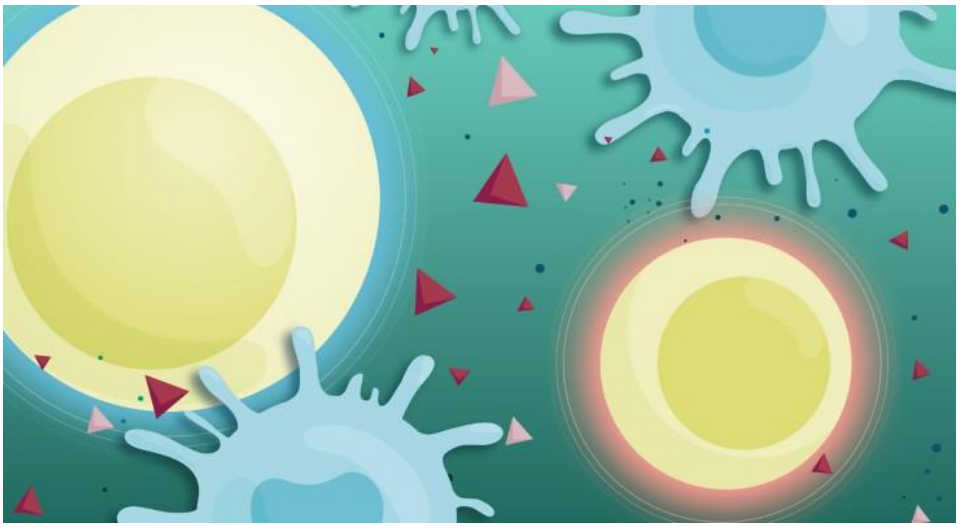
UNIVERSITÀ DEGLI STUDI DI PALERMO

Investigation of genetic factors and molecular targets influencing immunosenescence in Sicilian population: potential approaches for future immunotherapeutic interventions



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INVESTIGATION OF GENETIC FACTORS AND MOLECULAR TARGETS INFLUENCING IMMUNOSENESCENCE IN SICILIAN POPULATION: POTENTIAL APPROACHES FOR FUTURE IMMUNOTHERAPEUTIC INTERVENTIONS

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"*C'è una forza motrice più forte del vapore,*
dell'elettricità e dell'energia atomica: la tua volontà"

Albert Einstein

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(Poster Presentation)

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(Poster Presentation)

1ST EDITION. SUSTAINABLE DEVELOPMENT AND CIRCULAR ECONOMY. **Ligotti ME**. "Determinanti immunogenetici e razionalizzazione della terapia dell'infezione da HIV". December 13, 2018 – Palermo, Italy.

(Oral Presentation)

List of abstracts

1. **Ligotti, M.E**., Aiello, A., Accardi, G, Aprile, S., Bulati, M., Gervasi, F., Rizzo, S., Zareian, N., Caruso, C., Farzaneh, F., Candore, G. *Analysis of T and NK cell subsets in Sicilian population from young to supercentenarian: the role of age and gender*. (Manuscript under revision)

   ABSTRACT: Ageing dramatically affects number and function of both innate and adaptive arms of immune system, particularly T cell subsets, contributing to reduce vaccination efficacy, decreased resistance to infections and increased prevalence of cancer in the older people. In the present paper, we analysed the age-related changes in the absolute number of lymphocytes in 216 Sicilian subjects and, in a sub-cohort, in the percentages of T and NK cells. We compared these results with the immunophenotype of the oldest living Italian supercentenarian (111 years old). The results were also analysed by gender. The correlation between number/percentage of cells and age in all individuals and, separately, in males and females, was examined using a simple linear regression analysis. The results suggest that the different CD4+ and CD8+ subsets are not affected equally by age between females and males but we might speculate that gender may affect the response to CMV infection. The supercentenarian shows a CD4+ percentage with greater similarity to that of older females (79-88 years) than that of female belonged to the long-lived individuals (LLIs, > 90 years) group. Likewise, CD8+ percentages were in the mean ± SD of older females, above the range of female LLIs. Both the percentage of CD4+ and CD8+ naïve T cells were lower than those seen in young females, although they were very little (CD8+) or none (CD4+) different from those in older females. These suggests that supercentenarian has a naïve "younger" T cell profile comparable to that of an older female.

   AUTHOR KEYWORDS: ageing; immunosenescence; T cells; centenarian.

2. **Ligotti, M.E**., Aiello, A., Accardi, G, Amato. G., Caruso, C., Cascio, A., Colomba, C., Duro, G., Rao, M., Candore, G., Di Bona, D. *Do activating KIRs protect general population from HIV infection? A pilot study performed in Sicilian population.* (Manuscript submitted)

   ABSTRACT: Recent studies have shown that killer immunoglobulin receptors (KIRs) are associated with susceptibility to Human Immunodeficiency Virus (HIV) infection and HIV disease progression although some results were inconsistent. Therefore, in the present paper we analysed the role of KIR/HLA repertoire in the susceptibility to HIV in a sample of Sicilian population consisting of 60 blood donors as control population, chosen for their IgG positivity to Human Cytomegalovirus (HCMV), with no history of symptomatic disease, and 89 Sicilian patients with HIV infection. Logistic regression analysis shows that activating KIR2DS4-Full and HLA-A-Bw4 protect controls from HIV infection as well as haplotype Bx (respectively, Adj. OR, 5.82, $P<0.01$; Adj. OR, 0.14, $P<0.01$; Adj. OR, 0.09, $P<0.01$), whereas the ligand HLA-C1 (Adj. OR, 4.73, $P<0.01$) was a factor of susceptibility to infection. Present data and their interpretation are in line with the presence of activating natural cytotoxicity receptors in the vast majority of HIV-controllers and imply that general population, or at least blood donors, carry genetic markers for HIV resistance. It is intriguing because of we have previously demonstrated that the same population carries genetic markers against the occurrence of chronic hepatitis B.

   AUTHOR KEYWORDS: HLA, HIV, KIR, Logistic regression.

3. Pojero, F., Candore, G., Caruso, C., Di Bona, D., Groneberg, D.A., **Ligotti, M.E**., Accardi, G., Aiello, A. *The Role of Immunogenetics in COVID-19. (2021) Int. J. Mol. Sci. 2021, 22, 2636. https://doi.org/10.3390/ijms22052636*

   ABSTRACT: Coronavirus disease 2019 (COVID-19) is induced by SARS-CoV-2 and may arise as a variety of clinical manifestations, ranging from an asymptomatic condition to a life-threatening disease associated with cytokine storm, multiorgan and respiratory failure. The molecular mechanism behind such variability is still under investigation. Several pieces of experimental evidence suggest that genetic variants influencing the onset, maintenance and resolution of the immune response may be fundamental in predicting the evolution of the disease. The identification of genetic variants behind immune system reactivity and function in COVID-19 may help in the elaboration of personalized therapeutic strategies. In the frenetic look for universally shared treatment plans, those genetic variants that are common to other diseases/models may also help in addressing future research in terms of drug repurposing. In this paper, we discuss the most recent updates about the role of immunogenetics in determining the susceptibility to and the history of SARS-CoV-2 infection. We propose a narrative review of available data, speculating about lessons that we have learnt from other viral infections and immunosenescence, and discussing what kind of aspects of research should be deepened in order to improve our knowledge of how host genetic variability impacts the outcome for COVID-19 patients.

   AUTHOR KEYWORDS: AB0; COVID-19; HLA; immunogenetics; KIR; SARS-CoV-2

4. Di Bona, D., Pandey, J.P., Aiello, A., Bilancia, M., Candore, G., Caruso, C., Colomba, C., Duro, G., **Ligotti, M.E**., Macchia, L., Rizzo, S., Accardi, G. *The immunoglobulin γ marker 17 allotype and KIR/HLA genes prevent the development of chronic hepatitis B in humans*. (2020) 159 (2), pp. 178-182. Immunology. 2020 Feb;159(2):178-182. doi: 10.1111/imm.13133. Epub 2019 Nov 12. PMID: 31613998; PMCID: PMC6954734.

   ABSTRACT: Hepatitis B virus (HBV) infection causes a self-limiting disease in most individuals. However, < 10% of infected subjects develop a chronic disease. Genetic host variability of polymorphic genes at the interface of innate and acquired immunity, such as killer immunoglobulin-like receptors (KIR), their human leucocyte antigen (HLA) and IgG allotypes (GM), could explain this different clinical picture. We previously showed a protective role of the KIR2DL3 gene for the development of chronic hepatitis B (CHB), and a detrimental role of the KIR ligand groups, HLA-A-Bw4 and HLA-C2. We have expanded the previous analysis genotyping patients for GM23 and GM3/17 allotypes. The comparison of the patients with CHB with those who resolved HBV infection showed that the presence of GM17 allele virtually eliminated the risk of developing CHB (OR, 0·03; 95% CI, 0·004–0·16; P < 0·0001). In addition, the combination of GM17, KIR2DL3, HLA-A-Bw4 and HLA-C2 was highly sensitive to predict the outcome of HBV infection.

   AUTHOR KEYWORDS: hepatitis B virus; human leucocyte antigen; killer immunoglobulin-like receptor; γ marker.

5. Di Bona, D., Malovini, A., Accardi, G., Aiello, A., Candore, G., Ferrario, A., **Ligotti, M.E.**, Maciag, A., Puca, A.A., Caruso, C. *Taste receptor polymorphisms and longevity: a systematic review and meta-analysis*. Aging Clin Exp Res. 2020 Nov 10. doi: 10.1007/s40520-020-01745-3. Epub ahead of print. PMID: 33170488

ABSTRACT: Bitter taste receptors (TAS2R) are involved in a variety of non-tasting physiological processes, including immune-inflammatory ones. Therefore, their genetic variations might influence various traits. In particular, in different populations of South Italy (Calabria, Cilento, and Sardinia), polymorphisms of TAS2R16 and TAS238 have been analysed in association with longevity with inconsistent results. A meta-analytic approach to quantitatively synthesize the possible effect of the previous variants and, possibly, to reconcile the inconsistencies has been used in the present paper. TAS2R38 variants in the Cilento population were also analysed for their possible association with longevity and the obtained data have been included in the relative meta-analysis. In population from Cilento no association was found between TAS2R38 and longevity, and no association was observed as well, performing the meta-analysis with data of the other studies. Concerning TAS2R16 gene, instead, the genotype associated with longevity in the Calabria population maintained its significance in the meta-analysis with data from Cilento population, that, alone, were not significant in the previously published study. In conclusion, our results suggest that TAS2R16 genotype variant is associated with longevity in South Italy. © 2020, The Author(s).

AUTHOR KEYWORDS: Immune-inflammatory responses; Longevity; Meta-analysis; Taste receptors

6. Aiello, A., Accardi, G., Aprile, S., Caldarella, R., Cammarata, G., Carru, C., Caruso, C., Ciaccio, M., De Vivo, I., Gambino, C.M., **Ligotti, M.E.**, Vasto, S., Candore, G. *Pro-inflammatory status is not a limit for longevity: case report of a Sicilian centenarian*. Aging Clin Exp Res. 2020 Jun 23. doi: 10.1007/s40520-020-01628-7.Epub ahead of print. PMID: 32577916

   ABSTRACT: Most studies on centenarians represent them as the best model of ageing. They are defined “delayers”, if they exhibit age-related diseases between 80 and 99 years, “survivors” if they show clinically demonstrable diseases before the age of 80 years, and “escapers” when they attain their 100th year of life without any common age-associated pathologies. However, the extreme longevity is often characterized by not unique and unequivocal phenotype, as demonstrated by the centenarian population worldwide. Not all centenarians are alike, but everyone can represent a model of “positive biology”. The present paper shows the interesting case of CC, a female centenarian living in Sicily (Italy). CC has become centenarian in seemingly good health despite the presence of sub-optimal biochemical parameters. To study the peculiar features of this subject, anamnestic, cognitive, biochemical, genetic, and epigenetic data were collected. No age-related diseases have been detected with the exception of a supposed mild cognitive impairment. CC showed some laboratory signs of atrophic gastritis and a chronic status of inflammation, but her level of some microRNAs (miRNAs), with a role in the control of innate immunity and inflammation, was higher than female centenarians. Therefore, CC can be considered a case appealing and useful to provide new insights into the extreme phenotype represented by extreme longevity.

7. Aiello, A., Farzaneh, F., Candore, G., Caruso, C., Davinelli, S., Gambino, C.M., **Ligotti, M.E.**, Zareian, N., Accardi, G. *Immunosenescence and its hallmarks: How to oppose aging strategically? A review of potential options for therapeutic intervention*. Front Immunol. 2019 Sep 25;10:2247. doi: 10.3389/fimmu.2019.02247. PMID: 31608061; PMCID: PMC6773825.

   ABSTRACT: Aging is accompanied by remodeling of the immune system. With time, this leads to a decline in immune efficacy, resulting in increased vulnerability to infectious diseases, diminished

responses to vaccination, and a susceptibility to age-related inflammatory diseases. An age-associated immune alteration, extensively reported in previous studies, is the reduction in the number of peripheral blood naïve cells, with a relative increase in the frequency of memory cells. These two alterations, together with inflamm-aging, are considered the hallmarks of immunosenescence. Because aging is a plastic process, it is influenced by both nutritional and pharmacological interventions. Therefore, the role of nutrition and of immunomodulation in immunosenescence is discussed, due to the multifactorial influence on these hallmarks. The close connection between nutrition, intake of bioactive nutrients and supplements, immune function, and inflammation demonstrate the key role of dietary strategies as regulators of immune response and inflammatory status, hence as possible modulators of the rate of immunosenescence. In addition, potential options for therapeutic intervention are clarified. In particular, the use of interleukin-7 as growth factor for naïve T cells, the function of checkpoint inhibitors in improving T cell responses during aging and, the potential of drugs that inhibit mitogen-activated protein kinases and their interaction with nutrient signaling pathways are discussed. Finally, it is suggested that the inclusion of appropriate combinations of toll-like receptor agonists may enhance the efficacy of vaccination in older adults.

AUTHOR KEYWORDS: Aging; Immunomodulation; Immunosenescence; Immunotherapy; Nutrition.

8. Malovini, A., Accardi, G., Aiello, A., Bellazzi, R., Candore, G., Caruso, C., **Ligotti, M.E.**, Maciag, A., Villa, F., Puca, A.A. *Taste receptors, innate immunity and longevity: The case of TAS2R16 gene*. Int J Mol Sci. 2019 Feb 5;20(3):685. doi: 10.3390/ijms20030685. PMID: 30764515; PMCID: PMC6386818.

ABSTRACT: Background: Innate immunity utilizes components of sensory signal transduction such as bitter and sweet taste receptors. In fact, empirical evidence has shown bitter and sweet taste receptors to be an integral component of antimicrobial immune response in upper respiratory tract infections. Since an efficient immune response plays a key role in the attainment of longevity, it is not surprising that the rs978739 polymorphism of the bitter taste receptor TAS2R16 gene has been shown to be associated with longevity in a population of 941 individuals ranging in age from 20 to 106 years from Calabria (Italy). There are many possible candidate genes for human longevity, however of the many genes tested, only APOE and FOXO3 survived to association in replication studies. So, it is necessary to validate in other studies genes proposed to be associated with longevity. Thus, we analysed the association of the quoted polymorphism in a population of long lived individuals (LLIs) and controls from another Italian population from Cilento. Methods: The analysis has been performed on data previously obtained with genome-wide association study on a population of LLIs (age range 90-109 years) and young controls (age range 18-45 years) from Cilento (Italy). Results: Statistical power calculations showed that the analysed cohort represented by 410 LLIs and 553 young controls was sufficiently powered to replicate the association between rs978739 and the longevity phenotype according to the effect size and frequencies described in the previous paper, under a dominant and additive genetic model. However, no evidence of association between rs978739 and the longevity phenotype was observed according to the additive or dominant model. Conclusion: There are several reasons for the failure of the confirmation of a previous study. However, the differences between the two studies in terms of environment of the population adopted and of the criteria of inclusion have made difficult the replication of the findings. © 2019 The Author(s).

AUTHOR KEYWORDS: Bitter taste receptors; Case control study; GWAS; Innate immunity; Longevity; TAS2R16 gene

9. Zareian, N., Aprile, S., Cristaldi, L., **Ligotti, M.E.**, Vasto, S., Farzaneh, F. *Triggering of toll-like receptors in old individuals. Relevance for vaccination*. Curr Pharm Des. 2019;25(39):4163-4167. doi: 10.2174/1381612825666191111155800. PMID: 31713478.

   ABSTRACT: Aging is characterized by a general decline in a range of physiological functions, with a consequent increase in the risk of developing a variety of chronic diseases and geriatric syndromes. Additionally, increasing age is accompanied by a progressive decline in both innate and acquired immune system, referred to as immunosenescence. This impaired ability to mount an efficient immune response after exposure to microorganisms or vaccines represents a major challenge in acquiring protection against pathogens in aging. Therefore, there is still a great need for vaccines that are tailored to optimally stimulate the aged immune system, thus promoting more successful aging. Various strategies can be used to improve vaccine efficacy in old people. Despite this, meta-analyses have clearly shown that the magnitude of protection obtained remains lower in older adults. Recent studies show that stimulation of Toll-like receptors, using stimulatory ligands, can enhance vaccine efficacy by a number of mechanisms, including the activation of innate immune cells and the consequent production of inflammatory cytokines. Therefore, a possible strategy for more effective vaccination in the older population is the triggering of multiple TLRs, using a combined adjuvant for the synergistic activation of cellular immunity. Preliminary in vitro data suggest that in humans the presence of multiple TLR agonists can result in the greater stimulation of antigen-specific immune responses in immune cells both in the young healthy and in the immune senescent older donors. These data suggest that appropriately selected combinations of TLR agonists could enhance the efficacy of vaccination mediated immunity in older people.

   AUTHOR KEYWORDS: Aging; Cytokines; Dendritic cells; Immunosenescence; TLR; Vaccination.

10. Aiello, A., Accardi, G., Candore, G., Caruso, C., Colomba, C., Bona, D.D., Duro, G., Gambino, C.M., **Ligotti, M.E.**, Pandey, J.P. *Role of immunogenetics in the outcome of HCMV infection: Implications for ageing*. Int J Mol Sci. 2019 Feb 5;20(3):685. doi: 10.3390/ijms20030685. PMID: 30764515; PMCID: PMC6386818.

    ABSTRACT: The outcome of host-virus interactions is determined by a number of factors, some related to the virus, others to the host, such as environmental factors and genetic factors. Therefore, different individuals vary in their relative susceptibility to infections. Human cytomegalovirus (HCMV) is an important pathogen from a clinical point of view, as it causes significant morbidity and mortality in immunosuppressed or immunosenescent individuals, such as the transplanted patients and the elderly, respectively. It is, therefore, important to understand the mechanisms of virus infection control. In this review, we discuss recent advances in the immunobiology of HCMV-host interactions, with particular emphasis on the immunogenetic aspects (human leukocyte antigens, HLA; killer cell immunoglobulin-like receptors, KIRs; immunoglobulin genetic markers, GM allotypes) to elucidate the mechanisms underlying the complex host-virus interaction that determine various outcomes of HCMV infection. The results, which show the role of humoral and cellular immunity in the control of infection by HCMV, would be valuable in directing efforts to reduce HCMV spurred health complications in the transplanted patients and in the elderly, including immunosenescence. In addition, concerning GM allotypes, it is intriguing that, in a Southern Italian

population, alleles associated with the risk of developing HCMV symptomatic infection are negatively associated with longevity. © 2019 by the authors. Licensee MDPI, Basel, Switzerland.

AUTHOR KEYWORDS: Antibodies; Elderly; GM; HCMV; HLA; Immunosenescence; KIR; NK.

11. Accardi, G., Aprile, S., Candore, G., Caruso, C., Cusimano, R., Cristaldi, L., Bona, D.D., Duro, G., Galimberti, D., Gambino, C.M., **Ligotti, M.E.**, Mazzucco, W., Vasto, S., Aiello, A. *Genotypic and phenotypic aspects of longevity: Results from a sicilian survey and implication for the prevention and treatment of age-related diseases*. Curr Pharm Des. 2019;25(3):228-235. doi: 10.2174/1381612825666190313115233. PMID: 30864497.

    ABSTRACT: Background: It is well known that long living individuals are a model of successful ageing and that the identification of both genetic variants and environmental factors that predispose to a long and healthy life is of tremendous interest for translational medicine. Methods: We present the preliminary findings obtained from an ongoing study on longevity conducted on a sample of Sicilian long-lived individuals. Results: We review the characteristics of longevity in Sicily, taking into account lifestyle, environment, genetics, hematochemical values, body composition and immunophenotype. In addition, we discuss the possible implications of our data for the prevention and/or treatment of age-related diseases. Conclusion: As widely discussed in this review, the explanation of the role of genetics and lifestyle in longevity can provide important information on how to develop drugs and/or behaviours that can slow down or delay ageing. Thus, it will be possible to understand, through a "positive biology" approach, how to prevent and/or reduce elderly frailty and disability. © 2019, Bentham Science Publishers B.V.. All rights reserved.

    AUTHOR KEYWORDS:Age-related diseases; Body composition; Genetics; Immunosenescence; Longevity; Sicily.

12. Aiello, A., Candore, G., Accardi, G., Caruso, C., Colomba, C., Duro, G., Gambino, C.M., **Ligotti, M.E.**, Di Bona, D. *Translation of basic research into clinics: Killer immunoglobulin-like receptors genes in autoimmune and infectious diseases*. Curr Pharm Des. 2018;24(26):3113-3122. doi: 10.2174/1381612824666180911123249. PMID: 30205795.

    ABSTRACT: Killer immunoglobulin-like receptors (KIRs) regulate the activation of natural killer cells through their interaction with human leucocyte antigens (HLA). KIRs and HLA loci are highly polymorphic, and some of their combinations have been found to protect against viral infections or to predispose to autoimmune disorders. In particular, some activating KIRs profiles may be detrimental in autoimmune pathogenesis, and specific KIRs may be particularly aggressive in the clearance of different microorganisms, protecting individuals in the control of a given pathogen. So, considering that in the pathogenesis of many autoimmune disorders and infections innate immunity plays a key role, the recent development for KIRs characterization, diseases monitoring, and treatment becomes obvious. Here, we reviewed a growing body of evidence supporting the influence of KIRs variants and their interaction with ligands in the development of the main human autoimmune and viral diseases, highlighting the main applications in clinical practice. © 2018 Bentham Science Publishers.

    AUTHOR KEYWORDS: Autoimmune diseases; HLA ligands; Immunogenetics; KIRs; Translational medicine; Viral infections

List of abbreviations

AIDS	Acquired immune deficiency syndrome
APC	Antigen-presenting cell
ART	Antiretroviral therapy
CASAC	Combined adjuvant for synergistic activation of cellular immunity
CCR5/7	C-C chemokine receptor 5/7
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-guanosine
DC	Dendritic cell
DNA	Deoxyribo nucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
γδ T	Gamma-delta T cell
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin-like receptors
LPS	Lipopolysaccharide
mDC	Myeloid DC
MedDiet	Mediterranean diet
MHC	Major histocompatibility complex
MPLA	Monophosphorylated lipid A
MyD88	Myeloid Differentiation factor 88
NFκB	Nuclear factor kappa B
NK cell	Natural killer cell
NKT	Natural killer T cell
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate-buffered saline
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid DC
PRR	Pattern recognition receptor
rhu	Recombinant human
RMF	RMFPNAPYL
T_C	Cytotoxic T cell
TCR	T cell receptor
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{EMRA}	Terminally differentiated effector T cell
T_H1	Type 1 T helper cell
T_H17	Type 17 T helper cell
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
T_N	Naïve T cell
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
YF	Yellow fever

1. Introduction: Immunosenescence

Individual wellness is closely linked to a functional immune system, able to recognize and eliminate pathogenic microorganisms, infected, and cancer cells. For this purpose, the two arms of the immune system, non-specific innate and antigen-specific adaptive, act in concert to maintain a pathogen-free internal environment, either by direct contact or via the vast amount of elements that compose their secretome (*Fulop et al., 2014*).

Innate immunity, consisting mainly of monocytes, neutrophils, natural killer (NK) cells, and dendritic cells (DCs), provides a first preventive defence against pathogens, able to react within seconds, but lack of specificity. Innate mechanisms include phagocytosis, secretion of chemokines and inflammatory cytokines, activation of the complement system, production of acute phase proteins, and recruitment of immune cells.

The adaptive immunity, represented by B and T lymphocytes, is antigen-specific, develops a memory for repeated challenges but takes several days or weeks to develop (*Parkin and Cohen, 2001*). T cells are responsible for the cellular immune response, while the B cells are responsible for the humoral immune response. Changes in this interactive network of cellular interactions can lead to a malfunction and/or dysregulation of the immune system, which ultimately have an impact on the health span of individuals.

Advancing age and the onset of infections with agents that establish latency may negatively affect on the ability of the immune system to mount an effective response against pathogens. Various age-related functional impairments have been reported in immune response, and they are commonly known as “immunosenescence”. This term was coined by Roy Walford over four decades ago (*Walford, 1969*). He was the first to correlate the normal process of ageing and age-related diseases with the decline in immune capacity, specifically in humoral and cell-mediated immunity functions. Since then, the concept has evolved and, nowadays, immunosenescence is considered as a highly dynamic, multifactorial and multistage process, consisting of several changes in immune responses, both losses and gains in immune function, and a low level of chronic-persistent inflammation (*Fulop et al., 2016*).

Several factors contribute towards this phenomenon, including thymic involution with age, which leads to reduced thymic output in terms of naïve T cell numbers, and the lifelong antigen-exposure during persistent viral infections, which oblige certain T cell clones to monoclonally expand repeatedly over a lifetime (*Brunner et al., 2011*). Bone marrow, the primary site of hematopoiesis, also undergoes structural and functional changes with age, being inefficient to maintain the homeostasis for the delivery of new cells, including B cell precursors. Reduction in the ability to respond to new antigens, alterations in the interaction of the innate and the adaptive immune response,

telomere shortening, impaired DNA repair, and antioxidant mechanisms as well as persistent antigenic stress have all been associated with immunosenescence (*Aiello et al., 2019; Weiskopf et al., 2009*). These hallmarks of immunosenescence are extensively interconnected, implying that improvement or deterioration of one particular hallmark may impinge on others.

The well-documented multitude of changes in composition and responsiveness of the immune system between young and older are an undeniable evidence of the role of the ageing process in immunosenescence. However, it should be taken into account that the immune ageing process and its impact on the individual is highly context-dependent, particularly depending on "immunobiography", that is the combination of type, intensity, duration and repetition of individual antigenic stimulations (*Franceschi et al., 2017*). The different immunological histories partially explain the differences in clinical associations with the measured immune parameters between individuals and between populations (*Pawelec, 2018*). This makes it difficult to identify what immunosenescence really is and raises the question of whether biomarkers of immunosenescence should also be contextualised.

An extraordinary example of the non-ineluctability of ageing is represented by centenarians, individuals who, despite reaching the extreme limits of life, maintain relatively good health, being able to perform their routine daily life and to escape or delay age-related diseases. A great part of current knowledge about the immunosenescent phenotype and the contribution of the immune system to the processes leading to longevity results from the study of centenarians, who are considered the best model of successful ageing. Supercentenarians, *i.e.* people aged 110 years or above, represent a more selected subgroup compared with centenarians, but they are extremely rare, so difficult to analyse.

In addition to environmental stressor factors, including chronic infections such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV), even when viral replication is controlled, genetic inter-individual differences represent another variability factor involved in the outcome of immunosenescence.

Therefore, any intervention aimed at "rejuvenating" the immune status in old people should be targeted and individualized, taking into account the immunobiography and/or genetic differences between individuals.

This chapter aims to shed light on how ageing and various types of persistent viral infections, especially CMV and HIV, influence the senescence of the immune system and highlight the contribution of genetics in immune function. Immunosenescence is likely to be a major cause contributing to reduced potency of current vaccines as well as vaccine failure in the senior population.

Therefore, a potential option for therapeutic intervention to improve vaccine efficiency in older adults will also be explored.

1.1 Influence of advancing age on immune responses

Ageing is a complex, cumulative and progressive phenomenon, resulting from genetic, epigenetic, and environmental events interacting throughout life and characterized by changes in structure and decline in function of multiple cells, tissues and organs, including the immune system. Ageing is considered the most important risk factor for the development of many age-related chronic diseases, including cardiovascular diseases, cancer, neurodegenerative diseases, and diabetes, which shorten the lifespan and the healthspan (*Fulop et al., 2016*). Age-related alterations have been documented in both innate and adaptive arms of the immune system, where some immune responses are diminished, leaving others unchanged or exacerbated. However, innate immunity seems to be better preserved, while more severe age-dependent changes occur in the adaptive immune system.

Either they are a direct consequence of lifelong antigen exposure or a reflection of the progressive adaptation and compensation of the individual to environmental *stimuli*, overall, these alterations are implicated in the increased frequency and severity of infections, cancer, cardiovascular and neurodegenerative diseases, and lowered responses to vaccination in the old people.

1.1.1 Natural Killer cells

Natural killer (NK) cells are large granular lymphocytes, originally identified based on their spontaneous ability to lyse tumor cells without prior sensitization (hence their name) (*Kiessling et al., 1975; Herbeman et al., 1975*). Defined as sentinels of innate immunity, NK cells play an important role in the host's first line of defence against viral and tumor targets, providing immune surveillance and resistance to infection. Evidence of the critical role of NK cells in limiting viral infection prior to the induction of adaptive immune responses has been provided by clinical studies of individuals who are deficient in NK cells and/or their functions, leading to current infections by viral pathogens (*Orange, 2013*). NK cells have been shown to contribute to the control of CMV, hepatitis B virus (HBV), and HIV infections.

Consistent with their role in immune surveillance, NK cells are widespread throughout lymphoid and non-lymphoid tissues, but constitute just the 10-15% of circulating lymphocytes of healthy adults.

Distinct NK cell subsets have been defined in humans based on phenotypic and functional features. Specifically, NK cells are classically identified by expression of the cell-surface marker CD56 (also known as neural cell adhesion molecule) and absence of the lineage marker CD3. Based on their expression levels of the cell-surface markers CD56 and CD16 (also known as FcγRIIIA, low-affinity receptor for the Fc portion of immunoglobulin G), they can be also divided into two subsets. The

$CD56^{lo}CD16^{+}$ subset constitutes 90% of peripheral-blood NK cells and is mainly responsible for natural cytotoxicity by releasing cytoplasmic granules containing pore-forming protein perforin and serine protease granzymes B that lead to infected cell apoptosis by caspase-dependent and -independent pathways (*Paul and Lal, 2017*). By contrast, the $CD56^{hi}CD16^{-}$ subset constitutes less than 10% of circulating NK cells, shows minimal cytotoxic activity and is described as having a rather supporting role by predominantly secreting chemokines and cytokines. Among cytokines secreted by NK cells there are interferon (IFN)-γ, with both antiviral and immune enhancing capabilities, tumor necrosis factor (TNF)-α and granulocyte/macrophage colony-stimulating factor (GM-CSF), that can modulate the function of other innate and adaptive immune cells (*Paul and Lal, 2017; Rühle et al., 2016*). $CD56^{hi}CD16^{-}$ cells are particularly abundant in lymph nodes where they can differentiate to $CD56^{lo}CD16^{+}$ in the presence of inflammatory *stimuli* (*Solana et al., 2014*).

NK cell cytotoxic and secretory functions are tightly regulated by the balance of activating and inhibitory signals from an arsenal of membrane receptors, including killer cell immunoglobulin-like receptors (KIRs), randomly generated during NK cell differentiation and maturation (*Vidal et al., 2011*). Each NK cell, therefore, expresses its own repertoire of both inhibitory and activating receptors and the integration of their signals dictates the activation of NK cells as shown in Figure 1.1.

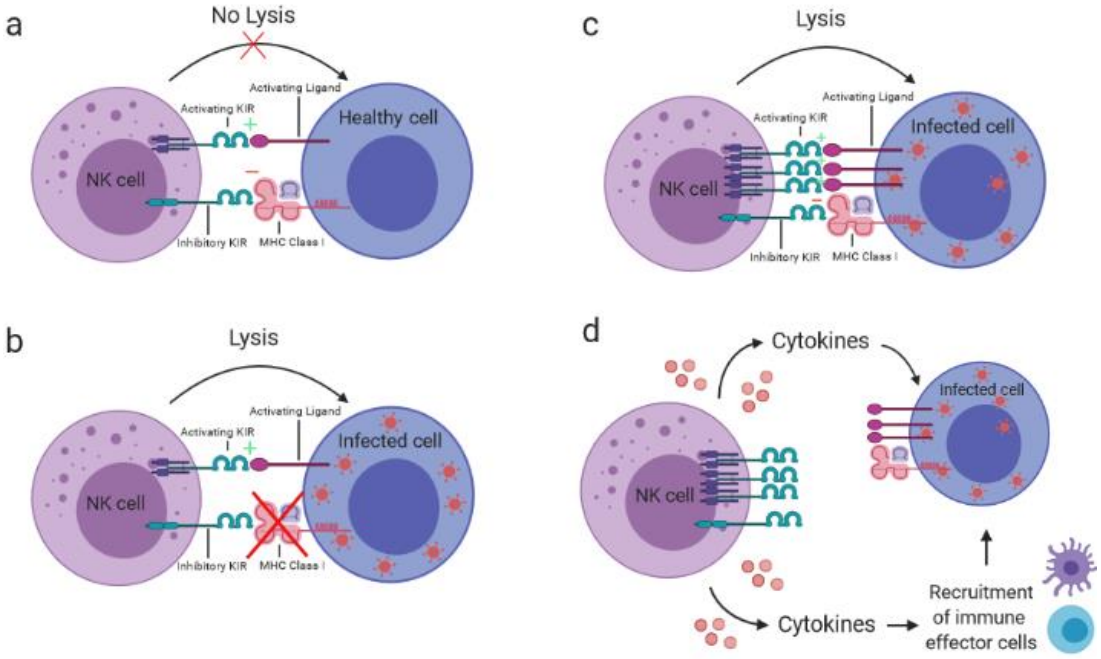

Figure 1.1. Natural killer cell recognition and killing of target cell. The nature of NK cell responses results from an integration of positive and negative signals received from multiple receptors, with a balance towards inhibition under normal conditions (**a**) but a shift to activation under conditions of infection and elimination of target cells directly through cell-mediated cytotoxicity mechanisms (**b,c**) or indirectly through secretion of pro-inflammatory cytokines (**d**). (Created with BioRender.com)

Age-associated changes in NK cell count, phenotype, and functions are directly attributed to the risk of several diseases and infections (*Bulut et al., 2020*).

With ageing, an increase in $CD3^{-}CD56^{+}$ NK cell frequency and absolute cell number has been observed. At the subset level, ageing is associated with an impaired $CD56^{hi}$/ $CD56^{lo}$ *ratio*, in particular with a decreasing fraction and cytokines production of $CD56^{hi}$ NK cell subset, probably due to limited production of its precursors, and with an expansion of cytotoxic $CD56^{lo}$ NK cells (*Solana et al., 2014; Gounder et al., 2018*). At the single-cell level, $CD56^{lo}$ NK cells have shown a lower lytic capacity with age, but their greater number compensates for this decrease. It has also been observed a decreased expression of cytotoxicity activating receptors with ageing, while there are discrepancies in the expression levels of NK inhibitory receptors (*Pera et al., 2015*). Although this remodelling of NK cell subsets may be a consequence of a compensation mechanism to reduce cellular cytotoxicity, it cannot be excluded that the decline of $CD56^{hi}$ NK cells may also contribute to the development of immunosenescence, since cytokines produced by this population are important for the activation of DCs and to promote inflammation interacting with monocytes (*Le Garff-Tavernier et al., 2010; Solana et al., 2012*). For example, activated NK cells secrete TNF-α, interleukin (IL)-8 and IFN-γ that amplify on-going innate immune response and influence the early phases of the adaptive immune response. The reduced secretion of these immune-modulatory cytokines can lead to a decrease NK cell-mediated enhancement of macrophage function, with consequent reduced anti-microbial immunity and increased susceptibility to intracellular pathogens, and a reduced NK-DC crosstalk, with impaired DC maturation, T cell polarization and reduced efficacy of vaccination. Simultaneously, the decreased cytotoxic activity of NK cells observed with ageing leads to an impaired killing of infected cells, with increased incidence/severity of infections (*Tariq et al., 2017*).

An innate T lymphocyte population, defined as $CD3^{+}CD56^{+}$, is named NKT cells. These cells are mostly $CD8^{+}$, expressing T-cell receptor (TCR)-$\alpha\beta^{+}$ that recognise peptides bound to classic HLA class I molecules and represent 1–11% of the peripheral T cell pool (*Almehmadi et al., 2014*). These cells show both cytotoxic and producing cytokines activities and have a significant role in the immune response against cancer (*Mocchegiani et al., 2009; Hassouneh et al., 2016*). Similarly to NK, NKT cell levels have been reported to increase significantly with age in peripheral blood of healthy subjects (*Almehmadi et al., 2014).*

1.1.2 Dendritic cells

Dendritic cells (DCs) are the most effective antigen presenting cells (APCs), specialized to uptake, process and present antigen to T cells. In their immature stage, DCs are ubiquitously distributed in

peripheral tissues with a role as sentinels characterized by high phagocytic capacity and low expression levels of major histocompatibility complex (MHC) and costimulatory molecules.

Upon stimulation with microbial products, inflammatory cytokines or CD40 binding, DCs engage a maturation program into fully functional APC, characterized by several morphological, phenotypical and functional changes, with downregulation of the phagocytic capacity and upregulation of costimulatory molecules, MHC and secretion of cytokines (*Agrawal and Gupta, 2011*). Mature DCs migrate to lymphoid tissues where they present processed antigens in their surface expressed MHC molecules to naïve T lymphocytes, generating an antigen-specific response (Figure 1.2).

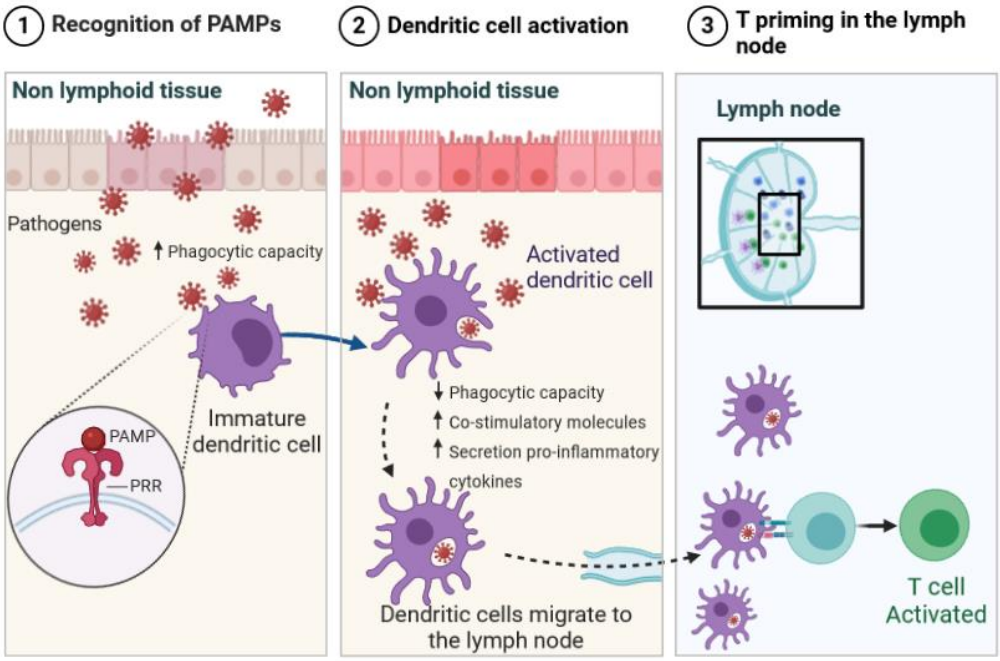

Figure 1.2. Antigen uptake, maturation and migration of dendritic cells to activate T cells. (**1**) After encounter with pathogens, specific PAMPs are recognized by PRRs of immature DCs, characterized by high phagocytic capacity. (**2**) After internalization of pathogen, immature DCs undergo the maturation process, which includes a decrease in antigen-capture activity, an increase in MHC and co-stimulatory molecule expression and migration into lymph nodes. (**3**) After the maturation process, mature DCs are capable of antigen presentation to naïve T cells, involving the presentation of processed peptides to T cells via MHC-TCR interaction, that lead to activation of T cell. (Created with BioRender.com)

With truly ingenious insight, Charles A. Janeway, Jr. predicted in 1989 that innate immune recognition is based on nonclonal, germline-encoded receptors, which he termed pattern recognition receptors (PRRs) (*Medzhitov, 2013*). These receptors were proposed to detect conserved microbial components, called pathogen-associated molecular patterns (PAMPs). Different PRRs react with specific ligands and lead to distinct anti-pathogen responses. Among them, Toll-like receptors (TLRs) play an important role in DC activation being capable of sensing a wide range of organisms, from bacteria to fungi, protozoa, and viruses (*Uematsu, 2008*). TLRs are type-I transmembrane

glycoproteins composed of extracellular, transmembrane and intracellular signalling domains that received their name from their similarity to the protein encoded by the toll genes, initially identified in *Drosophila melanogaster*. The extracellular domains have repeated leucine-rich repeat (LRR) motifs, which are typically 22–29 residues in length, responsible for binding to PAMPs (*Botos et al., 2011*). Variations in the LRR motifs of the different TLRs are thought to give specificity to the ligands they sense. The specific ligand-TLR interaction induces the dimerization of these receptors, activating downstream signalling events. The intracellular signalling domain is known as Toll/IL-1 Receptor (TIR) domain because shares homology with the signalling domain of IL-1R family members and is recognized by specific adaptor molecules also contain TIR domains which activate a specific signalling cascade (*O'Neill et al., 2007*).

In humans, the TLR family consists of 10 members expressed on the cell surface (TLR1, 2, 4, 5, 6, 10), binding lipids and proteins, or in endosomal compartments (TLR3, 7, 8, 9), responsible for the recognition of bacterial and viral nucleic acids generated through the autophagy process (*Chen et al., 2016*). Most of the TLRs are well characterised. However, the ligand and precise function of TLR10 remains in question (*Fore et al., 2020*). Examples of PAMPs include lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria for TLR4; imidazoquinoline compounds, such as R848, for TLR7/8; and CpG motif of bacterial and viral DNA for TLR9 (Table 1).

DCs are usually classified into myeloid DCs (mDCs), the primary source of IL-12, which is needed to drive a T helper type 1 (T_H1) response, and plasmacytoid DCs (pDCs), that produce high amounts of IFN-α/β, critical for antiviral responses (*Collin et al., 2013*). DC subsets show different patterns of expression of TLRs with different effects on activation (Table 1). For example, pDCs, specialized to respond to viral infection, highly express TLR7 and TLR9, the sensors for viral RNA and DNA, respectively. There are also differences in the type of pathway activated and the corresponding adapter molecule recruited. TLR signal transduction can be essentially divided into TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway and myeloid differentiation primary response 88 (MyD88)-dependent pathway. Specifically, recognition of PAMPs by all TLRs, except for TLR3, stimulates the recruitment of a set of intracellular adaptors containing TIR domains, including TIRAP and MyD88, leading to the activation of MAP kinases and the transcription factor NF-κB, to control the expression of inflammatory cytokine genes. The cytokines include IL-12 that promotes T_H1 response and NK cell activation, IL-10 with potent anti-inflammatory properties, the multifunctional TNF-α, and IL-1, IL-6, and IL-23 that promote T_H17 cell differentiation or proliferation (*Mills, 2011*). The activation of TLR7, 8, and 9, induce the MyD88-dependent signalling pathway, leading to the production of IFNs. TLR3 directly, and TLR4 through TRAM, recruit an adapter molecule TRIF, activating an alternative pathway (TRIF-

dependent pathway) that culminates in the activation of the transcription factor interferon-regulatory factor (IRF)-3, pivotal for the induction of type I IFNs (Figure 1.3) (*Kawai and Akira, 2007; Kawasaki and Kawai, 2014; Schreibelt et al., 2010*). TLR4 is the only TLR able to activate both MyD88- and TRIF-pathways. The signalling pathways associated with the activation of TLRs are therefore different with distinct biological responses. In this way, DCs are able to modulate the innate response based on the type of pathogen.

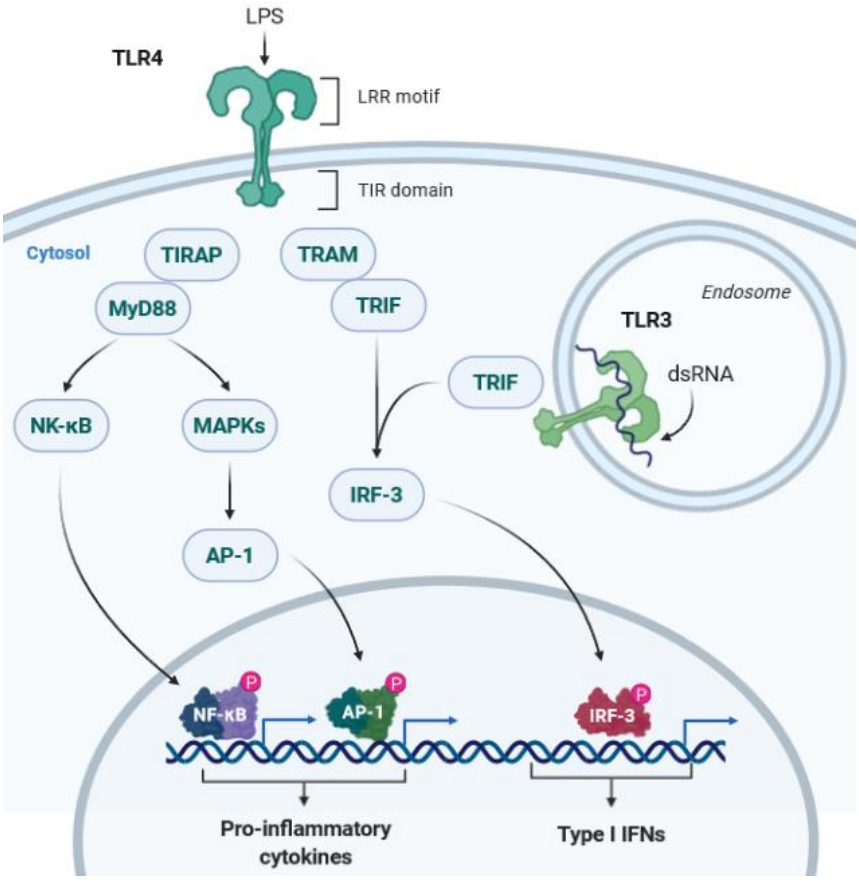

Figure 1.3. TLR signaling pathways. Schematic representation of TLR structure and the MyD88-dependent and TRIF-dependent pathway. (Created with BioRender.com)

Table 1. TLR expression and function on DC subtypes.

TLR	Location	DC subset	PAMPs recognized	Effector cytokines induced	References
TLR1	Cell surface	mDC, pDC	Peptidoglycan, lipopeptides	IL-6, IL-10, IL-12p70, TNF-α	(Schreibelt et al., 2010; Vidya et al., 2018)
TLR2	Cell surface	mDC	Peptidoglycan, haemagglutinin, ecc.	IL-6, IL-10, IL-12p70, TNF-α	(Mifsud et al., 2014)

TLR3	Endosome	mDC	dsRNA virus	IFN-α (intermediate), IL-12p70 (high)	(Mifsud et al., 2014)
TLR4	Cell surface	mDC	LPS, envelope proteins	IL-6, IL-10, IL-12p70	(Mifsud et al., 2014)
TLR5	Cell surface	mDC	Flagellin (flagellated bacteria)	TNF-α, IL-8	(Mifsud et al., 2014)
TLR6	Cell surface	mDC	Diacyl lipopeptides (mycoplasma), ecc	IL-6, IL-10, IL-12p70, TNF-α	(Mifsud et al., 2014)
TLR7	Endosome	mDC, pDC	Imidazoquinoline compounds, ssRNA	IL-12p70, type I IFNs	(Hornung et al., 2002; Mifsud et al., 2014; Kawasaki and Kawai, 2014)
TLR8	Endosome	mDC	Imidazoquinoline compounds, ssRNA	IL-12p70, type I IFNs	(Kawasaki and Kawai, 2014)
TLR9	Endosome	pDC	dsDNA viruses, CpG motifs from bacteria and viruses	IL-6, type I IFNs	(Hornung et al., 2002; Mifsud et al., 2014; Kawasaki and Kawai, 2014)
TLR10	Cell surface	Unknown	Uropathogenic bacteria, profillin-like molecule	Unknown	(Fore et al., 2020)

CpG, cytosine-phosphate-guanosine; dsDNA, double-stranded DNA; IFNs, interferons; IL, interleukin; LPS, lipopolysaccharide; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; ssRNA, single-stranded RNA; TLR, toll-like receptor; TNF, tumor necrosis factor.

There is controversial information concerning the effects of ageing on human DC activity and cytokine production. Several reports have demonstrated a numerical and functional decline especially on pDC with age, supporting the idea that DCs from aged individuals are less effective at activating T cells (*Grolleau-Julius et al., 2008; Orsini et al., 2012; You et al., 2013; Stervbo et al., 2015*).

However, other reports have shown a decline with age in the number of mDC, but not of pDC (*Della Bella et al., 2007; Agrawal et al., 2007*). In addition, a different expression and an age-related impairment function of some TLRs in older people, leading to altered TLR-mediated immune responses, has been observed. For example, TLR3 and TLR8 expression in mDCs and TLR7 and TLR9 expression in pDCs seem to be declined with increasing age (*Jing et al., 2009; Panda et al., 2010*). Several authors have reported an age-related reduction in IFN-γ and IL-6 production by pDCs obtained from older adults, after engagement of TLR7 or TLR9. These altered TLR-induced cytokine production has been attributed to an age-related decline in TLR expression and impaired intracellular signalling (*Agrawal and Gupta, 2011*). Instead, TLR2 and TLR4 surface expression in mDCs appears to be unchanged in healthy older with studies reporting increased, decreased or comparable production of TNF-α, IL-6, and IL-12 by aged mDCs in response to TLR stimulation (*Tariq et al., 2017; Shaw, 2019*). These differences probably reflect differences in assay system and experimental conditions and the mechanisms underlying these alterations remain incompletely understood. Age-

associated chronic inflammation and CMV reactivation throughout life have been considered as potential causes of age-related alterations in TLR functions. However, a close correlation between these factors has not yet been demonstrated (*Shaw, 2019*).

Either due to a reduction in their frequency or due to reduced expression of TLRs on mDC and pDC subsets, the antigen uptake, migratory capacity and response of DC to infections appear to be compromised with age, contributing directly or indirectly to the impaired ability of older people to respond to vaccination (*Crooke et al., 2019; Fuentes et al., 2017*).

In this context, due to their powerful immunostimulatory properties, TLR agonists are considered a promising strategy to increase vaccine efficiency in older individuals, inducing better DC activation and their production of cytokines (*Zareian et al., 2019*).

1.1.3 T lymphocytes

Unlike the innate immunity that is not specific to any pathogen, elements of the adaptive immune system respond to challenges with a high degree of specificity and memory. Both B and T cell compartments are affected by ageing but the T cell compartment undergoes the most substantial changes with age.

T cells comprise around 70% of the lymphocytes in human blood and can be divided into $CD4^+$ T cells (T helper or T_H), predominantly assisting other leukocytes in immunologic processes, and $CD8^+$ T cells (T cytotoxic or T_C), which the main function is to recognize and destroy infected or cancer cells.

T_H cells are the professional cytokine-producing cells, classified based on their cytokine profiles in T_H1, preferentially producing IFN-γ, and T_H2 cell clones that mainly produce IL-4 and IL-5. A third $CD4^+$ T cell population, namely T_H17, preferentially produces IL-17, an important cytokine for protective immunity against extracellular and intracellular pathogens (*Zhu, 2018*). $CD8^+$ T cells play a critical role in the clearance of intracellular pathogens by releasing cytotoxic molecules, such as granzymes and perforin, and to secrete cytokines, such as IFN-γ and TNF-α that further accelerate the innate and adaptive immune response against intracellular pathogens (*Mittrücker et al., 2014*).

$CD4^+$ and $CD8^+$ T cells can be classified into naïve T cells and antigen-experienced memory or effector T cells according to differential expression of surface molecules CD45RA, that plays a role in T-cell receptor (TCR) signal transduction, and the lymphoid homing receptor C-C chemokine receptor 7 (CCR7) (*Mahnke et al., 2013*).

Naïve T cells are generated in the thymus, the central organ of T-cell generation where each cell undergoes DNA rearrangement to generate a unique TCR, and migrates to the periphery, where they

continuously recirculate between secondary lymphoid organs and blood by expressing CCR7 (*van den Broek et al., 2018*). Functionally, T cells are considered naïve (T_N) until they encounter their cognate antigen in the periphery and they are $CD45RA^+CCR7^+$. In response to interacting with their cognate antigens in the context of HLA class I ($CD8^+$) and II ($CD4^+$) and co-stimulated by an APC (*e.g.*, DC), naïve T cells undergo rapid and robust proliferation and differentiate into different types of effector and memory cells (*Kaech and Cui, 2012; Mahnke et al., 2013*):

- $CD45RA^-CCR7^+$ Central Memory T cells (T_{CM}), which traffic to lymphoid tissues and produce IL-2. T_{CM} express the lymph node homing receptors CCR7 and have a high proliferative capacity but exhibit low cytotoxicity.
- $CD45RA^-CCR7^-$ Effector Memory T cells (T_{EM}), which can migrate to peripheral tissue sites and produce effector cytokines. $CD4^+$ T_{EM} cells are immediate producers of cytokines, such as IFN-γ, and $CD8^+$ T_{EM} cells are immediate producers of cytotoxic proteins.
- $CD45RA^+CCR7^-$ Terminally differentiated Effector Memory (T_{EMRA}), more frequently in the $CD8^+$ compartment. They generally display the shortest telomeres among T cells, express markers of senescence, and have low proliferative and functional capacity, indicating terminal differentiation.

After clearance of the pathogen, 90–95% of these effector $CD8^+$ T cells undergo apoptosis, but a small subset further differentiates into long-lived and functional memory cells that provide long-term protective immunity (*Ahn et al., 2018*).

The major age-associated differences are seen in the $CD8^+$ subset. In particular, the most commonly reported hallmarks of immunosenescence are a decreased absolute numbers and percentages of peripheral naïve $CD8^+$ T cells, a shrinkage of the T cell repertoire, higher numbers and proportions of memory T cells and a reversal CD4/CD8 T cell *ratio*.

Thymic involution is a developmentally programmed event in most mammals, occurring around puberty, characterized by a reduction in the overall size of the organ and a replacement by adipose tissue. A decrease in thymopoiesis and a progressive involution of the thymus has been demonstrated to be the reason for naïve T cell decline during ageing (*Ponnappan and Ponnappan, 2011*). The homeostatic peripheral proliferation of naïve T cells is thought to compensate for this loss but can lead to the outgrowth of certain T cell clones at the expense of others. It is universally reported that ageing leads to a decrease in naïve T cell populations, rendering the older highly susceptible to pathogens to which they have not been previously exposed but this condition is not *per se* predictive of early mortality.

Several studies have also shown an expansion of the memory T cell pool in old individuals. The shift of the T cell profile from being a "naïve profile", with less differentiated T cells, to an

"experienced profile", with more differentiated T cells, observed with ageing it is probably due to chronic or persistent infections, most commonly with CMV, which cause specific T cells to clonally expand through repetitive stimulation (*Pawelec, 2017*). It has been described that the highly differentiated effector-memory $CD8^+CD28^-$ T cells increased with age often consist of CMV-specific oligoclonal cells, supporting a relevant role of latent CMV infection in T cell immunosenescence (*Pera et al., 201*5). It is also important to consider that infections with different pathogens represent different settings in T cell subsets. Whether these accumulations of late-differentiated memory T cells contribute to frailty and mortality or are only adaptive responses to the persistent virus remains controversial (*Müller et al., 2017*). Concomitantly, the diversity of the naïve T cell repertoire tends to be lower in old individuals, critically affecting primary immune responses against pathogens (*Britanova et al., 2014; Britanova et al., 2016*).

Therefore, an immunosenescent profile is caused by a series of events that can occur independently but also converge in the achievement of physiological and immune ageing. As a result, old individuals are particularly susceptible to infections of newly arising pathogens, show impaired immunity to vaccination and increased development of age-related diseases. This impaired ability to mount an efficient immune response after exposure to pathogens or vaccines makes it necessary to develop targeted strategies to improve vaccine efficacy in older people, thus promoting more successful ageing.

1.2. Immunosenescence: the centenarian perspective

Centenarians, *i.e.*, subjects who have reached ten or more decades of life, escaping the common age-related diseases, are commonly considered the best model to study successful ageing and longevity *(Aiello et al., 2019*). The concept of successful ageing is multidimensional, encompassing domains of physical, functional, social, and psychological health. Overall, it is described as a condition characterised by avoiding illness and disability, having high cognitive, mental and physical functions, and being psychologically well adapted in later life (*Urtamo et al., 2019*).

Centenarians are able to repair damages and respond well to stressors. That is due to a combination of "positive features", *i.e*. intrinsic (genetic), extrinsic (environmental), and stochastic factors. In fact, centenarians exhibit peculiar immunologic characteristics, which contribute to their longevity:

- Presence of a well-preserved number of both $CD4^+$ and $CD8^+$ naïve T cells, despite the thymic involution (*Cossarizza et al., 1997*);
- Well-preserved NK cell number (especially $CD56^{dim}CD16^+$), comparable to those observed in young (*Caruso and Vasto, 2016; Aiello et al., 2019*);

- Satisfactory number of NKT cells with good cytotoxicity and INF-γ production activity (*Mocchegiani and Malavolta, 2004*).

A very interesting study has evaluated immune cell function in a cohort of centenarians after hospital admission. It was demonstrated that those centenarians who survived showed a better immune function, that includes higher NK cytotoxic activity, and higher lymphoproliferation in response to the mitogen PHA at the time of hospital admission, and higher IL-1β and IL-6 cytokine release by mononuclear cells after stimulation compared to centenarians whose did not survive (*Martínez De Toda et al., 2020*). According to these results, another previous study has shown that healthy centenarians produce higher IL-1β and TNF-α levels after stimulation compared to unhealthy ones (*Miyaji et al., 2000*).

Thus, centenarians represent a selected population, living 20–30 years longer than members of the same birth cohort despite being exposed to the same environmental conditions. Nevertheless, the sharp increase in life expectancy, coupled with the improvement in public health, improvements in diet and reduced exposure to infection and inflammation, has led to a strong increase in their global number during the 20th century. Although centenarians are becoming more common, they are exceptional subjects and, as such, are the central focus of research of "positive biology", with the aim to provide valuable information on the lifestyle to achieve healthy ageing (*Caruso et al., 2012*). However, supercentenarians are more extremely selected individuals and, therefore, extremely rare. This privileged group of individuals may represent the real extreme limit of ageing.

1.3. Immunosenescence: influence of chronic viral infections on immune responses

An important driver of remodelling of the innate and adaptive immune system over the lifespan is chronic immune activation by different pathogens.

Most primary viral infections are characterized by an acute phase during which new viral particles are produced in infected cells. Immune responses are mobilized to control viral replication and eliminate the pathogen. In the very early events of viral infection, there is a trigger of the innate immune response to virus with production of IFNs and the activation of cytolytic and immune regulatory mechanisms of NK cells. The following generation of virus specific cytotoxic T cells and B cells producing antibodies against the pathogen are part of an adaptive response that ultimately clears the virus. However, several viruses have developed strategies to avoid clearance by the immune system after primary infection (*Brunner et al., 2011).* Consequently, the virus is able to persist in the human host at low levels of viral replication, establishing a persistent infection that may be additive to age-dependent immune dysregulation, with serious impacts on health and longevity.

HIV can establish latency in resting $CD4^+$ T cells in people receiving antiretroviral therapy, but leads to several immunological changes that accelerate the ageing process, predisposing HIV-infected individuals to comorbidity linked to immunosenescence.

Latent CMV infection is usually asymptomatic in healthy adults but is able to lead to functional alterations of the immune system and can be a powerful and fatal pathogen in immunosuppressed people.

Several other viruses such as HBV are able to establish chronic infections in a proportion of infected persons, with adverse consequences as cirrhosis and eventually hepatocellular carcinoma in the case of HBV, whereas others eliminate the virus after primary infection.

1.3.1 Human Immunodeficiency Virus

HIV infection is one of the main causes of morbidity and mortality worldwide, with 38 million people living with HIV in 2019, whose 1.7 million newly infected and almost 700.000 HIV-related deaths. Untreated HIV replication causes a wide range of immunological alterations, leading to an increased risk of onset of opportunistic infections (*World Health Organization – HIV/AIDS*).

HIV is a member of the *Lentivirus* genus of the *Retroviridae* family and as such its infections show a chronic course of the disease, with a long period of clinical latency, and persistent viral replication (*Fanales-Belasio et al., 2010*). In the primary infection that typically occurs in the mucosa, HIV infects both innate and adaptive immune cells, including DCs and resident memory $CD4^+$ T cells, by recognition and binding of the viral envelope glycoprotein gp120 with the glycoprotein CD4 and the co-receptor CCR5 expressed on these cell surfaces. Following membrane fusion, HIV single-strand RNA is reverse transcribed into HIV DNA, which is integrated into the host DNA. The integrated viral DNA may then lie dormant, establishing lifelong reservoirs of HIV, or transcribed into a messenger RNA that is translated to produce new virus particles (*Fanales-Belasio et al., 2010).* Infected cells rapidly spread to lymph nodes, where the viral replication is responsible for the increase of viremia, reaching the viremic peak characteristic of acute infection, and the gradual decrease in $CD4^+$ T cell count (Figure 1.4).

In the absence of antiretroviral therapy (ART), plasma viremia typically peaks a few weeks later post-exposure, when HIV antibody levels become detectable (seroconversion). The immune system then achieves some degree of control, leading to a decline of viremia before reaching a viral set point, in which the level of HIV replication remains relatively stable (clinical latency), often for years. The level of the viral set point reflects the complex virus–host interactions and is an important determinant of the rate of disease progression in HIV-infected individuals who are not treated with ART. If left

untreated, HIV infection can induce damage of the thymus, primary lymphoid organ supplying new lymphocytes to the periphery, through thymocytes self-killing or disruption of the thymic stromal architecture, resulting in defective thymopoiesis and apoptosis of $CD4^+$ T cells (*Yep et al., 2004).* The progressive loss of $CD4^+$ T cells causes profound immunodeficiency that develops, more or less rapidly, in acquired immune deficiency syndrome (AIDS) (*Moir et al., 2011*). The normal absolute $CD4^+$ T cell count in adolescents and adults ranges from 500 to 1500 cells per mm^3 of blood. A person with HIV is considered to have progressed to AIDS when the number of their $CD4^+$ T cells falls below 200 cells/mm^3 and they develop one or more opportunistic infections regardless of their $CD4^+$ T cell count (*World Health Organization – case definitions of HIV*).

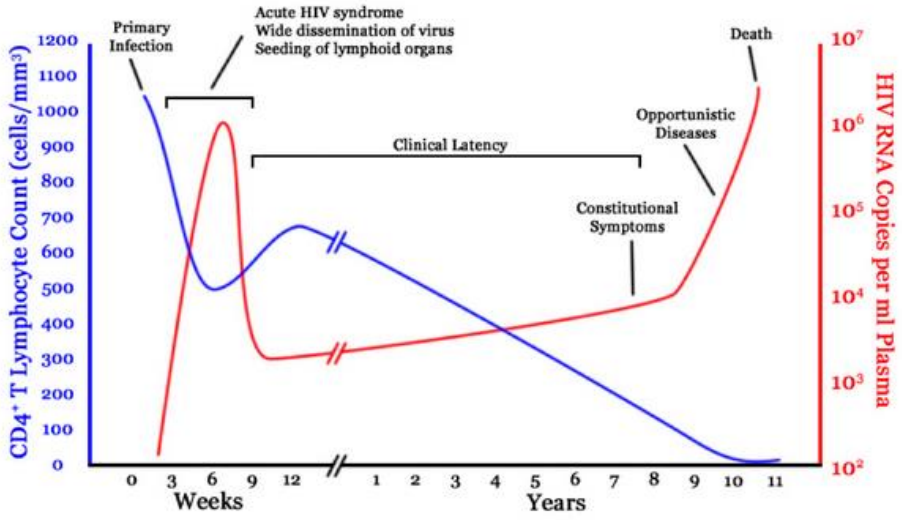

Figure 1.4. Natural history of HIV infection.

ART consists of the combination of at least three antiretroviral drugs and, when used appropriately, is highly effective, improving immune function and greatly reducing the risk of developing AIDS. However, ART is not curative and treated patients do not have completely restored health but increase the risk of developing non-AIDS complications, many of which are commonly associated with ageing (*Deeks and Phillips, 2009*).

For example, constant immune activation in response to the persistent HIV antigen leads to differentiation and accumulation of senescent $CD8^+$ T cells, with loss of costimulatory molecules CD28 and CD27, low CD4/CD8 *ratio* and release of inflammatory mediators by innate and adaptive activated cells that results in accelerated ageing in HIV disease (*Appay et al., 2019; Deeks, 2011; Desai and Landay, 2010*).

During the acute phase of infection, there is an activation of the cytotoxic compartment, as measured by the expression of CD38 and Ki-67, markers of activation and proliferation respectively. As the infection becomes more chronic, these activated T cells tend to decrease in number, with a

concomitant decrease in their cytotoxic activity, being replaced by the senescent $CD28^-$ T cells. Moreover, analysis of telomere length in HIV-infected individuals showed that $CD8^+CD28^-$ T cells from 40-year-old HIV positive persons have identical telomere length to that of centenarians, suggesting that patients' cytotoxic T cells that have undergone considerable proliferation resemble those that are found in healthy older adults (*Fülöp et al., 2017*).

All these events are directly related to the development of long-term HIV-associated complications and death.

Although HIV-specific adaptive response, especially cytotoxic T lymphocyte response, play a central role in the control of viral replication, several reports suggest that the earliest viral and immunological events occurring during the primary infection have an important impact on the course of HIV disease progression, highlighting the essential role of the innate immune system in controlling the infection (*Alter and Altfeld, 2009; Carrington and Alter, 2012*). In particular, a pivotal role has been observed for NK cells in antiviral containment during the early phases of HIV infection, while the adaptive immune response is developing. NK cells can contribute to the host immune response to HIV infection through cytotoxic and secreting chemokines and cytokines mechanism. After activation, NK cells release large amounts of cytokines and chemokines that induce inflammatory responses. Among these, CC-chemokines have been shown to inhibit CCR5-dependent entry of HIV to target cells, suppressing HIV replication *in vitro* (*Fauci et al., 2005*). In addition, HIV is known to cause downregulation of HLA-A and HLA-B expression in infected cells, which results in the suboptimal presentation of HIV peptides to cytotoxic T cells. However, the expression of HLA-C and HLA-E molecules is preserved, making these HIV-infected cells susceptible to cell-mediated lysis by NK cell expressing HLA-C-specific KIRs (*Fauci et al., 2005*). Moreover, several studies have described the influence of specific KIR-HLA combinations on the control of HIV viremia and disease progression, with differences depending on the population considered, confirming the strong contribution of the host's genetic factors and KIRs expression on NK cells in HIV progression.

1.3.2 Citomegalovirus

CMV is a wide-spread virus, reaching a prevalence of 100% in developing countries, and approximately 80% in industrialized societies, depending on subject age and socioeconomic status, with a higher prevalence in aged people (*Dowd et al., 2009*).

CMV is a double-stranded DNA virus belonging to the *Herpesviridae* family primarily residing in the myeloid cell compartment but known also to spread to other cell types, such as fibroblasts and smooth muscle cells. Its genome is of about 235 kb with *ca*. 200 genes, the largest genome of all viruses that infect humans. Transmission of the virus occurs through exposure to infectious body

fluids, including breast milk and blood. Primary CMV infection usually has an asymptomatic or subclinical course in immunocompetent hosts, after which the virus establishes lifelong persistence in cells of the myeloid lineage, but causes significant morbidity and mortality in immunosuppressed or immunosenescent individuals (*Gupta and Shorman, 2020*). The initial infection with CMV strongly activates both the innate and adaptive immune system in order to limit the replication of the virus. Specialized PRRs expressed on the surface of DCs and macrophages recognize viral glycoproteins or the viral genome, detecting the foreign nature of the virus very early after contact. These events lead to a release of IFNs and other cytokines and to adaptive immune system activation. Both T cells and antibodies specific for CMV seem to remain present throughout life (*Müller et al., 2017*).

Additionally, NK cells with their specific cytokine secretion and cytotoxic activity play a crucial role in CMV surveillance. Due to complex strategies of immune evasion, CMV establishes a latent state of infection that can be accompanied by recurrent episodes of viral reactivation, mostly in immunosuppressed or immunocompromised patients (Figure 1.5). For example, several CMV genes modulate the presentation of CMV peptides to T cells, inhibiting the loading of peptides onto HLA complexes or causing a dislocation and degradation of class I molecules into the cytosol. Changes in cell surface levels of HLA class I are sensed by NK cells through their receptors, including KIRs, rendering infected cells more susceptible to their attack (*Lin et al., 2007*).

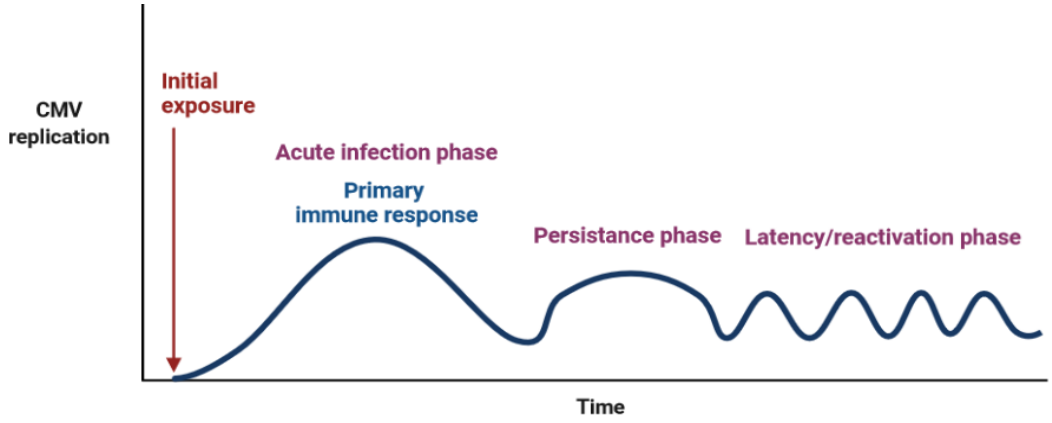

Figure 1.5. CMV infection phases in healthy individuals.

The cause-effect relationship between CMV and immunosenescence has been long studied but is not clear whether the infection causes long-term beneficial or deleterious immunological effects. Whether this is an adaptive or pathological phenomenon, it is clear that persistent CMV infection results in chronic stimulation of $CD8^+$ T cells, which expand clonally showing a late-stage differentiated effector memory phenotype that, after proper activation *stimuli*, can secrete cytokines,

and execute cytolysis. The absolute increase in memory T cells, called memory inflation, is observed only in CMV-seropositive older, demonstrating that ageing and CMV exert both distinct and joint influence upon blood T cell homeostasis (*Wertheimer et al. 2014*). This gradual increase in CMV-specific late-stage differentiated $CD8^+$ T cell population is weakly associated with early mortality and, in association with a decline of naïve $CD8^+$ T cells, with vaccine unresponsiveness (*Müller et al., 2017*).

However, other authors report that CMV infection enhances the immune response to influenza vaccination and to other pathogens, increasing antibody response, circulating levels of IFN-γ, and $CD8^+$ T cell sensitivity compared to uninfected individuals, and that accumulation of these CMV-specific effector memory cells may rather provide a survival advantage in the older population (*Derhovanessian et al., 2013; Pera et al., 2014; Furman et al., 2015*). These different effects of CMV infection in older may depend upon intra-individual factors, including health status and genetic background, and/or inter-individual factors in different human populations.

NK cells are key regulators of early CMV control. Individuals lacking NK cells and/or their functions suffer severe diseases and even death following infection (*Orange, 2012*). Since antiviral properties of NK cells are induced through interaction between NK cell receptors, including KIRs, and their ligands, it is not surprising that different KIR-HLA combinations are associated with different outcomes of CMV infection.

1.4. Role of genetic factors: focus on KIR and HLA

The different clinical outcome of CMV and of HIV suggests that, in addition to environmental factors, the genetic background is important for the development or the clinical course of these infectious diseases. Combinations of HLA and KIR genes have been associated with several autoimmune diseases, infectious diseases, and cancers.

KIRs are a family of activating or inhibitory transmembrane receptors expressed on NK cells and a subset of T lymphocytes (*Vilches and Parham, 2002).*

The KIR *locus* maps to chromosome 19q13.4, within the leukocyte receptor complex, and comprises a family of highly polymorphic genes that are tandemly arrayed over a segment of about 150 kb. To date, 15 KIR genes (2DL1 to 2DL5A/B, 3DL1 to 3DL3, 2DS1 to 2DS5, and 3DS1) and 2 pseudogenes (2DP1 and 3DP1) have been described. There are 4 framework genes (3DL2, 3DL3, 2DL4, and 3DP1) that are present with very few exceptions in all individuals (*Middleton and Gonzelez, 2010*). However, numerous haplotypes with different gene content and allelic diversity segregate in human populations, creating considerable diversity in the number of KIR genotypes observed in the population (*Shilling et al., 2002; Martin and Carrington, 2008*). The number of

officially named human KIR alleles has increased since the initial release and there are now over 1000 alleles, which code for over 500 unique protein sequences (*IPD-KIR database*).

Nomenclature of KIR gene is based on the structures of the molecules they encode, specifically on the number of immunoglobulin-like domains in the extracellular region (2D for two domains, 3D for three domains) and by the length of the cytoplasmic domain (L for long cytoplasmic tail and S for short ones) or P for pseudogenes (Figure1.6) (*Robinson et al., 2018*). All inhibitory KIRs have long cytoplasmic domains possessing an immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruit protein tyrosine phosphatases, critical for mediating inhibitory NK cell function. In contrast, the activating KIRs are characterized by short cytoplasmic domains associate with a transmembrane signalling adaptor protein, DAP12. This adaptor molecule contains an immunoreceptor tyrosine-based activation motif (ITAM), whose tyrosine residues become phosphorylated by protein tyrosine kinase following interaction of the receptor molecules with their ligands. Phosphorylated ITAMs are recognized and bound by other proteins containing a SH2 domain, inducing a signaling cascade resulting in NK cell activation (*Campbell and Purdy, 2011*).

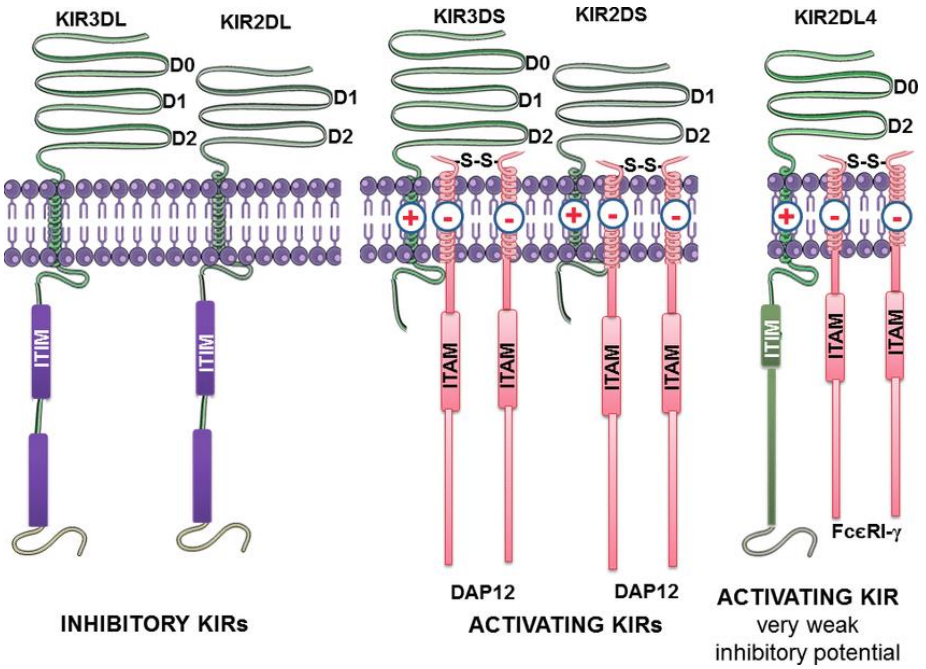

Figure 1.6. Structure of inhibitory and activating KIRs. DAP12, DNAX adaptor protein of 12 kDa; FcεRI-γ, high-affinity immunoglobulin epsilon receptor subunit gamma; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell immunoglobulin-like receptor. Figure from "Introductory Chapter: A Brief Overview on Natural Killer Cells" (doi: 10.5772/intechopen.72328)

Two basic haplotypes, designated A and B, have been identified based on gene content. Haplotype A is invariant in terms of gene content and is composed of five inhibitory genes (KIR2DL1, 2DL3, 3DL1, 3DL2, and 3DL3), one activating gene (KIR2DS4), and KIR2DL4, which may have both

inhibitory and activating capacity. The B haplotypes contain a more variable gene content with more activating KIR numbers (Figure 1.7) (*Blunt and Khakoo, 2020*). The frequency of these haplotypes varies substantially between populations, suggesting they have different functional properties that are subject to balancing selection.

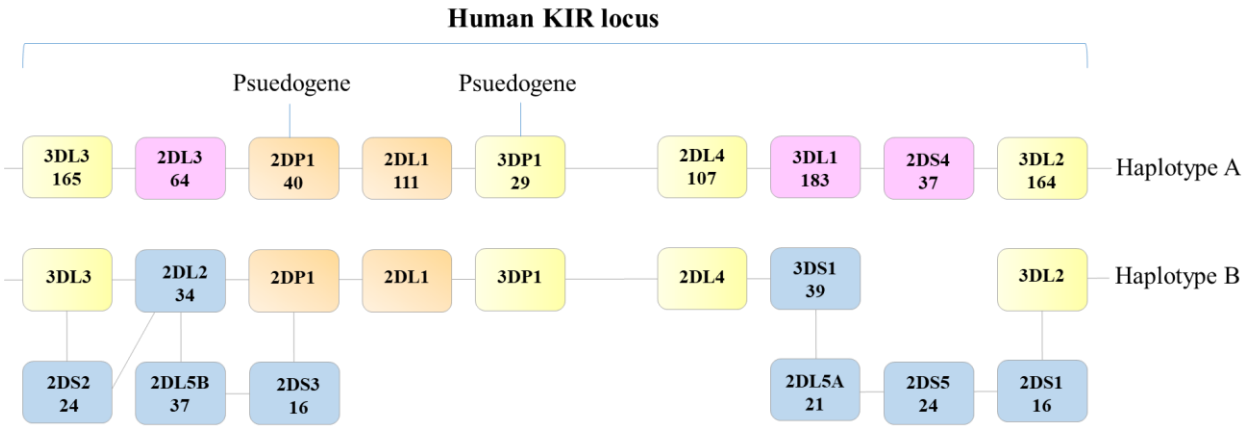

Figure 1.7. Human KIR locus. Haplotypes with identical gene content are further differentiated by polymorphism of the component genes: the box representing each gene shows the number of alleles. Framework genes are shown in yellow. Genes that can be present in both group A and group B KIR haplotypes are shown in orange, genes and/or alleles that are A haplotype-specific are shown in purple, those specific to group B KIR haplotypes are shown in blue. Data from IPD-KIR database.

The HLA class I (HLA-A, -B or -C) molecules, expressed on the surface of nearly every healthy nucleated cell, act as ligands for KIR receptors (see Table 2). The alleles of the HLA-C *locus* can be distinguished into two groups of ligands (C1 and C2) by the aminoacid present at position 80 of the alpha helix. HLA-C group 1 with asparagine at position 80 (HLA-C1^{Asn80}) provides the ligand for KIR2DL2 and KIR2DL3, whereas the HLA-C group 2 with lysine at position 80 (HLA-C2^{Lys80}) provides the ligand for KIR2DL1. Although KIR2DL2 and 2DL3 both bind HLA-C1^{Asn80} alleles, the affinity of these interactions may differ, and it is thought that KIR2DL3:HLA-C1^{Asn80} is a relatively weak interaction, whilst KIR2DL2:HLA-C1^{Asn80} is relatively stronger (*Biassoni et al., 2011*). KIR3DL1 has specificity for the HLA-Bw4 epitope present on many of the HLA-B alleles in addition to some HLA-A molecules (*Middleton and Gonzelez, 2010*). Similar to HLA-C, there is a sequence dimorphism at position 80 of the HLA-B Bw4 that affects its interaction with KIR3DL1. Allotypes with an isoleucine residue at position 80 (HLA-B Bw4^{Iso}) exhibit stronger inhibition through KIR3DL1 than Bw4 allotypes with threonine at position 80 (HLA-B Bw4^{Threo}). KIR3DL2 has as its ligand HLA-A3 and HLA-A11 allele families and HLA-G is the ligand for KIR2DL4.

The activating receptors KIR2DS1, 2DS2 and 3DS1 share sequence similarity in their extracellular domains with their corresponding inhibitory counterparts (KIR2DL1, 2DL2/2DL3, and 3DL1,

respectively) and are thought to share HLA ligand binding specificities as well (*Kulkarni et al., 2008*). Thus, binding of KIR2DS1 to HLA-C2^{Lys80} is of a similar specificity to that of KIR2DL1, but at a substantially lower affinity. Furthermore, although KIR2DS2 and KIR3DS1 share substantial sequence homology with their inhibitory counterparts, KIR2DL2/3 and KIR3DL1, respectively, binding to the relevant ligands are much weaker (*Sabouri et al., 2014*).

Table 2. KIR-HLA association.

Type	Receptors	Ligands
Activating receptors	KIR2DL4	HLA-G
	KIR2DS1	HLA-C2^{Lys80}
	KIR2DS2	HLA-C1^{Asn80}
	KIR2DS3	Unknown
	KIR2DS4	HLA-A11
	KIR2DS5	Unknown
	KIR3DS1	HLA-B Bw4^{Iso}
Inhibitory receptors	KIR2DL1 KIR2DL2	HLA-C2^{Lys80} HLA-C1^{Asn80}
	KIR2DL3	HLA-C1^{Asn80}
	KIR3DL1	HLA-B Bw4^{Iso} HLA-B Bw4^{Threo}
	KIR3DL2	HLA-A3,-A11

KIR, killer cell immunoglobulin-like receptor; HLA, human leukocyte antigen; Iso, isoleucine; Lys, lysine; Asn, asparagine; Threo, threonine.

Given the role of KIRs in the immune response and their extensive genomic diversity, it is conceivable that KIR gene variation and KIR-HLA association affect resistance and susceptibility to the pathogenesis of a number of diseases, such as infectious diseases and autoimmune/inflammatory disorders, through modulation of NK activation, cytotoxicity and cytokine release (*Aiello et al., 2018*).

1.4.1 KIR and KIR ligand in HIV progression

Several lines of evidence relate NK cell activation with the course of the infection as well as the acquisition of HIV.

In individuals infected with HIV, the intra-individual genetic combination of the activating KIR3DS1 with its putative ligand HLA-B Bw4^{Iso} was associated with slower progression to AIDS, lower mean viral load, and protection against opportunistic infections (*Martin et al., 2002; Qi et al., 2006*). The inhibitory counterpart of KIR3DS1, KIR3DL1, is characterized by highly polymorphic

alleles which leads to high variability in the expression of KIR3DL1. Co-expression of a KIR3DL1 high-expression allotype with HLA-B Bw4^{Iso} induced not only slowed disease progression, but also a protective effect against HIV acquisition (*Martin et al., 2007*). These results seem to contradict the model in which NK cell activation is protective. However they can be explained by the importance of KIR-HLA class I interaction in establishing tolerance to healthy cells as well as in the activation potential of mature NK cells. During their development, NK cells must engage at least one inhibitory NK cell receptor, which include KIR, with cognate HLA class I to achieve tolerance to self and become fully functional (*Pende et al., 2019*). The stronger inhibitory interactions conferred by KIR3DL1 during NK cell development can lead to a stronger NK cell reaction when ligand is downregulated during viral infection. Similarly, the presence of KIR2DL3 in combination with HLA-C1 was associated with lower viral load and diseases progression as well as HIV resistance (*Hens et al., 2016*), even if recent findings also associated co-carriage of KIR2DL3 and HLA-C1 with higher viral load and increased mortality rates (*Mori et al., 2015*).

Although some data are in contrast, probably related to the population analysed, the evidence suggests a strong contribution of the NK cell response in controlling HIV progression and confirming the key role played by KIR-HLA molecules.

1.4.2 KIR and KIR ligand in CMV outcome

NK cells provide a major defence against human CMV infection through the interaction of their surface receptors, including the activating and inhibitory KIRs, and HLA class I molecules. Studies from patients after kidney transplantation have showed the CMV reactivation rate in patients homozygous for the KIR A haplotype (virtually without activating KIRs) is higher than in patients with the B haplotype (with a variable number of activating KIRs), suggesting the importance of activating KIRs in the immune surveillance against CMV (*Hadaya et al., 2008; Stern et al., 2008*). According with these results, in other study immunocompetent patients with primary symptomatic CMV infection showed a higher frequency of the homozygous A haplotype (only KIR2DS4 as activating KIR) compared with controls with a previous asymptomatic infection. By logistic regression, the risk of developing symptomatic disease was associated with the homozygous A haplotype and the HLA-B Bw4^{Threo} allele (*Di Bona et al., 2014*). In a case study of a child with recurrent infections, mainly CMV, it was observed that all NK clones in the patient express KIR2DL1, which is able to interact with HLA-C2 molecule of the patient. This suggests that the strongly inhibitory KIR2DL1/HLA-C2 combination crippled NK cell activity and prevented the cells from mounting a protective response against CMV (*Gazit et al., 2004*).

These findings on the role of NK cells in the control of CMV infection should be of value in guiding efforts to reduce CMV-associated health complications in the older, including immunosenescence.

1.5. Vaccination in old age: TLR agonists as promising strategy

Infections, particularly those of the respiratory tract, can cause complications that could result in high morbidity and mortality among older people. Vaccination is one of the most effective medical interventions ever introduced, preventing millions of cases of infections worldwide every year. Better understanding of immune biology and advances in biotechnology have increased vaccine efficacy, while minimizing the side effects. However, vaccines are commonly less effective in providing protection in the older population, due to immunosenescence. Age-related immune impairments, such as innate dysfunction, constrictions in the naïve T-cell repertoire, humoral defects and co-infection with persistent viruses make older more susceptible to infections, particularly when there is no pre-existing immunity. For these reasons, strategies for improving the efficacy of vaccines for older people must be assessed in the context of the hallmarks of immunosenescence. A promising strategy to increase vaccine efficiency in older adults seems to be the incorporation of TLR agonists in vaccine formulations.

A vaccine adjuvant (from the Latin verb *adjuvare*, meaning to help) is a component designed to enhance and/or shape the specific immune response to a vaccine antigen, and has been incorporated in human vaccine formulations for more than 90 years. A variety of compounds with adjuvant properties have been discovered since then, and those employed in human vaccines licensed for use include alum, one of the oldest and most widely used adjuvant in human vaccines, oil-in-water (O/W) emulsions, TLR agonists or a combination of immunostimulants (*McKee and Marrack, 2017*).
TLRs agonist efficiencies as vaccine adjuvants rely mostly on the promotion of antigen uptake, presentation and maturation of DCs, their cytokine secretion and activation of T cells.

However, in older individuals an important consideration for the use of TLR agonists in vaccine formulations is the impact of ageing on TLR expression levels and their response to stimulation (see Paragraph 1.1.2). Therefore, adequate stimulation of TLRs with the aid of appropriate combination of agonists is likely to be particularly important in the older population. A new type of adjuvant formulation that could enhance vaccine efficacy in the older people consists of combined adjuvants for synergistic activation of cellular immunity (CASAC). Typically, the CASAC formulation incorporates two TLR agonists, for instance CpG-oligodeoxynucleotides (a TLR9 agonist) and polyI:C (a TL3 agonist), in combination with IFN-γ and the antigen. The latter may be individual or combinations of peptides for direct presentation by MHC-class I and II molecules, or longer peptides

and proteins for uptake, processing and presentation. Either is often given in a formulation of oil in water emulsion. The combined use of such combinations of immune stimulatory factors generate much stronger antigen-specific $CD8^+$ T cells responses (*Tye et al., 2015; Wells et al., 2008*).

Promising results were obtained in murine models of response to a tumor-associated self-antigens, where the simultaneous and synergistic activation of TLRs has enhanced DC activation, resulting in increased cellular immune responses to the antigen of interest. Comparative analysis of response to vaccination in young and aged, immune-senescent, mice with self-antigens or ovalbumin derived peptides in combination with the CASAC adjuvant has demonstrated substantially greater efficacy than vaccination with complete Freund's adjuvant (CFA) followed by incomplete Freund's adjuvant (IFA). Importantly, this greater efficacy of CASAC vaccination, as measured by the increased frequency, higher levels of cytokine expression and cytolytic activity in antigen specific $CD8^+$ T cells, was most pronounced in the aged, immune-senescent, mice.

To assess if these promising observations in animal models can be translated to humans, the ability of two combined TLR ligands, the imidazoquinoline R848 (TLR7/8 agonist) and MPLA (TLR4 agonist), to enhance the activation of DCs isolated from healthy aged and young human donors has been investigated. In both groups, the stimulation of DCs with the combination of TLR7/8 and TLR4 agonists induced higher levels of production of IL-12/p40 and TNF-α by mDCs, confirming their role in boosting the innate response of TLR agonists in humans. However, when the production of cytokines was compared between the young and the old donors, an increase of 5-10 fold in the production of IL-12/p40 by mDCs, as well as an increased amount of TNF-α by mDCs and pDCs isolated from the healthy old population of donors was observed (*Gambino et al. 2017*).

A more recent study in mice has clearly shown that the CASAC adjuvant can improve humoral and cellular immune responses against the recombinant HspX protein, a tuberculosis (TB) latency antigen able to stimulate immune responses resembling the bacillary latency stage of TB infection. Furthermore, in addition to promoting an increased antigen-specific IgG_1 and IgG_{2a} antibody response, as well as stronger $CD8^+$ and T_H1-driven immunity, adjuvanted HspX vaccine triggered a higher percentage of effector memory T-cells than those vaccinated without the CASAC adjuvant formulation (*Lew et al., 2020*).

Based on these results, it is evident that, despite the differences in their composition, adjuvants can contribute significantly to improving immune responses to vaccines. The further validation of these observations in the context of human immunosenescence, and specifically the impact of CASAC in the response of the aged population to preventative vaccinations against infectious agents, and therapeutic vaccinations against cancer associated antigens are now urgently needed.

2. Scientific Objectives

The main thread of my PhD thesis was to expand our knowledge on the impact of immunosenescence, as a result of the ageing process or the chronic viral infection. An objective that requires observation from different points of view, immunophenotypic and immunogenetic, in a homogenous population.

Literature data and genetic analyses performed by our group on CMV (Chapter 3.2) and HBV infections confirm the crucial role of KIR and their HLA ligand in control of viral infections. The impact of host genetic variation on outcome of HIV infection in the absence of combination antiretroviral therapy (cART) has been well documented.

An objective of this thesis, therefore, was to broaden the knowledge on the role of KIR/HLA repertoire in the susceptibility to HIV in a sample of a homogeneous Caucasian population from Sicily. The central hypothesis is that KIR/HLA repertoire has an important role in the susceptibility to HIV and in the disease progression in Sicilian immunologic responder patients on cART.

Another primary objective was to investigate the age-related changes in the absolute number and percentage of lymphocytes in Sicilian population, in correlation with gender and CMV and EBV serostatus, comparing these results with the immunophenotype of semi-supercentenarians and the oldest living Italian supercentenarian (111 years old).

Semi-supercentenarians and supercentenarians are extremely rare and, because of this, the main hypothesis was that they may have an immunological advantage that predisposes them to live longer than others.

Other important objective was to investigate the possibility of using a combination of two TLR agonists as vaccine adjuvant to triggering and shaping the adaptive immune response to vaccines

During my traineeship at the Professor Farzaneh's laboratories in King’s College London, I'm training in immune monitoring strategies and specifically the *in vitro* stimulation of human peripheral blood mononuclear cells (PBMCs) from Sicilian healthy donors for the production of antigen specific T cells against specific viruses and their associated antigens.

The main focus was then to better understand how PBMCs isolated from young and aged Sicilian healthy donors respond to a combination of TLR7/8 agonist R848, at 3 μg/ml, and TLR4 agonist MPLA, at 5 μg/ml, chosen from previous experiments, and CD40-L in response to stimulation with different pool of peptides.

Our three main hypotheses for this work were:

1. R848 + MPLA+ CD40-L combination (named as CASAC) will be powerful to stimulate the adaptive response to specific peptide compared with alum.

2. CASAC will be critical for enhance T cells response in immunosenescent older Sicilian healthy donors.
3. CASAC will be able to enhance adaptive responses to both previously encountered and new antigens.

For each of these issues, an in-depth examination of the literature was first conducted and the existing data summarise in review.

In the third section ("Results" Chapter), I will present the different studies, the main obtained experimental results and the resulting scientific papers and review. These data will be discussed in the fourth and final section ("Discussion and future perspectives" Chapter) of this thesis.

Given the employment restrictions imposed by ongoing pandemic of COVID-19, some experiments were subject to restrictions, others were not performed. This must be made aware before discussing the results.

3. Results

3.1. Immunosenescence and Its Hallmarks: How to Oppose Aging Strategically?

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REVIEW
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Immunosenescence and Its Hallmarks: How to Oppose Aging Strategically? A Review of Potential Options for Therapeutic Intervention

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Aging is accompanied by remodeling of the immune system. With time, this leads to a decline in immune efficacy, resulting in increased vulnerability to infectious diseases, diminished responses to vaccination, and a susceptibility to age-related inflammatory diseases. An age-associated immune alteration, extensively reported in previous studies, is the reduction in the number of peripheral blood naïve cells, with a relative increase in the frequency of memory cells. These two alterations, together with inflamm-aging, are considered the hallmarks of immunosenescence. Because aging is a plastic process, it is influenced by both nutritional and pharmacological interventions. Therefore, the role of nutrition and of immunomodulation in immunosenescence is discussed, due to the multifactorial influence on these hallmarks. The close connection between nutrition, intake of bioactive nutrients and supplements, immune function, and inflammation demonstrate the key role of dietary strategies as regulators of immune response and inflammatory status, hence as possible modulators of the rate of immunosenescence. In addition, potential options for therapeutic intervention are clarified. In particular, the use of interleukin-7 as growth factor for naïve T cells, the function of checkpoint inhibitors in improving T cell responses during aging and, the potential of drugs that inhibit mitogen-activated protein kinases and their interaction with nutrient signaling pathways are discussed. Finally, it is suggested that the inclusion of appropriate combinations of toll-like receptor agonists may enhance the efficacy of vaccination in older adults.

Keywords: aging, immunosenescence, immunomodulation, immunotherapy, nutrition

INTRODUCTION

People worldwide are living longer. In 2025, there will be about 1.2 billion people over the age of 60, increasing to 2 billion by 2050 (1). However, the increase in lifespan does not coincide with the increase in healthspan, i.e., the period of life free from serious chronic diseases and disability. In fact, the influence of aging on humans is responsible for physiological dysfunctions

in the different tissues, organs, and systems, including the immune system (2, 3). The age-related involvement of immune system leads to a progressive reduction in the ability to trigger effective antibody and cellular responses against infections and vaccinations. This phenomenon, called immunosenescence, a term coined by Roy Walford, is multifactorial, and affects both natural and acquired immunity, although T lymphocytes are dramatically affected (4). In fact, aging process more extensively affects acquired immunity than innate immunity (3, 5). Several factors, such as genetics, nutrition, exercise, previous exposure to microorganisms, biological and cultural sex, and human cytomegalovirus (HCMV) status can influence immunosenescence (3, 6–11).

Concerning sex, steroid hormones, linking to specific receptors, differentially modulate the immune system. In general, while estrogens increase the immune response, progesterone and androgens have immune suppressive actions. However, a few studies have analyzed the post-menopausal immune system (12). Therefore, it is unclear whether age-related changes in the immune system are different between men and women, although some data show that immunosenescence develops earlier in men than in women. This has been related to longer life expectancy of women (8, 13, 14). In addition, no evidence exists that males and females respond differently to therapeutic intervention against immunosenescence.

Many studies have emphasized the importance of viruses, such as herpes viruses, responsible for both latent and chronic infections, in shaping T cell compartments during aging (15). In particular, HCMV seropositivity seems related to many functional T cell changes. HCMV status has a greater impact than age on the immune system, because the virus contributes to shape the immune profile and function during normal human aging (16–18).

Understanding mechanisms of age-related disorders in immune regulation is important to identify more efficient strategies for immune rejuvenation and for effective induction of vaccination-mediated immunity in older individuals. Aging is a malleable process, affected by both nutritional and pharmacological interventions (19, 20). Therefore, immune system might also be prone to intervention. However, all possible therapies aimed at non-specifically "rejuvenating" the immune system might be counterproductive. In fact, the different parameters observed between young and older people could also be a product of the adaption vs. the exposome, i.e., all the stimuli that the immune system has undergone during life. Therefore, a targeted intervention for safe "rejuvenation" of the immune status in older people should be necessary (21).

Within the past years, numerous studies of underlying mechanisms of age-related immune decline have laid the groundwork for the identification of targeted approaches (5, 22–24). We will discuss below the most relevant strategies currently being investigated. We will also considered the role of nutrition in immunosenescence and in its counteraction in the section on dietary strategies currently being investigated. Further, we will examine the available data on growth factors [i.e., on interleukin (IL)-7], on monoclonal antibodies (MoAbs) that affect immune checkpoints, and on drugs that inhibit mitogen-activated protein kinases (MAPK) and their interaction with nutrient signaling pathways. These treatments, representing a promising therapeutic approach, will be treated in the section on clinical approaches. In the section on the other approaches in development, we will suggest that the inclusion of appropriate combinations of toll-like receptor (TLRs) agonists might enhance the efficacy of vaccination-mediated immunity in older adults. Finally, at the end of conclusion, we will outline possible future approaches.

SUMMARY OF IMMUNOSENESCENCE

Innate Immunity

The general picture of innate immunity in older people, which emerges from several studies, is that of the down-regulation of some functions and the up-regulation of others. We will discuss data on dendritic cells (DCs) due to their relevance for the immunotherapeutic approaches, including vaccination. For the other aspects of innate immunity in older individuals, see (3, 25, 26). Briefly, natural killer (NK) cell cytotoxicity is well-preserved in centenarians, and an increase in the actual number of NK cells is observed in healthy aging. Neutrophils show reduced function in bacteria phagocytosis and in the oxidative burst while macrophages show reduced chemotaxis and phagocytosis, and decreased cytokine production.

DCs, the most potent antigen presenting cells (APCs), can be divided into three subsets according to the expression of various markers (CD123, CD1c, CD141), one subset of plasmacytoid DCs (pDCs) and two subsets of myeloid DCs (mDCs) (27). Both pDCs and mDCs express TLRs that recognize conserved pathogen-associated molecular patterns (PAMPs) on microbes, and are key regulators of antimicrobial host defense responses. The type of TLR-activated DC determines the cytokine pattern (28).

There are discordant data on age-related changes in the frequency and absolute number of pDCs and mDCs. Regarding the ability to secrete cytokines upon stimulation, there are apparent inconsistencies in the available data for mDCs from older population. pDCs are instead characterized by a marked impairment of cytokine release in older people (27, 29). Recognition of microbial components by TLRs culminates in the secretion of type I interferons (IFNs) and cytokines that facilitate the coordination of innate to acquired immune responses. Peripheral blood mononuclear cells (PBMCs) isolated from older individuals ($\geq$65 years) exhibited a delayed and altered response to stimulation with TLR agonists compared with cells obtained from young adults ($\leq$40 years). This delayed response to agonists results in the reduced production of cytokines and chemokines (29). On the other hand, the addition of PAMPs to a subunit vaccine, triggering their corresponding pattern-recognition receptors (e.g., TLRs) improves vaccine efficacy in older humans and mice (25, 30–32). Accordingly, DCs together with naïve T cells represent the most restrictive elements for the immune response to primary viral infections in older people (33).

As the expression of TLRs remains constant during life, defects in signal transduction should be responsible for this impairment, as discussed by (24).

Acquired Immunity and the Hallmarks of Immunosenescence

The quality and quantity of the T and B cell responses change with increasing age, with consequent changes on the effectiveness of the immune response. This leads to an inadequate immune response against newly encountered antigens. The apparently inevitable consequence of this complex scenario is the reduced ability of older individuals to respond to novel antigens and to vaccines, resulting in an increased susceptibility to infection and in the development of age-related diseases, including cancer (3). As critically reviewed by (3, 6, 16), a number of longitudinal studies of octogenarians and non-agenarians performed in Sweden defined an immunological risk phenotype (IRP). Participants with the reversal of CD4/CD 8 T-cell ratio, a reduced proliferative response to mitogenic stimuli, and severe reduced B cells number showed reduced survival. Subsequently, the data were implemented and related to HCMV seropositivity, because HCMV seropositivity is closely related to the reversal of CD4/CD 8 T-cell ratio. In fact, as discussed below, persistent HCMV infection leads to chronic stimulation of CD8 T cells, which expand clonally showing an effector memory phenotype characterized by low CD28 expression. The IRP was present in around 15% of 85-years-olds in these studies at baseline. Follow-up of 2-, 4-, and 6-years mortality revealed significantly higher all-cause mortality in the IRP group than in the majority of other octogenarians and non-agenarians. However, this IRP was not confirmed in the Leiden 85-Plus study, a prospective population-based cohort study of individuals at the age of 85 years living in Leiden (NL). Thus, immune parameters associated with survival may vary in diverse populations at different ages (6). Therefore, we focus on the changes we have considered the hallmarks of immunosenescence, based on the literature data (6, 23).

The hallmarks of immunosenescence include: (i) a reduced ability to respond to new antigens; (ii) the accumulation of memory T cells; (iii) a lingering level of low-grade inflammation termed "inflamm-aging." Mechanistically, immunosenescence is only partially explained by organismal and cellular senescence. Therefore, these hallmarks of immunosenescence would be markedly affected by the history of the individual exposure to pathogens (6, 23).

The reduced ability to respond to new antigens is linked to a decreased number of peripheral naïve T and B cells (see last paragraph of this section). Naïve T cells are abundant in youth but may become "used up" by exposures to microorganisms over the course of life, hence differentiating into memory lymphocyte subsets. In addition, their number decreases following the involution of primary lymphoid organs, because age-related defects have been observed in their stroma. Some changes occur early in the developmental progression from hematopoietic stem cells (3). Thymus involution occurs at the time of puberty, and is characterized by atrophy and replacement by adipose tissue. This process seems related to the increase of sex hormones and to the decrease of IL-7, a hematopoietic growth factor secreted by stromal cells in the bone marrow and thymus. IL-7 exerts its action through the binding to a heterodimeric receptor composed of an α chain (IL-7Rα or CD127) and the common cytokine receptor γ chain (γc or CD132). CD127 is expressed on lymphoid lineage cells at different stages of development, whereas CD132 is shared with other cytokine receptors and expressed on most hematopoietic cells (34, 35). Irrespective of thymic activity, the naïve compartment only moderately decreases in size during the following life decades, while mostly maintaining overall diversity and distribution of clonal sizes. An abrupt contraction is seen in later life. Therefore, at the age of 50, T cell production is <10% of its previous peak levels. From an evolutionary point of view, this occurs because exposure to new pathogens is maximal during the first years of life, but less likely in later life when immune memory for previously encountered pathogens is both more prevalent and more significantly important for survival (3, 22, 36).

The life-long chronic antigen load causes the filling of the immunological space by a population of T lymphocytes with a late-differentiated phenotype and the shrinkage of the T cell repertoire. As previously stated, an age-related decrease in absolute number of peripheral blood naïve T cells is consistently found in all studies and in different human populations (22, 37). Due to the lifelong and chronic exposure to pathogens, T cells replicate several times and become late-differentiated effector memory T cells with features of replicative senescence (38). T cell senescence focuses on the phenotypic characteristics of individual lymphocytes and refers mainly to a low proliferative activity (39). Aging *per se* leads only to a relative accumulation of memory cell subsets, linked to the decrease in naïve cell populations. The absolute increase in memory T cells, called memory inflation, is observed only in older people infected by HCMV (40). These T cells do not express the co-stimulatory molecule CD28, required for the activation of T cells. The loss of CD28 occurs following cell proliferation, according to the observation that the $CD28^-$ T cells have shorter telomeres than $CD28^+$ cells. These CD28− cells express high levels of the adhesion molecule integrin CD11a/CD18 and have high levels of perforin and granzyme, responsible for the killing of the target cells. CD28 seems a good biomarker of immunosenescence, as further suggested by findings that late-differentiated $CD8^+/CD28^-$ T cells tend to accumulate particularly in older people, frail or affected by age-related diseases. These cells display a highly differentiated phenotype, expressing CD27, another co-stimulatory molecule, but not CD28 (however, in $CD28^+$ subset, $CD28^-CD27^-$ seem to be more frequent). They also carry short telomeres, lack telomerase and express negative signaling receptors, such as programmed cell death protein (PD)-1, which is involved in the down-regulation of the immune system (see paragraph on checkpoints inhibitors; the example of PD-1 and CTLA-4). Senescent T cells also express CD57 displaying a high cytotoxic potential, and killer cell lectin-like receptor subfamily G member 1. Late-stage memory senescent T-cells may also acquire new functions, such as suppressive activity, as demonstrated *in vitro*. In addition, they are producers of pro-inflammatory cytokines (17, 18, 41–47). However, a longitudinal study of 249 research participants followed for 10 years has strongly suggested that HCMV infection is not a primary causative factor in the age-related increase in systemic inflammation (48). Therefore, the accumulation of

memory T cells, especially late-stage differentiated $CD8^+$ cells is viewed as the result of depletion of the reservoir of naïve cells over time by contact with pathogens and their conversion to memory cells. However, the memory responses can be unsustained, because T cell memory established in humans during early age can deteriorate during the second half of life. The most obvious example of unsustained memory responses is the reactivation of latent varicella zoster virus (VZV) infection that manifests as herpes zoster. A steady decline of VZV-specific $CD4^+$ T cells over time has been documented, which is only very transiently boosted with zoster vaccination or reactivation (49). In contrast, high frequencies of antigen-specific T cells reactive to HCMV persist throughout life. T cell clones specific for HCMV dominate the repertoire in the older people and contribute to the contraction in diversity in the memory compartment (23).

Nearly 20 years ago, Looney et al. reported the dramatic impact of HCMV on the immune system of older people (50). This observation was subsequently described in numerous other studies (18, 46). In the latent state, the intermittent production of viral antigens prevents contraction of virus-specific T cells. Therefore, the virus is responsible for the generation of a large population of HCMV-specific $CD8^+$ T cells, with a significant increase in highly differentiated $CD8^+$ effector memory T cells, which expand clonally showing an effector memory phenotype characterized by low CD28 expression. As previously stated, this determines the phenomenon of memory cell inflation, leading to the emergence of vast populations of resting effector $CD8^+$ and, to a lesser extent, $CD4^+$ cells. In older people, one or a few clonal populations can occupy more than 25% of the entire $CD8^+$ cell pool (46, 51). These inflated HCMV-specific memory T cells maintain their efficient effector functions for the lifetime of the individual (40, 46, 52). Inflationary $CD8^+$ cells, after proper activation stimuli, can divide, secrete cytokines, and execute cytolysis, i.e., they are not exhausted. However, there may be a slight loss of control of HCMV replication in older compared with younger people. In fact, HCMV load in blood markedly increases in healthy people over the age of 70 years (53). Immune changes associated with HCMV may have significant impact during co-infection and vaccination, as well as on general and immunological fitness. However, the correlation between HCMV positivity and impaired responses is controversial because this relationship is observed in some but not all studies (54–56).

Persistent antigenic challenges lead to a poor response to newly encountered microbial antigens, as well as to a shift in the immune system toward an inflammatory, autoimmune, T helper (Th) 2 profile. In addition, the long-term chronic microbial burden induces progressive activation of macrophages, hence contributing to the chronic state of low-grade inflammation, inflamm-aging, another hallmark of immunosenescence (3, 9, 57). This term defines the systemic state of chronic low-grade inflammation considered a central biological pillar of the aging process and a common pathogenetic mechanism of age-related diseases, as well as a worse prognostic factor for all causes of death (9, 57–59). In the course of aging, there is a reduction in the ability to endure consequences of antigenic, chemical, physical, and nutritional triggers of inflammation. Chronic and low-grade inflammation can lead to tissue dysfunction and degeneration. Our immune system is quite efficient in fighting acute infections in young people, but not particularly efficient in responding to chronic stimuli, especially when they occur late in life. This leads to an increased production of pro-inflammatory cytokines and acute phase proteins (59, 60). Oxidative stress also plays an important role in determining and maintaining this low-grade inflammation, which, in turn contributes to oxidative stress (61, 62). Inflamm-aging results from the activation of signaling networks critical to inflammation, such as those regulated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor, particularly when combined with a variety of stimuli, such as senescent cells, obesity, circulating mitochondrial DNA, gut microbiota and diet triggering and sustaining inflammatory conditions (58, 63–68). However, as previously stated, immunosenescence represents the most important contributor to inflamm-aging, in turn, contributing to impaired immune responses. In fact, inflamm-aging is responsible for a high expression of micro (mi)-RNAs that interfere with B cell activation, driving tumor necrosis factor (TNF)-α production and inhibiting B cell activation as measured *in vitro* (69). Increased serum levels of TNF-α are also linked to a defective T cell response, in part due to reduced expression of CD28 (21). Accordingly, in monocytes, the pre-vaccination expression of genes related to inflammation and innate immune response is negatively correlated to vaccination-induced activation of influenza-specific antibody responses (70).

Age-related B cell changes are similar to those observed in T cell compartment and the effects on humoral immune response are detrimental as well. Age also affects B cell numbers and B cell repertoire diversity, as well as immunoglobulin isotypes and receptor repertoire with a decrease in specific humoral immune responses against new extracellular pathogens (71). Activated B cells isolated from older adults display a reduced induction of E47, a class I basic helix-loop-helix protein encoded by the E2A gene. This is the key transcription factor, for the induction of activation-induced cytidine deaminase (AID), involved in class switching and somatic hypermutation. The reduced expression of E2A might be responsible for the decreased avidity of antibodies and diminished antibody-mediated protection (72, 73). This defect might be linked to a reduced interaction with $CD40L^+$ T helper cells, because, in older adults, the memory/effector T cells show a reduced expression of CD40L, necessary for B cells cooperation (74). The reduced levels of E47 and AID mRNA in B cells from older individuals are also due to the reduced mRNA stability. It is due to the higher expression of the inflammatory mi-RNAs 16 and 155, which bind to the 3'-untranslated region of E47 and AID mRNA, respectively, inducing mRNA degradation (69). In addition to the decrease in circulating B lymphocytes, there is a shift from immunoglobulin produced by naïve cells (IgD, IgM) to immunoglobulin produced by memory B cells (IgG, IgA). This is accompanied by an impaired ability to produce high affinity protective antibodies against infectious agents and the shrinkage of the repertoire diversity. The reduced serum levels of IgM and IgD suggest a shift in the balance from the naïve (CD27) toward the memory compartment ($CD27^+$), although this is not observed in all studies (71, 75–77).

See **Figure 1** for the schematic changes occurring during aging.

DIETARY STRATEGIES CURRENTLY BEING INVESTIGATED

There is a mutual interaction between nutrition, intake of particular bioactive dietary components, immune function, and inflammatory status, hence a close relation with the hallmarks of immunosenescence (78, 79). Many existing data demonstrate the key role of foods as regulators of immune response and inflammatory status, hence as possible modulators of the rate of immunosenescence, particularly inflamm-aging (11, 80). Other data have demonstrated the importance of following a specific, even personally tailored, dietary pattern (81). However, the intricate cellular and molecular network of immune system makes difficult to identify targeted strategies to rejuvenate specific compartment of immunity. Starting from supplementation with a single nutrient, leading to the application of experimental dietary pattern, much progress has been made in this field. The main barrier to better clarity remains the wide heterogeneity among human beings, linked to different life-style and genetic factors that influence the rate of immunosenescence (6, 10, 11, 82). Data discussed below show that the main target of dietary strategies is inflamm-aging, because diet, biotics, and nutraceuticals can show anti-inflammatory and anti-oxidant properties.

Diet

The high rate of long living people and the low incidence of cardiovascular disease in many Mediterranean countries suggest the importance of a diet rich in fruits, vegetables, whole grains, legumes, and olive oil (probably the main anti-aging food in this area). The reduced consumption of animal proteins, in particular red and cured meat, is also important. The efficacy of this diet results as an attenuation of inflammation and oxidative stress, and from the maintenance of a condition of eubiosis of the microbiota, involved in the general improvement of immune response in these populations (68, 83–86). In particular, the Mediterranean diet down-regulates the levels of inflammatory mediators, such as soluble intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM-1),

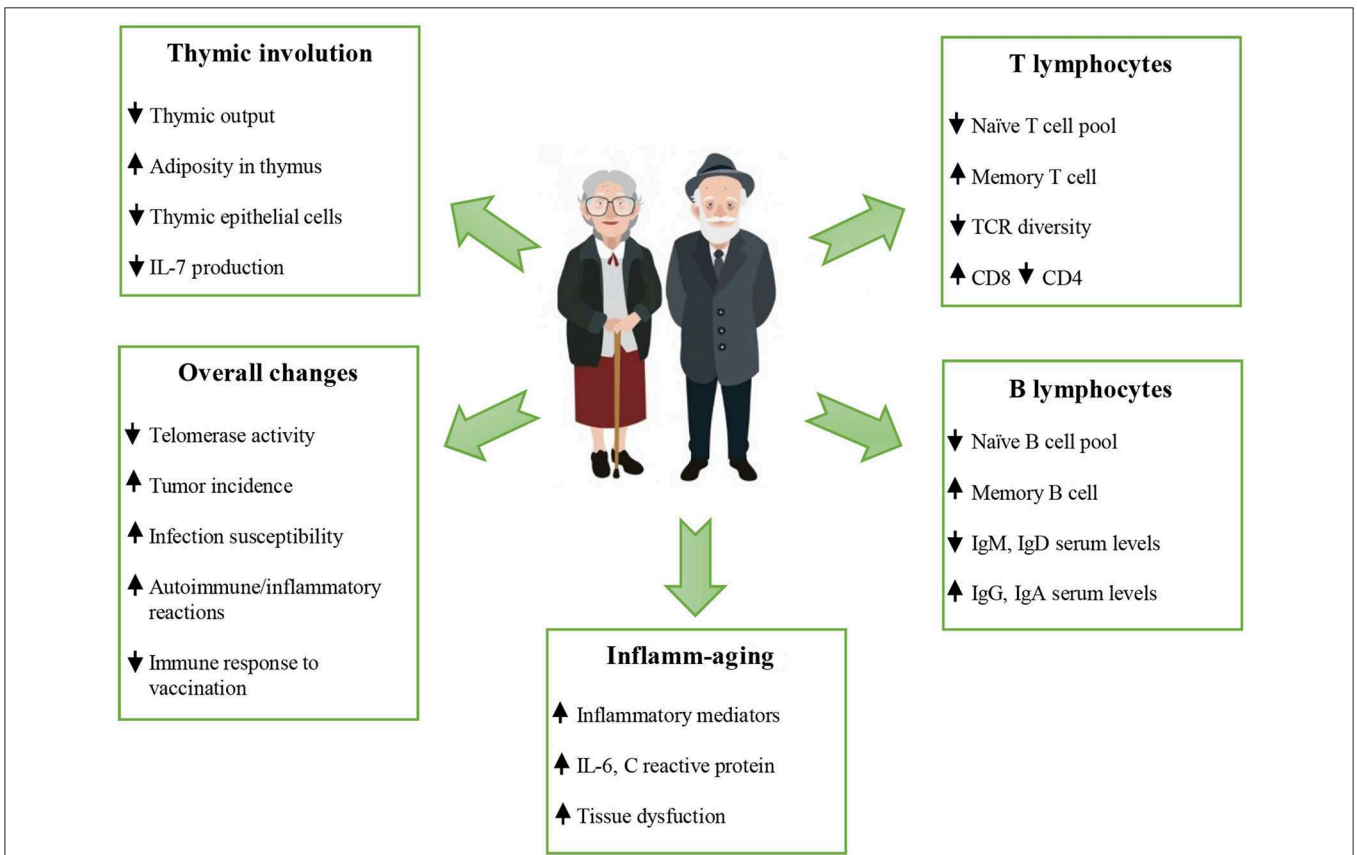

FIGURE 1 | Schematic changes occur during aging. The immunosenescence in older people is characterized by thymic involution, altered T and B cell responses, altered naïve/memory *ratio,* increased serum levels of IgG and IgA, a chronic low grade inflammation, and a poor response to newly encountered microbial antigens, including vaccines. The reduced ability to respond to new antigens, the accumulation of memory T cells and the lingering level of low-grade inflammation "inflamm-aging") are considered hallmarks of immunosenescence. See the text for the acronyms. ↑ increase; ↓ decrease.

C reactive protein, and IL-6, as well as many other biomarkers of inflammation (87–90).

A new interesting approach related to the possible reversion of immunosenescence is caloric restriction. NF-κB, mechanistic target of rapamycin (mTOR), and MAPK, pathways closely related to aging and inflammation are modulated by caloric restriction that downregulates the activation of IL-1β, IL-6, and tumor necrosis factor (TNF)-α genes, hence the pro-inflammatory state (20, 91, 92). More specifically, the results obtained by administration of different cycles of fasting, mimicking diet or long-term fasting influences inflamm-aging (20). These dietary patterns explicate their activity, particularly, during the refeeding period, reducing the rate of aging because of their antioxidant and anti-inflammatory effects, and possibly counteracting some other aspects of immunosenescence. The hypothetical explanation might be the disposal of damaged cells with growth of new functional cells.

Minor effects on immune function, after a brief starvation period of 72 hours, were seen in ten healthy, normal-weight, young volunteers. They showed an increase in suppressor cell numbers but no change in the number of peripheral blood leucocytes or in the differential counts (93). Unfortunately, these studies are severely limited by their complexity, further confounded by the small number of cases analyzed and the poor participants compliance (81).

New insights may also come from the use of caloric restriction mimetics, such as metformin, an activator of 5′ AMP-activated protein kinase (AMPK). It is a drug, typically administrated in type 2 diabetes but proposed as an anti-aging molecule for humans, such as the study called "Targeting Aging with Metformin" (94). Metformin can trigger AMPK, a pathway activated by energy depletion, i.e., by low levels of intracellular adenosine triphosphate (ATP), leading to the extension of healthy lifespan in model organisms (95). In mice with collagen-induced arthritis, metformin administration had an anti-inflammatory effect on arthritis due to the inhibition of Th17 cell differentiation, a subset of pro-inflammatory cells producing IL-17, and the upregulation of T regulatory cells (Tregs) differentiation along with the suppression of osteoclast differentiation (96, 97). Contrarily to caloric restriction, undernutrition, which is common in older people, is associated with an immunocompromised state, linked to altered T cell numbers, a reduced response to antigens, impaired release ofmediators, such as cytokines, and decreased phagocytosis and NK cell activity. This makes older people enable to trigger an efficient immune response to newly encountered pathogens. In such conditions of poor nutrition, the use of supplements, such as zinc, copper, iron, vitamins, nutraceuticals, and probiotics could be desirable and more appropriate than caloric restriction, as demonstrated by previous studies (98, 99).

Micronutrients

Nutritional status is crucial for the health status of older adults. Changes in phenotypic features, mainly loss of teeth and alterations in taste receptors, and gut disorders as well, determine a variation in both quality and quantity of food intake, contributing to general alterations in metabolism (100). Many studies have examined the influence of micronutrients and their influence on the enhancement of immune function in older adults (11, 79, 101, 102). Micronutrients, such as vitamins and minerals, are essential for the efficient performance of the immune system. They are needed in trace quantities, because the homeodynamic range is small, but the maintenance of a correct amount and balance is very rare in older people (often even in adults and young), both for scarcity and for excess due to unnecessary supplementation (78, 103).

One of the main micronutrients related to physiologic processes associated with immune system, and one of the main studied factors, is zinc. It is involved in many molecular processes, such as signal transduction, apoptosis, proliferation, and differentiation of cellular components of the immune system. Even slight deficiencies in zinc can have important consequences (104–107). Zinc deficiency can cause decreased levels of serum thymulin, a zinc dependent peptide hormone produced by thymic epithelial cells, with an activity that is progressively reduced with age, with a peak in pre-adolescence (78, 104, 108). The active form of thymulin induces the expression of markers of T lymphocyte activation, promoting T-mediated functions, acting both on the early and on the late phases of lymphocyte differentiation (109, 110). As shown in a randomized, doubleblind, placebo-controlled trial, after zinc supplementation for 12 months (45 mg elemental Zn gluconate/day), the incidence of infections was significantly lower, plasma zinc was significantly higher, and generation of TNF-α and oxidative stress markers was significantly lower in the zinc-supplemented participants than in the placebo group (both groups composed of 55–87 years old persons). Another doubleblind, randomized, controlled trial performed with zinc supplementation in old people (25 mg as zinc sulfate, once a day for 3 months, mean age of placebo group 80.6 ± 7.8, mean age of supplemented participants 79.5 ± 6.8) demonstrated increased levels of activated T helper and cytotoxic T lymphocytes, with a higher relative percentage of T cells with respect to the total circulating lymphocytes in zinc-supplemented older adults (105, 106, 111). Given the dose-dependent effect of zinc, both as a pro- and anti-oxidant, its presence in the normal range is essential for regulating the levels of reactive oxygen species (112). These studies highlight the importance of the zinc for immune function, but contrasting results exist, possibly reflecting the intrinsic complexities of this type of investigation (11).

Vitamin supplementation studies in older adults have demonstrated a role for vitamin E in the production of IL-2 as well as the activation induced T cell proliferation in naïve but not in memory T cells (78, 113–115). However, this response is variable, depending on genetics and immune functionality (102). Moreover, age-related oxidative stress, hence inflamm-aging, can be counteracted by vitamin C supplementation. In addition, it seems that this vitamin is involved in enhanced antibody generation and in differentiation and maturation of immature T-cells as well as of NK cells. Because vitamin C is water-soluble and humans have low storage capacity, its regular intake is up to 100-fold higher than that for many other vitamins (116, 117).

Probiotics, Prebiotics, and Symbiotics

The use of probiotics, prebiotics and symbiotics, i.e., the combination of pro and prebiotics, as immunomodulators, which act on microbiota, is very common. However, no strong cause-effect relation often exists between their use and specific end-points. Gut microbiota that plays an active part in healthy status, is compromised in older adults due to malnutrition, use of medications and immunosenescence itself. Therefore, the administration of specific strains of *Lactobacilli* and *Bifidobacteria* as probiotics as well as fructooligosaccharides, galactooligosaccharides, and other prebiotics, or the combination of both might constitute a benefit for immunocompromised people (118–122).

Data from supplementation studies with pro- or prebiotics in older adults show a control of inflammatory status because their use is responsible for a lower production of TNF-α, IL-1β, and IL-6 as well as an increase of anti-inflammatory cytokine IL-10, by PBMCs. In addition, these biotics improve the innate immune responses by the modulation of phagocytosis and cytotoxicity against specific bacteria, such as *Staphylococcus aureus*, increase activity of peripheral blood NK cells, and lower CD25 expression by resting T lymphocytes (123). However, the complexity of randomized controlled trials and lack of specific biomarkers in humans make difficult the reproducibility of the data (124). Moreover, healthy status, including absence of disease and nutritional status, seems to be crucial for their action as demonstrated by null results on immunomodulation after administration of prebiotics in older adults vaccinated with influenza or pneumococcal vaccines (125). Further studies are summarized in a very recent review by Suez et al., although in this case too, it is highlighted the weakness of the existing data (124). Therefore, a strong limitation linked to the study of these potential modulators is the lack of mechanistic studies that could reveal the molecular mechanisms underlying their action. This would allow a targeted and effective use, and would reduce the bias linked to individual variability and the conflicting existing results present in literature. Although meta-analyses and systematic reviews report interesting data, they cannot replace multicenter, randomized controlled clinical trials to address the relevance of the use of probiotics or the composition of the microbiota, both accompanied by molecular explanation of the observed evidence.

Nutraceuticals

Recently, various bioactive food components associated with health-related effects have been called nutraceuticals. These food compounds, mainly found in plant-based foods and fatty fish, have been implicated in offering physiological health benefits over and above basic nutritional requirements (126–128). Now, there is much interest in optimizing the immune response, and in reducing inflammation in older adults by increasing the intake of certain bioactive food agents (129, 130). Many studies have investigated how immune function and inflammation are directly affected by nutraceuticals. They provide evidence that increasing intake of some of them above the habitual and recommended dose levels can enhance some aspects of immune function, and reduce the level of inflammatory status, increasing cellular resistance to aging (131–133). Below, we examine the immunomodulatory effects of three classes of nutraceuticals, namely carotenoids, polyphenols, and polyunsaturated fatty acids (PUFAs), summarizing the most relevant nutritional studies on the reciprocal interactions between these dietary agents and immunosenescence.

Carotenoids are naturally occurring pigments found in most fruits and vegetables. They primarily exert antioxidant, hence anti-inflammatory, effects, but individual carotenoids may also act through other mechanisms, including immune-enhancing activities (134, 135). Jyonouchi et al. observed that lutein and astaxanthin increased the *ex vivo* antibody response of mouse splenocytes to T-cell antigens (136). Older adults supplemented with carotenoids (30 mg β-carotene, 15 mg lycopene and 9 mg lutein) had a shift to T cells expressing a mature phenotype and, in addition, higher IgA serum levels, and an increase in NK cells (137). Watson et al. report that higher doses of β-carotene (30 and 60 mg/day; instead of 15, 30, and 45) increase T helper cells and NK cells number (138). Although higher doses of carotenoids are not easily achievable in the diet of population, these findings suggest that low doses are insufficient to affect immune responses. Enhanced NK cell cytotoxicity was observed in participants treated with oral β-carotene and, similarly, long-term β-carotene supplementation increased NK cell activity in older adults (139, 140).

Dietary polyphenols are the biggest group of phytochemicals and they are defined as bioactive non-nutrient plant compounds. They are in fruits, vegetables, grains, and other plant foods, the consumption of which has been linked to reduction in risk of major age-related diseases (141, 142). In fact, as discussed below, their main action is the control of inflammation. Consumption of cocoa polyphenols rich in flavonoids (40 g/day) with 500 ml of skimmed milk, by participants at high cardiovascular disease risk (≥55 years), significantly reduced the expression of cell adhesion molecule very late antigen-4, CD40, and CD36 on monocytes. This treatment also lowered circulating levels of the inflammatory markers P-selectin and ICAM-1, compared with monocytes from the control group (only skimmed milk) (143). *In vitro* studies have shown that administration of olive oil polyphenols (caffeic acid and oleuropein glycoside) to human whole blood cultures stimulated with lipopolysaccharides significantly reduced IL-1β levels compared with stimulated control cultures that were not incubated with olive oil polyphenols. Interestingly, responses were inversely correlated to the dose (144). A small scale ($n = 23$) pilot study has shown that daily consumption of 12 green olives, containing oleuropein and hydroxytyrosol, significantly reduced serum IL-6 and malondialdehyde (a lipid peroxidation marker) levels after 30 days of consumption by healthy adults (90). Although several reviews have postulated potential beneficial effects of polyphenols on the immune response of older adults, there have been limited studies on this topic (145). However, the major effects of polyphenols are associated with increased release of IL-2 and IFN-γ, hence enhancing immune response (146). For example, resveratrol, a polyphenol typically found in red wine, grape skins, and berries, induces a significant increase in T helper

cells and in the delayed-type hypersensitivity response of aged rats (147).

In addition to carotenoids and polyphenols, several studies have also shown that dietary lipids can modulate the immune response. Fatty acids that have this role include the long-chain PUFAs of the omega-3 (n-3) and omega-6 (n-6) classes. n-6 PUFAs, derived from plants and land animals, have minimal effects on immune response. n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found mainly in fish and fish products and in some plants (flax seeds), have the most significant impact on immune cells. These have anti-inflammatory properties inhibiting the formation of eicosanoids and synthesis of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), chemokines (IL-8, monocyte chemoattractant protein 1), and adhesion molecules (ICAM-1, VCAM-1, selectins) (148). However, because dose, timing of administration, and participant age are important modulating factors of the effect of these molecules, contrasting results exist and only few studies focus on their use in older adults (149–154).

CLINICAL APPROACHES CURRENTLY BEING INVESTIGATED

Growth Factors

The various aspects of IL-7 physiology raise the possibility that reduction of this pleiotropic cytokine level could contribute to the age-related decrease in the absolute number of thymocytes and naïve T cells. Therefore, IL-7 might be used as a therapeutic agent to enhance thymopoiesis in lymphopenic patients or in older individuals, so counteracting the first hallmark of immunosenescence, i.e., the reduction of naïve T cells. In fact, the profound structural remodeling that characterizes the thymic involution also affects thymic epithelial cells with a consequent reduction in the intrathymic production of IL-7 (155, 156).

IL-7 produced by thymic epithelial cells provides survival and proliferative signals to immature double negative $CD4^{-}CD8^{-}$ thymocytes and promotes V(D)J recombination of the T cell receptor (TCR) γ-c locus (157). Mutations in the IL-7Rα or γc in humans lead to severe combined immunodeficiency, confirming the importance of the IL-7 signaling pathway in the development of T cells (158, 159). At later stages, the IL-7/receptor signaling complex is required for the homeostatic proliferation of naïve T cells in the periphery, exerting a higher effect in the cytotoxic T cell subsets. The high expression of IL-7Rα on naïve T cells allows the maintenance of the pool of these cells, but there are limited amounts of IL-7 under physiological conditions. Following the encounter with its cognate antigen, naïve T cells lose IL-7Rα expression and differentiate into effector T cells. IL-7R down-regulation guarantees an efficient use of the limited amount of IL-7 to naïve T cells that need it, driving their proliferation and preserving their phenotype (160). IL-7Rα is re-expressed at the memory stage, ensuring cell survival and proliferation in memory T cell pool too (156).

Interestingly, IL-7Rα chain is an integral component of the receptor for thymic stromal lymphopoietin (TSLP). TSLP provides normally a co-mitogenic activity that is less potent than that of IL-7 (161). However, to best of our knowledge no study has been performed on the possible role of TSLP in the treatment of immunosenescence.

In the first clinical trial in humans, patients with metastatic cancer (age range 20–59 years) treated with different doses of IL-7 showed a dose-dependent increase in circulating $CD4^{+}$ and $CD8^{+}$ lymphocytes along with a decrease in Tregs (162). Since then, numerous other clinical trials have used the administration of IL 7 for treating patients with various malignancies and chronic viral infections. In HIV-infected patients, with persistently low $CD4^{+}$ T-cell counts despite viral suppression, repeated cycles of recombinant human IL-7 induced a dose-dependent increase in circulating levels of both naïve and memory $CD4^{+}$ and mostly naïve $CD8^{+}$ T cells (163).

Therefore, some data suggest that IL-7 could have a therapeutic potential in improving the clinical outcome in settings that require enhanced immunological responses. However, in the complex scenario of aging, the immunorestorative properties of IL-7 may not be as great as initially hoped, most probably due to the deterioration of the thymus structure. The integrity of cortical and medullary thymic architecture and the presence of functional thymic epithelial cells are required to support and maintain thymopoiesis (164). Therefore, IL-7 effect on T cell development probably should require the preceding restoration of the thymic architecture.

Checkpoint Inhibitors; The Example of PD-1 and CTLA-4

The role of the immune checkpoint inhibitors, MoAbs that inhibit the expression of certain proteins made by T cells and some cancer cells, or antibodies that block the activation of inhibitory receptors, are pivotal for the management of cancer that occurs both in young and old patients. In fact, immune checkpoint inhibitors promote the immunological control of cancer cells by blocking the immune inhibitory responses that are evolutionary designed to prevent continuing immunological responses once an antigenic stimulus has been eradicated (165). However, there is a gap in the knowledge of the role of immune checkpoint inhibitors in the control of immune response in older patients because the data from randomized clinical trials are conflicting and often lack adequate statistical power.

The PD-1 and the cytotoxic T-lymphocyte antigen (CTLA)-4 are examples of checkpoint inhibitory receptors. The first regulates the inhibition and the fine-tuning of T cell responses. The second is a protein that contributes to the suppressor function of Tregs, mediating the inhibitory effect through the coordinated actions with the co-stimulatory receptor CD28. Activation of CD28 induces on lymphocytes and monocytes the expression of PD-1, which in turn interacts with its ligand (PD-L1) to regulate the balance between stimulatory and inhibitory signals needed for effective immune responses against antigens. This engagement leads to the inhibition of $CD28^{-}$ mediated co-stimulation, hence of TCR-mediated lymphocyte proliferation and cytokine secretion. The modulation of these pathways boosts anti-cancer immunity. Interestingly, the expression of PD-1

increases on T cells of older adults and its blockade partially restores T cells to functional competence (166–168).

The studies we discuss below are clinical studies based on the response to cancer. However, positive clinical data mean an increase in effector cell immune response, i.e., that therapy is in some ways targeting immunosenescence, or at least, dealing with the consequences of immunosenescence. In fact, immunosenescence influences the efficacy of the immune checkpoint inhibitors in older people (169); accordingly, the therapy is less efficient in patients ≥75 years (see below), probably due to a greater degree of immunosenescence. Consequently, there is limited evidence of successful therapy with immune checkpoint inhibitors in older adults, although a few observations of effectiveness in some patients are very encouraging. In the metastatic melanoma, for example, the use of the MoAb Nivolumab, a PD-1 inhibitor, alone or in combination with other antagonists, has survival benefits independently on age (170, 171). In another study, the administration of PD-L1 antibody Atezolizumab also shows positive results for all participants enrolled (172). In these studies, T cells of older adults were still able to respond to the blockade of their inhibitory receptors with a recovery of cytotoxic activity. Moreover, there is evidence about the efficacy of anti PD-1/PD-L1 MoAbs in older patients with non-small cell lung cancer (NSCLC) compared with chemotherapy. The benefit of immunotherapy in terms of response is stackable between younger and older patients (173).

Regarding the CTLA-4 use, several preclinical and clinical trials have reported the role of CTLA-4 inhibition in some kinds of cancer. In particular, the blockade with Ipilimumab can establish an anti-leukemic effect after allogeneic hematopoietic stem cell transplantation and can restore anti-tumor reactivity for patients with relapse (174). Although durable responses were observed, the efficacy of CTLA-4 inhibition needs to be confirmed. However, a recent meta-analysis analyzed the contextual administration of anti-CTLA-4 (tremelimumab and ipilimumab) and anti-PD-1 (nivolumab and pembrolizumab) molecules in four different settings: melanoma, prostate cancer, renal cell carcinoma, and NSCLC. The authors demonstrated a 37% reduction of the risk of death in favor of immune checkpoint inhibitors compared with control arm (175).

Recently, it has been demonstrated that the efficacy of the treatment with immune checkpoint inhibitors can be influenced by the composition of the host gut microbiota (176). As discussed above, the gut microbiota influences the immune system of the host. In fact, the interaction between specific microorganisms molecular pathways and immune cells can regulate local or systemic inflammation, hence influencing immune response (177). In particular, in cancer patients, the gut microbiota dysbiosis, caused by broad-spectrum antibiotic use, can be a contributor to immune checkpoint inhibitors resistance. In one study of 249 patients with NSCLC, renal cell carcinoma, and urothelial carcinoma treated with MoAbs against PD-1/PD-L1, a shotgun sequencing identified an overrepresentation of bacterial genera including *Akkermansia muciniphila* in responders to PD-1 inhibition compared with non-responders. In these patients, lymphocyte reactivity against *A. muciniphila* and IFN-γ production was significantly associated with survival (178). The analysis of 112 buccal and fecal samples from patients with metastatic melanoma also showed that the response to anti-PD-1 therapy depends on differences in the diversity and composition of the patient gut microbiota of responders vs. non-responders (179). These data demonstrated that, in responding patients, there was a relative abundance of bacteria of the *Ruminococcaceae* family. Moreover, in mice and patients, T cell responses specific for *Bacteroides* species, such as *thetaiotaomicron* or *fragilis* were associated with the efficacy of CTLA-4 blockade. On the contrary, tumors in antibiotic-treated or germ-free mice did not respond to CTLA blockade (180). Moreover, fecal microbiota composition of 26 patients with metastatic melanoma, using 16S rRNA, at time 0 and before each Ipilimumab treatment, was clustered on microbiota patterns. Baseline gut microbiota enriched with *Faecalibacterium* and other *Firmicutes* was associated with beneficial clinical response to Ipilimumab (181).

With the advent of immune checkpoint inhibitors immunomodulation is going to revolutionize the clinical management of at least some forms of cancer in older patients. In spite of several controversial points, some clinical trials suggest a significant benefit of immunotherapy in older patients, with the exception of patients ≥75 years that obtain less benefit from these treatments. Concerning this point, Metcalf et al. (25) have demonstrated that $CD28^-$ costimulation is required for the expansion of $PD\text{-}1^+$ CD8 T cells and effectiveness of PD-1 therapy in murine models of chronic viral infection and cancer. In addition, in lung cancer patients, PD-1+ CD8 T cells that proliferate in the peripheral blood after PD-1 blockade express CD28. Therefore, these data, which imply selective proliferation of $CD28^+$ cells by PD-1 therapy, highlight one mechanistic explanation why cancer patients older than 75 years may not respond as well to immunotherapy as younger patients. Understanding immune-regulatory functions is critical to implement integrative immunomodulatory strategies targeting checkpoints inhibitors.

Further studies of these checkpoints inhibitor functions might provide to be of great therapeutic value also in improving T cell responses to boost anti-microbial immunity and vaccine efficacy during aging as well. The combination of immunological, biochemical and systems biology data provides significant support for using PD-1 as an important target for therapeutic interventions of this type. In fact, studies carried out on HIV, hepatitis B and hepatitis C infections have shown that blocking the interaction PD-1/PD-L1 has a positive effect on the effector functions of T cells. Furthermore, future studies focusing on the elucidation of additive effects of blocking PD-1, other negative regulatory molecules, and immunosuppressive cytokines will help to identify combinatorial approaches that can improve T effector responses to vaccination and therapeutic interventions in older patients (182).

MAPK Pathway; Focus on p38 Regulation

Recently, the role of MAPKs pathways in the functional competence of the immune system has been demonstrated (183). The MAPK signaling pathways have been extensively studied in the context of oncogenic function and proliferative stimulus. However, these complex systems also regulate several

functions of the innate and acquired immunity. They are also involved in the production of pro-inflammatory cytokines, as well as in the intracellular signaling cascades initiated when a cytokine binds to its corresponding receptor (183). Three main subgroups of MAPKs are known: Erk, Jnk, and p38. These kinases can be targeted by small molecular weight compounds, which act to inhibit the phosphorylation of proteins, hence preventing their activation. Each one is separately regulated within individual cells (184) [for an overview of kinase inhibitors see (183)]. Understanding the immune-regulatory functions exerted by MAPK pathways is critical to implement integrative immunomodulatory strategies targeting these kinases.

The p38-MAPK pathway plays a pleiotropic role in cell survival, both sustaining proliferation, and inducing apoptosis in a cell type-specific manner, depending on the type of stimulus (185). The p38-MAPK pathway stimulates the positive regulation of Th1 differentiation and polarization. This pathway is not active in Th2 cells (186). The p38-MAPK pathway is critical for the production of inflammatory cytokines, positively regulating the production of IFN-γ in CD4 and CD8 cells (187, 188).

The studies discussed below have been performed *ex vivo* in mononuclear cells from mice and humans. They point out the possibility to affect the second hallmark of immunosenescence (the accumulation of memory T cells) through the regulation of p38 activation. p38 is generally absent in senescent human T cells. However, IFN-α signal can activate p38, triggering cellular senescence, and leading to inhibition of proliferation and telomerase activity in non-senescent T cells (189). It is also associated with alterations of energetic metabolism as well as autophagy. Autophagy, by inhibiting cell senescence, is a critical regulator of memory $CD8^+$ formation, and age-related autophagy defect is one of the explanations why $CD8^+$ T memory formation becomes defective in old age (38, 185). In 2009, Eisenberg et al. identified the use of spermidine, a polyamine compound, to promote longevity, via autophagy, using PBMCs as model. The authors monitored the survival cells using annexin V/7-AAD as co-staining. After 12 days, 50% of the cells survived after addition of spermidine. The rescuing effect did not involve inhibition of apoptosis, as the percentage of apoptotic cells was not influenced by spermidine. In fact, cell death, associated with membrane rupture, was indicative of necrosis (190). In immunosenescence models, $CD8^+$ T cell can be also rejuvenated in an autophagy dependent manner, using spermidine (191, 192). Low doses of a synthetic compound of natural spermidine significantly suppressed autophagy in human Jurkat T cell line. Moreover, the use of spermidine dramatically improved the $CD8^+$ T cell response to vaccination and infection in aged mice in an autophagy-dependent manner, contributing to the increased numbers of antigen-specific $CD8^+$ T cells (191).

Moreover, the effector memory $CD8^+$ T cells that express CD45RA, are not functionally exhausted. Indeed, they preserve the ability to secrete high levels of specific cytokines, such as IFN-γ and TNF-α. Furthermore, they only express low levels of key markers of exhaustion, such as PD-1. In these cells that present characteristics of immune senescence (decreased proliferation, lower telomerase activity, and increased presence of DNA damage), the simultaneous blockade of both p38-MAPK and PD-1 signaling supports their proliferation, both in young and in older human beings. Secretion of TNF-α in some populations of cells is reduced because of the contemporary arrest of p38-MAPK and PD-1 pathways. However, the telomerase activity in $CD8^+/CD45RA^+$ T cells is improved by blocking only the p38 pathway but not the PD-1 signaling, indicating that non-overlapping signaling pathways are involved (193, 194).

In addition to the inflammatory pathways that activate p38 through MAPK cascade by auto-phosphorylation, p38 can be associated with AMPK complex in response to chronic antigenic stimulation (see below, next paragraph).

The success of the studies using MAPK inhibitors, and kinase inhibitors in general, allows the possibility to analyze, and discover, the potential of these molecules in the treatment of immunosenescence, targeting the second hallmark. For example, a block at the level of p38-MAPK by sestrins causes age-related signaling defects in effector and memory $CD45RA^+/CCR7^-$ T cells (195, 196). Sestrins, the mammalian products of the Sesn1, Sesn2, and Sesn3 genes, are a family of stress sensing proteins (196). Lanna et al. proposed a possible role for sestrins in the control of the immune response, although this role has not yet been fully determined. Sestrins exhibit pro-aging activities in T senescent lymphocytes. The authors identified a complex named sestrin-dependent MAPK activation complex (sMAC) that simultaneously coordinates the activation of each MAPK that controls a functional response. The knockout of sMAC restored T cell activity (antigen-specific proliferation and cytokine production) from older humans, and enhanced responsiveness to influenza vaccination in the aged mice (196).

Examples of Nutrient Signaling Pathways: AMPK and mTOR

The mechanisms exposed above are distinct from another sestrin-inhibitory complex, containing GATOR and RAG A/B GTPase that involves the mTOR pathway (197–199). In particular, sestrins stimulate the activation of AMPK (by an unknown mechanism), inhibiting mTORC1 signaling. This suggests that the anti/pro-aging dichotomy of sestrin action in T cells vs. other cell types may depend on different sestrin-protein interactions (200).

In turn, senescent human $CD27^-/CD28^-/CD4^+$ T cells trigger AMPK to stimulate p38 recruitment, causing p38 auto-phosphorylation mediated by the protein scaffold TAB1. This pathway can inhibit telomerase activity, T cell proliferation, and expression of key components of the TCR signalosome. In the presence of low-nutrient levels and DNA-damage signaling the proliferative defect of senescent T cells is reversed by blocking AMPK-TAB1-dependent p38 activation (38). Moreover, in senescent $CD8^+$ T cells, p38-MAPK induces an increase in autophagy through interactions between a p38 interacting protein and autophagy protein 9, in a mTOR-independent manner, suggesting that p38-MAPK blockade reverses senescence via mTOR-independent pathway (185).

mTOR plays an important role in T cell activation and differentiation, especially of naïve $CD4^+$ T cells in their differentiation toward Th1 or Th17 phenotypes (201, 202). The

activation of mTOR signaling pathway is under the control of TCR/CD28 stimulation (201, 203). A growing body of research has highlighted mTOR inhibitors, i.e., rapamycin and everolimus, as promising treatments for several age-related pathologies, including immunosenescence, prolonging lifespan, especially in all four major animal models of aging: yeast, worms, flies, and mice (204, 205). The partial inhibition of mTOR could be beneficial for immune function in older people, although mTOR activity inhibits autophagy. At high doses, rapamycin is immunosuppressive, blocking both protein synthesis and cell division. In a clinical trial of over 200 older participants, they were assigned to a protocol including the use of mTOR complex 1 inhibitor everolimus, in different daily doses, for a 6-weeks period. Participants, after a 2-weeks drug-free interval, were challenged with the seasonal influenza vaccine. The two low-dose everolimus regimens improved immune function without causing serious side effects. Patients ameliorated their immune response, with improved hematopoietic stem cell function and a decreased proportion of PD-1$^+$ lymphocytes (206). In a subsequent follow-up study, combined BEZ235 (a dual ATP-competitive PI3K and mTOR inhibitor) and everolimus treatment for 6 weeks resulted in better infection control in older adults for a year after treatment had ended (207). However, rapamycin and Torin, another mTOR inhibitor, are also reported to suppress the anti-inflammatory effects of circulating glucocorticoids (208). These findings conflict with earlier studies showing the central importance of mTOR in innate immunity, specifically in the production of anti-inflammatory IL-10 and the suppression of pro-inflammatory cytokines IL-21 and IL-1β (209). The improved response after rapamycin treatment, which might involve a decrease in the percentage of PD-1 positive T cells, requires more detailed studies (207).

Data suggesting that nutrient signaling pathways may negatively influence lymphocyte function in aging indicate the possibility that inhibition of these pathways may enhance the activity of lymphocytes from older adults (210). Broad ranges of pharmacological agents with anti-immunosenescence properties have been identified and other trials with agents, such as rapamycin analogs are underway. Therefore, this represents a promising therapeutic approach to improving the health of older adults.

See **Figure 2** for the main clinical approaches in immunomodulatory interventions.

OTHER APPROACHES IN DEVELOPMENT

Other approaches focus on development of novel vaccines especially suited to raise protective immunity in older adults by overcoming the decrease in naïve cells. This approach includes high-dose vaccines, booster vaccinations, different immunization routes, and use of new adjuvant. The most used adjuvants are based on aluminum salts. These adjuvants induce the activation of APCs and strengthen the antigen immunogenicity by their slower release and higher persistence at the vaccination site. Another interesting compound is MF59, a squalene-based adjuvant, which increases the chemokine-dependent recruitment of APCs (211). However, adjuvants have shown only modest success (212). The most effective is generally considered complete Freund adjuvant, which can only be used in animals because it can cause a damaging skin inflammation (213). Therefore, there is an unmet need for new vaccine strategies for older people.

The development an identification of appropriate adjuvants and cytokines might effectively remedy defects in T cell functions from older adults, both directly and by better activation of DCs (214–216). Stimulation of TLRs by agonists seems to be a promising strategy to enhance vaccine efficacy, because TLR triggering can induce the production of cytokines by APCs, and can promote germinal center antibody production (217, 218). Age-related variations in cytokine production are seen in the APC isolated from older donors and efficient TLR stimulation may overcome the age associated TLR signaling dysfunction (219).

Triggering of multiple TLRs, using a combined adjuvant for synergistic activation of cellular immunity (CASAC), is an intriguing strategy. CASAC incorporates CpG (a single-strand oligodeoxynucleotide, characterized by motifs containing cytosines and guanines), which is a potent inducer of IFN-α by pDCs, in combination with polyI:C (a synthetic analog of viral dsRNA that targets TLR3, inducing the production of type I IFNs). CASAC also contains IFN-γ and MHC-class I and II peptides. This formulation results in potent cytotoxic T cell-mediated immunity in young mice. In fact, immunization with two or more TLR agonists, an activator anti-CD40 antibody, IFN-γ, and surfactants were sufficient to drive unprecedented levels of CD8 responses to peptides or protein antigens and highly polarized Th1 CD4 responses. CASAC stimulation activates both mDCs and pDCs with IL-12 secretion. This strategy is more effective than existing adjuvants and provides a technological platform for rapid vaccine development (213).

Accordingly, in aged mice, antigen specific CD8$^+$ T cell responses were stimulated after serial vaccinations with CASAC and a class I epitope, deriving either from ovalbumin or the melanoma-associated self-antigen, tyrosinase-related protein-2. Pentamer analysis revealed that aged, CASAC-vaccinated, animals had substantially higher levels of antigen specific CD8$^+$ T cells compared with mice vaccinated with complete/incomplete Freund adjuvant. Therefore, CASAC promoted significantly better functional CD8$^+$ T cell activity (220).

An approach able to overcome age-related defects in CD4 T cell responses *in vivo* comes from the ability of combined TLR ligands to induce the activation of peripheral blood DCs isolated from older healthy donors (29). Preliminary *in vitro* screening experiments suggest that, from the various TLR agonists tested, the condition that most effectively activates DCs is the combination of TLR7/TLR8 with TLR4. This TLR agonist combination induces significantly greater cytokine production than that induced by each of the individual agonists. This greater stimulation is probably due to the combined activation of both MyD88 and TRIF-dependent signal transduction pathways. Stimulation with the specific combination of TLR agonists, the imidazoquinoline R848 that targets TLR-7 and the monophosphoryl lipid A that targets TLR-4, induces significantly higher cytokine secretion by mDCs and pDCs from older

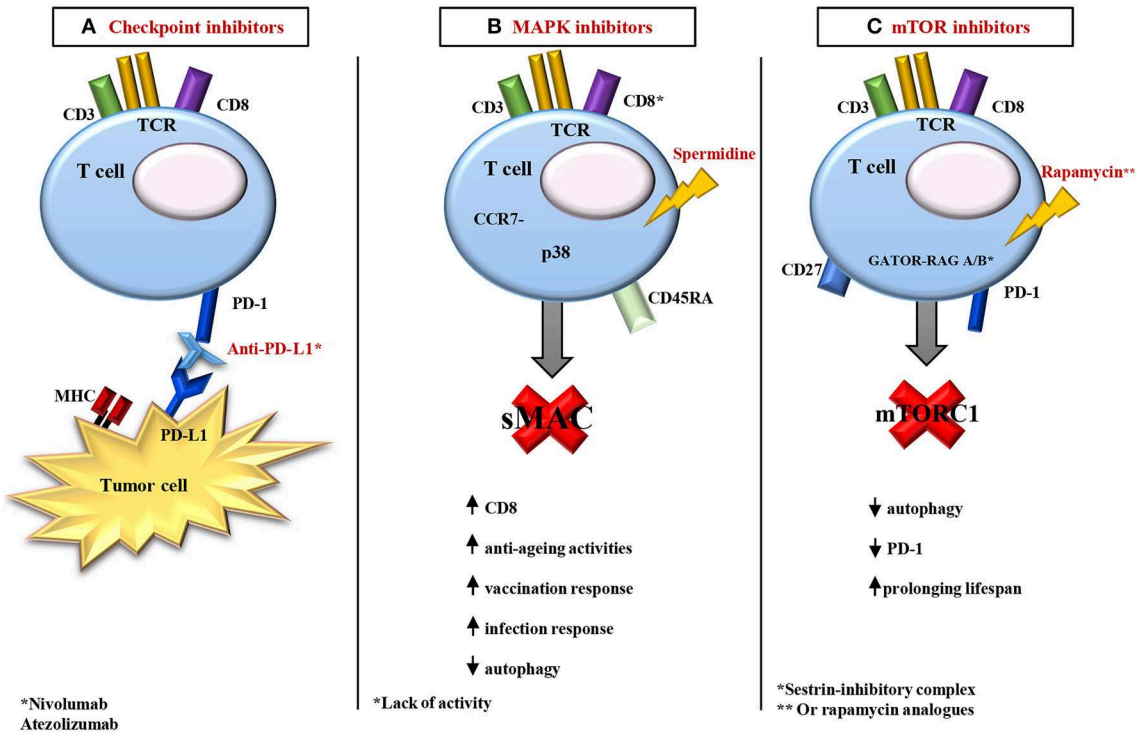

FIGURE 2 | Overview of the main strategies of immunomodulation. **(A)** In age-related diseases, in particular in some metastatic cancers, the use of the monoclonal antibody (such as Nivolumab, a PD-1 inhibitor) alone or in combination with other antagonists (PD-L1 antibody, like Atezolizumab) shows positive results on T cell activities in older adults. These cells became able to respond to the action of their inhibitory receptors with a recovery of cytotoxic activity. **(B)** In old models, with Lack of activity of $CD8^+$, it is possible to rejuvenate $CD8^+$ T cell responses in an autophagy dependent manner, using the polyamine compound spermidine. Spermidine induces autophagy and prolongs lifespan in model organisms. Moreover, in the $CD8^+/CD45RA^+/CCR7^-$ T cells, a block at the level of MAPK p38 by sestrins causes age-related signaling defects. The knockout of sMAC restores T cell activity (antigen-specific proliferation and cytokine production) in older humans, and enhances responsiveness to influenza vaccination in old mice. **(C)** GATOR and RAG A/B GTPase make a sestrin-inhibitory complex that involves the mTOR pathway. In particular, sestrins stimulate the activation of AMPK (by an unknown mechanism), inhibiting mTORC1 signaling. In addition, mTOR inhibitors, i.e., rapamycin and everolimus, are promising treatments for several age-related pathologies, including immunosenescence, prolonging lifespan. A soft inhibition of mTOR could be beneficial for immune function in older adults, although mTOR activity inhibits autophagy, and involves a decrease in the percentage of PD-1 positive T cells. See the text for the acronyms.

adults. This has potentially important implications, because the reduced production of cytokines by pDCs from older people, caused by defects in TLR signaling pathways, is associated with an ineffective antibody response to influenza vaccination (221). These findings highlight the efficient effect of adjuvants in the stimulation of cytokine production and point toward the potential use of appropriately selected combination of TLR agonists in future vaccination approaches for older adults to overcome the CD4 inability to respond.

CONCLUSION AND FUTURE APPROACHES

Until a few decades ago, a very small fraction of the population would reach 80 years of age. Now, in the Western world, this is a frequent event, with the average life expectancy for a newborn to have risen to 80 years in most Western European countries (1). However, the increase in lifespan does not coincide with increase in healthspan. The link between aging and disease is in part a reflection of the functional changes in the immune system of older people. Different factors contribute to the development of age-related immune dysfunction, but the epilog of an aged immune system is an increased propensity toward a reduced resistance to infection, poorer responses to vaccination, and the development of age-related diseases. The analysis of the contributing factors to this profound immune remodeling has revealed a complex network of alterations that influence both innate and acquired arms of the immune system. The diversity of cells, molecules and pathways involved in this remodeling, and their ability to influence each other, including the intra- and inter-individual variability of the immune response, make it hard to identify interventions that can be predicted to improve or, at least, to maintain the immune function in older adults. Within the past few years, numerous studies of the underlying mechanisms of age-related immune decline have laid the groundwork for the identification of targeted approaches; some of these have been discussed above, focusing on interventions able to target the hallmarks of immunosenescence. Possible other future approaches are reported below.

Taking into account the role of HCMV in the decrease of naïve T cells and increase of memory T cells, the reduction of the latent/lytic viral load, by vaccination and/or antiviral drugs, should be beneficial to diminish HCMV-associated

immunosenescence. Concerning the HCMV vaccine, Plotkin has published an extensive review. As pointed out by the author, as a result of 40 years of work, there are many candidate HCMV vaccines, including live recombinants, replication-defective virus, DNA plasmids, soluble pentameric proteins, peptides, virus-like particles and vectored envelope proteins. Therefore, we know the antigens needed in a HCMV vaccine, and that vaccination can be protective. To reach the goal of an effective HCMV vaccine, now we need a concentrated effort to combine the important antigens and to generate durable responses that will protect for a significant period. Interestingly, Plotkin emphasizes that aside from the two main targets for disease prevention, i.e., congenital infection and post-transplant disease, immunosenescence might be a target for vaccination mediated intervention, as well (222). Letermovir is an antiviral agent that inhibits HCMV replication by binding to components of the terminase complex. In patients undergoing hematopoietic stem cell transplantation, Letermovir daily prophylaxis is effective in preventing clinically significant HCMV infection when used through day 100 after transplantation, with only mild toxic effects and with lower all-cause mortality than placebo (223). However, there is no suggestion yet for the use of antiviral therapy as a strategy for prophylactic mitigation of immunosenescence.

Finally, possible future strategies to combat immunosenescence are represented by cellular and genetic therapies, including bone marrow transplantation and genetic reprogramming. In particular, genetically reprogramming cells into induced pluripotent stem cells can rejuvenate any cell type through telomere elongation, overcoming hurdles of replicative senescence (224).

SUMMARY

In the first part of the review we define immunosenescence and its relevance for the health of older persons, particularly in the context of acquired immunity. In the second part of the review we focus on the possible treatments to mitigate immunosenescence. First, we pay great attention to positive and negative effects of nutrition on immunosenescence. Then, we analyze the possible immunotherapeutic role of interleukin-7 as well as of checkpoint and mitogen-activated protein kinases inhibitors. Finally, we discuss a possible immunotherapeutic intervention to enhance the response of older adults to vaccines, i.e., the use of toll like receptor agonists. Therefore, we present a comprehensive review of several possible therapeutic interventions to alleviate immunosenescence.

AUTHOR CONTRIBUTIONS

AA, CC, SD, ML, and GA contributed to draft the manuscript. AA, FF, GC, CC, SD, CG, ML, NZ, and GA contributed to revising it. AA, CC, and GA wrote the final version.

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3.2. Genotypic and Phenotypic Aspects of Longevity

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REVIEW ARTICLE

Genotypic and Phenotypic Aspects of Longevity: Results from a Sicilian Survey and Implication for the Prevention and Treatment of Age-related Diseases

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Abstract: ***Background:*** It is well known that long living individuals are a model of successful ageing and that the identification of both genetic variants and environmental factors that predispose to a long and healthy life is of tremendous interest for translational medicine.

***Methods*:** We present the preliminary findings obtained from an ongoing study on longevity conducted on a sample of Sicilian long-lived individuals.

Results: We review the characteristics of longevity in Sicily, taking into account lifestyle, environment, genetics, hematochemical values, body composition and immunophenotype. In addition, we discuss the possible implications of our data for the prevention and/or treatment of age-related diseases.

Conclusion: As widely discussed in this review, the explanation of the role of genetics and lifestyle in longevity can provide important information on how to develop drugs and/or behaviours that can slow down or delay ageing. Thus, it will be possible to understand, through a "positive biology" approach, how to prevent and/or reduce elderly frailty and disability.

Keywords: Age-related diseases, body composition, genetics, immunosenescence, longevity, Sicily.

1. INTRODUCTION

Ageing leads to the incapacity to adapt to stress and to a decline in functional capacity. The ageing condition itself changes the performance of physiological systems and increases the susceptibility to death but new pieces of evidence suggest that the process is modifiable, thereby making it possible to delay age-related diseases [1, 2].

There are two main ways to become old, or having good and functioning health (successful ageing), either being with disability and age-related diseases (unsuccessful ageing). The latter concerns old people who develop one or more age-related diseases: neurodegenerative such as Alzheimer's disease (AD) and Parkinson's, metabolic diseases such as metabolic syndrome (MS), type 2 diabetes mellitus (T2DM), cardiovascular (CVDs) and cancer. Centenarians achieve successful ageing since most subjects reach the age of 100 or more without any age-related diseases, in good physical and mental condition. They represent the best model to study successful ageing and longevity although they have different genetic features and lifestyle [1, 3].

Centenarians are able to repair damages and respond well to stressors. That is due to a combination of "positive features", *i.e.* genetic and epigenetic characteristics and a favourable environment with social involvement [4]. Although many studies exist on centenarians, it has not yet been possible to identify the longevity signature. One of the reasons is that communities with a high number of centenarians are relatively rare in the world. These are five worldwide, and in 2004, Poulain *et al.* called them as blue zones (BZs) [5]. They are defined as a rather limited and homogenous geographical area where the population shares the same lifestyle and environment and its longevity has been proved to be exceptionally high. The validated BZs are so far in Sardinia (Italy), Ikaria (Greece), Okinawa (Japan), in Nicoya Peninsula (Costa Rica), and in California, the Loma Linda town where the Seventh-day Adventists live [6].

Such a wide distribution makes it difficult to repeat the data. This is probably due to the different genetic combinations, the genetic mosaic, and the interaction with the environment.

The increased ability to reach the age of 100 in Westernized countries over the last years and the reduction in the overall mortality clearly reflect the improvement of hygienic condition and quality of life, including the attention to diet and the advent of preventive medicine. The increased average life-span led to an increase in the number of nonagenarians and centenarians worldwide. It has also increased the number of elderly affected by age-related diseases [1, 7].

While the reason why human beings can develop a disease in late-life is a puzzle that is worth seriously studying, an even more fascinating enigma lies in understanding how some subjects are able to live for a century without a disease. For the prevention of

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age-related diseases, it would be interesting to develop medical interventions that would allow replicating the biology of centenarians in the average person.

In the last years, we have surveyed the population of some Sicilian inland towns, characterized by a high rate of centenarians to investigate the genetic and environmental patterns related to longevity [8, 9].

We present the preliminary findings obtained from an ongoing study on longevity conducted on a sample of Sicilian long-living individuals (LLIs), i.e. individuals that belong to the 5 percentile of the survival curve. We discuss data according to the available literature, for their possible implication for the prevention and/or treatment of age-related diseases.

2. LONGEVITY IN SICILY: AN OVERVIEW

A demographic survey performed in 2007 clearly showed that some small municipalities, with a low number of inhabitants, of unpolluted inland areas of Sicily were characterized by a reduced men mortality at the age of 80 and over. This implied an increase in the number of male centenarians in those zones. The rate of longevity was not increased in women, likely because of different conditions and educational level, implying different access to prevention or to health facilities [10].

A few years later, we conducted a pilot study in the Sicani Mountains, a chain of mountains sited in the Sicilian inland, where, at that time, the number of centenarians (people aged 100 to 107) per inhabitants was up to four-fold higher (10.37/10, 000) than the one documented for Italy (2.4/10, 000). In particular, male centenarians were 14 times more than in Italy (12.46/10, 000 inhabitants vs. 0.89/10,000 inhabitants). The nutritional assessment showed high adherence to the Mediterranean diet (MedDiet), with low glycaemic index and low animal protein intake. The meals were frugal and three times a day, comprising a small amount of carbohydrates and meat, while an abundant consumption of seasonal fruits and vegetables dressed with homemade extra virgin olive oil was reported. This is not surprising because, since the beginning of the 1990s, increasing evidence suggest that the MedDiet has a beneficial influence on several diseases (such as CVDs, MS, T2DM, atherosclerosis, cancer), favouring health and longevity at the same time. The evaluation of daily living activities, conducted on these subjects through instrumental activities of daily living, and the mini-mental state examination highlighted, for both genders, a good level of independence and cognitive functioning. The hematochemical profile and the evaluation of the main risk factors for age-related diseases confirmed their good health status, although a reduction in sight and hearing was measured. The importance of social engagement and light physical activities has to be highlighted, due to the mountainous region surrounding the village [8, 9, 11].

We conducted a survey in a different population of Sicily in mountains, including some villages in the Palermo province part of the Madonie Mountains, which partly confirmed the results obtained in Sicani Mountains. We documented the presence of 4.33 centenarians/10,000 inhabitants in the Madonie villages, as compared to 2.6 centenarians/10,000 in Italy. High rate of young people's emigration over time should be considered, thus to ascertain the true longevity rate, it is necessary to study the birth and death records. We checked the death age of about 37,000 newborns between 1881 and 1917 in a sample of five small municipalities (Petralia Soprana, Petralia Sottana, Geraci Siculo, Bompietro, Castellana Sicula) located in Madonie area. About 1,700 individuals died at the age of 90 years and over and about one hundred were centenarians. Therefore, the probability to reach 90 and 100 years old was of 4.6% and 0.22% respectively (Poulain, Busetta and Caruso unpublished observations). We cannot conclude that these small towns exhibit an exceptional level of longevity as that observed in Sardinia. However, the populations of these municipalities are experiencing higher longevity as compared to other places in Sicily and in Italy.

To better investigate the epidemiological context, we compared the Standardized Mortality Ratio (SMR) with respect to whole Sicily, of the two Palermo province Districts, namely Cefalù and Petralia Sottana, including the municipalities of interest, compared to the urban area of the province (Palermo city). In Table **1**, we present the ratios between the observed number of deaths in the population under study and the expected number of deaths, based on the age- and sex-specific rates of the population of Sicily at the last census available [12]. Estimates are provided separately for males and females. To this end, the regional Death Nominal Causes Register has been accessed with regard to the last available period 2004-2011 [13]. The SMRs estimates reported for Cefalù District and Petralia Sottana District were observed to be systematically lower than those reported for Palermo city. In particular, Cefalù District and Petralia Sottana District document statistically significant lower mortality rates for all causes of death and for all other selected death causes, except for diabetes mellitus in Petralia Sottana District and for lung cancer in Cefalù District, but only in females. Furthermore, Palermo City has a statistically higher mortality rate for all causes of death, for respiratory diseases and for lung cancer both in males and females, and for diabetes mellitus, only in males. Conversely, we documented a statistically lower mortality rate for circulatory system diseases in females. These mortality outcomes confirm that the Madonie municipalities belong to a zone with a high rate of longevity.

The ongoing survey based on preliminary analysis on the nutritional habits confirmed the possible association of longevity phenotype with a Mediterranean lifestyle but not during ageing or extreme longevity, rather during young age. In fact, long-lived people used to follow MedDiet but not during the ageing period, suggesting an interesting and effective role of epigenetics in the attainment of longevity.

Thus, longevity concerns people living in small villages, without pollution, likely because of different working conditions, different lifestyles, *i.e.* reduced smoking and alcohol abuse and MedDiet (presently or in the past). The reason because longevity has been observed particularly in small municipalities is not surprising. Individuals with greater access to social support and family network have better health care and lower levels of mortality, particularly when there are adult daughters in the family. All the LLIs studied in these two surveys lived on the mountains in multi-storey homes, thus throughout their life, they were constantly engaged in physical exercise. This is in agreement with the evidence obtained from studies that support the positive association between increased levels of physical activity, exercise participation and improved health in older adults [14]. Most LLIs individuals of the BZs in Sardinia, Costa Rica and Ikaria and in the Italian region of Cilento live on the mountains, with an environment characterized by a low degree of pollution. The average high slope of the terrain, quite common in the mountain zone, should be responsible for a long life with intense physical activity, hence improved cardio-respiratory fitness of the inhabitants. These populations have preserved a traditional lifestyle with an ideal social context as habitat, economic activity, intensive community and family support for their elderly, as well as the consumption of locally produced food. This has likely facilitated the accumulation of ideal conditions capable of limiting the factors that negatively affect health in the Western world [5]. As pointed out by Poulain *et al.* [5], the emergence of LLI phenotypes should be due to a balance between the benefits of traditional lifestyle and those of modernity as the increase in wealth and in better medical care.

In addition, prolonged and short repeated intense exercise can lead to significant reductions in human skeletal muscle mtDNA content, which might function as a signal stimulating mitochondrial

Table 1. Comparison of Standardized Mortality Ratio (SMR) for all causes and specified causes in males and females between Palermo City, Cefalù District and Petralia Sottana District, period 2004-201 (respect to whole Sicily).

MORTALITY		Cefalù District	Petralia Sottana District	Palermo City
		SMR (95%C.I.)	SMR (95%C.I.)	SMR (95%C.I.)
For All Causes	Male	89.4 (85.5;93.4)	85.1 (80.8; 89.6)	107.2 (105.8; 108.5)
	Female	87.2 (83.4; 91.1)	90.5 (86.0; 95.2)	102.4 (101.2; 103.7)
Diabetes Mellitus	Males	57.1 (43.3; 74.0)	79.6 (61.0; 102.1)	115.2 (108.7; 121.9)
	Females	69.8 (56.6; 85.3)	115. 9 (96.0; 138.6)	96.1 (91.3; 101.0)
Circulatory System Diseases	Males	90.3 (84.2; 96.7)	91.7 (84.7; 99.1)	97.2 (95.2; 99.3)
	Females	85.7 (80.3; 91.3)	92.4 (85.9; 99.2)	90.6 (89.0; 92.3)
Ischaemic Heart Diseases	Males	75.8 (65.6; 87.0)	76.3 (64.7; 89.3)	101.0 (97.4; 104.8)
	Females	51.0 (41.9; 61.4)	74.8 (61.9; 89.5)	90.4 (86.7; 94.2)
Respiratory Diseases	Males	78.7 (66.2;93.0)	59.4 (47.2; 73.7)	108.2 (103.3; 113.1)
	Females	71.9 (56.8; 89.7)	70.7 (53.4; 91.8)	112.3 (106.4; 118.4)
Lung Cancer	Males	78.5 (65.5; 93.3)	52.7 (40.7; 67.2)	121.7 (116.9; 126.7)
	Females	68.2 (44.1;100.7)	29.6 (12.7; 58.3)	151.2 (140.5; 162.4)
Breast Cancer	Males	-	-	-
	Females	71.9 (54.2; 93.6)	72.0 (51.5; 98.1)	95.3 (89.4; 101.4)

Font: DASOE (Department for Health Activities and Epidemiological Observatory Health Department - Sicily Region.) Health Atlas of Sicily 2004-2011 Monographic supplement, April 2012.). A district is an aggregation of municipalities used for health purposes.

Cefalù District (45274 people, 21974 males and 23300 females) includes nine small towns: Campofelice di Roccella, Castelbuono, Cefalù, Collesano, Gratteri, Isnello, Lascari, Pollina, San Mauro Castelverde; Petralia Sottana District (27546 people, 13121 males and 14425 females) includes nine small towns: Alimena, Blufi, Bompietro, Castellana Sicula, Gangi, Geraci Siculo, Petralia Soprana, Petralia Sottana, Polizzi Generosa. Palermo city (655875 people, 311121 males and 344754 females).

Statistical significance of SMRs has been explored by using the 95% confidence interval (data in the text).

biogenesis with exercise training and this component can positively influence health [15].

Older people may be encouraged to increase their activities if influenced by clinicians, family or friends, facilitating group-based activities and raising self-efficacy for exercise [16].

3. GENETICS OF LONGEVITY: APOE AND FOXO3A

LLIs are genetically predisposed to reach extreme ages. There are many possible candidate genes for human longevity, however, of the many genes tested, only APOE and forkhead box o3 (FOXO3*)* survived on association in independent populations [7, 17].

APOE expresses Apolipoprotein E, the principal cholesterol carrier that drives lipid transport and repairs injuries in the brain, and plays a central role in plasma lipoprotein metabolism and in lipid transport within tissues. APOE shows a genetic polymorphism determined by three common alleles known as ε2, ε3 and ε4, defined by combinations of genotypes of the single nucleotide polymorphisms (SNPs) *rs7412* and *rs429358*. *APOEε2* frequency fluctuates with no apparent trend while *APOEε3* is the most frequent allele in all the human groups, especially in populations with a long-established agricultural economy. The product of the three alleles differs in several functional properties. The frequency of the ancestral allele, *APOε4,* is higher in countries where foraging still exists, or food supply is (or was until the recent past) scarce and sporadically available. This allele, linked to elevated cholesterol and pro-inflammatory activity, is strongly associated with AD and to a lesser extent, with CVD. This allele could be identified as a 'thrifty' allele. The exposure of people carrying the *APOε4* allele to the new environmental conditions of the Western world should render them more prone to develop AD and CVD. *APO ε4 allele* has emerged as a negative candidate gene in longevity since Schachter *et al*. showed that French centenarians have a very low frequency of this allele. In addition, an increased frequency of the allele *APOEε2* in centenarians was observed [17-19].

Due to our small sample, we did not observe an association of *APOε2* allele with longevity. However, considering the detrimental *APOε4* allele, we did not find this allele in our LLI population.

Several studies have noted specific *FOXO3* SNPs associated with human longevity, particularly with *FOXO3A rs2802292* G-allele (G>T). Some studies support the role of *Daf-16* (ortholog of FOXO) in *C. elegans* longevity by protecting cells from oxidative stress that constitutes a nerve centre in ageing, increasing life-span [20]. Daf-16 is a transcription factor (TF) that modulates the expression of SOD2, acting as a free radical scavenger [21]. The role of FOXO3A in humans might be the same, acting as a TF on multiple homeostatic genes in response to decreased insulin/IGF-1 signalling and consequently increasing life-span [22]. It is conceivable to speculate that hyper or hypoactivation of this signalling pathway, due to genetic mutations that under or overexpress regulative molecules, leads to different expression of homeostatic genes.

A meta-analysis of over 7900 cases and 9500 controls confirmed the association of the G allele of the SNP *rs2802292* with exceptional longevity, especially in males [23]. This datum confirms the results of a previous one, including the sex-specific differences in the association of a genetic variation with survival in old age [24]. This is not surprising because males and females follow different strategies to attain longevity and several association studies show positive results only in males [25, 26]. The reason is multifactorial, with a socio-cultural component distinguished from biological trait linked to longevity.

In our analysis of this SNP in Sicilian LLIs, we did not observe an association with longevity, likely because of the relatively small number of LLIs. Another possibility refers to the diet of Sicilian LLIs, rich in vegetables and fruits and poor in refined sugars, responsible for a hypoactivation of insulin/IGF-1 pathway. However, analyzing survival to extreme longevity in four centenarian studies, Bae *et al*. showed that their results confirmed the previous association of common variants of *FOXO3* with older age, but these common variants did not modify the risk for mortality at ages beyond the oldest 1 percentile age of survival [27].

These two longevity-associated genes have been related to cardiovascular health. As previously stated, *APOE* has been linked to cardiovascular disease, but also *FOXO3A* has been implicated in coronary heart disease [18, 23].

Therefore, it is not surprising that in male Sicilian centenarians, recruited in a precedent survey, we observed a higher frequency of the anti-inflammatory alleles of CC chemokine receptor 5, 5-lipoxygenase, cyclo-oxygenase 2, Toll-like-receptor-4 and cytokine genes that protected them from CVD [28]**.** Genetic pro-/anti-inflammatory variations in innate immune response influence the susceptibility to all age-related diseases since these diseases have an inflammatory component. Pathogen load determines the type and intensity of inflammatory responses, according to the pro-inflammatory status and tissue injury, implicated in the pathophysiology of these diseases [29]. Adequate control of inflammatory responses might reduce the risk of these diseases, and, reciprocally, might increase the chance of extended survival in an environment with reduced pathogen load [30].

These data concern two essential points closely related to the achievement of successful ageing, *i.e.* the control of inflammation responsible for the development of age-related diseases and the control of nutrient sensing pathways (NSPs) [22, 31]. Control of inflammation can be pursued in different ways: i) anti-inflammatory treatments with statins and non steroidal anti-inflammatory drugs could be useful to counteract and reduce the age-dependent inflammatory status, preventing the development of age-related inflammatory diseases [32]; ii) probiotic treatment might also be useful since optimal gut microbiota plays a role as an anti-inflammatory agent [33]; iii) physical exercise since exercise-deprivation induces a cluster of physiological abnormalities, similar to MS (such as insulin resistance, impaired glucose uptake and hyperlipidemia) [34]; iv) anti-inflammatory diet, i.e. diet rich in fruit and vegetables and poor in meat and in refined sugar [35].

Diet rich in fruit and vegetables and poor in meat and in refined sugar also targets NSP responsible for downregulation of the signals that leads to the inhibition of FOXO, favouring the transcription of homeostatic genes involved in survival and longevity. Nutraceuticals, defined as "naturally derived bioactive compounds found in foods, dietary supplements and herbal products", are constituents of different dietary patterns, such as the Mediterranean and the Asiatic diets, and can modulate NSPs. They explicate their action as hormetins, activating cellular stress response pathways, like the nuclear factor (erythroid-derived 2)-like 2, and leading to the transcription of antioxidant genes [22].

In this context, extra virgin olive oil (EVOO) is a nutraceutical and functional food, an essential element of the MedDiet. Thanks to its bioactive compounds, EVOO modulates different processes linked to ageing and age-related inflammatory diseases [36-38].

4. PHENOTYPIC SIGNATURE

4.1. Hematochemical Values

A study published in 2008 evaluated laboratory parameters in a sample of 120 healthy centenarians and 381 old persons (between 65 and 85 years old) of Sicilian and Italian ancestry. Significant differences were observed in blood glucose, cholesterol and platelet levels, reduced in centenarians as compared to the old subjects, whereas blood urea nitrogen levels were found to be significantly increased in centenarians [39]. In the ongoing survey on Sicilian LLIs (mean age 101.3±4.9), preliminary results suggest no differences concerning lipid profile, glucose and insulin levels when compared to young (mean age 30.7 ±4.8) individuals, whereas creatinine was increased. As expected, several differences were observed between young and elderly (not LLIs), concerning albumin, insulin, and glycaemia (Ciaccio, Caldarella and Caruso, unpublished observations).

Unfortunately, the existing range of laboratory parameters is often not restricted for age with a possible under or overestimation of some values. The reason is due to the ageing process that itself is characterized by a low-grade chronic inflammatory status called inflamm-ageing [31] and reduced response to adaptive mechanisms. We hope that at the end of our study, there will be available reference values for old and oldest old people, allowing a better understanding of health or diseased states of these subjects.

4.2. Body Composition

In young and adults, the accumulation of abdominal fat mass is a risk factor for all-cause and cardiovascular mortality [40-42].

The progressive dysfunction of the white adipose tissue is an important hallmark of the ageing process, which in turn contributes to metabolic alterations, multi-organ damage and a systemic pro-inflammatory state. Obesity shares numerous biological similarities with the normal ageing process such as chronic inflammation and multi-system alterations. There is an interplay between accelerated ageing related to obesity and adipose tissue dysfunction; 'adipageing' illustrates the common links between ageing and obesity [43].

The obesity paradox is an inverse or null relationship between overweight and obesity and, conversely, a protection from fat accumulation, observed in elderly [44-49]. The characteristics of fat distribution in elderly involve the reduction of lean mass and redistribution of adipose tissue, thus maintaining the same body mass index (BMI) [50]. This remodelling of body composition and distribution leads to the hypothesis that the BMI range 18.5-24.9 (for a person with normal weight) could not be suitable for LLIs in which <23 values were associated with a greater mortality risk. This datum was not observed in the younger population in the overweight range [51].

The analysis of body composition (fat mass, fat-free mass, and total body water divided into intra and extra cellular) by bioimpedance (BIA) is a non-invasive technique to monitor health status. Its variation seems to be linked to oedema, response to a specific drug, clinical treatment, and the onset of age-related diseases, sarcopenia [52].

We performed an anthropometric evaluation with BIA 101 in several individuals (mean age of 101.5) recruited in the ongoing survey1 (Fig. **1** and Table **2** show the results of typical studies). The preliminary results demonstrate that this population has a mean BMI of 24.35 Kg/h2, and mean phase angle of 3.2° (mean ref. val. 6.5°). Na^+/K^+ ratio of subjects was above 1. All people followed a strict MedDiet during ageing but not at present. In aged people, abdominal fat accumulation is often a marker of resilience, better

functional reserve and lower subclinical disease prevalence, characterizing the "healthy cohort" effect.

The limit of BIA is the absence of a range of values for restricted population, as LLIs, with different body composition. One of the aims of our study was to identify a longevity anthropometric phenotype. Results of studies on adults and younger elderly led to an over- or underestimation of risks. Understanding the changes in body composition and distribution with ageing and their health implications is important for nutritional support and pharmacological treatment and for the development of appropriate health guidelines for the elderly.

4.3. Immunophenotype

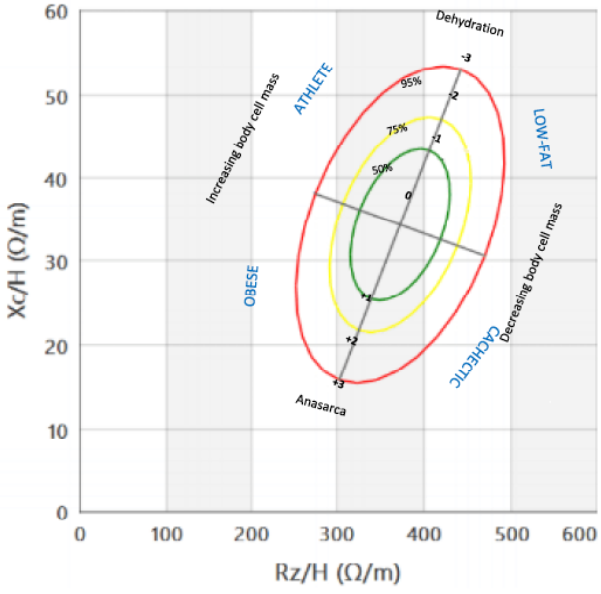
In the elderly, many alterations in innate and acquired immunity have been described and viewed as deleterious, as defined by the term "immunosenescence". Immunosenescence is a complex process involving multiple reorganizational and developmentally regulated changes, rather than the simple unidirectional decline of the whole function. Some immunological parameters are reduced in the elderly and, reciprocally, good function is closely related to health status. Whereas innate immunity is relatively well preserved in the elderly, acquired immunity is more susceptible due to both the functional decline associated with the passage of time and antigen burden to which an individual has been exposed during his/her life. This determines an increase in memory cells and a decrease in naïve cells able to fight new infections [53].

The centenarian's immune system shares characteristics of both young and elderly people. In our ongoing survey, we observe an increase in a subset of NK $CD56^{dim}CD16^{+}$ characterized by increased cytotoxicity, supporting the hypothesis that a well-preserved cytotoxic activity of NK cells represents a biomarker of healthy ageing and longevity. T cells show an increasing trend

Table 2. The table shows some anthropometric and bioelectrical impedance values of the two subjects depicted in Figure 1. Height and weight were measured barefoot and wearing light clothes. Body mass index (BMI) was calculated as weight (in kg) over height squared (in square meters). Fat Mass, free fat mass, Rz (resistance), Xc (reactance) and PhA (phase angle) are obtained by bioelectrical impedance.

Parameters	Centenarian	Old Subject
Age (year)	101.6	68
Sex	Male	Male
Height (cm)	160	180
Weight (Kg)	62.0	92.5
BMI (Kg/m^2)	24.2	28.5
Fat Mass (%)	27.5	41.3
Free Fat Mass (%)	72.5	58.7
Rz (Ω)	541	607
Xc (Ω)	32	35
PhA (°)	3.4	3.3

A)



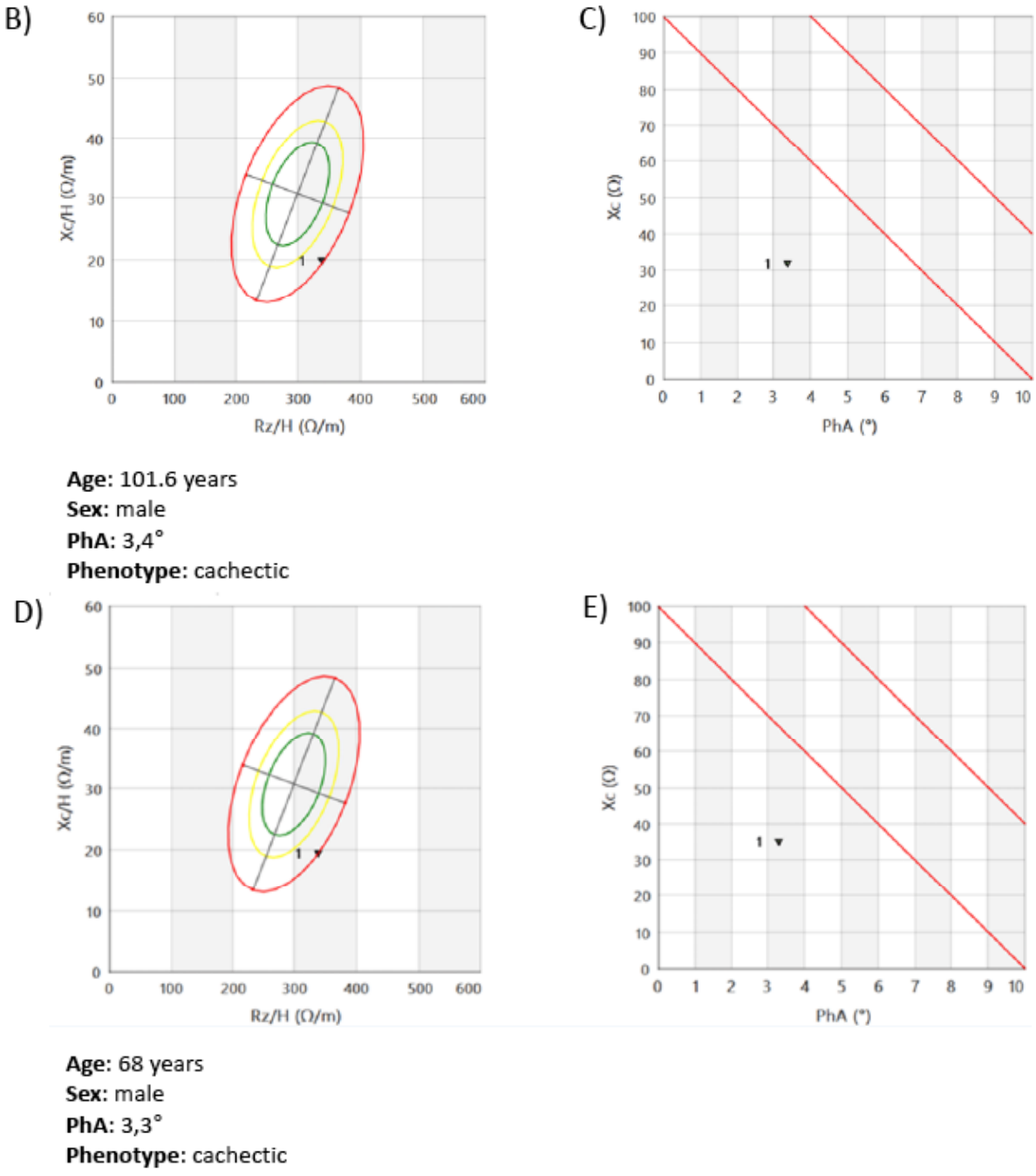


Fig. (1). Figure depicts reference nomogram, one of centenarian male and one of old man. The nomogram, or nomograph, is a two-dimensional diagram. It is a graphical, qualitative representation of a bi or multiple variables function. To get a nomogram, skin electrodes are placed on hand and foot of the same side of body, applying low-voltage and giving two-measures: resistance (Rz), indirectly correlated with amount of body fluids (the higher is Rz, the lower is total body water), and reactance (Xc, directly correlated with cell density in tissues) by human tissues, associated with body composition. A vector quantity, phase angle (Pa), is obtained from Rz and Xc. It permits to evaluate the quality of cell, depending on membrane integrity and body cell mass. Lower Pa is associated with low Xc and cell death or breakdown in selective permeability of membrane (people with low Pa have higher Na+/K+ ratio). The Fig. **A** shows reference nomogram, the Fig. **B** nomogram of centenarian male and Fig. **D** that of old man. Comparing **B** and **D**, referring to **A**, it is possible to speculate that both are cachetic and have similar body composition, although one is centenarians and other is elderly. The Fig. **C** and **E** show the phase angle for centenarian and for old, respectively. Also in this case it is possible to highlight similar results (3.4 and 3.3 respectively).

when compared with elderly, suggesting that this can be considered a predictive marker of longevity. This does not occur for B cells that are decreased, because of the preferential commitment of hematopoietic aged stem cells to the myeloid lineage [54, 55]. A limitation of these studies is the lack of appropriate controls, thus it is better to study centenarian offsprings (CO), which have a significant survival advantage compared to an appropriate control group, *i.e.* age-matched elderly whose parents died at an average life expectancy. In the previous survey, we performed several studies on Sicilian CO with the aim to track immune signatures in CO to test

the hypothesis that these individuals might have an immunological advantage, which may explain their longevity [56, 57].

Our findings documented that CO show significant positive differences in the numbers and proportions of both early- and late-differentiated $CD4^+$ and $CD8^+$ T cells, as well as potentially senescent $CD8^+$ T cells when compared with appropriate controls. This suggests that the acquired T-cell arm of the immune system is more "youthful" in CO than in controls. This might reflect a better ability to mount effective responses against newly encountered antigens, thus contributing to better protection against infection and to greater longevity. Also concerning the B branch, CO does not have the typical trend of memory/naïve B cell subsets observed in elderly people and this is in agreement with the higher levels of IgM in the serum of CO in comparison with data obtained in age-matched controls. This reservoir of naïve B cell might be another cause that makes CO able to keep fighting off new infections, hence prolonging their life [57].

The balance between positive and negative signals dictates the fate of individual T cells and the immune response [58]. Inhibitory molecules play an important role in regulating T cell activation and peripheral tolerance, in particular, the CD28 family, the major regulator of this critical balance. Cytotoxic T-Lymphocyte Antigen(CTLA)-4 is a component of the negative homeostatic control mechanism regulating T cell activation, mediating its inhibitory effects through the coordinated actions with CD28. PD-1 is induced on peripheral $CD4^+$ and $CD8^+$ T cells, B cells, and monocytes upon activation. Programmed cell death protein(PD)-1 signals inhibit T cell proliferation and Interleukin-2 production. CTLA-4 and PD-1 regulate the inhibition and fine-tuning of T cell responses and are used in boosting anti-cancer immunity. Further studies of their functions might be of great therapeutic value in boosting antimicrobial immunity and vaccine responses during ageing [59, 60]

CONCLUSION

In a heterogeneous population, such as the human population, the ability to maintain an adequate response to stressors within a range compatible with a state of good health should have a Gaussian distribution. Centenarians should be the extreme tail of this curve, representing the individuals able to maintain an adequate response to stressors and to repair the damage. They are the individuals better adapted to environmental conditions. In the generation studied here, these factors are represented by inflammatory age-related diseases as cardiovascular ones.

Success in increasing longevity in laboratory organisms has shown that ageing is not an immutable process. The time has come to make serious efforts to slow human ageing or to age successfully. As widely discussed in this review, the explanation of the role of genetics and lifestyle in longevity can provide important information on how to develop drugs and/or behaviours that can slow down or delay ageing. It will be possible to understand, through a "positive biology" approach that seeks to understand the causes of positive phenotypes, trying to explain the biological mechanisms of health and well-being, how to prevent and/or reduce elderly frailty and disability.

Interventions to slow ageing in humans have been the focus of biogerontology in the last decades. We know that different types of dietary restrictions are the possible solutions to increase lifespan in healthy condition. Physical exercise also contributes to control body weight and hematochemical parameters, maintaining them in the normal ranges. The human being is not a model animal, thus it is not easy to verify the efficiency of these approaches because few biomarkers have been identified to characterize healthy ageing, especially for LLI.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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3.3. Analysis of T and NK cell subsets in Sicilian population from young to supercentenarian: the role of age and gender

(Accepted)

Analysis of T and NK cell subsets in Sicilian population from young to supercentenarian: the role of age and gender

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Running title: Lymphocyte subsets from young people to supercentenarian

ABBREVIATIONS

CM, central memory

CMV, cytomegalovirus

EBV, Epstein-Barr virus

EDTA, ethylene diamine tetraacetic acid

EM, effector memory

FSC, forward scatter

NK, natural killer

PBMC, peripheral blood mononuclear cell

SSC, side scatter

TE, terminal effector

SUMMARY

Ageing dramatically affects number and function of both innate and adaptive arms of immune system, particularly T cell subsets, contributing to reduced vaccination efficacy, decreased resistance to infections and increased prevalence of cancer in the older people. In the present paper, we analysed the age-related changes in the absolute number of lymphocytes in 214 Sicilian subjects, and in the percentages of T and NK cells in a sub-cohort of donors. We compared these results with the immunophenotype of the oldest living Italian supercentenarian (111 years old). The results were also sorted by gender. The correlation between number/percentage of cells and age in all individuals and, separately, in males and females, was examined using a simple linear regression analysis. We did not record the increase in the rate of inversion of the CD4/CD8 *ratio* frequently reported as associated with ageing in literature. Our observation was the direct consequence of a flat average trend of $CD4^+$ and $CD8^+$ T cell percentages in ageing donors, even when gender differences were included. Our results also suggest that $CD4^+$ and $CD8^+$ subsets are not affected equally by age comparing females with males, and we speculated that gender may affect the response to CMV infection. The supercentenarian showed a unique immunophenotypic signature as regards the relative percentages of her T cell subsets, with $CD4^+$ and $CD8^+$ T cell percentages and $CD4^+$ naïve T cell values in line with those recorded for the octogenarian subjects. This suggests that the supercentenarian has a naïve "younger" T cell profile comparable to that of a >90 year old female.

INTRODUCTION

The immune system undergoes a complex and progressive functional remodelling during the ageing process. Age-related alterations have been documented in both innate and acquired arms of the immune system, where some immune responses are diminished, leaving others unchanged or increased[1,2]. One of the most commonly mentioned hallmarks of an aged immune system is a substantial decrease in naïve $CD4^+$ and $CD8^+$ T cells and their differentiation into memory lymphocyte subsets[2]. This phenomenon is the result of events (thymic involution and life-long antigenic stimulation) that can occur independently, but also converge in the achievement of an "experienced" T cell profile, *i.e.*, low proliferating cells that are highly active in effector cytokine production upon antigenic stimulation[3]. The involution of the thymus, the central organ of T-cell generation, starts at the time of puberty and is the main responsible for naïve T cell decline in the periphery and lymphoid organs during ageing[2,4]. While $CD4^+$ and $CD8^+$ naïve T cell percentages generally decline with age, gender-related differences were demonstrated. In particular, females have shown higher frequencies in both $CD4^+$ and $CD8^+$ naïve T cells, probably due to a more elevated thymic function compared to males[5].

Chronic stimulation by persistent viral infections, such as human cytomegalovirus (CMV) and, to some extent Epstein-Barr virus (EBV), could play a role in shaping age-related immune function, driving clonal expansion of specific T cells through repetitive stimulation. CMV is responsible for a persistent and latent viral infection that rarely causes obvious disease [6]. However, CMV seropositivity in older people has been included into an "immunological risk profile" predicting mortality[7] (see also below). Phenotypically, CMV is thought to be responsible for increasing the presence of effector memory (EM) and terminally differentiated effector (TE) T cells with phenotypic and

functional features of replicative senescence, especially in the cytotoxic compartment[8,9]. Indeed, as CMV infection is mainly controlled by cytotoxic T cells, an accumulation of high numbers of CMV-specific senescent $CD8^+$ T cells was observed with each cycle of viral reactivation[4]. A similar but slighter effect on clonal expansions of $CD8^+$ T cells has been observed in response to EBV infection in the Swedish population[7], although it does not seem to be confirmed in older people from West-Sicily[10]. In these subjects, the percentage of EBV-specific $CD8^+$ cells was significantly lower than in young, showing decreased levels of CD27 and CD28, but no increase in CD45RA[10].

Overall, the expansion of late-differentiated $CD8^+$ T cells with ageing has been associated with an age-related increase in the rate of inversion of the CD4/CD8 *ratio* (*i.e.,* <1 vs. an expected *ratio* between 1-5 and 2.5 or more) in older people[11]. This altered *ratio* is accordingly considered as a marker of frailty risk [7,12,13].

It becomes evident that the depicted alterations represent the direct consequence of lifelong antigen exposure or the reflection of the progressive adaptation and compensation of the individual to environmental *stimuli*. Overall, they are implicated in the increased frequency and severity of infections, cancer, and lowered responses to vaccination in older people, *i.e.,* the phenomenon called immunosenescence[1,2,7].

Innate immunity is also affected by age-related changes, as demonstrated by the increase in the overall absolute number and percentage of natural killer (NK) cells in the periphery in healthy older people. This reflects a decrease in cytokine-producing CD56 high ($CD56^{hi}$) cells and the increased presence of cytotoxic CD56 low ($CD56^{lo}$) cells[14-16].

Centenarians, individuals who have reached 100 years of age, are considered the best model of successful ageing as they avoid, delay or overcome age-related diseases, such as cancer, neurodegenerative and cardiovascular diseases[17]. Their relatively good

state of health implies a surprisingly active immune system, which has been therefore extensively analysed worldwide[18-20]. Becoming a centenarian was a rare phenomenon only a few decades ago. However, improvements in medical care and quality of life have led to a reduction in morbidity and mortality, with a consequent increase in life expectancy[17].

According to the database from the Italian National Institute of Statistics, as of January 1, 2019, in Italy the number of centenarians (100+ years old) was 14,456 (84% females), semi-supercentenarians (105+ years old) were 1,112 (87% females) and supercentenarians (110+ years old) were 21, representing a doubling in these numbers compared to 2009[21]. At the time of revising this paper, according to the Italian supercentenarians database, the validated supercentenarians are 12 and the oldest living person in Italy is a woman 111 years old, born and living in Sicily[22].

These demographic data show a significant gender difference in life expectancy, underlining the importance of a global analysis of an ageing-environment/gender interaction. Despite their biomedical relevance, gender differences seem to be still poorly considered and inadequately investigated in ageing studies[23].

Here, we investigated age-related alterations in the absolute numbers of lymphocytes among 214 Sicilian subjects (22-111 age range) and in the percentages of circulating lymphocyte subsets of 41 Sicilian donors, between 25 and 111 years of age, focusing on T and NK cells. Blood cells from a subgroup of 27 healthy donors including the oldest living Italian supercentenarian were used for a more complete dissection of T cell subsets. Data were also analysed according to gender. To investigate whether the differences observed in T cell subpopulations are attributable to previous CMV and EBV infections, the same cohort was screened for CMV and EBV seropositivity.

MATERIALS AND METHODS

Study cohort

Participants were recruited between 2017 and 2020 within the project "Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities (DESIGN)", funded by the Italian Ministry of Education, University and Research. The Ethics Committee of Palermo University Hospital (Sicily, Italy) approved the study protocol (Nutrition and Longevity, No. 032017). The study was conducted in accordance with the Declaration of Helsinki and its amendments. All study participants (or their caregivers) gave their written informed consent prior to enrolment. A total of 214 healthy donors (females: 121; males: 93) were recruited. All study participants were Sicilians, selected on the basis of their health status and aged between 22 and 111 years. Exclusion criteria were immunodepression, stroke, cancer, or the use of immunomodulatory drugs within the previous six months. In addition, on July 13, 2020 Mrs. Maria Oliva, the oldest living person in Italy, according to the Italian supercentenarians database[22], was recruited. To respect the privacy, all other donors were identified with an alphanumeric code. A database was created to handle the collected information. The participants underwent venipuncture in the morning, after a fasting period of 12 hours. The blood was collected in specific tubes containing ethylene diamine tetraacetic acid (EDTA) or no additives. Serum was separated by blood centrifugation of dry tubes and stored at -80°C before use. For more information about recruitment criteria, please see ref. n. 24.

Haematological parameters analysis

Whole blood was used for automated absolute leukocyte counts of 214 Sicilian subjects (22-111 age range), 121 females (mean age 68.82 years ± 26.26 SD), and 93 males (mean age 63.62 years ± 22.67 SD). Leukocyte, lymphocytes, neutrophil, and monocyte absolute numbers were counted at the Unit of Transfusion Medicine of University Hospital "Paolo Giaccone", Palermo, Italy.

Flow Cytometry analysis

Flow cytometry analysis was performed in a subgroup of 41 subjects (23 females and 18 males), aged 25-111 years. Peripheral blood was processed fresh to determine complete blood counts and to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were isolated at Laboratory of Immunopathology and Immunosenescence of the Department of Biomedicine, Neurosciences and Advanced Diagnostics (BiND) of University of Palermo (Italy) from whole blood, using Ficoll-Paque (GE Healthcare, South Plainfield, NJ, USA) density gradient centrifugation according to the manufacturer instructions within 6 hours. PBMCs were frozen in 90% foetal bovine serum and 10% DMSO and stored at −80 °C. Part of the samples were sent on dry ice to the King's College London, School of Cancer & Pharmaceutical Sciences, The Rayne Institute, where they were cryopreserved in liquid nitrogen tanks (−180 °C) up to the day of analysis. The remaining sample were stored at -70 °C in the Laboratory of Immunopathology and Immunosenescence for a few days until their characterization at the Specialistic Oncology Laboratory Unit of ARNAS Civico Hospital of Palermo.

For cell thawing, the cryopreserved vials were first transferred to a 37 °C water bath and washed with X-Vivo 15 (BioWhittaker, Walkersville, Maryland, USA). After centrifugation, the supernatant was discarded and PBMCs were resuspended in X-Vivo

15 for counting. Counts and viability were determined with a haemocytometer and trypan blue dye exclusion technique. With this method, dead cells appear blue and are distinguishable from viable cells. An average of >90% live cells were obtained for each count round.

For the analysis of T and NK cell subsets, PBMCs were thawed, washed, and counted.$1x10^6$ PBMCs were first stained with Fixable Viability Dye eFluor™ 780 (eBioscience, San Diego, California, USA), following manufacturer instructions, and next incubated with various combinations of monoclonal antibodies (Supplementary Table 1). As a negative control, human unstained cells were used. Single stain controls were used for the automatic calculation of the compensation matrix. A minimum of 500,000 cells per sample were analysed in LSR Fortessa (BD Biosciences, San Jose, California, USA), for the samples processed at King's College London (UK), and in Navios EX (Beckman Coulter, Brea, California, USA), for the samples processed in Palermo (Italy).

Lymphocyte subsets were identified through forward scatter (FSC) and side scatter (SSC), and further checked in the SSC/CD45 dot plot. An exemplificative schematic representation of the applied gating strategy for T and NK cell analysis is displayed in Figure 1. After setting the first gate in the FSC/SSC dot plot in the lymphocyte region, events were gated in the CD3/SSC dot plot, as recommended by Rühle *et al.*[25]. T cells were identified as $CD3^+$ events. After exclusion of NKT ($CD3^+CD56^+$) cells, T cells were gated in the CD4/CD8 dot plot to define helper and cytotoxic subsets[25]. $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells were finally explored for CD197 and CD45RA expression in order to describe the fraction of naïve ($CD197^+CD45RA^+$), CM ($CD197^+CD45RA^-$), EM ($CD197^-CD45RA^-$), and TE cells

($CD197^{-}CD45RA^{+}$)[26]. To complete T cell analysis, γδT cells were identified as $CD3^{+}\gamma\delta^{+}$, without distinction between the various subtypes. NK cells were identified in the "non-T" pool of lymphocytes ($CD3^{-}$, including NK and B cells) according to their positivity for CD56[25]. After gating in the CD56/CD16 dot plot, NK cells were classified in NK1 ($CD3^{-}CD56^{lo}CD16^{+}$) and NK2 ($CD3^{-}CD56^{hi}CD16^{-}$) as described elsewhere[25,27].

The analysed lymphocyte subsets are expressed as a fraction of the parental gated population, and reported as percentages in the graphics.

EBV and CMV serology

Specific IgG antibodies to EBV nuclear antigen (EBNA) and CMV in serum of 41 subjects (23 females and 18 males), aged 25-111 years, were quantified by chemiluminescence immunoassay using the LIAISON® EBNA IgG kit (DiaSorin, Saluggia, Italy) and the LIAISON® CMV IgG II kit (DiaSorin, Saluggia, Italy) respectively, as recommended by the manufacturer. Measurements were performed by LIAISON XL (DiaSorin, Saluggia, Italy). The range upper limit was sect at 180 U/mL for anti-CMV IgG and at 600 U/mL for anti-EBV IgG.

Statistical analysis

To analyse the percentages of T, NK, and NKT cells, flow cytometry data were analysed using FlowJo version 10.5.3 (Tree Star, Ashland, OR) and statistical analysis was performed with GraphPad Prism, version 8.1.2 (GraphPad Software, San Diego, California, USA).

Correlation between number/percent of cells and age in all individuals and in males and females were examined using a simple linear regression analysis. Figures were

plotted as scatterplots with a linear regression line and 95% confidence bands. For each statistical analysis, only p values <0.05 were considered significant.

RESULTS

Analysis of haematological parameters

The number of leukocytes in peripheral blood of 214 subjects was analysed according to age and gender. Correlation analysis showed that neither age nor gender affected leukocyte, neutrophil, and monocyte counts (data not shown).

In contrast, there was a significant decline in lymphocyte counts (Figure 2) for males (R^2=0.082, p=0.005) but not for both genders (R^2=0.016, p=ns) or female subjects alone (R^2=0.0005, p=ns).

Analysis of T cell subsets

For the analysis of total $CD4^+$ and $CD8^+$ T cells, PBMCs were isolated from the blood of 41 healthy donors, 23 females (aged 25-111 years) and 18 males (aged 26-102 years), for the analysis of the percentages of T cells. PBMCs from 27 healthy donors (aged 25-111 years), divided by age and gender as represented in Table 1 were used for a more complete analysis of T cell subsets.

To investigate the effects of age and gender on human lymphocyte subsets, the percentages of $CD4^+$ and $CD8^+$ T cells in PBMC pool were determined by flow cytometric analysis using the gating strategy described in Figure 1. The percentage of $CD4^+$ (Figure 3A) and $CD8^+$ (Figure 3B) T cells remained almost constant with ageing, even when gender differences were included in the analysis. In contrast to the reported age-related increase in the rate of inversion of the CD4/CD8 *ratio*[11], a flat average trend

of change in the CD4/CD8 *ratio* with age was recorded in all individuals merged together (Figure 4, R^2=0.010, *p*=ns) and analyzed according to gender (Figure 4, F: R^2=0.007, *p*=ns; M: R^2=0.0001, *p*=ns).

In order to investigate the impact of ageing upon T cell subsets, we examined age-related changes in the markers of differentiation within the $CD4^+$ and $CD8^+$ T cell subsets in PBMCs from a sub-cohort of 27 healthy donors, divided by age and gender as described in Table 1. Gating was performed on the CD4/CD8 dot plot, followed by $CD4^+$ or $CD8^+$ specific sub-gating according to CD45RA and CD197 (CCR7) expression as described above. An example of the applied gating strategy is displayed in Figure 5.

The percentages of $CD4^+$ naïve cells significantly declined with age in the combined group of males and females (Figure 5A, R^2=0.347, *p*=0.001), but this was attributable to a significant decrease in $CD4^+CD197^+CD45RA^+$ cells in female subjects (R^2=0.662, *p*=0.0002), while a constant trend was observed in the older males (R^2=0.039, *p*=ns). These results might suggest that older males were able to better maintain a naïve phenotype of $CD4^+$ T cell subset during ageing. However, it should be taken into account that the young females in our cohort showed a higher percentage of naïve $CD4^+$ T cells (ranging from 33% to 68%, mean 51.38±11.42) than their male counterparts (ranging from 22% to 41%, mean 27.8±7.45). Similarly, the significant increase in EM $CD4^+$ T subset observed in ungrouped individuals (R^2=0.345, *p*=0.001) was mainly ascribable to the female group (R^2=0.496, *p*=0.003), while a significant decrease in CM (R^2=0.163, *p*=0.033) $CD4^+$ T cells was recorded in males (R^2=0.380, *p*=0.024) but not in females. For TE $CD4^+$ T cell subset, no significant ageing dependent effects were detected.

Consistent with other reports[6,7,12,13], in our sub-cohort the percentages of $CD8^+$ naïve cells significantly declined with age (R^2=0.769, *p*<0.0001), being this piece of data

confirmed in both females (R^2=0.816, p<0.0001) and males (R^2=0.700, p=0.0013). Data showed a concomitant significant increase in the percentage of both TE (R^2=0.343 p=0.003; F: R^2=0.548, p=0.004) and EM (R^2=0.448, p=0.0004; F: R^2=0.438, p=0.014; M: R^2=0.482, p=0.018), and a significant decrease in CM (R^2=0.229, p=0.018; F: R^2=0.394, p=0.021) $CD8^+$ subsets (Figure 5B). Although naïve $CD8^+$ cells as well as the less represented CM $CD8^+$ subset declined dramatically with age, the concomitant increase in TE and EM $CD8^+$subsets contributed to the maintenance of an almost constant total $CD8^+$ T cell percentage with age.

An overview of the differences among young donors, older people and long lived individuals (LLI; aged >90 years) in the proportions of T cell subsets is depicted in the pie charts in Figure 5A and B.

In order to evaluate the general effect of persistent viral infection on T cell subset distribution, we screened our sub-cohort of 27 subjects for CMV and EBV seropositivity. As shown in Table 1, all individuals were EBV-seropositive, while 16.6% of young donors and 100% of older subjects and LLI were CMV-seropositive. Thus, it would be plausible to hypothesize that the progressive accumulation of T cells with a TE and EM phenotype in older individuals of our cohort might be directly influenced by CMV infection, but not by EBV infection. However, a CMV-specific T analysis should be conducted to confirm this inference.

Table 1. CMV and EBV serological status of the sub-cohort

Clinical features	Young (*n* = 12)	Older (*n* =9)	LLI (*n* = 6)
Age (Years)			
Mean±SD	28.69±3.47	82.2±2.60	99.55±5.27
Range	25-34	79-88	93-111
Gender			
Female	8 (66.6%)	4 (44.4%)	3 (50%)
Male	4 (33.3%)	5 (55.5%)	3 (50%)
CMV serological status			
CMV+ [n, %]	2 (16.6%; 1 F, 1 M)	9 (100%; 4 F, 5 M)	6 (100%, 3 F, 3 M)
Anti-CMV IgG titer (U/mL) Range	103-160	44-177	160->180
EBV serological status			
EBV+ [n, %]	12 (100%; 8 F, 4 M)	9 (100%; 4 F, 5 M)	6 (100%, 3 F, 3 M)
Anti-EBV IgG titer (U/mL) Range	25.4->600	17.8 ->600	20.48->600

CMV, Cytomegalovirus; EBV, Epstein-Barr virus; F, females; LLIs, long-lived individuals; M, males; n, total number; SD, deviation standard; U/mL, units per millilitre.

Analysis of γδT cells

We also analysed the γδT cell percentage from 25 healthy donors, 14 females (aged 31-102 years) and 11 males (aged 35-101 years), identified by their γδ expression on $CD3^+$ T cells. Correlation analysis in the combined group indicated that the percentage of γδT cells remained unchanged with age (Figure 6). However, when analysed separately, the male and female groups showed a reverse trend in the γδT cell percentages. Specifically, a lower trend in γδT cell percentages with age was observed in the female group, while in the males the fraction of these cells was increased.

Analysis of NK and NKT cells

NK and NKT cells from 40 healthy donors, 22 females (aged 25-101 years) and 18 males (aged 26-102 years) were analysed. NK cells (defined as $CD3^-$, $CD16^{negative\ to\ positive}$ and $CD56^+$) were divided into two subsets based on their CD56 and CD16 expression[25,27]. The $CD56^{lo}CD16^+$ subset (NK1, Figure 8A) is mainly responsible for

natural cytotoxicity by releasing cytoplasmic granules containing perforin and granzymes B. By contrast, the $CD56^{hi}CD16^{-}$ subset (Figure 8B) is described as secreting chemokines and cytokines.

An increase in the percentage of NK cells in healthy ageing has previously been reported, in association with a reduced fraction of $CD56^{hi}$ NK cell subset and an expansion of the $CD56^{lo}$ NK cells[15,16]. Accordingly, NK cell percentages in the peripheral blood of the older people showed a significant increase depending on age (Figure 7, R^2=0.124, p=0.025), becoming not significant when genders were analysed separately.

Besides, when $CD3^{-}CD56^{+}$ cells were subdivided into $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cells, there was a significant increase in cytotoxic NK cells in parallel with increasing age (Figure 8A, R^2=0.184, p=0.006). On the contrary, only a non-significant lower trend in the frequency of cytokine secreting NK cells in the older population was detected (Figure 8B, R^2=0.008, p=ns).

NKT cells, a cell type sharing some functional and phenotypic characteristics with NK cells[28], were determined by their CD3 and CD56 co-expression (Figure 9). Overall, a non-significant age-related increase was observed in the NKT cells (R^2=0.023 p=ns), mainly attributable to the female group.

Comparison with the T subset profile of a supercentenarian

The analyses of T cell subset included a cohort of 41 subjects with the oldest living Italian supercentenarian. In the graphs displaying gender merged analysis, the values for the supercentenarian are reported as a red dot. It is possible to note that in the $CD4^{+}$ T cell percentage graph the red dot (Figure 3, 66.6%) seems to be close at the upper limit of the 95% confidence interval, while in the $CD8^{+}$ T cell percentage graph the same point

(Figure 3, 22.35%) is localized at the lower limit line. The resulting CD4/CD8 *ratio* was 3.03 (Figure 4), just located on the regression line, in contrast to the other dots that are widely scattered around the cohort regression line. The values of both $CD4^+$ and $CD8^+$ naïve T cells for the supercentenarian were lower than those observed in the young females (Figure 5 A,B). However, compared to the individuals aged 90-100 years, the $CD4^+$ naïve T cell value for the supercentenarian (Figure 5A, 20.38%) is above the regression line, as in the case of values recorded for the octogenarian subjects. In contrast, the percentage of EM $CD4^+$ T cells for the supercentenarian (Figure 5A, 65.44%) seems to be above the line as the LLIs, differently from the octogenarian subjects whose values are clustered below. As regards the cytotoxic compartment, our Italian doyenne shows one of the highest values of EM $CD8^+$ T cells (Figure 5B, 74.32%) but a percentage of CM $CD8^+$ T cells (Figure 5B, 1.56%) comparable to their younger counterparts.

DISCUSSION

In the present study, we analysed the effect of ageing on the composition of immune system cell subsets, in correlation with gender and CMV and EBV serostatus, in a cohort of Sicilian donors, ranging from young individuals to a supercentenarian. We set our focus on T and NK cells. Our approach may be considered of some interest as it includes the immunophenotypic characterization of the supercentenarian immune system. Data about supercentenarian immune subsets are very rare in literature and, to the best of our knowledge, have only a transcriptomic focus[29].

In more than 15 years of research in the field, it is well documented that ageing is associated with a decrease in the absolute count of $CD4^+$ T lymphocytes with higher numbers reported for females, expansion of effector/memory $CD8^+$ T cells and

contraction of both naïve $CD4^+$ and $CD8^+$ T cell compartment[12,30-34]. These processes cannot be simply considered as the direct consequence of thymic involution since they are coupled with stimulation exerted by pathogens (especially by latent Herpes viruses) during the whole life of an individual[2,7-9,32,34-38]. The most notable manifestation of the listed events is represented by the inverted CD4/CD8 *ratio*. The probability to detect an inverted CD4/CD8 ratio increases with age and is more prominent in males than in females[11,32,33,39]. As stated in the Introduction, controversies still exist about this altered *ratio* since it is considered to be a marker of risk of frailty, although this association was not found in all populations studied[37].

The results of our study show a constant trend in the percentages of both $CD4^+$ and $CD8^+$ T cells with age. Consequently, we did not observe the described age-related increase in the rate of inversion of the CD4/CD8 *ratio*, whereas this value tends to increase in LLIs, and to decrease in the supercentenarian. As depicted by the linear regression graph, the total $CD4^+$ and $CD8^+$ T cell percentages in individuals aged 90-100 year old appear to exhibit different trends than those recorded in octogenarian subjects. Most 90-100 year old donors show lower $CD4^+$ T cell percentages, in line with data for the supercentenarian, and higher $CD8^+$ T cells percentages than octogenarian donors. It remains to be assessed if these fluctuations in $CD4^+$ and $CD8^+$ T cell percentages are correlated with the probability of reaching a successful ageing. Another possible explanation may be represented by other concomitant causes determining the reduced detection of the CD4/CD8 *ratio* inversion in our pool of donors, like different lifestyle and environmental factors such as diet, gut microbiota, access to health care, exposure to pathogens, pollution as well as genetic background[37].

As mentioned above, an age-related shift from antigen-inexperienced naïve T lymphocytes to antigen-experienced memory and effector T cells has been extensively reported. These phenomena are due to both long-term repeated exposure to antigens together with reduced thymopoiesis [2,5,12,35]. Our analyses demonstrated that age influences diversely lymphocyte subpopulations in the whole sampled population. Similar to previous reports[30-32], ageing was associated with a significant reduction in both naïve $CD4^+$ and $CD8^+$ T cells. Also, a significant reduction in CM $CD4^+$ and $CD8^+$ T cells was observed in our cohort, contradicting those reports recording an age-related increase in both T cell fractions[31,40,41]. Finally, a significant increase in EM $CD4^+$ and $CD8^+$ T cells and in TE $CD8^+$ T cells was observed, once again confirming previous reports documenting that the EM population increase with age[30,40,42].

Age-related differences in T cell subset distribution may partly be explained by the presence of chronic viral infections. Being CMV more frequent in older individuals of our cohort, CMV seropositivity may have influenced the T cell subset specific expansion and survival with age[30,40,42]. On the contrary, EBV infection seems to play no role, since all individuals in this study were EBV-seropositive. Indeed, accumulation of late-stage $CD8^+$ T cells, some of which may indeed be senescent and contribute to age-related diseases[43], are predominantly observed in CMV-seropositive older people, whereas older people infected with other persistent Herpes viruses, as EBV, do not show similar effects seemingly limited to CMV[38]. On the other hand, it has been claimed that a small proportion of CMV-infected individuals is able to counteract the pathological effects of the CMV-related accumulation of these cells, attaining longevity[44], as it seems to be happened in our LLIs including the supercentenarian.

Our data also demonstrated that γδT cells appeared to remain unchanged with increasing age. This is consistent with the demonstration by Argentati *et al*.[45], that the percentage of $CD3^+$ γδT cells in the peripheral blood was heterogeneous in the different age groups, with mean values not significantly different among the young donors, older subjects, and centenarians.

NK cell subsets are known to be differentially affected by ageing with a gradual decrease in the more immature $CD56^+$ NK cells, while the percentage and the absolute number of $CD56^+CD16^+$ NK cells have been variably reported as maintained, increased, or decreased in the older subjects and consistently increased in the centenarians[2,16,19,46,47]. Accordingly, our results showed a significant increase in cytotoxic NK cells with ageing, but no significant lower trend in the frequency of cytokine secreting NK cells in the older population. Finally, the number of circulating NK cells was slightly lower in females than in males.

NKT cells have been described to decrease in older people and increase in centenarians[19]. In our study, a slightly age-related increase was observed in the NKT cells, mainly attributable to the female group.

Besides these not-significant gender-related changes of NK and NKT cells, our data also suggest that the $CD4^+$ and $CD8^+$ T cell subsets are not affected by age in an equal manner between females and males. Indeed, the influence of gender was significant on various lymphocyte subpopulations. First, a significant age-related decline in the lymphocyte counts in males was identified. Males also showed significantly lower percentages of CM $CD4^+$ T cells. The reduction in naïve and the increase in EM $CD8^+$T cells were confirmed as significant in both genders, although the significance is higher in women, especially for the naive compartment. This is in contrast with a previous report[32],

where both very old and middle-aged females showed a higher percentage of naïve $CD8^+$ T cells than males. Females also exhibited a significant decrease in naïve $CD4^+$ and CM $CD8^+$ T cells.

Biological causes such as hormonal differences or the presence of two X chromosomes, in addition to other gender effects, could partially explain the age dependent differences in cell counts and percentages emerging comparing male and female donors in our study[48]. We might also speculate that gender could impact the response to CMV infection. In fact, it is well known that females are more resistant to infections[1,48].

A recent epigenetics study has confirmed that PBMCs of females and males significantly differed after 65 years of age. This analysis revealed that older females have higher genomic activity for B and T cells, thus suggesting that the age-related decline in T cells is greater in men. Older males, instead, have higher activity for monocytes and inflammation[49].

Centenarians are considered a model of successful ageing[17,50,51]. Therefore, supercentenarians, *i.e.* people who have reached 110 years of age, are a great model of successful ageing[29]. Their characteristics of delayed onset of age-related diseases and compression of morbidity[52,53] imply that their immune system remains functional[29]. Here, we analysed the immunological profile of the oldest living woman in Italy (111 years old), born and bred in Sicily, and compared her immunophenotype to that of Sicilian young, older and female LLIs donors.

From the graphs displaying values for naïve and EM $CD4^+$ T cells, it is interesting to note that the octogenarians seem to have higher percentages of naïve $CD4^+$ T cells than nonagenarians and centenarians and the supercentenarian seems to represent a meeting

point between these two groups. Conversely, nonagenarians and centenarians show higher percentages of EM $CD4^+$ T cells than octogenarians, but these values are similar to those reported for the supercentenarian. Our report is limited by the small number of analysed samples. However, it would be interesting to monitor these octogenarian subjects over time to see if with advancing age their immune profile in the context of $CD4^+$ T cells changes like that of their older counterparts and, especially, that of the supercentenarian.

Recently, circulating immune cells of supercentenarians have been analysed at single-cell resolution. This permitted the identification of $CD4^+$ T cells that have cytotoxic characteristics. This feature is truly unique to supercentenarians, as $CD4^+$ T cells generally have helper functions, but no cytotoxic activity[29]. This may represent an essential adaptation to achieve exceptional longevity by sustaining immune responses to infections and diseases.

Thus, an in-depth immunophenotypic and functional characterization of immune subsets of octogenarians followed over time could help to better understand if our experimental evidences, together with previously reported data, could be helpful in predicting the entity of changes affecting the immune system during ageing, and the probability of reaching an advanced age with a functional and adequately adapted defensive repertoire.

In conclusion, ageing dramatically affects both the relative presence and function of the different T cell subsets. These changes are likely to be among factors contributing to the reduced vaccination efficacy and decreased resistance to infections, as well as increased prevalence of cancer in the older population, *i.e.*, immunosenescence[1,2,7]. However, our knowledge of immunosenescence is likely to be still very incomplete. More

detailed data are needed, particularly on the immune phenotype of semi- and super-centenarians, to identify strategies that may counteract the effects of ageing on the immune system. A principal objective of such a kind of studies will be the identification of potential interventions that could reduce the incidence of and morbidity for age-associated diseases by better preservation and stimulation of functional immune competence in the course of chronological ageing.

Supercentenarians show a unique immunophenotypic signature as regards the relative percentages of their T cell subsets. Recent reports also confirm that their immune cells also show previously unknown functional properties[29]. It would be reasonable to think that the key of successful ageing may be encountered in the supercentenarians' uncommon immune characteristics. An internationally coordinated effort would be highly recommendable in order to extend the characterization of supercentenarians' immune system beyond the geographical borders, and to create an immunophenotypic and genomic database to share the details at a worldwide level. Such a type of global commitment may be beneficial in terms of sharing these rare pieces of information and of speeding up the extrapolation of a possible predictive "successful-ageing-profile".

DECLARATIONS

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DISCLOSURE

The authors have no competing interests or conflicts of interest to declare.

Authors contributions

MEL, FP performed statistical analysis of the data and drafted the manuscript. CC revised the manuscript and all authors contributed with the critical revising of the manuscript. MEL, MB, FG, NZ performed the cytometric analyses. FB, GG. performed the serologic analyses. MEL, CC, GC and FF conceived the study. CC was responsible with GA, AA, SA for patient recruitment and characterization. All authors checked statistical analysis of the data. All authors approved the final version of the manuscript

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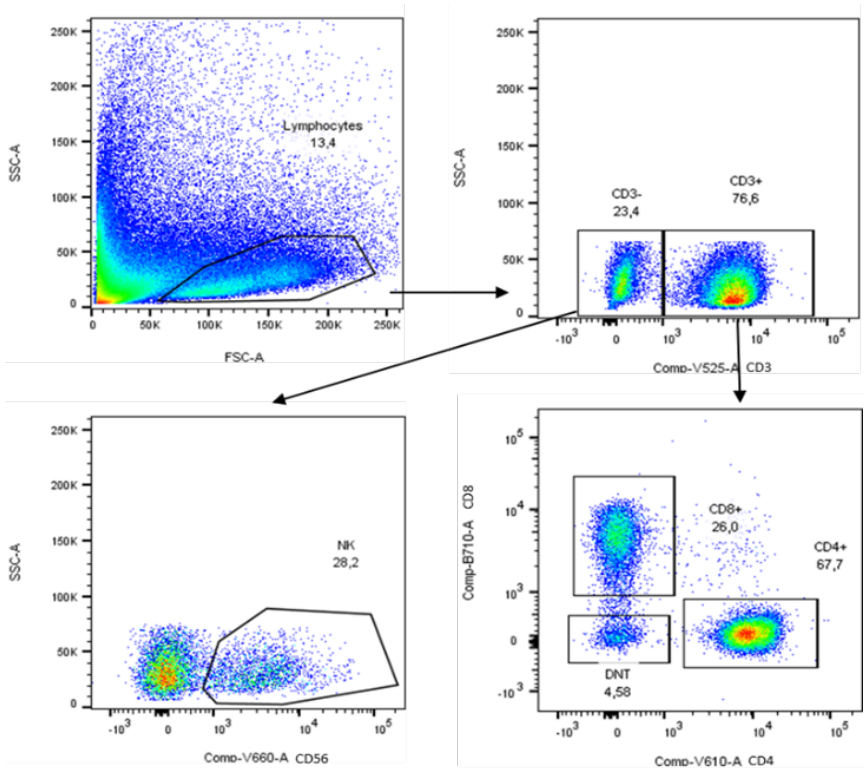


Figure 1. NK and T cell gating strategy. Gating strategies for the analysis of NK, $CD4^+$ and $CD8^+$ T cell subsets. The doublet exclusion on FCS-H vs FCS-A followed by SSC-H vs SSC-A is not shown. A representative donor is presented.

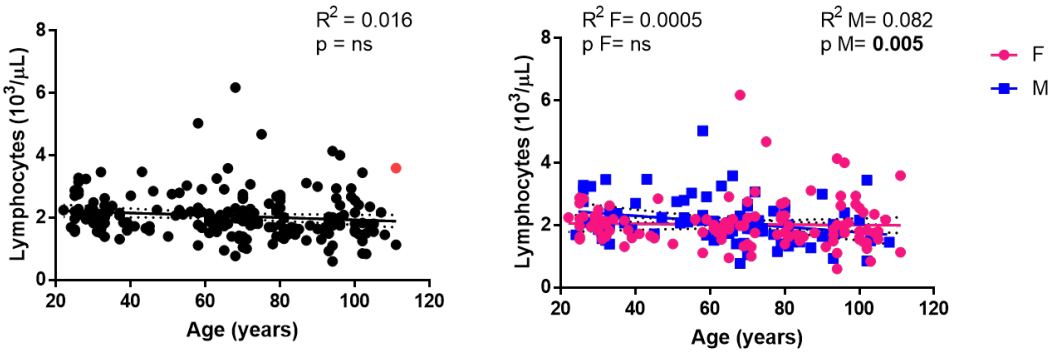


Figure 2. Correlations between lymphocyte counts and age. Linear regression analysis showing the relationship between lymphocyte count (10^3/µL) and age in all individuals (N = 214) (black line), males (N = 93) (blue line) and females (N = 121) (pink line). Each

point represents data from an individual healthy donor. The supercentenarian is shown in red in the graph on the left. The coefficient of determination and *p* values are shown on the graphs. R^2, R squared; ns, not significant; F, female; M, male.

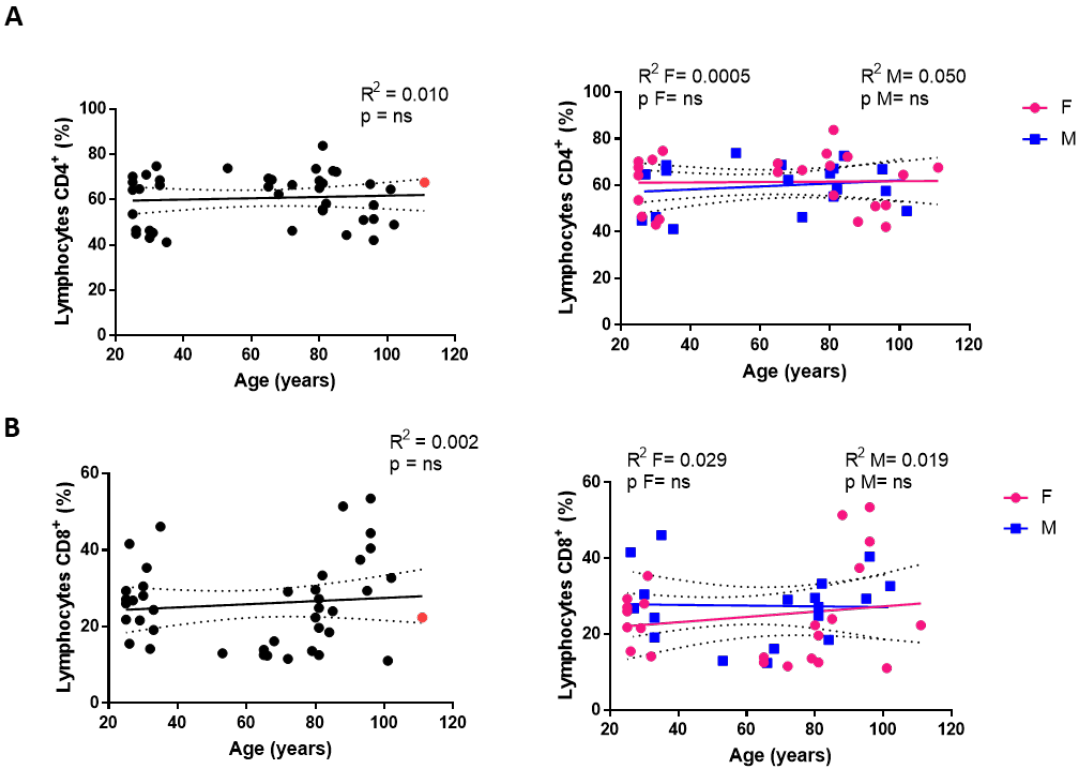


Figure 3. Analysis of CD4 and CD8 T cells. Linear regression analysis showing the relationship between lymphocytes $CD4^+$ % (**A**), $CD8^+$ % (**B**) and age in all individuals (N = 41) (black line), males (N = 18) (blue line) and females (N = 23) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. The supercentenarian is shown in red in the graphs on the left. R^2, R squared; ns, not significant; F, female; M, male.

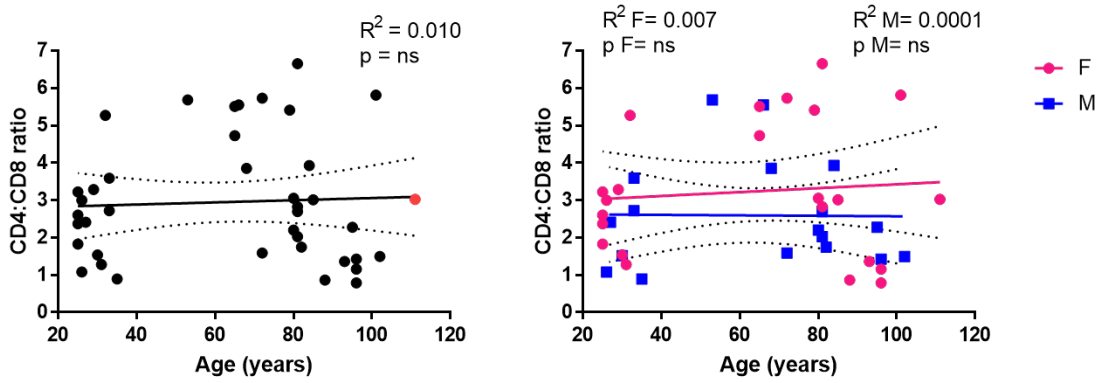
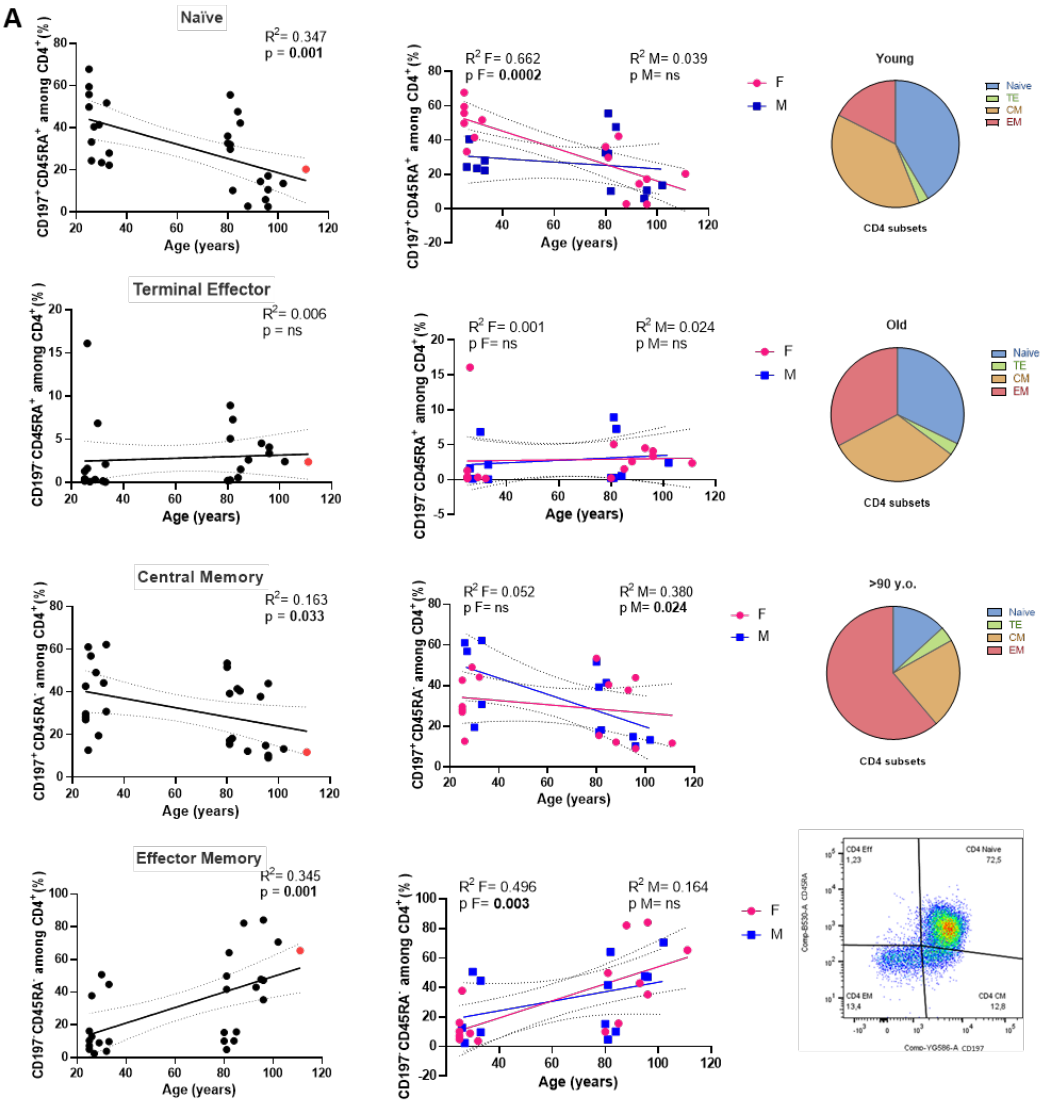


Figure 4. Correlations between the CD4/CD8 *ratio* and age. CD4/CD8 *ratio* of 41 donors (M = 18; F = 23) was calculated by dividing the CD4 T cell percentage by CD8 T cell percentage. The supercentenarian is shown in red in the graph on the left. R^2, R squared; ns, not significant; F, female; M, male.


A
Naïve
R^2= 0.347
p = 0.001
CD197+CD45RA+ among CD4+(%)
Age (years)
R^2 F= 0.662
p F= 0.0002
R^2 M= 0.039
p M= ns
F
M
Young
Naive
TE
CM
EM
CD4 subsets
Terminal Effector
R^2= 0.006
p = ns
CD197 CD45RA+ among CD4+(%)
R^2 F= 0.001
p F= ns
R^2 M= 0.024
p M= ns
Old
Central Memory
R^2= 0.163
p = 0.033
CD197+CD45RA- among CD4+(%)
R^2 F= 0.052
p F= ns
R^2 M= 0.380
p M= 0.024
>90 y.o.
Effector Memory
R^2= 0.345
p = 0.001
CD197 CD45RA- among CD4+(%)
R^2 F= 0.496
p F= 0.003
R^2 M= 0.164
p M= ns
CD4 Eff
1,23
CD4 Naive
72,5
CD4 EM
13,4
CD4 CM
12,8

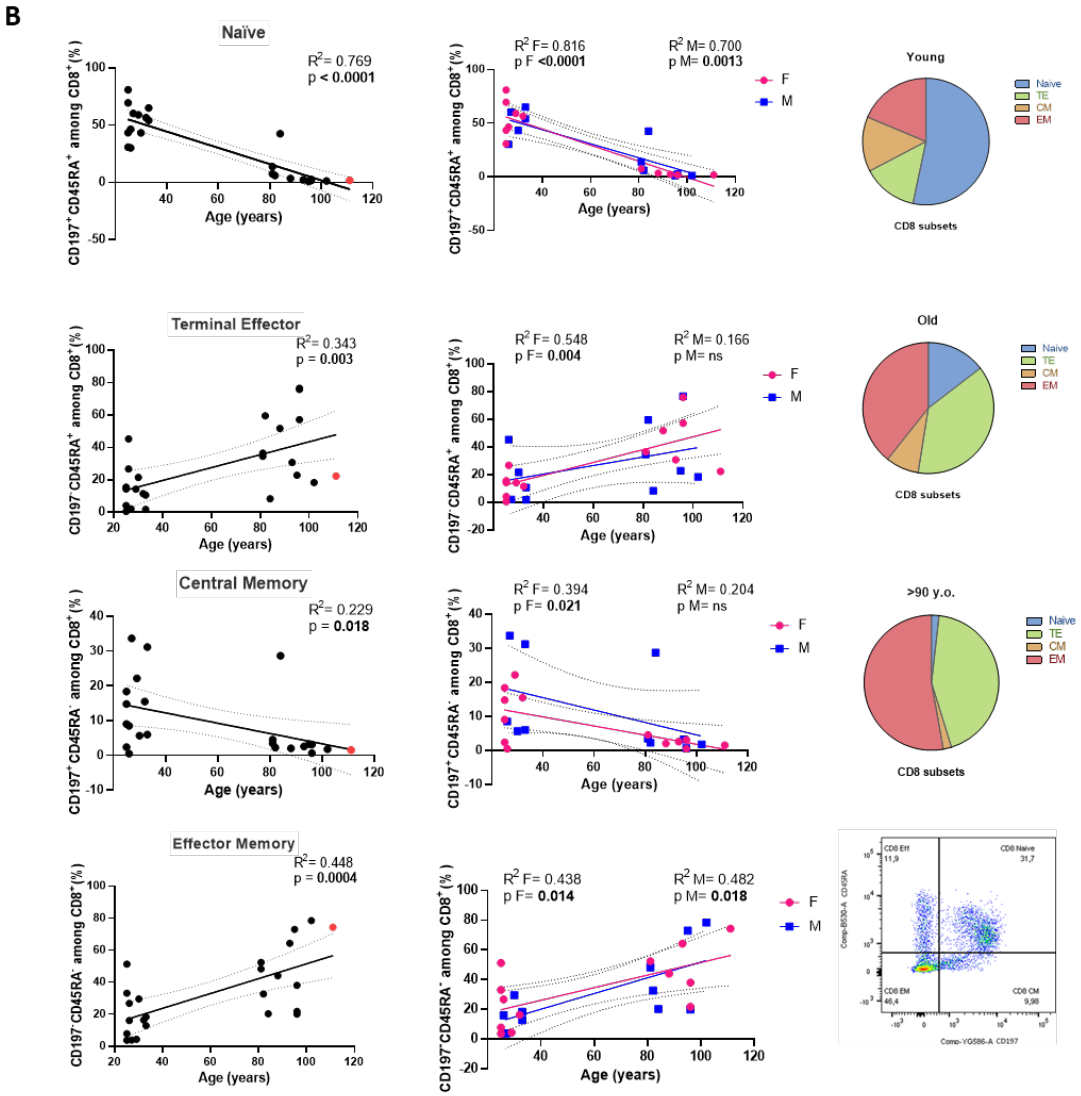


Figure 5. Analysis of CD4 and CD8 T cell subsets. Linear regression analysis shows the relationship between $CD4^+$ (**A**), $CD8^+$ (**B**) subsets and age in all individuals (N = 27) (black line), males (N = 12) (blue line) and females (F =15) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. The supercentenarian is shown in red in the graphs (A,B) on the left. R^2, R squared; ns, not significant; F, female; M, male. Right (**A** and **B**): representative FACS gating of (A) $CD4^+$ and (B) $CD8^+$ subsets. In the pie charts is depicted an overview of the differences among young donors, old people and LLI donors in the proportions of T cell subsets.

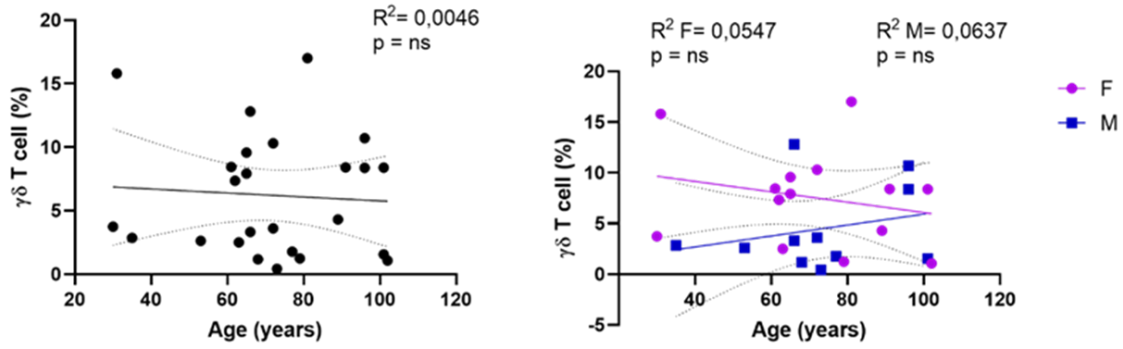


Figure 6. Analysis of γδT cells. Linear regression analysis shows the relationship between $CD3^+$ $\gamma\delta^+$ T cells and age in all individuals (N = 26) (black line), males (N = 12) (blue line) and females (N = 14) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. R^2, R squared; ns, not significant; F, female; M, male.

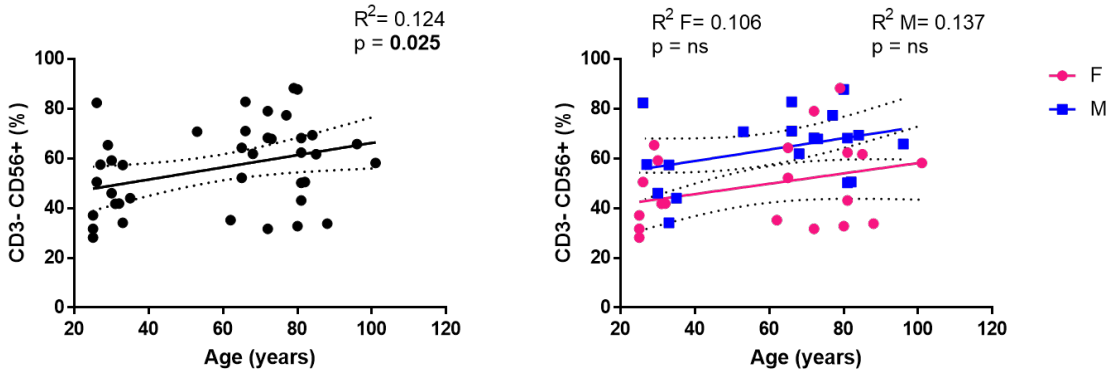


Figure 7. Analysis of NK cells. Linear regression analysis showing the relationship between $CD3^{-}CD56^{+}$ NK cells and age in all individuals (N = 40) (black line), males (N = 18) (blue line) and females (N = 22) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. R^2, R squared; ns, not significant; F, female; M, male.

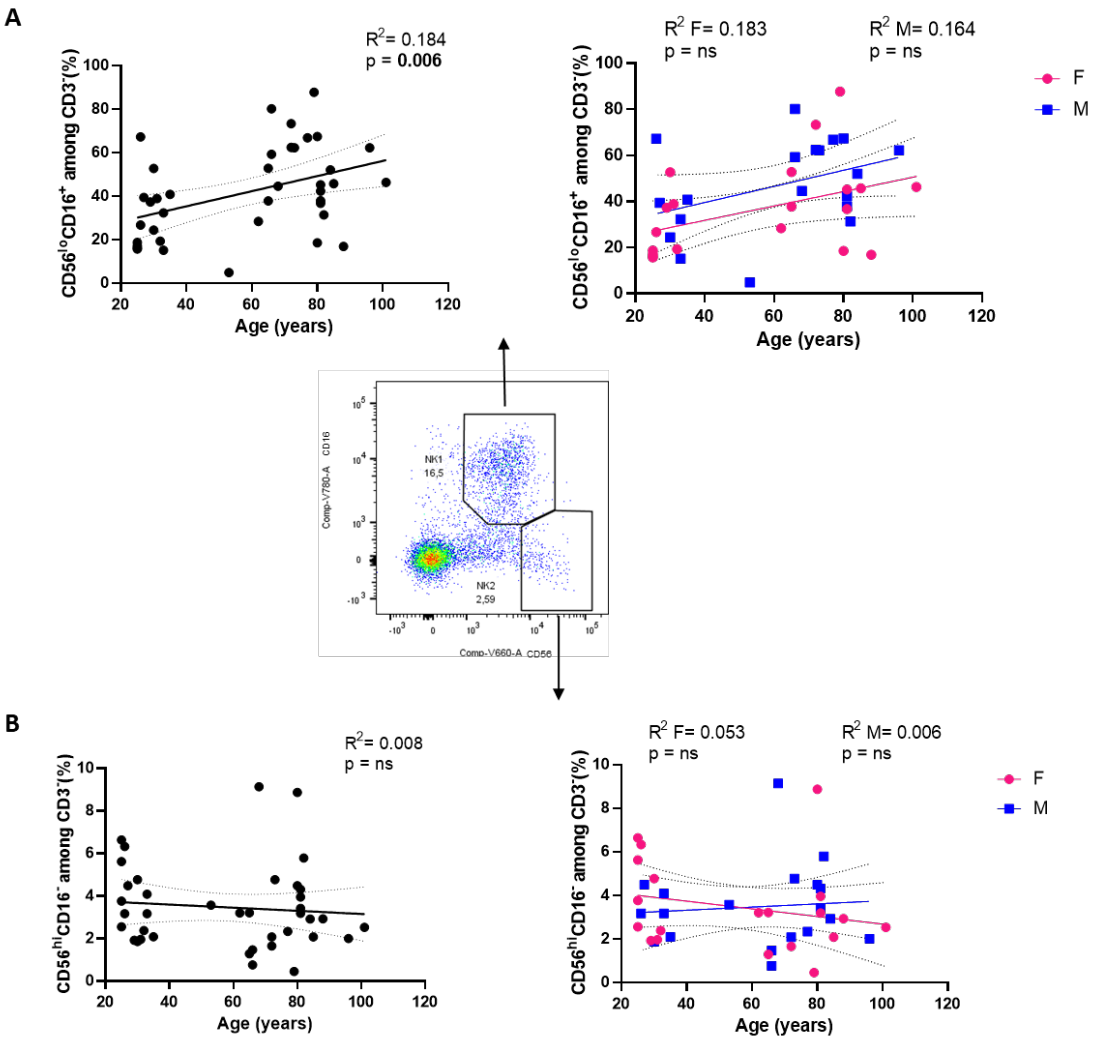


Figure 8. Analysis of NK subsets. Linear regression analysis showing the relationship between cytotoxic (**A**), secreting cytokines (**B**) NK cell subsets and age in all individuals (N = 40) (black line), males (N = 18) (blue line) and females (N = 22) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. Center: representative FACS gating of $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cell subsets; R^2, R squared; ns, not significant; F, female; M, male.

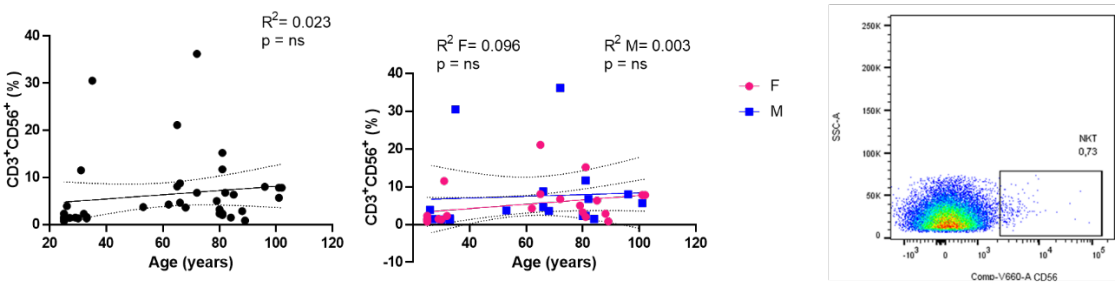


Figure 9. Analysis of NKT cells. Linear regression analysis showing the relationship between $CD3^+CD56^+$ NKT cells and age in all individuals (N = 40) (black line), males (N = 18) (blue line) and females (N = 22) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. Right: an example of the gating strategy for NKT cells. R^2, R squared; ns, not significant; F, female; M, male.

3.4. Role of Immunogenetics in the Outcome of HCMV Infection: Implications for Ageing

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Review

Role of Immunogenetics in the Outcome of HCMV Infection: Implications for Ageing

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Abstract: The outcome of host-virus interactions is determined by a number of factors, some related to the virus, others to the host, such as environmental factors and genetic factors. Therefore, different individuals vary in their relative susceptibility to infections. Human cytomegalovirus (HCMV) is an important pathogen from a clinical point of view, as it causes significant morbidity and mortality in immunosuppressed or immunosenescent individuals, such as the transplanted patients and the elderly, respectively. It is, therefore, important to understand the mechanisms of virus infection control. In this review, we discuss recent advances in the immunobiology of HCMV-host interactions, with particular emphasis on the immunogenetic aspects (human leukocyte antigens, HLA; killer cell immunoglobulin-like receptors, KIRs; immunoglobulin genetic markers, GM allotypes) to elucidate the mechanisms underlying the complex host-virus interaction that determine various outcomes of HCMV infection. The results, which show the role of humoral and cellular immunity in the control of infection by HCMV, would be valuable in directing efforts to reduce HCMV spurred health complications in the transplanted patients and in the elderly, including immunosenescence. In addition, concerning GM allotypes, it is intriguing that, in a Southern Italian population, alleles associated with the risk of developing HCMV symptomatic infection are negatively associated with longevity.

Keywords: antibodies; elderly; GM; immunosenescence; HCMV; HLA; NK; KIR

1. Introduction

Both environmental and genetic factors, as well as factors related to viruses, play a key role in determining the outcome of viral infections. Therefore, different individuals vary in their relative susceptibility to infections [1]. Regarding the genetic component of the response to viruses, the genes

involved are polymorphic most likely because of Darwinian selection. Indeed, as suggested for the first time by J.B.S. Haldane, the main infectious diseases were the most important selective forces in shaping our evolutionary history [2].

It is well known that human cytomegalovirus (HCMV) is an important pathogen from a clinical point of view, because it causes significant morbidity and mortality in immunosuppressed individuals such as the transplanted patients and the elderly, in turn exacerbating their immunosenescence [3,4]. It is, therefore, important to understand the underlying mechanisms of virus infection control.

HCMV is a member of the herpes virus family (type 5) that is ubiquitous in human populations. HCMV, the largest human virus, has 235 Kb, double-stranded linear DNA genome. HCMV is transmitted from human to human. There is no animal reservoir. The transmission occurs via saliva, urine, breastfeeding, placenta, sexual intercourse, blood and organ transplants. In transplanted patients, immunosuppressive drugs can increase the risk of infection and complications [4–7].

Clinically, primary HCMV infection is asymptomatic in the 90% of the infected and immunocompetent population. Symptomatic primary HCMV infection of the adolescent or adult usually results in a mononucleosis-like syndrome with malaise, fever, sweating, and abnormal liver function [8]. In any case, a latent infection that lasts a lifetime is established. In a review of literature, seroprevalence was found to range from 45% to 100%, being the highest in South America, Africa and Asia and the lowest in Western Europe and United States. Worldwide, seroprevalence among whites tended to be 20–30 percentage points lower than that of Caucasians. Persons of higher socioeconomic status were more likely to be HCMV seronegative. In addition, seroprevalence increases with age, reaching more than 85% by 80 years. Although in most studies the differences were small, men generally had lower seroprevalences than women [3,7,9,10].

Most people carry the virus in a latent form and are at risk of reactivation. HCMV infection establishes a long-lasting immunity (but HCMV is able to manipulate the immune system) that limits the replication of virus after reactivation from latency. The reactivation mostly occurs both in immunosuppressed patients (as those transplanted) and in immunocompromised patients (septic patients or elderly) [11,12]. All these conditions are characterized by an inflammatory state, and it has been suggested that inflammation can be responsible for reactivation [13].

Pattern recognition receptors such as Toll-like receptors recognize components of the virion, thereby triggering the innate immune response to the virus with the production and/or release of pro-inflammatory cytokines that in turn recruit and activate cells of innate immunity, including Natural Killer (NK) cells that destroy target cells infected by HCMV. There is also triggering of acquired immunity that involves both T and B lymphocytes. It results in a large cytotoxic CD8 T cell response that plays a key role in the control of primary HCMV infection and reactivation from latency. In addition, T helper CD4 lymphocytes, through the production of interferon-γ and their helper function for CD8 and B lymphocytes, play a role in the control of HCMV infection [14]. Due to coevolution within their hosts for millions of years, herpes viruses, including HCMV, are able to encode several proteins and microRNAs responsible for evading the host immune response, both during lytic infection and during latency. It allows HCMV to replicate and disseminate in the face of a competent immune system and to establish latency with periodical viral reactivation and virus shedding through the life of the host. HCMV products are, indeed, able to inactivate complement cascade, to mitigate the effects of interferon and to prevent apoptosis of infected cells. Other virus products are proteins homologue for cytokines, chemokines and their receptors as well as for fragment crystallisable γ receptor (FcγR). Lastly, virus products interfere with T and NK cells functions. [15,16].

HCMV is not only a master in immune evasion but also is responsible for the manipulation of immune system with ageing. In the latent state, the intermittent production of viral antigens prevents contraction of virus-specific T cells. Thus, a large population of HCMV-specific CD8+, and to a lesser extent, CD4+ T cells, is generated. That is responsible for the phenomenon of memory cell inflation leading to the emergence of vast populations of resting effector CD8+ and CD4+ cells. In the elderly, many alterations of innate and acquired immunity have been described and viewed as deleterious,

hence the term immunosenescence [17]. Age, followed by sex and the HCMV status, has the greatest impact on the immune system. HCMV is considered to contribute to shape the immune profile and function during normal human ageing. Accordingly, modulation of the immune response by HCMV may occur, resulting in less effective control of HCMV replication following virus reactivation [3,15,18]. However, inflationary CD8+ cells, after proper activation stimuli, can divide, secrete cytokines, and execute cytolysis, i.e., they are not exhausted. The production of inflammatory cytokines can contribute to the pro-inflammatory status of ageing, called inflamm-ageing [17]. Moreover, there is some evidence of a slight loss of control of HCMV replication in the elderly compared with younger people, as the HCMV load within blood increases markedly in healthy people over the age of 70 years [19]. There is some evidence of an association of HCMV seropositivity with increased mortality from cardiovascular disease, as a result of damage caused by the large expansion of cytotoxic CD4+CD28-negative T cell populations commonly seen in HCMV-infected individuals [20,21]. Immune changes associated with HCMV may have significant impact during co-infection and vaccination as well as on fitness [22,23]. Herpes virus latent infection should contribute to immune dysfunction in the elderly but may be beneficial during childhood-adult years [15,18].

In our review, we discuss recent advances in the immunogenetic control of HCMV-host interactions. We will focus on the role played by human leukocyte antigens (HLA) responsible for both humoral and cellular control, killer cell immunoglobulin-like receptors (KIRs), responsible for cellular control, after interaction with class I HLA, and γ marker (GM) immunoglobulin allotypes responsible for humoral control. Almost all of the papers concern the immunogenetic control of HCMV reactivation in transplanted patients. Data are also discussed considering the role of HCMV in ageing and immunosenescence.

2. HLA and HCMV

The HLA system encodes cell-surface proteins responsible for the regulation of the immune system. HLA genes are highly polymorphic, i.e., they have many different alleles, involved in fine-tuning of the acquired immune responses. HLA classes have different functions [24–26]. (I) Class I (HLA-A, -B and -C) presents peptides from inside the cell to the surface of the cell, including viral fragments if the cell is infected by a virus. It follows that the cell can be lysed by CD8+. (II) Class II (HLA-DRA, HLA-DRB1, HLA-DRB3–5, HLA-DQA, HLA-DQB, HLA-DPA and HLA-DPB) presents peptides from outside the cell to CD4+ cells, which in turn stimulate B lymphocytes to produce antibodies to that specific antigen. (III) Class III encodes components of the complement system and pro-inflammatory cytokines. The high polymorphism of class I and II molecules affects the peptide-binding groove, since it varies the amino acids sequences that can be housed within the peptide-binding pockets. Different HLA alleles possess different peptide-binding repertoires and it is difficult for disease-related proteins to escape detection. Therefore, it is not surprising that several infectious and autoimmune diseases are associated with different HLA antigens, responsible for different humoral or cellular immune responses [24–26]. However, in the last few years, it is becoming clear that Class I antigens can play a role as ligand for KIR. Therefore, several observed association of HLA class I antigen with infectious and autoimmune diseases can be explained by their role as ligands for KIR [26].

As mentioned previously, HCMV interferes with immune responses at different levels. Several proteins (or microRNA) produced by the virus can interfere with the functions of Class I and Class II molecules, i.e., antigen processing and presentation, e.g., they can induce their down regulation or their degradation [15,16].

Concerning the role of HLA in the control of HCMV infection, most studies regard HCMV infection/reactivation in patients after kidney transplantation. However, as reported by Futohi et al. [6] the results have been discordant. Therefore, no conclusion can be drawn from these studies, although in three papers out of eight an association with HLA-DR7 was reported [27–29].

As discussed by Caruso et al. [24], several methodological problems can explain the discrepancies observed in studies concerning association of HLA with diseases as follows. (I) Insufficient sample sizes to detect differences in HLA antigen frequencies. (II) Different inclusion with inappropriate mixing of data (cohort effect) and inappropriate control matching, i.e., lack of selection from the same target population. (III) The different genetic background of studied population. (IV) The lack of Bonferroni's correction for multiple comparisons.

However, in the previously quoted study [6], the presence of HLA-B8 allele had a protective effect for developing HCMV infection after kidney transplantation. It is intriguing that in a study performed on HCMV infection in Ireland, HLA-A1, HLA-B8 alleles were associated with HCMV seronegativity [5]. These alleles are carried by the 8.1 ancestral haplotype, i.e., an evolutionarily highly conserved haplotype, known to influence cytokines production with decreased type 1 responses in contrast to type 2 ones. It follows that humoral responses are enhanced in people carrying this haplotype [30].

These results are of some importance for the relationship between HCMV and ageing. In fact, this haplotype has been associated with male longevity [24,25], hence this datum strengths the suggestion of the detrimental role for HCMV in achieving successful ageing.

3. KIR and HCMV

KIRs, expressed on the membrane of NK cells and a minority of T lymphocytes, regulate the killing function of these cells by interacting with specific amino acid motifs, public epitopes, carried by some HLA class I molecules, and expressed on their targets. They are characterized by two or three extracellular domains and a short (S) or long (L) intracytoplasmic tail that respectively transduces either activating or inhibitory signals (see Figure 1). The KIR gene complex is characterized by multiple gene-content haplotypes, i.e., it is polygenic. KIR genes are organized in two basic haplotypes defined on the basis of gene content, and are termed A and B. Group A haplotypes are characterized by the absence of the following genes KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1, whereas group B haplotypes are characterized by the presence of one or more of the previous genes. Thus, the B haplotypes have more genes encoding activating KIR than A haplotypes. Moreover, KIR genes exhibit allelic variability, i.e., they are polymorphic. Furthermore, KIR expression on NK cells is stochastic, resulting in a significant variation of the NK cell repertoire among individuals and among populations. KIRs are able to detect cells infected by viruses or transformed, by binding the different class I allelic variants. Most inhibitory KIRs specifically recognize sets of HLA class I alleles; yet, the ligands for some of them and for most activating KIRs are unknown. Specific combinations of KIRs with their cognate HLA ligands have been associated with infectious diseases, autoimmune disorders, pregnancy outcomes and cancer. In addition, they are crucial in determining clinical outcomes of both hematopoietic stem cell and solid organs transplantation [31–34].

There is compelling evidence that NK cells play a crucial role in host defence against HCMV infection. Thus, it is not unexpected the interference by proteins or microRNA coded by HCMV with NK function. As an example, several proteins affect the expression of ligands of the NKG2D receptor, a transmembrane protein encoded by KLRK1 activating gene (not linked to KIR genes) whose ligands are induced-self proteins completely absent or present at low levels on the surface of normal cells, but overexpressed in infected or transformed cells [35]. Clearly, the degradation or the down regulation of HLA Class I antigens, previously reported, interferes with KIR [15,16]. However, as shown in Figure 1, these antigens can bind both activating and inhibitory KIRs.

The entire population of NK cells from a child with a novel immunodeficiency syndrome and recurrent HCMV infection expressed the KIR2DL1, with inhibitory function. Since the patient also possessed the KIR2DL1 ligand group, HLA-C2, it was suggested the possibility that the strong inhibitory KIR2DL1/HLA-C2 combination impaired NK cell activity and prevented the cells from mounting a protective response against HCMV [36].

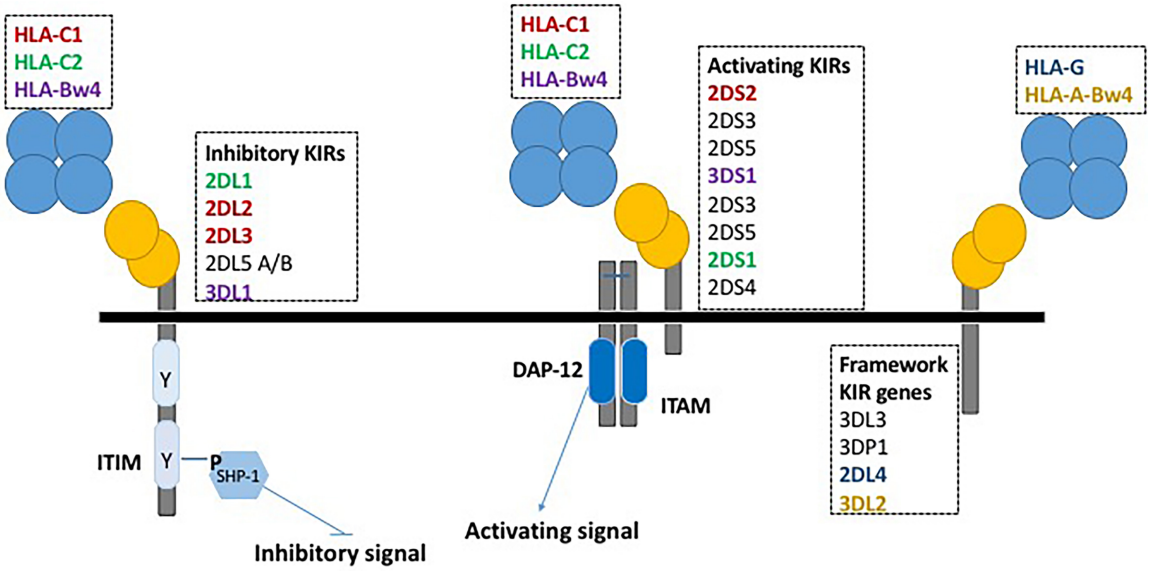

Figure 1. The figure shows the killer cell immunoglobulin-like receptor (KIR)/ human leukocyte antigens (HLA) interaction with inhibitory or activating effects. The activating receptors (↓) have short cytoplasmic tails lacking cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM). The tails interact with the adaptor molecule, the death associated protein (DAP) 12, and contain immunoreceptor tyrosine-based activation motif (ITAM) linked to protein tyrosine kinase activation pathways. On the contrary, the inhibitory KIRs (⊥) are characterized by the presence of ITIM, which recruits the SH protein-1/2 tyrosine phosphatases. In the figure, the receptors, inhibitory or activating, and the HLA ligands, that interact are marked with the same colour. The ligands of the receptors marked in black are unknown. The interaction between KIR3DL2 and HLA-A-Bw4 is inhibitory. The four blue spheres depict the four domains of the HLA complex. The two yellow spheres depict the two immunoglobulin-like extracellular domains of KIR. However, the figure does not show KIRs that have three immunoglobulin-like extracellular domains.

Several reports have documented a role for activating KIRs in the control of HCMV infection after hematopoietic stem cell or kidney transplantation. Activating KIR genes carried by kidney transplant-recipients have been shown to influence the rate of viral infection during the first year after transplantation. In fact, patients carrying more than one activating KIR gene (KIR BA or BB genotypes) showed a rate of infection and reactivation significantly lesser (20%) than that observed in patients with a KIR AA genotype (36%) (with KIR2DS4 as only activating KIR). Moreover, the number of activating KIR genes correlated with the degree of protection. This study supports a role for activating KIR in the control of HCMV infection after kidney transplantation [37]. The KIR/HLA genotypes and the rate of HCMV infection was analysed in 196 kidney transplant recipients. In kidney recipients, it was shown that, after transplantation, both the presence of activating KIR genes and the absence of the HLA-C ligands for inhibitory KIR were associated with a lower rate of HCMV [38]. After hematopoietic stem cell transplantation, whether the donor had either $\geq$5 activating KIR genes or KIR2DS2 and KIR2DS4 genes, there was a low risk of HCMV reactivation in the recipient [39]. Finally, in stem cell transplantations involving siblings where both donor and recipient were HCMV seropositive and the donor possessed a copy of the KIR haplotype B, the reactivation rate was 28%, whereas the HCMV reactivation rate was 65% if donor was homozygous for the KIR A haplotype, i.e., with only one activating KIR. A significant decreased risk of HCMV reactivation was also confirmed by multivariate analysis if the donor had more than one activating KIR gene [40]. All these results obtained in transplanted patients demonstrate the importance of activating KIRs in the immune surveillance against HCMV.

Interestingly, similar data were obtained in immunocompetent subjects, studying a cohort of 120 subjects with HCMV infection [41]. Indeed, evidence has been provided for a detrimental effect of the

AA haplotype or the HLA-Bw4^{T} group on the outcome of primary HCMV disease. The frequency of the homozygous A haplotype was higher in symptomatic patients than in controls. By logistic regression, the risk of developing symptomatic disease was associated with the homozygous A haplotype and the HLA-Bw4^{T} groups. These results show the key role played by KIR/HLA polymorphisms in the immunocompetent host, suggesting that HLA-Bw4^{T} group exerts its function by binding the inhibitory KIR3DL1 gene. With the aim to gain insight in the association of this combination and HCMV, our group analysed the distribution of HLA-B alleles, in symptomatic patients carrying HLA-Bw4^{T} group. We observed (Table 1) that, in symptomatic patients carrying HLA-Bw4T group, HLA-B*44 allele was over-represented, 55.6%, whereas its frequency in Sicily is 8.9% [42]. This result is intriguing since this allele is in linkage disequilibrium with HLA-DR7 [43], reported to be associated with infection/reactivation in patients after kidney transplantation [27,29].

Table 1. Distribution of HLA-B alleles in Human cytomegalovirus (HCMV) symptomatic patients positive for the different HLA-Bw4 groups. (The number of alleles is twice the number of subjects, as each person has two alleles—one maternal and one paternal).

HLA-Bw4 Groups	HCMV Patients (*N* = 31)	
	N	%
HLA-Bw4^{T}		
B*44	11	33.30
B*13	4	12.12
B*35	3	9.09
B*14	1	3.03
B*40	1	3.03
B*27	2	6.06
B*73	1	3.03
B*50	1	3.03
B*07	0	0
B*18	0	0
HLA-Bw4^{I}		
B*27	1	3.03
B*14	1	3.03
B*39	1	3.03
B*57	3	9.09
B*35	5	15.15
B*38	2	6.06
B*40	1	3.03
B*49	3	9.09
B*50	2	6.06
B*08	1	3.03
B*15	1	3.03
B*51	4	12.12
B*67	1	3.03
B*18	0	0
B*53	1	3.03
B*58	3	9.09
B*44	1	3.03
B*52	1	3.03
HLA-Bw4^{T}/HLA-Bw4^{I}		
B*44	4	12.12
B*13	0	0
B*49	1	3.03
B*53	1	3.03

Genomic DNA was extracted by a commercial kit (PureLink®® Genomic DNA, ThermoFisher Scientific, Waltham, MA, USA) from frozen mononuclear cells obtained in the previous study [41] from peripheral whole blood samples. The HLA-B loci genotypes were determined using the commercially available HLA Class I B Locus DNA Typing Tray kit (One Lambda, ThermoFisher Scientific Brand, California, USA), according to the manufacturer instructions.

Finally, the correlation of KIR gene distribution and the anti-HCMV antibody titer has been studied in the elderly. Analysis of the distribution of KIR genes showed a non-significant decreased frequency of inhibitory KIR2DS5 gene in the group with higher (>20 IU/mL) HCMV-specific IgG antibody levels [44,45]. Therefore, a study in a larger group of elderly is warranted.

4. GM Allotypes and HCMV

The term allotype refers to any genetic variant of a protein. In immunology, GM allotypes indicate allelic hereditary variants, encoded by autosomal codominant alleles that follow Mendelian laws of heredity, expressed on immunoglobulin constant region of γ1, γ2 and γ3 chains. GM allotypes are encoded by three very closely linked, highly homologous, immunoglobulin heavy gamma (IGHG) genes, on chromosome 14q32. Linkage disequilibrium in the GM system within an ethnic group is almost absolute and the determinants are transmitted as a group, i.e., haplotype. Each major ethnic group has a distinct array of several GM haplotypes [46,47].

These observations point towards a role for differential selection in the maintenance of GM polymorphism. Many lines of evidence point towards infectious diseases as the principal selective forces of natural selection [2]. GM allotypes have been shown to be associated with immunity to many infectious pathogens. They also influence the chance for survival from epidemics, such as typhoid and yellow fever [48]. Different mechanisms have been proposed to explain these associations [49].

There are inter individual differences in the level of anti-HCMV IgG antibodies, suggesting the existence of host immune response genes for this trait [50]. However, a genome-wide association study (GWAS) found no major genes for anti-HCMV antibody responsiveness [51]. As pointed out elsewhere [52,53], current GWAS do not evaluate GM genes because they are not included in the commonly used genotyping arrays. The extensive homology of IgG gene segments expressing various GM allotypes may have contributed to their exclusion from these arrays. Therefore, it is necessary to employ a candidate gene approach for evaluating the role of GM genes in the immunobiology of HCMV infection.

Using a candidate gene approach, the contribution of GM allotypes to the magnitude of antibody responses to HCMV glycoprotein B (gB), which is required for viral infectivity and is a major component of the viral envelope, was investigated. Results showed that two allotypes at the γ1 locus, GM3 and GM17, additively contributed to the level of IgG antibodies to gB. The homozygosity for the GM 17 allotype was associated with high, while the homozygosity for its allelic counterpart (GM 3) with low, anti-HCMV gB antibody levels, respectively. The heterozygotes exhibited intermediate levels of antibodies. GM 5 and GM 21 allotypes, which are in linkage disequilibrium with GM 3 and GM 17 allotypes, respectively, followed a similar pattern of anti-HCMV gB antibody responses [50].

Several mechanisms could account for the GM gene involvement in humoral immunity to HCMV: structural contribution to the idiotypes involved in HCMV immunity, contribution to the conformational modifications of antibody binding sites that could influence its affinity, and linkage disequilibrium of constant-region GM allotypes with the variable-region genes. Murine studies have clearly shown that differences in the amino acid sequences in the constant region affect the secondary structure of the antigen-binding site in the variable region. Amino acid substitutions that characterize GM allotypes cause structural changes in the constant region, which could impose structural constraints (conformation) on the variable region, resulting in variation in antibody affinity to HCMV. Thus, isotype restriction of antibodies to HCMV, which are predominantly IgG1 and IgG3, may reflect structural constraints imposed by the constant-region allotypes on the variable-region binding [46,49,54].

As previously stated, the HCMV has developed a large repertoire of highly sophisticated strategies, directed against almost every component of the human immune system, for evading host immunosurveillance [15,16]. One of these strategies involves the generation of three proteins, encoded by genes TRL11/IRL11, UL119-UL118 and RL13 that have functional properties of the FcγR [16,55]. The viral FcγRs essentially neutralize the constant region of anti-HCMV IgG antibodies that are

involved in effector functions, such as antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis and complement-dependent cellular cytotoxicity. This helps the virus to evade host immunosurveillance. GM allotypes have been shown to modulate this viral immunoevasion strategy. IgG1 proteins expressing the haplotype carrying GM3 allotype had significantly higher affinity for the TRL11/IRL11-encoded decoy FcγR than IgG1 expressing the haplotype carrying GM17 allotype. In contrast, the UL119-UL118-encoded FcγR has higher affinity for the IgG1 proteins expressing the GM17 haplotype than those expressing the GM3 haplotype. The affinity of the RL13-encoded FcγR to the allotypically disparate IgG1 proteins follows a similar pattern [56–58].

An interaction between HLA/KIR genes and GM allotypes in the control of HCMV infection was shown in Sicilian population. Particularly, it was shown the association of GM17/17 with the risk of developing symptomatic infection with a gene–dose effect, i.e., the association was observed only in homozygous people. The risk of symptomatic infection was also shown in people carrying GM23, without evidence of a gene–dose effect. In addition to these GM allotypes, HLA-C2 and HLA-Bw4^{T} groups remained independently associated with the risk of HCMV symptomatic infection in a multiple logistic regression analysis. Therefore, both a possible epistatic interaction of GM allotypes with HLA gene variants, and an independent effect of these GM allotypes can be hypothesized [59]. These results are not in contrast with the above reported association of GM17 allotype with an increased antibody response to HCMV [50], because this increased response should depend on a less efficient control of HCMV that determines a greater antigenic stimulation.

Regarding the relationship between HCMV and ageing, it is noteworthy that, in another Southern Italy population, GM3 allotype, the alternative allele of GM17, was significantly overrepresented in long-living individuals [60], strengthening the suggestion of the detrimental role for HCMV in achieving successful ageing.

To confirm and extend these observations, large scale studies involving GM allotypes and anti-HCMV antibodies should be conducted in long-living individuals and young controls.

5. Summary

Considering the profound effects of HCMV infection on health and the quality of life of immunosenescent individuals—either transplanted patients or old subjects—the identification of the mechanisms responsible for the immunogenetic control of HCMV infection should have important clinical implications. Therefore, the data discussed on the role of humoral and cellular immune responses in the control of HCMV infection are important in driving efforts to reduce the HCMV-associated health complications in old subjects and in transplanted patients. Further efforts in determining the crucial role of HLA, KIR and GM genes in HCMV control will greatly enhance our understanding of the immunogenetic aspects of HCMV infections, and to potentially apply this knowledge clinically, i.e., in the development of new vaccines and in the identification of new therapeutic targets.

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Abbreviations

FcγR	fragment crystallisable γ receptor
gB	glycoprotein B
GWAS	genome-wide association study

GM	genetic marker
HCMV	human cytomegalovirus
HLA	human leukocyte antigens
IGHG	immunoglobulin heavy gamma
KIRs	killer cell immunoglobulin-like receptors
NK	natural killer

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3.5. Translation of Basic Research into Clinics: Killer Immunoglobulin-like Receptors Genes in Autoimmune and Infectious Diseases

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REVIEW ARTICLE

Translation of Basic Research into Clinics: Killer Immunoglobulin-like Receptors Genes in Autoimmune and Infectious Diseases

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Abstract: Killer immunoglobulin-like receptors (KIRs) regulate the activation of natural killer cells through their interaction with human leucocyte antigens (HLA). KIRs and HLA loci are highly polymorphic, and some their combinations have been found to protect against viral infections or to predispose to autoimmune disorders. In particular, some activating KIRs profiles may be detrimental in autoimmune pathogenesis, and specific KIRs may be particularly aggressive in the clearance of different microorganisms, protecting individuals in the control of a given pathogen. So, considering that in the pathogenesis of many autoimmune disorders and infections innate immunity plays a key role, the recent development for KIRs characterization, diseases monitoring, and treatment becomes obvious. Here, we reviewed a growing body of evidence supporting the influence of KIRs variants and their interaction with ligands in the development of the main human autoimmune and viral diseases, highlighting the main applications in clinical practice.

Keywords: Autoimmune diseases, HLA ligands, Immunogenetics, KIRs, Translational medicine, Viral infections

1. INTRODUCTION

It is well known that translational medicine refers to the clinical application of scientific discoveries from laboratory studies, to improve human health by reducing incidence, morbidity, and mortality of diseases [1].

There is increasing evidence that natural killer (NK) cell-mediated immune-regulation is essential in the control of virus infection and autoimmune diseases. In particular, killer cell immunoglobulin-like receptors (KIRs), surface receptors expressed on NK cells as well as on a subset of CD8+ T lymphocytes, are implicated in the control of NK function [2].

The aim of this review will be to take advantage of the data obtained from basic research on the role of KIRs to transfer them into clinical practice. This will be done through a systematic review of the data obtained up to now, and some of the possible clinical applications, for the prevention or treatment of the before cited diseases.

In order to deeply understand the mechanisms that regulate the activation or the inhibition of NK cells, the review will start with an overview of the relationship between KIRs and their ligands.

KIRs receptors are specific for class I alleles of human leukocyte antigen (HLA). The polygenic nature of the KIR locus is functionally relevant since KIR genes code for receptors, which can either activate or inhibit both NK and CD8+ T cells [2].

The KIR gene cluster consists of a segment of about 150 kb that maps on chromosome 19q13.4, within the leukocyte receptor complex. To date, 15 KIR genes and 2 pseudogenes have been described. However, variability in gene content seems large due to non-allelic homologous recombination, gene duplication, and presence of a lot of polymorphisms. In fact, all levels of polymorphism contribute to KIR diversity, hence to differences in NK reactivity [3,4].

KIR genes are organized in two basic haplotypes defined on the basis of gene content. They are termed A and B. Haplotype A, uniform in terms of gene content is composed of one activating gene (KIR2DS4), five inhibitory genes (KIR2DL1, 2DL3, 3DL1, 3DL2 and 3DL3), and KIR2DL4 that may have both activating and inhibitory ability. Several A haplotypes are characterized by null variants of both KIR2DS4 and KIR2DL4, therefore they are not expressed on the cell surface [5,6]. Accordingly, these haplotypes do not have functional activating KIR.

The extraordinary differences in gene profiles observed in different ethnic groups are mostly due to the variable numbers of activating and inhibitory receptors carried by B haplotypes. The persistence of A and B haplotypes within the human population at different frequency suggests the occurrence of a balancing selection. Haplotypes B seems to be associated with improved reproductive fitness, whereas improved responses to pathogens seem to be associated with haplotype A [7-9].

Since most of the inhibitory KIRs are present on all or nearly all haplotypes, the variability among KIR haplotypes mostly depends on the presence or the absence of activating KIR [10]. The activating KIRs (i.e., KIR2DS and KIR3DS) have short cytoplasmic tails lacking cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM). The tails interact with the adaptor molecule DAP12 containing immunoreceptor tyrosine-based activation motif (ITAM) linked to protein tyrosine kinase activation pathways. The inhibitory KIR family is, instead, characterized by the presence of ITIM, which recruits the SH proteins -1/2 tyrosine phosphatases,

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hence preventing NK cells activation [11]. The activating KIRs stimulate NK/CD8 T lymphocytes cytokine secretion and target cell cytolysis may be generally beneficial in response to pathogens and cancer cells. Not all activating or inhibitory or receptors are present on the surface of NK or CD8 T cells and it is the balance of these signals that modulate NK cells cytotoxicity and cytokine release.

KIR ligands are represented by public epitopes present on subsets of HLA antigens. Thus, most inhibitory KIRs specifically recognize sets of HLA class I (i.e., HLA-A, B, and C) antigens; however, the ligands for some of them (e.g., KIR2DL5) and for most activating KIRs are unknown. HLA class I genes map to chromosome 6 and genes are extremely polymorphic, determining functional diversity, and generating variable susceptibility in response to pathogens and other diseases [12].

The inhibitory KIR2DL1, 2DL2, and 2DL3 recognize HLA-Cw alleles, divided into two groups on the basis of a dimorphism at position 80; HLA-C1 group has asparagine whereas HLA-C2 group has lysine at position 80 [13]. KIR2DL2 and KIR2DL3 are engaged by HLA- Cw antigens expressing asparagine at position 80 whereas KIR2DL1 binds to HLA- Cw antigens expressing lysine at position 80; KIR2DL3 is specific for HLA-B antigens with the Bw4 serologic specificity depending on isoleucine/threonine at positions 80 (HLA-$Bw4^{I/T}$), although it has also been reported low-affinity binding with Bw6. In addition, four HLA-A antigens (HLA-A*23/*24/*25/*32) carry the same epitope and the ligand is called HLA-A-Bw4 [14-16]. Furthermore, KIR2DL4 binds HLA-G, primarily expressed on foetal trophoblasts, cornea, and thymic endothelial cells and KIR3DL2 binds HLA- A*3 and HLA-A*11 [17,18].

The inhibitory receptors KIR2DL1, 2DL2-2DL3 and 3DL1 share sequence similarity in their extracellular domains with their corresponding activating counterparts (KIR2DS1, 2DS2 and 3DS1 respectively) and are thought to share HLA ligand binding specificities as well [19].

Combinations of HLA and KIR genes have been shown to be associated with several diseases such as autoimmune/inflammatory disorders, cancer, infectious diseases, transplantation, and reproduction. In these diseases, new data strongly suggests that the mechanisms involved are based on a continuum of activation to inhibition through different compound KIR/HLA genotypes [19]. Moreover, the strength of the interaction may also be determined by allelic variation [20]. In addition, since not all the presented peptides seem to allow KIR interaction, HLA-bound peptides can play a role in KIR/HLA ligand interactions [4].

KIR haplotype diversity that probably imparts a continuum from strong activation to relatively strong inhibition, suggests the pleiotropic effect of KIR on various diseases since a given KIR genotype may actually predispose to one disease and protects from another unrelated disorder.

In this regard, activating KIR profiles might be harmful in the pathogenesis of certain autoimmune diseases. However, this might be true for some autoimmune diseases and the opposite for other ones [21,22]. KIR associations with susceptibility to autoimmune disorders point to the short chain of the activating KIR. Moreover, the control of virus infections by NK cells has been very well reviewed [23]. Specific KIR genes can be particularly effective in the clearance of some virus, so their allelic polymorphism, as well as their presence in only a fraction of subjects, might explain the differences observed in the ability of the different subjects to control a given pathogen.

Moreover, it was seen that most of the difference in the receptors are located in the extracellular domains of KIR, affecting downstream pathways. These biological features of the receptors

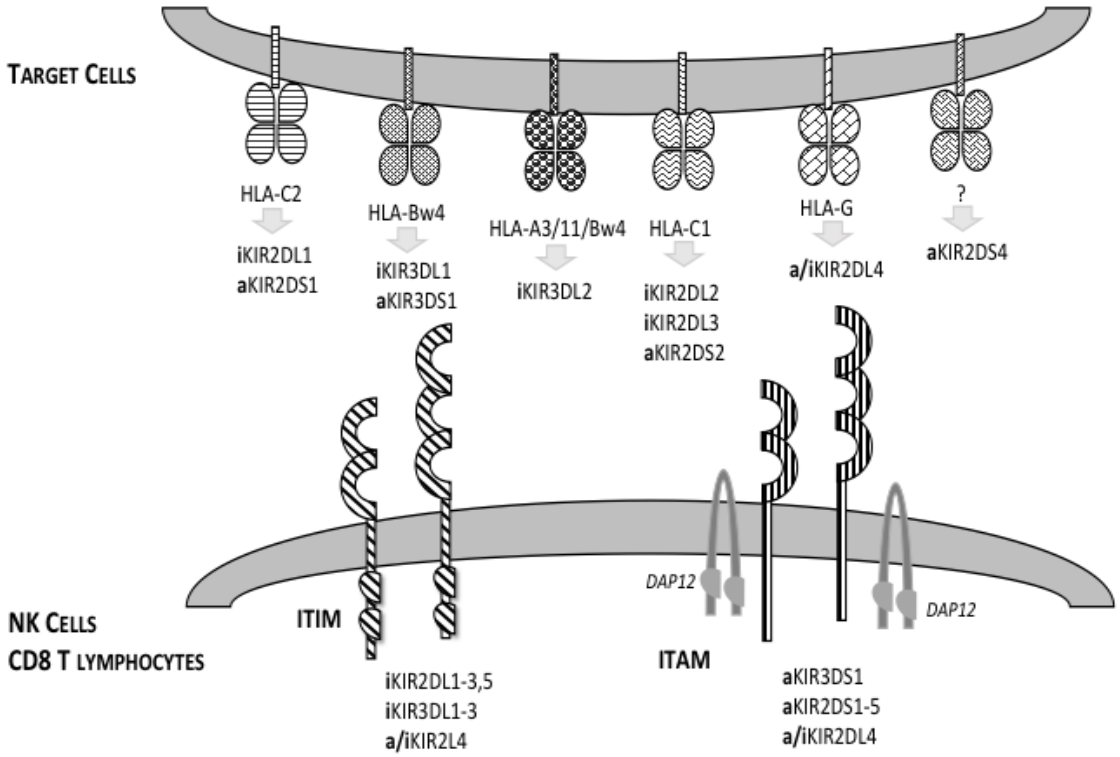

Fig. (1). The KIR/HLA interaction with inhibitory (i) or activating (a) effect. The figure shows the main interaction between activating (a) or inhibitory (i) KIR and HLA ligand.The final effect gives protection or susceptibility to autoimmune diseases or viral infection.
ITIM: immunoreceptor tyrosine-based inhibition motif.
ITAM: immunoreceptor tyrosine-based activation motif.

are suggested to play a prominent role in the outcome of virus infections, and possibly for risk assessment for autoimmune diseases [4].

In recent years, the manipulation of NK cell activity has grown, arising from both preclinical and clinical studies showing that it is possible to modulate the NK cell reactivity. The possibility to discover new drugs that can target NK cells is of great interest in order to switch from genotyping to clinical practice.

See Figure **1** for an overview of the KIR/HLA interaction.

2. KILLER IMMUNOGLOBULIN-LIKE RECEPTORS/HUMAN LEUKOCYTES ANTIGEN ASSOCIATION AND AUTOIMMUNE DISEASES

About 5% of the population of the Western countries is affected by different types of autoimmune disorders [22]. The multifactorial pathogenesis of autoimmune diseases remains unclear. However, the observed associations between the highly polymorphic HLA antigens and autoimmune disorders are strong [24]. However, some HLA associations with autoimmune disorders might be due to synergistic interactions between KIR and HLA class I ligands. Moreover, the association between KIR genes and some autoimmune diseases may be negative, because NK cell activation, suppressing or eliminating be cells and monocytes known to be involved in CD8+ generation, might play a protective role [25].

2.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) was the first disease in which it has been observed an effect of KIR genotype. In RA patients, where there is an expansion of CD4+ CD28null T cells responsible for endothelial damage, it was showed that these cells expressed KIR2DS2 in the absence of inhibitory KIR2DL2 [26]. In addition, KIR2DS2 frequency was shown to be increased in RA patients with vasculitis when compared both with controls and with RA patients without vasculitis. Interestingly, in patients with vasculitis, HLA-Cw*03, belonging to HLA-C1 group, a potential ligand for KIR2DS2, was also increased [27]. Therefore, it should be possible that a specific HLA- Cw*03 -peptide complex generated during RA vasculitis is recognized by KIR2DS2.

It has been reported that haplotypes B (that is rich in activating KIRs) [28] and KIR2DS1 alone [29] or in combination with HLA-Cw*06 (a C2 ligand for KIR2DS1) is associated with psoriasis [30]. Taking into account the results, the authors proposed a model where the susceptibility to psoriatic arthritis is influenced by a gradient of more activating to more inhibitory compound genotypes of KIR2DS and HLA-C. So, the genotypes conferring maximum inhibition (absence of activating receptors KIR2DS1 and KIR2DS2 and presence of both the inhibitory ligands, such as HLA-C1 and C2) are protective, whereas genotypes conferring highest activation (KIR2DS1 and/or KIR2DS2 with either HLA-C1 or C2 homozygosity) are associated with greatest susceptibility. However, the well-known association between psoriasis and HLA-Cw*06 might be explained by its role as KIR ligand.

2.2. Systemic Lupus Erythematosus

Moreover, the association between KIR gene polymorphisms and systemic lupus erythematosus (SLE) risk has been investigated by many case-control studies, but findings are not always consistent. SLE is known to be a multifactorial and highly polymorphic systemic autoimmune disease predominantly afflicting fertile age women. It is a complex interaction of genetic, environmental, and hormonal factors, companying a global loss of immune tolerance [31]. In 2007, Pellett et al., first reported that the frequency of KIR2DS1 was significantly increased in SLE patients compared with controls [32]. In addition, a recent meta-analysis shows that KIR2DL1 might be a potential risk factor for SLE in Caucasians, and KIR2DL3, KIR2DL5 might be protective factors for SLE in Asians [33], indicating that the association between KIR polymorphisms and the risk of SLE may be different in different ethnic populations. Moreover, the frequency of activating KIR profiles, like KIR2DS2/KIR2DS5/KIR2DS1 was significantly higher in SLE patients when compared to healthy persons; however, it was seen that various ethnic and environmental factors might influence susceptibility to disease, which is consistent with previous studies [34].

To gain insight into the role of KIR antigens in SLE, we undertook a study in our homogeneous Sicilian population. In addition, we determined plasma values of the anti-oxidant molecule taurine in all subjects. Our results showed a significant increase in the frequency of the KIR2DS2 gene in SLE patients when compared to healthy controls. We also showed a strong positive association between HLA-C1 ligand group and SLE. Following stepwise logistic regression analysis, HLA-C1 ligands remained significantly associated with the disease, whereas the KIR genes were no longer significant. Interestingly, SLE patients HLA-C1 group positive were demonstrated to show significantly decreased plasma levels of antioxidant marker taurine when compared to patients HLA-C1 group negative [35].

2.3. Systemic Sclerosis and Ankylosing Spondylitis

Several papers have examined systemic sclerosis (SSc) and ankylosing spondylitis (AS) patients to study the relation between autoimmunity and KIR. SSc is a chronic disease of the connective tissue characterized by the fibrosis of the skin associated with the damages of several organs such as lungs, heart, kidneys, and the gastrointestinal system, It affects all ethnic groups, and women are more susceptible than men [36]. KIR2DS2 was reported as a risk factor in the absence of KIR2DL2 in patients. Moreover, the KIR2DS3 gene was more frequent in SSc patients than in controls, the KIR2DL3 gene was instead less frequent in patients than in controls. Lastly, combining SLE and SSc, KIR2DS3 gene was more frequent in the patient group [37]. In Iranian patients, the interaction between KIR3DL1/HLA-$Bw4^{T}$ and KIR3DL1/HLA-A-Bw4 showed the role of the interplay between inhibitory KIR genes with HLA ligands as a critical index of SSc predisposition [38].

AS is a chronic inflammatory disease, characterized by a strong genetic association with HLA- B*27, that primarily affects the sacroiliac joint [39]. HLA-B*27 protein interactions with KIR have been implicated in the pathogenesis of AS. KIR3DL2 binds to B*27 heavy chain dimers, whereas KIR3DL1, for example, and possibly KIR3DS1, interact with classical B*27 [40]. Moreover, reduced HLA-Bw4 group, both with and without its inhibitory receptor KIR3DL1, has been suggested to influence the inhibitory effect of NK cytotoxicity, hence leading to continued damage in AS.

2.4. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disease, which involves the gut and includes Crohn's disease (CD) and Ulcerative colitis (UC). In IBD, NK cells have an important role in pathogenesis. Recently, it has been performed a meta-analysis of the six studies evaluating the association of the polymorphisms of KIR genes and the IBD risk (4 UC and 5 CD studies). The results showed a positive association between 2DL5 and 2DS1 and UC risk and a negative association between 2DS3 and CD risk [41].

Further work in this area will help to establish the role of KIR/HLA association in autoimmune disease development. However, some researches have been conducted to identify possible applied treatments regarding the main KIR-related autoimmune diseases and immunotherapies [42]. The choice of the appropriate therapy for patients with autoimmune diseases would be enhanced by diagnostic and prognostic markers able to predict the future severity of the disorder and monitor the response to the therapy. So, the invention of therapeutic methods, which are based on antibodies directed against specific inhibitory KIR, is

Table 1. Killer immunoglobulin-like receptor/human leukocyte antigen associations in autoimmune diseases.

Disease	KIR–HLA ligand pair	Effect
Rheumatoid arthritis	KIR2DS2/HLA-Cw*03	Susceptibility
Psoriasis	KIR2DS1/HLA-Cw*06	Susceptibility
	KIR2DS1-2DS2/HLA-C1-C2 homozygosity	Susceptibility
	KIR2DS1	Susceptibility
	KIR2DS1-2DS2–/ HLA-C1-C2+	Protection
Systemic lupus erythematosus	KIR2DS2; KIR2DS5; KIR2DS1; HLA-C1 group	Susceptibility
Systemic sclerosis	KIR2DS2 in the absence of KIR2DL2	Susceptibility
	KIR2DS3	Susceptibility
	KIR2DL3	Protection
Ankylosing spondylitis	KIR3DL1/HLA-B27	Susceptibility
	KIR3DL1+ or –/HLA-Bw4	Susceptibility
Ulcerative Colitis	KIR2DL5; KIR2DS1	Susceptibility
Crohn's disease	KIR2DS3	Protection

HLA human leukocyte antigen, KIR killer immunoglobulin-like, – absence, + presence.

For references, see text.

relevant to treat autoimmune disorders [43]. This method is not limited to any particular KIR inhibitory molecule target on T-cells, but it selects target cells [44]. Antibodies that derived from this invention could be administered to subjects at risk for autoimmune disorder.

Moreover, also the combination therapy to enhance NK cell-mediated cytotoxicity, in response to certain autoimmune disorders, could be an application in KIR studies. With this regards, it was seen that the lenalidomide, a drug initially used as a treatment for multiple myeloma, acts on signalling pathways mediated in NK cells by inhibitory KIRs [45].

An overview of the association between KIR/HLA genes and autoimmune diseases is shown in Table **1**.

3. KILLER IMMUNOGLOBULIN-LIKE RECEPTORS/ HUMAN LEUKOCYTES ANTIGEN ASSOCIATION AND VIRAL INFECTIONS

Several epidemiological studies have associated KIR/HLA genotypes with susceptibility to some infectious diseases such as human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), hepatitis C virus (HCV) and hepatitis B virus (HBV).

3.1. Human Immunodeficiency Virus

HIV is still a global public health problem because at the end of 2016, 36 million people were affected by HIV and 1.8 million people were infected in 2016. However, between 2000 and 2016, new HIV infections decreased by 39% and HIV-related deaths decreased by one third, with 13.1 million lives saved due to antiretroviral therapy over the same period [46]. New knowledge of the mechanisms of latent infection and of the relevance of reservoirs of infection could eventually lead to a better cure. It is receiving greater recognition of the role of immune activation in the pathogenesis of non-AIDS clinical events since they are the main causes of morbidity and mortality in people receiving antiretroviral therapy [47]. In individuals infected with HIV, the combination of KIR3DS1 with its presumable ligand HLA-Bw4^{I} was shown to be associated with slower progression to AIDS with lower mean viral load, and protection against opportunistic infections [48,49]. Moreover, in 25 HIV Asiatic exposed uninfected intravenous drug users, KIR3DS1 transcription was reported to be significantly higher than those of KIR3DL1 in KIR3DS1/3DL1 heterozygous individuals; in addition, it was observed an expansion of NK cells expressing KIR2DL3 in HLA-C1/C1 individuals, KIR2DS2−/2DL2− [50]. It was also observed that KIR3DS1 homozygosity was significantly increased in HIV exposed seronegative intravenous drug users and in HIV negative partners of serodiscordant couples [51]. Interestingly, in these subjects, it was also observed an increase of KIR AB haplotypes, characterized by increased numbers of activating KIR.

Recently, Martin et al. identified a variant of KIR3DL1 that modifies HLA-B*57 protection against HIV-1 [52]. HLA-B*57 control of HIV involves enhanced CD8+ T cell responses against infected cells, although an extensive heterogeneity exists in the level of HIV control among B*57+ individuals. Through a whole-genome sequencing, the authors showed that the protective effect of KIR3DL1 variant was restricted only on B*57:01 subtype and no effect was observed for the B*57:03. It also appeared an association of maternal KIR genetic variant with a reduction in perinatal transmission of HIV-1. In particular, maternal KIR2DL2 and KIR2DS5 were significantly associated with a reduction in perinatal HIV transmission in women with viral load <10000 copies/ml [53].

3.2. Hepatitis C Virus

The variability in the association between the innate host immune response and the HCV infection suggests that KIR and HLA play a role in HCV-related disorders. According to the World Health Organization, about 3% of the world's population is infected with HCV and 3 to 4 million individuals are infected each year. Although the new antiviral treatments are very promising, only a minority of patients heals from HCV infection whereas the remaining ones (60-85%) develop a chronic infection [54]. It has also been reported that NK cells play a role in the clearance of HCV infection [55]. Particularly, it has been shown that NK cells inhibit HCV replication and perform a targeted cytotoxic action since NK cells from healthy donors kill cells where HCV replicates and secrete IFN-γ [56.57].

Several studies, but not all, have demonstrated that KIR2DL3/HLA-C1 combination in homozygosis is associated with HCV clearance. This combination has also been associated with sustained virus response to anti-HCV therapy [43].

Moreover, it has been proposed a protective role for HLA-Bw4/KIR3DS1 against liver disease progression [43]. Previous results have also suggested that KIR2DL1/HLA-C2 may be responsible for a stronger inhibitory response than does KIR2DL3/HLA-C1 [58]. In a cohort of 407 HCV-seropositive, KIR2DL2, 2DS2, 2DL2/2DL3, and 2DL5A–/2DL5B1 were more frequent in individuals with HCV clearance than in patients with chronic infection. In particular, KIR2DL5A/2DL5B1 are associated with HCV spontaneous clearance, while KIR2DL3/2DL3, 2DL3/ 2DL31+HLA - C1, or C1C1 are associated with chronic infection. [59]. Moreover, the role of various KIR/KIR-ligand genotype on the outcome of HCV was analysed by flow cytometry also in people who inject drugs. In this study, the analysed population was a cohort of people who inject drugs from Germany, and the study was validated in a second cohort of people recruited in North America. Through a multivariate logistic regression analysis, KIR3DL1/HLA- Bw4T combination was demonstrated to be associated with spontaneous clearance of HCV infection in people who inject drugs. So, HLA-$Bw4^T$ and multiple copies in combination with KIR3DL1 are associated with protection against chronic hepatitis C in people who inject drugs by distinct mechanisms [60].

Finally, a meta-analysis of 16 studies of KIR genes and their HLA-ligands concerning KIR effect on the clearance of HCV infection, showed the association of KIR2DS3 with both spontaneous and treatment-induced clearance, and of HLA-C2 ligand with the inability to spontaneously erase the virus [61].

3.3. Human Cytomegalovirus

HCMV, member of the herpes virus family (type 5), is ubiquitous in human populations, but its prevalence in the different populations depends on socioeconomic status. So, it reaches a prevalence of 100% in Africa and Asia, and of 80% in the Western world. Primary HCMV infection is asymptomatic in the immunocompetent host, but a few individuals (<10%) develop clinical symptoms (malaise, fever, sweating, and abnormal liver function) [62]. NK cells play a crucial role in host defence against HCMV infection. In order to evade CD8+ lymphocytes response, HCMV encodes several proteins interfering with the expression of HLA class I antigens. This makes infected cells more susceptible to attack by NK cells [63].

In a case study, the whole NK population of a child with a new immunodeficiency syndrome and recurrent HCMV infection, expressed KIR2DL1 and the child also carried its ligand, HLA-C2, suggesting that this strong inhibitory combination (KIR2DL1/HLA-C2) suppressed NK activity, hence the cells were not able to mount an efficient immune response against HCMV [64]. On the other hand, a role for activating KIRs in the HCMV infection control has been documented after kidney or hematopoietic stem cells transplantation. In fact, the rate of HCMV reactivation in patients homozygous for the KIR A haplotype (known to do not carry almost activating KIRs) is higher than in patients with the B haplotype (known to carry a variable number of activating KIRs). This strongly suggests the importance of activating KIRs in the immune surveillance against HCMV [65-67].

Moreover, immunocompetent subjects carrying both homozygous A haplotype and the HLA-$Bw4^T$ allele have been shown to be, by logistic regression, at higher risk of developing symptomatic disease after primary HCMV infection [68]. In a further study, the same authors showed that there is an interaction between KIR/HLA and IgG allotypes genetic marker (GM) in the control of HCMV infection. These data on the role played of both cellular (KIR and HLA, hence NK) and humoral immunity (GM allotypes, hence immunoglobulins) in the control of HCMV infection should be of value in guiding efforts to reduce HCMV-associated health complications [69].

It has been reported that HCMV-specific CD8+ levels can predict the risk of post-transplant infection, and activating KIR genes have been related to protection [70]. Therefore, this dual role of NK cells in HCMV infection interrelation with rejection requires attention. Further phenotypic, functional, and genetic analyses of NK cells are required to gain insight into the pathophysiology of solid organ transplant complications. So, it will be possible to develop biomarkers with potential clinical value.

3.4. Hepatitis B Virus

It is estimated that 257 million people live with HBV infection (defined as positive for surface hepatitis B antigen). In 2015, hepatitis B caused 887,000 deaths, mostly due to complications (including cirrhosis and hepatocellular carcinoma) [71]. NK cells are activated in the early phase of the response to HBV, however, there is considerable population variability in rates of HBV infection. Despite the lack of detailed genetic and functional analyses that explore the KIR influences on HBV infection in large cohorts, the accumulated evidence supports the idea that activation of NK cells contributes to inflammation and hepatic injury during HBV infection in HBV transgenic mice, and in patients with HBV infection [72-74].

In a Turkish cohort, the frequencies of inhibitory KIR2DL3 and KIR3DS1 were higher in the healthy group than in the groups of chronic HBV patients and patients with spontaneous healing, whereas were not observed statistically significant differences for the frequencies of AA and Bx genotypes between the groups. Moreover, in chronic HBV patients, one copy of HLA-C1 alleles group was shown to be associated with cirrhosis and two copies of HLA-C1 alleles group, which resulted in inherently more potent NK cells, were shown to be associated with HCC. This suggests that NK cell activation should play a key role in the progression of cirrhosis to HCC. [75].

In a group of 202 Iranian HBV infected patients and 100 healthy controls, the frequencies of the KIR2DL5A, KIR2DS1, and KIR3DS1 genes were significantly increased in recovered subjects when compared with both control and patient groups. In addition, KIR2DL5, and KIR3DP1, a pseudogene, as well as HLA-Bw4 and HLA-A-Bw4 ligands groups, were increased in healed patients when compared with healthy controls. Finally, the KIR3DS1 + HLA-Bw4, KIR3DS1 + HLA-$Bw4^I$, and KIR3DS1 + HLA-A-Bw4 genotypes were significantly more frequent in subjects with resolved infection than both in healthy control and in patient groups. Also, this study suggested a role of the NK cells activation in the HBV clearance in infected people [76].

In a recent study, our group compared the frequencies of KIR and HLA genes/alleles in subjects with chronic hepatitis B (CHB) and subjects with resolved infection. The inhibitory KIR2DL3 gene was more frequent in subjects with resolved infection (98%) than in CHB (81%). The only other KIR gene expressed differently between subject with resolved infection and CHB was the KIR2DS4-Del, known to code for an inactive receptor. No difference was reported in the frequency of KIR haplotypes between the groups, so activating receptors should not play a role in the control of the infection [77]. Thus a combination of KIR/HLA gene/alleles is able to predict the outcome of HBV infection.

With the aim to gain insight in the association of this combination and CHB, our group analysed the distribution of HLA-A alleles, in patients and controls with resolved infection, carrying HLA-A-Bw4 group. We observed that HLA-A*24 allele is over-represented when compared to the frequency of HLA-A* 24 alleles in normal reference population (Table **2**). These results confirm and extend in vivo, data obtained in vitro with NK clones and with the binding of HLA- A*24 tetramers to KIR3DL1 [78].

Table 2. Distribution of human leukocyte antigen-A alleles in chronic hepatitis B patients and in subjects with resolved hepatitis B virus infection positive for HLA-A Bw4 and KIR3DL1.

	CHB patients (N = 33)		Resolved HBV infections (N=10)	
HLA-A alleles	**N**	**%**	**N**	**%**
A*1	3	9.09	1	10
A*2	7	21.21	2	20
A*3	3	9.09	1	10
A*11	5	15.15	1	10
A*23	4	12.12	2	20
A*24	28	84.85	10	100
A*25	3	9.09	0	0
A*26	1	3.03	0	0
A*29	2	6.06	1	10
A*30	3	9.09	0	0
A*31	0	0	0	0
A*32	2	6.06	0	0
A*33	0	0	1	10
A*34	0	0	0	0
A*36	0	0	0	0
A*43	0	0	0	0
A*66	2	6.06	0	0
A*68	0	0	1	10
A*69	1	3.03	0	0
A*74	1	3.03	0	0
A*80	1	3.03	0	0

HLA human leukocyte antigen, CHB chronic hepatitis B, HBV hepatitis B virus.

The number of alleles is twice the number of subjects, as each person has two alleles—one maternal and one paternal.

Genomic DNA was extracted by a commercial kit (PureLink® Genomic DNA, ThermoFisher Scientific, Waltham, MA, USA) from frozen mononuclear cells obtained in the previous study from peripheral whole blood samples. The HLA-A loci genotypes were determined using the commercially available HLA Class I A Locus DNA Typing Tray kit (One Lambda, ThermoFisher Scientific Brand, California, USA). The kit is based on the PCR-SSP method, performing 32 reactions for each individual according to the manufacturer's instructions.

It can be observed that, both in patients and controls, HLA-A*24 allele is over-represented, 28 alleles out of 66 and 10 out of 20, respectively. In fact, the gene frequency of HLA-A*24 allele, typed with serological methods was 79 x 10^{-3} in a panel of normal Sicilians (N=297) [90].

Finally, it has been recently reported an association between HBV virologic status and HLA class. The authors state that their results confirm the role of CD8+ T-cells in the control of HBV, suggesting that this effect is mostly driven by HLA-A restricted responses [79]. Based on these results, it is possible to suggest that this association might be also linked to the role of HLA-A antigens in the control of NK activity.

The data on virus and KIR suggest a role for pathogen selective pressure in determining KIR and HLA gene segregations. The influence of host genetic variations, particularly in these two polymorphic loci (HLA and KIRs), on viral infectivity, is becoming increasingly well accepted among infectious immunity researchers. Several mechanisms have been shown in different studies, which implicate the central role of these two molecules in anti-viral immunity. For instance, they work controlling the level of cytokine production and antibody responses, increasing the memory specific CD4+ T cell responses, impairing the CD8+ T cell reactions which increase recognizing, controlling the viral proteins, and predicts the autonomous resolution of viral infections.

Further efforts in determining the exact role of HLA/KIRs interaction in viral infections and understanding the molecular nature of this interaction with respect to the viral antigens as well as determining additional probable associations between HLA/ KIRs and other viral diseases will increase our capacity to find out the real place of immunogenetics in viral infections and to apply potentially this knowledge clinically.

An overview of the association between KIR/HLA genes and infectious diseases is shown in Table **3**.

Table 3. Killer immunoglobulin-like receptors/human leukocyte antigen associations in viral infections.

Disease	KIR–HLA Ligand Pair	Effect
HIV	KIR3DS1/HLA-Bw4-80^{I}	Slower progression
	KIR3DL1*004/HLA-Bw4	Slower progression
	KIR3DS1/HLA-B*57:01	Protection
	KIR3DS1	Reduced risk of infection
	KIR2DL2-2DS5	Reduction in perinatal HIV transmission
HCMV	KIR2DL1 expression on all NK cells	Recurrent CMV infection
	>1 activating KIR in donor in bone marrow transplantation	Protection from CMV reactivation in the recipient
	KIR A haplotype/HLA-Bw4^{T}	Higher risk of developing symptomatic disease
HCV	KIR2DL3/2DL3, 2DL3/2DL31+/HLA-C1-C1C1	Chronic infection
	KIR2DL3/HLA-C1 homozygosity	Resolution of infection
	KIR3DS1/HLA-Bw4	Protection
	KIR2DL5A–/2DL5B1	HCV spontaneous clearance
	KIR3DL1/HLA-Bw4 80^{T} KIR2DS3	Spontaneous clearance of infection in people treated with drugs Spontaneous and treatment-induced clearance of infection
HBV	KIR2DL3; KIR3DS1	Lower in chronic HBV patients
	KIR3DS1+/HLA-Bw4, KIR3DS1+/HLA-Bw4^{Iso}, KIR3DS1+/HLA-A Bw4	Common in recovered individuals

HIV immunodeficiency virus, CMV cytomegalovirus, HCV hepatitis C virus, HBV hepatitis B virus, HLA human leukocyte antigen, KIR killer immunoglobulin-like receptors, NK natural killer, – absence, + presence.

For references, see text.

4. DISCUSSION

Combinations of HLA class I and KIR variants have been associated with pathologies as autoimmunity, viral infections, pregnancy-related disorders, transplantation, and cancer, whereas contrasting results have been obtained in longevity studies [8,80-82]. Thus, interactions between KIR and HLA class I polymorphisms have probably been involved in human during incidences of epidemic infections and have affected reproduction and population expansion. These types of selection pressures might explain the functional coevolution of KIR with diverging HLA class I molecules and why KIR sequences, like the HLA loci, are highly polymorphic and rapidly evolving [48,83].

In general, KIR-HLA combinations determining lower levels of NK inhibition or stronger levels of NK activation are associated with an increased risk of autoimmune disorders. However, they tend to be protective against infectious diseases. Multiple factors as the limited understanding of KIR gene expression control, the high polymorphism of the KIR and HLA ligands, the oversimplification of the structural complexities of their interactions and the incomplete knowledge of KIR ligands complicate the interpretation of KIR-HLA disease association [84,85]. However, the reviewed data clearly demonstrate that susceptibility to both autoimmune and infectious diseases is modified by specific KIR/HLA ligand interactions.

Both to shape the KIR repertoire of fully functional NK cells and to establish self-tolerance KIR/HLA interactions are also essential. Indeed, NK education occurs mainly through the interactions between inhibitory KIRs and HLA class I molecules. This interaction determines the degree of response of NK cells to activating signals, like stress ligand and inflammatory cytokines. In addition, immunologic experiences can beyond modulate NK cells function, establishing adaptive, memory-like, NK cell populations [86].

The interactions between KIR and HLA, allelic-specific, strongly influence NK cell education, determining the threshold for NK reactivity, with an important impact on human health. The strength of this education is inversely correlated with the requirement for additional activating input (see Figure **2**). Uneducated NK cells do not bind to self HLA molecules and are also insensitivity for inhibition. None pattern of NK education can be classified as beneficial or detrimental, as their benefits are disease-specific [87]. In particular, in some autoimmune diseases, NK is thought to play both beneficial and detrimental roles. In RA and SLE, decreased numbers and function of NK lead up the onset of symptoms, hence NK are suggested to be important for controlling inflammation. However, although these observations support a regulatory role for NK cells in the control of autoimmune diseases, the involved mechanisms and the role of NK education remain to be studied (see below, control of inflammation by blocking KIR genes) [87].

New technologies to better define the KIR genotyping associated with the different diseases are fundamental to develop novel therapeutic approaches to treat several autoimmune diseases. Indeed, based on these genetic studies, antibodies directed against activating KIRs are currently tested in clinics. The blocking anti-KIR human monoclonal antibody 1-7F9 (IPH2101), directed against KIR2DL1 and KIR2DL2/3, is the most advanced compound targeting NK cells population [42]. It blocks their interaction with their HLA-C ligands (both HLA-C group 1 and group 2 allo-

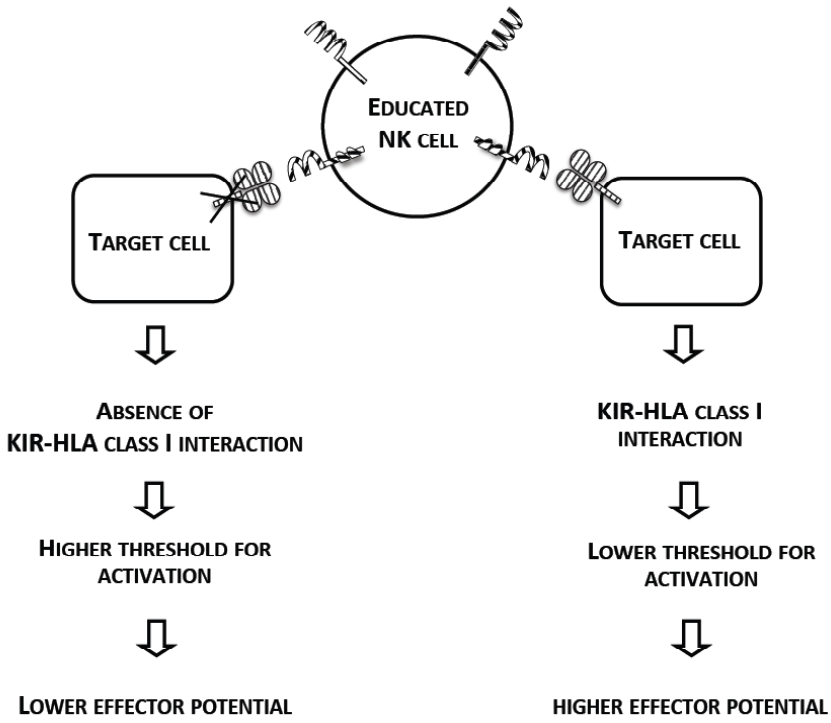

Fig. (2). Functional Outcome of Educated Natural Killer Cell–Target Interactions.
The figure shows the outcomes of the interaction between NK cells, which express KIRs, and target cell, which express or not HLA class I molecules. The presence or absence of KIR-HLA binding is able to influence the potential effector of NK cells.

types), hence breaking NK tolerance versus autologous cancer cells. So, its use can be proposed in anti-cancer immunotherapy rather in autoimmune diseases therapy [42]. On the other hand, the administration of an anti-KIR2DL1, 2, or 3 antibodies in mice transgenic for both KIR2DL3 and their HLA ligands, developed to study human KIR2DL1, 2 and 3 blockade, showed that the antibody is capable of inducing NK cells to effectively reduce or eliminate concanavalin A from T cell blasts, a model of inflammation. The results suggest that it can be beneficial to potentiate the activity of KIR2DL1, 2 and/or 3-positive NK cells as they can contribute to the removal of pro-inflammatory T cells [88].

Moreover, the understanding of the exact role of HLA/KIR interactions in control of viral infections could support the development of drug-resistant viruses, which may help to find strategies to improve therapeutic methods in viral infected people [89].

Further efforts and incremental experiments are necessary to specify KIR/ HLA role interaction in human disease, hence try to apply this knowledge in clinics, and to define a common thread of involvement of KIR across diseases sharing some etiological characteristics.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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3.6. Do activating Killer immunoglobulin receptors (KIRs) protect general population from Human immune deficiency virus (HIV) infection? A pilot study performed in Sicilian population.

(Manuscript submitted)

Do activating Killer immunoglobulin receptors (KIRs) protect general population from Human immune deficiency virus (HIV) infection? A pilot study performed in Sicilian population.

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Summary: Recent studies have shown that KIRs are associated with susceptibility to HIV infection and HIV disease progression although some results were inconsistent. Therefore, in the present paper we analysed the role of KIR/HLA repertoire in the susceptibility to HIV in a sample of Sicilian population consisting of 60 blood donors as control population, and 89 Sicilian patients with HIV infection. Logistic regression analysis shows that activating KIR2DS4-Full as well as haplotypes Bx were factors of resistance to infection, whereas the ligands HLA-C1 and HLA-A-Bw4 were factors of susceptibility to infection. Present data and their interpretation are in line with the presence of activating natural cytotoxicity receptors in the vast majority of HIV-controllers and imply that general population, or at least blood donors, carry genetic markers for HIV resistance. Our data imply that the general population, or at least the blood donors who are selected for their serological and molecular negativity towards hepatitis viruses B and C as well as HIV, are carriers of genetic markers that confer resistance to HIV. This is intriguing because previously we have demonstrated that the frequency of the HLA-A-Bw4 ligand group was significantly higher in Sicilian patients affected by chronic HBV hepatitis than in healthy controls represented by the same sample of blood donors of the present study.

Keywords: HLA**;** HIV; Immunogenetics; KIR

1. Introduction

Natural Killer (NK) cells are important components of the innate immune system that play a key role in host's first line of defence against viral and tumor targets. Their effector functions, including cytotoxic activity and chemokines and cytokines production, are regulated by the balance of signal transduction between the activating and the inhibiting receptors. Killer immunoglobulin receptors (KIRs) are expressed on the membrane of NK cells and regulate their killing function by interacting with specific ligands represented by some class I Human leukocyte antigens (HLA), expressed on their cell targets.

The alleles of the HLA-C and HLA-B *loci* can be divided into two groups of ligands according to the amino acid sequence determining the KIR-binding epitope. HLA-C alleles can be distinguished into HLA-C group 1 (C1) with asparagine at position 80 or HLA-C group 2 (C2) with lysine at position 80of the α -1 domain of the α helix. Among HLA-B alleles only those bearing the HLA-Bw4 motif serve as ligands for KIRs. Similar to HLA-C, HLA-Bw4 is divided into two groups based on the presence of isoleucine or threonine at positions 80 (HLA-Bw4$^{I/T}$). In addition, the same epitope is also found on 4 HLA-A antigens (HLA-A*23/*24/*25/*32) and the ligand is called HLA-A-Bw4 (Cerwenka and Lanier, 2001; Littera et al., 2017; Aiello et al., 2018; Aiello et al., 2019).

KIRs are characterized by two or three extracellular immunoglobulin-like domains and a short (S) or long (L) intracytoplasmic tail. All inhibitory KIRs are characterized by long intracytoplasmic tail, while the activating KIRs by short ones. KIRs recognize virus-infected or transformed cells by binding different class I allelic variants. The KIR system is polygenic because it is characterized by haplotypes that carry many genes. Haplotype A is invariant in terms of gene content and is composed of five inhibitory genes (KIR2DL1, 2DL3, 3DL1, 3DL2, and 3DL3), one activating gene (KIR2DS4), and KIR2DL4, which may have both inhibitory and activating capacity. Group B haplotypes embrace all other KIR haplotypes with different combinations of activating and inhibitory KIR genes with one or more of the following genes: KIR2DS1, 2DS2, 2DS3, 2DS5, 3DS1, 2DL2 and 2DL5. Therefore, haplotypes A have fewer activating genes than haplotypes B. Moreover, up to 70% of individuals of European ancestry homozygous for KIR haplotype A exclusively carry non-functional deletion variants of KIR2DS4 which means that they do not express activating KIRs on the NK cell surface (Aiello et al., 2018; Aiello et al., 2019; Bontadini et al., 2006; Lanier, 2008; Littera et al., 2017).

KIRs and/or their HLA ligands have been shown to affect susceptibility and resistance to infectious diseases, autoimmune/inflammatory disorders, and to be associated with variable cancer prognosis and response to treatment as well as with the outcome of both hematopoietic stem cell and kidney transplantation (Aiello et al., 2018; Aiello et al., 2019; Blunt and Khakoo, 2020; Di Bona et al., 2014; Di Bona et al., 2017; Gambino et al., 2018; Leone et al., 2017; Littera et al., 2017; Shaffer and Hsu, 2016).

Several lines of evidence relate KIR gene variation with the course of the infection as well as the susceptibility to human immunodeficiency virus (HIV) infection and HIV disease progression. According to a recent review (Hens et al., 2016), several studies supported the role of the activating KIR3DS1, in the presence or absence of its putative HLA-Bw4^{I} ligand, in the resistance of HIV infection and in a slower progression of the disease. Therefore, resistance to HIV-1 infection would be mainly related to the presence of KIR/HLA activating combinations, consisting of e.g., activating KIRs, group B haplotypes or KIR inhibitors in the absence of their ligands. However, studies examining disease progression reported both beneficial and harmful effects of activating KIR/HLA genotypes (Hens et al., 2016).

A more recent meta-analysis has contributed to the immunogenetic knowledge of this infectious disease, profiling the role of some KIR in HIV infection when infected patients were compared to HIV-exposed seronegative individuals. The lw number of studies precluded conclusions about Africans and Asians. Thus, their main findings were confined to patients of European ancestry, who were protected by two KIR polymorphisms (2DL3 and 3DS1S1) (Chaisri et al., 2019). KIR3DS1S1 has seemingly stronger support from functional studies than 2DL3. In the model proposed by authors, individuals carrying 3DS1 would lead to higher NK cell cytokine secretion to control early HIV-1 infection (Carr et al., 2007; Chaisri et al., 2019).

However, as NK cell activation is the result of a summation of activation and inhibition signals, it is difficult to determine which KIRs influence on the outcome of infection without an analysis of haplotypes and HLA ligands to distinguish combined effects. This kind of analysis could help to better understand the complex KIR's involvement in the innate immune responses of HIV acquisition. Therefore, further studies in homogeneous populations are mandatory as HIV continues to be a major global public health problem because an estimated 38.0 million people were living with HIV at the end of 2019 (HIV/AIDS - World Health Organization). New insights into the mechanisms of control of infection could lead to better care.

To gain insight into the mechanisms of the resistance/susceptibility to infection by HIV, in the present paper we analysed the role of KIR/HLA repertoire in the susceptibility to HIV in a sample of a homogeneous European population from Western Sicily, taking into account haplotypes and KIR/HLA combinations. All the patients were in treatment with antiretroviral therapy. As a control, we used blood donors, representative of the general population, to assess if there are differences in KIR or HLA-ligand repertoire between subjects with HIV infection and the general population.

2. Materials and Methods

Ninety-six Sicilian patients HIV positive, responders to treatment with antiretroviral therapy, were consecutively recruited at the "Paolo Giaccone" Palermo University Hospital, Sicily, Italy. As non-infected control group, 60 Sicilian blood donors (negative for anti-HIV I/II antibodies, anti-HCV antibodies, HBsAg, anti-HBc antibodies, and negative for HIV/HCV/HBV nucleic acids) already genotyped (Di Bona et al., 2014) were included. These subjects have been used as controls in previous studies (Di Bona et al., 2014; Di Bona et al., 2017). The mean age of the 96 patients with HIV was 51.5±11.8 years, with a prevalence of males (80.9%). The control population was younger (mean age, 39.2±11.5 years) with prevalence of male sex as well (males, 69.6%). The age difference is significant but in Italy the highest incidence of new HIV diagnoses is found in the 25-29 and 30-29 age groups (Notiziario dell'Istituto superiore della Sanità). The protocol study was approved by the Ethics Committee of the "Paolo Giaccone" Palermo University Hospital. The study was conducted in accordance with the Declaration of Helsinki and its amendments. All patients gave their written informed consent to participate as well as for sampling and banking of the biological material. Consent forms were administered by the physicians involved in the study.

Peripheral whole blood samples were collected, and genomic DNA was extracted from leukocytes by Maxwell® 16 Instrument (AS1000) using Maxwell® 16 LEV Blood DNA Kit (Promega Corporation, Madison, WI 53711, USA). Using the polymerase chain reaction sequence-specific primer technique, the DNA of cases and controls was genotyped for the presence of the 3 major KIR ligand groups: HLA-C1, HLA-C2 and HLA-Bw4, both HLA-B and HLA-A loci (Epitop-TYPE kit; BAG Health Care GmbH, Lich, Germany). KIR genotyping was performed for both inhibitory and activating KIRs using the KIR-TYPE kit (BAG Health Care GmbH, Lich, Germany). KIR and HLA gene (group) profiles were determined by the presence or absence of each KIR/HLA gene (group).

Crude comparisons of gene frequencies were made using 2x2 contingency tables, analysed by the χ^2 test, and crude ORs were also calculated. A complete case multiple logistic regression model was also considered to estimate Adj. ORs. The procedure started from a full model, including a set of variables as covariates encompassing KIR genes with their HLA ligands, except for those predictors having one unique value (zero-variance predictors). Both crude and Adj. ORs were calculated with 95% confidence interval (95% CI). We also used a Bonferroni correction as a guard against inflated family-wise error (that is the possibility of making at least one type I error) to further confirm the results of the logistic analysis. Statistical analyses were performed using Stata 14.2 package (Stata Corporation, College Station, Texas, USA). Level of significance was set at <0.05. Missing values were excluded and complete case analysis was performed.

3. Results

To assess if differences in KIR repertoire and in their HLA ligands may influence the susceptibility to HIV infection, in this study we compared the frequencies of these gene families between subjects with HIV infection and subjects from the general population, represented by blood donors.

There was a difference in the frequency of KIR haplotypes between the groups since haplotypes Bx (AB or BB) were more frequent in controls (93%) than in the HIV-infected patients (68%). This datum suggests that activating receptors, more present in haplotype B than in haplotype A (Aiello et al. 2018; Aiello et al., 2019), might play a role in the control of HIV infection (crude Odd Ratio (OR), 0.15, P=0.0002) (Table 1).

Accordingly, the activating KIR2DS4-Full gene was less frequent in HIV (11%) than in controls (45%) (crude OR, 0.15; P<0.01). All the other activating genes, except KIR2DS2, were also less frequent in HIV-infected subjects than in controls, although the differences did not attain statistical significance (Table 1). The inhibitory KIR2DL5A gene was less frequent in HIV-infected (34%) than in healthy blood donors (55%) (crude OR, 0.42; P<0.01). No difference in the frequencies of the other KIR inhibitor genes was found.

Regarding the KIR-ligand groups, the frequency of individuals with HLA-A-Bw4 was higher in the HIV group (34%) compared to controls (10%) (crude OR, 4.58, P<0.01). Similarly, the frequencies of HLA-C1 and

HLA-B-Bw4^{I} groups were higher in HIV patients than in controls (respectively, 73% vs. 42%, crude OR, 3.79, *P*<0.01 and 54% vs. 37%, crude OR, 2.02; *P*=0.038) (Table 1).

The KIR-HLA associations suggested by literature (Middleton and Gonzelez, 2010; Biassoni et al., 2011) were analysed and in Table 1 were reported combinations significantly different between patients and controls, i.e., 3DL2/HLA-A-Bw4 (inhibitory) and 2DS2/HLA-C1 (activating). The frequencies of both combinations were increased in patients.

Table 1. Frequencies of KIR, HLA ligands and KIR-HLA combinations among individuals with HIV and healthy controls.

Genetic Factor	Frequency HIV N_{tot} = 96 N (%)	Frequency Healthy blood donors N_{tot} = 60 N (%)	Crude-OR (95%CI)	*P*-value
KIR haplotypes				
AB+BB	65 (68)	56 (93)	0.15 (0.05; 0.45)	**0.0002**
KIR activating genes				
2DS1	30 (34)	29 (48)	0.54 (0.26; 1.12)	0.07
2DS2	53 (59)	27 (45)	1.79 (0.88; 3.68)	0.08
2DS3	30 (34)	27 (45)	0.62 (0.30; 1.28)	0.16
2DS4 Full	10 (11)	27 (45)	0.15 (0.06; 0.38)	**<0.0000 1**
2DS5	26 (29)	26 (43)	0.54 (0.25; 1.13)	0.07
3DS1	30 (34)	29 (48)	0.54 (0.26; 0.12)	0.07
KIR inhibitory genes				
2DL1	89 (93)	58 (97)	0.44 (0.09; 2.18)	0.30
2DL2	58 (60)	36 (60)	0.84 (0.40; 1,76)	0.63
2DL3	73 (76)	46 (77)	0.97 (0,45; 2.07)	0.92
2DL4	96 (100)	60 (100)	/	/
2DL5A	30 (34)	33 (55)	0.37 (0.19; 0.72)	**0.003**
2DL5B	30 (31)	15 (25)	1.24 (0.56; 2.79)	0,56
3DL1	88 (92)	56 (93)	0.79 (0.23; 2.73)	0.70
3DL2	96 (100)	59 (98)	/	0.20
3DL3	96 (100)	59 (98)	/	0.20
HLA groups				
HLA-C1	65 (73)	25 (42)	3.79 (1.79; 8.06)	**0.0001**
HLA-C2	64 (67)	36 (60)	1.29 (0.61; 2.70)	0.45
HLA-B-Bw4^{T}	23 (24)	12 (20)	1.28 (0.54; 3.21)	0.53
HLA-B-Bw4^{I}	48 (54)	22 (37)	2.02 (0.98; 4.19)	**0.038**
HLA-A-Bw4	30 (34)	6 (10)	4.58 (1.68; 14.37)	**0.0009**
KIR-HLA combinations				
3DL2/HLA-A-Bw4	30 (34)	6 (10)	4.58 (1.68; 14.37)	**<0.01**
2DS2/HLA-C1	39 (44)	13 (22)	2.82 (1.27; 6.46)	**<0.01**

Logistic regression analysis supported the effects of haplotype Bx (Adjusted (AdJ). OR, 0.09, $P<0.01$), ligands HLA-C1 group (Adj. OR, 4.73, $P<0.01$), HLA-A-Bw4 group (Adj. OR, 5.82, $P<0.01$) and KIR2DS4-Full (Adj.OR, 0.14, $P<0.01$). After Bonferroni's correction, all the variables remained significantly associated with susceptibility/resistance to infection ($P<0.01$ for all variables) (Table 2).

Table 2. Logistic regression predicting risk of disease.

Variable	Code	Adj. OR	95% CI	*P*-value
Haplotype	0: AA			
	1: Bx	0.09	0.01; 0.56	0.007
KIR2DS4-Full	0: Absent			
	1: Present	0.14	0,03; 0.55	0.001
HLA-C1	0: Absent			
	1: Present	4.73	1.83; 12.19	0.001
HLA-A-Bw4	0: Absent			
	1: Present	5.82	1.66, 20.46	0.007

Using Bonferroni correction the level of significance becomes 0.0125 (0.05/4=0.0125). With this more conservative threshold, all the variables remain significantly associated with the outcome ($P<0.01$ for all variables).

4. Discussion

Susceptibility to HIV infection has been shown by some studies, reporting differences in frequency in specific KIR genes and their cognate HLA ligands between HIV positive patients and exposed seronegative either partners or intravenous drug-addicted (Hens et al., 2016). Multiple meta-analytical treatments presented strong evidence of the protective effect (up to 81%) of the KIR genes (2DL3 and 3DS1S1) among exposed seronegative Caucasians (Chaisri et al., 2019). Interestingly, KIR3DS1 homozygosity was found to be significantly increased in HIV-exposed intravenous drug addicts and HIV-negative partners of sero-discordant couples when compared to HIV infected patients (Boulet et al., 2008). In addition, HIV-exposed seronegatives have shown a higher prevalence of KIR AB haplotypes, characterized by a higher number of activating KIR genes (Aiello et al. 2018; Aiello et al., 2019).

In HIV-infected patients, the KIR3DS1-HLA-Bw4^{I} combination has been associated with slower progression to Acquired Immunodeficiency Syndrome, lower mean viral load and protection against opportunistic infections (Martin et al., 2007; Qi et al., 2006). In addition, the results of Malnati *et al*., have suggested that co-expressed 2DS/3DS1 molecule network plays a key role in the immune control of HIV viremia levels, although the limited number of individuals studied did not allow a firm conclusion to be drawn (Malnati et al., 2017).

Overall, these results are consistent with our findings showing a lower frequency of both haplotype Bx and KIR2DS4-Full in HIV-infected patients compared to blood donors (respectively Adj. OR, 0.09 and 0.14). This suggests that a KIR balance shifted towards NK cell activation in the general population might be important as first line of defence against HIV and might reduce the rate of infection after exposure. Accordingly, the analysis of genotypic interactions revealed that the HLA-A-Bw4 ligand, more frequent in the HIV-infected group (Adj. OR 5.82), was always seemingly combined with the inhibitory KIR3DL2.

Concerning the other KIR ligands, present results showed that HLA-C1 group was significantly more frequent in HIV-infected patients (73%) than in controls (42%). Previously, it has been shown that a viral strategy to evade detection is to trigger inhibition of NK cells by maintaining HLA expression. Both Zika virus

and HIV induce the up-regulation of HLA-C expression on infected cells (Apps et al., 2013; Glasner et al., 2017; Zipeto et al., 2012). Furthermore, it has been suggested that HLA-C*01 prevalence correlates with Covid-19 spreading across Italy (Correale et al., 2020). Since HLA-C alleles interact primarily with NK cells and the vast majority of HLA-C molecules exhibit HLA-C1 or HLA-C2 groups epitope (Parham et al. 2012), this strategy enables escape from immune surveillance through HLA-C/KIR mediated inhibition of NK cells. In fact, KIR2DL1 and KIR2DL2 bind KIR-ligand HLA-C2 and HLA-C1 groups respectively, resulting in inhibition of NK cell activation (Khakoo et al., 2004; IPD - KIR Ligand Calculator). Accordingly, in our population of HIV-infected individuals, HLA-C1 group showed a higher frequency (73%) when compared to the same frequency the control population (42%), hence leading to stronger inhibition.

The small sample size is a limitation of the present study. However, we confirmed in a different setting the hypothesis that there can be a higher risk of HIV infection in susceptible individuals. Further studies, performed using the general population as control, will be necessary to confirm the evidence provided here.

Present data and their interpretation are in line with the distinctive persistence of effective induction of activating natural cytotoxicity receptor expression in elite controller and long-term nonprogressor patients (Marras et al., 2013). To the best of our knowledge, this is the first study showing KIR/HLA associations with HIV using as control not HIV-exposed seronegative individuals but a sample from the general population. Nevertheless, a note of caution should be added due to the relatively small number of HIV patients and controls studied.

Our data imply that the general population, or at least the blood donors who are selected for their serological and molecular negativity towards HBV, HCV, and HIV, are carriers of genetic markers that confer resistance to HIV. This is intriguing because the frequency of the HLA-A-Bw4 ligand group was higher in Sicilian patients affected by chronic HBV hepatitis (58%) than in healthy controls represented by the same sample of blood donors of the present study (10%) (crude OR, 12.38; P<.001) (Di Bona et al., 2017).

Conflicts of Interest: The authors declare no conflict of interest.

Author Contributions: "Conceptualization, AC, CCo, CC, GC, and DDB.; methodology AA, CC, GC, GD, and DDB.; software, MEL and DDB; validation, MEL, CC, and DDB; formal analysis, MEL, AA, GA, CC, and DDB.; investigation, MEL, AA, GA, GAm, and MR; resources, GAm, MR, AC, CCo, GD, GC; data curation, MEL, AA, GA, CC, and DDB; writing—original draft preparation, MEL,CC, and DDB.; writing—review and editing, MEL,CC, and DDB.; visualization, AC, CCo, CC, GC, and DDB; supervision, CC, GC, and DDB; project administration, CC, CCo, GC, and DDB; funding acquisition, CCO, GD, and AC. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.
Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to Privacy.

Abbreviations

Adj	Adjusted
HBV	Human epatitis B virus
HCV	Human epatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
KIR	Killer immunoglobulin receptor
NK	Natural killer cell
OR	Odd ratio

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3.7. Vaccination of the older adult: challenges and promises

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CHAPTER 8

c0100 # Vaccination in old age: Challenges and promises

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s0010 ## 8.1 Introduction

p0010 Aging is one of the main health challenges worldwide, and promoting healthy aging is a key global priority. The health of older people is threatened by their increased susceptibility to infectious disease and associated complications, which is related to many factors, especially the dysregulation of immunity generally termed "immunosenescence." This is believed to adversely affect the efficacy of vaccines, thus reducing the protection provided by most current vaccines in older people. Identifying the key factors responsible for reduced vaccination efficiency in older adults, and devising countermeasures to solve this problem, is essential for improving the outcomes of vaccination. This will allow for better protection against infections in this growing segment of the population (Caruso and Vasto, 2016).

p0015 In general, most available vaccines are not tailored for specific age or risk groups, such as that represented by old people. To address this problem, different approaches such as employing higher doses of vaccine, boosting vaccinations, and using adjuvants (for example, based on squalene) are being tested. However, due to immunosenescence, none of these approaches has so far been fully satisfactory, with the exception of an adjuvanted recombinant zoster vaccine (McElhaney et al., 2019).

p0020 Immunosenescence is likely to be a major cause contributing to reduced potency of current vaccines as well as vaccine failure in the senior population. However, the example quoted earlier (McElhaney et al., 2019), consisting of properly selected recombinant antigens, effective adjuvants, and suitable doses, shows that there may be no intrinsic reason why vaccine formulations for aged people cannot function highly effectively and mediate strong and long-lasting protection. Nonetheless, shingles is of course caused by reactivation of a latent herpesvirus, and requirements for protection are likely to be different from immune requirements for protection against de novo infections with novel acute viruses. As discussed by Aiello et al. (Aiello et al., 2019), the hallmarks of immunosenescence include a reduced ability to respond to new antigens, the accumulation of memory

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B and T cells for previously encounter antigens, and chronic low-grade inflammation (known as "inflamm-aging"). Although our understanding of the role of immunosenescence in the vaccination response in the senior population remains incomplete, and will be different for different infectious agents and different human populations, strategies for improving the efficacy of vaccines for older people must be assessed in the context of the hallmarks of immunosenescence.

p0025 This chapter is divided into two parts. In the first part, we will discuss the state of the art of vaccination as a preventive measure against infections in older adults. We will discuss the more relevant infections of old people that can be controlled by vaccines, i.e., influenza, pneumonia caused by *Streptococcus* (*S.*) *pneumoniae*, and herpes zoster. In the second part of this chapter, we will review the potential options for therapeutic intervention to improve vaccine efficiency in older adults.

s0015 8.2 The state of the art

s0020 8.2.1 Adjuvants

p0030 A vaccine adjuvant (from the Latin verb *adjuvare*, meaning to help) is a component designed to enhance and/or shape the specific immune response to a vaccine antigen, and has been incorporated in human vaccine formulations for more than 90 years. The first evidence of the use of adjuvants in vaccine formulations dates back to the early 1900s. At that time, Gaston Ramon observed that coadministration of diphtheria toxin with other compounds, such as starch and breadcrumbs, resulted in a significant increase of the antitoxin response to diphtheria (Ramon, 1925). A few years later, Jules Freund developed a powerful adjuvant, the complete Freund's adjuvant (CFA), consisting of a water-in-mineral oil emulsion with heat-killed *Mycobacteria* (Opie and Freund, 1937). Immunization with CFA results in strong antibody responses and cellular immunity. However, the use of CFA has been associated with injection site granuloma and necrotic abscesses, which limits its use to animal research. Incomplete Freund's adjuvant (IFA) is composed of just the mineral oil component of CFA, thus without the mycobacterial components. This adjuvant is highly effective in enhancing cytokine production and antibody responses, and is less inflammatory than the complete form. However, in this case, severe local reactions, including sterile abscesses and persistent painful granulomas, have been observed, hampering its use in humans (Miller et al., 2005).

p0035 A variety of compounds with adjuvant properties have been discovered since then, and those employed in human vaccines licensed for use include aluminum salts (aluminum oxyhydroxide, aluminum phosphate), oil-in-water (O/W) emulsions (for example, MF59), virosomes (Moser et al., 2011), Toll-like receptor (TLR) agonists, or a combination of immunostimulants (Table 8.1). Those currently employed are discussed later, and the others considered in the section Challenges and Promises.

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Q1 t9000 **Table 8.1**

Adjuvant name	Composition	Immune effects
Aluminum salts	Aluminum oxyhydroxide, aluminum phosphate	Promotes immune cell recruitment to the injection site; Increases local inflammation; Increases antigen uptake and local cytokine secretion by APCs.
MF59	Squalene O/W emulsion stabilized with Tween 80 and Span 85	Promotes: innate cells recruitment; antigen uptake by APCs; migration of cells to lymph nodes; local production of cytokines.
AS01	MPL and QS-21 with liposomes	Promotes: immune cell recruitment to the injection site; local production of cytokines; APC activation.
AS03	Squalene O/W emulsion with polysorbate 80 and α-tocopherol	Promotes local production of cytokines; Promotes recruitment of immune cell; Increases antigen uptake and presentation in draining lymph nodes.
AS04	Alum plus MPL	Promotes: immune cell recruitment to the injection site; APC activation via TLR-4.
Virosomes	Liposomes plus influenza antigens	Increases antigen uptake, processing and presentation by APCs.
MPL	A monophosphoryl lipid A preparation from LPS from *Salmonella minnesota*	Promotes APCs activation via TLR4.
CpG ODN	Synthetic single stranded DNA containing CpG dinucleotides	Promotes APCs activation via TLR9.

Continued

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Table 8.1 —cont'd

Adjuvant name	Composition	Immune effects
polyI:C	Synthetic double-stranded RNA polymer analog	Promotes APCs activation via TLR3.
Imidazoquinoline compounds	Imiquimod (R837), resiquimod (848)	Promotes APCs activation via TLR7/8.

APCs, antigen-presenting cells; *O/W*, oil-in-water; *LPS*, lipopolysaccharide; *MPL*, monophosphoryl lipid A; *TLR*, toll-like receptor; *ODN*, oligodeoxynucleotide; *polyI:C*, polyinosinic-polycytidylic acid.
References in the text.

p0040 Although each of these numerous types of adjuvant mediates their effects through a different mechanism of action with different molecular targets, all adjuvants mime some aspects of the natural response to pathogens. In particular, adjuvants should act by enhancing directly or indirectly antigen-presenting cell (APC) responses, primarily dendritic cells (DCs), and regulate differences within the activated subset. These events are reflected in the quality and magnitude of the adaptive immune response specific to vaccine antigens. In the context of vaccination of older people, achieving these immune outcomes is particularly important, because DC's function is also altered with aging (Aiello et al., 2019).

p0045 DCs, the most potent APCs, are classified into three major subsets with different biomarkers and distinct functional activities, namely, two subsets of myeloid DCs (mDCs) and one subset of plasmacytoid DCs (pDCs). mDCs are the primary source of the interleukin (IL)-12, needed to drive a T helper type1 (Th1) response, and produce other cytokines, including IL-6 and tumor necrosis factor (TNF). In contrast, pDCs produce large amounts of type I interferons (IFN-α/β), critical for antiviral responses (Collin et al., 2013). Several studies of the effects of aging on the immune system in mice and humans support the idea that DCs from aged individuals are less effective in activating T-cell activations (Grolleau-Julius et al., 2008; You et al., 2013), showing a numerical and functional decline, especially the pDCs (Jing et al., 2009; Oh et al., 2019). Therefore, adjuvants would need to counteract the immunosenescence hallmark "reduced ability to respond to new antigens" at the DCs level (Aiello et al., 2019).

p0050 Despite the crucial immune-modulating role of adjuvants, most currently used vaccines are usually less effective at inducing an adequate cellular response in older than in younger adults (Weinberger, 2018). As an example, aluminum salts, commonly referred to as alum, represent one of the oldest and most widely used adjuvants in human vaccines (McKee and Marrack, 2017; Clements and Griffiths, 2002), although their precise mechanism of action is only now beginning to be elucidated. Initially, it was proposed that adsorption to alum increases antigen persistence, creating a depot effect at the site of

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injection (Kool et al., 2012; Marrack et al., 2009). However, several further studies have disproved this theory, showing that resection of the injection site 2 h after administration does not affect the magnitude and kinetics of antigen-specific adaptive immune responses (Hutchison et al., 2012). It transpired that, in mice, alum increases immune cell recruitment to the injection site (Goto et al., 1997), DC's antigen uptake and their migration to the draining lymph node (Morefield et al., 2005). In addition, although unable to directly activate DCs, at the site of injection, alum induces the release of some endogenous signal of cellular damage, activating the NOD-like receptor protein 3 (NLRP3) inflammasome with consequent caspase-1-dependent local, but not systemic, secretion of cytokines IL-1β and IL-18 by DCs (Eisenbarth et al., 2008; Del Giudice et al., 2018). Despite the long-term success as a vaccine adjuvant, also due to its safety profile and ability to enhance immune response to a range of antigens, aluminum salts do not seem to perform optimally in old subjects (Yam et al., 2016).

p0055 Two powerful adjuvants able to induce stronger immune responses are adjuvant system (AS)03 and MF59, O/W emulsions containing squalene, a precursor to cholesterol, both used in influenza vaccines as adjuvants (Wilkins et al., 2017). MF59 was the first novel adjuvant approved for use in human vaccines after alum. When injected into the muscle, MF59 promotes a local activation of neutrophils, eosinophils, monocytes, macrophages, and DCs that respond by producing several chemokines, including C—C motif chemokine ligands (CCL)2, CCL3, and CCL4. AS03 seems to have similar effects. This local chemokine gradient promotes the recruitment of monocytes and immature DCs from blood to the site of injection, resulting in a more efficient uptake, processing, and transportation of the antigen to the lymph nodes (O'Hagan et al., 2013; Dupuis et al., 2001). Studies in mouse models have shown that recruited cells also internalize MF59 and then transport it to the lymph node, where the adaptive immune response to vaccine antigen is triggered through the activation of T and B cells (Khurana et al., 2011).

p0060 Immune response activation by MF59 is more powerful with higher levels of antigen-specific antibody production, increased innate immune cell recruitment, and cytokine production than accomplished by alum. In addition, compared with nonadjuvanted influenza vaccines, MF59-adjuvanted trivalent influenza vaccine elicited a significantly greater antibody response both in the young and in adults $\geq$65 years of age (Frey et al., 2014), although several local and systemic adverse reactions, albeit mild and transient, were more common after MF59-adjuvanted vaccination (46%) compared with the nonadjuvanted vaccine group (33%) (Kostova et al., 2013).

p0065 The available evidence suggests that MF59 is more effective than nonadjuvanted vaccines or vaccines with alum, and it could be an excellent ally in enhancing the efficacy of vaccines. However, future studies need to investigate the safety and the risk of adverse reactions related to MF59-adjuvanted vaccines in populations particularly vulnerable and at risk of developing severe complications, such as old people.

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s0025 ## 8.2.2 Influenza

p0070 Older adults are particularly vulnerable to respiratory and emerging virus infections. Acute respiratory viral infections are a major cause of death and disability in old people. Among these, seasonal influenza is by far the most important also for complications such as pneumonia and cardiovascular disease, which require hospitalization and increase mortality during the winter season. Even in the current SARS-CoV-2 pandemic, ongoing at the time of writing, one should not lose sight of the fact that influenza is a highly dangerous pathogen. Thus, an estimated 50%–70% of influenza-related hospitalizations are of adults over the age of 65 each year, with 70%–90% of deaths related to influenza occurring among this same age group. Many old people hospitalized during ´flu outbreaks led active lives before but do not fully recover thereafter. In addition to severe acute disease and death, flu in older adults also causes functional disabilities as long-term sequelae. Up to 12% of hospitalized old patients will require, once discharged, a higher level of care in daily life than they required before the disease (Kostova et al., 2013).

p0075 Vaccines against influenza are usually either split-virus or subunit formulations that contain distinct antigens each year determined by the World Health Organization on the basis of surveillance data on predicted circulating strains. They are standardized according to the content of virus hemagglutinin (HA) for each of three different strains (A/H1N1, A/H3N2, B), or sometimes four. There are egg-, cell-based, and recombinant flu vaccines in common use. Recombinant baculovirus-expressed HA protein avoids the possible generations of mutations associated with virus egg adaptation. Current seasonal anti-flu vaccines confer beneficial effects on older people, reducing disease with typical flu symptoms by approximately 50%–60% and influenza-related complications by approximately 30%–50%, depending on the major circulating strains of the season and accuracy of prediction. However, in this age group, vaccine efficacy and efficiency is commonly lower than in younger adults, although there is some debate regarding how these statistics are reported (Osterholm et al., 2012; Russell et al., 2018; Treanor, 2016; Andrew et al., 2019). In fact, the clinical efficacy of influenza vaccines is difficult to evaluate, because many variables must be taken into account, such as population types, different epidemiological criteria, and virus-related factors such as the different virulence of the different strains and in particular the possible discrepancy between vaccine, and circulating viral strains. The issue of "original antigenic sin" and the state of health of the individual are also of crucial importance. It must be borne in mind that in most studies, laboratory flu diagnosis is not made, but flu-like disease is referred to such that certainty of influenza infection is not guaranteed (Weinberger, 2018). In addition, immune changes associated with human cytomegalovirus (HCMV) seropositivity might have a significant impact on vaccination, although this point is controversial because this relationship has not been observed in all studies (Aiello et al., 2019; Merani et al., 2017). However, on the basis of meta-analyses, it can be concluded that, in old people, protection is reduced when compared with younger ones (Beyer et al., 2013).

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p0080 Humoral immunity is known to play an important role in preventing influenza virus transmission and infection, and immunogenicity of influenza vaccines is usually measured by HA inhibition (HAI) assay, which quantifies antibodies specific for the virus HA. A greater number of older adults fail to seroconvert, i.e., to have the fourfold increase in postvaccination antibody titer, relative to their younger counterparts that is one of WHO criteria for assigning responsiveness, with seroconversion rates ranging from 10% to 30% in older adults compared with 50%–75% in younger subjects (although it can sometimes be the case that older people already have a high antibody titer due to previous exposures and thus cannot increase titers 4-fold, resulting in erroneous classification as nonresponders). In particular, older adults may fail to generate protective HAI antibody titers compared with younger adults (Weinberger, 2018; Crooke et al., 2019). Cellular immunity is also strongly associated with protection against influenza, as some older adults have been shown to remain protected against infection even in the absence of robust antibody responses (McElhaney et al., 2016). However, further studies are needed in order to fully understand the effects of immunosenescence on cellular immunity to influenza (Crooke et al., 2019). In this context, it is likely that HCMV has a larger impact on T-cell immunity than on humoral immunity (Haq et al., 2017).

p0085 In an effort to improve clinical outcome following influenza vaccination, vaccine formulations have been licensed specifically for use in old people. That led to the licensing of trivalent vaccines containing the O/W emulsion adjuvant MF59 (adjuvanted vaccines) (Ansaldi et al., 2008), or higher doses of 60 μg instead of 15 μg of HA per dose (high-dose) (Robertson et al., 2016), and of a vaccine administered via the intradermal instead of the intramuscular route (Holland et al., 2008). Trivalent influenza vaccines contain an A(H1N1)-like influenza virus, an A(H3N2)-like influenza virus, and a B-like influenza virus. In addition, high-dose quadrivalent vaccines became available, as two different B strains had circulated in parallel for several years (Smetana et al., 2018). Annual vaccination against influenza is recommended, as the composition of the vaccine changes in order to reflect currently circulating virus strains and many virulent new strains that infect humans jump species barriers (Trucchi et al., 2017; Weinberger, 2018).

p0090 A large trial demonstrated that vaccination with adjuvanted vaccine reduced the risk of hospitalization for influenza or pneumonia in old people during the peak of the influenza season by 25% relative to vaccination with nonadjuvanted vaccine. The routine use of adjuvanted vaccine in older people would provide an important clinical benefit over the traditional nonadjuvanted vaccines (Mannino et al., 2012). In a study including residents of long-term care facilities, the risk of influenza-like disease was calculated for nonadjuvant *vs.* adjuvant vaccine recipients, also stratifying for chronic cardiovascular, respiratory, and renal diseases. The risk was higher for the nonadjuvant vaccine recipients and highest for those with respiratory disease and cardiovascular disease. Therefore, it was concluded that the MF59-adjuvanted vaccine provides superior clinical protection

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among old people, especially those with chronic diseases (Iob et al., 2005). While the mechanisms of action are not completely understood, MF59 is believed to enhance innate immune responses and stimulate germinal center reactions (Cioncada et al., 2017).

p0095 A recent meta-analysis of studies reporting influenza vaccine efficacy against laboratory-confirmed hospitalized influenza among adults showed that influenza vaccines provided a moderate protection against influenza-associated hospitalizations among adults. They seemed to provide low protection among old people in seasons where vaccine and circulating A(H3N2) strains were antigenically variant (Rondy et al., 2017).

p0100 Influenza viruses have a huge impact on public health. Current influenza vaccines need to be updated annually and protect poorly against antigenic drift or novel emerging subtypes. Vaccination against influenza can be improved by inducing more broadly protective immune responses. Numerous candidate vaccines are being investigated, which utilize different antigens, such as conserved regions of the surface proteins HA and neuraminidase or internal viral proteins. The most promising universal influenza vaccine candidates are likely those that induce both broad humoral and cell-mediated responses (Wiersma et al., 2015).

s0030 8.2.3 Streptococcus pneumoniae

p0105 Bacterial pneumonia is another respiratory disease often more likely to be fatal in older people. The disease results from infection with *S. pneumoniae*, a common bacterial commensal, which frequently colonizes the upper respiratory tract. There are more than 90 different strains, many of which cause disease, but only a few of which are responsible for invasive pneumococcal infections. Pneumococcal infections are contracted by transmission by the air, via direct contact with the respiratory secretions, or saliva, or contact with healthy carriers of this bacterium, which can nest in the back of the nose or in the throat. While colonization is generally benign, migration of *S. pneumoniae* into the lower respiratory tract may lead to a pronounced disease progression. Pneumonia, bacteremia and sepsis, and meningitis are the most serious forms of infections. The mortality rates associated with pneumococcal disease vary between 15% and 30% among old people (Crooke et al., 2019). It has been calculated that between 2004 and 2040, as the American population will increase by 38%, pneumococcal pneumonia hospitalizations will increase by 96%, because population growth is fastest in older age groups experiencing the highest rates of pneumococcal disease (Wroe et al., 2012).

p0110 Human studies have found that people >65 years old have significantly lower antibody titers against many of the common pneumococcal serotypes and diminished opsonization activity compared with younger adults. Therefore, antibody titers wane over time, and there may also be functional deficiencies in antibody responses against pneumococcal antigens. While humoral immunity is primarily thought to mediate protection from disease, there are conflicting reports regarding age-related changes of T-cell responses against pneumococcal infection (Crooke et al., 2019).

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p0115 Both young children and older adults are affected by invasive pneumococcal disease. However, it represents only a fraction of the adult pneumococcal disease burden. By comparison, nonbacteremic community-acquired pneumonia (CAP) makes up the vast majority of pneumococcal disease in adults and older people. However, vaccination recommendations for *S. pneumoniae* are different in different countries. In fact, some countries still recommend the polysaccharide vaccine, while others recommend the conjugate vaccine alone or followed by the polysaccharide vaccine one year later (Weinberger, 2018).

p0120 For many years, the 23-valent polysaccharide vaccine has been used for older people. However, polysaccharides are T cell-independent antigens, so they induce an IgM response without adequate immunological memory. Conjugation can overcome this limitation via binding of the pneumococcal glycans to diphtheria toxoid (Musher et al., 2011). Therefore, conjugated vaccines have been developed for the vaccination of children. Around the year 2000, 7-valent and 10-valent conjugate vaccines were introduced for childhood immunization. Reduction in children of disease incidence and carriage of the serotypes included in the vaccines followed, which has determined decreased transmission of these serotypes and, therefore, also disease incidence in the older age group. However, serotype replacement also resulted in increases in the incidence of pneumococcal disease caused by other serotypes, not included in the conjugated vaccines, both in children and in older adults (Hanquet et al., 2010; Weinberger, 2018). Thus, a 13-valent conjugate vaccine has been introduced also for older adults. The polysaccharide is bound to carrier protein CRM197 and adsorbed on aluminum phosphate. CRM197 is a genetically detoxified form of diphtheria toxin. A single mutation at position 52, substituting glutamic acid for glycine, causes the ADP-ribosyltransferase activity of the native toxin to be lost (Malito et al., 2012). The vaccine induces both B- and T-cell responses as well as mucosal immunity, i.e., suppression of carriage of nasal serotypes by the vaccine. In a large randomized trial in older adults (> 65 years), 75.0% fewer first episodes of vaccine-type strain invasive pneumococcal disease and 45.6% fewer first episodes of vaccine-type strain CAP requiring hospitalization occurred in the vaccine group compared with placebo (Bonten et al., 2015). However, similar effects, i.e., serotype replacement, have been observed for the 13-valent vaccine (Esposito and Principi, 2015; Weinberger, 2018).

p0125 The phenomenon of serotype replacement has provided the impetus for the development of a new-generation recombinant protein and whole-cell pneumococcal vaccines with the potential to provide serotype-independent protection. To this end, universal pneumococcal vaccines would also be very useful, as there are approximately 90–100 serotypes of *S. pneumoniae*. Currently, vaccine manufacturers try to increase the number of serotypes included in conjugated vaccines, but antibody responses to polysaccharides will probably always be serotype-specific. Several pneumococcal proteins have been identified as potential universal vaccine candidates. They are highly conserved in all clinically relevant serotypes and elicit potent immune responses in animal models. Additionally, whole-cell inactivated vaccines, live-attenuated vaccines, and combinations of protein and polysaccharide components are being investigated (Feldman and Anderson, 2014).

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s0035 8.2.4 Varicella zoster virus

p0130 Almost all adults are latently infected with varicella zoster virus (VZV). The primary infection, which usually occurs in childhood, manifests as chickenpox and live-long latency is established afterward. Before the introduction of routine childhood vaccination against VZV, nearly 100% of the adult population was exposed to VZV during the lifetime, establishing latent infection within dorsal root ganglia (Gershon and Gershon, 2013). Reactivation of the virus is usually controlled by virus-specific T-cell responses, whereas several observations suggest that antibody responses do not play a significant role in protective immunity against VZV (Crooke et al., 2019). Following immunosenescence, the possible reactivation of VZV is responsible for herpes zoster disease. Accordingly, herpes zoster incidence shows an age-related increase, and 50% of cases are in individuals over 85 years of age (Crooke et al., 2019; Haq et al., 2017; Weinberger, 2018). In a fraction of patients, acute episodes of herpes zoster are followed by postherpetic neuralgia (PHN), characterized by long-lasting severe pain after the resolution of the zoster rash. The incidence of this complication is higher in older zoster patients, where it occurs in approximately one-third of the cases. Particularly in older patients, PHN is associated with significant deficits in the ability to carry out daily activities (Mallick-Searle et al., 2016).

p0135 Vaccine development efforts have proven very effective in combating herpes zoster in older adults. Two vaccine formulations have been licensed for clinical use, but clinical responses differ significantly between the two vaccines (Crooke et al., 2019). A live-attenuated vaccine that induces both T-cell and antibody responses has been licensed since 2016 (Weinberger, 2018). In people older than 60 years, it reduces the incidence of herpes zoster by 51% and the incidence of PHN by 66% (Oxman et al., 2005). Unfortunately, efficacy was found to significantly decline as age at the time of vaccination increased, decreasing to 41% in adults >70 years of age and 18% in individuals ≥80 years of age. Moreover, established protection waned over time, dropping to 21.1% for the prevention of herpes zoster and 35.4% for PHN 7–10 years after vaccination (Schmader et al., 2012; Morrison et al., 2015). A second dose of the vaccine more than 10 years after the first dose resulted in a cellular immune response higher after boosting, suggesting that a repeated vaccination of older individuals at appropriate intervals could be beneficial (Weinberger, 2018).

p0140 Both the immunogenicity and safety of a vaccine containing the viral glycoprotein E (gE) in combination with the liposome-based AS01B (MPL and QS21, see Section 8.3.1) have been documented in older adults (Chlibek et al., 2013). The assessment of safety and efficacy of the recombinant zoster vaccine in older adults was evaluated using pooled data from almost 17,000 subjects aged at least 70 years old. The estimate of vaccine efficacy against herpes zoster was 90% in the 70- to 79-year-old subjects and 89% in those aged 80 years and older, and 88.8% for the prevention of PHN over a 3.7-year follow-up period (Cunningham et al., 2016). The excellent performance of this vaccine is probably due to the development of a specific memory Th1-type response against the

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viral gE stronger than those present in subjects receiving the live-attenuated vaccine, since this VZV subunit promotes cell-to-cell interactions that lead to cell fusion in the pathogenesis of virus infection and reactivation. (Levin et al., 2018; Cunningham et al., 2018). According to studies performed in mice, MPL, in both the AS04 and AS01B adjuvanted combinations, stimulates TLR4 on antigen-presenting cells. QS21 stimulates inflammasomes in macrophages of lymph nodes draining the muscle injection site, with a subsequent increase in the number of activated resident lymph node dendritic cells and those derived from immigrating blood monocytes. These dendritic cells present the gE antigen to T cells (Didierlaurent et al., 2017).

p0145 The high efficacy of this recombinant vaccine is exceptional among vaccines given to older adults. Compared with live vaccine, it is distinguished by boosting robust and persistent memory responses. The AS01B adjuvant is critical for the magnitude of the Th response and probably plays a role in its persistence (Takeda and Akira, 2015). This effect is likely due to its ability to improve antigen presentation; to reverse memory T-cell exhaustion, an immunosenescence hallmark; and, likely, to reduce regulatory T-cell activity (Gershon and Gershon, 2013).

s0040 8.3 Challenges and promises

s0045 8.3.1 TLR agonists

p0150 As previously mentioned, a promising strategy to increase vaccine efficiency in older adults seems to be the incorporation of TLR agonists in vaccine formulations. The human TLR family consists of 10 members expressed on the cell surface (TLR1, 2, 4, 5, 6, 10) and in intracellular compartments (TLR3, 7, 8, 9) on sentinel cells, especially DCs, which recognize specific highly conserved microbial components, called pathogen-associated molecular patterns (PAMPs) (Chen et al., 2016). Each TLR recognizes specific PAMPs, and, in particular, TLRs located on the cell surface bind lipids and proteins, while TLRs located on the endosomal compartments are responsible for the recognition of bacterial and viral nucleic acids (Chen et al., 2016). Examples of PAMPs include lipopolysaccharide (LPS) for TLR4, imidazoquinoline compounds, such as R848, for TLR7/8 and CpG motif of bacterial and viral DNA for TLR9 (Guy, 2007; Kornbluth and Stone, 2006).

p0155 TLRs agonist efficiencies as vaccine adjuvants rely mostly on the promotion of antigen uptake, presentation, and maturation of DCs, their cytokine secretion and activation of T cells. DC subsets show different patterns of expression of TLRs with different effects on activation but, generally, binding of PAMPs to TLRs triggers an intracellular MyD88 or TRIF-dependent signaling cascade that induces maturation of immature DCs to professional APCs, increasing expression of major histocompatibility complex (MHC) class II and costimulatory molecules, and secretion of several cytokines (Schreibelt et al., 2010). Due to their powerful immunostimulatory properties, TLR agonists are considered important vaccine adjuvant candidates and some of them are indeed currently in use

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or being tested as adjuvants (Table 8.1). In the context of aged individuals, however, an important consideration regarding their use in vaccines is the influence of aging on TLR responsiveness and expression levels. For example, TLR3 and TLR8 in mDCs and TLR7 in pDCs seem to be expressed at lower levels in healthy older people than in young individuals, whereas TLR2 and TLR4 surface expression in mDCs appears to be unchanged with increasing age (Jing et al., 2009; Panda et al., 2010). The different expression of some TLRs in old people is associated with an altered TLR-mediated immune response. Several studies show an age-associated reduction of TLR-induced type 1 IFN(—I) production in pDCs in response to influenza virus and CpG ODN (Weinberger, 2018) and other stimuli (Shodell and Siegal, 2002; Qian et al., 2011), as well as defects in TLR-induced IL-12p40, IL-6, and TNF-α production in mDCs (Panda et al., 2010), impairing host defense to viral infections. Age-related increased oxidative stress can also contribute to impaired TLR-induced IFN-I response by aged pDCs (Stout-Delgado et al., 2008). For these reasons, most TLR agonists are administered in a combined treatment, as in the case of the AS01, a liposome-based vaccine adjuvant containing the TLR4 agonist 3-O-desacyl-4′-monophosphoryl lipid A (MPL), a detoxified derivative of LPS extracted from *Salmonella minnesota*, and the saponin QS-21, extracted from *Quillaja saponaria molina* (Didierlaurent et al., 2017). The two components of AS01, MPL acting via TLR4 and QS-21 in an NLRP3 inflammasome-dependent manner, act synergistically to strongly stimulate innate responses and to induce the highest antigen-specific T-cell response. Following intramuscular injection in mice, AS01 coinjected with antigen induces a rapid and transient local production of cytokines, mostly chemokines attracting circulating monocytes and granulocytes, ≥8.7-fold higher than antigen injection alone. In addition, AS01 coadministered with antigen does not persist at the injection site but is rapidly transported to the lymph nodes where AS01 enhances the recruitment of innate immune cells from the injection site and increases expression of T-cell stimulatory molecules in both recruited and lymph node-resident DCs. No increase in antigen uptake by DCs has been observed following vaccination with AS01-adjuvanted vaccine. Thus, AS01 acts by recruiting a large number of activated and cytokine-secreting APCs at the injection site and lymph node level, ensuring a favorable environment particularly efficient in T-cell priming (Didierlaurent et al., 2017).

p0160 Another adjuvant system, AS04, is a combination of aluminum salts with MPL. In mouse models, AS04 induces a rapid transient cytokine production and innate immune cell recruitment. When injected with a specific antigen at the same injection site within 24 h, AS04 is able to promote an increased number of activated antigen-loaded DCs and monocytes, which then leads to the activation of antigen-specific immune adaptive responses. Although the induction of cytokine secretion by DCs is mainly due to the action of the TLR agonist, the presence of alum in the AS04 formulation seems to prolong the MPL-mediated cytokine response (Didierlaurent et al., 2009). When used in combination, therefore, MPL and alum synergize, ensuring a more effective response than either alone.

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p0165 With this in mind, a new type of adjuvant formulation that could enhance vaccine efficacy in older people consisting of combined adjuvants for synergistic activation of cellular immunity (CASAC) was formulated, incorporating two TLR agonists, CpG-oligodeoxynucleotides (TLR9 agonist) and polyI:C (TL3 agonist), IFN-γ, and MHC-class I and II peptides in an O/W emulsion (Tye et al., 2015). Promising results were obtained in murine models where the simultaneous and synergistic activation of TLRs has enhanced DC activation, resulting in increased cellular responses to the antigen of interest. Immunosenescent old and young mice were vaccinated with CASAC or CFA/IFA adjuvant, with a class I epitope, and the specific CD8+ T-cell response was subsequently evaluated, assessing frequency, by MHC pentamer staining, cytotoxicity, and intracellular production of IFN-γ. These analyses have shown that, in both young and aged mice, vaccination with CASAC generated higher frequencies and stronger responses of antigen-specific CD8+ T cells compared with mice vaccinated with CFA/IFA (Tye et al., 2015). To assess if these promising observations in animal models can be translated to humans, the ability of two combined TLR ligands, R848 (TLR7 agonist) and MPL (TLR4 agonist), to enhance the activation of DCs isolated from human healthy aged and young donors has been investigated. In both groups, the stimulation of DCs with the combination of TLR7/8 and TLR4 agonists induced a higher production of IL-12/p40 and TNF-α by mDCs, confirming their role in boosting the innate response of TLR agonists in humans. However, when the production of cytokines was compared between the young and the old donors, an increase of 5- to 10-fold in the production of IL-12/p40 by mDCs, as well as an increased amount of TNF-α by mDCs and pDCs isolated from old people, was observed (Gambino et al., 2017). Based on these results, it is evident that, despite the differences in their composition, adjuvants can contribute significantly to improving immune responses to vaccines in immunosenescent individuals but, since most of studies in this field have been done in mouse models, further studies are needed to test which combinations can offer the same protective effects in humans.

s0050 8.3.2 Virosomes, viral vectors, reverse vaccinology

p0170 Virosomes are particles structurally and functionally similar to viruses, although they are assembled in vitro. Immunopotentiating reconstituted influenza virosomes (IRIVs), the most commonly used form of virosome, are spherical vesicles consisting of unilamellar phospholipid membrane incorporating virus (HA, by which the membrane fusion activity of the native virus is preserved, and neuraminidase) (Zurbriggen, 2003). Mimicking both viral morphology and antigenic presentation, but unable to replicate, virosomes can deliver these antigens directly to their targets, enhancing antigen uptake, processing, and presentation by APCs. Virosomal HA promotes binding at the APC surface followed by receptor-mediated endocytosis and fusion of viral and endosomal membranes. A wide range of antigen types have been combined successfully with influenza virosomes and

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their location in the vesicles determines the processing and presentation pathway. Vaccine antigens cross-linked on the surface remain in the endosomal compartment after the fusion event and are thereby presented to the CD4+ T cells by MHC class II molecules, generating primarily antibody responses. Antigens encapsulated within the lumen of the virosome are delivered to the cytosol upon membrane fusion and are presented to the CD8+ T cells via the MHC class I pathway (Schumacher et al., 2004). Virosomal technology is approved for use in humans, and there are several clinical trials that demonstrate the immunogenicity and the high safety profile of IRIV for all age groups, including older people and immunocompromised individuals (Glück et al., 1994; Zanetti et al., 2002).

p0175 Other promising tools for vaccines are viral vectors, designed to take advantage of the natural efficiency of viruses at entering and transducing their own genome into host cells for their replication. By replacing nonessential viral genes with exogenous genes of therapeutic interest, viral vectors are able to transduce their cellular target at the immunization site, resulting in high levels of synthesis of the immunogen, characteristics that give rise to robust humoral and cellular immune responses (Bouard et al., 2009). Compared with virosomes that deliver vaccine antigens directly into a host cell, viral vectors enable intracellular antigen expression, ensuring highly efficient induction of both humoral and T-cell responses. To ensure safety for human use, most viral vectors are rendered unable to replicate through targeted gene deletion. For example, in adenovirus-based vectors, the genomic regions required for viral replication are deleted and replaced with the target gene (Ura et al., 2014).

p0180 Adenovirus is one of the most commonly exploited viral vectors for vaccine development, but the main disadvantage of the clinical use of human adenovirus is the presence of preexisting specific immunity against the vector, due to a previous exposure to the virus and leading to the production of neutralizing antibodies that reduce vaccine efficacy. This obstacle has been surmounted by replacing the human virus with replication-deficient chimpanzee adenoviruses (Ewer et al., 2016). In addition to adenoviruses, several other viral vectors have been shown to be successful in clinical trials. Modified vaccinia Ankara (MVA) virus is a highly attenuated strain derived from the vaccinia virus, belonging to the poxvirus family. MVA was rendered replication-deficient by the deletion of roughly 15% of its original genome (Choi and Chang, 2013). When used as a vaccine vector, MVA shows an excellent safety profile and induces potent humoral and cellular antigen-specific responses, especially in the cytotoxic T subset (Ewer et al., 2016).

p0185 The particular characteristics of viral vectors have been exploited for the development of alternative vaccination strategies to elicit potent T-cell responses specific for highly conserved viral antigens. A similar approach could provide protection against antigenically distinct viruses. This strategy is particularly relevant for vaccinations against viruses with pandemic potential, as in the case of influenza virus where its rapid evolution, due to

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an accumulation of mutations within antigenic sites, causes an escape from serological host immunity conferred by a previous infection or vaccination (Coughlan et al., 2018). Current seasonal influenza vaccines induce subtype-specific responses and offer no heterosubtypic immunity against novel subtypes or other emerging viruses. An approach that aims to enhance preexisting memory T-cell responses against highly conserved influenza antigens, such as nucleoprotein (NP) and matrix protein 1 (M1), rather than to stimulate naïve lymphocytes de novo may be particularly beneficial in the older people, characterized by reduction in naïve T-cell output and expansion of selected memory T-cell clones (Olsson et al., 2000; Antrobus et al., 2012). Based on these premises, the safety and immunogenicity of the vaccine MVA expressing the NP and M1 antigens (MVA-NP + M1) in people aged 50–85 years was assessed. MVA-NP + M1 was shown to be immunogenic, boosting influenza-specific CD4 + and CD8 + memory T-cell responses. Surprisingly, no significant differences were observed in the induction of the immune responses between young and old subjects, unlike what was observed for seasonal influenza vaccination (Antrobus et al., 2012; Boraschi and Italiani, 2014). These observations provided the basis for a subsequent phase I study in which MVA-NP + M1 was tested in combination with a ChAdOx1 vector carrying the same influenza antigens (ChAdOx1 NP + M1). The authors showed that a two-dose MVA/ChAdOx1 regimen was highly immunogenic, increasing preexisting T-cell responses to influenza antigens in both young and older adults. Also, in this case, both vaccines were well tolerated, demonstrating again the safety of the use of viral vectors in older people (Antrobus et al., 2012; Pawelec and McElhaney, 2018).

p0190 A significant step forward that revolutionized the classic vaccine concept is reverse vaccinology, a genome-based approach to vaccine design. Sequencing the whole genome of the pathogen allows the identification of new candidate vaccine antigens, without the need for growing microorganisms. Once identified, antigens are expressed and screened in animal models (Rappuoli, 2000). The first recently licensed vaccine using reverse vaccinology is a vaccine against meningococcus B (MenB), which has shown to be highly effective in preventing MenB disease in infants (Parikh et al., 2016). This innovative approach breaks new ground for vaccine development and, in the future, could be exploited to enhance the immune responses of immunosenescent subjects.

s0055 8.3.3 Interleukin-7

p0195 One of the best-studied and accepted age-related immune alterations is the reduction in the number and repertoire diversity of peripheral naïve T cells, as a result of progressive thymic involution, characterized by thymic epithelial space atrophy and replacement with adipose tissue (Lynch et al., 2009). All these phenomena lead to a reduction in the T-cell reservoir necessary for protection against newly encountered microbial antigens, including vaccine antigens. Strategies reported to prevent or reverse thymic atrophy

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include the use of IL-7, a pleiotropic cytokine produced by thymic epithelial cells with a wide range of functions. In particular, IL-7 binding to its specific heterodimeric receptor activates an interconnected intracellular signaling network critical for the development of T-cell lineages in the thymus and for the development and survival of naive T cells and CD4 and CD8 memory cells in the periphery (Barata et al., 2019). Therapeutic benefit of the use of IL-7 has been widely demonstrated in animal experiments, where IL-7 administered pharmacologically induced a transient expansion of naïve and memory T cells with low toxicity (Phillips et al., 2004; Okoye et al., 2005). In a trial in HIV-infected individuals treated with antiretrovirals, but with persistently low CD4+ T-cell counts, IL-7 increased both naïve and memory CD4+ and mostly naïve CD8+ T-cell counts (Lévy et al., 2012).

p0200 Considering the critical role of IL-7 in maintaining T-cell compartment homeostasis, the possibility of using IL-7 therapy to improve vaccine responses has been studied. Administration of IL-7 during immunization in mice led to increased expansion of antigen-specific effector and memory cytotoxic T cells, accompanied by increased death of effector cells during the contraction phase (Melchionda et al., 2005; Colombetti et al., 2009). These studies in the mouse suggest that IL-7 could be used for improving the adaptive response to vaccine antigens. However, the progressive thymic involution observed with age places an obstacle to the immune-restorative action of this pluripotent cytokine. It is reasonable to think that, in the case of older people, early restoration of the thymic architecture is required so that the IL-7 can act to its full potential (Aiello et al., 2019).

s0060 8.3.4 Inhibitors of mitogen-activated protein and adenosine monophosphate-activated protein kinases as therapeutic interventions for vaccination improvement

p0205 The role of mitogen-activated protein kinase (MAPK) and adenosine monophosphate-activated protein kinase (AMPK) pathways has been recently demonstrated in the functional competence of the immune system (Chi and Flavell, 2010; Silwal et al., 2018). The first is mainly involved in the production of cytokines, as well as in the intracellular signaling cascades initiated when a cytokine binds to its corresponding receptor. The second is a pathway activated by energy depletion, i.e., by low levels of intracellular adenosine triphosphate, leading to the extension of healthy lifespan in model organisms. Both are implicated in the regulation of T-cell immunosenescence hallmarks that are the reduced ability to respond to new antigens and the accumulation of memory T cells. In particular, sestrins, the mammalian products of the Sesn1, Sesn2, and Sesn3 genes, are a family of stress-sensing proteins that can bind these kinases, inhibiting them (Lanna et al., 2017). It was proposed a possible role for sestrins in the control of the immune response, although this has not yet been fully determined. Indeed, sestrins show pro-aging activities in senescent T lymphocytes through the sestrin-dependent MAPK activation complex.

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This simultaneously coordinates the activation of each MAPK that controls a functional response, and its knockout restored T-cell activity (antigen-specific proliferation and cytokine production) from older humans, and enhanced responsiveness to influenza vaccination in aged mice (Lanna et al., 2017). Ex vivo, the inhibition of AMPK and MAPK via these small-molecules overcomes human T-cell senescence in human HCMV-specific T cells, and restores T-cell proliferation, IL-2 production, and cytotoxicity (Lanna et al., 2014).

p0210 Other approaches include the role of small-molecule kinase inhibitors (SMKIs), consisting of four classes of kinase inhibitors targeting tyrosine, serine/threonine, dual (that can phosphorylate either tyrosine or serine/threonine residues), and lipid kinases. These are compounds that include marketed drugs and drugs in development that have the potential to exercise a dual synergistic effect as immune-system modifiers of influenza infection and vaccination in older adults (Wu et al., 2016). Along with those approved by Food and Drug Administration, a large number of other SMKIs have been enrolled in clinical trials at different phases for the treatment of human malignancies and neoplastic disorders with immune-stimulatory properties. In particular, five of these have already proven efficacious in restoring T-cell functions in preclinical models and human senescent T cells. These include MAPK inhibitors with known anti-T-cell aging capacities in vitro and in vivo in murine models of influenza vaccination and with potential anti-inflamm-aging properties (Lanna et al., 2017; Lanna et al., 2014). Moreover, some of these SMKIs have already demonstrated their potential for direct incorporation into (injectable) vaccine formulations. As an example, doramapimod, a MAPK-p38 inhibitor, binds p38α, has low inhibitory action on molecules that regulate T-cell activation and effector functions, such as Raf, Fyn, and Lck, and does not show a significant inhibition of ERK and Syk. Also, an ERK inhibitor, FR18024, possesses strong anti-T-cell senescence activities and was shown to improve clinical parameters in murine models of viral infections (Sreekanth et al., 2014).

p0215 The possibility of using bioactive compounds derived from olive oil as inhibitors of inflamm-aging, the third hallmark of immunosenescence (Aiello et al., 2019), might also be tested in vitro for the improvement of influenza vaccination response together with the inhibitors previously described (Gambino et al., 2018). It was demonstrated that inflamm-aging plays an important role in compromising the immune responses by way of inducing high expression of some microRNAs that interfere with B-cell activation. In vitro, this drives TNF-α production and inhibits B-cell activation (Frasca et al., 2015). Increased serum levels of TNF-α are also linked to a defective T-cell response, in part due to reduced expression of CD28 on T cells (Ponnappan and Ponnappan, 2011). Moreover, in monocytes, the prevaccination expression of genes related to inflammation and innate immune response is negatively correlated with vaccination-induced activation of influenza-specific antibody responses (Nakaya et al., 2015). Phenolic compounds of olive oil include around 30 molecules, some with strong

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antioxidant and anti-inflammatory properties. Their nutraceutical properties counteract the pathophysiology of age-related diseases, such as cardiovascular diseases, arthritis, neurodegenerative diseases, with a relevant role in many antiaging strategies. The mechanisms of action involve the scavenging of radical oxygen species, inhibition of mast cell degranulation, and inhibition of cyclooxygenases 1 and 2, positively counteracting chronic low-grade inflammation.

p0220 Hydroxytyrosol exhibits a promising antioxidant potential in protecting mononuclear cells against 2,3,7,8-tetrachlorodibenzo-p-dioxin, an external stressor. The acetyl derivatives of phenolic compounds are more efficient compared with native molecules, with a higher rate of cellular internalization. Peracetylation of oleuropein and its derivatives may improve their capacity to permeate the molecular membrane. In vivo, the application of olive oil derivatives by parenteral administration has been shown to mitigate inflammation and oxidative stress and the parenteral administration of oleuropein aglycone after challenge of the pleural cavity with carrageenin, a strong inflammatory agent, fully abrogated induced inflammation in a mouse model (Impellizzeri et al., 2011). It was also seen that parenteral treatment with oleic derivatives reduced malondialdehyde, a marker of lipid peroxidation, in rat brains (Rizzo et al., 2017).

s0065 8.4 Conclusion and future perspectives

p0225 This chapter summarizes data regarding the immunogenicity and efficacy of influenza, pneumococcal, and herpes zoster vaccines currently in use for older people, and provides a perspective on possible new methodological approach for future vaccine development specifically for this age group. Considering the differences between young and older peoples' immune systems, successful development of new vaccines specifically tailored for the aged population will require a deeper knowledge of the mechanisms of immunosenescence. This is not a simple challenge for vaccinologists, also in light of the fact that immune responses in aged people are significantly influenced by the individual past history of exposures and immunity. In fact, the immunological experience and other stresses that individuals encounter over their lifetimes shape their ability to respond to external stimuli, such as vaccinations. In addition, many other factors influence outcomes, including nutrition, physical exercise, drug treatments, the microbiota, and chronic diseases. On the other hand, methodological and technological advances allow modern vaccination approaches that are opening up the possibility of developing new vaccines against practically any pathogen. This is relevant to address major and upcoming global threats like antimicrobial resistance, responsible for at least 700,000 deaths each year. Vaccines would be a promising solution against antimicrobial resistance for several reasons.

p0230 Unfortunately, antibiotics become quickly obsolete because resistance emerges immediately after their introduction, while vaccines allow lasting protection against infections. In addition, while antibiotics target only certain metabolic targets, vaccines

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can elicit a broad multitarget immune response by reducing the likelihood of evolving resistant mutations (Andreano et al., 2019; Tagliabue and Rappuoli, 2018).

p0235 It is to be hoped that these new approaches will be soon available also for the preparation of SARS-CoV-2 vaccines to protect against COVID-19, an acute requirement at the time of writing. It is amazing the rapidity with which SARS-CoV-2 was sequenced and vaccine candidates produced for testing in a matter of weeks. Broadly speaking, these vaccines group into several different "platforms," among them old standbys such as inactivated or weakened whole viruses, genetically engineered proteins, and the newer mRNA technology. One such vaccine designated mRNA-1273 developed by NIAID scientists and their collaborators at the biotechnology company Moderna, Inc., based in Cambridge, Massachusetts, was the first to enter clinical trials. This investigational vaccine directs cells to express a viral protein that it is hoped will elicit a robust immune response. The mRNA-1273 vaccine has shown promise in animal models, and there is the first trial to examine it in humans (Cohen, 2020). Furthermore, a chimpanzee adenovirus-vectored vaccine, ChAdOx1, developed by Oxford University's Jenner Institute has been shown to induce a strong immune antibody response up to the 56th day of the ongoing testing. These are the preliminary results of phase 1–2 of testing which involved 1077 healthy adults. The vaccine showed an acceptable safety profile, and homologous boosting increased antibody responses. These results, together with the induction of both humoral and cellular immune responses, support large-scale evaluation of this candidate vaccine in an ongoing phase 3 program (Folegatti et al., 2020). However, vaccine design for the older adult will need to overcome the challenges of immunosenescence and aim to stimulate a broad T- and B-cell response tailored to restoring reduced immune function in the older population (Pawelec and Weng, 2020).

s9015 Note added in proof

p9015 At the beginning of 2021 in United States, in United Kingdom and in European Union are used three anti-SARS-CoV-2 vaccines, two are based on mRNA technology and the third is an adenovirus-vectored vaccine. The antigen is represented by protein Spike that plays the key role for entering the cells. Concerning the BNT162b2 mRNA COVID-19 vaccine, 37,706 subjects (16–91 years, median 52 years) completed the trial; a two-dose regimen of BNT162b2 conferred 95% protection against COVID-19. Safety over a median of 2 months was similar to that of other viral vaccines (Polack et al., 2020). As regards the mRNA-1273 SARS-CoV-2 Vaccine, 29,148 subjects (>18 years) completed the two-dose regimen trial. The vaccine showed 94.1% efficacy at preventing COVID-19, including severe disease. Aside from transient local and systemic reactions, no safety concerns were identified (Baden et al., 2021). Regarding the third vaccine, overall vaccine efficacy across groups was 70.4% in 11,636 subjects (18–55 years) which completed the two-dose regimen trial. ChAdOx1 nCoV-19 had an acceptable safety

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profile and has been found to be efficacious against symptomatic COVID-19 in this interim analysis of ongoing clinical trials (Voysey et al., 2021).

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3.8. Triggering of Toll-like Receptors in Old Individuals. Relevance for Vaccination

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REVIEW ARTICLE

Triggering of Toll-like Receptors in Old Individuals. Relevance for Vaccination

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Abstract: Aging is characterized by a general decline in a range of physiological functions, with a consequent increase in the risk of developing a variety of chronic diseases and geriatric syndromes. Additionally, increasing age is accompanied by a progressive decline in both innate and acquired immune system, referred to as immunosenescence. This impaired ability to mount an efficient immune response after exposure to microorganisms or vaccines represents a major challenge in acquiring protection against pathogens in aging. Therefore, there is still a great need for vaccines that are tailored to optimally stimulate the aged immune system, thus promoting more successful aging. Various strategies can be used to improve vaccine efficacy in old people. Despite this, meta-analyses have clearly shown that the magnitude of protection obtained remains lower in older adults. Recent studies show that stimulation of Toll-like receptors, using stimulatory ligands, can enhance vaccine efficacy by a number of mechanisms, including the activation of innate immune cells and the consequent production of inflammatory cytokines. Therefore, a possible strategy for more effective vaccination in the older population is the triggering of multiple TLRs, using a combined adjuvant for the synergistic activation of cellular immunity. Preliminary *in vitro* data suggest that in humans the presence of multiple TLR agonists can result in the greater stimulation of antigen-specific immune responses in immune cells both in the young healthy and in the immune senescent older donors. These data suggest that appropriately selected combinations of TLR agonists could enhance the efficacy of vaccination mediated immunity in older people.

Keywords: Aging, cytokines, dendritic cells, immunosenescence, TLR, vaccination.

1. INTRODUCTION

People worldwide are living longer. In 2025, there will be about 1.2 billion people over the age of 60, increasing to 2 billion by 2050 [1]. Mechanisms driving this phenomenon are the demographic and epidemiologic transitions. The increased number of aged people is closely related both to reduced birth rate and to the decreased rate of deaths, as well as the epidemiological shift from infectious diseases to non-communicable diseases. The implications of this demographic change are enormous and affect all aspects, social, political, and economic of human life. Furthermore, the health of older people deteriorates with increasing age; hence the increased incidence of diseases such as cancer, cardiovascular disorders and neurodegeneration. It is essential to learn more about the aging process and to understand the intricate connections between aging and disease. This is not to increase longevity *per se*, but rather because such studies could help to understand how to age successfully [2, 3].

Aging is, in fact, a time-dependent functional decline that, due to a diminished homeostatic ability, reduces responsiveness to environmental stimuli; generally is associated with an increased predisposition to illness and death. A complex remodelling of tissues and organs, in response to time-dependent exposure to biological and environmental stressors, plays a key role in the detailed phenotype of old age [4]. In particular, many age-associated alterations in innate and acquired immunity have deleterious effects, which are collectively referred to as immunosenescence, a term coined by Roy Walford [5, 6].

Indeed, in 1969, Roy Walford published his landmark book, "The Immunologic Theory of Aging" [7]. Briefly, he hypothesized that the faulty immune processes play a relevant role in the aging of humans and of all mammals. Therefore, he was the first to note and promote the power of modern immunological approach as a tool for the analysis of aging [5, 8]. Research has repeatedly confirmed the insightful predictions made by Roy Walford regarding the role of the immune system in various pathologies associated with aging [5]. Indeed, in accordance with Roy's original hypothesis on the role of immunosenescence in human aging, there is evidence that the previously identified diseases of aging are closely linked to dysregulation of the immune function and excessive inflammation [5, 9]. In fact, immunosenescence also includes a well characterized and profound modification of the cytokine network. A key feature of this phenomenon is the increase in pro-inflammatory mediators [9]. These circulating inflammatory molecules are associated with a low grade, chronic inflammation, called inflamm-aging [9].

However, immunosenescence is a complex process involving several developmentally regulated changes, rather than a simple unidirectional decline of the whole function. Nevertheless, in older adults there is a decrease in a range of immunological functions, associated with reduced health; reciprocally, immune competence

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correlates with a range of characteristics associated with a good health status [6].

The impact of aging on the immune system typically includes intrinsic defects within immune cells as well as alterations in the number and activity of the different lymphocyte subsets, and possibly defects in the microenvironment of the lymphoid organs. This phenomenon results in a reduction in naive T and B cells, thus a reduced repertoire of both cellular and immunological responses in the aged population. This reduced complexity in the range and efficacy of response by the immune effector cells to pathogens, underpins the reduced T cell cytotoxicity, proliferation and cytokine production, as well as defective memory responses in the older population [6, 10-12]. Consequently, aged individuals exhibit an increased incidence of infectious disorders and cancer, both associated with reduced immunological surveillance and protection, as well as an increased incidence of autoimmune disease as a consequence of enhanced inflammatory immune functions [9]. In addition, the aged immune system does not efficiently respond to stimuli; therefore, current vaccines are less effective in the older population [12]. Thus, the aging process affects more extensively the acquired, rather than the innate immunity [6, 10]. Age-related changes in antigen uptake, processing and presentation, as well as functional defects of T cells, lead to reduced antibody responses, as well as suboptimal antigen-specific cellular immunity [13].

2. VACCINES IN OLD PEOPLE

Research in immunological aging seeks not only to understand the age-related disorders of immune regulation, but also to identify new efficient strategies for immune rejuvenation and for effective vaccination induced immunity in older people. The severity of many infections is higher in the aged adults. In addition, infectious diseases are frequently associated with long-term consequences such as the onset of frailty and consequent impairment in activities of daily life and loss of independence [14, 15]. Therefore, there is still a great need for vaccines tailored to optimally stimulate the aged immune system in order to promote successful aging [13]. Higher antigen dose, alternative routes of administration, and the use of adjuvants are all strategies to improve vaccination efficacy in old people. However, the already employed strategies induce only moderately higher antibody concentrations in old people vaccinated for influenza [16]. Thus, the identification of more effective adjuvants should be able to enhance the impaired immune responses in order to revert or slow down the age-associated erosion in immunity and to promote healthier aging [12, 13].

Adjuvants are molecules that can stimulate both the non-specific innate immune responses and the direct or indirect activation of antigen presenting cells (APCs), primarily dendritic cells (DCs) [17], by stimulating their recruitment to the site of vaccination, antigen uptake, processing and presentation. Adjuvants are therefore able to promote both the innate and adaptive immunological responses. There has been enormous progress in the last twenty years in the development of new vaccine adjuvants. These adjuvants are made up of different components, such as aluminium salts, emulsions such as MF59 and AS03, both of which are squalene-based, and toll-like receptor (TLR) agonists or a combination of immunostimulants such as detoxified forms of lipopolysaccharides such as MPL. Most adjuvants induce the early activation of innate immunity, in turn potentiating acquired immune responses against the antigens present in the vaccine. Some of these new adjuvants are in clinical use, showing excellent performance in the prevention of infectious diseases, such as influenza and in cervical cancer caused by subtypes of human papilloma virus (HPV) [18].

However, the ineffective induction of T cell mediated immunity in older people remains a persistent challenge for vaccine development. Thus, there is a need for a more efficient and sophisticated adjuvant that will complement novel vaccine strategies for older people. The development and identification of appropriate adjuvants and cytokines might effectively remedy defects in the aged T cell functions, both directly and by better activation of DCs [19, 20].

Various strategies can be used to improve the antibody responses caused by vaccines in older people. Some of these have been used for the anti-influenza vaccination. These include intradermal vaccines [21], high-dose vaccines [22] and vaccines with the squalene based oil-in-water MF59 emulsion as an adjuvant. MF59 contains a synthetic muramyl peptide shown to have significant immunostimulatory activity and low toxicity [23]. These enhanced vaccines show slightly higher immunogenicity in older people when compared with the standard inactivated vaccines [24]. Nevertheless, meta-analyses have clearly shown that the magnitude of protection is lower in older adults than in young people [25, 26].

3. THE DENDRITIC CELLS

DCs are the most potent APCs, specialized in the uptake, processing, transport and presentation of antigens to T cells. After their activation in the periphery, DCs migrate to lymphoid tissues where they interact with T and B cells to initiate and shape the acquired immune responses. According to the expression of various markers, DCs can be divided into three subsets. The CD123 marker characterizes plasmacytoid DCs (pDCs). They possess the ability to produce high levels of type I Interferons (IFN-α/β). In contrast, myeloid DCs (mDCs) express the CD11c marker and are divided into two subsets: CD1c+ mDCs and CD141+ mDCs. Upon stimulation, mDCs secrete mainly interleukin (IL)-6, IL-12, and tumor necrosis factor (TNF)-α [17].

Specific subsets of TLRs are expressed by both innate and adaptive arms of the immune system, including monocytes, macrophages, NK, B, T and dendritic cells. Both pDCs and mDCs, recognize conserved pathogen-associated molecular patterns (PAMPs) on microbes, hence they are key regulators of antimicrobial host defense responses. Recognition of PAMPS by TLRs culminates in the secretion of type I IFNs and pro-inflammatory cytokines that facilitate the linkage of innate to acquired immune responses. Deficiencies in human TLR signalling leads to increased severity of multiple immunological disorders, including sepsis, immunodeficiencies, atherosclerosis and asthma [27].

The cytokine pattern is determined not only by the type of TLR activated, but also by the type of cell. As an example, TLR-7 stimulation induces the expression of IFN-α by pDCs. They are associated with innate antiviral immunity and the development of acquired immunity. By contrast, TLR-7 stimulation induces the expression of IL-12 from mDCs, important for the induction of a T helper (Th)-1 response. Type I IFNs enhance antigen cross-presentation, T cell proliferation, DC maturation and NK cell activation [28].

Data on the influence of aging on human DC activity and cytokine production, in response to *in vitro* stimulation, shows either comparable or reduced DC function in older people. Tan *et al*., [29] report that human DCs isolated from both young and aged individuals exhibit comparable activation in response to most TLR ligands, and are equally capable of direct and cross-presentation of antigens to T cells *in vitro*. On the contrary, You *et al*., [30] demonstrate a reduced production of TNF-α by DCs from old people in response to LPS stimulation. However, in older people there is invariably a marked reduction in cytokine release by pDCs.

As previously mentioned, recognition of microbial components by TLRs induces also the secretion of cytokines. Blood mononuclear cells isolated from young individuals exhibited a quicker and faster response to stimulation with TLR agonists compared with cells obtained from older adults. This resulted in an increased production of cytokines and chemokines [19]. On the other hand, the addition of PAMPs to a subunit vaccine, in order to induce the stimulation of specific TLRs, improves vaccine efficacy in older

people [19, 20, 31, 32]. Thus, DCs and naïve T cells represent the most restrictive elements for the immune response to primary viral infections in older people [33]. Defects in signal transduction appear to be responsible for this impairment since the expression levels of different TLRs remain constant during life [13].

Over the last decade, TLR agonists have emerged as novel vaccine adjuvants [34]. Since TLR stimulation can induce both the production of cytokines by APCs, and the antibody production by germinal centre B cells [13, 35], TLR agonists would be expected to offer a promising strategy to enhance vaccine efficacy. Cytokine production by APCs shows age-related variations, but efficient TLR stimulation may overcome the age-associated TLR signalling dysfunction [36].

Previous studies have shown that the stimulation of human DCs, present in blood mononuclear cells, with two or more TLR agonists, can induce the sustained secretion of IL-12p70. This causes a T1 polarization of the naive T cells, at significantly higher levels than stimulation with single TLR agonists. Activation of DCs with specific combinations of TLRs induces the synergistic production of Th1 polarizing cytokines, up-regulation of co-stimulatory markers of DC and down-regulation of programmed death-ligand 1 (PD-L1) expression [37, 38].

4. DATA FROM MURINE MODELS

A possible strategy for enhanced vaccination efficacy is the activation of multiple TLRs, using a combined adjuvant for synergistic activation of cellular immunity (CASAC). As an example the single-strand oligodeoxynucleotide, characterized by motifs containing cytosines and guanines (CpG), a potent inducer of IFN-α by pDCs, is incorporated in CASAC. This adjuvant also contains a synthetic analogue of viral dsRNA (polyI:C - polyinosinic-polycytidylic acid) that targets TLR3, inducing the production of type I IFNs. In addition, there are IFN-γ and MHC-class I and II peptides. Immunization of young mice with the CASAC adjuvant (containing two or more TLR agonists, anti-CD40, IFN-γ, and surfactant) produced high levels of CD8 responses to peptide or protein antigens and highly polarized Th1 responses [39, 40]. CD40 signalling was required for CD8 expansion but it could be substituted with a concomitant CD4 Th response in place of anti-CD40. Triggering of these pathways activated the migration and activation of mDCs and pDCs and the secretion of IL-12. Therefore, cross-presentation can be exploited to induce potent cytotoxic responses and long-term memory to peptide/protein antigens. When combined with a tumor-associated peptide from tyrosinase-related protein 2, this combined adjuvant approach effectively halted tumor growth in an *in vivo* melanoma model and was more effective than anti-CD40 and a single TLR agonist. Antitumor immunity was associated with long-lived effector memory CD8 T cells specific for the naturally processed and presented tumor antigen; tumor protection was partially but not entirely dependent on CD8 T cells. Thus, CASAC vaccine formulation with two or more TLR agonists, anti-CD40, IFN-γ, and surfactant is more effective than existing adjuvants and provides a technological platform for rapid vaccine development [39, 40].

In the old immunosenescent mice, serial vaccinations with CASAC or Freund's complete / incomplete adjuvant (CFA/IFA) and a class I epitope, derived from ovalbumin (SIINFEKL, SIL) or melanoma auto-antigen, tyrosinase-related protein-2 (SVYDFFVWL, SVL) were used to get antigen specific CD8+ T cell responses. The analysis conducted by quantification of increase in the antigen specific T cells (by MHC/antigen pentamer staining) demonstrated that the immune senescent animals vaccinated with CASAC/SIL had substantially higher frequencies of CD8 + T cells specific for H-2K(b)/SIL compared with the CFA/IFA vaccinated groups. Similarly, higher frequencies of H-2K(b)/SVL-pentamer^{+} and IFN-γ^{+} CD8^{+} T cells were detected in the aged, CASAC+SVL-vaccinated mice than in their CFA/IFA-vaccinated counterparts. In both antigen settings, CASAC promoted significantly better functional CD8+ T cell activity. These studies demonstrate that functional CD8+ T cells, specific for both foreign and tumour-associated self-antigens, can be effectively induced in aged immunosenescent mice using the novel multi-factorial adjuvant CASAC [40].

5. PRELIMINARY HUMAN DATA

Based on these promising results in mice, the ability of combined TLR ligands to induce the activation of peripheral blood DCs isolated from human aged donors has been investigated. Preliminary *in vitro* screening experiments suggested that the combination of TLR7/TLR8 with TLR4 was the most efficient in activating DCs. This combination induced significantly greater cytokine production than that induced by each of the individual agonists. This greater stimulation is probably due to the combined activation of both MyD88 and TRIF-dependent signal transduction pathways [41]. Stimulation with the specific combination of TLR agonists, imidazoquinoline R848, a TLR-7 ligand, and monophosphoryl lipid A, a TLR-4 ligand, induced significantly higher cytokine secretion by mDCs and pDCs from old people. Notably, the combination of R848 and MPLA induce 5-10 fold higher production of IL-12/p40 in CD141+ mDCs isolated from over 75 years old donors compared with their young counterparts (less than 40 years old). In addition, the increased levels of TNF-α were also observed in CD1c+ mDCs and pDCs from these older donors, in response to R848 and MPLA stimulation. These differences were statistically significant when compared with their unstimulated counterparts. These results have potentially important implications, since the reduced production of TNF-α by pDCs from older people, caused by defects in TLR signalling pathways, is associated with an ineffective antibody response to the influenza vaccination [42]. Thus, impaired production of cytokines by older DCs could result in a weak response to vaccination and might contribute to the dysregulation of DC-induced T cell proliferation in the older people.

The involvement of TNF-α in DC-induced T cell proliferation is also evident from clinical data in rheumatoid arthritis patients, showing that the treatment with anti-TNF-α antibodies causes poor stimulation of T cell activity by DCs [43,44].

CONCLUSION

Due to the increase in the human life span, the immune system must defend the organism for several decades longer than foreseen by evolution; hence it has to work efficiently for a considerable number of years. Old people suffer more frequently from severe infections and experience poorer outcomes from these infections as compared with younger adults. However, vaccine-induced immune responses are frequently less effective in the old people when compared with younger adults [6,10,12,13]. Thus, there is an increasing need to develop new vaccination strategies in order to ensure an enhancement of the immune defense against infections of the older people, as a preventive measure to promote a successful aging.

Data obtained in model studies demonstrate that functional antigen specific CD8+ T cells can be effectively generated in aged immunosenescent mice using the novel multi-factorial adjuvant CASAC. Our preliminary human *in vitro* data highlight a similarly efficient CASAC-mediated stimulation of cytokine production by antigen-specific T cells, indicating the potential use of appropriately selected combination of TLR agonists in future vaccination approaches for the older people.

Interestingly, triggering of TLRs and other pattern recognition receptors by PAMPs and other agonists induces long lasting epigenetic changes in innate immune cells, including DCs. This results in trained immunity, *i.e.*, an enhanced response to a second challenge by the same or unrelated agent [45]. This opens a new avenue in vaccine development based on the use of multiple, synergistically

acting, immune modulators for the stimulation of broader and longer lasting protection against pathogens.

CONSENT FOR PUBLICATION

All authors have provided consent to the publication of the manuscript in Current Pharmaceutical Design.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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3.9. Assessing the efficacy of combined molecular adjuvant CASAC in enhancing the T cell response in young and aged Sicilian healthy donors

(*Study in progress)*

3.9.1 Introduction

The immunological competence to generate protective immunity upon vaccination progressively declines with advancing age. Due to their immunosenescence status, in the older population vaccines are commonly believed to be less effective in providing protection. Thus, there is a need for more efficient and sophisticated adjuvants that will complement novel vaccine strategies for the older. To this end, we have investigated the effect of the combined molecular adjuvant CASAC, incorporating two complementary TLR agonists and CD40-L, in enhancing the T cell response in aged Sicilian healthy donors.

3.9.2 Materials and Methods

Study Population

A cohort of twenty young healthy donors (9 males, 11 females; range, 25-35 years) and twenty-four aged healthy donors (10 males, 14 females; range, 80-92 years) from Sicily were recruited. Health status and current medication were assessed by a questionnaire to exclude individuals with infection, inflammation, a history of cancer, autoimmune disease, and taking immunoregulatory drugs in the last 6 months. Written informed consent was obtained from all healthy donors in accordance with the Ethic Committee of Palermo University Hospital. All the data were entered in a database to perform statistical analyses.

Blood Collection and isolation of PBMCs

For each healthy donors, whole blood was collected by venepuncture in 3 vacutainer tubes with ethylenediaminetetraacetic acid (EDTA) (3 ml and 6 ml) and 2 plain clot activator vacutainer tubes (6 ml). Serum was obtained by centrifugation of the whole blood in plain tubes at 1500g for 10 min and 2 aliquots of 500 µl per donor were stored at -80°C for serological tests. 2 aliquots of 500 µl of whole blood in EDTA per donor were also stored at -80°C.

PBMCs were isolated at the Laboratory of Immunopathology and Immunosenescence of the Department of Biomedicine, Neurosciences and Advanced Diagnostics of University of Palermo, within 6 hours from whole blood in EDTA tubes, using Ficoll-Paque (GE Healthcare, NJ, USA) density gradient centrifugation according to the manufacturer's instructions. PBMCs were frozen in 90% foetal bovine serum (Sigma-Aldrich, USA) and 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA) at a concentration of $4x10^6$ cells/ml and stored at −80 °C. Samples were sent on dry ice to the King's College London (KCL), School of Cancer & Pharmaceutical Sciences, The Rayne Institute, where they were cryopreserved in liquid nitrogen tanks (−180 °C) until the day of analysis (Figure 3.1).

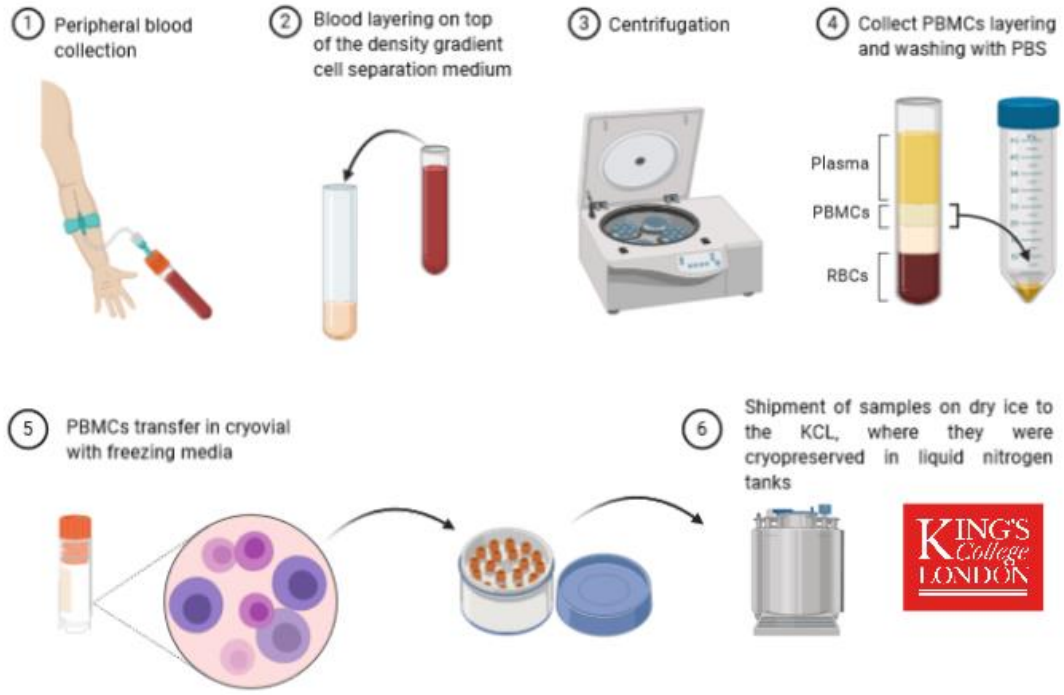

Figure 3.1. PBMCs isolation and cryopreservation protocol. To isolate PBMCs, peripheral blood was collected from healthy donors (**1**), layered on top of a density gradient cell separation medium (**2**), and centrifuged (**3**). PBMCs were collected and washed with PBS before counting (**4**). Subsequently, cells were frozen in a freezing medium (**5**) and, once collected, sent to KCL (**6**). (Created with BioRender.com)

Complete blood count

Whole blood in 3 ml of EDTA tubes was used for automated absolute counts of red blood cells, leukocytes, lymphocytes, neutrophils, and monocytes, performed by Unit of Transfusion Medicine of University Hospital "Paolo Giaccone", Palermo.

CMV serology

Specific IgG responses against CMV were measured serum of all donors in cooperation with King's College London. Serum were analyzed with the human anti-cytomegalovirus antibody (IgG) ELISA Kit (Cusabio, China).

HLA-A and HLA-C typing

Peripheral whole blood samples were collected, and genomic DNA was extracted from leukocytes by a commercial kit (PureLink® Genomic DNA, ThermoFisher Scientific, Waltham, MA, USA). HLA-A and HLA-C profiles of a subgroup of donors were obtained by PCR-SSP (Micro SSP Generic HLA Class I A Locus DNA Typing Tray and Micro SSP Generic HLA Class I C Locus DNA Typing Tray), performing 12 and 16 reactions respectively for each individual, according to the manufacturer's instructions.

T cell activation

In order to compare T cell responses of healthy older and young donors to TLR agonist stimulation, we first performed several experiments to select the best epitope peptide pools for precise monitoring of functional T cell responses. All experiments were performed at King's College London. Several test experiments were conducted to define and optimize the stimulation protocol with frozen PBMCs from three healthy donors from NHS Blood and Transplant (NHSBT) UK.

In these preliminary experiments, it was tested different concentrations of four peptide pools (ProImmune, Oxford, UK) and two Yellow Fever (YF) peptides at a concentration of 10 µg/ml, listed in Table 3 and chosen based on data from the literature. All peptides used in this study were resuspended in DMSO at final concentrations below of 1 % (v/v) to avoid toxicity and stored at -20 °C. Peptides were thawed the day of the assay and diluted with PBS buffer to the required concentration. Heteroclitic MelanA was used as a positive control. For each healthy donor, the following conditions were tested:

1) No stimulation (blank);
2) Peptides only (at different concentrations);
3) MelanA as a positive control.

Table 3. Pool of peptides and their concentrations tested

Peptide pool name	Epitope source and HLA allele restriction	Concentrations tested (µg/ml)	Remarks
ProMix CEF	32 peptides, each corresponding to a defined HLA class I-restricted T cell epitope from cytomegalovirus, Epstein-Barr virus and influenza	0.5, 1, 1.5 and 2	25 µg/peptide
ProMix CEFT	24 peptides, each corresponding to a defined HLA class II-restricted T cell epitope from cytomegalovirus, Epstein-Barr virus, influenza and tetanus toxoid	4, 5, 6 and 8	25 µg/peptide
ProMix CMV	14 peptides, each corresponding to a defined HLA class I-restricted T cell epitope from cytomegalovirus	1, 3, 5 and 7	25 µg/peptide
ProMix Influenza	17 peptides, each corresponding to a defined HLA class I-restricted T cell epitope from influenza	1, 2.5, 5, 7.5 and 9.5	25 µg/peptide
Yellow fever	HLA-A*02:01- restricted	10 µg/ml	2 mg lyophilized peptide
Yellow fever	HLA- C*06:02- restricted	10 µg/ml	2 mg lyophilized peptide

Each experiment consisting of 2 weeks of *in vitro* stimulation and the protocol includes (Figure 3.2):

- Stimulation of PBMCs ($1x10^6$ cells/well) with specific peptides and addition of recombinant human (rhu) IL-4 and rhu IL-7 to promote T cell differentiation and survival/ proliferation (day 0-1);
- Maintenance every 2 days approximately with rhu IL-2 to promote T cell proliferation;
- Re-stimulation with specific peptides (day 7);

On the last day of culture (day 14), for each condition/well, the cells were divided and transferred into two wells of a 96-well plate and stimulated overnight in the presence of a mixture of CD107a antibody and protein transport inhibitor Golgi Plug:

- one well with specific peptide at the same final concentration as used during two weeks of culture;
- the other well with the non-specific WT1 derived peptide RMFPNAPYL (RMF) as the control.

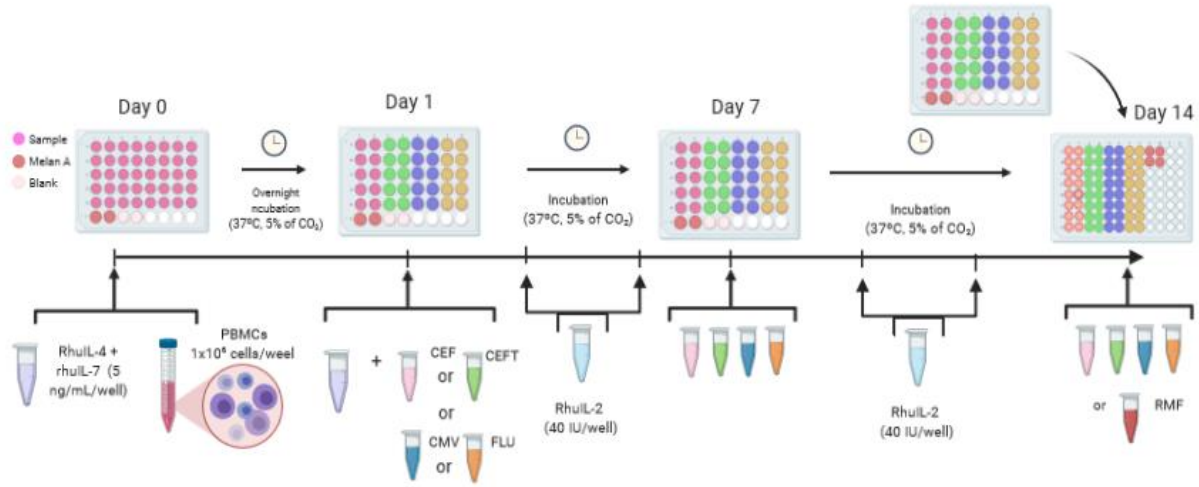

Figure 3.2. T cell stimulation with specific peptide timeline. (Created with BioRender.com)

For each peptide pool, the two concentrations with the best performance, in terms of cytokines production and magnitude of T cell responses, were tested in the presence or absence of CASAC combination consisting of two TLR agonists (InvivoGen, San Diego, USA) (Table 4) and CD40-L (Miltenyi Biotec, Bergisch Gladbach, Germany), a member of the TNF superfamily important in T cell-APC interaction.

Table 4. Agonists of TLRs

Agonist	TLR	Description	Concentrations
Resiquimod (R848)	TLR7/8	An imidazoquinoline compound with potent anti-viral activity. This low molecular weight synthetic molecule activates immune cells via the TLR7/TLR8 MyD88-dependent signaling pathway.	3 µg/ml
Monophosphoryl Lipid A (MPLA)	TLR4	A low-toxicity derivative of LPS that retains its immunostimulatory properties.	5 µg/ml

For each pool of peptide, the following conditions were tested:

- Peptides only;
- Peptides + CD40-L (1µg/ml);
- Peptides + R848 + MPLA + CD40-L (1µg/ml)

Separately, the same conditions were tested with CD40-L at a concentration of 0.5 µg/ml. Both TLR agonists and CD40-L were added to the culture on the same day of the peptides (day 1 and day 7, Figure 3.2), except the last one (day 14, Figure 3.2). After 2 weeks, the cellular supernatant was collected and cryopreserved and cells were transferred to FACS tubes and stained with antibodies.

Once the protocol was optimized, the cells of the healthy Sicilian donors were tested with the most promising combination of peptide pool and CASAC.

Besides, in order to evaluate the function of TLR agonists in the enhancing of the primary immune response against never encounter peptides, PBMCs from Sicilian donors were stimulated with YF peptides, focus on $CD8^+$ T cell response. Sicilian people are not immunized for YF, because it is not endemic to Europe but affects the areas of South America and Africa. Thus, the use of YF peptides is ideal to explore *in vitro* the effects of immunosenescence on a primary infection and the role of CASAC in enhancing this response. Immune responses to stimulation with YF peptides in combination with CASAC were compared with CMV stimulation, which trigger a secondary immune response, and with Alum stimulation, one of the oldest and most widely used adjuvant in human vaccines (*Clements and Griffiths, 2002; McKee and Marrack, 2017*). The concentration of Alum was chosen based on data from the literature.

Flow cytometry analysis

Flow cytometry analysis was performed at the King's College London, School of Cancer & Pharmaceutical Sciences, The Rayne Institute. For cell thawing, the cryopreserved vials were first transferred to a 37 °C water bath and then washed with X-Vivo 15 (Bio Whiteaker, Maryland, USA).

After centrifugation, the supernatant was discarded and the PBMCs were re-suspended in X-Vivo 15 for counting. Counts and viability were determined with a haemocytometer and the trypan blue dye exclusion technique. With this method, dead cells appear blue and are distinguishable from viable cells (>90%).

Baseline panel

For the analysis of T and NK cell subsets, $1x10^6$ PBMCs were first stained with Fixable Viability Dye eFluor™ 780 (eBioscience), following manufacturer's instructions, and next incubated with a surface staining antibody cocktail (Table 5). For negative control, human unstained cells were thawed. Single color controls were used for automatically calculated compensation factors. A minimum of 500,000 cells per sample were analysed by LSR Fortessa (BD Biosciences).

Lymphocyte subsets were obtained with forward scatter (FSC) and side scatter (SSC). An example of the applied gating strategy for the analysis of T and NK cells is displayed in Figure 3.3. NK cells were identified as $CD3^-CD56^+$ and T cells by their CD3 expression. Thus, T_H and T_C cells were distinguished by their differential CD4 and CD8 expression, respectively.

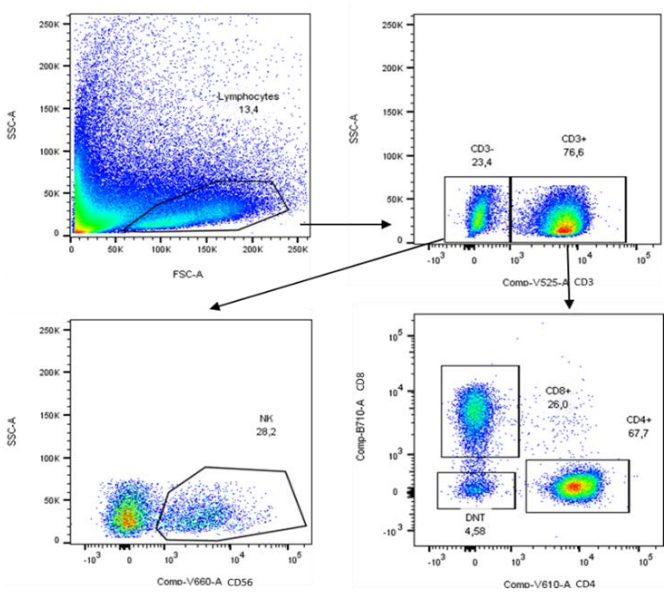

Figure 3.3. NK and T cell gating strategy. Gating strategies for the analysis of NK, $CD4^+$ and $CD8^+$ T cell subsets. The doublet exclusion on FCS-H vs FCS-A followed by SSC-H vs SSC-A is not shown. A representative donor is presented.

Table 5. Antibody cocktail for T and NK cell baseline counting

Specificities	Clone	Fluorochrome	Provider
CD3	OKT3	BV510	BioLegend
CD4	SK3	BV605	BioLegend
CD8	RPA-T8	PC5.5	BioLegend
CD16	3G8	BV785	BioLegend
CD45RA	HI100	FITC	BioLegend
CD56	5.1H11	BV650	BioLegend
CD197 (CCR7)	G043H7	PE	BioLegend

T cell activation panel

T cell subsets and their activation status were analyzed after two weeks of cell culture. For this, 96-well U-bottom plate was centrifuged (5 min, 300 g) and supernatant was carefully removed. Next, PBMCs were stained with Fixable Viability Dye eFluor™ 780 (eBioscience). After 20 min of incubation, PBMCs were washed in PBS and surface stained with an extracellular antibody cocktail (Table 6) for 20 min on ice and in the dark. After incubation, staining was stopped by washing PBMCs twice with PBS. Then, stained cells were fixed by re-suspending them in Cytofix/Cytoperm (BD, USA) buffer. After an incubation of 15 min on ice in the dark, fixed cells were washed twice with Perm/Wash buffer (1X). Cells were directly centrifuged (5 min, 300 g) and supernatant was carefully removed. In order to permeabilize the cells, cell pellets were incubated with Perm/Wash buffer for 15 min on ice. Next, PBMCs were stained with an intracellular antibody cocktail, listed in Table 6, for 30 min on ice and in the dark. After a final washing step with Perm/Wash buffer, cells were centrifuged and pellets re-suspended in 200 µL of PBS. Samples were acquired on a LSR Fortessa (BD Biosciences). An example of a gating strategy for intracellular markers is shown in Figure 3.4.

Table 6. Antibody cocktail for T cell activation

Specificities	Clone	Fluorochrome	Provider
Extracellular staining			
CD3	OKT3	BV510	BioLegend
CD4	SK3	BV605	BioLegend
CD8	RPA-T8	PC5.5	BioLegend
Intracellular staining			
CD137	4B4-1	PE/Dazzle 594	BioLegend
IFN-γ	4S.B3	BV650	BioLegend
TNF-α	MAb11	Alexa Fluor 700	BioLegend

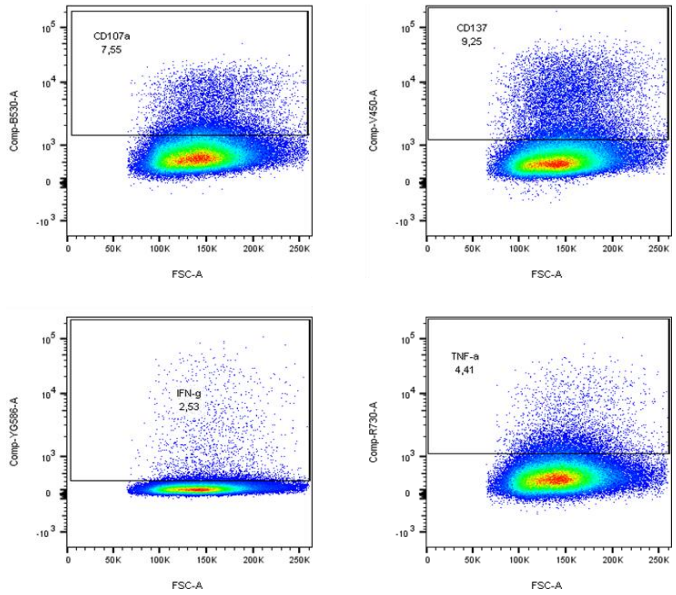

Figure 3.4. Intracellular T cell gating strategy. Gating strategies for the analysis of $CD8^+$ T cell degranulation (CD107a), activation (CD137) state, and IFN-γ and TNF-α secretion. A representative donor is presented.

Statistical analysis

FACS data was exported into fcs-data files and analyzed using FlowJo version 10.5.3 (Tree Star, Ashland, OR). All FACS data were properly compensated using matching single-stain controls. Gated data was exported into data Excel tables. Statistical analysis and graphics were performed with GraphPad Prism, version 8.1.2 (GraphPad Software).

3.9.3 Results

Preliminary results showed higher overall cytokine production and survival rate following CEF and CMV stimulation – UK donors

In the first experiments were used human PBMC from NHSBT UK in order to optimize the T cell stimulation protocol. In particular, these experiments were aimed at selecting the best concentrations of the 4 peptide pools in terms of IFN-γ and TNF-α secretion by both $CD4^+$ and $CD8^+$ T cells, degranulation ($CD107a^+$), activation ($CD137^+$) state, and live cell number. Based on data obtained (not shown), for each pool of peptide the following concentrations were chosen and tested in the presence or absence of CASAC:

- CEF at a concentration of 2 and 2.5 µg/ml;
- CEFT at a concentration of 4 and 6 µg/ml;
- CMV at a concentration of 1 and 5.5 µg/ml;
- FLU at a concentration of 5 and 7.5 µg/ml.

After 3, 9 and 11 days of culture, the cells were examined by microscope. The stimulated T cells show a change in morphology, with the formation of large clusters, well known to occur between DCs and stimulated T cells (*McLellan et al., 1999*) (Figure 3.5).

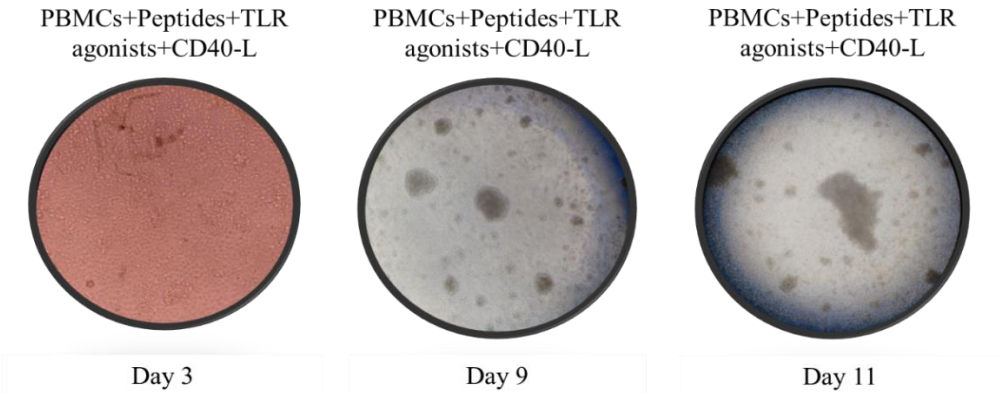

Figure 3.5. Formation of clusters following T cell activation. The figure shows bright field images (40X) of PBMCs stimulated with specific peptide in combination with TLR agonists and CD40-L for 3, 9 and 11 days. The results are representative of at least three independent experiments with similar findings.

After two weeks of culture, unstimulated and stimulated PBMC were stained with the same panel of 1st experiment (Table 5) and 1 viability marker.

From data analysis by FlowJo, CD40-L showed a similar effect to both 0.5 and 1 µg/ml, thus the lowest concentration was chosen. As shown in Figures 3.6, CASAC combination (TLR agonists and CD40-L) seems to stimulate higher overall cytokine production by $CD4^+$ and $CD8^+$ T cells when combined with the CEF pool at a concentration of 2 µg/ml.

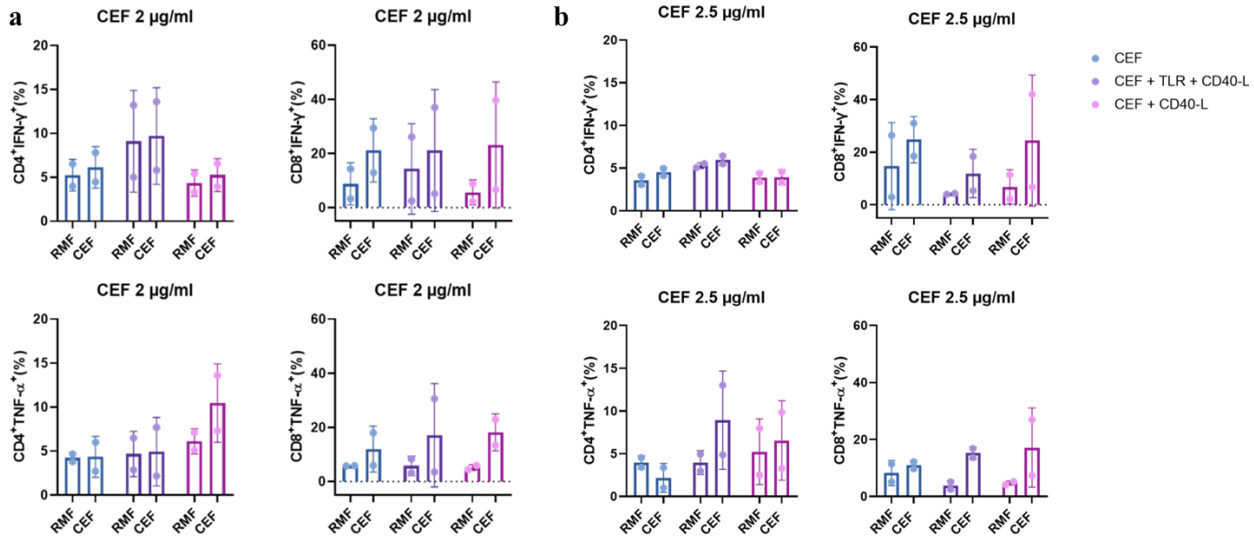

Figure 3.6. Quantification of IFN-γ and TNF-α production by $CD4^+$ and $CD8^+$ T cells after 2-week culture with the CEF pool at a concentration of 2 (**a**) and 2.5 µg/ml (**b**). Column bars represent mean of n = 2 UK donors. Each point represents an individual condition.

CEFT pool has induced markedly lower cytokine production, particularly of IFN-γ, by both T cell subsets especially at a concentration of 4 µg/ml (Figure 3.7).

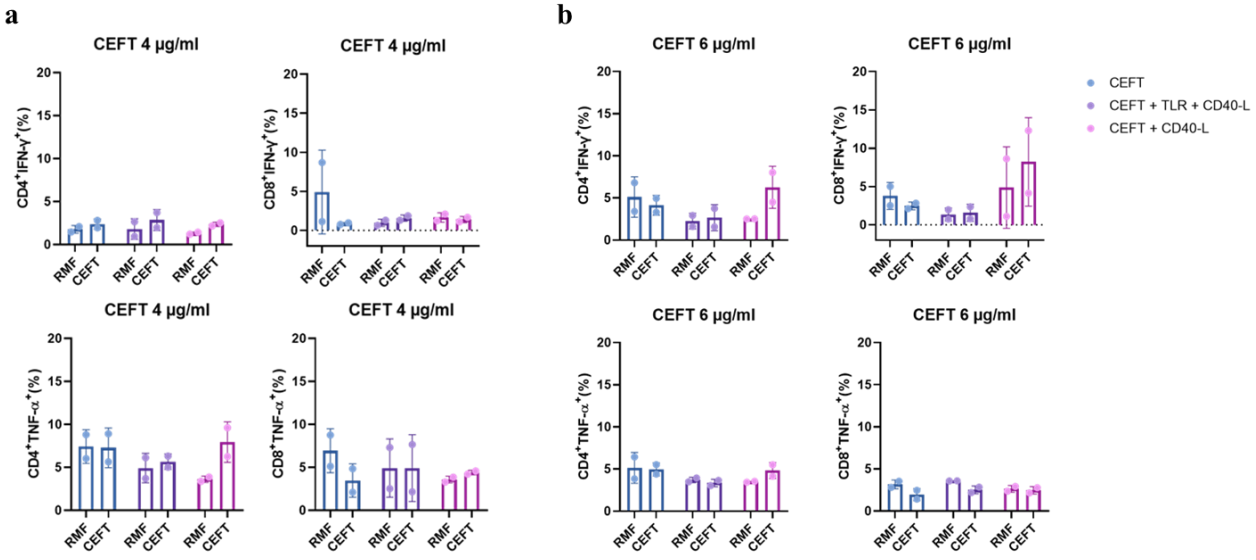

Figure 3.7. Quantification of IFN-γ and TNF-α production of $CD4^+$ and $CD8^+$ T cells after 2-week culture with the CEFT pool at a concentration of 4 (**a**) and 6 µg/ml (**b**). Column bars represent mean of n = 2 UK donors. Each point represents an individual condition.

Comparing all culture conditions, the CMV pool at 1 µg/ml concentration has stimulated a satisfactory amount of cytokines in the "peptide only" condition (mean % >10 for $CD4^+TNF\text{-}\alpha^+$, $CD8^+TNF\text{-}\alpha^+$, and $CD8^+IFN\text{-}\gamma^+$), while lower amounts were detected when cells were stimulated with CMV in combination with CASAC (mean % <10), especially in the cytotoxic compartment (Figure 3.8,a).

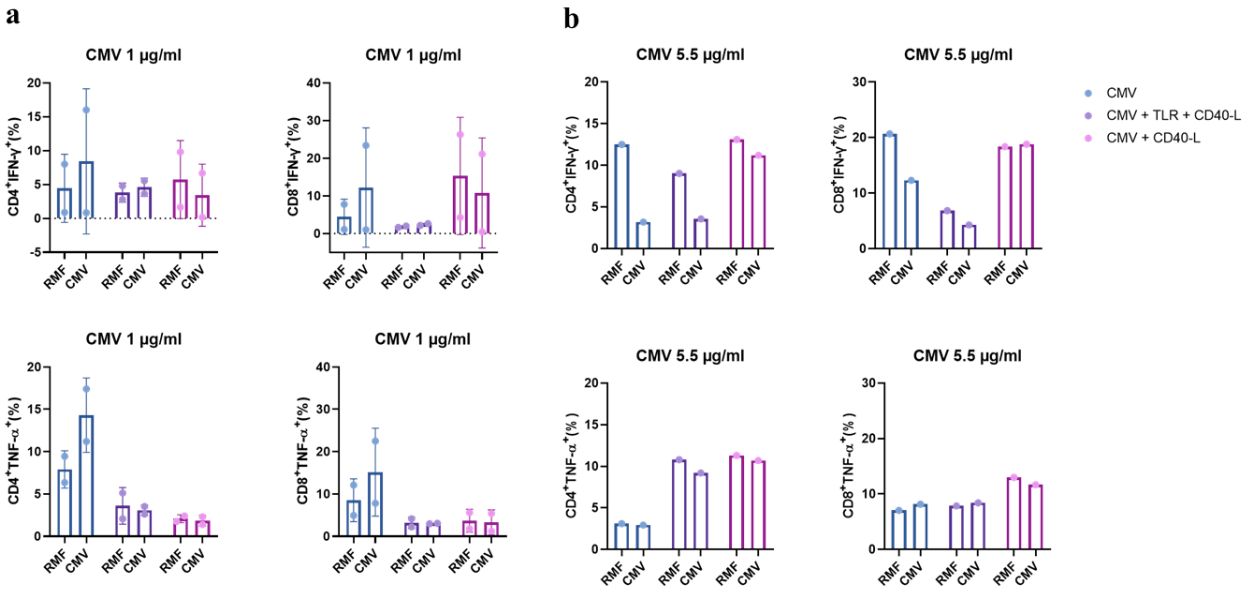

Figure 3.8. Quantification of IFN-γ and TNF-α production of $CD4^+$ and $CD8^+$ T cells after 2-week culture with the CMV pool at a concentration of 1 (**a**) and 5.5 µg/ml (**b**). Column bars represent mean of n = 2 UK donors for CMV 1 µg/ml and represent value of n=1 UK donor for CMV 5.5 µg/ml . Each point represents an individual condition.

Likely, this may be due to over-stimulation of the cells, resulting in activation-induced cell death (AICD) where a fraction of the population is lost upon activation. Confirming this, at the concentration of 5.5 µg/ml, there is a significant decrease in IFN-γ production by both T cell subsets and a comparable amount of TNF-α secreted compared to the lower concentration (Figure 3.8,b).

Here and for FLU peptides pool, one UK donor was excluded from the analysis as it disproportionately influencing the scaling of the data due to probably too few cells in culture.

Finally, FLU at a concentration of 7.5 µg/ml, compared with 5 µg/ml concentration, has induced a higher production of IFN-γ by $CD8^+$ T cells when alone (% >20) but a lower production of IFN-γ when combined with CASAC (% <10), while TNF-α^+ cell percentages were almost constant (Figure 3.9,a e b). Also in this case, it can be assumed that there was excessive stimulation.

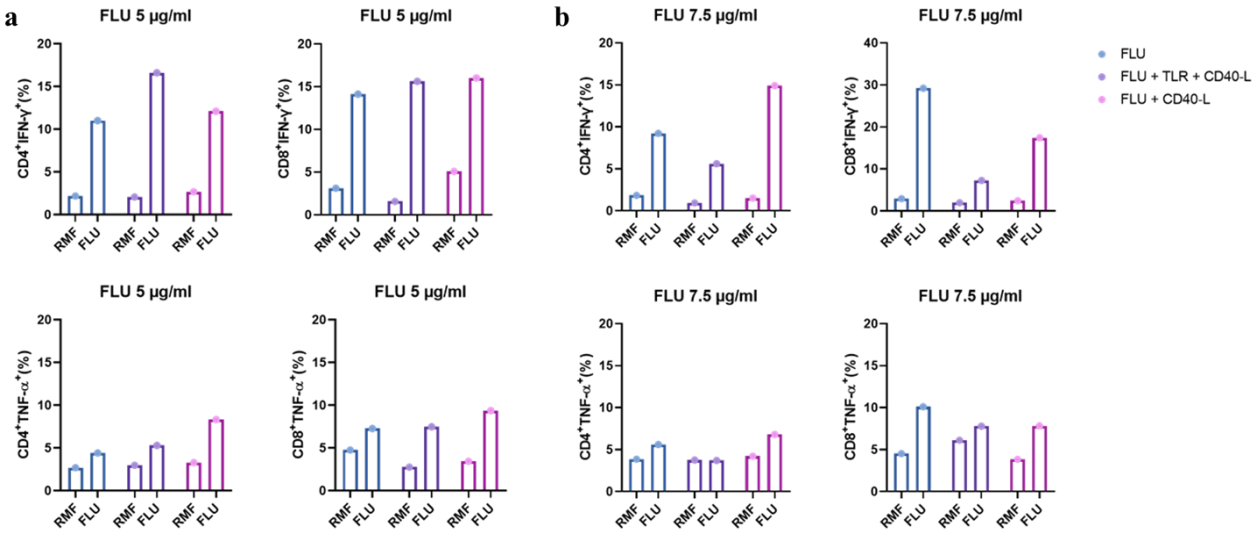

Figure 3.9. Quantification of IFN-γ and TNF-α production of $CD4^+$ and $CD8^+$ T cells after 2-week culture with the FLU pool at a concentration of 5 (**a**) ad 7.5 µg/ml (**b**). Column bars value of n = 1 UK donors. Each point represents an individual condition.

In order to clarify this aspect, for each donor and each condition, it was compared to the number of live cells, identified by staining with Live/dead and subtracting background by the matching un-stimulated sample.

As shown in Figure 3.10, cells stimulated with CEF and CMV show a higher survival rate than other peptides. Since CEFT did not show great performance in cytokine production and, together with FLU, showed the lowest number of live cells, these two peptide pools were excluded from the next experiments with CASAC combination. CEF has shown a similar effect to both 2 and 2.5 µg/ml, the lowest concentration was chosen. CMV performance was better at the lowest concentration. For this reason, the CMV pool at a concentration of 1 µg/ml was chosen.

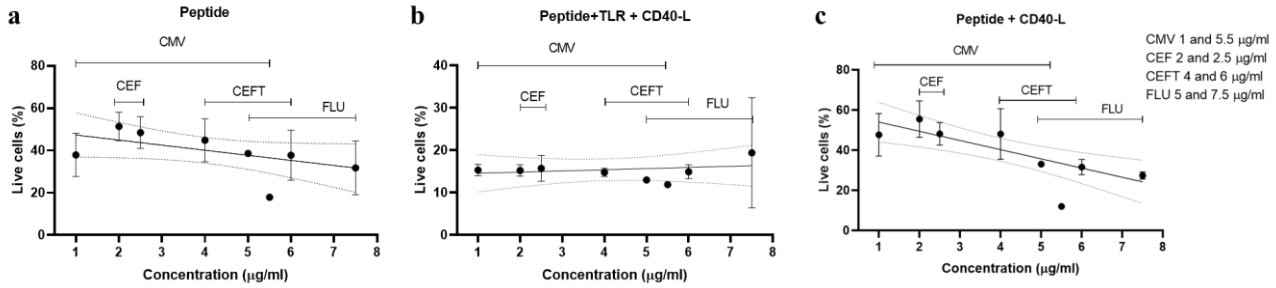

Figure 3.10. Percentages of live cells in correlation with different conditions after 2-week culture. Data were plotted as scatterplots with a linear regression line with 95% confidence bands. Each point represents an individual condition.

After optimizing the protocol, we proceeded to in vitro stimulation of PBMCs from healthy Sicilian donors, focusing in the function of TLR agonists in the enhancing of primary immune response against never encounter peptides.

Sicilian donors

A cohort of twenty young healthy donors (9 males, 11 females; range, 25-35 years) and twenty-four aged healthy donors (10 males, 14 females; range, 80-92 years) from Sicily were recruited.

Pharmacological status

All donors were questioned about their health status in order to evaluate both pharmacological therapy and the prevalence of diseases in both groups. No young donor was affected by any pathology and did not take drugs. 50% of the older were on cardiovascular and hypotensive therapy (Table 7).

Table 7. Pharmacological status of donors

	Young N=20	**Older N=24**
Cardiovascular therapy, n (%)	0 (0)	12 (50%)
Hypotensive therapy, n (%)	0 (0)	12 (50%)
Lipid-lowering therapy, n (%)	0 (0)	10 (41.7%)
Hypoglycaemic therapy, n (%)	0 (0)	3 (12.5%)

Haematological parameters and baseline immunophenotype

Blood cell count was determined in all donors. As reported in Table 8, all the values were in the normal range.

Table 8. Blood cell count

Haematological parameters (unit of measurement, reference values)	Young, N=20 mean ±SD	Older, N=24 mean ±SD
Leukocytes (10^3/µL, 4-11)	6.16±1.08	6.13±1.23
Lymphocytes (10^3/µL, 1-5)	2.19±0.42	1.84±0.48
Neutrophils (10^3/µL, 2-8)	3.22±1.12	3.53±1
Monocytes (10^3/µL, 0.16-1)	0.47±0.1	0.54±0.2

Data were expressed as mean ± standard deviation (SD).

For the analysis of total $CD4^+$ and $CD8^+$ T cell subsets a day 0 of cell culture (Figure 3.2), PBMCs isolated from the blood of a sub-cohort of 21 healthy donors, 12 young (6 females, 6 males) and 9 older (4 females, males) were thawed, counted and stained with specific surface markers (Table 9).

To investigate the effects of age and gender on human lymphocyte subsets, the percentages of $CD4^+$ and $CD8^+$ T cells were determined in PBMCs by flow cytometric analysis using the gating strategy described in Figure 3.3. The percentage of $CD4^+$ and $CD8^+$ T cells slightly increased with age and this was reflected in a not significant change in the CD4/CD8 *ratio* with age (Table 9).

Table 9. Percentage of $CD4^+$, $CD8^+$, NK and NKT cells

Leukocyte subpopulations	Young, N=20 mean±SD	Older, N=24 mean±SD	*p*-value
$CD3^+$ T cells (%)	66.82±11.43	62±9.01	0.324
$CD3^+CD4^+CD8^-$ T_H cells (%)	61.59±10.72	65.27±11.44	0.459
$CD3^+CD8^+CD4^-$ T_C cells (%)	24.83±7.36	27.11±10.94	0.573
CD4:CD8 *ratio*	2.74±1.09	2.90±1.65	0.783
$CD3^-CD56^+$ NK cells (%)	46.98±16.35	57.42±17.64	0.177
$CD3^-CD56^{lo}CD16^+$ cytotoxic NK cells (%)	27.44±15.14	39.70±15.94	0.088
$CD3^-CD56^{hi}CD16^-$ secreting cytokine NK cells (%)	3.83±1.65	4.30±2.02	0.560
$CD3^+CD56^+$ NKT cells (%)	1.96±0.88	3.14±1.90	0.152

Data were expressed as mean ± standard deviation (SD). The CD4:CD8 *ratio* was calculated by dividing the percentage of $CD4^+$ T cells by the percentage of $CD8^+$ T cells. *p*-value was obtained by unpaired t test. See the text for the acronyms.

Within the $CD3^-$ population, NK cells were divided into two subsets based on their CD56 and CD16 expression. The $CD56^{lo}CD16^+$ subset is mainly responsible for natural cytotoxicity by

releasing cytoplasmic granules containing perforin and granzymes B. By contrast, the $CD56^{hi}CD16^{-}$ subset is described as secreting chemokines and cytokines. NK cell percentages in the peripheral blood of older showed an age-related increase, due to an increase in both subsets, especially in the cytotoxic NK subset (Table 9). There was also a higher percentage of NKT, determined by their CD3 and CD56 co-expression, in older than in young people.

Using a combination of surface markers, *i.e.* CD45RA and CD197 (CCR7), it was also investigated the difference in the distribution of T cell subpopulations in our cohort.

Consistent with other reports, the percentages of $CD8^{+}$ naïve cells significantly decline with age (p=0.0003, Figure 3.11,e), with a concomitant significant increase in the percentage of both TE (p=0.008, Figure 3.11,f) and EM (p=0.013, Figure 3.11,g), and a non-significant decrease in CM (p=0.338, Figure 3.11,g) $CD8^{+}$ subsets. Also in $CD4^{+}$ T cell compartment the percentage of naïve and CM cells decrease and EM and TE cells increase in the older group, but these differences were not significant.

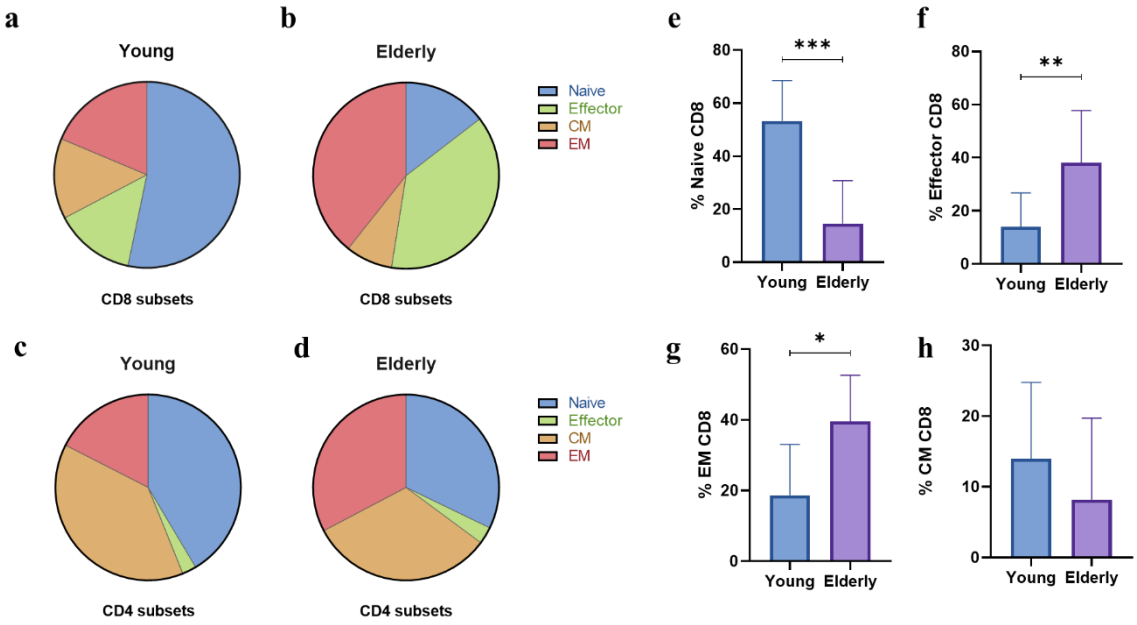

Figure 3.11. Analysis of $CD4^{+}$ and $CD8^{+}$ T cell subsets. p-value was obtained by unpaired t test. *=$p \leq 0.05$; **=$p \leq 0.01$;***=$p \leq 0.001$. See the text for the acronyms.

CMV seropositivity

CMV is a member of a widely distributed subgroup of Herpesviruses. CMV can infect people of all ages, but its seroprevalence increases with age, ranging from 50% in adults by the age of 40 to 90% in 75–80-years old subjects (*Al Mana et al. 2019*). In immunocompetent adults, CMV infection rarely causes disease but results in a latent infection that can cause chronic immune activation.

In order to explore immunophenotype differences in correlation with CMV exposure, which may influence the response to *in vitro* antigenic stimulation, it was investigated serum CMV IgG positivity in both age groups. It was found that 100% of the older group and 25% of young donors were CMV seropositive (Table 10).

Table 10. CMV seropositivity

	Young **N=20**	**Older** **N=24**
CMV positivity, n (%)	5 (25%)	24 (100%)

Preliminary data from Sicilian donors

In order to evaluate how PBMCs isolated from Sicilian healthy older donors respond to CASAC stimulation compared to Alum and how young and older differ in their magnitude of responses, PBMCs from young (< 35 years) and healthy aged two Sicilian donors (> 90 years) (see "Discussion and future perspectives" Chapter) were thawed, washed, counted and *in vitro* stimulated, following the previously mentioned stimulation protocol.

In this experiment, we aimed to evaluate both the response to stimulation with naïve peptides and peptides previously encountered. To assess the activation of secondary immune responses, an old CMV seropositive donor was also selected.

Although containing epitope from cytomegalovirus, the CEF pool of peptides was excluded because it included other epitopes that could interfere with the nature and magnitude of cytokine production. In this experiment, for each donor, the conditions therefore were:

- CMV 1µg/ml + R848 (at 3 µg/ml) + MPLA (at 5 µg/ml) + CD40-L (0.5 µg/ml);
- CMV 1µg/ml + Alum (10 µg/ml);
- YF HLA-A*02:01 (4µg/ml) + YF HLA- C*06:02 (0.5µM) + R848 (at 3 µg/ml) + MPLA (at 5 µg/ml) + CD40-L (0.5 µg/ml);
- YF HLA-A*02:01 (4µg/ml) + YF HLA- C*06:02 (0.5µM) + Alum (10 µg/ml).

A young CMV seronegative donor was selected, and both young and older were positive for at least one HLA between HLA-A*02:01 and HLA- C*06:02.

After two weeks of culture, CASAC combination has triggered increased production of IFN-γ and TNF-α by T cells, especially by $CD8^+$, in both young and older donors (Table 11).

Table 11. Percentage of $CD4^+$ and $CD8^+$ secreting TNF-α and IFN-γ in young and older healthy donors

Culture conditions	$CD4^+$ TNF-α^+ (%)		$CD4^+$ IFN-γ^+ (%)		$CD8^+$ TNF-α^+(%)		$CD8^+$ IFN-γ^+ (%)	
	Y	**O**	**Y**	**O**	**Y**	**O**	**Y**	**O**
RMF CMV+TLRs+CD40-L	2.60	0.35	0.47	0.56	4.52	0.86	10.4	2.65
CMV+TLRs+CD40-L	7.26	0.92	0.40	2.69	6.57	0.82	8.94	3.38
RMF CMV+Alum	3.70	0.76	0.17	0.28	3.73	0.36	10.6	3.02
CMV+Alum	4.90	0.91	0.44	0.26	5.41	0.36	13.2	1.39
RMF YF+TLRs+CD40-L	0.47	0.84	0.09	0.22	2.10	0.21	14.5	2.34
YF+TLRs+CD40-L	4.19	2.12	0.27	0.34	5.77	0.99	12.0	2.66
RMF YF+Alum	1.58	0.85	0.20	0.15	3.70	0.92	12.7	2.90
YF+Alum	0.79	0.43	0.11	1.12	1.59	0.68	10.2	6.14

CMV= cytomegalovirus peptides; O=older; RMF= RMFPNAPYL; TLR=toll-like receptor agonist; Y=young; YF=yellow fever peptides.

3.9.4 Conclusions and future directions

Given the employment restrictions imposed by the ongoing pandemic of COVID-19, these experiments were suspended. However, our preliminary results highlight the effect of the CASAC adjuvant in increasing the T cell response, through activation of circulating DCs, to both naïve and peptide stimulation previously encountered. Further experiments will need to be performed to confirm the potential use of this appropriately selected combination of TLR agonists in future vaccination approaches for the older.

4. Discussion and future perspectives

Immunosenescence is a result of a combination of events that can occur independently or converge in the achievement of significant quantitative and qualitative changes in the immune system, which becomes less able to defend the body against pathogens and to develop immunity after vaccination.

My PhD research activity was focused on exploring and deepening the two main factors responsible for the immunosenescent phenotype, ageing and chronic viral infections. This part of the study had an exploratory character and started with general initial assumptions.

Increasing age is accompanied by a progressive decline in both innate and acquired immune system, which favors the onset of many diseases. According to this view, ageing and age-related diseases become part of a *continuum* where the extreme is represented by centenarians, who largely avoided or postponed most chronic age-related diseases and are characterized by decelerated ageing (*Franceschi et al., 2018*). Improved health care, hygiene, and healthier lifestyles have led to a remarkably increase in the world's centenarian population in the last decades (*Borras et al., 2020*). Thus, the second aim of my thesis was to study how ageing affects the immunological profile of the Sicilian population, extending the analysis to a cohort ranging from the young to the supercentenarian. In this observational analysis, it was also examined whether, in a changing society, centenarians are now the new 80.

As summarised in Chapter 3.1, immunosenescence is commonly measured by biomarkers, called hallmarks of immunosenescence, which are parameters commonly found in the older population. These include an inverse CD4/CD8 *ratio*, loss of naïve T cells, increased frequency of T_{ERMA} T cells, and a lingering level of low-grade inflammation. Possible treatments to mitigate the effects of immunosenescence include dietary strategies. Numerous studies show the power of food to regulate the immune system, and much attention is paid to the Mediterranean diet (MedDiet), particularly rich in fruit, vegetables, legumes and extra virgin olive oil. The efficacy of this diet results as attenuation of inflammation and oxidative stress and is involved in the general improvement of the immune response.

The study discussed in Chapter 3.2 confirms the importance of adherence to the MedDiet, especially at a young age. A survey of the population living in Sicilian mountain villages in the province of Palermo has shown that the Madonie is an area with a high rate of longevity. Preliminary analysis of the nutritional habits has shown that long-lived people used to follow MedDiet but not during the ageing period, suggesting an interesting and effective role of epigenetics in the attainment of longevity. Added to this is greater access to social support and family network and a constant

engagement in physical exercise that contributes to control body weight and hematochemical parameters, maintaining them in the normal ranges, thus promoting successful ageing.

Within this context, the analysis of the immune phenotype of the Sicilian population could provide important information on how to develop new strategies to slow down or postpone ageing.

In the study described in Chapter 3.3, it was analysed the effect of ageing on the composition of the innate and adaptive immune system, in correlation with gender and CMV and EBV serostatus, in a cohort of Sicilian donors, ranging from young to a supercentenarian. The analyses resulting from this study confirm the literature reports, *i.e.* ageing has a dramatic effect on the immune system, as demonstrated by the age-related shift from antigen-inexperienced naïve T lymphocytes to antigen-experienced effector, although not memory, T cells observed. In order to evaluate the general effect of persistent viral infection on T cell subset distribution, our cohort was screened for CMV and EBV seropositivity. Being CMV more frequent in older individuals, CMV seropositivity may have influenced the T cell subset specific expansion and survival with age. On the contrary, EBV infection seems to play no role, since all individuals in this study were EBV-seropositive.

Despite these similarities with literature, compared to that previously reported our Sicilian donor cohort differs in some aspects. For example, it is commonly known that with advancing age there is an inversion of the CD4/CD8 *ratio*, characterised by a decrease of the $CD4^+$ T cell number and a parallel increase of $CD8^+$ T cells. However, it was observed a constant trend in the percentages of both $CD4^+$ and $CD8^+$ T cells with age, even when gender differences were included. Consequently, we did not observe the described age-related increase in the rate of inversion of the CD4/CD8 *ratio*, whereas this value tends to increase in LLIs, and to decrease in the supercentenarian.

This is interesting because, although the sample size is small, we can clearly observe different trends of the total $CD4^+$ and $CD8^+$ T cell percentages between individuals aged 90-100 year old and octogenarian subjects. Most 90-100 year old donors show lower $CD4^+$ T cell percentages and higher $CD8^+$ T cells percentages than octogenarian donors. Even more interesting, the percentages of $CD45RA^+CCR7^+CD4^+$ naïve T of our supercentenarian, the oldest living woman in Italy, were more similar to the octogenarians than to individuals aged 90-100 years. These results support the theory that the immune system of the supercentenarians has adapted to achieve exceptional longevity by sustaining immune responses to infections and diseases. Nevertheless, some limitations should be considered in our study. First, and mostly due to the rarity of subjects, the sample size is small. More detailed data are needed, in particular on the immune phenotype of semi- and super-centenarians, in order to identify strategies that can counteract the effects of ageing on the immune system. Second, this study was limited by the amount of blood collected for immunophenotypic analysis from the older population, due to ethical concerns. Due to the limited number of PBMCs isolated, it was not

possible to conduct an in-depth analysis of T subsets for all donors. For these reasons, parallel recruitment of healthy donors is being carried out in order to expand the sample size. For cytofluorimetric analysis, it was used fresh blood instead of frozen PBMCs.

Currently, 5 young donors (range age 29-33), 6 older (range age 61-73), 2 semi-supercentenarians (107 and 108 years old) have been recruited. Despite the number of donors currently recruited is still small (recruitment is in progress), a preliminary statistical analysis was carried out in order to check whether the previous trend is confirmed or not (Supplementary Table 1). In line with the data previously presented and with what has been reported in the literature, naïve T cells decrease dramatically with advancing age, with a concomitant increase in the percentage of TE, highly significant in $CD4^+$ T cells. Another notable data is the significant increase observed in activated $CD4^+PD1^+$ T cells, a possible symptom of chronic activation of the immune system. These data also confirm the significant increase in NK cells with ageing and add a drastic, albeit not significant, reduction in the percentage of B cells.

The role of the host's genetic factors in the outcome of viral infections was investigated, in order to identify potential protective genetic profile useful to predict disease susceptibility or progression. In particular, my research has focused on HIV infection, one of the world's most fatal infectious disease, for which there is no cure. HIV infection shows immune parallelism with ageing in its clinical manifestations. Therefore, identifying factors involved in an efficient anti-HIV innate immunity could be vital in order to plan optimal customized therapeutic strategies and, thus, to reverse or at least reduce the associated immunosenescent profile.

The study described in Chapter 3.6 investigated the effect of particular KIRs and their HLA ligands on susceptibility and clinical outcome in a Sicilian cohort of HIV-infected adults, responders to treatment with antiretroviral therapy.

The results of this study reveal that HIV-positive individuals show an overall prevalence in KIR inhibitors and their respective ligands, compared to the healthy donor population. Specifically, HIV-positive individuals showed a lower percentage of all activating KIR, except KIR2DS2, although only KIR2DS4 was significantly lower (crude OR, 0.15; $p<0.01$). Conversely, no difference in the frequencies of the KIR inhibitory genes was found, except for the KIR2DL5A significantly less frequent than in controls (crude OR, 0.42; $p<0.01$). The significantly lower frequency of KIR2DS4, the only activator gene of haplotype A, and the comparable presence of inhibitory genes are reflected in a significantly higher Bx haplotype compared to HIV-seronegative individuals (crude OR, 0.19; $p=0.01$).

A similar trend was observed in the KIR ligand group. HLA-A Bw4, a ligand of inhibitor KIR3DL2, was significantly more frequent in the HIV group than in controls (crude OR, 4.58; $p<0.01$), as were HLA-C1^{Asn80} and HLA-B Bw4 (respectively, crude OR, 3.79, $p<0.01$ and crude OR, 2.02; $p=0.038$). Although the latter two are able to bind both inhibitory and activating KIRs, the HLA-C1:KIR2DL2/3 and HLA-B Bw4:KIR3DL1 interactions are stronger than their activating counterpart.

The important role of activating KIR in controlling infection is also supported by studies on CMV infection, as discussed in Chapter 3.4 and 3.5. CMV infection can lead to significant morbidity and mortality in immunosuppressed or immunosenescent individuals, such as the transplanted patients and the older, respectively. However, several studies have shown that the absence of the HLA ligand for inhibitory KIR and the presence of activating KIR genes or Bx haplotype in patients with a kidney transplant were both associated with a lower rate of CMV infection after transplantation (*Hadaya et al., 2008; Stern et al., 2008*). On the contrary, the HLA-Bw4^{Threo} groups and the homozygous A haplotype were associated with the risk of developing the symptomatic disease (*Di Bona et al., 2014*).

These studies are consistent with our finding showing a KIR balance shifted towards NK cell inactivation in HIV-infected patients. Although limited by a small number of patients and controls recruited, this study hopes to contribute to knowledge on the role of host immunogenetic factors in susceptibility to this epidemiologically important infectious disease.

It is commonly known that immunosenescence leads to a reduced ability of the older person to respond to vaccination against either infectious agents. However, it must be emphasised that older people are only less able, not unable, to respond to commercial vaccine formulations. This means that the aged immune system, if properly stimulated, could still respond adequately to vaccination. Another notable example is SHINGRIXTM vaccine for varicella zoster, with an efficacy of over 80%, even in the 80+ population (*Witkowski et al., 2020*). Clearly, the use of appropriate adjuvant can make all the difference.

Another part of my PhD research activity was aimed at testing a new potential strategy for more effective vaccination in the older.

As widely discussed in Chapter 3.7 and Chapter 3.8, a promising strategy to increase vaccine efficiency in older adults seems to be the incorporation of TLR agonists in vaccine formulations. TLRs agonist efficiencies as vaccine adjuvants rely primarily on the promotion of antigen uptake, presentation, and maturation of DCs, including their cytokine secretion and activation of T cells. Due to their powerful immune-stimulatory properties, TLR agonists are considered important vaccine adjuvant candidates and some of them are indeed currently in use or being tested in clinical studies

as adjuvants. Promising results were obtained in murine models where the simultaneous and synergistic activation of TLRs has enhanced DC activation, resulting in increased cellular responses to the antigen of interest (*Tye et al., 2015*). To assess if these promising observations in animal models can be translated to humans, the ability of two combined TLR ligands, the imidazoquinoline R848 (TLR7/8 agonist) and MPLA (TLR4 agonist), to enhance the activation of T cells isolated from human healthy aged and young donors has been investigated.

Preliminary *in vitro* experiments with NHSBT UK donors described in Chapter 3.9 suggest that, from the various combinations tested, the CMV peptide pool at a concentration of 1 μg/ml in combination with TLR7/TLR8 and TLR4 agonists + CD40-L was the most effective condition that activates T cells, in terms of cytokine production and cell survival. This TLR agonist combination + CD40-L induced greater cytokine production in both $CD4^+$ and $CD8^+$ T cells than that induced by Alum, one of the oldest and most widely used adjuvant, in CMV-seropositive healthy Sicilian old donor. This greater stimulation is probably due to the combined activation of both MyD88 and TRIF-dependent signal transduction pathways and the activation of CD40-CD40L pathway. This has potentially important implications because defects in TLR signalling pathways are associated with an ineffective antibody response to vaccination in older people (*Panda et al., 2010*).

One of the main age-related immune changes is the reduction of the pool of naïve T cells, crucial for the recognition of new antigens the immune system has never encountered, initiating an immune response and allowing the body to react to unfamiliar pathogens, bacteria, and infections. Due to their senescent immunophenotype, the older are also more vulnerable to encountering new pathogens. To evaluate the function of TLR agonists in the enhancing of the primary immune response against never encounter peptides, PBMCs from Sicilian healthy young and old donors were stimulated with YF peptides. TLR agonists induced higher overall cytokine production upon stimulation with YF peptides in both young and old donors compared to stimulation with Alum. As expected, the effects of TLRs stimulation were more pronounced in the young subject, especially in TNF-α production by both $CD4^+$ (4.19% with TLR agonists vs 0.79% with Alum) and $CD8^+$ (5.77% with TLR agonists vs 1.59% with Alum) T cells. Although less evident, the old donor also showed higher TNF-α production by $CD4^+$ (2.12% with TLR agonists vs 0.43% with Alum) and $CD8^+$ (0.99% with TLR agonists vs 0.68% with Alum), while lower IFN-γ production was observed.

TNF-α plays several biological functions during the immune response to vaccines, including activation of the vascular endothelium with increase of vascular permeability, stimulation of immature DC and NK cells to secrete large amounts of IFN-γ, activation of macrophages and killing of some target cells, and so on (*Visser, 2011*).

Thus, the preliminary results of this study demonstrate the importance of proper T cell stimulation in old subjects. In particular, they highlight the efficient effects of an appropriately selected combination of TLR agonists in stimulating both primary and secondary responses by an ageing immune system.

This lays the foundation for further future studies to expand the sample size and point towards the potential use of this selected combination of TLR + CD40-L agonists in future vaccination approaches for older adults.

Collectively, this study confirms the importance of an integrated view of immunosenescence that includes the age-related aspect in the strict sense and the contribution of chronic viral infections.

The analysis of the KIR/HLA genotype in HIV-positive individuals confirmed the importance of genetic background in the outcome of viral infections. Given the current global pandemic crisis, could be important to investigate the contribution of the KIR/HLA repertoire on the outcome of COVID-19 infection.

The analysis of the immunophenotype of the Sicilian older has highlighted the importance of using a correct model of successful ageing which, given the increase in the global number of centenarians, should be represented by supercentenarians. The immune system of such extremely longevous individuals who have successfully survived the daily battle against environmental insults could provide important information on the determinants of longevity.

This study further shows that, although the response to vaccination is reduced with ageing, a better naïve and secondary response was achieved triggering of TLRs in older. These preliminary data provide a first promising insight into the possibility of incorporation of TLR agonists in vaccine formulations to increase their efficiency in aged individuals. However, further experiments will be necessary to increase the sample size. One of my future objectives, indeed, is to proceed with new experiments in KCL, including an overnight protocol in order to analyse the response of DCs to TLR agonists.

5. References

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Supplementary Table 1.

	Young, N=5 **mean ±SD**	**Older, N=6** **mean ±SD**	**Semi-supercentenarian N=2** **mean ±SD**	***p*-value summary**	***p*-value multiple comparisons**
Monocyte count (10^3/µL)	0.47±0.13	0.55±0.19	0.71±0.31	0.3679	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
Monocyte (%)	7.36±1.36	7.1±2.78	9.0±1.41	0.5806	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
Lymphocyte count (10^3/µL)	2.33±0.25	2.11±0.54	2.14±0.03	0.6812	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
Lymphocyte (%)	33.20±8.04	27.58±8.54	31.35±10.25	0.5639	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD3^+$ (%)	64.50±7.06	67.62±13.51	67.85±12.52	0.886	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD3^+CD4^+$ (%)	37.76±8.92	40.72±7.72	32.75±0.35	0.4781	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD3^+CD4^+$ count (10^3/µL)	0.88±0.23	0.87±0.28	0.70±0.003	0.6756	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD4^+$ $PD1^+$	19.60±6.41	30±13.54	58.20±21.50	0.0131	**C vs. O** = 0.046 **C vs. Y** = 0.010 **O vs. Y** = ns
$CD4^+$ Naïve (%)	42.88±11.44	35.92±9.45	17.75±8.83	0.0449	**C vs. O** = ns **C vs. Y** = 0.036 **O vs. Y** = ns
$CD4^+$ CM (%)	36.74±18	47.13±3.11	40.15±4.45	0.3665	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD4^+$ EM (%)	18.96±11.22	14.15±7.05	24.55±6.43	0.3648	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD4^+$ TE (%)	1.41±1.23	2.84±2.95	17.55±2.05	<0.0001	**C vs. O** <0.0001 **C vs. Y** <0.0001 **O vs. Y** = ns
$CD3^+CD8^+$ (%)	24.02±5.25	23.50±12.17	31.75±10.96	0.5863	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD3^+CD8^+$ count (10^3/µL)	0.56±0.12	0.53±0.39	0.68±0.24	0.8396	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD8^+$ $PD1^+$	21.64±8.11	30.57±17.30	19.05±15.63	0.4857	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD8^+$ Naïve (%)	46.28±28.77	27.52±13.86	17±11.31	0.2166	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD8^+$ CM (%)	9.78±5.69	24.53±14.29	16.65±9.40	0.1404	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD8^+$ EM (%)	17.38±10.40	14.08±11.85	6.15±0.21	0.4785	**C vs. O** = ns

$CD8^+$ TE (%)	26.96±18.42	32.03±19.73	60.20±20.93	0.1622	**C vs. Y** = ns **O vs. Y** = ns **C vs. O** = ns
$CD3^-CD56^+$ NK cell (%)	11.40±3.62	14.68±5.74	28.50±13.44	0.0263	**C vs. Y** = ns **O vs. Y** = ns **C vs. O** = ns **C vs. Y** = 0.022
NKT cells (%)	2.02±4.51	3.18±3.68	0	0.6086	**O vs. Y** = ns **C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
$CD3^-CD19^+$ B cell (%)	11.98±5.41	11.75±9.68	3.45±0.63	0.3951	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns
γδ T cells (%)	4.19±1.76	3.25±3.26	3.60±2.97	0.8522	**C vs. O** = ns **C vs. Y** = ns **O vs. Y** = ns

C=semi-supercentenarian; O=older; Y=young; SD=standard deviation. Absolute numbers and cellular percentages were compared using Tukey's multiple comparison test. Statistical analysis was performed with GraphPad Prism, version 8.1.2 (GraphPad Software). For each statistical analysis, only *p* values under 0.05 were considered significant. See the text for the other acronyms.