSO. Subsequently, the T-cells were stained for surface markers and intracellular cytokines. To define the CMV-reactive T-cells, the flow data was gated on singlet lymphocytes (as shown in Figures 1 and 2) and subsequently gated on CD3+CD8+ cells and CD3+CD4+. The plots show intracellular cytokine staining results for a single patient. CMV-reactive T-cells were defined as IFN-γ+ TNF-α+.

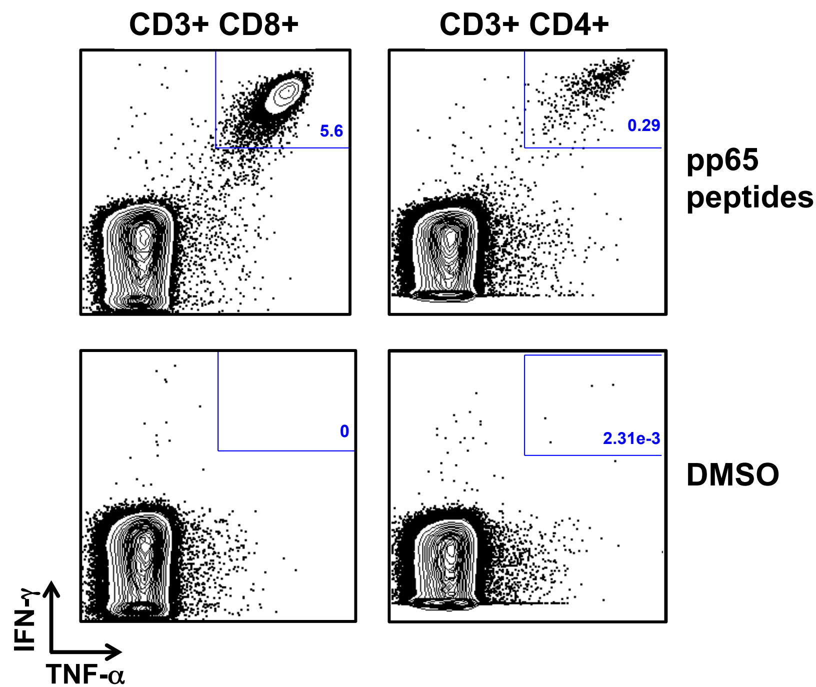
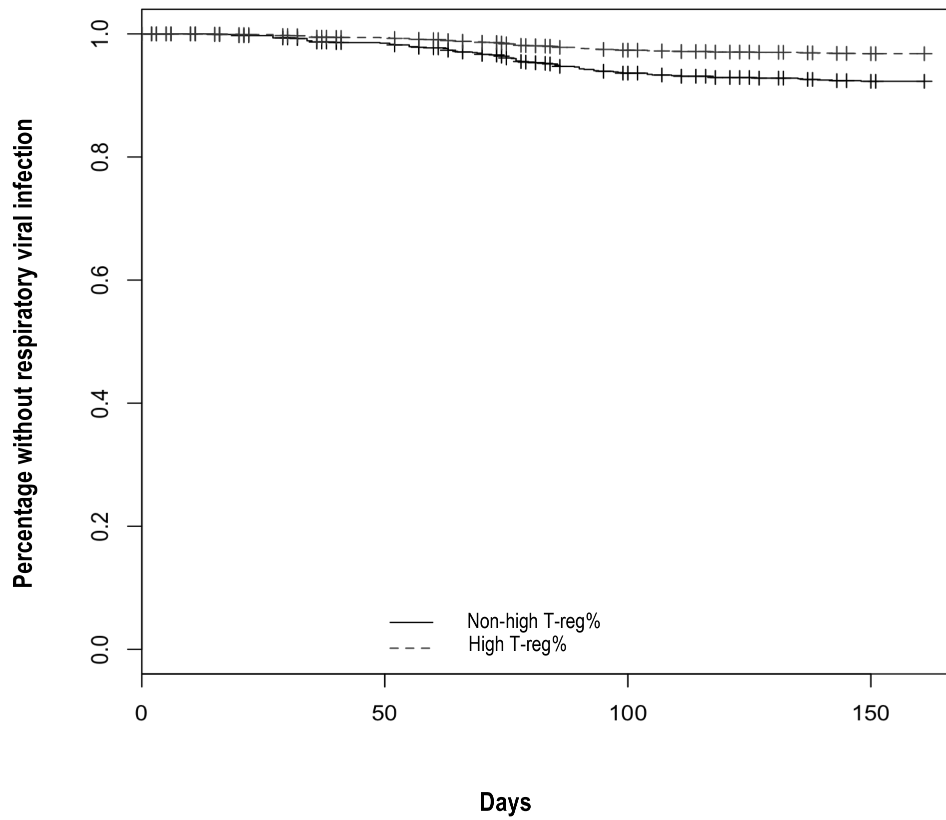
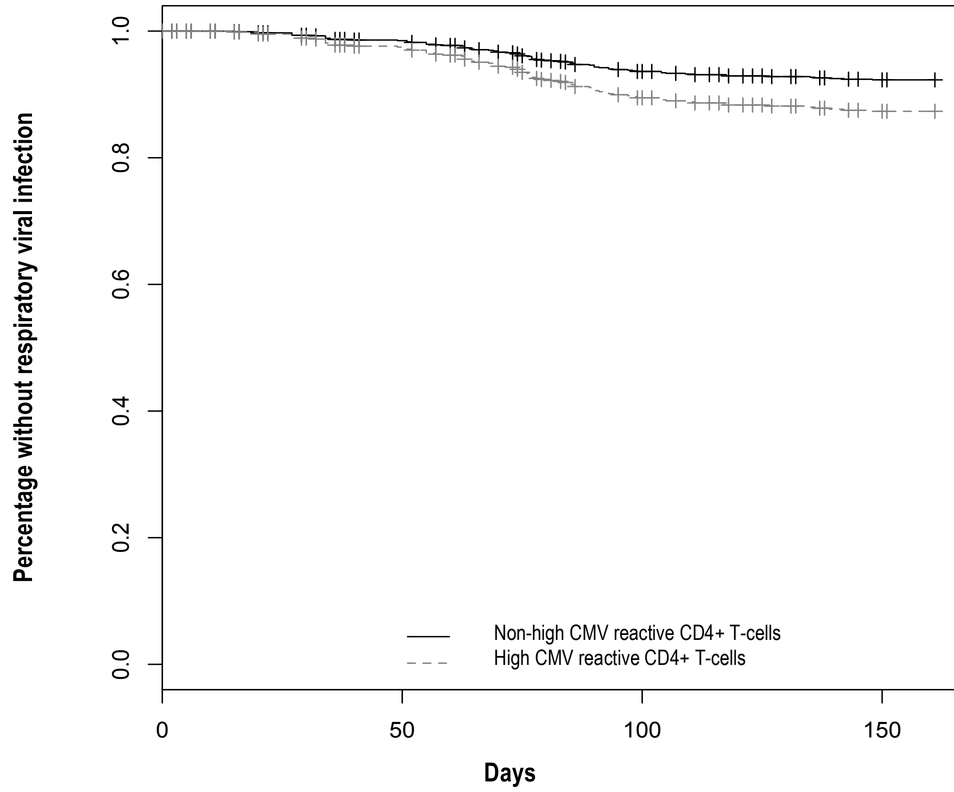


Figure 4: Time to respiratory viral infection stratified by a) T-reg%, adjusted for age, sex, frailty and CMV-reactive CD4+ T-cell%) and b) CMV-reactive CD4+ T-cell%, adjusted for age, sex, frailty and T-reg%.

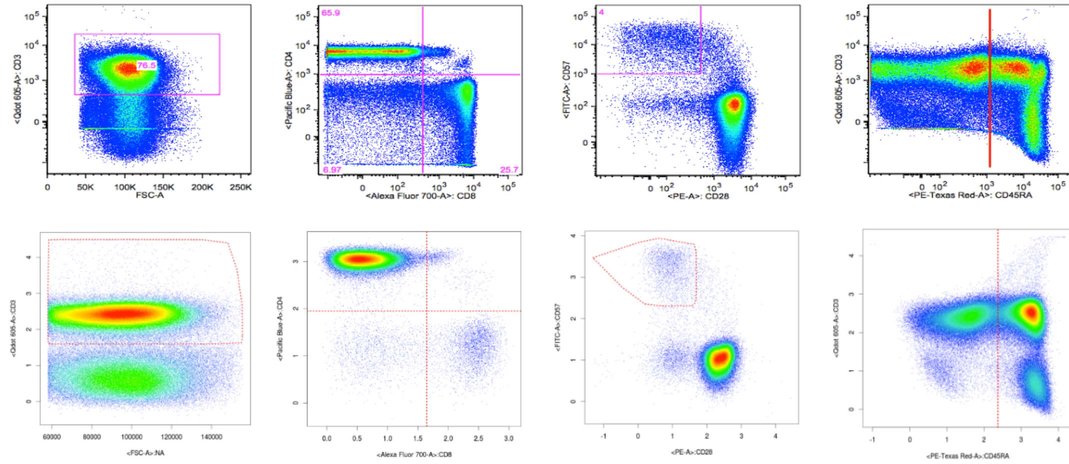
a)



b)



Supporting Information Figure 1: Comparison of automated versus manual gating for T-cell phenotypes. The same gating hierarchy was used for manual (top row) and automated (bottom row) approaches for the T-cell panel as described in the text. Similar results were obtained for both methods.



CHAPTER 4

Thesis manuscript 3: *Immune Biomarkers Predictive of Mortality in Elderly Nursing Home Residents*

PREFACE TO CHAPTER 4

In this chapter, we explored associations between immune phenotypes and risk of mortality using a Cox proportional hazards model adjusted for age, sex, frailty status and clustering at the level of the nursing home. In multivariable analysis, high levels of CMV-reactive CD4+ T-cells but not CMV-reactive CD8+ T-cells were associated with increased risk of mortality within 1-year in individuals aged 65-84 years. Other immune phenotypes including CD4+ T-cell subsets, CD8+ T-cell subsets and CD4+/CD8+ T-cell ratio less than one were not individually predictive of mortality within 1-year.

The student contribution to this study included study conception, data cleaning, data preparation, statistical analysis and drafting the manuscript. Co-authors include Robin Parsons, Fern Botelho and Jamie Millar who analyzed the laboratory peripheral blood mononuclear cell (PBMC) specimens under the supervision of Dr. Bramson; Drs. McNeil, Fulop and McElhaney were local site investigators and responsible for recruiting participants and coordinating laboratory specimens and provided critical review of the manuscript; Dr. Andrew provided the Clinical Frailty Scale and its interpretation and use throughout the study and critically reviewed the manuscript; Dr. Walter provided statistical expertise and provided critical review of the manuscript; Dr. Devereaux provided critical review of the manuscript; Mehrnoush Malekesmaeili provided interpretation of the PBMC flow cytometry and was supervised by Dr. Brinkman; and Drs. Loeb and Bramson were involved in conception of the study, aided in data interpretation, and provided critical review of the manuscript. Dr. Loeb provided funding support for the study.

This manuscript has been formatted for the Journal of Gerontology: Medical Sciences, and will be submitted once the manuscript presented in Chapter 3 (Immune Biomarkers Predictive of Respiratory Viral Infection in Elderly Nursing Home Residents) is accepted for publication.

Immune Biomarkers Predictive of Mortality in Elderly Nursing Home Residents

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Abstract

Objective: To determine among elderly nursing home residents whether immune phenotypes associated with immunosenescence were predictive of mortality within 1-year.

Methods: Residents ≥65 years from 32 nursing homes in four Canadian cities were enrolled between September 2009 and October 2011, and followed for one year. Following enrolment, peripheral blood mononuclear cells (PBMCs) were obtained and analysed by flow cytometry for CD4+ and CD8+ T-cell subsets (CD45+CCR7+, CD45-CCR7+, CD45-CCR7-, CD45+CCR7-, CD28-CD57+) and CMV-reactive CD4+ and CD8+ T-cells. A Cox proportional hazards model, adjusted for age, sex and frailty, determined the relationship between immune phenotypes and time to death.

Results: 1072 residents were enrolled; the median age was 86 years and 72% were female. In total, 151/1072 (14%) of the cohort died within 1-year. In multivariable analysis there was a significant negative multiplicative interaction between age and CMV-reactive CD4+ T-cell% (HR 0.39, 95% CI 0.19 – 0.78), by which high CMV-reactive CD4+ T-cell% was associated with mortality in participants aged 65 – 84 years (HR 2.25, 95% CI 1.29 – 3.92) but had no differential effect in participants aged 85-104 years (high CMV-reactive CD4+ T-cell% HR 2.89, 95% CI 0.86 – 4.92 versus low CMV-reactive CD4+ T-cell% HR 3.30, 95% CI 1.99 – 5.50) relative to those aged 65 – 84 years

with low CMV-reactive CD4+ T-cells (reference category, HR 1.0). No other significant factors were identified.

Conclusions: In elderly nursing home residents, high CMV-reactive CD4+ T-cells were associated with an increased risk of mortality within 1-year in those aged 65-84 years but had no differential effect in those aged 85-104 years. The relationship between CMV-reactive CD4+ T-cells and mortality should be explored further.

Key words: Immunosenescence, immune biomarker, mortality, nursing home, frailty.

Introduction

Dysfunctional changes to the immune system that arise with age are known as immunosenescence [1]. Out of all immune compartments, T-cell phenotypes appear most influenced by age [1]. A reduction of naïve T-cells and a corresponding increase in memory T-cells subsets including higher terminally differentiated memory T-cells (CD8+CD45+CCR7-) [2] and senescent cells (CD8+CD28)[3-5] have been observed with ageing. These changes are considered indicators of poor immunological function and it is postulated that immunosenescence is associated with increased risk of mortality [6].

Accordingly, immune biomarkers predictive of mortality have been sought. The most extensively studied immune biomarker is the immune risk profile, defined as a CD4+/CD8+ T-cell ratio less than 1. The immune risk profile was initially found to be predictive of mortality in the OCTO study, a longitudinal Swedish cohort of octogenarians [7]. It was hypothesized that this inverted ratio was related to an expansion of the CD8+ T-cell compartment due to higher numbers of poorly functional senescent CD8+ T-cells, possibly driven to exhaustion by cytomegalovirus (CMV) infection [7]. This hypothesis was supported by the findings in a subsequent OCTO study and a second aging Swedish cohort study, the NONA study, where the immune risk profile was associated with CMV infection [8, 9]. However, not all studies have found the immune risk profile to be predictive of death. In both a Spanish cohort study (n=151, 39 died) and an English cohort study of community dwelling elderly (n=425, 153 died) there was an association between the immune risk profile and mortality in unadjusted analysis (Spanish study HR 3.23, 95% CI 1.42 – 7.33 and English study HR 1.56, 95% CI 1.09 –

2.22), but not once the results were adjusted for potential confounding variables (Spanish study HR 0.65, 95% CI 0.18 – 2.28 and English study HR 1.38, 95% CI 0.96 – 1.98) [10, 11]. A nested case control study of elderly women in Baltimore (n= 122, 61 died) found no association between the CD4+/CD8+ T-cell ratio and mortality (p=0.71) [12]. Thus it remains unclear whether the immune risk profile is able to consistently predict mortality in aged cohorts.

Whether other immune phenotypes associated with immunosenescence are predictive of mortality is also unknown as the few studies investigating these associations have not used newer immune phenotyping that can better delineate the naïve and memory T-cells pools [11-15]. Furthermore, to our knowledge, the association between mortality and regulatory T-cells (T-regs), a subset of immune cells responsible for the regulation of the immune response and thought to be influenced by immunosenescence [16], has not been investigated.

The nursing home elderly are a group that are at high risk of mortality [17]. Knowledge of immune biomarkers predictive of mortality could help identify elderly nursing home residents at highest risk of dying, thereby potentially providing an opportunity for intervention. To this end, we sought to identify immune biomarkers predictive of mortality within 1-year in an elderly nursing home cohort.

Methods

Subjects and Setting

Details of our prospective cohort study have been published previously [18]. Briefly, elderly participants were recruited from 32 nursing homes in four study cities: Halifax, Nova Scotia; Sherbrooke, Quebec; Hamilton, Ontario and Vancouver, British Columbia during September and October 2009, 2010 and 2011. Nursing home residents ≥65 years of age were eligible. Exclusion criteria consisted of the following: residents on immunosuppressive medications (including cancer chemotherapy, oral corticosteroid use >21 days, methotrexate, post-transplant medications and/or anti-cytokine or B-lymphocyte depletion therapies), participants expected to die within 30 days (as determined by the supervising physician) and residents who planned to refuse vaccination against influenza. All participants (or their legally appointed guardian in the event they were not competent to provide consent themselves) provided written informed consent. The study had Research Ethics Board approval from each participating institution and nursing home.

Research personnel abstracted baseline demographics from the participants based on an interview, examination and chart review. In addition to age and sex, frailty status was ascertained given its known association with mortality [17]. Frailty has traditionally been a difficult construct to measure [19]. However, recent advances have led to the development of scales capable of accurately measuring frailty [20]. The Clinical Frailty Scale is a reproducible scale that has been validated in the nursing home setting [17]. It is an 8-point scale ranging from very fit (1) to very severely frail (8) [17]. Following enrolment, peripheral blood mononuclear cells (PBMCs) were drawn. Participants were

followed for one year from the time of PBMC blood draw. Date of participant death was obtained using the nursing home records.

Peripheral Blood Mononuclear Cell Analysis and Flow Cytometry

Methods of PBMC analysis and flow cytometry have been previously described in detail [18]. Briefly, PBMCs were isolated and frozen using a validated common standard operating procedure [21]. T-cell immune phenotypes were determined by thawing patient PBMCs [22] and placing an aliquot (0.5–16x10^6 cells/stain) in round-bottom 96-well plates with anti-CD3-Qdot605, CD8-Alexa Flour 700, anti-CD4-Pacific Blue, anti-CD45RA-PE Texas Red, anti-CD28-PE, anti-CD57-FITC, anti-CCR7-PE Cy7. T-regs were identified using anti-CD3-FITC, anti-CD4-Pacific Blue, anti-CD127-PerCP-Cy5.5, anti-CD25-PE, and anti-FoxP3-AlexaFluor700. We defined the T-cell subsets as follows: naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-), terminally differentiated (CD45RA+CCR7-) and senescent (CD28-CD57+). T-regs were defined as CD4+CD25hiCD127loFoxp3+. Antibody staining was performed using a Beckman Coulter Biomek NX^p Laboratory Automation Workstation (Beckman Coulter, Ontario) [23], followed by analysis using an LSR II flow cytometer with a high-throughput sampler (BD Biosciences, NJ, USA). T-regs were analyzed using FlowJo 9.6 (Treestar Inc, Ashland, OR). T-cell subset analysis employed an automated gating strategy using the flowDensity algorithm [18, 24].

As previously described, CMV-reactive T-cells were identified by stimulating PBMCs with a pool of overlapping peptides spanning the immunodominant pp65 protein

of CMV (PepTivator pp65, Miltenyi Biotec) [18, 22]. CMV-reactive T-cells were identified as CD3+ CD4+ or CD8+ IFN-γ+ TNF-α+.

Statistical analysis

The distributions of immune phenotypes and age were skewed; we therefore summarized these continuous variables using medians and interquartile ranges (IQR). The age and sex for those who had PBMCs obtained and those who did not were compared using Mann-Whitney U and chi-square tests as appropriate. Complete case analysis of immune phenotypes was planned if there was <10% missing data for each variable [25].

Cox proportional hazards models were constructed to explore the relationship between immune phenotypes and time to death, first using unadjusted hazard ratios (HR) and 95% confidence intervals (CIs). The natural log of each immune phenotype was analyzed as a continuous variable. The CD4+/CD8+ ratio was analyzed both as a continuous variable (natural log transformed) and also as a categorical variable (using ≥1 as the reference category). *A priori*, it was decided that age, sex and frailty would be included in the final model, given their potential for confounding with the effects of primary interest in this population [26]. Age was re-scaled by dividing by 10 and analyzed as a continuous variable to improve interpretability. Sex and frailty were analyzed as categorical variables and entered into the model as dummy variables; the reference categories were male and Clinical Frailty Score level 4, respectively. The possibility of multiplicative interactions were first explored by assessing each individual

immune phenotype, age and their product in the Cox proportional hazards model, and then between each individual immune phenotype, sex and their product in the Cox proportional hazards model.

Covariates with a p-value of <0.20, in univariable analysis and interaction terms with a p-value of <0.05 were evaluated for inclusion in the final multivariable model. If the multiplicative interaction term remained significant in the multivariable analysis, the magnitude of corresponding additive interactions was also quantified using the relative excess risk due to interaction (RERI) measure [27]. The final model was determined using backwards elimination. Bootstrap estimates of the standard error for each regression coefficient in the final model were also obtained for greater accuracy. The bootstrap estimates were resampled 1200 times and the CI's were calculated using the bias corrected and accelerated distribution method [28]. A sandwich variance estimator was used to account for the clustering effect at the level of the nursing homes [29]. The proportional hazards assumption for continuous variables was explored graphically by plotting partial residuals against time to event and tested by regressing the partial residuals against time. The proportional hazards assumption for categorical variables was examined by a log-minus-log graph to assess if the plotted lines were approximately parallel. The presence of multicollinearity was examined using the variance inflation factor (VIF); presence of multicollinearity was defined as VIF >10.

As an additional exploratory analysis, hierarchical agglomerative cluster analysis of the participants using immune phenotypes was performed. Cluster analysis is a tool that can be used to identify homogeneous subgroups of participants within a set of data

[30]. Ward's method with squared Euclidian distance was used [30]. The maximum number of cluster variables allowable in a cluster analysis is calculated using 2^m, where m is the number of cluster variables [30]. Thus all immune phenotypes could not be included in the same cluster analysis. To reduce the number of factors, only immune phenotypes with p-values <0.20 in univariable analyses were chosen. The covariates were standardized prior to the cluster analysis. The association between cluster terms and mortality was then tested using Chi-squared.

P-values and 95% CIs were constructed using 2-tailed tests. P-values <0.05 were considered statistically significant. Cox proportional hazard model analyses were performed using R, version 3.0.2 and the R commander package [31, 32]. Cluster analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois).

Results

Initially, 1165 residents were enrolled in the study. Of these, 1087 (93%) had PBMCs obtained. There was no statistically significant difference in age (median, 86 [IQR 80 – 90] versus 85 [IQR 79-89] without PBMCs, p=0.42) or sex (%female, 72% versus 64% without PBMCs, p=0.12) between those that did and did not have PBMCs obtained. Fifteen participants (1%) withdrew before the end of the study. Thus, 1072 was our final sample size.

Baseline characteristics of the 1072 participants are summarized in Table 1. The median age was 86 years (IQR 80 – 90 years) and the ages ranged from 65 – 102 years;

eight persons were ≥100 years. Most of the cohort (93%) had at least one co-morbidity, and almost half (44%) scored either 7 or 8 on the Clinical Frailty Scale, which is defined as severely or very severely frail. The median CD4+/CD8+ T-cell ratio was 3.95 (IQR 2.33 – 6.76) and only 47/1072 (4%) of the cohort had a ratio <1.0. The remaining immune phenotype medians and IQRs are summarized in Table 1.

In total, 151/1072 (14%) of the cohort died within 1-year. The cumulative death rate over time is displayed in Figure 1. The proportional hazards assumption was satisfied for all covariates included in the model. In univariable analyses, increasing age was associated with a significantly increased risk of mortality within 1-year (per increase in 10 years, HR 1.83 95% CI 1.46 – 2.30 and dichotomized, 65-84 years 44/468 (9%) versus 85- 104 years 107/602 (18%), HR 3.30 95% CI 1.99 – 5.50). For each 1% increase in the natural log of the following immune parameters, mortality within 1-year was reduced: naïve CD8+ T-cell% (HR 0.82, 95% CI 0.70 – 0.96), central memory CD4+ T-cell% (HR 0.74, 95% CI 0.55 – 0.99) and T-reg% (HR 0.62, 95% CI 0.44 – 0.86). However, after adjusting for age, sex and frailty, only CD4+ central memory T-cell% (HR 0.73, 95% CI 0.54 – 0.98) and T-reg% (HR 0.65, 95% CI 0.47 – 0.90) remained associated with mortality within 1-year. There was no statistically significant association between the CD4+/CD8+ T-cell ratio <1.0 and mortality within 1-year (CD4+/CD8+ T-cell ratio ≥1.0 139/1017 (14%) versus CD4+/CD8+ T-cell ratio <1.0 11/47 (23%), HR 1.73, 95% CI 0.90 – 3.31)(Table 2).

Exploration of potential interactions between age and each immune phenotype revealed a significant multiplicative interaction between age and CMV-reactive CD4+ T-

cell% (p=0.01). There were no other significant interactions between sex and any immune phenotypes. The following parameters were evaluated for inclusion in the multivariable model adjusted for age, sex and frailty: naïve CD8+ T-cell%, effector memory CD8+ T-cell%, CD4+ T-cell%, naïve CD4+ T-cell%, central memory CD4+ T-cell%, CD4+/CD8+ T-cell ratio <1.0, T-reg%, CMV reactive CD4+ T-cell% and the interaction between age and CMV reactive CD4+ T-cell%. There was no evidence of multicollinearity between these covariates. Only age, sex, frailty, CMV-reactive CD4+ T-cell and the interaction between age and CMV-reactive CD4+ T-cell% remained in the final model (Table 3). The bootstrap confidence intervals were wider than the original estimate, however, CMV-reactive CD4+ T-cell% and the interaction between age and CMV-reactive CD4+ T-cell% remained statistically significant (Table 3). There was a significant negative multiplicative interaction between age and CMV-reactive CD4+ T-cell% (HR 0.39, 95% CI 0.19 – 0.78) as well as a corresponding negative additive interaction (RERI -1.66, 95% CI -3.32 to 0) (Table 4).

The cluster analysis was performed using the following immune parameters: naïve CD8+ T-cell%, effector memory CD8+ T-cell%, CD4+T-cell%, naïve CD4+ T-cell%, central memory CD4+ T-cell%, T-reg% and CD4+/CD8+ T-cell ratio (<1.0 and ≥1.0). Cluster analysis can only be performed on participants with complete data (i.e. no observations missing); thus data from 1051 participants were available for the cluster analysis. The initial cluster analysis grouped the participants into 3 groups (Table 5) and there was no statistically significant difference between mortality percentages between groups (Cluster 1: 35%, Cluster 2: 15% and Cluster 3: 13%, p=0.06). Cluster 1 contained

almost all (96%) of the participants from the cohort with a CD4+/CD8+ T-cell ratio <1.0 and contained no participant with a CD4+/CD8+ T-cell ratio ≥1.0. The mortality percentage and the immune phenotype medians in Cluster 2 and Cluster 3 looked similar, thus as an additional *post hoc* exploratory analysis, they were combined and compared to Cluster 1 (Table 5). There was a statistically significant difference in the mortality percentage between Cluster 1 and Cluster 2 and 3 combined (Cluster 1: 35% versus combined Cluster 2 and 3: 14%, p=0.03). However, Cluster 1 was not predictive of mortality when compared to the combined Cluster 2 and 3 in the Cox proportional hazards model in unadjusted analysis (Cluster 1 HR 1.85, 95% CI 0.96 - 3.57, p=0.07) or analysis adjusted for age, sex and frailty (Cluster 1 HR 1.61, 95% CI 0.76 – 3.33, p=0.21).

CMV-reactive CD4+ T-cell% was not included in the cluster analysis based on the *a priori* defined method of variable selection. However, as a *post hoc* exploratory analysis, it was added to the cluster analysis. The results were similar to the original cluster analysis; the cluster analysis grouped participants into 3 groups and there was no statistically significant difference between mortality percentages between groups (Cluster 1=9/39 (23%) died; Cluster 2=36/275 (13%) died; Cluster 3=79/531 (15%) died, p=0.25). In Cluster 1, all 39 participants had a CD4+/CD8+ T-cell ratio <1.0 and Cluster 2 and Cluster 3 each only contained 1 participant with a CD4+/CD8+ T-cell ratio <1.0.

Discussion

In this prospective cohort study of elderly nursing home residents, high levels of CMV-reactive CD4+ T-cells but not CMV-reactive CD8+ T-cells were associated with increased risk of mortality within 1-year in individuals aged 65-84 years in multivariable analysis. Other immune phenotypes including CD4+/CD8+ T-cell ratio <1.0 were not individually predictive of mortality within 1-year but a selection of immune phenotypes were able to cluster residents into groups based on risk of mortality.

To our knowledge, this is the first study to evaluate the association between CMV-reactive CD4+ and CD8+ T-cells and mortality. CMV-reactive CD8+ T-cells have previously been shown to be elevated in the elderly and associated with CD8+ T-cell subset oligoclonality [33, 34], supporting the hypothesis that CMV is a driver of immunosenescence [1, 6]. However, these studies did not examine death as an outcome [33, 34]. In our study, CMV-reactive CD8+ T-cells were not predictive of mortality. Instead, we found CMV-reactive CD4+ T-cells to be predictive of mortality in individuals aged 65-84 years. Unexpectedly, this finding did not extend into the very old (85-104 years), which we speculate could be because old age is such an overwhelming predictor of mortality. Although few studies have described the effect of CMV on CD4+ T-cell compartment in aging, CMV positivity has been shown to be associated with large numbers of virus-specific CD4+ T-cells in the elderly [35] as well as increase in CD4+ memory T-cells [35, 36].

Based on existing literature [33, 34] and the theory of immunosenescence [1, 6], we hypothesized that CMV-reactive CD8+ T-cells would be associated with mortality and it was unexpected that they were not. However, there was little evidence of an

expanded CD8+T-cell compartment relative to the CD4+ T-cell compartment in most people; only 4% of individuals in our study cohort had a CD4+/CD8+ T-cell ratio <1.0. In addition, CD4+/CD8+ T-cell ratio <1.0 was not predictive of mortality in our study. Although the immune risk profile was predictive of mortality in two longitudinal Swedish cohorts of elderly people [7, 9], there have been at least two other studies in addition to ours, where the CD4+/CD8+ T-cell ratio <1.0 was not associated with mortality [10, 11]. These studies were cohort studies of community dwelling elderly; one in Cambridge, UK (n=425) [11] and one in Sevilla, Spain (n=151)[10]. In both, CD4+/CD8+ T-cell <1.0 was associated with time to death in unadjusted analyses (English study HR 1.56, 95% CI 1.09 – 2.22 and Spanish study HR 3.23, 95% CI 1.42 – 7.33), but not in multivariable analyses (English study HR 1.38, 95% CI 0.96 – 1.98 and Spanish study HR 0.65, 95% CI 0.18 – 2.28)[10, 11]. A third nested case control study of elderly women in Baltimore, Maryland (n=61 cases and n=61 controls matched by age, concurrent illness, use of hormone replacement therapy and frailty status) found no difference between the CD4+/CD8+ T-cell ratios between cases and controls (p=0.71)[12].

When we conducted an exploratory cluster analysis to group residents using a selection of immune phenotypes, one cluster had essentially all the participants with a CD4+/CD8+ ratio <1.0 from the cohort. The mortality in this group was statistically higher than the other two clusters (when they were combined), but the cluster was not predictive of mortality within 1-year in the Cox proportional hazards model. In contrast to Cox proportional hazards, cluster analyses fails to take follow-up time or censoring into account and for that reason the results from Cox proportional hazards are preferred.

However, although CD4+/CD8+ T-cell ratio <1.0 was not predictive of mortality in multivariable analysis in our cohort, and the cluster defined by a CD4+/CD8+ T-cell ratio <1.0 was not predictive of mortality, our cluster analysis suggests that there was a small subgroup of individuals in our cohort, defined by a CD4+/CD8+ T-cell ratio <1.0, that may have a different risk of mortality than the rest of the group. It is possible that reasons for non-significance in the Cox proportional hazard model could be due to the small size of this subgroup and resultant lack of power. We speculate that the current immunosenescence concept, the expansion of the CD8+ T-cell compartment due to an accumulation of poorly functional senescent CD8+ T-cells, possibly driven to exhaustion by CMV infection is responsible for the adverse outcomes seen in the elderly, may only apply to a select subgroup, possibly based on genetic make-up. In contrast, our multivariable analysis suggests that mortality for others is predicted by an expansion of the CMV-reactive CD4+ T-cells. This finding could explain the differences between the Swedish cohort, a relatively genetically homogeneous population where the CD4+/CD8+ T-cell ratio <1.0 is predictive of mortality [7, 9], and our study and others conducted in Canada, the United States and the United Kingdom (generally more genetically heterogeneous populations [37]) where the CD4+/CD8+ T-cell ratio <1.0 has not been found predictive of mortality [11, 12].

In our study, CD4+ and CD8+ T-cell subsets associated with immunosenescence [1] were not predictive of mortality. Our findings are consistent with another study, conducted in a cohort of community dwelling elderly women, which also did not find any association between CD4+ and CD8+ T-cell subsets (all p-values >0.10)[12]. We also

found no association between T-regs and mortality. Although T-regs were highly associated with mortality in univariable analysis, they were not associated with mortality in the final multivariable model, demonstrating the importance of adjusting for other immune phenotypes in studies of immune biomarkers.

Limitations of our study include the fact that we could not confirm the cause of death in the vast majority of cases as autopsies are seldom performed in this population. Thus, it remains possible that immune phenotypes associated with immunosenescence are only predictive of mortality in selected cases such as those with infectious etiologies. In addition, our results were obtained in an elderly nursing home population and may not be generalizable to community dwelling elderly.

In conclusion, in elderly nursing home residents, high CMV-reactive CD4+ T-cells but not CMV-reactive CD8+ T-cells were predictive of increased risk of mortality within 1-year in those aged 65-84 years. The immune risk profile and other CD4+ and CD8+ T-cell subset immune phenotypes associated with immunosenescence were not associated with mortality in our cohort. The importance of CMV on CD4+ T-cells in immunosenescence should be explored further.

Conflict of Interest Statement

All authors report no conflict of interest.

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Table 1: Baseline characteristics and immune phenotype distributions of elderly nursing home residents.

	Total n=1072
Demographics, Co-morbidity and Frailty	
Age (years) [n(%))	
65-74	131 (12)
75-84	337 (31)
85-94	508 (47)
≥95	96 (9)
Sex (Female) [n(%)]	776 (72)
Prior co-morbidity [n(%)]	
COPD	186 (17)
Coronary artery disease	346 (32)
Diabetes	290 (27)
Heart failure	148 (14)
Stroke	273 (25)
Dementia	511 (48)
≥5 medications [n(%)]	966 (90)
Frailty [n(%)]	
4	76 (7)
5	174 (16)
6	354 (33)
7	460 (43)
8	8 (1)
Immune phenotypes	
CD8+ T-cell [median (IQR)]	
CD8+ T-cell%	18.66 (12.1 – 27.76)
Naïve CD8+ T-cell%	1.10 (0.60 – 1.82)
Central memory CD8+ T-cell%	0.47 (0.26 – 0.85)
Effector memory CD8+ T-cell%	6.37 (3.81 – 10.51)
Terminally differentiated CD8+ T-cell%	8.95 (4.72 – 14.80)
Senescent CD8+ T-cell%	5.87 (2.40 – 11.58)
CMV-reactive CD8+ T-cell%	0.32 (0.03 – 1.53)
CD4+ T-cell [median (IQR)]	
CD4 T-cell%	74.53 (62.92 – 82.53)
Naïve CD4+ T-cell%	13.22 (6.90 – 22.85)
Central memory CD4+ T-cell%	12.22 (8.07 – 16.74)
Effector memory CD4+ T-cell%	31.24 (24.27 – 39.01)
Terminally differentiated CD4+ T-cell%	8.46 (4.93 – 14.04)
Senescent CD4+ T-cell%	1.66 (0.28 – 4.54)
CMV-reactive CD4+ T-cell%	0.06 (0.006 – 0.40)
Ratio	
CD4+/CD8+ T-cell ratio [median (IQR)]	3.95 (2.33 – 6.76)
CD4+/CD8+ T-cell ratio <1.0 [n%)]	47 (4)
T-reg [median (IQR)]	
T-reg%	2.73 (2.12 – 3.45)

Table 2: Predictors of mortality within 1-year in univariable analyses and after adjustment for age, sex and frailty.

	HR (95% CI) Unadjusted	P-value	HR (95% CI) Adjusted for Age, Sex and Frailty	P-value
Demographics n (%)				
Age (per 10 years)	1.83 (1.46 – 2.30)	<0.001	1.85 (1.46 – 2.33)	<0.001
Sex				
Male	Reference		Reference	
Female	0.99 (0.72 – 1.37)	0.97	0.86 (0.62 – 1.19)	0.37
Frailty				
4	Reference		Reference	
5	0.63 (0.29– 1.36)	0.24	0.66 (0.30 – 1.43)	0.29
6	1.47 (0.65 – 3.34)	0.35	1.50 (0.61 – 3.65)	0.38
7 or 8	1.38 (0.64 – 3.01)	0.41	1.38 (0.61 - 3.13)	0.44
Immune Phenotypes [Median (IQR)]				
CD8+ T-cell				
CD8+ T-cell	0.90 (0.69 – 1.18)	0.44	0.89 (0.71 – 1.12)	0.32
Naïve CD8+ T-cell	0.82 (0.70 – 0.96)	0.02	0.89 (0.76 – 1.05)	0.18
Central memory CD8+ T-cell	0.91 (0.76 – 1.10)	0.33	0.92 (0.75 – 1.12)	0.40
Effector memory CD8+ T-cell	0.85 (0.70 – 1.03)	0.09	0.90 (0.43 – 1.88)	0.78
Terminally differentiated CD8+	1.0 (0.82 – 1.20)	0.97	0.96 (0.81 – 1.13)	0.59
Senescent CD8+ T-cell	0.99 (0.89 – 1.10)	0.88	0.96 (0.88 – 1.05)	0.39
CD8+ CMV stimulated T-cell	1.02 (0.93 – 1.11)	0.72	1.0 (0.91 – 1.09)	0.96
CD4+ T-cell				
CD4+ T-cell	0.53 (0.29 – 0.97)	0.04	0.56 (0.31 – 1.01)	0.06
Naïve CD4+ T-cell	0.90 (0.79 – 1.02)	0.10	0.91 (0.80 – 1.03)	0.15
Central memory CD4+ T-cell	0.74 (0.55 – 0.99)	0.04	0.73 (0.54 – 0.98)	0.03
Effector memory CD4+ T-cell	0.98 (0.50 – 1.91)	0.95	0.90 (0.43 – 1.88)	0.78
Terminally differentiated CD4+ T-cell	0.91 (0.75 – 1.10)	0.31	0.90 (0.73 – 1.11)	0.33
Senescent CD4+ T-cell	1.07 (0.96 – 1.20)	0.22	1.03 (0.92 – 1.15)	0.61
CD4+ CMV stimulated T-cell	1.04 (0.96 – 1.12)	0.31	1.01 (0.93 – 1.10)	0.83
Ratio				
CD4+/CD8+ T-cell ratio	1.01 (0.84 – 1.21)	0.95	1.02 (0.87 – 1.18)	0.83
CD4+/CD8+ T-cell ratio <1.0	1.73 (0.90 – 3.31)	0.10	1.51 (0.73 – 3.12)	0.27
T-reg				
T-reg	0.62 (0.44 – 0.86)	0.005	0.65 (0.47 – 0.90)	0.01

Table 3: Predictors of mortality within 1-year in multivariable analyses; original and bootstrap estimates.

	HR (95% CI) Final Model	**HR (95% CI) Bootstrap Estimate**
Age (per 10 years)	1.32 (1.02 – 1.71)	1.35 (0.90 – 1.98)
Sex		
Male	Reference	Reference
Female	0.86 (0.63 – 1.17)	0.85 (0.57 – 1.28)
Frailty		
4	Reference	Reference
5	0.68 (0.33– 1.43)	0.69 (0.27 – 2.18)
6	1.44 (0.62 – 3.37)	1.52 (0.72 – 3.53)
7 or 8	1.34 (0.62 – 2.86)	1.40 (0.71 - 3.66)
CD4+ CMV stimulated T-cell	3.45 (1.40 – 8.48)	3.40 (1.04 – 11.46)
Age*CD4+ CMV stimulated T-cell	0.39 (0.19 – 0.78)	0.87 (0.76 – 1.00)

Table 4: Predictors of mortality within 1-year in multivariable analyses*.

	HR (95% CI)**				RERI (95% CI)
CD4+ CMV T-cell and Age	Age 65-84 and low/absent CD4+ CMV T-cells	Age 65-84 and high CD4+ CMV T-cells	Age 85-104 and low/absent CD4+ CMV T-cells	Age 85-104 and high CD4+ CMV T-cells	
	1.00	2.25 (1.29 – 3.92)	3.30 (1.99 – 5.50)	2.89 (0.86 – 4.92)	-1.66 (-3.32 to 0.00)

*Results for age and CMV-reactive CD4+ T-cell% dichotomized for ease of interpretation.
**Adjusted for age, sex and frailty.

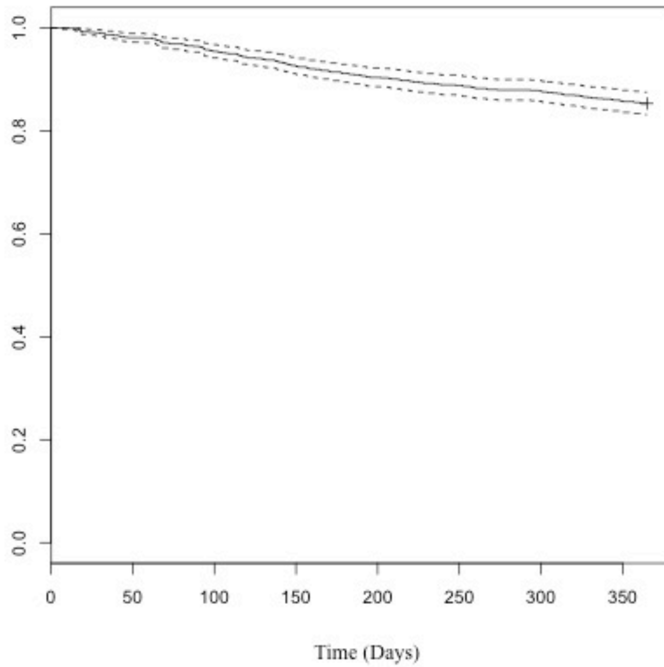
Table 5: Selected immune phenotypes stratified by 3-cluster solution and 2-cluster solution.

Immune phenotype	Cluster 1 n=44 Died=35%	Cluster 2 n=497 Died=15%	Cluster 3 n=510 Died=13%	Cluster 1 n=44 Died=35%	Cluster 2 and 3 combined n=1007 Died=14%
CD8+ naïve T-cells [median (IQR)]	1.25 (0.85 – 2.02)	0.97 (0.55 – 1.70)	1.18 (0.67 – 1.94)	1.25 (0.85 – 2.02)	1.10 (0.60 – 1.82)
CD8+ effector memory T-cells [median (IQR)]	16.26[a] (10.51 – 24.56)	9.33 (6.06 – 13.28)	4.21 (2.52 – 6.21)	16.26[a] (10.51 – 24.56)	6.11 (3.74 – 9.96)
CD4+ T-cells [median (IQR)]	38.69[b] (34.44 – 42.57)	65.44 (57.52 – 73.34)	81.45 (76.47 – 86.67)	38.69[b] (34.44 – 42.57)	75.19 (65.04 – 82.91)
CD4+ naïve T-cells [median (IQR)]	3.33[b] (1.82 – 7.14)	8.31 (4.52 – 12.89)	22.13 (14.58 – 29.88)	3.33[b] (1.82 – 7.14)	13.88 (7.59 – 23.31)
CD4+ central memory T-cells [median (IQR)]	5.96[b] (4.08 – 9.24)	9.79 (6.62 – 13.82)	15.31 (11.56 – 19.86)	5.96[b] (4.08 – 9.24)	12.57 (8.46 – 16.97)
T-reg [median (IQR)]	1.69[b] (1.20 – 2.14)	2.31 (1.83 – 2.97)	3.22 (2.64 – 3.90)	1.69[b] (1.20 – 2.14)	2.78 (2.17 – 3.48)
CD4+/CD8+ T-cell <1.0 [n(%)]	44 (100)	2 (0.40)	0 (0)	44 (100)	2 (0.20)

[a]Fourth (highest) quartile

[b]First (lowest) quartile

Figure 1: Cumulative risk of death before 1-year of follow-up. Solid line represents the hazard rate and dotted lines represent the 95% confidence interval.



CHAPTER 5

Thesis manuscript 4: *Immune Biomarkers Predictive of Frailty in Elderly Nursing Home Residents*

PREFACE TO CHAPTER 5

In this chapter, we investigated immune phenotypes associated with frailty in elderly nursing home residents using a multi-level modeling approach. In elderly nursing home residents, higher naïve CD4+ T-cells, and higher effector memory CD8+ T-cells predicted lower levels of frailty, and higher central memory CD8+ T-cells predicted higher levels of frailty.

The student contribution to this study included study conception, data cleaning, data preparation, creation of the Frailty Index and validation of the Frailty Index, statistical analysis and drafting the manuscript. Co-authors include Dr. Andrew who aided in the creation and validation of the Frailty Index and critically reviewed the manuscript; Robin Parsons, Fern Botelho and Jamie Millar who analyzed the laboratory peripheral blood mononuclear cell (PBMC) specimens under the supervision of Dr. Bramson; Drs. McNeil, Fulop and McElhaney were local site investigators and responsible for recruiting participants and coordinating laboratory specimens and provided critical review of the manuscript; Dr. Walter provided statistical expertise and provided critical review of the manuscript; Dr. Devereaux provided critical review of the manuscript; Mehrnoush Malekesmaeili provided interpretation of the PBMC flow cytometry and was supervised by Dr. Brinkman; and Drs. Loeb and Bramson were involved in conception of the study, aided in data interpretation, and provided critical review of the manuscript. Dr. Loeb provided funding support for the study.

This manuscript has been formatted for the journal Mechanisms of Ageing and Development and will be submitted once the manuscript presented in Chapter 3 (Immune

Biomarkers Predictive of Respiratory Viral Infection in Elderly Nursing Home Residents) is accepted for publication.

Immune Biomarkers Predictive of Frailty in Elderly Nursing Home Residents

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Abstract

Objective: To determine whether immune phenotypes associated with immunosenescence were predictive of frailty in elderly nursing home residents.

Methods: Residents ≥65 years from 32 nursing homes in four Canadian cities were enrolled (September 2009 to October 2011). Following enrolment, frailty was assessed using the Frailty Index and peripheral blood mononuclear cells (PBMCs) were obtained and analysed by flow cytometry for CD4+ and CD8+ T-cells and subsets (CD45+CCR7+, CD45-CCR7+, CD45-CCR7-, CD45+CCR7- and CD28-CD57+), T-regs and CMV-reactive CD4+ and CD8+ T-cells. Multilevel linear regression analysis was performed; the outcome was frailty, there was a random effect of nursing homes, and age, sex and immune phenotypes (natural log transformed) were fixed effects.

Results: 1072 residents were enrolled; the median age was 86 years and 72% were female. The mean Frailty Index was 0.44, SD 0.13. Higher numbers of naïve CD4+ T-cell% (CD4+CD45+CCR7+)(p=0.001) and effector memory CD8+ T-cell% (CD8+CD45-CCR7-)(p=0.019) were associated with a lower mean Frailty Index whereas higher numbers of CD8+ central memory T-cell% (CD8+CD45-CCR7+) were associated with a higher mean Frailty Index (p=0.017).

Conclusions: In elderly nursing home residents, naïve CD4+ T-cells, effector memory CD8+ T-cells and central memory CD8+ T-cells were predictive of frailty.

Key words: Immunosenescence, immune biomarker, frailty, nursing home

Introduction

Frailty is a syndrome that arises due to accumulating comorbidity across domains leading to loss of an individual's reserve; accordingly, increased frailty is associated with increased risk of death [1]. The pathophysiological mechanisms that lead to frailty are not well understood, but it has been hypothesized that frailty is associated with immunosenescence, the dysfunctional changes to the immune system that arise with age [2].

Changes to the immune system associated with immunosenescence include a reduction in the number of naïve T-cells due to thymic involution [3], and an increase in CD8+ memory T-cells subsets including higher numbers of terminally differentiated memory T-cells (CD45+CCR7-) [4] and senescent cells (CD28-CD57+) [5, 6], cells that are all considered to be indicators of poor immunological function [7]. Senescent T-cells are highly associated with latent cytomegalovirus (CMV) and it has been theorized that CMV may accelerate immunosenescence due to disproportionate increase in CMV-reactive CD8+ T-cells, leading to higher CD8+ T-cells relative to the CD4+ T-cell compartment [8]. Indeed, an immune risk profile characterized by a CD4+/CD8+ ratio less than 1 has been found to be predictive of mortality in two Swedish cohorts [9, 10].

The few studies addressing the association between immunosenescence and frailty have given conflicting results. Studies using data from the Women's Health and Aging Studies cohort (n=635) found that women with the highest levels of CMV antibody had a greater incidence of frailty [11] and frail women (n=28) had significantly higher CD8+ T-cells, senescent CD8+ T-cells, and lower CD4+ T-cells than pre-frail (n=75) and non-frail

women (n=24) [12]. However, in a cross-sectional study of community-dwelling individuals aged 80 years and older (n=567), those having CMV antibody were actually less frail in adjusted analysis (OR 0.37, 95% CI 0.15 – 0.91) [13]; also a cross-sectional study of elderly people 85 years and older (n=552) found no association between frailty and CMV serostatus (OR 0.74, 95% CI 0.42 – 1.28) or CD4+/CD8+ ratio less than 1 (OR 0.98, 95% CI 0.58 – 1.66)[14] in adjusted analyses.

The goal of this study was to determine whether immunosenescence-related immune phenotypes are associated with frailty, and to identify potential candidate immune biomarkers that could be investigated in future studies as predictors for change in frailty status over time. To this end, we sought to identify immune biomarkers predictive of frailty scores in elderly nursing home residents in a cross-sectional analysis.

Methods

Subjects and Setting

Details of our study have been described previously [15]. Briefly, elderly participants were recruited from 32 nursing homes in Halifax, Nova Scotia; Sherbrooke, Quebec; Hamilton, Ontario and Vancouver, British Columbia during September and October 2009, 2010 and 2011. Nursing home residents ≥65 years of age were eligible. Exclusion criteria were the use of immunosuppressive medications (including cancer chemotherapy, oral corticosteroid use >21 days, methotrexate, post-transplant medications and/or anti-cytokine or B-lymphocyte depletion therapies) or expected death

within 30 days (as determined by the supervising physician). All participants (or their legally appointed guardian in the event they were not competent to provide consent themselves) provided written informed consent. The study had Research Ethics Board approval from each participating institution and nursing home.

The Frailty Index Score

There are at least 3 measures of frailty that have been validated in nursing homes, including the Frailty Index, the Cardiovascular Health Survey (Frail-CHS, also known as Fried frailty) and the Clinical Frailty Scale [16]. The Frailty Index was selected for this study as it is the most precise measure of frailty in nursing home populations [16]. The Frailty Index is a ratio that is derived by counting the number of deficits and dividing it by the number of items considered; the more deficits a person has, the higher the level of frailty [17]. The deficits included in the Frailty Index are not standardized; however creation of each Frailty Index requires a minimum of 30 items, all related to health status and they must cover different components of health [18]. The ability of the Frailty Index to predict mortality is reproducible across studies, despite the different deficits that might be selected [19]. The Frailty Index used in this study was developed following standard procedures [18]. The thirty variables used to calculate the Frailty Index and their coding can be found in the Appendix. Trained research personnel abstracted baseline demographics as well as the Frailty Index variables using patient interviews, examinations and chart reviews.

Peripheral Blood Mononuclear Cell Analysis and Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were drawn following enrolment and at the same time frailty was measured. Methods of PBMC analysis and flow cytometry have been previously described in detail [15]. Briefly, PBMCs were isolated and frozen using a validated common standard operating procedure [20]. T-cell immune phenotypes were determined by thawing patient PBMCs [21] and placing an aliquot (0.5–16x10^6 cells/stain) in round-bottom 96-well plates with anti-CD3-Qdot605, anti-CD8-Alexa Flour 700, anti-CD4-Pacific Blue, anti-CD45RA-PE Texas Red, anti-CD28-PE, anti-CD57-FITC, anti-CCR7-PE Cy7. T-regs were identified using anti-CD3-FITC, anti-CD4-Pacific Blue, anti-CD127-PerCP-Cy5.5, anti-CD25-PE, and anti-FoxP3-AlexaFluor700. We defined the T-cell subsets as follows: naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45-CCR7-), terminally differentiated (CD45RA+CCR7-) and senescent (CD28-CD57+). Antibody staining was performed using a Beckman Coulter Biomek NX^p Laboratory Automation Workstation (Beckman Coulter, Ontario) [22], followed by analysis using an LSR II flow cytometer with a high-throughput sampler (BD Biosciences, NJ, USA). T-regs were analyzed using FlowJo 9.6 (Treestar Inc, Ashland, OR). T-cell subset analysis employed an automated gating strategy using the flowDensity algorithm [15, 23].

As previously described, CMV-reactive T-cells were identified by stimulating PBMCs with a pool of overlapping peptides spanning the immunodominant pp65 protein of CMV (PepTivator pp65, Miltenyi Biotec) [15, 21]. CMV-reactive T-cells were identified as CD3+ CD4+ or CD8+ IFN-γ+ TNF-α+.

Statistical analysis

The Frailty Index was calculated for each resident and a histogram was created to determine whether the score was approximately normally distributed. To confirm construct validity, the Frailty Index score was compared to the Clinical Frailty Scale using ANOVA testing. The Frailty Index is a continuous measure whereas the Clinical Frailty Scale is categorical. The factors in the ANOVA were Clinical Frailty Scale 4, 5, 6 and 7/8. Participants were nested within nursing homes, thus, to appropriately account for the potential clustering effect of nursing homes, a multilevel modelling approach was taken [24, 25]. First, an intercepts-only model was generated which allowed the proportion of variation in the response variable (Frailty Index) associated with the clustering within the nursing homes to be calculated using intra-class correlation. Then, the following immune phenotypes percentages were added to the intercepts-only model as fixed effects: CD4+ T-cell, naïve CD4+ T-cell, central memory CD4+ T-cell, effector memory CD4+ T-cell, terminally differentiated CD4+ T-cell, senescent CD4+ T-cell, CMV-reactive CD4+ T-cell, CD8+ T-cell, naïve CD8+ T-cell, central memory CD8+ T-cell, effector memory CD8+ T-cell, terminally differentiated CD8+ T-cell, senescent CD8+ T-cell, CMV-reactive CD8+ T-cell and CD4+/CD8+ ratio. The final model was determined using appropriate backwards elimination of the fixed effects, while retaining the random effect of nursing homes. *A priori*, it was decided that age and sex would be included in the final model, given their potential for confounding with the effects of primary interest in this population [19]. The possibility of multicollinearity was

examined using the variance inflation factor (VIF); presence of multicollinearity was defined as VIF >10. Age was entered into the equation as a continuous variable and rescaled by dividing by 10, to enhance interpretation and sex was entered as a dichotomous variable with female as the reference category. The values of the immune phenotypes were natural log transformed to reduce the skewness seen in the original distributions, and were analyzed as continuous variables. CD4+/CD8+ T-cell ratio was entered as a dichotomous variable (<1.0 and ≥1.0) with the ratio <1.0 as the reference category. To ensure that there were no severe violations of regression assumptions by the final model, the residuals were plotted against the predicted values to check that the variance of the residuals was constant and that there was a mean of zero, and to identify possible outliers.

To test the robustness of our results, a sensitivity analysis was planned. The main analysis was repeated, but excluded any nursing home with fewer than 10 participants.

P-values using 2-tailed tests and 95% CIs were constructed. P-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois).

Results

In total, 1165 residents were enrolled in the study and of these, PBMC were obtained from 1087 (93%). Fifteen participants (1%) withdrew before the end of the study, leaving 1072 as our final sample size. The median age was 86 years (interquartile

range [IQR] 80 – 90 years) and the ages ranged from 65 – 102 years. Eight persons were ≥100 years. 776 (72%) were female. Participants were recruited from 32 nursing homes; 5 in Hamilton, Ontario; 4 in Sherbrooke, Quebec; 9 in Vancouver, British Columbia and 14 in Halifax, Nova Scotia. The median number of participants enrolled at each site was 17 (range from 3 to 135); 7 nursing homes had less than 10 residents in the study. The medians and corresponding IQRs for each immune cell type tested in this study are shown in Table 1. Only 47 (4%) of residents had a CD4+ /CD8+ T-cell ratio <1.0.

The Frailty Index was approximately normally distributed (Figure) with a mean of 0.44 and a standard deviation of 0.13. Frailty Index means of nursing homes ranged from 0.30 – 0.59. The 99th percentile upper limit of the Frailty Index distribution was 0.72. In ANOVA testing, the mean Frailty Index significantly increased as the Clinical Frailty Scale score increased [mean Frailty Index when: Clinical Frailty Scale 4 = 0.30 (SD 0.10); Clinical Frailty Scale 5 = 0.35 (SD 0.09); Clinical Frailty Scale 6 = 0.42 (SD 0.11); Clinical Frailty Scale 7 or 8 = 0.51 (SD 0.12), p<0.001], satisfying construct validity.

In the main analysis, the intra-class correlation from the intercepts only model (Table 2) was 0.18 [0.003/(0.003+0.014)] meaning 18% of the variability in the Frailty Index was associated with differences between nursing homes. Results of the final multilevel model are found in Table 2. Increasing age per 10 years was associated with a higher mean Frailty Index (p=0.002) whereas sex had no statistically significant effect (p=0.706). Higher numbers of naïve CD4+ T-cell% (p=0.001) and effector memory CD8+ T-cell% (p=0.019) were associated with a lower mean Frailty Index whereas higher numbers of CD8+ central memory T-cell% were associated with a higher mean

Frailty Index score (p=0.017). There were no obvious violations of regression assumptions by the final model. The results from the sensitivity analysis were almost identical to the main analysis (Table 2).

Discussion

In this multilevel linear regression analysis of immune biomarkers predictive of frailty, we found that higher levels of naïve CD4+ T-cell% and effector memory CD8+ T-cell% predicted lower levels of frailty and higher levels of central memory CD8+ T-cell% predicted higher levels of frailty in elderly nursing home residents. To our knowledge, this is the first study to describe the association between these immune phenotypes and frailty.

Naïve CD4+ T-cells are responsible for orchestrating immune response upon antigen stimulation, and hence having fewer naïve CD4+ T-cells may reduce an individual's ability to respond to immune threats [26]. It is therefore not surprising that frail individuals, individuals with less reserve and more deficit accumulation [1] have lower naïve CD4+ T-cells. Both naïve CD8+ T-cells and naïve CD4+ T-cells decline with age due to thymic involution, however naïve CD4+ T-cells decline far more slowly than naïve CD8+ T-cells [4]. An association between CD8+ naïve T-cells and frailty may not have been found due to a floor effect; elderly nursing home residents may all have very low CD8+ naïve T-cells. This hypothesis was supported by the fact that the naïve

CD8+ T-cells counts were far lower and with a narrower distribution than naïve CD4+ T-cells.

Effector memory T-cells are responsible for immediate effector function in response to known antigenic stimuli, whereas central memory T-cells have little or no effector function but can self renew and differentiate into effector cells in response to antigenic stimuli [27]. Both effector memory CD8+ T-cell and central memory CD8+ T-cell numbers are known to increase with age [4]. Consistent with our study findings, effector memory CD8+ T-cells are more common in the blood than central memory CD8+ T-cells [27]. While we cannot explain why having higher central memory CD8+ T-cells in the blood would be associated with higher levels of frailty, central memory CD8+ T-cells may not be an ideal immune biomarker, given that most are located in the lymph nodes and tonsils and not the blood [27].

Studies examining the association between CMV sero-status have been conflicting [11, 13, 14]. We chose not to examine CMV sero-status as a predictor, but instead focused on CMV-reactive T-cells. Neither CMV-reactive CD4+ T-cells nor CMV-reactive CD8+ T-cells were associated with frailty in our study. Thus, although CMV has been postulated as a driver of immunosenescence [8], and higher CMV-reactive CD4+ T-cells have been associated with poor outcomes in elderly people including increased risk of respiratory viral infection and mortality [15, 28], CMV-reactive T-cells do not appear to be associated with frailty in elderly nursing home residents.

Our results differed from those of a prior study of frailty in community dwelling elderly women, which found that frail women (n=28) had lower CD4+ T-cells (p=0.02),

higher CD8+ T-cells (p=0.03) and higher senescent CD8+ T-cells (p=0.05) and found no differences between CD4+CD45RA+ (p=0.60) and CD8+CD45RO+ T-cells (p=0.38) when compared to non-frail (n=24) and pre-frail (n=75) women [12]. Reasons for differences between the two studies may be due to the differing populations (community dwelling versus nursing home), differing measures of frailty (Fried versus Frailty Index), and the use of immune phenotyping by the other study that did not allow complete separation of naïve and memory T-cells [29]. Our results are similar to a cross-sectional study of elderly people at least 85 years and older that defined frailty using both the Fried score and the Frailty Index, and which found no differences between CD4+/CD8+ T-cell ratio less than 1 [14]. This study did not analyze naïve and memory CD4+ and CD8+ T-cells individually as predictors of frailty.

We found that frailty varied substantially across nursing homes and future studies of frailty in nursing homes should incorporate statistical techniques to account for this clustering effect; failure to do so risks finding a difference when no difference exists (Type 1 error) [24]. We used the Frailty Index to measure frailty because of its precision in the elderly nursing home population [16]. Although we did not use the original Rockwood Frailty Index [17], we created our Frailty Index according to standard procedures [18], and we were satisfied with its construct validity when compared to the Clinical Frailty Scale. In addition, the upper limit of 99th percentile of the Frailty Index in our study was 72%, similar to other studies that have used the Frailty Index [19].

Although our study had many strengths, including the large number of participants, detailed immune phenotyping, and a statistical analysis that adjusted for

known confounders including other immune phenotypes and accommodated the clustering effect of nursing homes, limitations included its cross-sectional design. Future study is needed to determine whether the immune biomarkers identified in our study predict decline in frailty in elderly nursing home residents. If these associations are validated in a prospective cohort study, immunotherapeutic interventions could be sought and studied [30].

In conclusion, we found that higher levels of naïve CD4+ T-cell% and effector memory CD8+ T-cell% predicted lower levels of frailty and higher levels of central memory CD8+ T-cell% predicted higher levels of frailty in elderly nursing home residents. These results, particularly those of naïve CD4+ T-cells and effector memory CD8+ T-cells provide insights into how immunosenescence may contribute to frailty. A cohort study should be designed to determine whether these immune biomarkers predict change in frailty over time.

Conflict of Interest Statement

All authors report no conflict of interest.

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Table 1: Summary of the distribution of immune phenotypes.

	Total n=1072
Immune phenotypes [median (IQR)]	
CD8+ T-cell	
CD8+ T-cell%	18.66 (12.1 – 27.76)
Naïve CD8+ T-cell%	1.10 (0.60 – 1.82)
Central memory CD8+ T-cell%	0.47 (0.26 – 0.85)
Effector memory CD8+ T-cell%	6.37 (3.81 – 10.51)
Terminally differentiated CD8+ T-cell%	8.95 (4.72 – 14.80)
Senescent CD8+ T-cell%	5.87 (2.40 – 11.58)
CMV-reactive CD8+ T-cell%	0.32 (0.03 – 1.53)
CD4+ T-cell	
CD4+ T-cell%	74.53 (62.92 – 82.53)
Naïve CD4+ T-cell%	13.22 (6.90 – 22.85)
Central memory CD4+ T-cell%	12.22 (8.07 – 16.74)
Effector memory CD4+ T-cell%	31.24 (24.27 – 39.01)
Terminally differentiated CD4+ T-cell%	8.46 (4.93 – 14.04)
Senescent CD4+ T-cell%	1.66 (0.28 – 4.54)
CMV-reactive CD4+ T-cell%	0.06 (0.006 – 0.40)
Ratio	
CD4+/CD8+ T-cell ratio	3.95 (2.33 - 6.76)
T-reg	
T-reg%	2.73 (2.12 – 3.45)

Table 2: Multilevel linear regression model of Frailty Index; intercepts only and final model for the main analysis and sensitivity analysis.

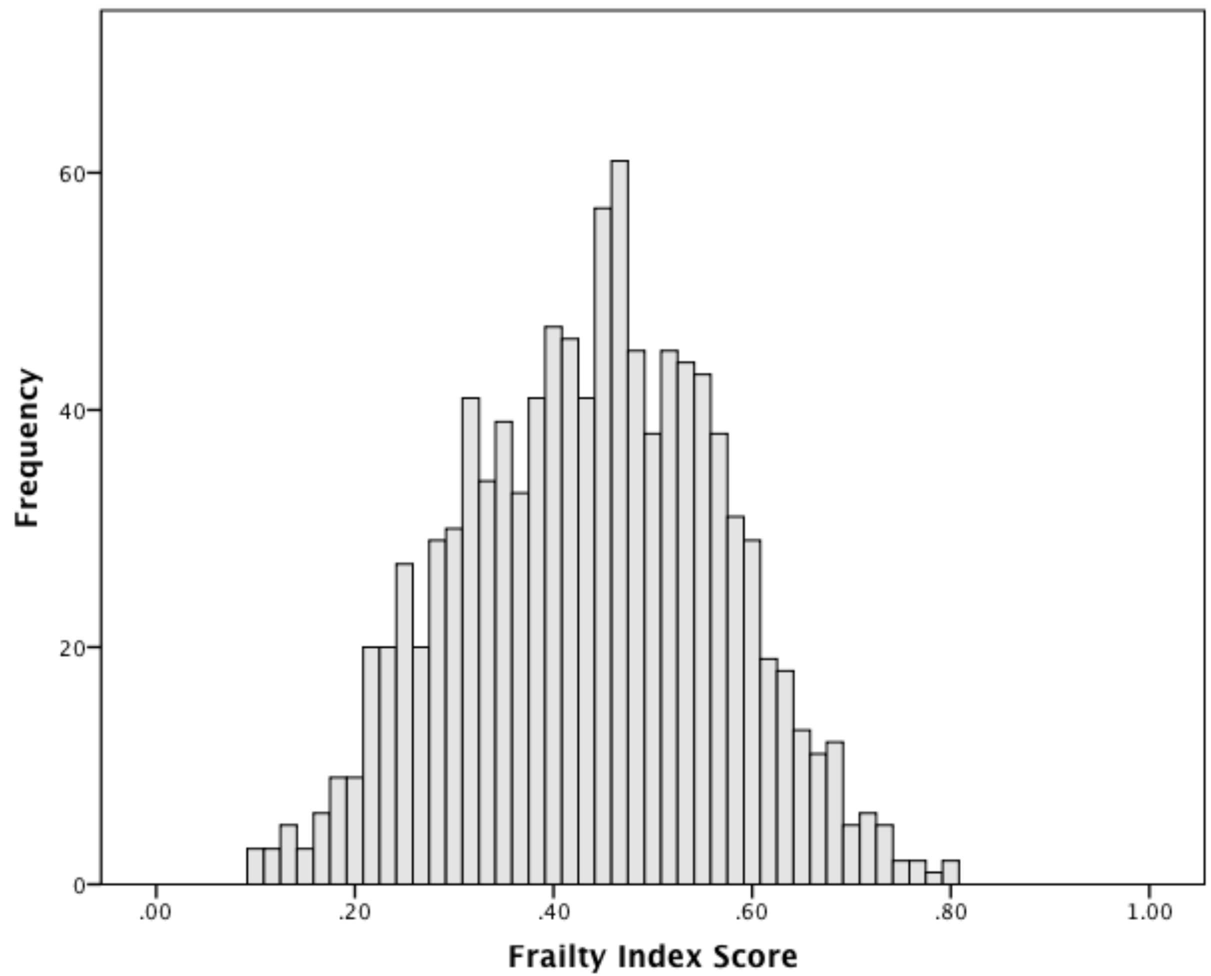
	Intercepts Only Model*			**Final Model****		
Main Analysis						
Variable	Estimate	SE	P-value	Estimate	SE	P-value
Fixed-effect participant-level variables						
Intercept γ_{00}	0.431	0.012	<0.001	0.373	0.058	<0.001
Age/10 γ_{10}				0.017	0.006	0.002
Sex γ_{20}				0.004	0.010	0.706
CD4+ naïve T-cells γ_{30}				-0.020	0.006	0.001
CD8+ central memory T-cell γ_{40}				0.014	0.006	0.017
CD8+ effector memory T-cells γ_{50}				-0.019	0.008	0.019
Random-effect variables						
Intercept variance μ_{0j}	0.003	0.001	0.007	0.003	0.001	0.009
Residual e_{ij}	0.014	0.001	<0.001	0.014	0.001	<0.001
Sensitivity Analysis						
Fixed-effect participant-level variables						
Intercept γ_{00}	0.433	0.012	<0.001	0.383	0.058	<0.001
Age/10 γ_{10}				0.017	0.006	0.004
Sex γ_{20}				0.003	0.010	0.261
CD4+ naïve T-cells γ_{30}				-0.019	0.006	0.001
CD8+ central memory T-cell γ_{40}				0.014	0.006	0.017
CD8+ effector memory T-cells γ_{50}				-0.021	0.008	0.011
Random-effect variables						
Intercept variance μ_{0j}	0.003	0.001	0.010	0.003	0.001	0.012
Residual e_{ij}	0.014	<0.001	<0.001	0.014	<0.001	<0.001

SE: standard error

*Model equation: $Y_{ij} = \gamma_{00} + \mu_{0j} + e_{ij}$

** Model equation: $Y_{ij} = \gamma_{00} + \gamma_{10} + \gamma_{20} + \gamma_{30} + \gamma_{40} + \gamma_{50} + \mu_{0j} + e_{ij}$

Figure: Histogram summarizing the distribution of the Frailty Index in the elderly nursing home residents.



Appendix: Variables used to calculate the Frailty Index.

Variable	Coding
Help bathing	1=Dependent 0=Independent
Help dressing	1=Dependent 0.5=Needs some help 0=Independent
Help transfers	1=Dependent 0.5=Needs some help 0=Independent
Help with mobility	1=Dependent 0.5=Needs some help 0=Independent
Help eating	1=Dependent 0.5=Needs some help 0=Independent
Help grooming	1=Dependent 0=Independent
Help using toilet	1=Dependent 0.5=Needs some help 0=Independent
Help up/down stairs	1=Dependent 0.5=Needs some help 0=Independent
Help with medications	1=Dependent 0.5=Needs some help 0=Independent
Cognition	1=Dementia 0.5=Cognitive impairment, no dementia 0=Within normal limits
Weight loss	1=Loss 0=Stable/gain
Falls	1=Yes 0=No
Mood	1=Depressed/anxiety 0=Within normal limits
Help with finances	1=Dependent 0.5=Needs some help 0=Independent
Continent of bladder	1=No 0=Yes
Continent of bowel	1=No 0=Yes
Skin ulcer	1=Yes 0=No
Skin edema	1=Yes 0=No
Chronic obstructive pulmonary disease	1=Yes 0=No
Diabetes	1=Yes 0=No

Heart failure	1=Yes 0=No
Stroke	1=Yes 0=No
Cancer	1=Yes 0=No
Coronary artery disease	1=Yes 0=No
Hypertension	1=Yes 0=No
Polypharmacy (≥5 medications)	1=Yes 0=No
Balance	1=Impaired 0=Within normal limits
Hearing	1=Impaired 0=Within normal limits
Vision	1=Impaired 0=Within normal limits
Speech	1=Impaired 0=Within normal limits

CHAPTER 6

CONCLUSION

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CONCLUSION

In this final chapter, I provide an overview of the findings within each thesis manuscript chapter, discuss the implications of the results and propose future directions of research.

Summary of Research

In the first thesis manuscript (Chapter 2), I characterized T-cell and natural killer (NK) cell immune phenotypes in elderly nursing home residents by first comparing their immune phenotypes to those of healthy adults and then explored how age, sex, frailty and nutritional status influenced immune phenotypes in the elderly nursing home residents.

In elderly nursing home residents, there were fewer naïve CD8+T-cells, more terminally differentiated CD8+T-cells, and more senescent CD8+ T-cells, consistent with the remodeling of the T-cell compartment seen in community dwelling elderly [1-3]. Although there was no difference between total CD8+ T-cells between the two groups, there were higher numbers of CD4+ T-cells in the nursing home elderly compared to healthy adults. This was likely why the nursing home elderly group had a higher CD4+/CD8+ T-cell ratio than healthy adults and only 6% of elderly nursing home residents had a CD4+/CD8+ T-cell ratio less than one.

There were higher T-regs and higher mature NK cells in the elderly nursing home group compared to the healthy elderly, consistent with immunosenescence studies including community dwelling elderly [4-6]. The very frail and malnourished elderly

people had lower median T-regs and higher senescent NK cells than those that were less frail and had better nutritional status.

We concluded that differences in naïve CD8+ T-cells, terminally differentiated CD8+ T-cells, T-regs and NK cells subsets in the nursing home elderly when compared to healthy adults were similar to studies involving community dwelling elderly. However, the CD4+/CD8+ T-cell ratio was higher in the elderly nursing home group.

In the second thesis manuscript (Chapter 3), I identified immune phenotypes associated with immunosenescence that predicted risk of respiratory viral infection during the ensuing winter respiratory viral season using a Cox proportional hazards model adjusted for age, sex, frailty status and clustering at the level of the nursing home. In multivariable analysis, high CMV-reactive CD4+ T-cells were associated with an increased risk of respiratory viral infection and high T-regs were associated with a reduced risk of respiratory viral infection. In contrast to prior hypotheses [7], CD8+ T-cells were not found to be predictive of respiratory viral infection in multivariable analysis. To our knowledge, this is the first study to identify immune biomarkers predictive of respiratory viral infection in elderly people.

T-regs have been consistently observed to be higher in elderly people when compared to younger healthy adults [6], and the prevailing hypothesis had been that the accumulation of T-regs represented an adverse adaptation, potentially increasing risk of infection in the elderly [6, 8]. Instead, in our study, higher levels of T-regs were associated with reduced risk of respiratory viral infection. Little is known about the role of T-regs in preventing acute respiratory viral infection in humans [9]. In mice, T-regs

appear to play an important role in controlling the immune response to respiratory viral infection [10-12]. We speculate that elevated levels of T-regs may suppress immune pathology associated with anti-viral immunity. The relationship between T-regs and risk of infection should be an important focus of future research.

CMV is hypothesized to be an important driver of immunosenescence [13]. We therefore explored the association between CMV-reactive T-cells and the risk of symptomatic respiratory viral infection. Having higher CMV-reactive CD4+ T-cells, but not CMV-reactive CD8+ T-cells, was associated with an increased risk of respiratory viral infection. This was the first study to find an association between CMV-reactive CD4+ T-cells and risk of infection.

In the third thesis manuscript (Chapter 4), I explored associations between immune phenotypes and risk of mortality using a Cox proportional hazards model adjusted for age, sex, frailty status and clustering at the level of the nursing home. In multivariable analysis, high levels of CMV-reactive CD4+ T-cells but not CMV-reactive CD8+ T-cells were associated with increased risk of mortality within 1-year in individuals aged 65-84 years. Other immune phenotypes including CD4+ T-cell subsets, CD8+ T-cell subsets and CD4+/CD8+ T-cell ratio less than one were not individually predictive of mortality within 1-year.

As a secondary analysis, I performed an exploratory cluster analysis using similarities between immune phenotypes to group nursing home residents. Using this approach, immune phenotypes were able to cluster essentially all participants with a CD4+/CD8+ ratio less than one from the cohort together, and similar to the results in the

OCTO study [14], the mortality in this group was statistically higher than the other two clusters, when they were combined. This finding raises the possibility that while having a low CD4+/CD8+ T-cell ratio is not predictive of mortality for most people within our cohort, there is a small select subgroup where a low CD4+/CD8+ T-cell ratio may be predictive of mortality.

In the fourth manuscript (Chapter 5), I investigated immune phenotypes associated with frailty in elderly nursing home residents using a multi-level modeling approach. In elderly nursing home residents, higher naïve CD4+ T-cells, and higher effector memory CD8+ T-cells predicted lower levels of frailty, and higher central memory CD8+ T-cells predicted higher levels of frailty. Central memory CD8+ T-cells may not be an ideal immune biomarker given that most are located in the lymph nodes and tonsils and not the blood [15]. These results, particularly those of naïve CD4+ T-cells and effector memory CD8+ T-cells may provide insight into potential biological mechanisms of frailty. For example, naïve CD4+ T-cells are responsible for orchestrating immune response upon antigen stimulation [16]. Thus having fewer naïve CD4+ T-cells may reduce an individual's ability to respond to immune threats [16] thereby contributing to the risk of accumulating deficits. Similarly, reduced numbers of effector memory T-cells (cells responsible for immediate effector function in response to known antigenic stimuli [15]) could contribute to an increase in risk of accumulating deficits.

Importance of the Research and Future Directions

In this thesis, I sought to identify immune biomarkers predictive of clinical outcomes. The reasons for doing this were three-fold. First, immune biomarkers could help identify residents at highest risk of the specified outcome and therefore provide potential opportunities for prevention. The second was to help guide future research of novel prevention strategies and the third was to provide insight into potential mechanisms. In the following paragraphs, I highlight the importance of the findings using this framework and outline potential future research directions.

In the second and third thesis manuscripts (Chapter 3 and 4), we found high CMV-reactive CD4+ T-cells but not CMV-reactive CD8+ T-cells to be predictive of adverse outcomes including respiratory viral infection in elderly nursing home residents and mortality in nursing home residents aged 65 – 84 years. We are unaware of any other study linking CMV-reactive CD4+ T-cells to increased risk of respiratory viral infection or mortality.

CMV-reactive CD4+ T-cells could be used to identify nursing home residents at highest risk of respiratory infection. Heightened surveillance of those at risk could be performed during the highest risk periods of respiratory viral infection to help prevent nursing home outbreaks and transmission to healthcare workers. This prevention strategy could be tested in a cluster randomized trial where nursing homes are randomized to receive targeted respiratory viral surveillance of nursing home residents with high CMV-reactive CD4+ T-cells or routine surveillance. The primary outcome would be symptomatic respiratory viral infection in residents and healthcare workers. However,

this trial may not be feasible. Although CMV reactive CD4+ T-cells predicted increased risk of respiratory viral illness (HR 1.69, 95% CI 1.03 - 2.78), the lower limit of the confidence interval was just above one and thus it may not be an ideal biomarker for this strategy. An immune biomarker with better predictive ability would be preferred.

CMV-reactive CD4+ T-cells could also be used to identify nursing home residents ages 65-84 years at risk of dying within 1-year. A multi-disciplinary intervention aimed to reduce risk of mortality in nursing home residents with high CMV-reactive CD4+ T-cells could be designed and tested in a randomized controlled trial. However, this trial may not be feasible. Although high CMV-reactive CD4+ T-cells predicted increased risk of death in those aged 65-84 years, it had no differential effect in those aged 85-104 years. An ideal biomarker would be predictive of mortality in all age groups. Furthermore, it may be difficult to design an intervention to reduce the risk of mortality in this frail elderly population. A more practical use of CMV-reactive CD4+ T-cells could be to help healthcare workers prognosticate and allow nursing home residents and their families prepare for death.

The results of our studies investigating immune biomarkers predictive of respiratory viral infection and mortality suggest that the interplay between CMV and CD4+ T-cells could be important in immunosenescence and a deeper understanding of its potential mechanistic role should be explored. It is known that CMV positivity is associated with large numbers of virus-specific CD4+ T-cells in the elderly [17] and an increase in CD4+ memory T-cells [17, 18] but we are the first to describe an association between CMV-reactive CD4+ T-cells and clinical outcomes.

If CMV infection is established as a contributing cause of immunosenescence, emerging vaccines designed to prevent CMV infection [19] could prove to be a useful strategy to prevent respiratory viral infection and/or mortality; however this study design would be difficult to power as only individuals not yet infected with CMV (only 10% of elderly nursing home residents in our first thesis manuscript [Chapter 2]) would potentially benefit. As an alternative prevention strategy, anti-viral therapy to reduce reactivation of CMV could be tested as an intervention in a randomized controlled trial to prevent respiratory viral infection (or mortality in those aged 65-84 years) using existing anti-CMV therapy including ganciclovir or valganciclovir [20].

Another important finding of this thesis is that despite immunosenescence theory and literature [7, 21, 22] the CD4+/CD8+ T-cell ratio less than one was not predictive of mortality in this cohort. However, our cluster analysis suggests that there is a small subgroup of individuals, defined by a CD4+/CD8+ T-cell ratio less than one that may have a higher risk of mortality than the rest of the group. We speculate that the current immunosenescence dogma, that mortality in the elderly is related to the expansion of the CD8+ T-cell compartment due to an accumulation of poorly functional senescent CD8+ T-cells, possibly driven to exhaustion by CMV infection, may only apply to a select subgroup, possibly based on genetic make-up but that CD4+/CD8+ T-cell ratio less than one is not predictive of mortality in most in our cohort. These results were exploratory and thus should be interpreted with caution, however, this hypothesis could help explain the discrepant findings between the Swedish cohorts [21, 22] and other cohort studies

conducted in Canada, the United States and the United Kingdom where the CD4+/CD8+ T-cell ratio less than one has not been found predictive of mortality [23, 24].

Having high T-regs predicted lower risk of respiratory viral infection. This study was designed to determine association and not causation, but it suggests that the role of T-regs in preventing respiratory viral infection should be explored further. Currently little is known about the role of T-regs in preventing viral respiratory infection [9]. Murine data has focused on the response of T-regs during respiratory vial infection [10, 11]. Potential mechanisms and biological plausibility of how high T-regs could prevent viral respiratory infection could be explored using mouse models.

If mechanisms are identified, T-regs could be investigated as a novel way to prevent respiratory viral infection. The modulation of T-regs for the purpose of therapy is a growth area of research [25], and strategies that boost circulating T-regs could first be tested as a way to prevent respiratory viral infection in mice. Ultimately, the goal would be to determine whether T-reg boosting strategies prevented respiratory viral infection in elderly nursing home residents in a randomized controlled trial; however there are many research questions to address prior to this research being feasible.

Last, a prospective cohort study should be designed to determine whether naïve CD4+ T-cells and effector memory CD8+ T-cells could predict change in frailty over time. The ability to predict declines in frailty would provide an opportunity to test multi-disciplinary interventions designed to slow advancing frailty.

In our thesis studies, there were several examples where significant findings in unadjusted analysis became non-significant after adjusting for potential confounders

including age, sex and frailty as well as other immune phenotypes. For example, although high CD8+ terminally differentiated T-cells and CD8+ senescent T-cells were associated with an increased risk of respiratory viral infection in univariable analyses, these CD8+ T-cell subsets became non-significant in multivariable analysis and were not included in the final model. Failure to account for potential confounders would have led to falsely concluding that the prevailing immunosenescence hypothesis (i.e. that high CD8+ terminally differentiated T-cells and CD8+ senescent T-cells were associated with increased risk of respiratory viral infection [26]) was correct. We also accounted for the clustering effect of nursing homes in each analysis. In the fourth thesis manuscript (Chapter 5), almost a fifth of the variability in frailty as measured by intra-class correlation was associated with differences between nursing homes. Failure to account for clustering when there is evidence of even low values of intra-class correlation can substantially increase the risk of finding a difference when no difference exists (Type 1 error)[27]. These examples highlight the need for a robust statistical approach in any future study of immune biomarkers and outcomes.

Limitations

The studies described in this thesis had many strengths including a large sample size of nursing home residents, detailed prospectively collected clinical information including frailty, as well as comprehensive immune phenotyping. However, there were limitations to our approach. First, we limited our immune phenotypes to T-cell subsets as T-cells are most affected by immunosenescence [7]. We acknowledge that all

components of the immune system are altered during immunosenescence, and we did not evaluate innate immune system predictors or humoral immune system as potential predictors of clinical outcomes. Second, immune phenotypes are descriptive and do not provide insights into the functionality of the immune cells [28]. Examining the functionality of the T-cell subsets would be an important next step, which we were unable to do due to cost limitations and limited patient material. Last, we used immune phenotypes from PBMCs as biomarkers to predict clinical outcomes. Immune phenotypes provide a snapshot of the immune system at the level of the peripheral blood, and are easily accessible, but may not represent the distribution of immune cells centrally, or at a site of infection (i.e. the lungs during a respiratory infection).

Conclusion

The studies described throughout this thesis have furthered our knowledge of immunosenescence. Using rigorous methodology and through a variety of statistical techniques, we have discovered new associations between immune phenotypes and clinical outcomes, which will be further explored in future research programs. For example, the role of T-regs in preventing respiratory viral infection will be an important area of future research and could lead to novel prevention strategies. Additionally, many of our findings failed to support prevailing immunosenescence hypotheses such as the association between CD8+ T-cell subsets and risk of respiratory viral infection and the expansion of CD8+ T-cells relative to CD4+ T-cells (CD4+/CD8+ T-cell ratio <1.0) as a predictor of mortality. CMV-reactive CD4+ T-cells but not CMV-reactive CD8+ T-cells

were predictive of respiratory viral infection and mortality suggesting that CMV-reactive CD4+ T-cells may be more important in the pathogenesis of immunosenescence than CMV-reactive CD8+ T-cells. Insights into the relationship between naïve CD4+ T-cells, effector memory CD8+ T-cells and frailty should be investigated and tested as predictors of change in frailty.

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