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The Immune System in the Oldest-Old
Clinical and Immunological Studies in
the NONA Immune Cohort

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Labor omnia vincit

Abstract

The oldest-old (people aged 80 or older) constituted 5 % of the population in Sweden in 2000, an increase from 1.5 % fifty years earlier. The immune system undergoes dramatic changes at high age, sometimes referred to as “immunosenescence”. However, the natures of these changes, and in particular, their clinical consequences are incompletely understood. In a previous longitudinal study, a set of immune parameters were identified and termed immune risk phenotype (IRP) because of an association with increased mortality. The IRP consists of changes in the T lymphocyte compartment, in particular an inverted CD4/CD8 ratio. The IRP was found to be associated with cytomegalovirus (CMV) infection, which through expansions of cytolytic anti-viral CD8 cell responses was ascribed a role in the development of IRP. The general aim of this thesis was to increase the knowledge of changes in the immune system and their clinical consequences in the oldest-old. The population-based random sample of the longitudinal NONA-Immune Study (n = 138, mean age 90 years at baseline) was used for all investigations.

In paper I, the effects on sample size of various exclusion protocols for immune studies of the elderly was examined. The commonly used SENIEUR protocol, selecting individuals representing ‘normal ageing’, excluded 90 % of nonagenarians. Based on different protocol criteria, individuals were grouped into ‘very healthy’, ‘moderately healthy’ or ‘frail’. The prevalence of CMV was similar across the groups. Further, differentiated CD8 populations associated with CMV, *i.e.* those expressing CD56, CD57 and CD45RA while lacking expression of CD27 and CD28, were equally distributed across the groups of the oldest-old, but were, as expected, significantly increased in the elderly compared to a middle aged control group. The findings showed that lymphocyte subsets associated with IRP might serve as significant biomarkers of ageing independent of the overall health status, also supporting the notion that immunological studies of the oldest-old should be done in population-based non-selected populations.

The IRP and the presence of low-grade inflammation, for example increase of IL-6 in plasma, constitute major predictors of 2-year mortality in the oldest-old. In paper II, the CD4/CD8 ratio and IL-6 were found to predict 97 % of observed survival and 57 % of deaths over 2 years. The impact of IRP and IL-6 on 2-year survival was independent of age, sex and several diseases. The longitudinal design allowed temporal evaluations, suggesting a sequence of events starting with IRP and leading to inflammation in the decline state.

Four-year mortality in the oldest-old (paper III) was found to be mainly related to markers of inflammation and IRP. Individuals with both inverted CD4/CD8 ratio and high IL-6 level had significantly higher 4 year mortality (82 %) compared to individuals with CD4/CD8 ratio ≥ 1 and low IL-6 level (29 %) at baseline. The presence of IRP and increased IL-6 level showed some associations with presence of diseases; in particular, IL6 was associated with the presence of cognitive impairment. However, despite being strong predictors of mortality, IRP and IL-6 could not be linked to any specific cause of death, probably due to the multi-factorial nature of these factors.

The prevalence of antinuclear antibodies (ANA) in the oldest-old was higher compared to younger controls (paper IV). The difference across age was most pronounced in men, showing low levels at younger age, whereas the prevalence among the oldest-old men reached a similar level as in women. There was no association between the presence of ANA and IRP, CMV status or health status in the oldest-old.

Table of contents

POPULÄRVETENSKAPLIG SAMMANFATTNING	5
ORIGINAL PUBLICATIONS	7
ABBREVIATIONS	8
INTRODUCTION	11
Demographics	11
Ageing	12
Immunosenescence	15
Immune risk phenotype (IRP) and T cells - Findings from the OCTO Immune study	16
B cells and antibodies	18
Natural killer cells and Natural killer T cells	19
Neutrophil Granulocytes	20
Clinical implications of immunosenescence	23
Cytomegalovirus	25
Anti-viral T cell responses	27
Inflammation	29
AIMS OF THE THESIS	31
General aim	31
Specific aims	31
MATERIALS AND METHODS	33
Ethics	33
Subjects	33
Paper I	33
Paper II.....	33
Paper III.....	34
Paper IV.....	34

Selection protocols for immunogerontological studies.....	35
SENIEUR protocol	35
OCTO Immune protocol	37
Measures and procedure in the NONA Immune Study	39
Medical records (Paper I-IV).....	39
Cognitive function (Paper I-IV)	40
Self reports	40
Medication usage	41
Weight and length measurement.....	41
Clinical infections	41
Death certificates and cause of death (Paper III)	41
Cell separation and sorting	41
Analysis of cytokine production	42
Clinical chemistry laboratory analysis	42
Measurement of antinuclear antibodies	43
Analysis of antibodies to Cytomegalovirus	44
Flow cytometry analysis of surface protein expression.....	45
Phenotypic markers on lymphocytes (CD surface markers).....	45
Statistics	47
RESULTS AND DISCUSSION	49
Paper I.....	49
Paper II	50
Paper III.....	51
Paper IV	54
Concluding remarks	56
Future perspectives.....	57
Acknowledgements	59

References..... 61

Populärvetenskaplig sammanfattning

I Sverige, liksom i många andra länder, har det under förra århundradet skett en markant ökning av andelen äldre individer i befolkningen. Andelen äldre-äldre (här definierade som individer äldre än 80 år) har ökat från 1.5 % till 5 % av befolkningen under de senaste femtio åren. Den förväntade livslängden ökar ständigt tack vare en mängd faktorer, däribland minskad dödlighet i infektionssjukdomar och hjärtkärlsjukdomar. I hög ålder sker dramatiska förändringar av immunförsvaret. I Jönköping startade under 1980-talet en longitudinell studie av åttiåringar (OCTO studien, av engelskan octogenarian, dvs. åttiåringar) som undersöktes vid upprepade tillfällen med avseende på förändringar i immunsystemet. Resultaten visade att förändringar i antalet av vissa typer av vita blodkroppar kunde knytas till 2-års dödlighet. De förändringar som knöts till denna risk benämndes immun risk profil (IRP) och bestod i en ökning av så kallade cytotoxiska CD8 T lymfocyter och minskning av så kallade T-hjälpar CD4 lymfocyter. Immun risk profilen visade sig vara associerad till förekomst av cytomegalovirus (CMV). CMV är ett vanligt virus, smitta sker vanligtvis under tidiga barnaåren och 60–90 % av vuxna är smittade. Efter primärinfektion håller sig viruset gömt för kroppens immunförsvaret. För att hålla detta virus under kontroll så reagerar immunförsvaret med dels antikroppsproduktion (humoral immunitet) och dels med ett cellulärt svar (cellmedierad immunitet). I studier av äldre-äldre har man sedan 1970-talet vanligtvis använt sig av olika protokoll för att inkludera individer med god hälsa för immunologiska studier. Ett sådant protokoll är SENIEUR protokollet vilket är mycket strikt och utesluter i princip alla individer med medicinering eller individer med känd sjukdom inklusive de med avvikelser i vissa laboratorieanalyser.

Huvudsyftet med studierna har varit att öka kunskapen om immunsystemet hos äldre-äldre och vilka kliniska konsekvenser förändringar i immunsystemet ger upphov till. Syftet med första studien var att använda olika selektionsprotokoll på ett icke-selektat populationsbaserat urval av nittioåringar ($n = 138$) från Jönköpings kommun och för att studera olika T lymfocyt populationer i relation till hälsotillstånd. SENIEUR protokollet uteslöt 90 % och endast 13 av 138 blev kvar och klassificerades som 'very healthy'. Ett annat protokoll (tidigare använt i OCTO studien) exkluderade 65 % av de ursprungliga individerna och de kvarvarande 38 individerna klassificerades som 'moderately healthy'. De resterande, som inte uppfyllde kriterierna i något av protokollen, klassificerades som 'frail'. Av de 138 individerna hade 22 (16 %) en IRP och förekomsten av IRP var densamma i grupperna med olika hälsotillstånd. Det förelåg statistiska skillnader mellan en medelålders kontrollgrupp och de äldre-äldre med avseende på olika T lymfocyt populationer. Den sammanslagna gruppen av äldre-äldre hade högre förekomst i blodet av olika populationer av CD8 T-lymfocyter jämfört med en yngre kontrollgrupp. Däremot var förekomsten densamma i äldre-grupperna med olika hälsotillstånd. Dessa lymfocyt populationer är alltså associerade till ålder och förekomst av CMV infektion, men de är oberoende av hälsotillstånd. Studien visar att dessa lymfocyt förändringar fungerar som markörer för åldersförändringar i immunsystemet oavsett hälsotillstånd. Vidare är det viktigt att studier på äldre-äldre görs på breda populationsbaserade material.

Syftet med den andra studien var att korrelera IRP och inflammation till 2-års dödlighet. Med inflammation avses bland annat förhöjda nivåer i blod av C-reaktivt protein (CRP) och interleukin-6 (IL-6). Studien visade att IL-6-nivåer och förekomst av IRP kunde förutsäga 97 % av observerad överlevnad och 57 % av dödligheten under en 2-års period.

I den tredje studien undersöktes IRP och låggradig inflammation i relation till 4-års dödligheten, och deras inverkan på sjuklighet och dödsorsak. Individer med en kombination av hög nivå av IL-6 och förekomst av IRP hade signifikant högre 4-års dödlighet (82 %) jämfört med individer utan IRP och låg nivå av IL-6 (29 %) vid start av studien. Förhöjd nivå av IL-6 var kopplad till framför allt förekomst av demens. Däremot kunde varken IL-6 eller IRP knytas till specifik dödsorsak. Anledningen till att IL-6 och IRP inte är kopplade till specifik dödsorsak, trots att de är så starka prognostiska faktorer, kan vara att de är markörer för flera olika processer, som alla var för sig är kopplade till dödsorsak.

Syftet med fjärde studien var att fastställa förekomsten av en viss typ av autoantikroppar, antinukleära antikroppar (ANA), hos äldre-äldre i jämförelse med en yngre kontrollgrupp. Förekomsten av ANA var signifikant ökad hos äldre-äldre jämfört yngre kontroller. Skillnaden var mest uttalad för män, som har en låg förekomst i unga år, medan de äldsta männen nådde ungefär samma nivåer som hos kvinnor. Inget samband hittades mellan förekomst av ANA och sjuklighet, IRP eller förekomst av CMV.

Original publications

I. Bengt-Olof Nilsson, Jan Ernerudh, Boo Johansson, Per-Eric Evrin, Sture Löfgren, Frederick G. Ferguson, Anders Wikby

Morbidity does not influence the T-cell immune risk phenotype in the elderly: Findings in the Swedish NONA Immune Study using sample selection protocols.

Mechanisms of Ageing and Development 124, 469-476, 2003.

II. Anders Wikby, Bengt-Olof Nilsson, Rosalyn Forsey, Julie Thompson, Jan Strindhall, Sture Löfgren, Jan Ernerudh, Graham Pawelec, Frederick Ferguson, Boo Johansson

The immune risk phenotype is associated with IL-6 in the terminal decline stage: Findings from the Swedish NONA immune longitudinal study of very late life functioning.

Mechanisms of Ageing and Development, 127, 695-704, 2006.

III. Bengt-Olof Nilsson, Jan Strindhall, Sture Löfgren, Rosalyn Forsey, Julie Thompson Boo Johansson, Jan Ernerudh, Anders Wikby

The Immune Risk Phenotype and IL-6 among Nonagenarians and Associations with Morbidity and Mortality: Findings from the Swedish NONA Immune Longitudinal Study.

Manuscript

IV. Bengt-Olof Nilsson, Thomas Skogh, Jan Ernerudh, Boo Johansson, Sture Löfgren, Anders Wikby, Charlotte Dahle

Antinuclear antibodies in the oldest-old women and men.

Journal of Autoimmunity, 27, 281-288, 2006.

Abbreviations

ACTH	Adrenocorticotrophic hormone
ADL	Activity of Daily Living
AIDS	Acquired Immunodeficiency Syndrome
ALAT	Alanine Aminotransferase
ANA	Antinuclear Antibodies
ANOVA	One-way analysis of variance
APA	American Psychiatric Association
ASAT	Aspartate Aminotransferase
BMI	Body Mass Index
CCR7	Chemokine receptor 7
CD	Cluster Designation
CMV	Cytomegalovirus
Con A	Concanavalin A
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DSM	Diagnostic and Statistical Manual of Mental Disorders
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot Assay
ENA	Extractable Nuclear Antigens
ESR	Erythrocyte sedimentation rate
EURAGE	Concerted Action Programme on Ageing of the European Community
F-ANA	Fluorescence-ANA
fMLP	formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GrA	Granzyme A
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
ICD	International Classification of Diseases
IgG	Immunoglobulin G
IL	Interleukin
IRP	Immune Risk Phenotype
Jak	Janus kinase
LPS	Lipopolysaccharide

MCTD	Mixed Connective Tissue Disease
MHC	Major Histocompatibility Complex
mIg	Monoclonal Immunoglobulin
MIR	Memory-In-Reality
MMSE	Mini-Mental State Examination
NADH	Nicotinamide Adenine Dinucleotide
NK cells	Natural Killer cells
NKT cells	Natural Killer T cells
NONA	Nonagenarians
OCTO	Octogenarians
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PMT	Photo-multiplier
pp65	phosphoprotein 65
PWM	Pokeweed mitogen
SENIEUR	from SENIorEUropean
SLE	Systemic lupus erythematosus
STAT	Signal transducers and activators of transcription
T1	Baseline measurements NONA Immune
T2	2-year follow-up NONA Immune
T3	4-year follow-up NONA Immune
Th	T helper cell
TLR	Toll like receptor
TNF	Tumour Necrosis Factor
WBC	White blood cell
WHO	World Health Organization

Introduction

Demographics

Since the beginning of the 20th century, there has been a dramatic change in the demographics of the world generally, and in the Swedish population specifically. There has been an increase in the total world population from 1.65 billion people in 1900 to nearly 6 billion people almost 100 years later (Kinsella and Phillips, 2005). In Sweden, the population has increased by 3.7 million inhabitants to 8.8 million, between 1900 and 1998. More than 17 % of Sweden's population is aged 65 or older (65+), a doubling from 8 % in 1900. The oldest-old (people aged 80 or older) constituted 5 % of the population in Sweden in 2000, an increase from 1.5 % fifty years earlier. In many countries the oldest-old are the fastest growing age segment of the population (Statistiska centralbyrån, 1999). Since 1950, the number of centenarians has doubled each decade in industrialized countries, and the odds of living to the age of 100 have risen from 1 in 20 million to 1 in 50 for females in low-mortality nations such as Sweden and Japan (Vaupel and Jeune, 1995). Life expectancy for Swedes at the beginning of the 20th century was 55 years and 53 years for females and males respectively; this increased to 82 and 77 years respectively by 1997 (Statistiska centralbyrån, 2000). The general belief has been that human death rates increase with age in an exponential manner. Today this picture is now changing, due to new knowledge from studies carried out in various countries, where reliable data has shown that the rate of increase in the mortality rate tends to slow down among the oldest-old. A study from 28 countries between 1950 and 1990 found a tendency for a greater decline in mortality in more recent years amongst the oldest-old (Kannisto, 1994). It is primarily the decrease in deaths caused by circulatory diseases among older people that has led to this fall in the mortality rate (Statistiska centralbyrån, 2007). In Europe in 1900, women outlived men by two or three years. Today, the average gap between the sexes is approximately seven years, although lower in Sweden. Men have a lower life expectancy at birth and also in later life; this is the reason why women outnumbered men in older age groups. However, the explanation for this gender difference in life expectancy is still eluding scientists. The ageing of the world population during the last century is related to a health transition that has been occurring throughout the world — there has been a shift from high to low fertility, an increase of life expectancy at birth and at older ages, and a transition from infectious diseases to non-communicable diseases and chronic conditions.

There was an increase in the population in the county of Jönköping, from 1750–1900, from around 103 000 to approximately 202 000 inhabitants. Today, the county of Jönköping has about 320 000 inhabitants, and whereas the city of Jönköping had about 23 000 inhabitants in 1900, this is now 80 000 approximately (Statistiska centralbyrån, 1999). The sample of the oldest-old in this thesis comes from the population living in the municipality of Jönköping at the end of the 1990s.

Ageing

Ageing is not purely a matter of increasing years, but a process of “adding life to years, not years to life”. When the desired outcomes of ageing are maximized and the undesired ones are minimized, we are in a process of successful ageing. A model of successful ageing was created by Rowe & Kahn in 1987, where three main interacting components are needed to reach a state of successful ageing (Rowe and Kahn, 1997). One of the components was related to avoiding disease and disability, another component was related to the maintenance of cognitive and physical functions, and the third component was related to an active engagement with life.

One fundamental question in ageing research is whether humans and other species possess an unchangeable life-span limit. The observed maximum age at death in Sweden rose from 101 years during the 19th century to about 108 years in the 1990s. The rise in the maximum age at death during this time period is more than 70 % attributable to a reduction in death rates above the age of 70. Another minor explanation for this rise is attributable to a larger size of cohorts. Results from twin studies had found that genetic variation may account for only 25 % of the variation in longevity (Herskind et al., 1996).

At the beginning of the 20th century infectious diseases dominated adult mortality. Since then, there has been a shift from infectious disease; coronary heart disease and cancer dominate adult mortality today. It has been hypothesized that physiological or metabolic “programming” occurs at critical periods during early development and this determines the development of pathological phenomena later in life (Barker et al., 1989). There is an observed relationship between low birth weight and the risk of cardiovascular disease later in life. This “foetal origins hypothesis” is one, but not the only explanation for the development of chronic diseases later in life. In the 19th century, infectious diseases such as smallpox, tuberculosis, and whooping cough had a big impact on infant mortality and later adult mortality. This cohort influence on adult mortality has lost its effect due to the transition from infectious diseases to chronic diseases.

Ageing is an inevitable process that affects humans and all living organisms. Ageing can be defined as a result of the gradual deterioration of normal physiological functions, probably as a result of changes made to cells, tissues, organs and organ-systems. These changes would have a direct impact on the functional ability of the organism as a whole.

The following five criteria for normal ageing have been adapted from Strehler (Strehler, 1962):

Universal—changes affect all individuals of a species

Cumulative—changes increase over time

Progressive—a series of gradual changes

Intrinsic—changes that are not related to environmental factors

Deleterious—changes which compromise normal biological functions

A problem still not resolved in ageing research today is how to distinguish normal ageing from underlying disease. There exists hitherto no unifying theory of ageing; instead many different theories have been proposed to explain the process of ageing. The ageing theories have been grouped into two categories and several subcategories - see Table 1. A separate theory cannot explain all observations, and some observations are best explained by another theory. The theories can be viewed as pieces in a jigsaw puzzle, which together can bring a better understanding of the complexity of ageing. Following is a brief explanation of some of the theories of ageing.

Table 1 Theories of ageing

A. Programmed theories
1. Endocrine theory
2. Programmed senescence theory
3. Immunological theory
B. Damaged/Error theories
1. Free radicals theory
2. Somatic mutation theory
3. Living theory
4. Cross linking theory
5. Wear and tear theory
6. Error theory

The programmed theories explain the ageing process by the concept of an internal biological clock. In the endocrine theory hormones act as the biological clock to control the pace of ageing. The production of several hormones has been observed to decline with age, e.g. human growth hormone, oestrogen and testosterone. According to the damaged/error theories, external or environmental forces are the culpable forces that damage the internal cells and organs. Free radicals, by-products of normal metabolism, can damage proteins, membranes, and nucleic acids etc. All this damage causes ageing, according to the free radical theory. Genetic mutations caused by damage or arising spontaneously (somatic mutation theory),

occur and will accumulate with increasing age. These mutations may result in the production of defective proteins, with harmful effects on the cell. According to the rate of living theory, ageing is the by-product of metabolism. The accumulation of waste products in non-dividing cells impedes the normal function of the cell. A higher rate of metabolism in the organism leads to a shorter life span and vice versa. According to the wear and tear theory, cells have vital parts that wear out like parts in a machine. DNA undergoes continuous damage throughout life, and the ability to repair certain types of damage is related to the life span of its species. Errors in the mechanisms that synthesise protein will lead to faulty proteins that accumulate in the cell and cause damage to cells, tissues and organs.

Immunosenescence

This term was coined in 1969 by Walford in his book entitled, 'The Immunologic Theory of Aging', and refers to the immune system's diminished function with age (Walford, 1969). Today, the term embraces many different changes in human ageing. Immunosenescence may be defined as a "constellation of age-related changes in the immune system, resulting in greater susceptibility to infection and a reduced response to vaccination" (Grubeck-Loebenstein et al., 2009). It can be described as the functional deterioration of the immune system with age. Both the evolutionary older innate and the evolutionary younger adaptive immune systems are influenced by immunosenescence. DelaRosa defined immunosenescence as "the state of age associated dysregulation that contributes to morbidity and mortality due to the greater incidence or reactivation of infectious diseases as well as possibly autoimmune phenomena and cancer" (DelaRosa et al., 2006). Immunosenescence can also be referred to as the 'physiological ageing' of the immune system. In 1995, Franceschi proposed the re-modelling theory of ageing; according to this theory immunosenescence is not a random deteriorative process influencing the immune system (Franceschi et al., 1995): The evolutionary older innate system is preserved or negligibly affected, and is in some cases almost up-regulated, in contrast to the most evolutionary recent adaptive immune system, that deteriorates with age (Ottaviani and Franceschi, 1997). This theory proposes that there is a continuous adaptation (re-modelling) of the body to the deteriorative changes occurring over time. Immunosenescence can be visualized as the result of the continuous encounter of the immune system with a variety of antigens, such as microbial ones, but also food and self molecules. Antigens can be regarded as a sort of stressor of the immune system, and immunosenescence is the consequence of continuous attrition caused by chronic antigen stress (Franceschi et al., 2000a). An accumulation of memory and effector T cells (Weng et al., 2009; Wikby et al., 2002), a reduction of naive T cells (Fagnoni et al., 2000), a shrinkage of T cell repertoire (Ouyang et al., 2003) and a reduction of the immunological space are some of the characteristics of immunosenescence.

Infections have been one of the most important causes of death in the past, and a vigorous immune system is vital for survival. When human lifespan was less than 50 years, the role of immunosenescence was negligible. This lifespan has continuously increased during the last century; however, the role of infections as a major health problem has decreased. According to the evolutionary theory of ageing, the beneficial effects of the immune system early in life become detrimental later in life, in a period not foreseen by evolution (De Martinis et al., 2005).

The ability to mount a strong immune response is vital for survival in infectious environments, but the long-term consequences of the related unintentional damage can be severe (i.e. immunopathology). A genetic predisposition to weak inflammatory activity (e.g. low tumour necrosis factor, high interleukin-10) is advantageous for longevity, provided individuals escape succumbing to infection (Franceschi et al., 2007). It has been proposed that

the increase in life expectancy at older ages over history may not just be due to progress in hygiene and medical care, but be directly due to reduced inflammation during early life, leading to increases in morbidity and mortality, as a result of chronic conditions in old age (Crimmins and Finch, 2006).

The immune system is also influenced by the endocrine system and vice versa; immunosenescence modulates the endocrine system, and endocrinosenescence changes the endocrine system (Straub et al., 2000).

Immune risk phenotype (IRP) and T cells - Findings from the OCTO Immune study

Immune studies had previously been carried out for individuals in their 60s to 70s, and so information about immune system changes was inadequate for the oldest-old population. In addition, studies with a longitudinal design were not common. A step to overcome this lack of knowledge in the immune system of the oldest-old was taken when the longitudinal OCTO Immune study was launched in the late 1980s (Wikby et al., 1994). One aim was to provide a better understanding of processes and mechanisms related to changes of the immune system regulation in very late life. Another aim was to identify presumptive predictors for subsequent mortality and clinical parameters related to the morbidity seen in later life.

The OCTO Immune study started in 1989, when an immunological study was added to the OCTO Longitudinal study, which began in 1987. The OCTO Immune study was a collaboration between researchers at the Institute of Gerontology and the Department of Natural Science and Biomedicine, School of Health Sciences, the Department of Microbiology, Ryhov Hospital, Jönköping and the Department of Veterinary Science, Penn State University, USA, and ended in 1997, when the vast majority of participants were deceased.

Blood samples were drawn for the analysis of immune system parameters.

- Complete blood cell count
- Three colour flow cytometry for cell surface markers (CD) on B and T cells
- Interleukin 2 production
- Proliferative response of peripheral blood mononuclear cells using a mitogen (Concanavalin A, ConA) stimulation assay
- Cytomegalovirus and Herpes simplex serology

Cluster analyses were used to group individuals according to similarities in T cell mitogen response, and the percentages of CD3, CD4, CD8 and C19 positive cells. The identified groups were then compared with respect to their association with survival / non-survival. This

analysis of immune data at baseline revealed a cluster predictive of subsequent 2-year mortality (Ferguson et al., 1995). Individuals in this cluster were characterized by immune parameters that consisted of elevated high levels of CD8+ T cells, low levels of CD4+ T cells, poor proliferative response to mitogen, as well as low levels of CD19+ B cells. Individuals with this profile, Immune Risk Profile or Immune Risk Phenotype (IRP), showed significantly increased 2-year mortality compared to individuals with Non-IRP, both at baseline and two years later (Ferguson et al., 1995; Wikby et al., 1998). A summary of 2-year survival / non-survival in IRP / non-IRP individuals in the OCTO Immune Longitudinal Study is shown in Table 2.

Table 2 Survival versus non-survival in IRP and Non-IRP individuals. P-values were assessed by Chi-square testing

Survival	IRP	Non-IRP	P <
1989–1991			
Survivors	5	59	.001
Non-survivors	9	16	
1991–1993			
Survivors	9	34	.05
Non-survivors	9	11	

Individuals with an IRP at baseline, or those moving into IRP during follow-up, were examined in 1997, eight years after baseline of the OCTO Immune Longitudinal Study. Of the 30 individuals that were classified as IRP individuals by using cluster analysis, twenty-two (73 %) had a CD4/CD8 ratio below one, compared to six of 62 (9 %) with CD4/CD8 ratio above one. Thus, IRP could be defined by using only the inverted CD4/CD8 ratio, since this sole marker was strongly associated with the IRP defined by the cluster of parameters (Wikby et al., 1998).

In the last follow-up of the OCTO Immune Longitudinal Study, in 1997, various subsets of CD8 T cells were included in the study. Changes were found in a number of T cell subsets, with significant increases in the number of CD8+CD28- cells, in particular, indicating that differentiated effector / memory CD8+ cells are disproportionately represented in this cell population.

Interestingly, an inverted CD4/CD8 ratio was found to be associated with the occurrence of persistent CMV infection (Olsson et al., 2000). No evidence was found for a relationship between an inverted CD4/CD8 ratio and the presence of other viruses, such as Herpes simplex and Epstein Barr virus, implying a unique impact of human cytomegalovirus (HCMV) on the

immune system. This notion is supported by Looney, who also found an association with HCMV seropositivity and an increased number of CD8+CD28- T cells (Looney et al., 1999).

The findings suggest that the changes in T cell balance among IRP individuals, at least to some extent, is produced by the generation of CD8+ effector / memory cells against HCMV, and subsequent homeostatic decreases in the CD4+ and CD4/CD8 ratio. This notion was supported by tetramer technology, demonstrating significant expansion of CD8+ T cells, specific for the CMV_{A2/NLV} peptide in HLA-A2 individuals, to be associated with both age and the IRP (Ouyang et al., 2004).

B cells and antibodies

There are several differences in the humoral immune system between young and elderly individuals. Serum immunoglobulin levels change during ageing. In a study of over 75 000 individuals in different age groups, IgG and IgA levels were raised in the oldest-old, in contrast to IgM levels, which were lower in the oldest-old, compared to younger individuals (Ritchie et al., 1998). The capacity to recover from viral and bacterial infections is associated with good humoral immune responses, as shown by increasing levels of specific antibodies following infection. The ability to respond to new antigens is decreased in the elderly. The ability of influenza, hepatitis B and pneumococcal vaccines to induce protection is lower in the elderly (> 65 years), compared to younger people, and the antibody duration is shorter in the elderly (Goodwin et al., 2006; Looney et al., 2001; Melegaro and Edmunds, 2004). A CD8+ T-lymphocyte subpopulation, characterized by IL-4 production, was found to be increased in the elderly with a good response to influenza vaccination, compared to non-responders (Schwaiger et al., 2003). This specific lymphocyte population has not been reported in individuals < 40 years of age but is present in about one third of individuals > 60 years of age. However, the implications of this finding for influenza and other vaccinations are unclear.

There is also an age-related increase in the prevalence of monoclonal immunoglobulin (mIg). About 50 % of elderly people, as well as old mice, have detectable circulating mIg, of which about 50 % react with autoantigens (Weksler, 2000). Many investigators have reported that the prevalence and/or the levels of circulating autoantibodies are increased in elderly individuals, as previously reviewed (Ramos-Casals et al., 2003; Tomer and Shoenfeld, 1988). However, the prevalence of thyroid autoantibodies (organ-specific antibodies) in centenarians was similar to that found in individuals less than 50 years of age. Whether the increased prevalence of autoantibodies reflects normal immunosenescence is, however, controversial. An alternative suggestion is that the increased occurrence of autoantibodies in the elderly rather reflects increased morbidity (Candore et al., 1997), whereas the repertoire of naturally occurring autoantibodies remains constant from an early age throughout life (Lacroix-Desmazes et al., 1999). Several studies have shown that the total number of circulating B cells

decreases with increasing age (Cossarizza et al., 1997; Utsuyama et al., 1992). Human studies indicate that an age-related decline in the generation of B-lymphocyte precursors may drive an ageing population of peripheral antigen experienced B cells with an increased life span (Johnson and Cambier, 2004). The number of B cells with a memory phenotype is increased in the elderly compared to younger individuals, as a decreased number of naive B cells are found in the elderly (Colonna-Romano et al., 2006). As the number of naive B cells decreases with age, the diversity of antibody production is affected. Using spectratype analysis, an analysis used for B-cell repertoire studies, a loss of diversity was shown in the B-cell repertoire in NONA Immune individuals. This loss of diversity was correlated with health status and survival (Gibson et al., 2008).

Natural killer cells and Natural killer T cells

Natural killer cells, NK cells, are a subpopulation of lymphocytes that are cytotoxic and are an important component of the immune response against viral infection and tumours. NK cells are particularly important in immunosurveillance against CMV. Primary immunodeficiency in NK cells is rare, and those children born with NK deficiency are very vulnerable to common viral infections such as CMV (Eidenschenk et al., 2006). Low NK cell activity has been associated with the risk of infection and death due to infection (Ogata et al., 2001; Ogata et al., 1997). NK cells have the capacity to distinguish between normal and damaged cells, as well as self- and foreign cells. NK cells also participate in the regulation of the immune response by their production of cytokines and chemokines. NK cells are characterized by lacking CD3 and the expression of variable amounts of CD16, CD56, and CD57. They can be divided into two functional groups based on the level of CD56 expression. If the expression of CD56 is high, they belong to the CD56^{bright} population, which are major cytokine producers, whereas CD56^{dim} cells, with low CD56 expression, exhibit a greater cytotoxic capacity (Solana and Mariani, 2000). CMV has developed mechanisms for viral immune evasion; mechanisms to escape recognition by, and activation of, NK cells. CMV is able to modulate the innate and the adaptive immune response at every step of its life cycle (Rajagopalan and Long, 2005). Several age-related alternations in NK cell function have been found; however, contradictory data exist, due to the different selection criteria of the studied populations. In studies of centenarians, an extremely rare group who probably reach this age because of well-preserved defence mechanisms, the overall cytotoxicity of the NK cells was not significantly affected, compared to a young control group (Sansoni et al., 1992). In general, however, there is evidence of a decreased cytotoxicity per NK cell in the elderly, which is compensated by an increasing numbers of NK cells (Mariani et al., 1994).

Natural killer T cells, NKT cells, constitute a minor lymphocyte population that displays features of both NK and T cells. They were earlier divided in two groups based on their CD1d restriction: the CD1d restricted classical NKT cells and the non-CD1d restricted non-classical NKT cells. The latter group is now called NKT-like cells. The non-classical NKT cells account for 5–20 % of total T lymphocytes in human peripheral blood, compared to the

classical NKT cells that account for less than 0.1 % of T cells in peripheral blood (Miyaji et al., 1997; Molling et al., 2005). NKT cells are involved in the regulation of immune responses in cancer, autoimmunity and bacterial infections. They can influence the outcome of both innate and adaptive processes by their capacity to rapidly produce immunomodulatory cytokines early in the course of immune response (Jing et al., 2007; Peralbo et al., 2007). Very little information is available about age-related changes in these cell subsets. A study of 101 healthy elderly people (mean age = 78.1 years), showed a significant decline in a subset of NKT cells, but an increase in Th2 cytokine secretion from the subset of NKT cells. This increase in Th2 cytokine production in the elderly may help understand the suggested age-related shift from Th1 to Th2 cytokine response and propose that the age-related changes in NKT cells may contribute to immune senescence (Jing et al., 2007).

Neutrophil Granulocytes

Polymorphonuclear leukocytes (neutrophils) are key effector cells of the innate immune system. They are short-lived cells and die by apoptosis spontaneously within 12–24 h of their release from the bone marrow. The bone marrow produces 10^{11} neutrophils per day to maintain the homeostasis of the neutrophils. This production is controlled by two colony stimulating factors and by interleukin-3 (IL-3). In infections, the survival of neutrophils depends on factors such as lipopolysaccharide (LPS), complement and pro-inflammatory cytokines. The neutrophil has several receptors for complement, IgG Fc, IL-8, Granulocyte-Macrophage-Colony-Stimulating Factor (GM-CSF), formyl-methionyl-leucyl phenylalanine (fMLP), and also toll-like receptors (TLR). The weakening of the immune system also affects the innate immune system and the neutrophils. Individuals aged > 65 years display a predisposition to inflammation and infection, combined with an increase in morbidity and mortality, compared to younger individuals (Leng et al., 2005). Studies of the function of neutrophils have resulted in diverging outcomes. One reason may be due to how the aged subjects and their control group were selected. Thus, the SENIEUR protocol was created to clearly separate age-related from non age-related alternations of the immune system (Ligthart et al., 1984). This protocol sets the criteria for selecting healthy elderly persons for immunogerontological studies. This document will provide a glimpse of the major alteration found in the function of neutrophils in the aged. Table 3 summarizes the main findings of age-related changes of neutrophils in ageing, with appropriate references. Chemotaxis is found to be reduced or not altered with ageing. Although the number of neutrophils is normal or increased in the elderly, impairments in phagocytic capacity, accompanied by reduced intracellular killing, are seen. The phagocytic capacity of *Escherichia coli* and *Staphylococcus aureus* was significantly reduced in neutrophils from elderly donors fulfilling the SENIEUR protocol, compared to neutrophils from younger donors. With increasing age, a significant reduction was found in the intracellular production of reactive oxygen after stimulation with *S. aureus*. In contrast, no difference in reactive oxygen production was found after stimulation with *E. coli* (Wenisch et al., 2000). These results may have clinical importance in the treatment of the elderly who have infections (Whitelaw et al., 1992)). Furthermore,

neutrophils from elderly donors cannot be rescued from apoptosis when incubated with GM-CSF, G-CSF, IL-2 or LPS; these extend the lifespan of neutrophils in younger donors. Decreased Jak/STAT activation by GM-CSF stimulation is an example of changes in signal transduction that occurs in the neutrophils of the elderly (Fortin et al., 2007).

Increased total white blood count (WBC) has been associated with increased all-cause mortality (Ruggiero et al., 2007) and cardiovascular mortality (Margolis et al., 2005; Weijenberg et al., 1996). It is unclear if the increased numbers of neutrophils are causative of the increase in mortality, or are rather an indication of ongoing low grade inflammation (Bovill et al., 1996).

Table 3 Age-related changes of neutrophils

Parameter/function	Stimulant	Effect	References
Number of circulating neutrophils		↑	(Chatta et al., 1993) ↔, (Born et al., 1995) ↔, (Cakman et al., 1997) ↑
Number of PMN precursor cells in bone marrow		↔	(Chatta et al., 1993) ↔
Proliferative response of precursor to...	G-CSF	↓	(Chatta et al., 1993) ↓
	GM-CSF	↔	(Chatta et al., 1993) ↔
	IL-3	↔	(Chatta et al., 1993) ↔
Phagocytosis	Opsonized bacteria	↓	(Wenisch et al., 2000) ↓
Chemotaxis		↓	(Biasi et al., 1996) ↔, (Esparza et al., 1996) ↔, (Niwa et al., 1989) ↓, (Wenisch et al., 2000) ↓ (Fulop et al., 2004) ↓
Adhesion molecules	CD11a	↔	(Esparza et al., 1996) ↔, (Butcher et al., 2001) ↔
	CD11b	↔	(Rao, 1986) ↔, (Esparza et al., 1996) ↑, (Butcher et al., 2001) ↔
	CD15	↑	(Esparza et al., 1996) ↑
	CD62L	↓	(De Martinis et al., 2004) ↓
Neutrophils (%) with		↓	(Butcher et al., 2001) ↓, (Lipschitz et al., 1991) ↓
CD 16 expression		↓	(Fulop et al., 2004) ↔
Expression of TLR2, TLR4		↔	
Oxidative burst after fMLP stimulation		↓	(Biasi et al., 1996) ↓, (Braga et al., 1998) ↓, (Tortorella et al., 2000) ↓, (Lord et al., 2001) ↔ ↑, (Butcher et al., 2001) ↔ ↑, (Fulop et al., 2004) ↓
Ca ²⁺ mobilization after fMLP stimulation		↓	(Varga et al., 1988) ↓, (Fülöp et al., 1989) ↓, (Lipschitz et al., 1991) ↓
Intracellular level of Ca ²⁺ in resting PMN		↑	(Varga et al., 1988) ↑, (Mohácsai et al., 1992) ↑, (Wenisch et al., 2000) ↑
Capacity to be rescued by...	G-CSF	↓	(Tortorella et al., 1998) ↓
Jak/STAT activation	GM-CSF	↓	(Fülöp Jr et al., 1997) ↓, (Tortorella et al., 1998) ↓
After Fas activation	GM-CSF	↓	(Fortin et al., 2007) ↓
	IL-2	↓	(Fülöp Jr et al., 1997) ↓
	LPS	↓	(Fülöp Jr et al., 1997) ↓, (Tortorella et al., 1998) ↓

(↔): unaltered (↓): significantly decreased, (↑): significantly increased, (↑): not significantly increased

Clinical implications of immunosenescence

Immunosenescence has a clinical impact on e.g. vaccine efficacy, immunological memory, risk of infectious diseases, autoimmunity and cancer. The immune response to several vaccines, e.g. influenza (Goodwin et al., 2006) and hepatitis B (Looney et al., 2001), is significantly reduced in elderly compared to younger individuals. A lower protection of influenza vaccination has been correlated with increased numbers of CD8⁺ CD28⁻ T cells (Goronzy et al., 2001; Saurwein-Teissl et al., 2002; Trzonkowski et al., 2003).

Infectious diseases are increasing in incidence and severity in the elderly (Gavazzi and Krause, 2002). Important infections include influenza, pneumococcal infection and urinary tract infections caused by Gram-negative bacteria (Yoshikawa, 2000). Reactivation of latent infection, such as tuberculosis and herpes zoster, is also more common in the elderly. Increased morbidity and mortality to bacterial and viral infection (Bender, 2003; Falsey and Walsh, 2005; High et al., 2005) are regarded as consequences of immunosenescence. The relationship between an increased incidence of infections with ageing and immunosenescence can be primary. As the number of naive T cells decreases, the possibility to mount an effective response to new pathogens also decreases. Dysregulation of the immune system also leads to a decrease in specificity, as well as to loss of memory. Susceptibility to infections is also influenced by concomitant diseases, medications, psychological status and nutritional status. However, the direct nature of the association between a dysregulated immune system and increased susceptibility to infections with ageing is still unknown.

Several reports have been published about the relationship between mortality and the immune response in elderly individuals. Murasko reported that elderly individuals with a lack of response to three mitogens (the T cell mitogens ConA and phytohaemagglutinin (PHA), and the T-dependent B-cell mitogen, pokeweed mitogen (PWM)), were associated with a doubled risk of dying during the next 2 years, compared to elderly people who did respond to mitogens (Murasko et al., 1987). In a study of individuals older than 80, it was found that those who were anergic to a panel of mitogens had a 2-year mortality rate of 80 %, compared to 35 % in those who were non-anergic (Roberts-Thomson et al., 1974). A third study found that non-survival was related to anergy, and all caused mortality in initially 273 healthy individuals, 60 years of age and older. Anergy was defined as decreased, delayed hypersensitivity response to four common recall antigens (Wayne et al., 1990).

The increased incidence of autoimmune disease (Ramos-Casals et al., 2003) and inflammatory conditions (Hasler and Zouali, 2005; McGeer and McGeer, 2004) are also associated with immunosenescence. There is an increasing prevalence of autoantibodies with

ageing. However, it is not clear whether autoantibodies are innocent bystanders of the ageing process or whether they play an important role in chronic diseases of ageing, such as atherosclerosis (Liang and Gabriel, 2007).

The occurrence of malignancy increases with age, but only up to the age of about 85 years (Bonafè et al., 2001). One hypothesis suggests that alterations in immune surveillance accompanying immunosenescence may be the cause of increasing cancer incidence with ageing. The reason for the stabilisation in cancer incidence in the oldest-old remains unsolved, but might correlate with an increase in effector T cells and the proinflammatory milieu as ageing progresses (Fulop et al., 2005). However, a direct demonstration of a causal link between immunosenescence and tumours is still missing. Interventions to restore the dysregulated immune system with ageing are important issues for future research. An overview of possible interventions has been published by Fülöp in 2007 (Fülöp et al., 2007). Possible interventions to restore the dysregulated immune system include strategies to decrease the antigenic load, restore thymic output, modulate T cell functions, increase exercise, improve nutrition and install hormone treatment. Several exercise intervention trials have shown improvements in some immune functions in the elderly, but not in frail individuals. Improvements after exercise interventions include greater lymphocyte IL-2 production, greater antibody production after immunization, improved NK cytotoxicity and a reduction in inflammatory markers such as CRP and IL-6 (Senchina and Kohut, 2007).

Cytomegalovirus

The immune risk phenotype was found to be associated with human cytomegalovirus (HCMV) but not to other viruses such as Herpes simplex or Epstein Barr virus. HCMV has also been shown to have a central role in the ageing of the immune system (see below), and is therefore described in more detail. Human cytomegalovirus (HCMV) is a ubiquitous β -human herpesvirus type 5. It is the largest of the herpesviruses, with a genome encoding about 165 genes (Davison et al., 2003), and the mature virions range in diameter from 200–300 nanometers. HCMV is composed of an outer envelope, the tegument, the nucleocapsid and an internal core consisting of protein and a doubled stranded linear DNA (Chen et al., 1999). The most abundant tegument protein is the lower matrix phosphoprotein 65 (pp65) (Varnum et al., 2004). Transmission of HCMV can be through placental transfer, breastfeeding, sexual contact, blood transfusion, saliva, solid-organ transplantation or hematopoietic stem cell transplantation (Sia and Patel, 2000). Primary infection is normally asymptomatic in immunocompetent individuals, after which the virus establishes lifelong latency within the host. The exact site of latency has, despite many attempts, not been definitely determined, but appears to be in cells of the myeloid lineage, including monocytes and granulocytes (Sinclair and Sissons, 2006). Reactivation of HCMV from latency can be detected in response to immunosuppression, inflammation, infection or stress (Kutza et al., 1998; Mutimer et al., 1997; Prösch et al., 2000). In immunocompromised patients and in the foetus, infection can cause an array of damaging clinical effects. HCMV is a major infectious problem in stem cell transplantation, solid-organ transplantation, and it used to be a problem in human immunodeficiency virus (HIV) infected individuals prior to the introduction of highly active antiretroviral therapy. A CD4+ T-lymphocyte count below 100 cells / μ L is a major risk factor for HCMV disease in HCMV seropositive patients (Palella et al., 1998).

Both the innate and adaptive immune systems play important roles in the defence against HCMV. Natural killer cells (NK cells), a part of the innate immunity, have been shown to contribute to the recovery from HCMV infection in renal transplant patients (Venema et al., 1994). Children born without NK cells are very vulnerable to common viral infections such as CMV (Eidenschenk et al., 2006). The role of antibodies in protection against and control of HCMV is not fully understood, however women with preconceptual immunity to HCMV transmit infection to the foetus at a lower rate than women with primary infections (Fowler et al., 1992; Stagno et al., 1986). The HCMV replication is predominantly controlled by the cell-mediated immune response, where HCMV specific CD8+ T lymphocytes, CD4+ T lymphocytes and $\gamma\delta$ T lymphocytes are important for controlling the infection. The important role of CD8+ T cells has been shown in bone marrow transplants, where the development of HCMV-specific CD8+ responses after transplantation correlate with protection (Li et al., 1994; Reusser et al., 1991). A large proportion of CD8+ T cells are engaged in the anti-HCMV response. The proportion of CD8+ T cells in peripheral blood, specific for HCMV antigens, increases with age, from a median of 10 % in healthy virus carriers up to 40 % in elderly individuals (Crough et al., 2005; Gillespie et al., 2000; Khan et al., 2004; Sylwester et

al., 2005). It is intriguing that one common virus has such a big impact on the cell-mediated immunity in humans. The specific T cell response recognizes a variety of structural antigens, early and late, and in addition virus-encoded immunomodulators (Elkington et al., 2003; Manley et al., 2004; Sylwester et al., 2005). The unique long (UL83) antigen, also known as pp65, is one of the most immunodominant antigens, to which HCMV specific CD8+ T lymphocytes respond (Boppana and Britt, 1996). The impact of chronic HCMV infection on T cell homeostasis has been investigated in a numbers of studies, but studies of primary infection have been more difficult to conduct. Primary HCMV infections in healthy individuals are difficult to identify, which is why studies have been conducted in cohorts with a high incidence, such as early childhood, or in renal transplantation from a seropositive donor to a seronegative recipient (Gamadia et al., 2003; Miles et al., 2007).

Diagnosis of HCMV infection, primary or persistent, can be made by the detection of antibodies of the type IgM or IgG. The introduction of a new technique for the detection of the viral genome, the polymerase chain reaction (PCR), is today a routine diagnostic method for molecular diagnosis in various fields of virology and microbiology (Drew, 2007), and can also be used for the monitoring of viral CMV DNAemia. Various techniques have been developed for monitoring the immune response to viral infections, such as MHC class I/peptide multimers, enzyme-linked immunospot (ELISPOT) and flow cytometric intracellular cytokine staining. The heavy chain of MHC class I molecules are bound to a tetramer labelled with a fluorochrome. A synthetic peptide is loaded to the binding pocket of the class I molecule. This complex binds only to those T cells (CD8+) that recognize both the MHC class I molecule and the corresponding peptide. The labelled cells can then be detected and enumerated by flow cytometry (Ogg and McMichael, 1998).

This monitoring of the cell-mediated immune system is particularly important in the transplant setting (Engstrand et al., 2000). These techniques can, of course, also be used in the investigation of the cell-mediated immune system in the elderly (Gillespie et al., 2000).

The seroprevalence of HCMV increases with age, however differences have been found in seroprevalence in populations of different socio-economic conditions or ethnicity. The seroprevalence of HCMV in the population of the USA was 36.3 % in children 6–11 years old, and increased to 90.8 % in subjects ≥ 80 years old (Staras et al., 2006). Sixteen year old Swedish girls had a HCMV seroprevalence of 45 % (Andersson-Ellström et al., 1995), compared to 55 % in a middle-aged group and 87 % in nonagenarians (Wikby et al., 2002). Two studies, from Spain (de Ory et al., 2004) and Germany (Lübeck et al., 2010) respectively, found declining seroprevalence, in contrast to a Swedish investigation of increasing seroprevalence in children over a 30 year period (Svahn et al., 2006).

Anti-viral T cell responses

The general course of a primary T cell response to a viral infection can be summarized, as follows: Naive virus specific T cells encounter viral antigens processed by antigen presenting cells, such as dendritic cells, in local draining lymph nodes. The dendritic cells have phagocytosed virus-infected cells or cell fragments at a peripheral site, and have moved to a local lymph node for presentation to the specific T cell. The specific T cells, both CD4 T-helper cells and CD8 T-cytolytic T cells, undergo an extensive clonal expansion. The numbers of naive precursor specific CD8 T cells range from about 100 to 500, and during an extensive clonal expansion they can expand 1000 fold (Badovinac and Harty, 2002). After this proliferation and activation of effector functions (such as cytokine production and expression of cytotoxic granules), the effector T lymphocytes leave the lymph node and migrate to the site of infection, guided by chemotactic gradients detected by surface receptors, in combination with local changes in adhesion molecule expression on blood vessel endothelium. Subsequently, when the T cells have fulfilled their effector function, the expanded virus-specific T cells undergo a contraction phase in which more than 90 % of the cells are lost. The remaining T cells constitute a long-term memory T cell pool.

Various classifications of T cells have been proposed, based on the expression of cell surface markers. Phenotypic and functional separation of memory and effector human CD8+ T cells, based on the expression of CD45RA and CD27, was proposed by van Lier (Hamann et al., 1997). Naive cells express CD45RA (CD45RA+) and CD27 (CD27+), memory cells express CD27 but not CD45RA (CD45RA-), effector T cells express CD45RA but not CD27. In 1999, Sallusto originally proposed another classification of T cells, based on the surface expression of the chemokine receptor 7 (CCR7) and CD45RA. T cells were classified into either central memory (CD45RA-CCR7+) or effector memory T cells, expressing CD45RA but not CCR7 (Sallusto et al., 1999). The chemokine receptor 7 (CCR7), is a secondary lymphoid organ homing marker, associated with subsets of T-lymphocytes (Campbell et al., 1998; Sallusto et al., 1999).

A hypothetical model of CD8+ T cell differentiation (Table 4) was proposed in 2002 (Appay et al., 2002), comprising four different phenotypes of CD8+ T lymphocytes. Lymphocytes that have not encountered their cognate antigen have a naive phenotype. They express the cell-surface markers, CD28 and CD27, which are co-stimulatory molecules involved in the regulation of T cell activation and in the generation of antigen-primed cells, respectively (Riley and June, 2005; van Lier et al., 2003). Granzyme A and perforin are cytolytic effector molecules that are stored in cytolytic granules, and are the actual functional molecules for killing target cells. When T cell activation occurs, there is a shift in the expression of CD45RA+ to CD45RA-. HCMV specific CD8+ T cells that are generated early after HCMV infection express perforin and granzymes, and have been shown to have a direct ex vivo cytotoxicity. These functional cytolytic cells have lost expression of CCR7 and CD45RA.

Then, after the primary infection, T cells are selected to become memory cells, expressing CD45RA+ but lacking CD27/CD28.

This hypothetical model for CD8+ T cell differentiation has recently been reviewed (Appay et al., 2008), underscoring the need to further establish the sequence of differentiation into different T cell subsets in humans, and also pointing to the need for improving consensus on the nomenclature of T cells (Appay et al., 2008).

Table 4 Hypothetical model of CD8+ T cell differentiation (adopted from Appay et al. 2002)

	Phenotype	Markers
Antigen-inexperienced cells	Naive	CD28+CD27++ GrA- Perforin- CD45RA+CCR7++
Antigen-experienced cells	Early	CD28+CD27(++→) + GrA+ Perforin+ CD45RA-(+)CCR7(+→) -
	Intermediate	CD28-CD27+ GrA+ Perforin+ CD45RA-(+)CCR7-
	Late	CD28-CD27- GrA+ Perforin+ ↔ ++ CD45RA (+)-CCR7-

GrA: Granzyme A, - : negative, + : positive, ++ : high expression. Phenotypes in brackets represent intermediate or minor populations

The impact on T cell homeostasis and the differentiation phenotype of HCMV-experienced CD8+ T cells in chronic HCMV infection have been examined in a number of studies. The main CD8+ effector T cell population during acute HCMV infection shows a CD45RA-CD45RO+ CD27+ CD28+/- CCR7- phenotype. Two main HCMV specific CD8+ T cell populations exist in chronic HCMV infection: CD45RA- CD45RO+ CD27- CD28- CCR7- effector memory or CD45RA+ CD45RO- CD27- CD28- CCR7- terminally differentiated effector T cells re-expressing CD45RA (Appay et al., 2002; Gamadia et al., 2003).

A feature of the CD8+ T cell response to HCMV is a reduction in the naive T cell pool and an accumulation of an oligoclonal T cell repertoire (Day et al., 2007; Price et al., 2005). An accumulation of HCMV-specific CD8+ T cells occurs with age, and it may represent almost 40 % of the CD8+ T cell pool (Crough et al., 2005; Gillespie et al., 2000; Khan et al., 2004; Ouyang et al., 2003; Sylwester et al., 2005). Similar changes in the HCMV-specific CD4+ T

cells have recently been shown (Pourghesari et al., 2007). The expansions of CD8+ HCMV specific T cells are consistently oligoclonal or monoclonal, and express a highly differentiated effector memory (CD28- CD57+ CCR7-) phenotype (Khan et al., 2002).

A lower success rate in HCMV seropositive elderly individuals to influenza virus vaccination has been shown (Grubeck-Loebenstien et al., 2009; Trzonkowski et al., 2003). HCMV has also been shown to be a cofactor that enhances the progression of HIV infection to AIDS (Griffiths, 2006). The immune system has to keep HCMV under control; however, the virus constantly challenges the immune system (Stowe et al., 2007). Studies have shown that there is a continuous human T cell response to latent HCMV infection. This is displayed by continuous expansion and contraction of the HCMV-specific CD8+ T cells (Crough et al., 2005; Dunn et al., 2002). However, the role of CD4+ T cells in the control of HCMV infection is not the focus of this document.

Inflammation

There is compelling evidence for an increased systemic inflammatory situation in the oldest-old (Franceschi et al., 2000b) with increased circulating levels of tumour necrosis factor (TNF), interleukin 6 (IL-6) and C-reactive protein (CRP) (Bruunsgaard et al., 1999a; Fagiolo et al., 1993; Forsey et al., 2003; Koenig et al., 1999; Wei et al., 1992). IL-6, considered a major cytokine in ageing, is mainly regarded as a pro-inflammatory cytokine, although it indeed is a pleiotropic cytokine being involved, e.g. in B cell differentiation and T helper (Th) differentiation (Bertolini and Benson, 1990; Diehl and Rincón, 2002). IL-6 is involved in the induction of acute-phase CRP, and both IL-6 and CRP are clearly associated with morbidity affecting elderly, like cardiovascular disease, dementia and type-2 diabetes (Kravitz et al., 2009; Kuo et al., 2005; Remarque et al., 2001; Tarkowski, 2002; Volpato et al., 2001). Other age-related conditions such as osteoporosis and frailty are known to be associated with elevated levels of IL-6 (Bruunsgaard and Pedersen, 2003; Ding et al., 2008).

Aims of the thesis

General aim

- To increase knowledge of immune changes and their clinical consequences in the oldest-old population.

Specific aims

- To evaluate the effect of various exclusion protocols on the sample size of a population- based sample of the oldest-old (Paper I).
- To asses T cell populations in relation to health status (Paper I).
- To examine IRP in relation to low-grade inflammation and 2-year mortality and to evaluate the sequence of events of immune changes in the oldest-old (Paper II).
- To evaluate the relative importance of IRP and low-grade inflammation in relation to 4-year mortality (Paper III).
- To examine associations of IRP and IL-6 with morbidity and cause of death (Paper III).
- To determine the prevalence of ANA and its relation to health status, CD4/CD8 ratio and CMV serology in the oldest-old (Paper IV).

Materials and Methods

Ethics

The studies were approved by the ethics committee of Linköping.

Subjects

Paper I

Subjects for the NONA Immune study were recruited from among participants in the Swedish NONA Longitudinal study. In the NONA Longitudinal study, participants were investigated with regard to how health, the activities of daily living (ADL) and the application of care changed over time (Bravell et al., 2007). The sample was drawn from the mid-sized municipality (122 000 inhabitants) of Jönköping, located in the south central part of Sweden. Based on available population register information in September 1999 of all individuals permanently residing in Jönköping, a non-proportional sample was drawn with the aim of recruiting equal numbers of individuals from three birth cohorts: 1905, 1909 and 1913. Due to the limited number of available subjects in the oldest-old birth cohort, a few were also included from the birth cohorts of 1904 and 1906. A group of 300 people was randomly selected, and 157 people could and wanted to participate in the NONA Longitudinal study. In 1999, 138 individuals were included in the present NONA Immune study. Inclusion criteria based on health status were not used in the NONA Immune study. For comparisons, a sample ($n = 18$) of middle-aged women and men were drawn from among staff working in the laboratories at the Ryhov Hospital in Jönköping. The characteristics of the NONA Immune individuals are shown in Table 5.

In paper I, the base line data of clinical and laboratory parameters are used.

Paper II

The individuals in the NONA Immune study ($n = 138$) were longitudinally examined for 2-year survival in 2001. During the 2-year follow-up, 40 individuals were deceased, and another 14 declined to participate in the second-year follow-up in 2001. Thus, there were 84 participants left for the 2-year follow-up, with a mean age of 91.6 years; 69 % of the participants were women.

Paper III

In this report from the NONA immune longitudinal study, we examine 4-year mortality in relation to base-line levels of a set of laboratory parameters, as well as morbidity and cause of death in a population-based sample of oldest-old individuals (n=138). After 4 years, 71 individuals (51.4 %) were deceased, leaving 67 individuals, of whom 70 % were women.

Paper IV

In paper IV, the prevalence of antinuclear antibodies in the oldest-old was evaluated in comparison to healthy blood donors. Frozen sera from healthy blood donors were compared with frozen plasma samples from individuals included in the present NONA Immune study (n = 138), and in addition individuals from the earlier OCTO Immune study. The OCTO Immune study started in 1989, when an immunological study was added to the OCTO Longitudinal study, which started in 1987. Census data from the municipality of Jönköping was used to recruit a non-proportional sample that comprised 100 individuals in each of four birth cohorts: 1897, 1899, 1901 and 1903. Of the initial 324 people examined at baseline, 96 were deceased and 15 declined to participate in the following OCTO Immune study. In the OCTO Immune study, several exclusion criteria were used to exclude individuals who were institutionalized, had cognitive dysfunction or were on a drug regimen that may influence the immune system. Finally, 110 met the inclusion criteria, and for the present study, plasma samples were available from 97 individuals. The 200 blood donors, equal numbers of women and men, were recruited from the University Hospital of Linköping, Sweden. The mean age of the blood donors was 41 years, with a range between 18–68 years (Table 5).

Table 5 Subjects included in the thesis

		Age (years)	Women (n)	Men (n)	Paper
NONA Immune study			97	41	I–IV
	1904	95	3	2	
	1905	94	16	10	
	1906	93	11	0	
	1909	90	31	16	
	1913	86	36	13	
OCTO Immune study			63	34	IV
	1897	92	11	7	
	1899	90	14	8	
	1901	88	13	7	
	1903	86	25	12	
Blood donors		18–68	100	100	IV
Controls		32–59	12	6	I

Selection protocols for immunogerontological studies

SENIEUR protocol

Knowledge of the human immune system has expanded enormously during the last decades. Most of the studies in the 1970s and earlier have been done on material obtained from healthy young people, mostly blood donors (Ligthart et al., 1984). Studies of the human immune system in individuals ≥ 65 years, immunogerontological studies, have earlier often led to contradictory results. One reason was the non-standardized selection of subjects to be examined, which seemed to lack ways of excluding individuals with underlying diseases, which might influence the immune system and thus the result of the study. A protocol, the SENIEUR (SENIorEURopean) protocol, was developed in 1984 by a working party within the framework of EURAGE (Concerted Action Programme on Ageing of the European Community). This protocol was an attempt to solve the problem through the admission of “apparently healthy” individuals or those “without overt disease”. The basis for the SENIEUR protocol in immunological studies is clinical information and laboratory data (Table 6).

Table 6 Exclusion criteria (Ligthart et al., 1984)

A. *Clinical information* (including follow-up at 2 weeks)

1. Infection
2. Inflammation
3. Malignancy
4. Other conditions which influence the immune system

B. *Laboratory data* (findings outside age-dependent reference range)

Erythrocyte sedimentation rate (ESR), haemoglobin, mean corpuscular volume, leucocyte count with differentiation
Urea, alkaline phosphatase, glucose, ASAT, ALAT
Protein and immunoelectrophoresis
Urine analysis: protein, glucose, sediment

C. *Pharmacological interference*

1. Prescribed medication for treatment of defined disorder
 2. Medication with known influence on the immune system
-

A. *Clinical information*

1. Infection. This includes all kinds of infection; most frequent are respiratory and urinary infections. All such individuals to be excluded. The follow up after 2 weeks is to make sure that the primary examination was not done during the incubation period of an infectious process.

2. Inflammation. Acute or chronic inflammation is usually disclosed at the time of taking the medical history, during examination and laboratory tests.

3. Malignancy. All malignancies, present or past, lead to exclusion. The problem with silent diseases like prostatic cancer, which is often asymptomatic, but might be present in a large amount of men above 80 years of age, is that they cannot always be excluded.

4. Other conditions which influence the immune system. Arteriosclerosis, which is present in virtually every individual above 65 years of age, does not lead to exclusion. However, a condition that is a manifestation of arteriosclerosis, such as a stroke or a myocardial infarction, leads to exclusion if the condition has happened in the last 6 months. Cardiac insufficiency, if treated, will lead to exclusion. Hypertension leads to exclusion if treated. Dementia is a reason for exclusion, due to the problem of taking a medical history, and also to obtain an informed consent. For malnutrition the SENIEUR protocol proposes the use of body mass index (BMI) for exclusion purposes. A BMI below 22 for men and below 20 for women leads to exclusion due to malnutrition. Drug abuse and alcoholism are also causes for exclusion.

B. *Laboratory data*

The reference values used in the SENIEUR protocol are based on a study of healthy volunteers aged 70 years, and included in a longitudinal study in Göteborg, Sweden (Landahl et al., 1981). Values outside the reference range lead to exclusion (Table 7).

C. *Pharmacological interference*

The use of drugs, prescribed or self-administered, is and will always remain a problem in immunological studies in the elderly. It is not realistic to exclude all individuals using drugs, which is why the SENIEUR protocol has two categories of drug use leading to exclusion. Prescribed medication for the treatment of a defined disease: The use of diuretics for cardiac insufficiency leads to exclusion, due to the use of a drug for a defined disease. However the use of a diuretic for hypostatic oedema, which is not a defined disease, is not a cause for exclusion. Medication with a known influence on the immune system, such as anti-

inflammatory drugs, hormones and analgesics will lead to exclusion. Vaccination given in the last 6 weeks before the examination will cause exclusion from the study.

The aim of the SENIEUR protocol was to exclude endogenous and exogenous influences on the immune system, and also to standardize the population for the study. By applying strict criteria, a vast majority of individuals will be excluded, leaving a small number of individuals for a closer examination of the ageing process itself.

Table 7 Laboratory reference values used in the SENIEUR protocol

	Women	Men
ESR (mm)	2–34	1–30
Haemoglobin g/L	119–159	130–171
Mean corpuscular volume (fL)		80–100
Leucocyte count (x 10 ⁹ /L)		4.0–10.0
Leukocyte differentiation	normal differentiation pattern according to local standard	
Urea (mmol/L)	2.3–9.2	2.6–9.7
Alkaline phosphatase (μkat/L)	1.4–4.7	1.3–4.5
ASAT (μkat /L)	0.17–0.60	0.21–0.58
ALAT(μkat /L)	0.09–0.49	0.07–0.55
Glucose (mmol/L)		
fasting		4.0–7.2
postprandial		< 11.0
Urine analysis		
Protein		max. trace
Glucose		max. -
<u>Sediment</u>	<u>per high power field</u>	
Leucocytes		< 6
Erythrocytes		< 6
Granular casts		< 2

OCTO Immune protocol

This protocol was created in 1989, for exclusion criteria in the OCTO Immune study. The aim of the protocol was to reduce confusion between ageing, pathology and medication, and to secure reliable self-reports on psychosocial variables. The exclusion criteria in the OCTO Immune protocol are less rigorous than for the SENIEUR protocol, and result in a lower exclusion of potential candidates for the study. Individuals were included if they: (a) were non-institutionalized, (b) had normal or only mild cognitive dysfunction, according to neuropsychological tests (Johansson et al., 1992), and (c) were not on a drug regimen with cytotoxic agents, drugs affecting the endocrine function, immunosuppressants, hormones

(ACTH and corticosteroids), drugs used in the therapy of infectious diseases (antibiotics, sulphonamides, immunoglobulin, and drugs used in the therapy of viral diseases and tuberculosis), anti-inflammatory and anti-rheumatoid drugs (glucocorticoids, non-steroid anti-inflammatory rheumatoid agents, specific anti-rheumatic agents), and calcium channel blockers. In addition all individuals for the study were also screened for information about their health status. No malnutrition, serious heart disease, infectious disease processes, or significant cancers were present.

Measures and procedure in the NONA Immune Study

The NONA immune study examines a population-based random sample without excluding individuals due to compromised health, but instead to include a continuous evaluation of various individual health parameters. Information on morbidity and medication usage, past or present, was obtained by a thorough review of all available medical records and self-reports. Trained registered nurses conducted an examination in the subject's place of residence, including tests for cognitive function and a structured questionnaire-based interview, comprising medication usage. The whole procedure took about 3 hours, including breaks, for those who were able to participate in all parts.

Medical records (Paper I-IV)

Informed consent for reviewing medical records was given by the subject or a near relative, if the subject was unable to comprehend the meaning of the request. Medical records for the period 1970–1999 were requested from hospitals, outpatient clinics and primary health care centres to get a full coverage of the entire period, with information about past and present diseases and medication usage. The International Classification of Diseases (ICD) was used for the recording of the subjects' diseases and conditions. The history of ICD goes back to the 19th century, but a modern international classification was developed after the Second World War by WHO, by introducing causes of morbidity for the first time. The present ICD-10 came into use in WHO Member States as from 1994 and was used for the NONA Immune study. Diagnoses and conditions reported earlier than 1994 were transformed to the corresponding ICD-10 classification. Almost all of the subjects had been residents in the municipality of Jönköping for many decades, and due to the very low migration rate in the subjects, no individuals were lost during the 2-year follow-up (Paper II). The diagnosis of cardiac insufficiency was a clinical diagnosis, based on dyspnoea and oedema referable to heart disorders, typically treated with diuretics or digitalis (Nilsson et al., 2002). Information was well documented for the 21 individuals with a history of malignancy; 9 breast, 4 prostate, 2 colon, one gastric and one gynaecologic cancer, 2 with malignant melanoma and 2 with myelodysplastic syndrome. Information about cognitive dysfunction was scanty in the medical records, and was further investigated by tests (see below). Chronic pulmonary disease was present in 16 individuals and, in addition, asthmatic disease was present in 6 individuals. Diabetes mellitus was present in 15 individuals; one was on dietary treatment and, of the remaining 14, 7 were on combination treatment with insulin and anti-diabetic drugs. Rheumatoid arthritis was diagnosed in 12 individuals, based on typical clinical findings in addition to a positive blood test. The diagnosis of hypothyroidism was based on blood tests for thyroid function, and was present in 8 individuals. The diagnosis of pernicious anaemia was based on anaemia and B12 deficiency. Polymyalgia rheumatica, a diagnosis based on clinical signs and symptoms showing a beneficial response to steroid treatment, was grouped together with temporal arteritis, which is another systemic inflammatory disorder. Polymyalgia rheumatica/temporalis arteritis was present in 7 individuals of the NONA

Immune sample. Body mass index (BMI) was based on weight and length measurements in the subject's place of residence, or from information in their medical records. Four individuals were considered to be malnourished according to the BMI criteria used in the SENIEUR protocol. Parkinson's disease and epilepsy were present in 3 individuals, respectively. Ulcerative colitis was present in 2 individuals, and one had schizophrenia.

Cognitive function (Paper I-IV)

Information about cognitive function was scanty in the medical records. Cognitive dysfunction was consequently based on a neuropsychological battery of tests performed in the subject's place of residence. The Mini-Mental State Examination (MMSE) (Folstein et al., 1975) is one of the most commonly used tests to identify cognitive impairment in epidemiological studies. The test consists of five different parts; section 1: orientation, section 2: memory (part 1), section 3: attention and calculation, section 4: memory (part 2) and section 5: language, writing and drawing. The maximum score is 30 points, and a score below 23–24 points is used in epidemiological studies to indicate dementia. Individuals with cognitive dysfunction generally score less than 26 points, but the test is not a test for Alzheimer's disease, since there can be other causes for a weak performance and a low score.

In the Memory-In-Reality (MIR) test (Johansson, 1988/89) the participants are asked to remember 10 common everyday household objects, and where they placed these objects in a replica of an apartment. After about 30 minutes the participants were asked to recall the 10 items, or to recognise items that were not recalled. Finally they are asked to place the items in the same place as they did previously. The score is the sum of free recall plus recognition.

These tests, together with overall medical information (Johansson et al., 1997), were used to divide the NONA Immune subjects into three cognitive status categories: cognitive intact, mild cognitive dysfunction or questionable cases (cases not fully meeting DSM-IV criteria for dementia; APA, 1994), and dementia (according to DSM-IV criteria; APA, 1994). Twenty individuals met the criteria for dementia and another 20 were classified with mild cognitive dysfunction.

Self reports

The subjects were interviewed for current and previous diseases, using an extensive list of disease categories, and in addition an open question about 'other' diseases and impairments.

Medication usage

Information about current medication came from medical records, through the individual themselves, or through a carer, if the individual was unable to provide the information.

Weight and length measurement

Weight and length measurements were done in the subject's place of residence.

Clinical infections

Registration of previous infections was done by reviewing the medical records for the entire period 1970–1999. All infections from baseline to first follow-up in 2001 were registered (Paper II). Comparisons were made between survivors and non-survivors, and CD4/CD8 ratio over or below one.

Death certificates and cause of death (Paper III)

In paper III, IL-6 and IRP were related to cause of death in the NONA Immune study. Of the 138 individuals from the baseline of 1999, 71 were deceased after 4 years (51.4 %). The cause of death classification was based on death certificates, of which only one was after autopsy. The definite cause of death was corrected in one case, due to complementary information in the medical records. Four groups were then constructed for the cause of death: (i) cardiovascular disease, including cerebrovascular disease, (ii) infection, (iii) cancer, and (iv) other causes.

Cell separation and sorting

Blood was drawn from the NONA participants in the morning between 8:00 and 10:00, in their place of residence. Whole blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes, and plasma was prepared by centrifugation, and stored at -80 °C. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep® (Nycomed Diagnostica, Oslo, Norway). The counting of PBMC and the differentiation between lymphocytes and monocytes was done in a haematology analyzer and, in addition, microscopic examination was carried out for control purposes.

Analysis of cytokine production

Plasma samples were thawed and levels of circulating IL-6 and IL-10 were measured by commercial ELISA kits. All samples were measured in duplicate. The lower detection level for the high sensitivity assay was 0.156 pg/mL.

Clinical chemistry laboratory analysis

A summary of all chemical laboratory analyses is shown in table 8. All analysis was done with routine automated multianalyser instruments. Immunoturbidimetric and immunonephelometric methods were used for protein analysis. Both methods measure the turbidity in a solution, where reaction between antigen and the corresponding antibody results in the formation of immune complexes. The solution becomes turbid, and a light sent through the solution is scattered depending on the degree of turbidity. The reduction (absorbance) in the intensity of light is measured by a photometer, and is correlated to the antigen concentration in the solution (immunoturbidimetric method). The nephelometric method is also based on the scattering of light for large complexes, in this case immune complexes of IgG (or IgA or IgM) and corresponding anti-human IgG (or IgA or IgM) antibodies. The light scattering is proportional to the immunoglobulin concentration in the sample. The methodology for urea analysis is based on the production of ammonia by the enzyme urease. The ammonia and oxoglutarate are converted to glutamate in a NADH depending reaction. The oxidation of NADH causes a change in absorbance, which is proportional to the urea concentration in the sample. Creatinine was analyzed by a modified Jaffè method.

Table 8 Clinical laboratory analysis of whole blood and plasma

Method	Analyte	Analyser
Immunoturbidimetric method using specific antibodies against the analyte	P-Albumin P-CRP P-Cystatin C	Advia 1650 (Bayer)
Immunonephelometric method using specific antibodies against the analyte	P- α 1-Antitrypsin P-Haptoglobin P-IgA P-IgG P-IgM P-Orosomucoid P-Transthyretin	Image 800 (Beckman)
Jaffè method – modified	P-Creatinine	Advia (Bayer)
Enzymatic method	P-Urea	Axon (Bayer)

Measurement of antinuclear antibodies

Indirect immunofluorescence was used in paper IV for investigating the prevalence of antinuclear antibodies (ANA) in the OCTO Immune and NONA Immune samples, in comparison with blood donors. Positive samples were further analysed for the presence of double-stranded (ds) DNA antibodies by immunofluorescence microscopy and all samples were screened by enzyme-immunoassay (EIA) for the presence of antibodies against extractable nuclear antigens (ENA). Positive tests were confirmed with double radial immunodiffusion.

Principle of method: Immunofluorescence is used to detect autoantibodies that react to various nuclear and cytoplasmatic proteins. ANA is used to describe these antibodies, regardless of the components to which they bind. The technology for the indirect fluorescent antibody assay dates back half a century (Weller and Coons, 1954). HEp-2 cells, from a cell line established from human laryngeal carcinoma in a 56 year old Caucasian male (Moore et al., 1955), are extensively used as an antigen source for the detection of ANA. HEp-2 cells are fixed on a slide and plasma or serum samples are applied; the autoantibodies present bind to the specific antigen. This binding is visualized by applying a secondary fluorescent anti-human IgG antibody. The fluorescent dye (fluorescein isothiocyanate) emits light (yellow-green fluorescence) at a lower wavelength when it is excited by a high energy blue incident light at a higher wavelength. The slides are inspected under a fluorescence microscope and different patterns of fluorescence can be distinguished, (for details see table 9). The most common pattern is homogenous, where the antibodies are directed to histone proteins and/or dsDNA. ANA-positive samples are then end-point titrated to the highest titre where the sample is still positive.

This study used the prevalence of ANA only, and not the specific patterns of fluorescence. The detection of antibodies against ENA is performed using a commercial EIA, which in the present study screened for several nuclear antigens, i.e. Sm, snRNP, SS-A(Ro), SS-B(La), Jo-1 and Scl-70. The wells are coated with human purified extractable nuclear antigens (ENA) and antibodies bind to the specific antigen, if present in the sample. In a second step, the enzyme labelled secondary antibody binds to the antigen-antibody complex. The enzyme labelled complex converts the added substrate to form a coloured solution, and the rate of colour formation is a function of the amount of antibodies in the sample.

The double radial immunodiffusion in agarose gel (Ouchterlony) is used to confirm the presence of specific ENAs. Antigen and antibodies in separate wells are allowed to diffuse into the gel and form antigen-antibody complexes that precipitate in the gel and become visible to the eye (Ouchterlony, 1949).

The ANA method is included in quality control programmes to check for accuracy of the method. Internal controls are used to check for stability of the method.

Table 9 Common ANA immunofluorescence staining patterns, associated antigens, and clinical association (Karlsson-Parra et al., 1994)

Staining Pattern	Antigen	Clinical association			
		SLE	MCTD	Systemic sclerosis	Sjögren's syndrome
Homogenous	dsDNA	+			
	Histone	+			
	DNA/histone	+			
Speckled	Sm	+			
	RNP	+	+		
	Scl-70			+	
	SS-B(La)				+
Nucleolar	SS-A(Ro)				+
	fibrillarin			+	
	PM-Scl			+	
	RNA-polymerase I			+	

Frozen plasma samples from individuals in the OCTO/NONA Immune studies, and frozen sera from blood donors, were used for analysis of ANA. The storage temperature was between - 70 °C and - 80 °C. The storage time was, for the OCTO Immune sample, mean 13 years, compared to less than 3 years for the NONA Immune sample. The storage time for the blood donors' samples was 4 years. It is of crucial importance that the cryo process and the storage-time do not have a significant effect on the concentration and structure of the immunoglobulins. In a recent Norwegian study of serum proteins and long-term storage stability (Gislefoss et al., 2009), there was no significant difference in concentrations of IgG with respect to storage time up to 25 years, using a storage temperature of only - 25° C. The stability of thyroid autoantibodies during long-term storage (up to 23 years) was examined in a study of blood samples collected from pregnant women (Männistö et al., 2007). They found that the concentration of thyroid autoantibodies increased significantly with increased storage time. Importantly, however, levels stayed under the upper limit of reference interval if samples were stored less than 10 years.

Analysis of antibodies to Cytomegalovirus

An immunoassay was used for the detection of IgG anti-CMV antibodies in the OCTO and NONA individuals. The immunoassay consists of very small microparticles, coated with CMV antigen, in a liquid suspension, which is placed in the wells of a plastic microtiter plate. The serum samples are incubated and bound antibodies are visualized using enzyme linked anti-human-IgG antibodies ('conjugate'). A colourless substrate is enzymatically converted to

a coloured product. The quantification of CMV is based on the enzyme dependent colour reaction, where the colour intensity is proportional to the amount of bound conjugate, and thus to the amount of CMV in the serum sample.

Flow cytometry analysis of surface protein expression

Three-colour flow cytometry was used at baseline and at follow-ups in the NONA Immune Study for the determination of surface markers (cluster designation, CD) on lymphocytes. Light scattering properties and light excitation and emission of fluorochrome molecules are the basic principles of flow cytometry that generate information on cell size (forward scatter), cell granularity (side scatter) and cell surface marker expression (emission of light). Cells labelled with fluorochrome conjugated antibodies pass through a conical flow chamber by means of a sheath fluid, and are then hydro-dynamically focused to the middle of the stream. As the cells pass, one by one, through the intercepting light source, they scatter light and the fluorochromes are excited to a higher energy state; they then emit light of longer wavelength. The scattered light and the emitted light are detected by photomultiplier tubes (PMTs), each specific to a certain emitting light signal. The PMTs convert the light into electrical pulses, which are processed and provide a graphical presentation, as one or two-parameter histogram or dot-plots. Quality control was performed on a daily basis using calibrating beads, and internal control was performed to check consistency for CD markers included in more than one tube; this resulted in a variation coefficient of 5–7 %.

Phenotypic markers on lymphocytes (CD surface markers)

The following CD markers were used in the NONA Immune study, paper I–IV:

CD3 is a complex of membrane glycoproteins that associates with the T cell antigen receptor, and is necessary for T cell receptor surface expression and signal transduction. It is a marker for T cells.

CD4 is a membrane glycoprotein which serves as a co-receptor in MHC class II-restricted antigen induced T cell activation. It is expressed on T helper cells, but, to a lesser extent, also on some monocytes.

CD8 is a membrane glycoprotein which serves as a co-receptor in MHC class I restricted antigen induced T cell activation and is expressed on T cytotoxic cells and on a proportion of Natural Killer T (NKT) cells.

CD27 is a membrane glycoprotein which serves as a co-stimulatory molecule. It is present on CD4 and CD8 cells and is strongly increased after T-cell activation. CD27 is often used as a marker for memory B cells.

CD28 is a membrane glycoprotein which is present on CD4 and CD8 cells. It is a co-stimulatory molecule binding CD80/CD86 molecules on antigen presenting cells. Engagement of the CD28 receptor triggers the T-cell to activation.

CD45 is a membrane-associated enzyme, tyrosine phosphatase, which is expressed on all leukocytes. CD45 is an essential regulator of T and B cell antigen receptor-mediated activation. CD45RA, an isoform of CD45, is present on naive T cells, NK cells and B cells. Another isoform is CD45RO, which has been considered as a marker for memory T cells.

CD56 is a membrane glycoprotein expressed on NK and NKT-cells and also on subpopulations of CD8 T cells.

CD57 is a membrane glycoprotein carrying a carbohydrate epitope that contains a sulfoglucuronyl residue. It is expressed on NK cells and subsets of T-cells.

Lymphocyte subpopulations can be investigated by using a combination of fluorochrome conjugated antibodies against these CD markers.

Statistics

All statistical analyses were conducted using SPSS version 10–15 (SPSS Inc. Chicago, IL).

In paper I, One-way Analysis of Variance (ANOVA) was used for comparisons across groups. *Post-hoc* tests were applied to examine differences between groups.

In paper II, Parametric Student's t-tests (normally distributed parameters), and non-parametric Mann–Whitney U-tests (parameters with skewed distribution) were employed for comparisons of independent groups. Non-parametric tests were used for body weight, due to outliers. Correlation analysis was performed using the Pearson's correlation coefficient. χ^2 and logistic regression analyses were used to analyse 2-year survival/non-survival in categories of individuals with a CD4/CD8 ratio (less than 1.00 and greater than 1.00) and IL-6 (using log transformed data to obtain normal distribution). Logistic regression was conducted with CD4/CD8 ratio and IL-6 as independent variables and with 2-year survival (survival or deceased) as dependent variable. Also, an interaction term (CD4/CD8 ratio \times IL-6) was included in the model. Age, sex, cardiovascular disease, chronic obstructive pulmonary disease, malignancy, anaemia, rheumatoid arthritis, diabetes, hypothyroidism, cognitive impairment, CRP, transthyretin and albumin were entered as covariates.

In paper III, correlation analyses were performed using the Pearson's and the Spearman's correlation coefficients for normally and non-normally distributed data, respectively. Student's t-tests (normal distributed parameters) and non-parametric Mann-Whitney U-tests (parameters with skewed distribution) were employed for comparison of independent groups, and data was presented as mean and median, respectively. χ^2 analysis was used for comparisons between survivors and non-survivors of individuals with CMV IgG positivity and F-ANA IgG \geq 200. ANOVA was used for comparisons across groups. *Post-hoc* tests were applied to examine differences between groups. Kaplan-Meier survival analysis was used to analyse 4-year survival or non-survival in categories of individuals by CD4/CD8 ratio (< 1 and ≥ 1) and by IL-6 level (\geq or $<$ median level of 3.15g/mL).

In paper IV, the χ^2 test for contingency table was used for comparison between groups. p values < 0.05 were considered significant.

Results and discussion

Paper I

First, the effect of two different sample selection protocols was examined in a sample of nonagenarians ($n = 138$). The commonly used SENIEUR protocol for immunological studies in the elderly (here slightly modified) excluded 90.6 % of the subjects and the OCTO-Immune protocol excluded 64.5 % of the subjects. Thus, remarkably, only 9 % of a population-based sample of oldest-old remained after exclusion according to the SENIEUR protocol (Paper I, Table 3). The original protocol, suggesting additional laboratory analysis for exclusion, would likely have led to an even higher exclusion rate. Although it is known that the SENIEUR protocol leads to a high exclusion rate (Ligthart et al., 1984) no previous study has thoroughly evaluated the exclusion rate in a population-based sample of the oldest-old. The value of the SENIEUR protocol in immunogerontological studies for distinction between 'ideal ageing' and clinical reality has been under debate (Ershler, 2001). Indeed, it is preposterous that immune studies of the oldest old should entirely be restricted to the 10 % healthiest individuals, although this is an interesting group itself. Rather, it has been suggested to use SENIEUR individuals as a reference population to assist in illuminating the changes in the immune system in the elderly (Castle et al., 2001).

A second aim of this study was to evaluate the occurrence of CMV and the distribution of lymphocyte populations across health status in the oldest-old. We therefore created three health groups based on the results from the two selection protocols: very healthy ($n = 13$, fulfilling the SENIEUR protocol); moderately healthy ($n = 38$, fulfilling the OCTO-Immune protocol but not the SENIEUR protocol); frail ($n = 87$, the remaining individuals). The findings were compared with a middle aged group ($n = 18$). As expected, the whole group of oldest-old, compared with the middle-aged group, showed increased numbers of certain CD8+ populations, such as, CD8+CD57+CD28-, CD8+CD45RA+CD27-, and CD8+CD56+CD57+ subsets (Paper I, Table 6). These CD8+ subsets represent different forms of lately differentiated cells (Khan et al., 2002), i.e. cells that are associated with immunosenescence and the presence of CMV. Interestingly, the numbers of these IRP-associated cell populations did not differ between the three health status groups (Paper I, Table 5), and, in addition, there was no difference in the prevalence of positive CMV serology between the three health groups. Furthermore, there was no significant difference in the numbers of IRP individuals across the health status groups. Thus, 2 of 13 'very healthy', 4 of 38 'moderately healthy' and 16 of 87 'frail' individuals showed IRP status as determined by a CD4/CD8 ratio below 1 (Chi-square $p = .54$). Taken together, our results indicate that lymphocyte subsets associated with IRP might serve as significant biomarkers of ageing, independent of the overall health status. This notion is corroborated by results in non-inbred

mice showing that clusters of immune markers can predict longevity independently of health conditions (Miller, 2001).

The CD8⁺ T cells have an important role in the control of CMV infection. The naive CD8⁺ T cells undergo an extensive clonal expansion following the encounter with CMV, resulting in expansion of subpopulations of CD8⁺ T cells. Naive CD8⁺T cells express CD45RA and the co-stimulatory molecules CD28 and CD27. During differentiation to memory cells they lose expression of CD45RA (Hamann et al., 1999). Effector T cells re-express CD45RA but do not express CD28 and CD27 (Hamann et al., 1999). The expression of CD56 and CD57 on CD8⁺ T cells are markers of cytolytic effector function (Ohkawa et al., 2001; Pittet et al., 2000). Accordingly, the expanded CD8⁺ cells noted in this study of base-line levels in the NONA Immune cohort, comprised of CD8⁺CD57⁺CD28⁻, CD8⁺CD45RA⁺CD27⁻, and CD8⁺CD56⁺CD57⁺, representing late stages of CD8 differentiation. At the time of the study, three-colour flow cytometry was used, allowing a limited number of marker combinations. By using 6-colour flow cytometry, which is currently used in our lab, it would have been possible to further delineate CD8 T cell subpopulations.

Paper II

The relations between IRP, inflammatory activity, morbidity and 2-year mortality were examined longitudinally in the NONA Immune sample (n = 138). Data from baseline (T1), 2-year follow-up (T2) and 4-year follow-up (T3) were used. After two years (T2), 40 individuals had died and another 14 declined to participate in this follow up, leaving 84 individuals to be examined. Of these, 22 (26 %) had died after another 2 years (T3). In the following (paper II), survivors and non-survivors refer to the situation at T3.

Increased inflammatory activity was found at T2 for non-survivors at T3 compared to survivors (Paper II, Table 1). Thus, non-survivors showed increased plasma levels of IL-6, CRP and decreased plasma levels of albumin. As expected, associations were found in between inflammatory markers such as plasma levels of IL-6 and other markers of inflammation, and similarly, plasma levels of CRP were associated with albumin and transthyretin, and levels of transthyretin were associated with albumin. With regard to cellular abnormalities, there were increased relative numbers of individuals at T2 showing an inverted CD4/CD8 ratio in non-survivors at T3 compared with survivors. Interestingly, no association was found at T2 between the CD4/CD8 ratio and the inflammatory markers, and furthermore, logistic regression showed significant main effects for the CD4/CD8 ratio and IL-6, but no major interactions between these two parameters. IRP and low-grade inflammation were independently the major predictors of 2-year mortality from T2 to T3. Low-grade inflammation could be defined by any of the markers IL-6, CRP, albumin, or transthyretin. The CD4/CD8 ratio and the IL-6 level collectively predicted 57 % of the deaths and 97 % of the observed survival over 2 years. IL-6 was the strongest predictor of survival. The impact of IRP and IL-6 on 2-year survival was independent of age, sex and several diseases, as shown

in the logistic regression model. The finding of association between IRP and mortality risk is in line with results from the OCTO longitudinal study and from the Healthy Ageing Study of younger elderly UK people (Ferguson et al., 1995; Huppert et al., 2003; Wikby et al., 1998). Inflammation is also a known prognostic factor, and the over-all results in our study are in agreement with previous findings that low-grade inflammation can predict mortality (Bruunsgaard et al., 2003; Klonoff-Cohen et al., 1992; Reuben et al., 2002). Several studies have shown that an increase in IL-6 levels is a characteristic component of age-associated pathological processes such as Alzheimer's disease (AD), osteoporosis, type-2 diabetes and cardiovascular disease (Ershler and Keller, 2000). However, in this study IRP and IL-6 were predictive of mortality in a manner not significantly affected by prevalent diseases, including AD, cardiovascular disease and type-2 diabetes.

By examining IRP individuals (showing a CD4/CD8 ratio < 1 at baseline) in relation to data on inflammatory parameters at T1 and T2 it was possible to evaluate the temporal development of inflammation in IRP individuals. IRP non-survivors (dead at T3) showed only a minor inflammatory activity at baseline (T1) (Paper II, Table 2), but interestingly, they developed increased inflammatory activity at follow-up (T2). Non-IRP individuals (showing a CD4/CD8 ratio > 1), on the other hand, showed increased inflammation in non-survivors compared to survivors both at base-line (T1) and at follow-up (T2).

Studies have indicated that increases in IL-6 levels are associated with the increased amounts of fat tissue and loss of muscle mass that occurs with normal ageing (Pedersen et al., 2003). The age-related decline in lean body mass with decreased muscle strength, functional disability and loss of bone mineral density, known as the syndrome of frailty, is characterised by a negative protein balance in the skeletal muscle (Ershler and Keller, 2000). Accordingly, we found a median weight loss of 2 kg across the measurement occasions (T1 to T2) in IRP non-survivors (at T3), as compared to unchanged and normal median weight in IRP survivors. These results, with weight loss occurring in the group developing inflammation at T2, support that IL-6 has direct catabolic effects and/or indirect effects by blocking anabolic effects of insulin in the development of frailty (Roubenoff, 2003). Frailty might be initiated by infectious disease, malnutrition or stressful events associated with increased levels of IL-6 (Roubenoff, 2003). It has also been shown that ageing is associated with a prolonged inflammatory activity with regard to circulating IL-6 during an infectious disease (Bruunsgaard et al., 1999b) and that malnutrition is a major risk factor for infection and that infections per se can induce malnutrition (Fulop et al., 2005).

Paper III

The 4-year mortality (from baseline, T1, to 4-year follow up, T3) of the NONA Immune sample ($n = 138$) was examined in relation to a broad set of laboratory parameters and morbidity. Associations were examined between the cause of death and laboratory and

clinical parameters. At 4-year follow-up, 71 individuals were deceased. The remaining life expectancy for individuals at age 86 to 94 is between 5 years to 3 years (Statistiska centralbyrån, 2007), respectively, implicating that 4 years later, approximately 50 % of the individuals would be expected to have died. This figure corresponds well with the finding of 51 % deceased after 4 years in our population-based sample.

By examining a broad panel of laboratory parameters at base-line in the NONA Immune cohort, we showed that 4-year mortality was associated mainly with parameters either related to inflammation or to abnormalities in T cell populations (Paper III, Table 1). The CD4/CD8 ratio was significantly lower in non-survivors and furthermore, the majority of individuals (17 of 22) with CD4/CD8 ratio < 1 were non-survivors. T cell related abnormalities also included increased numbers of terminally differentiated CD8+CD28- cells. With regard to inflammatory parameters, plasma levels of albumin were significantly lower, and levels of CRP, haptoglobin, orosomucoid and IL-6 were significantly higher in non-survivors compared to survivors.

Other parameters influencing 4-year mortality included a lower haemoglobin concentration (in women), which could, among other possible causes, be influenced by inflammation. Urea and Cystatin C levels (in men) were also related to 4-year mortality, plausibly by being markers for kidney insufficiency. However, Cystatin C has also shown firm correlations with inflammation (Shlipak et al., 2005).

We also found that BMI was lower in non-survivors compared with survivors. A lower BMI, at least in part, may be mediated by a catabolic state. Interestingly, inflammation could be one important contributing factor in the development of catabolism in the oldest-old (Ershler and Keller, 2000; Pedersen et al., 2003). Thus, inflammation may be associated with the lower BMI observed in oldest-old individuals.

Taken together, these findings confirm and extend previous knowledge that inflammation and T cell abnormalities are the major factors contributing to mortality in later life.

By correlation analyses between laboratory parameters and 4-year mortality, we showed that IRP and IL-6 were the factors most strongly associated with mortality. However, these markers did not correlate with each other. Since IL-6 and IRP also have been emphasized in the literature, we used them to stratify the sample into risk groups based on the absence or presence of one or both risk factors.

Kaplan-Meier survival curve analysis (Paper III, Figure 1) confirmed that both IRP (CD4/CD8 ratio < 1) and IL-6 (\geq median level of 3.15 pg/mL) groups were independently associated with increased 4-year mortality rates; 73 % and 64 %, respectively, compared to individuals without these risk factors (29 %). Individuals with both IRP and inflammation, the “Double risk” group, showed an even higher 4-year mortality of 82 %, indicating an additive risk if both inflammation and T cell abnormalities is present. The results are in line with our

previous findings that IRP and IL-6 are independently associated with mortality (Wikby et al., 2006), which is further confirmed in the present study by the lack of correlation between CD4/CD8 ratio and IL-6 levels.

Age-related diseases such as cardiovascular disease, type-2 diabetes mellitus, osteoporosis, Alzheimer's disease and frailty have previously shown associations with elevated levels of IL-6 (Alexandraki et al., 2006; Carlsten, 2005; Cesari et al., 2003; Ding et al., 2008; Forsey et al., 2003; Spranger et al., 2003; Yaffe et al., 2003). In the present study, cognitive dysfunction and dementia were more common in the "IL-6" and "Double risk" (High IL-6 level and low CD4/CD8 ratio) groups, thus confirming the association between IL-6 and the presence of dementia (Paper III, Table 2). For example, 16 of 20 individuals with dementia had IL-6 levels above the median level. Exclusion of dementia did not change the 4-year mortality differences between the various risk groups, indicating that IL-6 itself and not dementia is most important for the increased 4-year mortality. For cardiac insufficiency there was a tendency for a low prevalence in the "No risk" group and higher and similar prevalence in the other groups. This finding thus corroborates an association between cardiovascular disease and IL-6, but it also points to a possible association between IRP and cardiovascular disease. CMV infection is a major driving force in the development of IRP (Wikby et al., 2002), but CMV itself has also been implicated in the pathogenesis of cardiovascular disease (Gredmark et al., 2007; Muhlestein et al., 2000; Zhu et al., 2001). Hence, CMV could alone, or by its association with IRP be associated with cardiovascular disease. The hazard ratio for 7-year mortality risk among Finnish older adults with stable cardiovascular disease was 2.23 (95 % CI, 1.32–3.79) in the highest HCMV IgG quartile compared to the lowest quartile; supporting an independent association between HCMV IgG antibody level and 7-year mortality (Strandberg et al., 2009). This warrants further studies of HCMV activity and of possible intervention strategies.

Despite strong associations with mortality, it is not previously known whether IRP and IL-6 are associated with the specific cause of death. One might expect an association between increased levels of IL-6 and death by cardiovascular disease and dementia, whereas IRP, with its profile of a functional T cell deficiency could be associated with death caused by infection or cancer. However, in the present study we found no evidence for an association between IL-6 or IRP and the specific cause of death when grouped into the categories of cardiovascular disease (including cerebrovascular disease), infection and cancer (Paper III, Table 3). This lack of association suggests that IL-6 and IRP are related to survival but not to cause of death. This is supported by a Finnish investigation of community elderly with stable cardiovascular disease and 7-year mortality risk. They found no association between the distribution of causes of death and HCMV IgG antibody level (Strandberg et al., 2009). One obvious possible limitation of these studies is the difficulty of determining cause of death, in particular when post-mortem examinations are not carried out.

IL-6 and IRP were chosen as factors reflecting inflammation and T cell abnormality, respectively. One reason for the lack of association between IL-6 and IRP and specific cause of death is that both markers are likely to represent a broader spectrum of risk factors. IL-6 is not only involved in the induction of inflammation, but as a true pleiotropic cytokine (Ershler, 1993) it is for example also involved in B cell differentiation, Th cell polarization, bone and muscle metabolism and sickness behaviour (Bertolini and Benson, 1990; Bluthe et al., 2000; Diehl and Rincón, 2002; Ershler and Keller, 2000; Pedersen et al., 2003). Thus, IL-6 can be linked to a variety of conditions, in addition to cardiovascular disease and dementia, for example to osteoporosis and frailty. Likewise, IRP may reflect a broad spectrum of risk factors since IRP has been linked with CMV-infection, which may be a significant contributor in the development of immunosenescence being associated with increased susceptibility to infectious disease, cancer, but also with the development of cardiovascular disease (Söderberg-Naucler, 2006). In addition, high in vivo plasma levels of IL-6 and IRP are associated with a lower response to immune stimulation, as shown e.g. in a situation of chronic stress (Bosch et al., 2009; Sjögren et al., 2006). A lower response in IL-6 might mirror a functional defect in innate immunity, hence implying that not only IRP, but also IL-6 contribute to an increased risk of infections and tumours. These multiple properties of IRP and IL-6 may in fact explain why these factors stand out as the most important predictors of mortality. Accordingly, it may not be surprising that these parameters are not directly linked to specific causes of death. In addition, it is likely that the cause of death with increasing age becomes more and more random. The risk of dying at younger age is low, and death is more likely caused by specific diseases or events, whereas the ageing process implies an increasingly higher risk of dying (indeed the total risk of dying is 100%), and for a frail oldest-old individual, the specific cause of death is more random among multiple candidates which alone or more likely in combination contribute to terminate life.

Paper IV

The prevalence of antinuclear antibodies (ANA) was investigated in the oldest-old. Plasma samples from the NONA Immune (n = 136) and the OCTO Immune (n = 97) studies were compared with serum samples from younger healthy controls (blood donors, n = 200). An abnormal level of ANA as measured by immunofluorescence (F-ANA) was defined as a titre of $\geq 1:200$, based on the 95 % percentile of a reference population of women.

The prevalence of abnormal F-ANA was 8 % in the NONA Immune group (women and men), which is significantly higher compared to 1.5 % in healthy blood donors (women and men). Individuals in the NONA Immune Study were not excluded due to compromised health (Nilsson et al., 2003), in contrast to the OCTO Immune Study, where individuals were selected according to health status. The difference between the two samples is the frail group, which is not included in the OCTO sample. Thus, the prevalence of abnormal ANA was first examined across different health groups (Paper IV, Table 2), showing no significant

differences; 9.3 % in the frail group, 8.1 % in the moderately healthy group, 0 % in the very healthy group, respectively. Furthermore, presence of F-ANA was not associated with the occurrence of autoimmune disease. This lack of association between ANA-occurrence and health status is in line with other reports (Andersen-Ranberg et al., 2004; Formiga et al., 2008). Since there was no association between the health status and the presence of F-ANA in the NONA Immune sample, the OCTO Immune and the NONA Immune samples were pooled for the further investigations.

In total 10.1 % of the oldest-old women and 6.7 % of the oldest-old men were F-ANA positive at a titre of $\geq 1:200$. Elderly women had a higher prevalence of F-ANA than healthy female blood donors (3.0 %). For men, there was a more pronounced difference between the elderly and the younger men (prevalence 0 %, Paper IV, Fig. 2). Thus, at younger age, there is a significant difference between women and men with regard to the prevalence of F-ANA. In contrast, at high age, this difference has almost disappeared.

In a Finnish study, 12.3 % of the nonagenarians had an abnormal ANA titre (defined as $\geq 1:160$), similar to the results in our study. Interestingly, they found no difference in IL-6 or CRP concentrations between ANA negative and positive individuals, indicating that the presence of ANA is not associated with an inflammatory response. Furthermore, there was no difference in 4-year mortality between ANA negative and positive individuals (Hurme et al., 2007). In our study, neither the presence of CMV nor the presence of IRP was associated with the occurrence of ANA. Taken together, it does not seem that the presence of ANA is a major factor for morbidity or mortality at high age. However, the increased frequency of ANA in the elderly, in particular in men, must be taken into account when evaluating ANA in a clinical setting.

Concluding remarks

- The commonly used SENIEUR protocol, selecting individuals representing ‘normal ageing’, excludes 90 % of nonagenarians for immune studies. Morbidity in terms of very healthy, moderately healthy or frail does not influence T lymphocyte alterations associated with the immune risk phenotype (IRP). These findings support the concept of performing immunogerontological studies on non-selected population based samples.
- IRP and low-grade inflammation were the major predictors of 2-year mortality in the oldest-old. The impact of IRP and IL-6 on 2-year survival was independent of age, sex and several diseases. The longitudinal design allowed temporal evaluations, suggesting a sequence of events starting with IRP and leading to inflammation in the decline state.
- 4-year mortality in the oldest-old was mainly associated with parameters related to inflammation or T cell abnormalities. The presence of IRP and increased IL-6 level showed some associations with presence of diseases; in particular, IL6 was associated with the presence of cognitive impairment. However, despite being strong predictors of mortality, IRP and IL-6 could not be linked to any specific cause of death, probably due to the multi-factorial nature of these factors.
- The prevalence of antinuclear antibodies (ANA) in the oldest-old was higher compared to blood donors. The difference across age was most pronounced in men, with low levels at younger age, whereas the prevalence among the oldest-old men reached similar level as in women. There was no association between the presence of ANA and CD4/CD8 ratio, CMV status or health status in the oldest-old.

Future perspectives

Studies in octogenarians and nonagenarians have shown an association between IRP and CMV positivity. About 16 % of individuals in those studies had an IRP at inclusion and IRP individuals showed an increased mortality risk. Important questions in further studies include:

- Does IRP exist in a younger population?
- Do younger individuals with IRP have an increased mortality risk?
- Is there an association between IRP and CMV in younger individuals?

To address these questions, another study has already started. Hexagenarians (60 to 70 years of age) are recruited to participate in a longitudinal immune study.

Another important area that needs to be further investigated is how an optimal immune response to CMV is mounted and what the nature of such a response is. CMV contains several antigens, and it is not known if there are certain epitopes that confer protection. Neither is the role of the antibody response versus the T cells response settled. Furthermore, it is likely that NK cells and regulatory T cells, populations that undergo marked changes in ageing, could influence the development of CMV infection and thereby the development of IRP. Therefore, a longitudinal study including a broad set of lymphocyte populations could elucidate the interactions and roles of these cells in relation to IRP and CMV.

The ultimate question is whether immune changes in the oldest-old can be prevented or reduced. With regard to CMV, it would be reasonable to try to vaccinate in order to reduce the CMV burden. However, such a vaccine has not been approved, and there is a lot of basic knowledge that needs to be established before a safe CMV vaccine can be used. The most obvious task would be to vaccinate adult infected individuals in order to strengthen the immune response against CMV, thereby hopefully diminishing the further CD8 clonal expansions. An even more drastic strategy would be to vaccinate early in order to prevent primary CMV infection, and thereby avoiding the effect of the CMV immune response that is seen in the elderly. However, it is completely unknown what would be the consequences of these strategies. One must remember that there is a co-evolution of the human and CMV genomes. It would, however, be very interesting to evaluate the effects of vaccination in the elderly, where a more prominent negative effect is seen. With regard to the vitality of the T cell compartment, a continuous production of T cells would be desired. Therefore, trials have been started with treatment using IL-7, a growth factor for T cell development in the thymus, and growth hormone, also with T cell supportive effects. Since inflammation is so strongly linked to mortality in the oldest-old, strategies to reduce inflammation would also be advantageous. Psychosocial stress is known to induce inflammation, and steps to reduce stress, albeit difficult, could be rewarding.

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