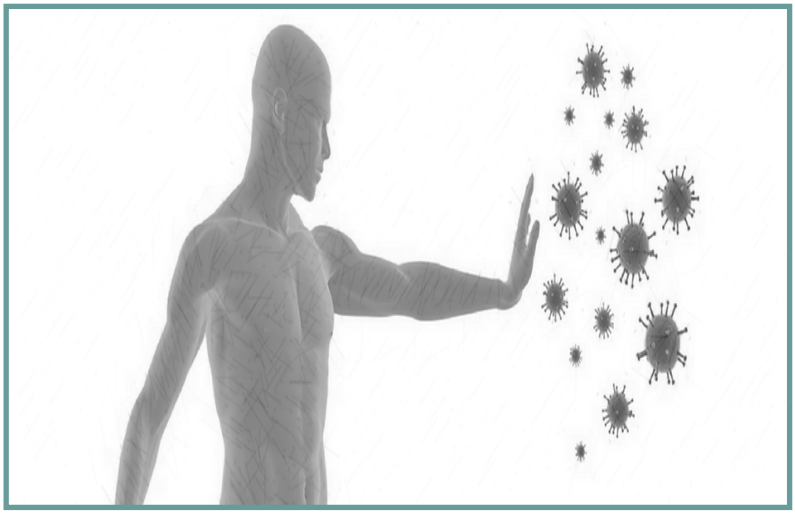
UNIVERSITÀ DEGLI STUDI DI PALERMO

Molecular approaches in autoimmunity and ageing: potential implications for future therapies



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MOLECULAR APPROACHES IN AUTOIMMUNITY AND AGEING: POTENTIAL IMPLICATIONS FOR FUTURE THERAPIES

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Abstract of papers produced during PhD course

1. **C.M. Gambino**, D. Di Bona, A. Aiello, C. Carru, G. Duro, G. Guggino, A. Ferrante, A. Zinellu, C. Caruso, G. Candore , G. Accardi. *HLA-C1 ligands are associated with increased susceptibility to systemic lupus erythematosus* (Manuscript in press).

Abstract

Recently, the role of killer cell immunoglobulin-like receptor (KIR) in autoimmune diseases has received increasing attention. The present study was undertaken to determine the association of KIR genes and the human leukocytes antigen (HLA) ligands with Systemic Lupus Erythematosus (SLE) and accompanying oxidative stress. Presence or absence of 17 KIR and 5 HLA loci was performed using the polymerase chain reaction-sequence specific primer (PCR-SSP) method by case-control study. A total of 45 SLE patients, and 60 healthy controls, all of Sicilian descent, were enrolled. Plasma values of the anti-oxidant molecule Taurine were determined in all subjects by capillary electrophoresis UV detection.

The carrier frequency of the KIR2DS2 gene was significantly increased in SLE patients compared to healthy controls (73.3 versus 45.0%; OR=3.36; 95% CI=1.46–7.74; p=0.005) suggesting a role of KIR2DS2 gene in the susceptibility to disease. We also observed a strong positive association between the presence of HLA-C1 alleles and the disease (82.2% in SLE patients versus 41.7% in controls; OR=6.47, 95% CI=2.58–16.26; p<0.0001). Interestingly, we found that SLE patients HLA-C1 positive showed significantly decreased plasma levels of antioxidant activity marker Taurine (69.38 ± 28.49 μmol/L) compared to SLE patients HLA-C1 negative (108.37 ± 86.09 μmol/L) (p=0.03). Logistic regression analysis confirmed the effect of the HLA-C1 allele in SLE patients (OR=7.06, 95% CI= 0.07–2.19; p=0.002). In conclusion, HLA class I locus C was significantly associated with an increased risk of SLE as well as an increased oxidative stress status overall in SLE patients.

2. **C.M. Gambino**, D. Di Bona, G. Accardi, A. Aiello, C. Carru, G. Duro, B.G. Gioia, G. Guggino, C. Schinocca, A. Zinellu, C. Caruso, G. Candore. *Increased Malondialdehyde levels and Intima Media Tickness in Systemic Lupus Erythematosus: the role of uncoupling protein 2 -866G/A gene polymorphism.* (Manuscript submitted)

Abstract

Increased oxidative stress potentially leads to accelerated atherosclerosis and consequently cardiovascular diseases, the main cause of death in Systemic Lupus Erythematous (SLE). We studied the association of uncoupling protein (UCP)2 genetic variants, a gene involved in the mitochondrial production of reactive oxygen species, and oxidative stress with SLE and the presence of atherosclerosis. Genetic analysis of the UCP2 -866 G/A and UCP2 Ins/Del polymorphisms was performed in 45 SLE patients and 36 healthy controls by RFLP-PCR. Oxidation status was determined by measuring malondialdehyde (MDA) levels. Presence of subclinical atherosclerosis was investigated by evaluation of intima-media thickness using echo-colour-Doppler carotid ultrasound examination. Allelic and genotypic frequencies of the SNPs analysed were evaluated by gene count. Significant association was found between UCP2 -866A allele and susceptibility for SLE ($p = 0.001$). Higher levels of MDA were found significantly increased in SLE patients (MDA, 5.05 ± 3.36 μmol/L) compared to normal controls (MDA, 2.79 ± 0.89 μmol/L) ($p< 0.0001$). Our results suggest that -866G/A UCP2 polymorphism is associated with SLE causing increased ROS levels, that in turn result in increased MDA levels responsible of accelerated atherosclerosis.

3. G. Guggino, M. Lo Pizzo, D. Di Liberto, A. Rizzo, P. Cipriani, P. Ruscitti, G. Candore, **C.M. Gambino**, G. Sireci, F. Dieli, R. Giacomelli, G. Triolo, F. Ciccia. *Interleukin-9 over-expression and T helper 9 polarization in systemic sclerosis patients*. Clin Exp Immunol. 2017, 190(2):208-216.

Abstract

T helper 9 (Th9) cells and interleukin (IL)-9 are involved in the pathogenesis of several autoimmune diseases. The exact role of IL-9 and Th9 cells in patients with systemic sclerosis (SSc) have not yet been studied adequately. IL-9, IL-9R, transcription factor PU.1 (PU.1),

IL-4, thymic stromal lymphopoietin (TSLP) and transforming growth factor (TGF)-b expression were assessed in skin and kidney biopsies of SSc patients and healthy controls (HC) by immunohistochemistry (IHC). The cellular source of IL-9 was also analysed by confocal microscopy analysis. Peripheral IL-9-producing cells were also studied by flow cytometry. The functional relevance of IL-9 increased expression in SSc was also investigated. Our results demonstrated a strong expression of IL-9, IL-9R, IL-4, TSLP and TGF-b in skin tissues of patients with both limited and diffuse SSc**.** IL-9 expression was observed mainly in the context of skin infiltrating mononuclear cells and keratinizing squamous epithelium. IL-9 overexpression was also observed in renal biopsies of patients with SSc. IL-9 producing cells in the skin were identified as Th9 cells. Similarly, Th9 cells were expanded and were the major source of IL-9 among SSc peripheral blood mononuclear cells (PBMC), their percentage being correlated directly with the modified Rodnan skin score. Infiltrating mononuclear cells, mast cells and neutrophils expressed IL-9R. In in-vitro studies stimulation with rIL-9 significantly induced NET (neutrophil extracellular traps) release by dying cells (NETosis) in neutrophils, expansion of mast cells and increase of anti-systemic scleroderma 70 (Scl70) production by B cells. Our findings suggest that Th9 cells and IL-9 could be implicated in the pathogenesis of SSc.

4. **C.M. Gambino**, S. Vasto, K. Ioannou, G. Candore, C. Caruso, F. Farzaneh. *Triggering of Toll-like receptors in the elderly. A pilot study relevant for vaccination.* Proceedings of the Symposium Updates in Pathobiology: Causality and Chance in Ageing, Age-related diseases and Longevity. Edited by Accardi & Caruso. 2017; pp 79-90
   Abstract
   The impaired ability of the elderly to mount an efficient immune response after exposure to microbes or vaccines represents a major challenge in protection against pathogens in ageing. Recently studies have shown that stimulation of Toll-like receptors (TLRs), 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. Since TLR stimulation is a key regulator of the type and magnitude of the immune response, we evaluated cytokine production in dendritic cell populations upon stimula tion with two complementary TLR agonists, R848 and MPLA. Our preliminary results demonstrate that TLR activation by this combination of agonists can significantly enhance the activation of

dendritic cells in the peripheral blood isolated from healthy elderly donors. This data suggest that the inclusion of appropriate combination of TLR agonists may enhance the efficacy of vaccination in the elderly.

5. Aiello, G. Accardi, G. Candore, **C.M. Gambino**, C. Caruso, D. Di Bona. *The importance of the interactions between KIRs and HLA ligands in the development of human autoimmune and viral diseases*. Proceedings of the Symposium Updates in Pathobiology: Causality and Chance in Ageing, Age-related diseases and Longevity. Edited by Accardi & Caruso. 2017; pp 91-110

   Abstract

   Killer immunoglobulin-like receptors (KIRs) regulate the activation of natural killer cells through their interaction with human leucocyte antigens (HLA). KIR and HLA loci are highly polymorphic, and certain KIR/HLA combinations have been found to protect against viral infections or to predispose to autoimmune disorders. In particular, some activating KIR profiles may be detrimental in autoimmune pathogenesis, and specific KIR genes may be particularly aggressive in the clearance of different microorganisms, protecting individuals in the control of a given pathogen. Here we reviewed a growing body of evidence purporting the influence of KIR polymorphism and KIR-HLA interaction in the development of the main human autoimmune and viral diseases.

6. D. Di Bona, G. Accardi, A. Aiello, M. Bilancia, G. Candore, C. Colomba, C. Caruso, G. Duro, **C.M. Gambino**, L. Macchia, J.P. Pandey. Association between GM, HLA, and Killer Immunoglobulin-like Receptors and the natural course of hcmv infection: a pilot study performed in sicilian population. Immunology, 2017.

   Abstract

   Natural killer (NK) cells provide a major defence against cytomegalovirus (HCMV) infection through the interaction of their surface receptors, including the activating and inhibitory killer immunoglobulin-like receptors (KIRs), and human leukocyte antigens (HLA) class I molecules. Also GM allotypes, able to influence the NK antibody-dependent cell-mediated cytotoxicity (ADCC), appear to be involved in the immunological control of virus infections, including HCMV. In some cases, their contribution requires epistatic interaction with other genes of the immune system, such as HLA. In the present report, with the aim to gain insight into the immune mechanisms

controlling HCMV, we have studied the possible associations among humoral and NK response, and HCMV infections. In a previous study we assessed whether the KIR and HLA repertoire might influence the risk of developing symptomatic (N=60) or asymptomatic (N=60) disease after primary HCMV infection in the immunocompetent host. In the present study, the immunocompetent patients with primary symptomatic HCMV infection were genotyped for GM 3/17 and GM 23 allotypes, along with the 60 subjects with a previous asymptomatic infection as controls. Notwithstanding the presence of missing data record, advanced missing data recovery techniques were able to show that subjects carrying the GM23 allotypes, both in homo- and heterozygosity, GM17/17, HLA-C2 and Bw4T KIR-ligand groups are associated with the risk of developing symptomatic infection. Our findings on the role of both cellular and humoral immunity in the control of HCMV infection should be of value in guiding efforts to reduce HCMV-associated health complications in the elderly, including immunosenescence, and in transplantation.

7. **C.M. Gambino,** G. Accardi, A. Aiello, G. Candore, G. Dara-Guccione, M. Mirisola, A. Procopio, G. Taormina, C. Caruso. *Effect of extra virgin olive oil and table olives on the immune-inflammatory responses: potential clinical applications*. Endocr Metab Immune Disord Drug Targets. 2017

Abstract

Extra virgin olive oil (EVOO) is the common element between the Mediterranean countries. It can be considered a nutraceutical and functional food, thanks to its bioactive compounds. It can act and modulate different processes linked to ageing and age-related diseases related to a common chronic low grade inflammation. Depending on the cultivar, the growth conditions, the period of harvesting, the productive process and time of product storage, EVOO could contain different amount of vegetal components. Of course, the same is for table olives. Many studies, on model organisms, cell culture and humans demonstrated the effect of specific molecules obtained from EVOO on the modulation of specific cytokines and anti-oxidants. The aim of our review is to summarize the effects of EVOO and table olives on the immune-mediated inflammatory response, focusing our attention on human studies and speculating the potential application of specific components as therapeutic adjuvant, supplements or drugs. Moreover, we will highlight the importance of the daily consumption of both EVOO and table olives in the context of a Mediterranean

dietary pattern and the different action on immune-inflammatory biomarkers, depending on the olive tree cultivar.

8. A. Aiello, D. Di Bona, G. Candore, C. Carru, A. Zinellu, G. Di Miceli, A. Nicosia, **C.M. Gambino**, P. Ruisi, C. Caruso, S. Vasto, G. Accardi G. *Targeting Aging with Functional Food: Pasta with Opuntia Single-Arm Pilot Study.* Rejuvenation Res. 2017

   Abstract

   Interventions to extend life span represent the new perspective in aging investigation. Healthy dietary habits are important modifiable factors that can favor a healthy aging phenotype. Many studies have demonstrated benefits for metabolic syndrome and type 2 diabetes mellitus resulting from the traditional Mediterranean foods. Opuntia Ficus Indica (OFI), widespread in the Mediterranean basin, belongs to the Cactaceae family. It is known for its antioxidant and anti-inflammatory properties. Moreover, products containing extracts from OFI fruits or cladodes have been used to control obesity and other metabolic parameters, such as glycemia and lipid profile. The aim of this study was to analyze the antioxidant and anti-inflammatory effect of pasta with 3% of OFI cladode extracts added to show its beneficial effect in human health. We performed a single arm longitudinal intervention study in 42 healthy volunteers, administrating 500 g/week of this functional pasta for 30 days. Our pasta had antioxidant and anti-inflammatory properties with putative effect on the aging process and related metabolic diseases. We also demonstrated a hypoglycemic effect. The results are preliminary, but it is possible to speculate that our pasta could be considered an effective food for the prevention of age-related metabolic disorders.

9. Aiello, G. Accardi G., Candore, **C.M. Gambino**, M. Mirisola, G. Taormina, C. Virruso, C. Caruso. *Nutrient sensing pathways as therapeutic targets for healthy ageing.* Expert Opin Ther Targets. 2017.

   Abstract

   In the present paper, the authors have discussed anti-aging strategies which aim to slow the aging process and to delay the onset of age-related diseases, focusing on nutrient sensing pathways (NSPs) as therapeutic targets. Indeed, several studies have already demonstrated that both in animal models and humans, dietary interventions might have a positive impact on the aging process through the modulation of these pathways. Areas covered: Achieving healthy aging is the main challenge of the twenty-first century because lifespan is increasing, but

not in tandem with good health. The authors have illustrated different approaches that can act on NSPs, modulating the rate of the aging process. Expert opinion: Humanity's lasting dream is to reverse or, at least, postpone aging. In recent years, increasing attention has been devoted to anti-aging therapies. The subject is very popular among the general public, whose imagination runs wild with all the possible tools to delay aging and to gain immortality. Some approaches discussed in the present review should be able to substantially slow down the aging process, extending our productive, youthful lives, without frailty.

10. G. Accardi, A. Aiello, **C.M. Gambino**, C. Virruso, C. Caruso, G. Candore. *Mediterranean nutraceutical foods: Strategy to improve vascular ageing.* Mech Ageing Dev. 2016.

Abstract

Ageing is characterized by a decline in all systemic functions. A greater susceptibility to apoptosis and senescence may contribute to proliferative and functional impairment of endothelial progenitor cells. They play an important role in neo-angiogenesis and endothelial repair. Vascular ageing is associated with changes in the structure and functions of vessels' wall. There are many possible causes of this damage. For sure, inflammation and oxidative stress play a fundamental role in the pathogenesis of endothelial dysfunction, commonly attributed to a reduced availability of nitric oxide. Inflammageing, the chronic low-grade inflammation that characterizes elderly people, aggravates vascular pathology and provokes atherosclerosis, the major cardiovascular disease. Nutraceutical and molecular biology represent new insights in this field. In fact, the first could represent a possible treatment in the prevention or delay of vascular ageing; the second could offer new possible targets for potential therapeutic interventions. In this review, we pay attention on the causes of vascular ageing and on the effects of nutraceuticals on it.

11. G. Accardi, A. Aiello, V. Gargano, **C.M. Gambino**, S. Caracappa, S. Marineo, G. Vesco, C. Carru, A. Zinellu, M. Zarcone, C. Caruso, G. Candore. *Nutraceutical effects of table green olives: a pilot study with Nocellara del Belice olives*. Immun Ageing. 2016

Abstract

The aim of this study was to analyse the nutraceutical properties of table green olives Nocellara del Belice, a traditional Mediterranean

food. The Mediterranean Diet has as key elements olives and extra virgin olive oil, common to all Mediterranean countries. Olive oil is the main source of fat and can modulate oxidative stress and inflammation, whereas little is known about the role of olives. Moreover, emerging evidences underline the association between gut microbiota and food as the basis of many phenomena that affect health and delay or avoid the onset of some age-related chronic diseases. In order to show if table green olives have nutraceutical properties and/or probiotic effect, we performed a nutritional intervention, administering to 25 healthy subjects (mean age 38,3), 12 table green olives/day for 30 days. We carried out anthropometric, biochemical, oxidative stress and cytokines analyses at the beginning of the study and at the end. Moreover, we also collected fecal samples to investigate about the possible variation of concentration of Lactobacilli, after the olives consumption.

Our results showed a significant variation of one molecule related to oxidative stress, malondialdehyde, confirming that Nocellara del Belice green olives could have an anti-oxidant effect. In addition, the level of interleukin-6 decreased significantly, demonstrating how this food could be able to modulate the inflammatory response. Moreover, it is noteworthy the reduction of fat mass with an increase of muscle mass, suggesting a possible effect on long time assumption of table olives on body mass variation. No statistically significant differences were observed in the amount of Lactobacilli, although a trend towards an increased concentration of them at the end of the intervention could be related to the nutraceutical effects of olives.

These preliminary results suggest a possible nutraceutical effect of daily consumption of green table olives Nocellara del Belice. To best of our knowledge, this is the first study performed to assess nutraceutical properties of this food. Of course, it is necessary to verify the data in a larger sample of individuals to confirm their role as nutraceuticals.

List of Abbreviation

ATP	Adenosine triphosphate
AID	Autoimmune disease
ANA	Antinuclear antibody
APC	Antigen presenting cells
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated antigen
DC	Dendritic cell
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IFN	Interferon
KIR	Killer immunoglobulin-like receptor
Kb	Kilobase
MHC	Major histocompatibility complex
MDA	Malondialdehyde
mDC	Myeloid DC
MS	Multiple sclerosis
NK	Natural Killer
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed death-1
PRR	Pattern recognition receptor
pDC	Plasmacytoid DC
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SSc	Systemic sclerosis
SLE	Systemic lupus erythematosus
TH	T helper
T1DM	Type 1 diabetes mellitus

TLR Toll-like receptor

TNF Tumor necrosis factor

Chapter 1

INTRODUCTION

1.1 Immunosenescence and autoimmune diseases: an overview

Ageing is recognized as an important factor in the development of autoimmune diseases (Hasler P et., 2005).

With advancing age, the immune system undergoes a complex series of remodeling events involving almost all compartments – both the innate and the adaptive system, and gradually declines. This condition, noted as immunosenescence, is characterized by a dysfucntion in immune response and an increase in the inflammatory background. Overall, this phenomenon is noted as "inflammaging", a term coined by Franceschi (De la Fuente et al., 2009; Franceschi et al., 2007; Ramos-Casals et al., 2003). There is also an increased reactivity towards self or endogenous antigens resulting in a greater production and release of autoantibodies. Consequently, individuals have more propensity towards developing autoimmune disorders with age. Interestingly, autoimmunity may also develop in young adults by premature immunosenescence events (Colmegna et al., 2008; Valenzuela et al., 2002; Koetz et al., 2000; Wagner et al., 1998; Schmidt et al., 1996).

Autoimmune diseases (AIDs) are a heterogeneous group of human pathological conditions of more than 80 different chronic disorders affecting from 3% to 10% of the general population (https://www.niaid.nih.gov). Their pathogenesis typically involves a complex interaction among environmental and genetic factors, as we will discuss in the following subsection.

The term autoimmunity refers to a specific adaptive immune response against own cells and tissues. A frequent mistake is to consider a

disease as autoimmune only due to the fact that it involves immune reactions harming tissues or organs. Typically, most of autoimmune diseases include the presence of autoantibodies and an association to Major Histocompatibility Complex (MHC), also known as human leukocyte antigen genes (HLA) in humans (Morris et al., 2014).

Autoantibodies represent the most characteristic immunological alteration in the pathogenesis of AIDs. They can be directed against a variety of nuclear or cytoplasmic antigens. The most common are the antinuclear antibodies (ANA), a group of heterogeneous molecules targeting multiple distinct nuclear components. This acronym identifies non-organ specific immunoglobulin class A (IgA), G (IgG), and M (IgM) which may be present in different pathological conditions.

Autoantibodies are important markers for the diagnosis of AIDs and some of them may be present for many years before disease onset and thus are not merely a consequence of inflammation (Ronnelid et al., 2005; Arbuckle et al., 2003; Rantapaa-Dahlqvist et. Al, 2003).

Interestingly, presence of autoantibodies is also increased in elderly subjects, suggesting that these autoantibodies may reflect the expression of a damaged tissue process (Candore et al., 1997).

AIDs express a wide variability in terms of age of onset, targeted tissues, and response to immunosuppressive treatments and are distinguished in organ-specific and non-organ specific or systemic, based on the localization of the disease. In organ-specific autoimmune diseases, the autoimmune responses are specifically directed against antigens present only in a specific tissue/organ and end-organ damage can be mediated by antibodies and/or T cells. The organ specific category includes Hashimoto's thyroiditis, Graves' disease, multiple sclerosis (MS), type 1 diabetes mellitus (T1DM), etc. Whereas, in systemic autoimmune

diseases, the autoimmune response targets self-antigens expressed in many organs and/or tissues, and end-organ injury is typically mediated by autoantibodies and, less commonly, T cells. Some of the more notable examples in this group are systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), primary Sjögren's syndrome and dermatomyositis.

Autoimmune diseases progressively provoke the complete tissue destruction due to the persistence of chronic inflammatory response. Immune system is subjected to constant action of costimulatory molecules, cytokines and chemokines that modulate recruitment, trafficking and *in situ* localization of activated lymphocytes (Rotondi et al., 2007). The final result is the rupture of the physiological mechanisms that control autoreactive lymphocytes, with loss of tolerance or immunological ignorance (Rotondi et al. 2007; Makay and Rosen, 2001).

1.1.1 Autoimmune diseases in this thesis

The papers in this thesis are focused on SLE and SSc, two severe and complex chronic autoimmune diseases characterized by a broad spectrum of clinical manifestation, multiple-organ involvement, and an unpredictable course of disease often leading to remarkable morbidity and mortality (Desbois and Cacoub, 2016; D'Cruz et al., 2007).

SLE is a chronic inflammatory diseases with different clinical manifestations, in which latency phases alternate with periods of extreme aggression. It is characterized by the massive production of autoantibodies directed against numerous nuclear, cytoplasmic and cell surface antigens, leading to formation of immune-complexes, whose tissue deposition causes inflammation and damage to various organs and

in particular to the connective tissue of the kidney (Yee et al., 2003). The disease shows a marked predilection for the female sex, with a female/male ratio 9:1. The onset of the disease can occurs at any age, nevertheless, young woman seem to be more susceptible to develop SLE.

SSc, also termed scleroderma, involves the connective tissue of skin and internal organs. The disease is characterized by severe and diffuse endothelial cell damage and by overproduction and accumulation of collagen and other extracellular matrix proteins, resulting in thickening of the skin and fibrosis of the affected organs. Consequently, there is a remarkable heterogeneity in the clinical features and course of the disease, leading to high morbidity and mortality.

The disease affects primarily female, with a female/male ratio varying from 3:1 to 14:1 according to different studies. It can affect people of all age, however subjects between the third and fifth decade of life are preferentially hit.

SLE and scleroderma can cross-over or have overlapping symptoms making difficult the diagnosis of disease. Both diseases impact critically on the quality life of patients and their ability to carry out daily activities due to pain, decreased physical functioning, and fatigue (Poole and Brandenstein, 2016; Poole et al., 2015; Schmeding and Schneider, 2013; Malcus Johnsson et al., 2008). Even though enormous advances in the diagnosis and the treatment of autoimmune diseases has been performed, a lot is unknown regarding which genes, pathways and processes are involved.

1.2 The immune system

The immune system is a complex network of cells, tissues, and organs that protect the body against potentially harmful disease caused by pathogens, like bacteria or viruses. It is amazingly complex and effective.

A working well immune system is able to response to external harms distinguishing between the body's own cells (self) and foreign cells (non self). The delicate equilibrium between an efficient immune reaction to non-self and the need to preserve the self from being attacked, is one the most fascinating and important action of the immune system. Any failure in this fragile balance could lead to autoimmune phenomenon and more predisposition to infectious diseases.

The processes of the immune system are extremely intricate and versatile. We will briefly described only immune aspects that are relevant for this thesis.

1.2.1 Innate and adaptive immunity

The immune system is traditionally divided into innate and adaptive. Innate immune system represents the first line of defence. It constitutes of mechanical barriers like skin and mucosa, and immune cells including macrophages, dendritic cells, neutrophils, mucosal epithelial cells. All these molecules express pattern recognition receptors (PRRs) on their cellular surface, able to recognize pathogen-associated molecular patterns (PAMPs) (Broz and Monack, 2013). Some of these cells can also function as a link with the adaptive immune system and activate the antigen specific adaptive response.

Recognition of PAMPs triggers multiple signaling pathways that lead to the activation of the inflammatory responses, secretion of cytokines by leucocytes, activation of phagocytosis, production of reactive oxygen

species (ROS) and other antimicrobial peptides. The most important class of innate PRRs is Toll-like receptors (TLRs), that will be widely discussed at paragraph 1.5.

Overall, innate immune cells remove microorganisms through several mechanisms:

- phagocytosis and intracellular killing of pathogens recognized by PRRs or complement receptors;
- cytokine and chemokine production;
- killing by Natural Killer (NK) cells, antimicrobial peptides, complement membrane attack complex;
- antigen presentation to cells of the adaptive immune system.

Innate immune response makes a crucial contribution to the activation of adaptive immunity. This last can recognize and remove those pathogens that resist the innate system.

Adaptive immune system is classically divided into humoral and cell-mediated. The humoral immunity is mediated by B lymphocytes and defends the body against extracellular microbes, whereas cell-mediated immunity is primarily directed toward intracellular microbes and is mediated mainly by T lymphocytes: T-helper (TH) cells and cytotoxic T cells (CTLs). T lymphocytes also include regulatory T (Treg) cells, which main role is to inhibit immune response and maintain tolerance.

Activated B-and T-cells are the effector cells that execute the killing response of the adaptive immune system. They act through different mechanisms. Activated B cells either produce antibodies that can bind to antigens, or survive in the immune system for long periods of time (up to years) in order to quicken the reaction of the immune system in case of exposure to the same antigen.

T cells can either enhance the immune response by cytokines production (TH cells, also known as CD4+ T cells), or destroy infected cells by perforin and granzyme as well as Interferon (IFN)-γ secretion (CTLs, also known as CD8+ T cells). TH lymphocytes differentiate to a variety effector cells, including the classical TH1 and TH2 cells and the most recently discovered TH17, TH9 and T follicular helper. TH1 cells induce cell mediated response by releasing IFN-γ that causes macrophage activation; TH2 cells secrete a pattern of cytokines that stimulate humoral immune response; while, TH9 and TH17 cells are associated with AIDs and chronic inflammation (Pan et al., 2013; Ghoreschi et al., 2011).

T cells recognize appropriate peptides only presented by MHC molecules, expressed on the surface of professional antigen presenting cells (APC), which are able to digest and process pathogen components and bind pathogen-derived peptides to MHC molecules.

Generally, all nucleated cells express on their cell surface MHC class I molecules, which can present self-derived intracellular peptides or digested from infecting virus, while MHC class II are present mainly on APCs. T cells identify MCH-peptide combination through surface molecules called CD (cluster of differentiation) molecules and get activated. T Helper cells with CD4+ molecules on its surface recognize MHC class II molecules. The interaction induces B-cells to produce antibodies, and macrophages to kill ingested microbes. T-cells having CD8+ molecules on their surface bind class I MHC molecules and become CTLs lysing other infected cells that present the antigen.

Many other cell types have an important role in immunity as dendritic cells (DCs). They are the most potent APCs and are specialized for the

uptake, processing, transport and presentation of antigens to T cells (Collin M et al, 2013). After their activation in the periphery, DCs migrate to lymphoid tissues where they interact with T and B cells to initiate and shape acquired immune responses.

DCs in human blood are defined as Lineage 1 (CD3, CD14, CD16, CD19, CD20, and CD56)-negative and HLA-DR-positive cells and can be divided into plasmacytoid DCs (pDCs), and myeloid DCs (mDCs), according to the expression of various markers. In particular, pDCs are characterized by the expression of CD123 marker and present the capacity to produce a high level of IFN-α/β.

Recent evidence observes that aberrant human pDC functions may be one of the triggering causes of autoimmune and inflammatory diseases (Takagi et al., 2016). While a remarkable decrease of pDC numbers in peripheral blood is observed in older subject, with consequent impairment of IFN-α secretion in response to virus (Canaday et al. 2010; Sridharan et al., 2010; Jing et al. 2009).

These results suggest that pDCs, may be considered as key players both in autoimmune than in ageing process, in the exact opposite way.

1.2.2 Breakdown of self-tolerance

The concept of autoimmunity appears within scientific scenario in 1900, when the immunologist Paul Ehrlich coined the term of "horror autotoxicus" to emphasize the unwillingness of the organism to endanger itself by formation of toxic autoantibodies (Silverstein, 2005). Only fifty years later, the first murine model of autoimmunity, the New Zealand black mouse, was described. Subsequently, thyroid autoantibodies were demonstrated in autoimmune thyroiditis and it was understood that failure

of recognizing and/or reacting against self-cells is the underlying cause of immune responses in AIDs (Rose and Witebsky, 1956).

The ability of the immune response to trigger a reaction against any foreign antigen, but not respond to substances normally present in the organism itself, is defined as self-tolerance. The strategy is based on the production of a large repertoire of lymphocytes with different antigen-specific receptors, and inactivation or killing of those reactive against self-antigens, without altering the maturation of reactive lymphocytes for non-self-antigens (Romagnani, 2006).

Immune system displays multiple levels of regulation to eliminate or inactivate self-reactive clones of lymphocytes. These processes can occur both during cells development and in periphery (Alpdogan and van den Brink, 2012). Classically, mechanisms of immunological tolerance are divided into central and peripheral tolerance.

Central tolerance develops in generative lymphoid organs: bone marrow for B cells and thymus for T cells, respectively. During this stage, self-reactive immature lymphocyte clones which recognize self-antigens are typically inactivated or eliminated by apoptosis. In particular, T-cells go through selective processes, where T cells with low-affinity to self-peptide-MHC complexes survive and migrate from the cortex to the medulla of thymic. This process is referred as positive selection. The remaining T cells, which receptors bind too strongly or do not even recognize MHC complexes, are destined to die through apoptosis (negative selection) (Starr et al. 2003).

B immature lymphocytes go through similar pathways. They have to be able to produce efficient membrane bound Ig-molecules to survive. When they recognize self-antigens with high affinity they will undergo a process called receptor editing, where they produce a new B-cell receptor

and if they are still self-reactive will be eliminated by apoptosis. However, maturation of T lymphocytes occurs predominantly during fetal life, when some tissues are not yet formed and some human antigens are not available in the thymus to operate the negative selection of T lymphocytes. In fact, numerous clones of mature lymphocytes can escape the negative selection process and therefore become potentially autoreactive.

Peripheral tolerance is important for maintaining tolerance towards autoantigens that are expressed in the secondary lymphoid tissues. Peripheral tolerance towards autoreactive T lymphocytes may occur through three main mechanisms (Makay and Rosen, 2001) (Figure 1):

- anergy: T cells encounter with self-antigen might lead to intrinsic functional inactivation remaining alive in a long-term hyporesponsive state. This mechanism can occur when APCs express MHC class II molecules but none costimulatory molecules (such as CD80 and CD86) on them surface (Powell JD. 2006). Another mechanism may occur when the inhibitory molecule cytotoxic T lymphocyte associated antigen (CTLA-4), present on activated T cells, bind the co-receptor of APCs subtracting it from the link with the activating molecule CD28 (Alegre et al., 2001). CTLA-4 binds to CD80 and CD86 with higher affinity than CD28. Genetic CTLA-4 deficiency shows autoimmunity and lethal lymphoproliferative disorders (Perez et al., 1997; Tivol et al., 1995).

  Another candidate for regulating anergy induction is programmed death-1 (PD-1) molecule, an immunoinhibitory receptor. Animals deficient for PD-1 or its ligand PDL1 and PD-L2 exhibit a

breakdown of peripheral tolerance and demonstrate autoimmune disorders (Keir et al., 2006; Nishimura et al., 1999);

- Clonal deletion: self-reactive lymphocytes engaged by self-MHC complexes are killed by apoptosis, a process noted as 'Activation induced cell death' (AICD) which is regulated by the Fas (CD95) signaling pathway (Brunner et al., 1995).
- Suppression: immunity responses to autoantigens are inhibited by Treg that produce transforming growth factor (TGF)-β and IL-10 cytokines, which, in turn, block the activation and the functions of the effector T lymphocytes. These cells include natural killer T cells (NKT) and T cells CD4 + CD25 + Foxp3 + (Alpdogan and van den Brink, 2012).

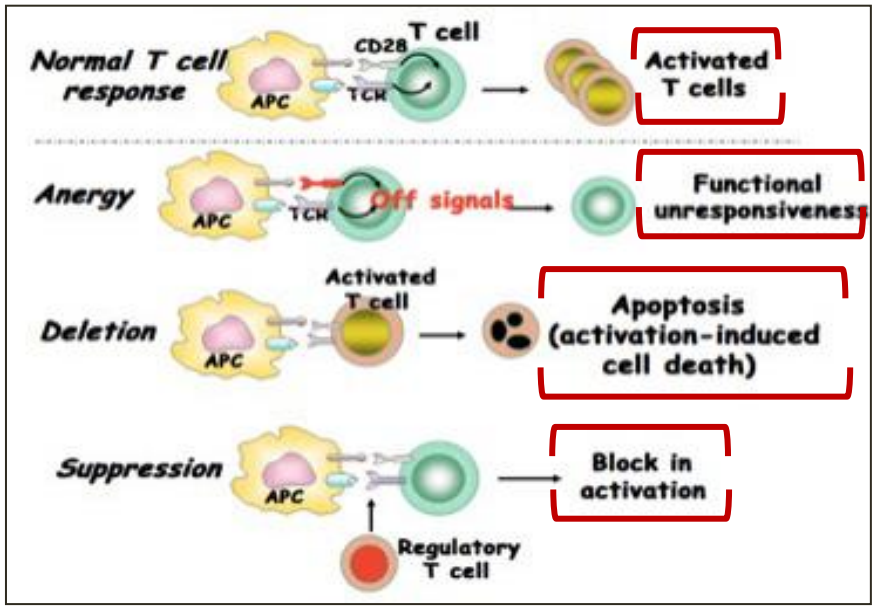

Figure 1: Mechanisms of peripheral tolerance. Mature self-reactive lymphocytes that recognize self-antigens in peripheral tissues are inactivated (anergy), killed (deletion) or suppressed.

Autoimmunity emerges when self-tolerance mechanisms fail in the predisposed individual. Breakdown of self-tolerance usually occurs as a

consecutive series of many events. Loss of tolerance towards autoantigens may be due to both an abnormal selection of autoreactive lymphocytes, that is a defect of the central tolerance, and altered presentation of autoantigens to immune system cells, that is an abnormality of peripheral tolerance. Moreover, some infectious agents can share cross-reactive antigens with self-antigens and induce an immune response that can also affects self-cells. This phenomenon is noted as molecular mimicry (Blank et al., 2007). The result is the production of autoreactive T cells, which provoke tissue cytolysis and inflammatory cytokines release. In addition, autoantibodies also mediate tissue damage through immune-complex induction, phagocytosis of antibody-tagged cells, and interfering with normal cell functions. Prolonged inflammatory responses cause further extensive organ damage.

1.2.3 Immunosenescence: changes of the immune system of older people

Immunosenescence refers to the progressively deterioration of immune function that occurs with advancing age. On the contrary, ageing in good condition seems directly correlated with a good functioning of the immune system.

The changes of immune system in elderly depend on environmental and genetic factors, but also on antigenic load which individuals are exposed during the course of life (Pawelec e Larbi, 2008; Van Baarle et al., 2005).

The most obvious changes that affect immune system during ageing are:

- thymic involution, determining the reduction of T cells production;

- lymphocyte remodeling, affecting particularly T cells compartment;
- altered cytokine production, that concerns with altered secretion of pro- and anti-inflammatory cytokines.

According to the Remodeling Theory in Ageing formulated by Franceschi, ageing is not simply a general deterioration of immune system, but a dynamic process of global restructuring that involves all subsystems, taking place during whole life span (Franceschi et al., 2000). In this remodeling vision, some mechanisms deteriorate over time, whereas others improve their performance and others still can remain essentially unchanged. Some cellular clones expand enormously while others diminish dimension, characterizing the cell repertoire of each individual.

Studies in cohorts of elderly subjects, report a gradual transition from $CD45RA^+$ naive T cells towards $CD45RO^+$ memory T cells. Thus, there is a progressive decline of virgin lymphocyte repertoire, and a progressive increase in effector and/or memory cells, especially in CTLs repertoire (Pawelec and Larbi, 2008; Pawelec et al., 2002). This phenomenon translate into reduced responsive to immune stimulation and vaccination in aged subjects, which consequently, become likely prone to develop cancer, chronic inflammatory diseases and autoimmune disorders (Fulop T et al., 2013; Reber et al.,2012).

The decline of naive T cell is the result of thymic involution in combination with ongoing differentiation of naive into memory T cells or antigen-specific effector (Appay et al., 2010). The reduction of the immunological space and decrease in virgin T lymphocyte number can be considered the main features of the immunosenescence.

Another remarkable modification of immune system with age, is the progressive accumulating age-dependent of the number of highly differentiated CD28- T cells, especially within the CD8+ T-cell subset in the elderly, associated with the disappearance of CD28+ T lymphocytes (Effros, 2000; Fagnoni et al., 1996). Clones lacking CD28 co-stimulatory molecule are not able to undergo clonal expansion.

With advancing age, it is also observed a high frequency of Treg cells and an imbalance in Treg response as well as in IL-17 production (Rosenkranz et al., 2007).

Ageing also impacts on B lymphocytes, both in percentage and absolute number (Buffa et al., 2011; Colonna Romano et al., 2003; Franceschi et al., 1995). Although the number of B cells is decreased, the serum concentration of IgG, IgA is increased. This apparent paradox could be explained by clonal expansion of memory B, due to the persistence of viral antigens.

In association with previous evidence showing defects in Treg cell activity in autoimmune disorder, Blair et al., (2010) observed that a subpopulation of B cells, called regulatory B cells ($CD19^+$ CD24hiCD38hi) involving in interleukin (IL)-10 production, loss their suppressive capacity in elderly people, producing less IL-10.

Another modification that accompanies ageing is the increase of $CD19^+CD5^+$ B lymphocytes serum levels, important producers of IgA, that also contribute to several autoimmune disease (Karim et al., 2017).

Finally, other changes that occur in immune system involve innate immunity including highly conserved processes such as chemotaxis, phagocytosis, DCs and NK activity. For example, it has been documented an age-related expansion of NK cells with high activity, probably due to

the increase body's need to cope with viral and bacterial infections (Solana et al., 2012).

Thus, with advancing age, lymphocytes undergo complex and pleiotropic alterations. These last concern both remodeling and modification of cellular function. The most dramatic changes occur in adaptive compartment and are associated with increased incidence of infectious and autoimmune diseases, as well as, cancer in elderly subjects (Caruso et al., 2004; Pawelec et al., 2000; Effros, 2001). In addition, increased production of cytokines and chemokines plays a central role in the generation of the "inflammed" milieu, typical of ageing.

1.2.4 Autoimmunity in elderly

Autoimmunity and AIDs among elderly people reflect changes of immune system function in ageing.

According to immunological theory of ageing, introduced by Roy L. Walford in 1969, immune system loses efficiency and experiences widespread dysfunction. This is evidenced by immune reactions against one's own body proteins (Diggs 2008). In particular, autoimmunity in elderly could be explained through contrasting phenomena which happened in immunosenescence. On the one hand, the decline in naive T-cells and accumulation of memory T-cells may lead to their activation against "neoantigens"; on the other hand, autoantibody production lead to tissue damage causing the development of autoimmune conditions. As mentioned above, increased levels of $CD19^{+}CD5^{+}$ B lymphocytes in the elderly population induce poly-reactive autoantibodies production leading to an imbalance of the control mechanism in the immune response against self-antigens (Bulati et al., 2011; Weksler 2000).

Nevertheless, even there is an increase in autoimmunity in the elderly, this does not always translate into an increase in AIDs. Indeed, higher prevalence of both organ- and non-organ-specific autoantibodies have been reported among healthy centenarians (people with age range 101 to 106 years) when compared with younger individuals. The main increase was observed in autoantibodies such as ANAs, anti-cardiolipin antibodies as well as anti-thyroid antibodies; suggesting that, autoimmunity in long-lived individuals may be not an autoimmune response but the result of damaged tissue process (Candore et al., 1997).

This apparent paradox may be explained by the presence of protective factors, exerting an anti-ageing effect in older subjects. For example, some studies found increased frequency of peripheral Treg cells, which play an immunosuppressive role *in vitro*, by expressing high levels of IL-10 and thus, protecting against the insurgence of autoimmune disorders (Oviedo-Orta et al., 2013; Simone et al., 2008;).

1.3 Genetic aspects

Family and twin studies have long suggested that genetic factors play an important role in autoimmunity. The first associations between MHC gene and autoimmune diseases were discovered in the 1970s and still today they are the strongest risk genetic factors for autoimmunity onset (Mulder, 1974; Grumet et al., 1971). Moreover, genome-wide association studies (GWAS) and other linkage analysis identified genes influencing susceptibility for developing of AIDs, that are located outside the HLA region. Technological advances allowed the identification of more than 200 genetic loci associated with autoimmune disorders, many of which have been associated with one or more AIDs (Diaz-Gallo et al., 2012; Cho and Gregersen, 2011). Some of the strongest risk genes for AIDs

outside the HLA region include the following genes: protein tyrosine phosphatase, non-receptor type 22 (PTPN22), nucleotide-binding oligomerization domain-containing protein 2 (NOD2); insulin, CD25, IL-23 receptor; IL-10; CTLA4, autophagy protein 16.

Nevertheless, the genetic susceptibility of AIDs is extremely variable, ranging from very high in ankylosing spondylitis to almost negligible in SSc (Selmi et al., 2012). Actually, the National Human Genome Research Institute's Catalog of Published GWAS (www.ebi.ac.uk/gwas/), updated on September 2016, indicates 4.4395 single-nucleotide polymorphism for AIDs (MacArthur et al., 2017). Nevertheless, from the genetic point of view, the underlying cause of autoimmunity is still unknown.

1.3.1 The Major Histocompatibility Complex

It has been mentioned before that the main genetic region linked to AIDs is the MHC loci (Cho and Gregersen, 2011; Rioux et al., 2009).

The MHC is a group of highly polymorphic genes, encoding for cellular antigens involved in discrimination between self and non-self and in antigen recognition by T lymphocytes. This system is present in all vertebrate with different name. In humans it is called HLA and is located on the short arm of 6 chromosome (Figure 2), where it occupies a length of about 3500 Kilobase (Kb).

HLA system consists of three regions, which, starting from the centromere, are arranged in order class II, class III and class I. The class I region, is divided into classic and non-classical HLA-I loci. Classic loci are divided into A, B and C, and codify for class I antigens having function to present processed endogenous peptides CD8 CTLs. On the

contrary, the non-classical HLA-I loci are divided into E, F and G and codify for minor proteins of unknown function. Class I molecules are expressed on all nucleated cells.

The class II region contains the locus D, subdivided into the sub-regions DP, DQ and DR, which codify for the class II molecules, and a minor locus, DM, also correlated with class II MHC. Class II proteins present exogenous peptides to CD4 TH and are expressed exclusively on B lymphocytes, macrophages, dendritic cells and Langherans cells.

The class III region, is located between the class I and II. It codifies for immunological molecules not related to HLA called class III antigens, including components of complement system, the 21-hydroxylase enzyme and protein involved in immune response as tumor necrosis factor (TNF)-α and -β.

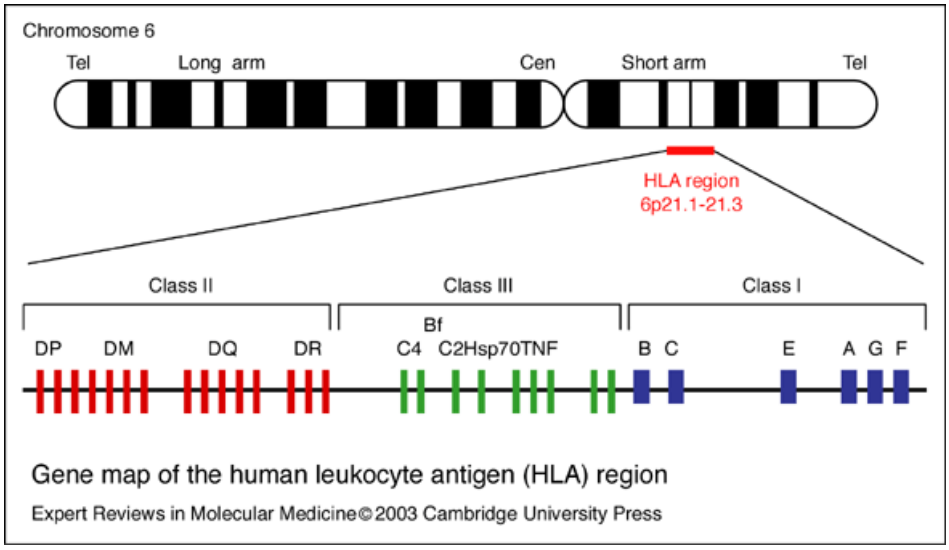

Figure 2: Localization and organization of the HLA complex on human chromosome 6. The number of different alleles is shown in this figure by the height of the bars. The figures are the numbers of HLA alleles currently officially assigned by the WHO.

The antigen binding sites show high levels of variation. This peculiarity derives from the high multi allelism of involved genes: for every gene there are many different allelic forms that, in turn, codify for as many MHC molecular variants. For this reason, it is a rare event find individuals with identical molecules within a species (Hughes and Yeager, 1998).

Over the past decades, advances in genotyping technology have determined the powerful associations between specific allelic variants within the MHC class I and/or II locus and some autoimmune diseases. Association between HLA-B8 genotype and SLE is the first direct evidence that supports the genetic contribution to disease (Grumet et al, 1971). Further studies have shown that HLA-DR2 (DRB1*15:01) and HLA-DR3 (DRB1*03:01) class II genes, may be the most consistent genetic risk factors associated with SLE in Caucasian populations, with DRB1*03:01 allele, in linkage disequilibrium with HLA-B8 (Schur et al, 1982). This contribution is predominantly at levels of autoantibodies production (Graham et al., 2007). Since then, variation within the MHC has been found to be associated with almost every autoimmune disease, as well as several inflammatory and infectious diseases. Thus, HLA typing can be considered a good tool to screen susceptibility to certain autoimmune diseases. Nevertheless, results of some HLA association studies on autoimmune disease have often produced conflicting or irreproducible results.

In Table 1 are reported the main association between systemic autoimmune diseases and HLA. Some autoimmune diseases show a preferential association with an antigen encoded by B locus, others with those of the C locus, still others with DR or DQ antigens. Moreover, some diseases are associated with different combined HLA molecules

expressed at various loci (class-I and/or class-II) rather than the result of one HLA variant only. In addition, associations as yet observed are different among ethnic groups. For example, association between some HLA-DRB1 alleles and SLE susceptibility, in several ethnic groups has been reported: DRB1*03:01 and *15:01 in European (Morris et al., 2012), *15:03 in African-American (Suggs et al., 2011), *08:02 in Hispanic and *15:01 and *15:02 in Asian populations (Reveille et al., 1998).

Of particular note is that certain haplotypes, like HLA-DRB1*03:01 and DRB1*15:01 in SLE, HLA-DRB1*04 in RA, and HLA-DRB1*03:01–DRB1*04 in T1DM, occur commonly in the general population, resulting in low relative risks for these diseases and raising more questions about the significance of their genetic contributions. Fascinatingly, Candore et al., (2007, 2006), reported that classical ancestral haplotype 8.1 (HLA-A1-B8-DR3-DQ2) may modify immune response in many way. This haplotype is most common in Caucasian and has been associated with several autoimmune diseases; authors observed that people with 8.1 carrier have decreased neutrophil chemotaxis and lower serum levels of IgG2, suggesting that ancestral haplotype 8.1 take to slower clearance of the infectious agent and hence to a lasting present of it. Thus, the persistence of infectious agents could cause an increase of autoantibodies production with higher risk of cross-reactions.

Table 1. Association between main autoimmune diseases and HLA alleles.

Autoimmune disease	HLA-DRB1 associated allele		References
	Susceptibility	Protection	
Ankylosing spondylitis	HLA-B*27	—	*Akkoc et al., 2017*
Celiac Disease	HLA-DQA1*05 HLA-DQB1*02 HLA-DQB*03:02	—	*Megiorni and Pizzut, 2012i*
Myasthenia Gravis	HLA-DRB1*03	—	*Avidan et al., 2014*
Multiple Sclerosis (MS)	HLA-DRB1*03 HLA-DRB1*08 HLA-DRB1*15	HLA-DRB1*10 HLA-DRB1*14	*Cree et al., 2014* *Silva et al., 2007*
Psoriasis or Psoriatic Arthritis	HLA-DRB107	—	*Ho et al., 2008*
Rheumatoid Arthritis (RA)	HLA-DRB1*01 HLA-DRB1*04 HLA-DRB1*10	HLA-DRB1*13	*Furukawa et al., 2014*
Systemic Lupus Erythematosus (SLE)	HLA-DRB1*03 HLA-DRB1*08 HLA-DRB1*15	HLA-DRB1*09 HLA-DRB1*13	*Morris et al., 2012* *Furukawa et al., 2014*
Systemic Sclerosis (SSc)	HLA-DRB1*01 HLA-DRB1*08 HLA-DRB1*11	HLA-DRB1*07 HLA-DRB1*15	*Flam et al., 2015* *Gladman et al., 2005*

1.3.3 KIR and KIR ligands

Combinations of HLA and Killer immunoglobulin-like receptor (KIR) genes have been associated in several autoimmune inflammatory disorders. The pathogenic mechanism, involves an incessant interaction between inhibition and activation signals, through various KIR/HLA genotypes (Kulkarni et al., 2008).

KIRs are surface receptors expressed by NK cells and a subset of CD8 T lymphocytes, binding specifically defined alleles of HLA class I.

The KIR gene cluster consists of a segment of about 150 Kb situated on chromosome 19q13.4 within the leukocyte receptor complex. KIR genes are extremely polymorphic, determining functional diversity, and generating variable susceptibility in response to pathogens and other diseases. To date, 15 KIR genes and 2 pseudogenes have been described. According to their function, they can be divided into activating and inhibitory KIRs. The activating KIRs stimulate NK/CD8 T lymphocytes cytokine secretion and cytolysis of target cell, generally, in response to microorganisms and tumor cells. On the contrary, the inhibitory KIR family prevent NK cells activation. Not all inhibitory or activating receptors are present on the surface of NK or CD8 T cells; the balance of these signals modulates NK cells cytotoxicity and cytokine release.

KIR genes are organized in two basic haplotypes that have been defined on the basis of gene content, and are termed A and B (Figure 3). The A haplotype is uniform 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. Interestingly, many A haplotypes possess null variants of both KIR2DS4 and KIR2DL4 that are not

expressed on the cell surface (Hsu et al., 2002; Witt et al., 2000). Accordingly with this, these haplotypes do not have functional activating KIR. The B haplotypes contain variable numbers of activating and inhibitory receptors and are the primary contributors to the extraordinary differences in gene profiles observed in distinct ethnic populations.

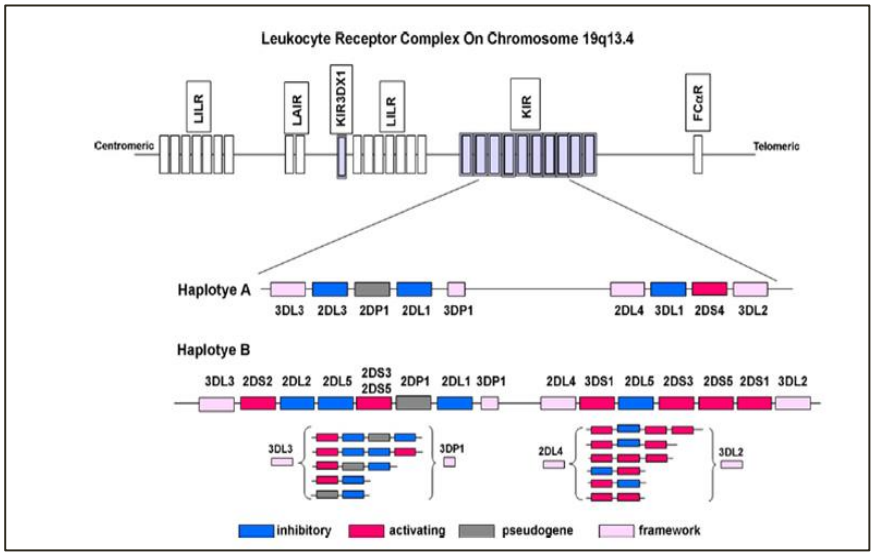

Figure 3: KIRs genetic organization. KIR genes are encoded within a 150 Kb stretch of the 1 Mb long extended LRC on chromosome 19. A and B KIR haplotypes are shown in the figure, where pink boxes indicate framework genes, green boxes pseudogenes, blu boxes indicate inhibitory KIR and purple boxes represent activating KIR genes.

The diversity of KIR haplotypes, which likely imparts a continuum from relatively strong inhibition to strong activation, suggests the pleiotropic nature of KIR on different diseases. In fact, some KIR genotype may confer protection against one disease and, at the same time, predispose to another unrelated disorder.

In this regard, it is imaginable that activating KIR profiles may be detrimental in autoimmune pathogenesis, potentially aggravating the disease process, although, this may be true for only certain autoimmune

diseases and quite the opposite for others (Baxter and Smyth, 2002; Flodstromet et al, 2002). In accordance with this hypothesis, a growing number of studies report that KIR expressed on NK cells may play an important role in autoimmune disorders.

The first association between KIR genotype and AIDs, was observed in RA. Patients with RA display expanded CD4 T lymphocytes responsible of tissue damage. Interestingly, these cells show activating KIR2DS2 in the absence of inhibitory KIR2DL2 (Namekawa et al, 2000). Further, frequency of KIR2DS2 was increased in RA patients with vasculitis in comparison to normal controls and RA patients without vasculitis. HLA-Cw*03, an HLA-C1 allotype, and therefore a putative ligand for KIR2DS2, was also increased in subjects with vasculitis, although this was not true for other C1 alleles (Yen et al, 2001). Thus, it is possible that KIR2DS2 recognizes a specific HLA-Cw*03-peptide complex generated during RA vasculitis.

Activating B haplotypes of KIR (Suzuki Y et al, 2004) and KIR2DS1 alone (Luszczek et al, 2004) or in combination with HLA-Cw6 (a C2 ligand for KIR2DS1) have been reported to associate with psoriasis (Holm et al, 2005). Based on the data, a model was proposed in which a gradient of more activating to more inhibitory compound genotypes of KIR2DS and HLA-C appear to influence susceptibility to psoriatic arthritis. So, genotypes conferring highest activation (KIR2DS1 and/or KIR2DS2 with either HLA-C1 or C2 homozygosity) are associated with greatest susceptibility, whereas 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) were protective. Other association between KIR gene polymorphisms and AIDs

has been investigated by many case-control studies, especially for SLE. However, current results are inconsistent and population specific.

Table 2 indicates KIR-HLA association with AIDs actually known.

Table 2 reports the main association between KIR-and autoimmune diseases.

Autoimmune disease	KIR-HLA associated allele		References
	Susceptibility	Protection	
Systemic Lupus Erythematosus (SLE)	KIR2DS1 KIR2DS2 KIR2DS5	KIR2DL5	*Pellet et al.,2007* *Kimoto et al., 2010*
Systemic Sclerosis (SSc)	KIR2DS2/KIR2DL2(-) KIR2DS3	—	*Romano E et al, 2011* *Tozkir JD et al, 2016*
Rheumatoid Arthritis (RA)	KIR2DS2/HLA-Cw*03	—	*Yen JH et al, 2001*
Multiple Sclerosis (MS)	KIR2DS1 KIR2DL5 KIR3DS1/HLA-Bw*04	KIR3DL1/HLA-Bw*04	*Garcia-Leon et al., 2011*
type I diabetes	HLA-DRB1*03 HLA-DRB1*04	HLA-DRB1*07 HLA-DRB1*11	*Singh et al., 2016*
Psoriasis or Psoriatic Arthritis	KIR2DS1/HLA-Cw*06 KIR2DS1 KIR2DL5 KIR haplotype B	—	*Luszczek et al., 2004* *Suzuki et al., 2004*

1.4 Oxidative stress

The term oxidative stress indicates the alterations that occur in tissues, and cells when these are exposed to an excess of oxidizing agents (Sies, 1991; Sies et al., 1985).

All aerobic organisms display a delicate balance between the production of oxidizing agents, as reactive oxygen species (ROS), and antioxidant defense, which prevent and/or repair damage.

ROS are molecules having a single unpaired electron in their outermost shell of electrons and this feature makes them highly reactive. They are continuously produced by our body through numerous biochemical processes, including mitochondrial respiration, and metabolism of foreign compounds.

ROS may play a beneficial or deleterious role, depending on their own concentration and localization. The positive effects of ROS are observed at low or moderate concentration, during protective mechanisms against infectious agents and cellular signal transduction. On the contrary, harmful effects are observed when there is an overproduction of ROS or a deficiency of antioxidant systems.

So, in a normal cellular environment, ROS are essential for life, but in case of excessive production they could become deleterious, due their oxidizing activity on lipids, proteins, and nucleic acids (Cornelli et al., 2000). The result of oxidative reactions is the production of several molecules of remarkable clinical importance, as they appear to be good indicators of oxidative state, as malondialdehyde (MDA).

Nowadays, it is widely accepted that oxidative stress plays a significant role not only in accelerating the physiological process of ageing (Jha et al., 2009) but also in the pathogenesis of numerous autoimmune diseases by enhancing the inflammation, inducing apoptotic cell death, and

breaking down the immunological tolerance (Shah et al., 2014; Ortiz et al., 2013). Conventionally, patients with AIDs exhibit an excessive oxidative stress or a defective antioxidant defence. Recently, oxidative stress has been proposed as a contributory mechanism of autoimmune skin diseases. Data confirm that oxidative stress is related to disease activity and that HLA allele status has a significant influence on it (Shah et al., 2016). In addition, increased lipid and protein oxidation and lowered anti-oxidant defenses have been associated with severity of illness in SLE patients (Scavuzzi et al., 2017).

1.4.1 The uncoupling protein family

Uncoupling proteins (UCPs) are mitochondrial anion carriers, receiving increased attention as regulator molecules of ROS in the last years.

UCP family contains about 40 different carriers and transporters; some of them play a key role in reduction of mitochondrial membrane potential and dissipating metabolic energy (Mailloux and Harper, 2011). Particularly, these proteins uncouple substrate oxidation from adenosine triphosphate (ATP) synthesis, releasing energy as heat and causing a lowering in ROS formation. Hence, the uncoupling of mitochondrial oxidative phosphorylation may represent the first line of defense against oxidative stress (Mailloux and Harper, 2012; Brand, 2005).

To date, five UCPs have been described in mammals, from UCP1 to UCP5.

UCP1 (4q28.31), the first identified, is expressed almost exclusively in brown adipose tissue and plays an important role in thermogenesis induced by cold and nutritional changes.

UCP2 and UCP3 share a common region on chromosome 11q13. UCP2 has been found in several tissues, including, immune cells, while UCP3 is mainly present in the skeletal muscle (Donadelli, 2014). They are involved in a number of postulated functions in energy regulation, including ROS production and control of the immune response (Arsenijevic, 2000).

Finally, UCP4 and UCP5 are mainly expressed in central nervous system and, in a lower concentration, in other tissues (Hoang, 2012).

Unlike UCP1, the physiological role of the other UCPs is not yet completely known.

Scientific evidence highlights a strong correlation between mitochondrial uncoupling and chronic inflammatory disorders involving alterations in cellular homeostasis, as well as ageing process (Rose et al.,2011; Souza et al., 2011; Avesani et al., 2008).

It is widely accepted that UCP2 controls macrophage activity and ROS production (Arsenijevic et al., 2000). Its functional involvement in immune cell response was demonstrated in a wide variety of diseases both in mice and humans.

In a murine model of autoimmune diabetes, Ucp2-Knockout mice showed increased infiltration of lymphocytes and highly inflammatory macrophages into pancreatic islets, suggesting a role of UCP2 in the adaptive response (Emre et al., 2007).

Further genetic analyses on common functional variant in the promoter of human UCP2 gene (−866G/A) reinforce the results in animal studies. The A allele is related to lower expression of UCP2 and it was associated with development of inflammatory diseases such as diabetes and/or obesity (Huriyati et al., 2016; Baturin et al., 2015; Diano and Horvath, 2012).

All these conditions share a chronic oxidative state with important consequences for health.

Mitochondrial function is crucial in these processes, as they are the principal cellular sites controlling energy metabolism and redox state.

Therefore, UCPs acting as a sensor of mitochondrial oxidative stress, constitute an important component of local feedback mechanisms generally implicated in protective activities controlling ROS production and regulating redox-sensitive cytosolic signaling pathways.

1.4.2 Lipid peroxidation

Lipids are easy target for free radicals, due to the widespread presence of intermolecular double bonds.

The lipid oxidative action proceeds by a free radical chain reaction mechanism called lipoperoxidation. Particularly, free radicals can activate the lipid peroxidation process that develops through three consequential phases: initiation, propagation, and termination.

Consequences of peroxidation depending on the lipid fraction involved. If the lipids involved are those of the plasma membrane, there will be structural alterations as loss of fluidity and membrane permeability, and functional changes, as loss of enzymatic functions, that disrupt the entire cellular metabolism (Vance et al., 2002).

Lipid peroxidation generates several highly reactive molecules, one of the main ones is MDA (Miyamoto et al., 2007; Therond et al., 2006). These by-products are harmful to other constituents present inside and outside the cell, such as nucleic acids and proteins, causing alteration of cellular function (Del Rio et al., 2005). Due to its high reactivity, MDA react with deoxyadenosine and deoxyguanine in DNA causing cellular cytotoxicity and, forming mutagenic compounds, precursors of carcinogenesis

(Marnett LJ, 1999). In addition, MDA react with lipoproteins and enzymes altering their physicochemical features as well as immunogenicity. Studies *in vivo*, reported that similar changes generate neoantigens that, in turn, could elicit an immune response leading to antibody production and formation of immune complex. So, it is reasonable to suppose that MDA may be involved in an autoimmune response through alteration of self-molecules.

1.4.3 Antioxidant defence

Antioxidants are different enzymes or molecules, able to counteract free radicals damaging action, and therefore exert a protective action on cellular integrity. They are distinguished in primary, which prevent or delay oxidation by removing or inhibiting oxidizing agent, and secondary, whose function is to stop the oxidation once started.

Antioxidant activity consists in capturing the high energy unpaired electron of the radical oxidant species, transforming themselves into a radical, but with lower oxidizing charge. Generally, an antioxidant performs its action in synergy with other antioxidants

The antioxidant defense system is regularly distributed both at extracellular and intracellular level. In the first case, it consists of a set of molecules such as albumin, bilirubin, uric acid, and various exogenous antioxidants introduced with food (ascorbate, tocopherol, polyphenols, etc.). Extracellular antioxidant defense systems are represented by thiol group, characterized by the presence of a highly reactive sulfhydryl group (-SH). An important antioxidant system is represented by taurine, a sulfur amino acid present at high concentrations in tissues exposed to elevated levels of oxidative stress. Biochemical and nutritional role of taurine has only been known in recent times. It is well accepted that taurine plays an

important antioxidant activity in the immune system, protecting cells from oxidative stress (Schaffer et al. 2009; Wang et al. 2009) Therefore, the primary role of taurine is to protect cells against oxidative injury.

Taurine has been defined as a functional nutrient, that could protect against cardiovascular events and diabetes mellitus. In fact, changes in plasma levels of taurine in biological fluids and tissues have a close relationship with onset of different diseases and it seems that an increase in taurine levels is inversely correlated with the onset of coronary diseases (DiNicolantonio et al., 2017).

1.5 Toll-like receptors: new approaches in autoimmunity and ageing

Recently, stimulation of TLRs using positive and/or negative ligands has been recognized as promising strategy both to enhance vaccine efficacy in the elderly, by activation of innate immune cells and production of inflammatory cytokines, than for the treatment of autoimmunity by modulation of IFN-α/β and IL-10 production in patients with autoimmune disorders.

Briefly, TLRs are type I transmembrane proteins able to recognize proteins, lipids, and polysaccharides of microbes or nucleic acids from intracellular pathogens, according to their expression profiles (Kawai and Akira, 2006).

To date, ten human and twelve murine have been characterized, TLR1 to TLR10 in humans, and TLR1 to TLR9, TLR11, TLR12 and TLR13 in mice, the homolog of TLR10 being a pseudogene.

TLRs are usually present on cellular surface of several immune cells, except for TLR3, TLR7, TLR9, which are localized in the endosomal

compartment (Nishiya and De Franco, 2004). Stimulation of TLRs initiates signal transduction pathways, that lead to inflammatory cytokines or interferon-regulatory factors production (Uematsu and Akira, 2007; Kawai and Akira et al., 2005). The exact response initiated by each TLR depends on the recruitment of specific adaptor protein, which contain a TIR domain (e.g., MyD88, TIRAP, TRIF, or TRAM) (Kawai and Akira, 2010). MyD88 is used by all TLRs, except by TLR3. The signal transduced culminate in NF-kB activation, leading to inflammatory cytokine production. On the other hand, TLR3 and TLR4 use TRIF to activate an alternative pathway leading induction of type I IFN and inflammatory cytokine productions (Figure 4).

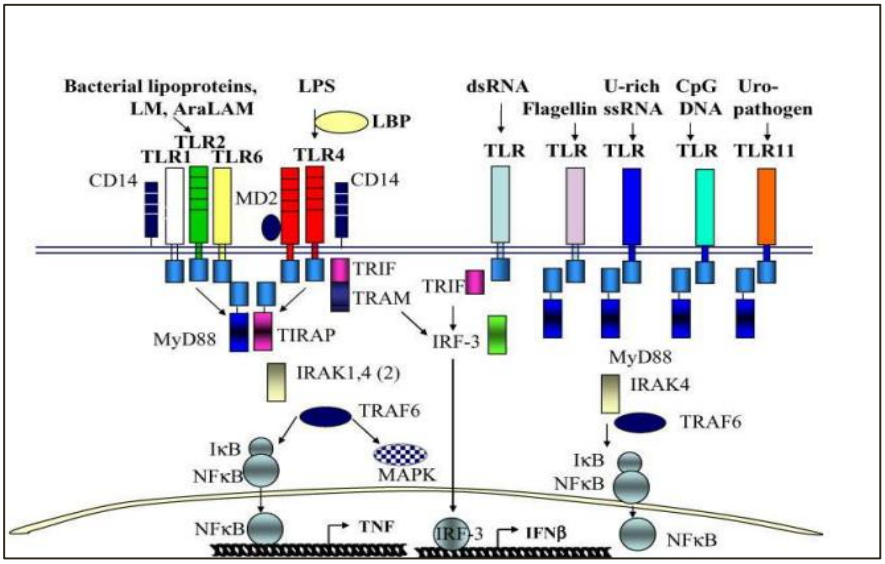

Figure 4: TLRs and their microbial ligands. Schematic representation of TLR structure and the major TLR ligands. TLR signaling is mediated through adaptor molecules such as MyD88, TIRAP, TRIF or TRAM.

Scientific evidence reported that deficiencies in human TLR signaling, lead to increased severity of several diseases, including sepsis, immunodeficiencies, atherosclerosis and asthma (Cook DN et al, 2004). On the contrary, prolonged activation of TLRs, are involved in the development of autoimmune disorders, like SLE (Duffy and O'Reilly, 2016). In this regard, positive or negative regulation of these receptors and their signaling pathways by using specific ligands, may be a promising strategy for the treatment of senescent phenotype and AIDs.

Currently, stimulation of TLRs using adjuvants has been recognized as promising strategy to enhance vaccine efficacy in the elderly through production of inflammatory cytokines by stimulated APCs, as demonstrated in animal models (Tye et al, 2015).

TLR agonists are actually evaluated as potential adjuvants for vaccine development (Mbow et al., 2010). Adjuvants are molecules that stimulate the non-specific, innate immune response, inducing the recruitment and activation of APCs at the site of vaccination. Using TLR agonists as adjuvants in vaccines for elderly has provided promising results. Studies on murine models, stimulated with TLR agonists like CpG (TLR9), poly(I:C) (TLR3) or pam3CSK4 (TLR1/2), showed a functional antigen-specific T cell responses to vaccination, both in young than aged mice (Tye et al., 2015; Maue et al., 2009). In addition, preclinical studies in autoimmune conditions have evaluated TLR-blocking agents to down modulate their over-activation and reduce the excessive inflammation (Gao et al., 2017). Moreover, lupus-prone murine models, which overexpress TLR7 show higher production of autoantibodies and display an autoimmune phenotype; while, deletion of TLR7 reduces autoantibodies frequency and inflammatory cytokines, such as IL-6 and INF-α, improving disease symptoms (Kono et al., 2009; Lee et al., 2008).

These findings shed a new light on how pro- and anti-inflammatory immune responses can be determined in vivo by ligands acting on DCs.

Chapter 2

OUTLINE OF THE THESIS

In AIDs, particularly in SLE and SSc, early diagnosis, can be difficult to identify, because of complex and heterogeneous presentation of symptoms, and unpredictable course. In addition, the initial symptoms may include signs or symptoms common among different AIDs.
Pathogenesis of these diseases has still not clear, but a strong genetic association with disease susceptibility is well accept. HLA seem to play a central role in the susceptibility to autoimmune disease, nevertheless, other factors as oxidative stress, KIR and inflammatory cytokines have a relevant role in disease induction and progression, particularly in predisposed individuals.

The aim of this thesis was to observe molecular and genetic variants connected to inflammatory and oxidative status, typical of autoimmune disorders, in order to increase our knowledge about pathogenesis and to find possible diagnostic markers and/or therapeutic targets for improving diagnosis and treatment of these severe diseases. Indeed, for every new tiny discovery, there is a potential for understanding the pathogenesis which helps in finding biomarker profiles that define each diseases and perhaps also to more individualized treatment regimens.

As second part of this thesis, relying on recent evidence, reporting TLR stimulation as important tool to determine the magnitude and quality of the acquired immune response, we evaluated cytokine production in DCs populations after stimulation with combined molecular adjuvants, incorporating complementary TLR agonists. This mechanism may be useful in the new development of vaccine strategy both in young than in elderly. Moreover, the results may open future strategic perspective to adopt in autoimmunity. Indeed, if on the one hand triggering of TLRs may induce increased secretion of inflammatory cytokines to enhance T cell response against pathogens; on the other hand, using TLRs regulators could confer an anti-inflammatory polarizing profile to DCs that could be beneficial in patients with AIDs.

In **chapter 3** we investigate the role of KIR genes and their HLA ligands in SLE and accompanying oxidative stress. We also performed a meta-analyses of the papers that reported KIR gene frequencies in SLE patients and controls and our results, in order to validate our observations.

In **chapter 4** we study the association of UCP2 genetic variants, and oxidative stress and atherosclerosis, in patients with SLE and their age-matched healthy controls to evaluate whether any differences exists that could be related to increase oxidative stress of people genetically susceptible for autoimmunity.

In **chapter 5** we examine the systemic signs of immune-inflammatory response in SSc. We analyze the IL-9/IL-9R axis expression in the inflamed skin of SSc patients and its accompanied expansion of pathogenic Th9 cells in inflamed skin and in the peripheral blood of SSc patients.

In **chapter 6** we investigate *in vitro* the ability of combined TLRs ligands to enhance pro-inflammatory cytokines environment in the elderly and in

centenarian offspring, useful for increasing the success rate of vaccination during ageing, comparing results with those obtained by the analysis of their young controls.

Finally, in **chapter 7**, a summary and general discussion of the results are presented.

Chapter 3

HLA-C1 LIGANDS ARE ASSOCIATED WITH INCREASED SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS

(Manuscript in press)

HLA-C1 LIGANDS ARE ASSOCIATED WITH INCREASED SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS.

SHORT TITLE: KIR AND LINGANDS ASSOCIATION WITH SLE

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Key Words: HLA, KIR, NK, SLE.

Abbreviations: CI, Confidence Interval; HLA, Human Leukocyte Antigen; KIR, Killer cell Immunoglobulin-like Receptor; NK, Natural Killer; OR, odds ratios; PCR-SSP, Polymerase Chain Reaction-Specific Sequence Primer; SLE, Systemic Lupus Erythematosus;

ABSTRACT

Recently, the role of killer cell immunoglobulin-like receptor (KIR) in autoimmune diseases has received increasing attention. The present study was undertaken to determine the association of KIR genes and the human leukocytes antigen (HLA) ligands with Systemic Lupus Erythematosus (SLE) and accompanying oxidative stress. Presence or absence of 17 KIR and 5 HLA loci was performed using the polymerase chain reaction-sequence specific primer (PCR-SSP) method by case-control study. A total of 45 SLE patients, and 60 healthy controls, all of Sicilian descent, were enrolled. Plasma values of the anti-oxidant molecule Taurine were determined in all subjects by capillary electrophoresis UV detection. The carrier frequency of the KIR2DS2 gene was significantly increased in SLE patients compared to healthy controls (73.3 versus 45.0%; OR=3.36; 95% CI=1.46–7.74; p=0.005) suggesting a role of KIR2DS2 gene in the susceptibility to disease. We also observed a strong positive association between the presence of HLA-C1 ligands group and the disease (82.2% in SLE patients versus 41.7% in controls; OR=6.47, 95% CI=2.58–16.26; p<0.0001). Stepwise logistic regression analysis supported the effect of the HLA-C1 ligands in SLE patients (OR=7.06, 95% CI= 0.07–2.19; p=0.002), while the KIR genes were no longer significant. Interestingly, we found that SLE patients HLA-C1 positive showed significantly decreased plasma levels of antioxidant activity marker Taurine (69.38 ± 28.49 μmol/L) compared to SLE patients HLA-C1 negative (108.37 ± 86.09 μmol/L) (p=0.03). In conclusion, HLA-C1 ligands group was significantly associated with an increased risk of SLE as well as an increased oxidative stress status overall in SLE patients.

1. Introduction

SLE is a chronic inflammatory disease, which mostly affects young women, characterized by an overproduction of autoantibodies to nuclear antigens. Some of these autoantibody assemble in immune-complexes and affect skin, kidneys, haematological tissues, joints, and serosal membranes, causing different clinical manifestations [1,2].

SLE is considered as a multi-factorial disease with strong contributions from genetic and environmental factors. In particular, genes located in the HLA region as HLA-DRB1*03:01 (DR3 allelic group) and HLA-DRB1*15:01 (DR2 allelic group) confer most of the genetic susceptibility for the development of the disease in all Caucasian population, as previously reported [3,4]. Nevertheless, numerous genes outside the HLA region also contribute to increased genetic risk, getting more difficult to understand the aetiology of the disease [5,6]. Accordingly, a common functional variant in the promoter of human Uncoupling Protein-2 gene (−866G>A), confers susceptibility for SLE (unpublished observations).

Recently, Spada et al. [7] have suggested the possible role of NK cells in SLE pathogenesis, showing a deregulation of NK cell activity and altered cytokine production from these cells. It is well known that KIRs are crucial for NK regulation through their interaction with HLA Class I molecules [8]. According to their function, KIR can be divided into inhibitory KIR (KIR2DL1-4, KIR2DL5A, KIR2DL5B, and KIR3DL1-3) and activatory KIR (KIR2DS1-5, and KIR3DS1). KIR2DL4 is involved in both inhibitory and activatory signals. KIRs bind specifically defined alleles of HLA-C, HLA-B, or HLA-A [9].

KIR and HLA are highly polymorphic molecules, with some HLA-KIR combinations or KIR haplotypes having a propensity toward higher activation or lower levels of inhibition of NK cells, affecting immune response. Accordingly combinations of HLA alleles and KIR genes have been associated with several diseases such as infectious diseases, inflammatory disorders, cancer [10-12].

In addition, a growing number of studies report that KIR expressed on NK cells may play an important role in autoimmune disorders including SLE [13-23]. However, current results are inconsistent and population specific, as reported by a recent meta-analysis [24] and only a paper reported data on HLA ligands [18].

The aim of this study is to verify if KIR polymorphisms and their known HLA ligands influence the susceptibility or resistance to SLE in our homogeneous population. Moreover, we performed meta-analyses of the genes, which we found associated with SLE to validate our results.

2. Materials and Methods

2.1 Patients and controls

Forty-five Caucasoid Sicilian patients with SLE (40 females and 5 males), age range 21–63 years (41.46 ± 10.89), were consecutively enrolled (so due to chance the female/male ratio was slightly different from the expected) at Rheumatology Unit of the Palermo University Hospital according to the American College of Rheumatology 1997 revised criteria [25]. SLE activity was calculated at the time of blood sampling. Baseline information including age, involved organs, duration and severity of the disease, smoking, body mass index and hypertension were collected by face-to-face interviewing. The control group consisted of 60 healthy individuals, age range 22-64 years (39.25 ± 11.48) with no history of autoimmune diseases. Both patients and controls were born in West Sicily as their parents and grandparents, so our population was genetically homogenous. The suitability of SLE sample size was checked (http://ps-power-and-sample-size-calculation.software.informer.com/) on the basis of the results of a previous study on KIR and SLE [15,26]. The Ethic Committee of Palermo University Hospital approved the study protocol, conducted in accordance with the Declaration of Helsinki and its amendments. Informed consent was obtained for collection of samples from all patients and controls.

2.2 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). KIR and HLA profiles were obtained by PCR-SSP, performing 28 reactions for each individual according to the manufacturer's instructions. The KIR genotyping was performed using KIR-TYPE kit (BAG Health Care GmbH). Fourteen KIR genes (2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1) and 2 pseudogenes (2DP1, 3DP1) were investigated. For HLA class I ligands, a KIR/HLA ligands kit was used (Epitop-TYPE kit; BAG Health Care GmbH, Lich, Germany). DNA of cases and controls was genotyped for the presence of the following KIR ligands groups: HLA-C1, HLA-C2, HLA-B-Bw4-80T (threonine at position 80), HLA-B-Bw4-80I (isoleucine at position 80) and HLA-A-Bw4. KIR gene profiles were determined by the presence or absence of each KIR gene.

The HLA-Cw genotypes were determined using the commercially available HLA Class I C Locus DNA Typing Tray kit (One Lambda, Thermo Fisher Scientific Brand, California, USA). The kit is based on the PCR-SSP method. To detect the specific HLA-Cw alleles, primer mixes are already available in the kit, and PCR amplification is performed according to the manufacturer's instructions .

2.3 Taurine analysis

It was conducted in collaboration with the University of Sassari. Plasma values of the anti-oxidant molecule taurine were determined by capillary electrophoresis UV detection as previously described [27].

2.4 Meta-analysis

The primary source of studies addressing the role of KIR genes in SLE was the PUBMED database limited to English language literature. The medical subject headings used for PUBMED

search were: “systemic lupus erythematosus” or “SLE,” killer cell immunoglobulin-like receptors” or “KIR”. Last search was updated on May 31, 2017. The abstracts found were read to identify papers reporting of the frequencies in Caucasoid controls and patients of KIR genes found positive in our study. The papers were read in their entirety to assess their appropriateness for inclusion in the meta-analysis. Extraction of data was performed independently by CC, CMG and DDB who compared results and agreed on a consensus; disagreements were settled by discussion.

2.5 Statistics

The comparisons of frequencies of KIR genes and haplotypes between case and control groups were tested by contingency tables (χ^2 test) constructed to determine statistical differences of the two groups analysed. The data were tested for goodness of fit between the observed and expected genotype and haplotype values and their fit to the Hardy–Weinberg equilibrium. The magnitudes of risk associations are reported by odds ratios (OR) and confidence intervals (95% CI). Statistical analysis was performed by using Graphpad Prism Software (Sand Diego, CA, USA). The p value of = 0.05 was adopted as the significance limit. A logistic regression model was also carried out, in order to derive a reduced and easily interpretable model for predicting onset. The unpaired Welch's correction of Student’s t test was used for analysis of two nonparametric quantitative data.

For meta-analysis the data were analysed using Review Manager, version 5.1, a statistical software package for managing and analysing all aspects of a Cochrane Collaboration systematic review (The Cochrane Collaboration, Oxford, UK, 1999). The overall OR between the frequencies of genes in both cases and controls was estimated with models based on both fixed-effects and random-effects assumptions. The fixed effects model considers only within-study variability. The random effects model uses weights that include both the within-study and between-study variance. Because of the high heterogeneity between the populations of most of the studies included in this

meta-analysis, we have presented the results of random-effects models that are the most conservative ones [28]. The 95% CI of the OR was also calculated.

3. Results

3.1 Genetic analysis

To assess if genetic variants of KIR and their HLA ligands play a role in SLE we first compared the frequencies of these genes in patients with SLE and controls (Table 1). We observed a significantly higher frequency of the activating KIR2DS2 gene in patients compared to healthy controls (73.3% vs. 45.0%, p=0.005; OR=3.36, 95% CI=1.46 – 7.74) suggesting a role of KIR2DS2 gene in the susceptibility to disease. A significant trend was also observed for KIR2DL5B gene. In particular, we found higher frequency of KIR2DL5B in SLE patients compared to controls (48.9% vs. 25.0%, p=0.01; OR=2.87, 95% CI=1.25 – 6.56) (Table 1). No statistical differences were found for other KIR genes (Table 1).

Two broad haplotypes of KIR genes have been defined on the basis of gene content. The A haplotype contains a single surface activating KIR gene, KIR2DS4, which is present as a null allele in 80% of cases, and 5 inhibitory KIR genes (KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2, and KIR3DL3). In contrast, the B haplotype is characterized by variable numbers of activating and inhibitory genes [29]]. No significant differences in the frequency of the two haplotypes (haplotype B included both homo- and heterozygotes, i.e. BB or AB) were observed between the two groups (data not shown).

The combinations of HLA class I and KIR variants contribute to both innate and acquired immune responses, hence they might influence susceptibility to autoimmune diseases [30]. To this end, we analysed all known HLA class I KIR ligands. We observed a strong positive association between the presence of HLA-C1 ligands and the disease. Particularly, we found that the HLA-C1 ligands were present at the ratio of 82.2% in SLE patients versus 41.7% in controls (p<0.0001; OR=6.47, 95% CI=2.58 – 16.26) (see Table 1).

Regarding the HLA-KIR interaction, alleles of HLA-C1 group are the ligands of KIR2DL2, KIR2DL3, and KIR2DS2 receptors [8]. The combination of KIR2DL2/2DL3 genes and HLA-C1 ligands was detected significantly higher in patient group compared to controls (60.0% vs. 28.3%, p = 0.001, OR=3.79, 95 % CI=1.67 – 8.60 and 62.2% vs. 30.0 %, p = 0.001, OR=3.84, 95 % CI=1.70 – 8.70, respectively). Moreover, the presence of both KIR2DS2 and HLA-C1 ligands also exhibited a higher frequency in SLE group than KIR2DS2 separately (62.2% vs. 21.7 %, p< 0.0001, OR=5.95, 95 % CI=2.52- 14.08), as reported in Table 1 and Table 2. Similar findings were reported for the combination of KIR2DS2 gene and HLA-A-Bw4 in SLE patients compared to controls (24.4% vs. 3.3%, p= 0.002, OR=9.38, 95 % CI=1.96 – 44.89) (Table 2).

All genotypes are shown in supplementary Tables 1 and 2.

To determine which Cw allele in C1 group is involved in the susceptibility, we determined the frequencies of HLA-Cw alleles in patients and controls. No significant difference between SLE patients and healthy controls was observed (supplementary Table 3). This suggests that the increased frequency of HLA-C1 ligands in lupus is probably due to the synergic presence of all alleles belonging to this group showing the same epitope.

3.2 Meta-analysis

Due to the discordant results present in literature [13-23] we decided to perform a meta-analysis of the papers that reported the gene frequencies in patients and controls of KIR and HLA genes shown to be associated with SLE in the present report.

Three studies on the association between 2DSD2 gene and SLE were identified in Caucasoid populations by our search strategy, i.e. the studies of Akhtari et al. [18], Pellet et al., [16] and Tozkir et al., [15]. The pooled summary, including data of the present report, OR for the genotypic comparison between the patients versus the controls is 1.30 (95% CI=0.80-2.12.) with no statistical significance using the random-effects model.

Two studies on the association between 2DL5B gene and SLE were identified in Caucasoid populations by our search strategy, i.e. the studies of Akhtari et al. [18] and Tozkir et al., [15]. The

pooled summary, including data of the present report, OR for the genotypic comparison between the patients versus the controls is 1.83 (95% CI=0.67-4.96) with no statistical significance using the random-effects model.

Unfortunately, as previously stated, only a previous study has been performed on the frequencies of HLA ligands [18], so it was not possible to perform a meta-analysis.

3.3 Logistic regression

However, as a next step a logistic regression model was carried out, in order to derive a reduced and easily interpretable model for predicting the onset of the disease. By logistic regression analysis, the only significant association remained with HLA-C1 ligands (p=0.002; OR=7.06, 95% CI= 0.07 – 2.19).

3.4 Taurine and KIR/HLA

As further analysis we investigated the implication of HLA-C1 ligands in oxidative stress in SLE patients. To this end, we compared the mean values of the anti-oxidant molecule Taurine [27] according to HLA-C1 ligands, using Student's t-test with Welch correction.

Taurine is a sulphur amino acid present at high concentration in tissues exposed to elevated levels of oxidative stress. It is now accepted that taurine plays an important antioxidant activity in the immune system, protecting cells from oxidative stress [31,32].

In our study the plasma levels of antioxidant activity marker Taurine were found significantly increased in SLE patients HLA-C1 negative (108.37 ± 86.09 μmol/L) compared to SLE patients HLA-C1 positive (69.38 ± 28.49 μmol/L) (p=0.03) (Figure 1). No significant differences were observed, analysing data according to Cw alleles (data not shown).

4.Discussion

SLE is a chronic inflammatory disease, affecting mostly young women, characterized by a deregulated immune response. This determines an overproduction of autoantibodies, some of which assemble in immune-complexes and affect multiple organs, resulting in a wide spectrum of different clinical manifestations. Multiple predisposing factors including hormonal factors, environmental, and, genetics are responsible for the onset of the disease [1,2].

Our genetic association study shows that the KIR2DL5B inhibitory and KIR2DS2 activating receptor genes, as well as the KIR ligand group HLA-A-Bw4 and HLA-C1 are associated with the disease. Activating KIR2DS2 gene was found to be significantly higher in SLE patients (OR=3.36, p=0.005), suggesting a role in the susceptibility to disease, as previously reported by Toloza et al. [33] in SLE patients with vascular arterial events. Similar results have been obtained by Pedroza et al. [19] reporting activating KIR gene-based KIR2DS2+/KIR2DS5+/KIR2DS1+ profiles in patients with SLE. However, our data are different from the report by Pellet et al. [16] and Hou et al. [17] which observed, on the contrary, a significant increase in the frequency of KIR2DS1 in the absence of KIR2DS2 gene in Caucasian and Asian patients with SLE, respectively.

A significant association was also observed between KIR2DL5B gene and SLE susceptibility. KIR2DL5B is the last functional KIR identified in the human genome, for which no ligands have yet been recognized. Since its discovery, it was defined as an inhibitory "orphan" KIR receptor [34] and its function and importance in human health are poorly understood. Recently, Tozkir et al. [15] suggested that the presence of KIR2DL5B might have a role in the pathogenesis of autoimmune disorders such as systemic sclerosis and SLE. In line with these data, we found that KIR2DL5B is mainly expressed in SLE patients respect to controls (OR=2.87, p=0.01).

The results of our analysis comparing SLE to healthy controls showed that the frequency of HLA-C1 ligands group was more frequent in case (82.2%) than in control group (41.7%), suggesting a detrimental role in the pathogenesis of SLE.

Moreover, our stratification analysis on combination of KIR and their ligand suggested that the some receptor–ligand combinations were positively associated with SLE. The KIR2DL2/HLA-

C1, KIR2DL3/HLA-C1 and KIR2DS2/HLA-C1 combinations were reported to be positively associated with SLE. The study by Tozkir et al. [15] presented the combination of KIR2DS2/HLA-C1 as a strong significant risk factor in SLE (this combination existed in 30% of the controls and 53.3% of the patients, p= 0.03). Our results, in agreement with data from Tozkir et al. [15], may suggest that the activating function is probably the major factor interfering in the pathogenesis of SLE.

However, analysing pooled data with results of previous studies we failed to find correlations between KIR2DL5B and KIR2DS2 genes with susceptibility to SLE (P>0.05). As previously mentioned, only a study has been performed on the frequencies of HLA ligands [18], so it was not possible to perform a meta-analysis.

Stepwise logistic regression analysis supported the effect of the HLA-C1 ligands in SLE patients (OR=7.06, p=0.002), while the KIR genes were no longer significant.

To best of our knowledge, in addition to the strong association of SLE susceptibility with HLA class II, the present study is the first reporting significant association between this disease and the HLA class I locus C.

Some report suggests that KIR genes might play a role in disease susceptibility in autoimmune diseases such as rheumatoid arthritis through their interaction with HLA class I molecules[35]. KIR and HLA interaction is crucial for the NK cell regulation. These cells participate in the immune response and T cell activation. Recently, it has been suggested that NK not only exert cell-mediated cytotoxicity against infected or cancer cells, but are also able to promote or suppress functions of other immune cells by secretion of cytokines and chemokines [36]. Thus, it is reasonable suppose that their over activation or dysfunction might be associated with pathogenesis of autoimmune diseases.

Our results showed that KIR and, in particular, HLA-C1 ligands might to be associated with the onset of the disease. The mechanism by which HLA-C1 is involved in SLE pathogenesis is not

known. Nevertheless, we can suppose that HLA-C1 induces NK cell activation through its KIR and thereby promote the autoimmune process.

The detrimental effects of the activating HLA-C1 ligands are also confirmed by data of oxidative stress. We found that SLE patients HLA-C1 negative showed higher levels of antioxidant activity marker Taurine compared to SLE patients HLA-C1 positive.

Taurine is the most abundant sulphur amino acid involved in many fundamental biological functions such as anti-oxidative and anti-inflammatory effects [37]. Studies in animal models and in humans reported that modification in Taurine levels plays a major part in the development of chronic inflammatory and degenerative diseases as diabetes, cardiovascular diseases and ageing [38-41], nevertheless, a few studies have investigated this molecule in autoimmune diseases. However, according to a recent work, Taurine attenuates oxidative stress and alleviates cardiac failure in type I diabetic rats [42]. So, it is reasonable to suppose that it may be equally involved in oxidative stress in SLE, contributing to pathogenesis of the disease.

No significant association has been observed with the other parameters considered in the analysis (data not shown).

In conclusion, we used a genetic approach to provide the first evidence for a direct association of HLA-C1 ligands with SLE pathogenesis in Sicilian population, suggesting its possible role as a strong risk factor marker of the disease. The binding of HLA-C1 ligands to their receptors might induce NK cells activation and an increase of oxidative stress status that translate into SLE progression. Further studies on the genotyping, expression, and function of KIR receptors and their HLA ligands should be done to fully determine the role of these ligands and receptors in the onset of the disease.

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DDB, GG, and AF collected the data; CMG, CiCa and AZ performed the experiments and compiled the data for the summation and analysis. CMG, DDB, and CaCa designed the study. CMG and CaCa wrote

the paper. All authors analyzed the data, reviewed the paper, approved the final version and agreed to submit the paper.

Disclosure

The authors state that they have no disclosure to declare.

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Table 1. Comparison between KIR and HLA genes frequencies in SLE patients and healthy controls

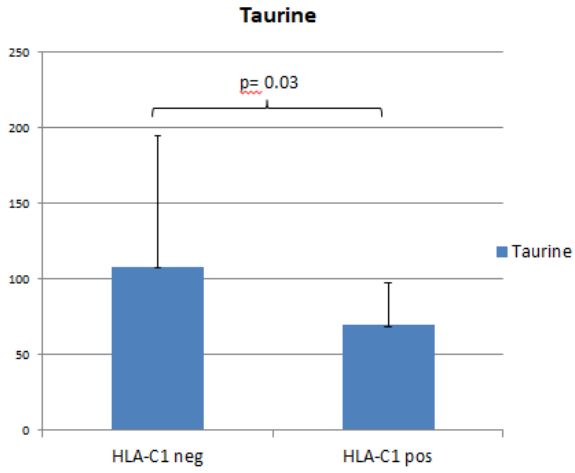
	SLE (n=45)		Control (n=60)			
KIR gene	**%**	**n**	**%**	**n**	**p**	**Odd Ratio (95% CI)**
Inhibitory						
2DL1	95.5	43	96.7	58	ns	
2DL2	71.1	32	60.0	36	ns	
2DL3	80.0	36	76.7	46	ns	
2DL4	100	45	100	60	ns	
2DL5A	44.4	20	55.0	33	ns	
2DL5B	48.9	22	25.0	15	**=0.01**	2.87 (1.25 – 6.56)
3DL1	86.7	39	93.3	56	ns	
3DL2	100	45	98.3	59	ns	
3DL3	100	45	98.3	59	ns	
Activating						
2DS1	44.4	20	48.3	29	ns	
2DS2	73.3	33	45.0	27	**=0.005**	3.36 (1.46 – 7.74)
2DS3	46.7	21	45.0	27	ns	
2DS4*001	26.7	12	45.0	27	ns	
2DS4*003-007	64.4	29	66.7	40	ns	
2DS5	42.2	19	43.3	26	ns	
3DS1	44.4	20	48.3	29	ns	
HLA gene						
HLA-A-Bw4	28.9	13	10.0	25	ns	
HLA-B-Bw4-80I	48.9	22	36.7	22	ns	
HLA-B-Bw4-80T	28.9	13	20.0	12	ns	
HLA-C1^{Asn80}	82.2	37	41.7	25	**<0.0001**	6.47 (2.58 – 16.26)
HLA-C2^{Lys80}	68.9	31	60.0	36	ns	

Table2. KIR genotypes detected in SLE patients and healthy controls

Genetic Factor	SLE n=45 (%)	Controls n=60 (%)	P	Odd Ratio (95% CI)
HLA-C1C1	14 (31.1%)	8 (13.3%)	**=0.03**	2.93 (1.10 – 7.79)
HLA-C1C2	23 (51.1%)	17 (28.3%)	**=0.025**	2.64 (1.18 – 5.95)
HLA-C2C2	8 (17.8%)	19 (31.7%)	Ns	
2DL2 + HLA-C1	27 (60.0%)	17 (28.3%)	**=0.0014**	3.79 (1.67 – 8.60)
2DL3 + HLA-C1	28 (62.2%)	18 (30.0%)	**=0.0014**	3.84 (1.70 – 8.70)
2DS2 + HLA-C1	28 (62.2%)	13 (21.7%)	**<0.0001**	5.95 (2.52- 14.08)
2DL2 + HLA-C1C1	10 (22.2%)	6 (10.0%)	ns	
2DS2 + HLAC1C1	11 (24.4%)	3 (5.0%)	**=0.007**	6.15 (1.60- 23.61)
2DL1 + HLA-C2	29 (64.4%)	35 (58.3%)	ns	
2DS1 + HLA-C2	15 (33.3%)	19 (31.7%)	ns	
2DS2 + HLA-C2	22 (48.9%)	18 (30.0%)	ns	
2DS2 + HLA-C2C2	5 (11.1%)	9 (15.0%)	ns	
HLA-B-Bw4-80T – HLA-B-Bw4-80T	9 (42.2%)	9 (15%)	ns	
HLA-B-Bw4-80T- HLA-B- Bw4-80I	18 (40.0%)	19 (31.6%)	ns	
HLA-B-Bw4-80I-HLA-B- Bw4-80I	4 (8.8%)	3 (5.0%)	ns	
3DL1 + HLA- B-Bw4-80T	12 (26.7%)	11 (18.3%)	ns	
3DL1 + HLA-B- Bw4-80I	20 (44.4%)	21 (35.0%)	ns	
3DS1 + HLA- B-Bw4-80I	8 (17.8%)	12 (20.0%)	ns	
2DS2 + HLA-A-Bw4	11 (24.4%)	2 (3.3%)	**=0.002**	9.38 (1.96 – 44.89)

LEGEND TO FIGURES

Figure 1. Analysis of Taurine levels according to the presence or absence of HLA-C1 ligands. SLE patients HLA-C1 negative exhibit significantly higher levels of plasma Taurine compared to SLE patients HLA-C1 positive (p=0.03).


Taurine
250
200
150
100
50
0
p= 0.03
Taurine
HLA-C1 neg
HLA-C1 pos

Chapter 4

INCREASED MALONDIALDEHYDE LEVELS AND INTIMA MEDIA TICKNESS IN SYSTEMIC LUPUS ERYTHEMATOSUS:THE ROLE OF UNCOUPLING PROTEIN 2 -866G/A GENE POLYMORPHISM

(Manuscript submitted)

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Increased Malondialdehyde levels and Intima Media Tickness in Systemic Lupus Erythematosus: the possible role of uncoupling protein 2 -866G/A gene polymorphism.

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Keyword:	Cardiovascular Disease, Systemic Lupus Erythematosus, Oxidative stress
Abstract:	Background: increased oxidative stress potentially leads to accelerated atherosclerosis and consequently cardiovascular diseases, the main cause of death in Systemic Lupus Erythematous (SLE). Objectives: to gain insight into these mechanism, we studied the association of uncoupling protein (UCP)2 genetic variants, a gene involved in the mitochondrial production of reactive oxygen species,and oxidative stress with SLE and the presence of atherosclerosis. Methods: genetic analysis of the UCP2 -866 G/A and UCP2 Ins/Del polymorphisms was performed in 45 SLE patients and 36 healthy controls by RFLP-PCR. Oxidation status was determined by measuring malondialdehyde (MDA) levels. Presence of subclinical atherosclerosis was investigated by evaluation of intima-media thickness using echo-colour-Doppler carotid ultrasound examination. Results: allelic and genotypic frequencies of the SNPs analysed were evaluated by gene count. Significant association was found between UCP2-866A allele and susceptibility for SLE (p=0.001). Higher levels of MDA were found significantly increased in SLE patients (MDA, 5.05±3.36 μmol/L) compared

	to normal controls (MDA, 2.79±0.89 μmol/L) (p< 0.0001). Conclusion: our results suggest that -866G/A UCP2 polymorphism is associated with SLE causing increased ROS levels, that in turn result in increased MDA levels responsible of accelerated atherosclerosis.

Increased Malondialdehyde levels and Intima Media Tickness in Systemic Lupus Erythematosus: the possible role of uncoupling protein 2 -866G/A gene polymorphism.

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Running title: MDA and IMT in SLE: role of UCP2 SNP

Background: increased oxidative stress potentially leads to accelerated atherosclerosis and consequently cardiovascular diseases, the main cause of death in Systemic Lupus Erythematous (SLE). **Objectives:** to gain insight into these mechanism, we studied the association of uncoupling protein (UCP)2 genetic variants, a gene involved in the mitochondrial production of reactive oxygen species, and oxidative stress with SLE and the presence of atherosclerosis. **Methods:** genetic analysis of the UCP2 -866 G/A and UCP2 Ins/Del polymorphisms was performed in 45 SLE patients and 36 healthy controls by RFLP-PCR. Oxidation status was determined by measuring malondialdehyde (MDA) levels. Presence of subclinical atherosclerosis was investigated by evaluation of intima-media thickness using echo-colour-Doppler carotid ultrasound examination. **Results:** allelic and genotypic frequencies of the SNPs analysed were evaluated by gene count. Significant association was found between UCP2-866A allele and susceptibility for SLE (p=0.001). Higher levels of MDA were found significantly increased in SLE patients (MDA, 5.05±3.36 μmol/L) compared to normal controls (MDA, 2.79±0.89 μmol/L) (p< 0.0001). **Conclusion:** our results suggest that -866G/A UCP2 polymorphism is associated with SLE causing increased ROS levels, that in turn result in increased MDA levels responsible of accelerated atherosclerosis.

Key words: atherosclerosis, IMT, lupus, MDA, oxidative stress

Introduction

Systemic Lupus Erythematosus (SLE) is a complex and severe autoimmune inflammatory disease of unknown aetiology, characterized by a perturbed immune response. The result is an overproduction of autoantibodies to nuclear components, some of which assemble in immune-complexes and affect multiple organs, resulting in a wide spectrum of different clinical manifestations including renal, cardiovascular, neuropsychiatric, and musculoskeletal damage[1]. Multiple predisposing factors including hormonal factors, environmental, and, genetics interact to contribute to SLE pathogenesis[2].

Gender is clearly the strongest risk factor for the disease that predominantly affects premenopausal females with an incident rate to favour women over men of 9:1 [3].

The genetic contribution was described the first time by studies of human leukocyte antigen (HLA) region. Association of certain alleles and haplotypes with SLE have been already identified, e.g. the HLA-DRB1*0301 (HLA-DR3) and HLA-DRB1*1501 (HLA-DR2) alleles confer significant risk for the development of the disease in all Caucasian populations, as stated in our previous reports [4,5].

Moreover, genome-wide linkage analysis and other association studies identified genes influencing susceptibility for developing SLE that are located outside the HLA region. At present, over forty susceptibility single nucleotide polymorphisms (SNPs) have been described, concerning different pathways[6,7]. However, current understanding of genetic risk is still complicated, indicating that other, as-of-yet unidentified genes, as well as gene-gene interaction are involved in SLE pathogenesis [8].

Over the past four decades, medical treatment and diagnostic resources have significantly improved survival in SLE patients, with life expectancy increasing from 50% in the fifties to the current 90% [9,10]. Nevertheless, morbidity and mortality in these patients is still higher that of comparable demographic groups, with an increased risk, up to 17-fold, of developing a cardiovascular disease (CVD)[11]. To note, cardiovascular disorders in SLE patients often occur

during reproductive age, the period in which women usually are protected from coronary heart disease[12]. In particular, Manzi et al. noted that SLE female aged 35-44 years have a 50-fold increased risk of myocardial infarction when compared to women without SLE participating in the Framingham Offspring study[13].

Cardiovascular events are often associated with subclinical accelerated atherosclerosis, and it cannot be fully explained by traditional cardiovascular risk factors that can be present in SLE patients as a result of disease itself or as a consequence of treatment[14,15].

Interesting, recent advances in molecular biology provide important links between pro-inflammatory genetic marker and atherosclerosis, suggesting that this pathological condition is a peculiar form of inflammation, influenced both by environmental and genetic factors[16]. So, it is reasonable to suppose that other conditions like the chronic inflammatory status as well as the increased oxidative stress observed in SLE[17] are important contributing factors to accelerated atherosclerosis, hence to increased cardiovascular morbidity and mortality rate in these patients[18]. To date, there is no effective treatment to prevent cardiovascular events in SLE. Therefore, it is essential to understand the role of oxidative stress in premature vascular damage to better identify and treat this potentially devastating complication of the disease.

Numerous studies have documented that reactive oxygen species (ROS) are key drivers of the atherosclerotic process[19,20]. The primary targets of ROS are double bonds in polyunsaturated fatty acids in the cell membrane. So, they are able to induce lipid peroxidation, which may result in more oxidative damage in SLE. One of the products of lipid peroxidation is malondialdehyde (MDA), which modifies low density lipoprotein (LDL) particles taken up in monocytes/macrophages and could play a role in atherogenesis [21,22].

Recently, Rahman et al. reported that SLE patients show low levels of IgM antibodies against MDA and that it is associated with more disease activity including CVD[23], suggesting a role of MDA and consequently of ROS in accelerated atherosclerosis in SLE. In addition, increased levels of MDA have been observed in association with many clinical features of SLE

like lupus nephritis and tissue damage[24]. However, further studies are necessary to better understand the clinical role of MDA in SLE in order to ameliorate some clinical manifestations of this inflammatory disease by targeting lipid peroxidation with dietary or pharmacological antioxidants [25,26].

One of the factors receiving in the last years increased attention as a regulator of ROS is uncoupling protein (UCP) 2. This ubiquitously inner mitochondrial membrane protein is a plausible negative regulator of ROS production, since it dissipates the electrochemical gradient that drives ATP synthesis, decreasing ATP production by the mitochondrial respiratory chain and is involved in the regulation of lipid metabolism. A common functional variant exists in the promoter of human UCP2 gene (−866G/A), with the A allele being associated with low levels of UCP2 expression and a number of cardiovascular risk factors, including LDL size and asymptomatic carotid atherosclerosis in the middle-aged women [27,28].

Taking into account the role of UCP2 in ROS regulation and its role in the protection against oxidative stress, we investigated whether -866G/A, and Ins/Del UCP2 gene polymorphisms, previously described in association with other inflammatory diseases such as diabetes and/or obesity[29-31], are also associated with SLE and their relationship with cardiovascular complications. This potentially relevant association has never been explored, although it might contribute to the understanding of the possible mechanisms linking oxidative stress to cardiovascular risk in SLE.

Materials and Methods

Participants

The study group included 45 SLE patients (40 females and 5 males),age range 21–63 years (41.46 ± 10.89), who were consecutively admitted to rheumatologic department at the University Hospital of Palermo and recruited according to the American College of Rheumatology 1997 revised criteria[32]. SLE activity was calculated at the time of blood sampling when anti-DNA and anti-ANA were performed. Baseline information including age, involved organs, duration and

severity of the disease, smoking, Body Mass Index (BMI) and hypertension were collected by face-to-face interviewing.

The control group consisted of 36 healthy individuals (34 females and 2 males), age range 22-55 years (38.19 ± 7.27) with no history of autoimmune diseases and who were negative for anti-DNA and anti-ANA assays. The patient and control groups were matched according to gender, age and race. All healthy donors were interviewed and examined for coronary artery disease. Individuals with a history of, or having a family with, hypertension, autoimmune disorders, hypercholesterolemia or CVD were excluded from the study. The study protocol, conducted in accordance with the Declaration of Helsinki and its amendments, was approved by the Ethic Committee of Palermo University Hospital.

Taking into account the central role played by MDA, in our study, the suitability of this sample size was checked (http://ps-power-and-sample-size-calculation.software.informer.com/) on the basis of the results of our previous study on MDA[26].

Samples collection

Blood samples were collected in specific blood collection tubes containing ethylenediaminetetraacetic acid for molecular and oxidative analyses and in serum tubes with no additives. Plasma and sera were separated from whole blood by low-speed centrifugation at 2,500 rpm for 15′ at 4 °C. After separation, the samples were stored at −80 °C for further assays.

SNPs Genotyping

Genomic DNA was extracted from peripheral blood leucocytes using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). SNPs were detected by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) method. DNA fragment analogous to −866 G/A polymorphism (rs659366) in UCP2 gene was amplified with the primers 5'-CACGCTGCTTCTGCCAGGAC-3' (forward) and 5'-AGGCGTCAGGAGATGGACCG-3' (reverse). Genomic DNA (100ng) in a total volume of 50 µL was used for the PCR, using the Hot Start Taq DNA polymerase (Bioline, London, UK) according to the manufacturer.

Amplification conditions consisted of an initial denaturation at 95 °C for 1 minute followed by 35 cycles of denaturation at 95 °C for 15 seconds; annealing at 65 °C for 15 seconds; extension at 72 °C for 10 seconds. The PCR products were digested by 1 µL *Mlu*I restriction enzyme (Thermo Scientific Inc., Waltham, Massachusetts, USA) as described[33]. Digestion fragments were resolved on 2% agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium Inc., CA, USA) and visualized under ultraviolet illumination.

The −866A/A genotype was indicated by a single 369 base-pair (bp) fragment as result of loss of *Mlu*I site, whereas, the wild-type −866 G/G genotype was digested into 297 and 72 bp fragments. Evaluation of the 45 bp Ins/Del polymorphism in the 3'-untranslated region (UTR) of exon 8 of the UCP2 gene was detected by PCR using primers that have been described elsewhere[34]. We used the following primers 5'-CAGTGAGGGAAGTGGGAGG-3' (forward) and 5'-GGGGCAGGACGAAGATTC-3' (reverse).

Amplification conditions consisted of an initial denaturation at 95 °C for 1 minute followed by 35 cycles of denaturation at 95 °C for 15 seconds; annealing at 60 °C for 15 seconds; extension at 72 °C for 10 seconds. The PCR products of 457 bp (Insertion allele) or 412 bp (Deletion allele) were resolved on 2% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Freemont, CA, USA) and visualized under ultraviolet light.

HLA Class II Genotyping

HLA-DR and DQ alleles were amplified using sequence specific oligonucleotide primers (PCR-SSP) following the method previously described[35]. For genotyping HLA class II region was used the Micro SSP HLA DNA Typing kit (One Lambda Inc., Canoga Park, CA). Procedure of PCR mix amplification and PCR cycling parameters was in concordance with kit manufacturer's instructions and PCR products were identified on 2.5% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc.) and visualized under ultraviolet light.

Malondialdehyde assay

This analysis was conducted in collaboration with the University of Sassari. Thiobarbituric acid reactive substances (TBARS) were determined according to the method described by Esterbauer and Cheeseman[36]. TBARS methodology measures MDA and other aldehydes produced by lipid peroxidation induced by hydroxyl free radicals. For the measurements, plasma was mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid and heated at 95 °C in thermoblock heater for 25 minutes. TBARS were determined by measuring the absorbance at 535 nm. A calibration curve was obtained using standard MDA and each curve point was subjected to the same treatment as that of the samples.

Autoantibodies test

These assays were conducted at laboratory of clinical diagnosis of autoimmunity diseases at the Department of Transfusion Medicine of University Hospital of Palermo.

The presence of antinuclear antibodies (ANA) was assessed by the HEp-2000 indirect fluorescent antibody (IFA) test system (ImmunoConcepts, Sacramento. CA, USA) in accordance with the manufacturer's instructions. Briefly, HEp-2000 cells were incubated with sera of case and control groups, diluted in phosphate buffered saline (PBS) for 30 minutes, and unbound antibody was washed away in PBS for 10 minutes. Bound antibodies were detected with FITC-labelled anti-human IgG antibody provided with the kit. We considered positive at a cut-off titre of ≥1:80. Each HEp-2000 slide was scored under a fluorescent microscope for the presence of ANA and for the pattern of ANA binding by two independent observers unaware of the clinical details of the subjects. Logarithm values of the ANA titre were used during analysis, to eliminate bias from extremely high titre results.

Autoantibodies to double-stranded DNA (anti-dsDNA)were determined using an anti-dsDNAIFA kit test system (ImmunoConcepts) in accordance with the manufacturer's instructions. Briefly, slides with *Crithidia luciliae* were incubated with sera of SLE patients diluted 1:10 in PBS for 30 minutes, and unbound antibody was washed away in PBS for 10 minutes. Bound antibodies

were detected with FITC-labelled anti-human IgG antibody provided with the kit. Each slide was scored under a fluorescent microscope for the presence or absence of anti-dsDNA antibodies.

Intima-media thickness (IMT) assessment

Of 45 SLE patients, only 34 were agreed for a complete echocardiographic evaluation of both common carotid arteries according to the American Society of Echocardiography guidelines[37]. The echocardiographic evaluations were performed with a duplex ultrasound B-mode and color-coded duplex sonography using a 10-MHz scanning frequency with subjects in the supine position with their heads turned to the opposite side of the transducer. Doppler echocardiography studies were performed by a radiologist with a high experience in echocardiography and a high inter-study reproducibility, which was unaware of the clinical data. The images were recorded into a computerized database.

IMT was defined as the distance between the media-adventitia interface and the lumen-intima interface. We measured IMT twice on each side (right and left) and a mean value of both these measurements was obtained for each side, with the highest value (right or left) considered for the analysis.

Statistical analysis

Differences of anthropometric, biochemical and genetic variables between SLE subjects and control group as well as effects of genotypes and HLA on clinical parameters were tested using Graphpad Prism Software (Sand Diego, CA, USA). Results are expressed as means ± standard deviation, or frequencies as appropriate. Logarithmic transformation was made for skewed variables as ANA titre. The unpaired Welch's correction test was used for analysis of two nonparametric quantitative data. Allele and genotype frequencies among groups were estimated by gene counting. The Fisher's exact test (2x2 table) and Chi-squares test (3x3 table) was used to detect significant changes in genetic variables between groups respectively. Standard allelic odds ratios (OR) with 95% confidence interval (CI) were calculated. Multivariate analysis was conducted by linear regression to investigate the effect of these polymorphisms on intima-media thickness. All

reported *p* values were two-sided, and *p* values lower than 0.05 has been considered to be statistically significant.

Results

Patient characteristics

The demographic features and laboratory findings of SLE patients and healthy controls are shown in Table 1. Blood glucose and triglyceride levels resulted similar between the two groups of subjects (data are not shown). No significant differences were observed in mean age, gender, BMI, and high-density lipoprotein (HDL) among groups. Lower significantly LDL levels were observed in SLE patients compared with control group ($p< 0.0001$), although the values of this last are comprised into the normal range. The majority of patients were taking hydroxychloroquine and corticosteroids at the time of blood sampling. Only few patients were taking an additional immunosuppressant such as azathioprine or mycophenolate.

Genotype and allele distributions

In order to demonstrate the role of UCP2 in the pathophysiology of SLE and to support our hypothesis that genetic factors involved in oxidative stress regulation may play a key role in SLE and its complications, we compared genotype and allele frequencies of the selected SNPs between 45 patients and 36 controls. All genotypes were in agreement with those predicted by the Hardy Weinberg equilibrium. The polymorphisms of UCP2 (rs659366 and 45 bp Ins/Del) were genotyped by PCR-RFLP in all subjects. The allele and genotype frequency distributions were present in Table 2.

Significant differences were found for the UCP2 -866G/A. Interestingly, we observed that the rs659366 UCP2 polymorphism seemingly confers susceptibility for SLE. The percentage of the protective -866 G/G UCP2 genotype has a frequency of only 6.67% (3) in cases versus 38.89% (14) controls ($p=0.001$). Moreover, -866 G/A and A/A genotypes (dominant model) were associated with increased risks of diseases as compared with G/G genotype (OR=8.9; 95% CI, 2.31-34.36;

p=0.0007). The association of the UCP2 –866 G/A polymorphism with SLE was also observed in the allele comparison, the frequency of -866A allele was higher in SLE patients (52.22%) than in control (34.72%) (OR=2.05; 95% CI, 1.08-3.89; p= 0.027) as compared with G allele.

In most studies, the minor A allele has been reported to reduce UCP2 transcriptional activity, suggesting a reduced protection against ROS production[38]. In line with this hypothesis, Oberkofler et al., showed that the -866 A allele is associated with an increased risk of asymptomatic carotid atherosclerosis in middle-aged women[28]. However, literature data have been conflicting, reporting either decreased[39] or increased[40,41] UCP2 mRNA levels associated with -866A allele.

No statistical significant differences were detected for the 45-bp Ins/Del polymorphism in the 3'-UTR of UCP2 between cases and controls. However, allelic comparison showed higher frequency of Ins allele was in SLE patients (33.33%) than in control(19.45%) (OR=2.07; 95% CI, 0.99-4.3; p=0.05) as compared with Del allele.

Various studies reported that UCP2 Ins/Del polymorphism is associated with obesity with the UCP2 Ins allele associated with increased BMI[42]. In our study, we found an association with Ins allele with SLE development. However, this significance is not confirmed by dominant model (OR=1.75; 95% CI, 0.70-4.34; p=0.078).

Stratifying the SLE patients according to these genotypes, no statistical significant differences were detected in their anthropometric and biochemical characteristics. Data concerning oxidative stress and IMT are shown below.

Comparison of MDA levels between SLE patients and healthy controls

To understand the role of oxidative stress in the progression of SLE, we measured MDA levels, marker of lipid peroxidation, in case and control groups. Higher levels of MDA were found significantly increased in SLE patients (MDA, 5.05 ± 3.36 µmol/L) compared to normal controls (MDA, 2.79 ± 0.89 µmol/L) (p< 0.0001), as shown in Table 1. Our results, similar to the previous report of Perez et al.[43] indicate that the lipid cell membrane was attacked in SLE confirming the increased oxidative stress in these patients. MDA can be considered a good marker of oxidative

stress of the disease providing a basis for designing appropriate antioxidant interventions to prevent or alleviate SLE clinical manifestations.

Rahman et al.[23] and Yoon et al.[44] reported a significantly association between oxidative stress markers and carotid artery IMT in healthy male subjects. In our study, linear correlation analysis showed moderate but significant association between MDA levels and the IMT in all SLE subjects (r=0.383, 95% CI, 0.05-0.64; p=0.02). Gender-specific comparison confirmed the significantly correlation between MDA levels and IMT in women with SLE (r=0.416, 95% CI, 0.06-0.67; p=0.02) but not in men with SLE (r= -0.647, 95% CI, -0.99-0.83; p=0.35), likely due to a few men in our sample. However, our results confirm that MDA can be considered a marker for atherosclerosis in female SLE patients.

Association of UCP2 -866 G/A gene polymorphism with oxidative stress and their implication in IMT

Given the overrepresentation of UCP2 -866A in patients and the potential protective effect of -866G allele in SLE susceptibility (OR=8.9, Table 2), we evaluated its biological effect in influencing the grade of oxidative stress. Thus, we assessed the eventual significant differences in plasma levels of MDA between patients and controls.

As reported in Table 3, when we analysed all subjects enrolled in the study for rs659366 UCP2 polymorphism, we observed significantly higher MDA levels in subjects with A allele than their G counterparts (p=0.04). This phenomenon was not significant examining only SLE patients and there was no association between genotype and MDA levels in control group.

Elevated levels of plasma oxidative stress markers associated with polymorphic variant in the promoter of UCP2 gene, the -866G/A polymorphism, have been previously reported as predictor of future risk of coronary heart disease events in a cohort of diabetic patients[45]. Thus, increased oxidative stress values associated with the UCP2 genotype may be involved in cardiovascular disorder.

To gain insight into this topics, we investigated the role of UCP2 SNPs and oxidative stress in IMT, a well-established predictor of cardiovascular disease events[46], in SLE group. Unfortunately, missing data for IMT evaluation has limited the power of this study to investigate the association of UCP2 -866G/A and Ins/Del polymorphisms with atherosclerosis development as measured by IMT thickness in SLE subjects (p=0.75 and p=0.07; respectively).

Nevertheless, stratifying SLE patients according to pathological IMT values[46], (IMT value >1 mm) we observed higher plasma MDA levels in patients with pathological IMT value compared to SLE subjects with IMT<1 mm (MDA, 4.12±1.43 and 6.02 ± 4.2 for IMT<1 mm and IMT>1 mm, respectively; p=0.05), data shown in Table 4.

Previously, we have evaluated the frequencies of genes HLA-DR in Sicilian population according to longevity and observed that HLA haplotypes, playing an important roles in the regulation of the immune system, are population specific, being heavily affected by the population-specific genetic and environmental history[35]. So, as further analysis we investigated the implication of HLA system in oxidative stress and cardiovascular risk in SLE patients.

To this end, we compared the mean values of the main studied parameters according to HLA haplotype, using Student's *t*-test with Welch correction. Again, we observed a significant association between oxidative stress and HLA haplotype. Higher levels of MDA were found significantly increased in SLE patients DR2 negative (MDA, 5.46 ± 3.47 µmol/L) compared to SLE patients DR2 positive (MDA, 3.65 ± 1.25 µmol/L) (p=0.014). No significant association has been observed with the other parameters considered in the analysis (data not shown).

Discussion

This is the first report suggesting that a common functional variant in the UCP2 gene as well as HLA genotype is associated with increased oxidative stress in SLE patients. In particular, such data support a role for UCP2 (and hence the mitochondrial electron transport chain) in the regulation of ROS generation, and highlight its potential impact upon cardiovascular risk.

We suppose that the -866G/A UCP2 polymorphism in SLE causing increased ROS levels, results in a pro-oxidation environment, which in turn could result in decreased antioxidant activity and increased MDA levels responsible of accelerated atherosclerosis[47]. Wang et al. showed that markers of lipid oxidation correlate with worsening disease status in SLE supporting the role of oxidative stress in the pathogenesis of SLE[48]. Here, we suggest that MDA as oxidative stress marker may have a potential role in the future to measure oxidative stress in SLE patients and evaluate the use of antioxidant interventions and their efficacy.

Several limitations should be considered when interpreting the current results. Firstly, we did not correct p values for multiple comparisons. However, standard corrections, such as Bonferroni's, do more harm than good to sound statistical inference in biomedical research[49]. Rather than relying on the validity of statistical corrections, the best way to determine whether our observations have a biological basis (and are not a result of chance occurrences) would be to repeat these studies using an independent study population. The main limitation is the relatively small sample size and the fact that the participants were from the same geographic area may limit the applicability of our results to the determination of the role of UCP2 and oxidative stress in atherosclerosis risk in SLE. So, the present findings in an Italian population need to be replicated in independent studies to determine whether the 866G/A UCP2 promoter polymorphism influences MDA levels and whether it is truly implicated in the development of atherosclerosis in individuals at risk. Finally, we cannot exclude the possibility that the 866G/A polymorphism of UCP2 is not itself responsible for the observed association with increased MDA levels, but instead it is in linkage disequilibrium with an unknown causative variant in a distal regulatory site or with an unidentified causative polymorphism in a gene different from, but close to, the UCP2 gene. Further studies would be much more robust with a larger sample size and with mechanistic studies examining UCP expression levels and MDA production.

Notwithstanding these limitations, we believe our study offers important information for investigators studying the link between oxidative stress and cardiovascular risk in patients with SLE.

Declaration of Conflicting Interests

The authors testify that they have no actual or potential conflicts of interest including any financial, personal, or other relationships with other people or organizations within three years from the beginning of the submitted work that could inappropriately influence, or be perceived to influence, their work.

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Table 1. Difference of anthropometric profile, lipid profile, and oxidative stress profile in SLE and healthy subjects.

Variables	SLE (n= 45)	Healthy Controls (n= 36)	p value
Age (y)	41.46 ± 10.89	38.19 ± 7.27	N.S.
Sex (female/male)	40/5	36/2	N.S
Body mass index (kg/m^2)	22.46 ± 3.46	23.06 ± 3.1	N.S.
HDL (mg/dL)	54.06 ± 14.16	57.78 ± 13.17	N.S.
LDL (mg/dL)	54.31 ± 25.94	81.17 ± 27.62	< 0.0001
Smokers (never,past/current)	29/16	28/8	N.S.
Hypertension, n (%)	15 (33.33)	0 (0)	
Treatment, n (%)			
Hydroxychloroquine	22 (48.89)	0 (0)	
Corticosteroids	9 (20)	0 (0)	
Statins	2 (4.44)	0 (0)	
Azathioprine	4 (8.89)	0 (0)	
Aspirin	3 (6.67)	0 (0)	
Mycophenolate	5 (11.11)	0 (0)	
MDA (μmol/L)	5.05 ± 3.36	2.79 ± 0.89	< 0.0001

Values are expressed as mean ± SD; HDL high density lipoprotein, LDL low density lipoprotein, MDA malondialdehyde, N.S. not significant

Table 2. Frequency of UCP2 polymorphisms in SLE patients and matched controls

UCP2 polymorphisms	SLE patients (n=45)		Healthy controls (n=36)		OR (95% CI)	P value (3x2 table) (2x2 table)
-866 G/A	**N**	**%**	**n**	**%**		
G/G	3	6.67%	14	38.89%	------	**= 0.001**
G/A	37	82.22%	19	52.78%		
A/A	5	11.11%	3	8.34%		
G	43	47.78%	47	65.28%	0.49 (0.26 – 0.92)	**= 0.027**
A	47	52.22%	25	34.72%		
Dominant model						
G/G	3	6.6%	14	39%	0.11 (0.03– 0.43)	**= 0.0007**
G/A + A/A	42	93.4%	22	61%		
Recessive model						
G/G+ G/A	40	88.9%	33	91.67%	0.72 (0.16 - 3.27)	**N.S.**
A/A	5	11.1%	3	8.3%		
45 bpIns/Del						
Del/Del	21	46.7%	24	66.67%	-------	**N.S.**
Ins/Del	18	40%	10	27.78%		
Ins/Ins	6	13.3%	2	5.56%		
Del	60	66.67%	58	80.55%	0.48 (0.23 – 1.03)	**= 0.05**
Ins	30	33.33%	14	19.45%		
Dominant model						
Del/Del	21	46.67%	24	66.67%	0.44 (0.18 - 1.08)	**N.S.**
Ins/Del + Ins/Ins	24	53.33%	12	33.33%		
Recessive model						
Del/Del + Ins/Ins	39	85%	34	95%	0.38 (0.07 - 2.02)	**N.S.**
Ins/Ins	6	15%	2	5%		

OR = odds ratio; CI = confindence interval, N.S. = not significant.

Fisher's exact test used for 2X2 table and Chi-squares test used for 3x3 table.

Table 3. Association of UCP2-866 gene polymorphism with MDA values.

	SLE patients (N=45)		Healthy controls (N=36)		All Subjects (N=81)	
	UCP2-866A carriers	UCP2-866G carriers	UCP2-866A carriers	UCP2-866G carriers	UCP2-866A carriers	UCP2-866G carriers
MDA (μmol/L)	5.04 ± 3.19	4.06 ± 1.29	2.80 ± 0.89	2.79 ± 0.91	4.17 ± 2.85	3.25 ± 1.12
P value	N.S.		N.S.		=0.04	

Values are expressed as means ± SD; N.S. not significant

Table 4. Lipid, oxidative stress and UCP profile in SLE according the absence/presence of pathological values of IMT

Parameters	IMT ≤ 1 mm	IMT > 1 mm	P value
MDA	4.12 ± 1.43	6.02 ± 3.84	= 0.05
Total cholesterol	169.81 ± 45.94	185.16 ± 51.39	N.S.
HDL	55.72±17.04	54.08 ± 8.19	N.S.
LDL	48.86 ± 20.19	64.75 ± 38.07	N.S.
BMI	21.53 ± 2.86	24.20 ± 3.4	=0.02
Smokers (%)	29.41	11.76	N.S.
UCP2 -866A/G Genotype (%)			
GG	5.88	2.94	N.S.
GA	55.88	29.41	
AA	2.94	2.94	
UCP2 Ins/Del Genotype (%)			
Del/Del	29.41	20.58	
Ins/Del	32.35	5.88	N.S.
Ins/Ins	2.94	8.82	

Values are expressed as means ± SD; N.S. not significant

Chapter 5

INTERLEUKIN-9 OVER-EXPRESSION AND T HELPER 9 POLARIZATION IN SYSTEMIC SCLEROSIS PATIENTS

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Interleukin-9 over-expression and T helper 9 polarization in systemic sclerosis patients

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Summary

T helper 9 (Th9) cells and interleukin (IL)-9 are involved in the pathogenesis of several autoimmune diseases. The exact role of IL-9 and Th9 cells in patients with systemic sclerosis (SSc) have not yet been studied adequately. IL-9, IL-9R, transcription factor PU.1 (PU.1), IL-4, thymic stromal lymphopoietin (TSLP) and transforming growth factor (TGF)-β expression were assessed in skin and kidney biopsies of SSc patients and healthy controls (HC) by immunohistochemistry (IHC). The cellular source of IL-9 was also analysed by confocal microscopy analysis. Peripheral IL-9-producing cells were also studied by flow cytometry. The functional relevance of IL-9 increased expression in SSc was also investigated. Our results demonstrated a strong expression of IL-9, IL-9R, IL-4, TSLP and TGF-β in skin tissues of patients with both limited and diffuse SSc. IL-9 expression was observed mainly in the context of skin infiltrating mononuclear cells and keratinizing squamous epithelium. IL-9 over-expression was also observed in renal biopsies of patients with SSc. IL-9 producing cells in the skin were identified as Th9 cells. Similarly, Th9 cells were expanded and were the major source of IL-9 among SSc peripheral blood mononuclear cells (PBMC), their percentage being correlated directly with the modified Rodnan skin score. Infiltrating mononuclear cells, mast cells and neutrophils expressed IL-9R. In *in-vitro* studies stimulation with rIL-9 significantly induced NET (neutrophil extracellular traps) release by dying cells (NETosis) in neutrophils, expansion of mast cells and increase of anti-systemic scleroderma 70 (Scl70) production by B cells. Our findings suggest that Th9 cells and IL-9 could be implicated in the pathogenesis of SSc.

Keywords: IL-9, ILC2, systemic sclerosis, Th9

Introduction

Systemic sclerosis (SSc) is a chronic autoimmune disorder in which abnormal immune responses lead to activation and expansion of autoreactive effector immune cells, resulting in fibrosis of multiple organs [1]. Accumulating data suggest that infiltrating $CD4^+$ T helper (Th) cells, essentially skewed towards Th2 and Th17 phenotypes, play important roles in essentially mediating inflammation and fibrosis progression [1,2].

Recently, interleukin (IL)-9, a pleiotropic cytokine produced mainly by Th9 cells, has been demonstrated to participate actively in the induction of tissue fibrosis and in the pathogenesis of different autoimmune disorders [3,4]. The differentiation of Th9 cells requires a balance of signals, including IL-4 and transforming growth factor (TGF)-β [5], and the epithelial cytokine thymic stromal lymphopoietin (TSLP) [6], resulting in the induction of the transcriptional factor PU.1 that, in concert with interferon (IFN) regulatory factor 4 (IRF4), directly binds the IL-9 promoter and activates gene expression in Th9 cells [7]. Once differentiated, Th9 cells require activation of the IL-25/IL–17RB axis to produce high levels of IL-9 [8,9]

Increased expression of Th9 polarizing cytokines and of IL-25 has been demonstrated previously in patients with SSc [10–12], but the expression of IL-9 and Th9 cells has not yet been studied adequately. In the present study, we

Table 1. Baseline characteristics

Patients	Limited *versus* diffuse SSc	Modified Rodnan skin score	Pulmonary involvement	Pulmonary hypertension	Autoantibodies	%Th9
1	Limited	18	Yes	Yes	Scl-70	0·72
2	Diffuse	27	Yes	None	Scl-70	0·89
3	Diffuse	24	None	None	ANA	0·92
4	Diffuse	33	Yes	None	Scl-70	1
5	Limited	15	None	None	ANA	0·69
6	Diffuse	23	None	None	ANA	0·76
7	Limited	24	None	Yes	ACA	0·60
8	Limited	12	None	Yes	ACA	0·81
9	Limited	9	None	Yes	ANA	0·73
10	Limited	16	None	Yes	ACA	1
11	Diffuse	38	Yes	None	Scl-70	0·87
12	Limited	12	Yes	None	ANA	0·60
13	Limited	15	Yes	None	ANA	0·79
14	Diffuse	19	Yes	None	Scl-70	0·88
15	Limited	22	None	Yes	ANA	0·62
16	Limited	26	Yes	None	ANA	0·67
17	Limited	12	None	None	ACA	0·66
18	Limited	14	Yes	None	ANA	0·72
19	Limited	28	Yes	None	ANA	0·78
20	Diffuse	26	None	None	ACA	0·85
Controls (mean ± s.e.m.)	–	–	–	–	–	0·2040 ± 0·05384
Patients (mean ± s.e.m.)		–	–	–	–	0·7780 ± 0·02751

SSc = systemic sclerosis; Th9 = T helper type 9; s.e.m. = standard error of the mean.

analysed the expression of IL-9 and Th9 cells in SSc. We further examined the relationship of Th9 cells with specific subsets of SSc and the expression of Th9 relevant cytokines, including IL-17A, TGF-β1, TSLP and IL-4 and the role of IL-9 in modulating the function of IL-9R-expressing cells. The aim of the study was to evaluate Th9/IL-9 expression in different tissues of SSc patients as a possible player in the pathogenesis of SSc, thus representing a potential therapeutic target.

Patients and methods

Skin biopsy specimens of sclerotic cutis and peripheral blood were obtained after informed consent from 20 patients with SSc diagnosed according to the 2013 classification criteria for systemic sclerosis [13] (13 patients with limited and seven with diffuse SSc) (18 female and two male, mean age = 42 ± 12 years, mean disease duration = 34 ± 12 months) and 20 healthy controls (age- and sex-matched) who underwent surgery for non-cutaneous disease evaluation. Kidney samples were also obtained from an additional eight patients with SSc in whom renal biopsy was performed for hypertensive renal crisis attributable to SSc or persistent renal impairment (serum creatinine value > 2 mg/dl plus a diastolic blood pressure > 110 mmHg), or otherwise inexplicable presence of blood and/or protein in the urine. Renal biopsies were also obtained from five controls with isolated urinary alterations but normal kidney histology. Modified Rodnan skin score was calculated to assess the extension of skin involvement [14]. Pulmonary involvement was defined as a forced vital capacity < 70% of predicted, as measured by a dry spirometer, or by the evidence of amorphous or reticulonodular infiltrates in high-resolution computed tomography scans (HRCT). Pulmonary hypertension (PH) was also evaluated [pulmonary artery pressures (PAPs) > 25 mmHg and peak tricuspid regurgitant jet velocity (TRV) >2·7 m/s] according to Denton *et al.* [15]. Table 1 shows the baseline characteristics of the patients. Age- and sex-matched healthy donors (HD) were enrolled as controls. Collection of samples was approved by the ethical committee and the Institutional Review Board of the University of Palermo and informed consent was obtained from each patient and controls in accordance with the Helsinki Declaration.

Immunohistochemistry

Tissue samples were fixed immediately with 4% formaldehyde and embedded in paraffin. Immunohistochemistry analysis was performed on 5-μm-thick paraffin-embedded sections. Following rehydration, antigen was unmasked for 45 min at 95°C using Dako Target retrieval solution (pH 6.0) (Dako, Glostrup, Denmark). Endogenous peroxidase was blocked for 10 min with Dako peroxidase blocking reagent, and non-specific binding was blocked for 20 min

with Dako protein block. The primary antibodies were added and incubated for 1 h at room temperature. Isotype-matched irrelevant antibodies were used as a negative control [mouse immunoglobulin (Ig)G1 monoclonal antibody (mAb) (ab27479) and rabbit IgG polyclonal antibody (ab27472); Abcam, Cambridge, UK)]. Following three washes with Tris-buffered saline, slides were incubated for 30 min with peroxidase-conjugated Dako EnVision polymer. After three further washings, peroxidase activity was visualized using diaminobenzidine chromogen (Dako), and slides were counterstained lightly with haematoxylin before dehydration and mounting in DePex (VWR International, Radnor, PA, USA). Sections were analysed by two experienced scientists (A. R., F. C.) who were blinded with regard to subject group. The number of positive cells was determined by counting immunoreactive cells on photomicrographs obtained from three randomly obtained high-power microscopic fields (×400 magnification) under a DM2000 optical microscope, using a DFC320 digital camera (Leica, Wetzlar, Germany). To address specifically the nature of IL-9-producing cells, triple staining for Th17 and Th9 cells were performed on paraffin-embedded skin sections. Sections were treated with fluorescein isothiocyanate (FITC)-, Rhodamine Redor Cy-5-conjugated anti-mouse or anti-rabbit antibodies (Invitrogen, Carlsbad, CA, USA) plus RNasi (200 ng/ml) and counterstained using (4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Paisley, UK]. Confocal analysis was used to acquire fluorescence staining. A list of the antibodies used is shown in Supporting information, Table S1.

Isolation of peripheral blood mononuclear cells and flow cytometry

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and antibiotics from 15 SSc patients (10 with limited SSc and five with diffuse SSc) and 10 healthy controls. Cell viability (trypan blue dye exclusion) was always $> 95\%$. Cells were activated with anti-CD3/CD28 beads (ratio 1 : 2) for 6 h and then stained with mAb. Flow cytometry analysis was performed using a FACSCanto (BD Biosciences). At least 50 000 cells (events), gated on lymphocyte region, were acquired for each sample. Data were analysed with FlowJo software programs (FloJo, TreeStar Inc., Ashland, OR, USA). A list of the antibodies used is listed in Supporting information Table S1.

Cell cultures

Mast cells and B cells were isolated with microbeads (anti-CD117 and CD19, respectively) (positive selection) and magnetic-activated cell sorting techniques (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Neutrophils were isolated from peripheral blood using the density gradient separation method with a mixture of sodium metrizoate and dextran 500 and lysis of residual erythrocytes. Cells were cultured in 24-well flat-bottomed plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 1×10^6 cells in 1 ml of RPMI-1640 medium with 10% FCS, 2 mM L-glutamine, 20 nM HEPES and 100 U/ml penicillin/streptomycin with or without recombinant IL-9 (rIL-9) (10 ng/ml) (R&D Systems, Minneapolis, MN, USA). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. After incubation cells were fixed, stained with myeloperoxidase (MPO) and counterstained with DAPI. Confocal analysis was used to acquire fluorescence staining.

Statistical analysis

Student's *t*-test or the non-parametric Mann–Whitney *U*-test was used to calculate the statistical significance between groups. *P*-values less than 0·05 were considered significant.

Results

IL-9 is up-regulated in skin specimens of SSc patients

We first investigated whether IL-9 was up-regulated in skin specimens of SSc patients. As shown in Fig. 1, IL-9 expression was increased in both diffuse and limited SSc patients. In particular, IL-9 expression was detected mainly between infiltrating cells, endothelial cells and keratinizing squamous epithelial cells of skin of SSc subjects (Fig. 1a–c,l). The cellular source of IL-9 was represented prevalently by Th9 cells, as demonstrated by the co-localization of IL-9 and the transcriptional factor PU.1 (Fig. 1e–h). IL-9-producing Th17 cells were also found in the skin specimens of SSc patients (Fig. 1e–h). According to IL-9 over-expression, IL-9R was expressed intensely among infiltrating cells and keratinizing squamous epithelial cells (Fig. 1i–k,m). With regard to the expansion of Th9 cells, we next evaluated the expression of Th9-inducing cytokines in skin specimens of SSc. As shown in Fig. 2a–i, increased expression of IL-4, TSLP and TGF-β was detected in in skin tissues of patients with SSc, especially in those with diffuse disease.

The IL-25–IL-17RB pathway has been demonstrated to be important in the induction of IL-9 production by Th9 cells [9]. As shown in Fig. 2l–s, we found over-expression of IL-25, IL-17RB and of the specific tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) in the skin of SSc patients compared to controls. IL-9 over-expression was also observed in the inflamed glomeruli obtained from SSc patients (Supporting information, Fig. S1). In particular, SSc patients displayed intense staining in the context of mesangial glomerular cells with weak tubular epithelial cells positivity (Supporting information, Fig. S1).

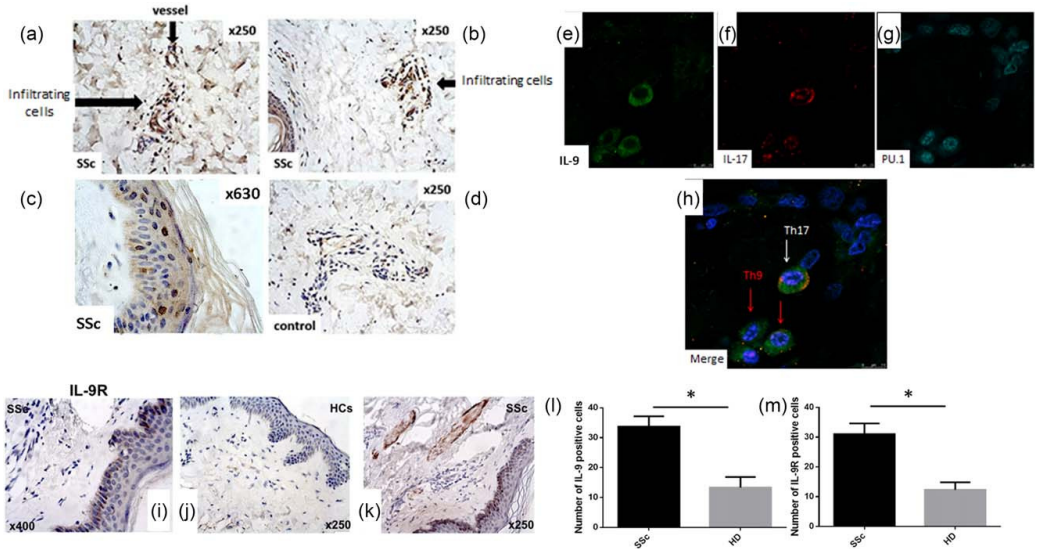

Fig. 1. Interleukin (IL)-9 and IL-9R expression in the skin of systemic sclerosis (SSc) and HD. IL-9 expression in the skin of SSc patients (a–c) and controls (d). Single staining for IL-9 (e), IL-17 (f) and transcription factor PU.1 (PU.1) (g). (h) Merged triple staining for IL-9, IL-17 and PU.1 in the skin of SSc patients. IL-9R expression in the skin of SSc patients (i,k) and controls (j). Quantification of IL-9 and IL-9R-producing cells from SSc patients and controls (l,m). Original magnification ×400 (i), ×250 (a,b,d,j,k), ×630 (c). Data are expressed as mean ± standard error of the mean (s.e.m.); $^*P < 0{\cdot}05$.

Conversely, HC showed intense tubular epithelial cell positivity (Supporting information, Fig. S1). These findings indicate that the Th9 pathway is activated strongly in SSc patients.

Phenotypical analysis of IL-9-producing cells among PBMC of SSc patients

The expression of IL-9 by different subsets of T helper cells was also analysed by FACS analysis on cells isolated from

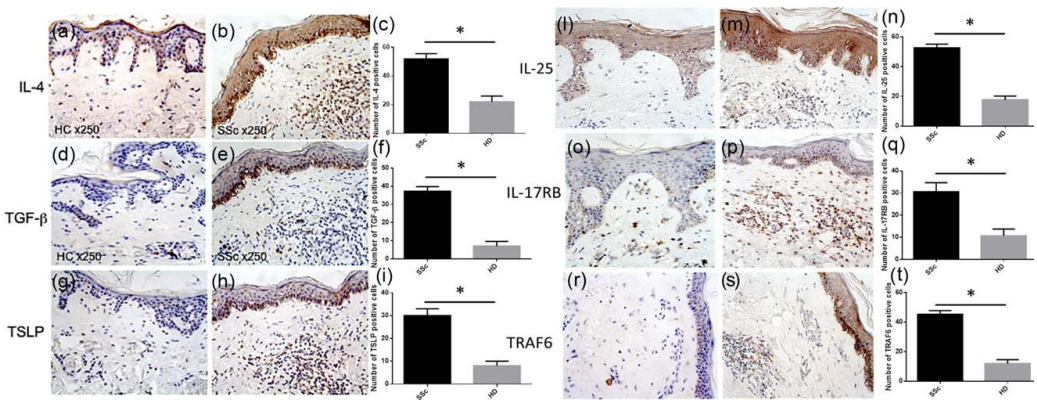

Fig. 2. Interleukin (IL)-4, transforming growth factor (TGF)-β, thymic stromal lymphopoietin (TSLP), IL-25, IL-17RB and tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) expression in systemic sclerosis (SSc) and healthy donors (HD). IL-4, TGF-β, TSLP, IL-25, IL-17RB and TRAF6 expression in the skin of SSc patients (b,e,h,m,p,s) and controls (a,d,g,l,o,r). Quantification of IL-4-, TGF-β–, TSLP-, IL-25-, IL-17RB- and TRAF6-producing cells from SSc patients and controls (c,f,i,n,q,t). Original magnification ×250. Data are expressed as mean ± standard error of the mean (s.e.m.); $^*P < 0{\cdot}05$.

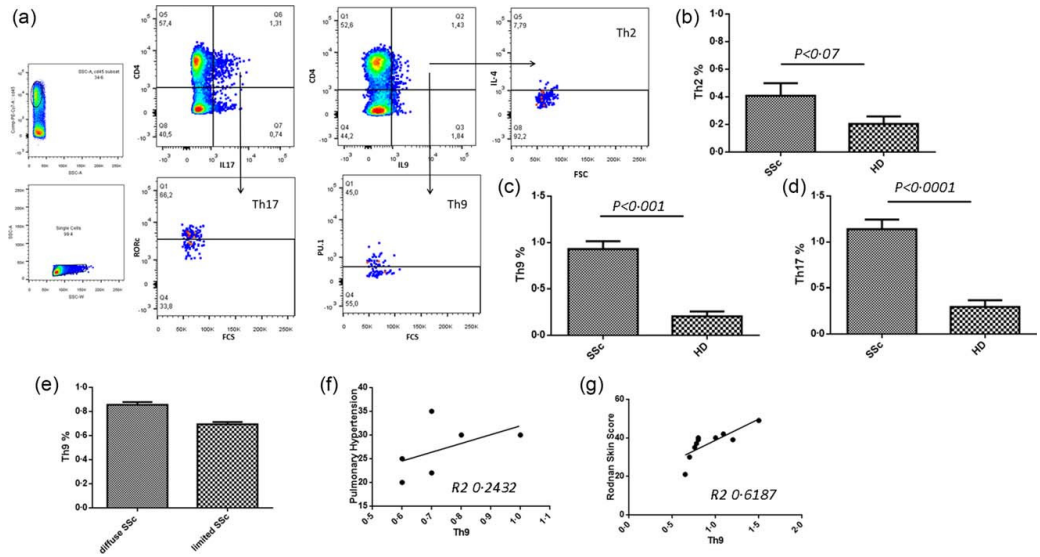

Fig. 3. Percentage of T helper type 9 (Th9), Th17 and Th2 in systemic sclerosis (SSc) and healthy donors (HD). Representative dot-plot analysis and gating strategy in an SSc patient (a). Percentage of Th2 (b), Th9 (c) and Th17 (d) cells in SSc and HD. Mean percentage of Th9 cells in patients with diffuse or limited disease (e). Direct correlation of Th9 percentage in SSc and pulmonary hypertension (f) and Rodnan skin score (g). Data are expressed as mean ± standard error of the mean (s.e.m.).

the peripheral blood of patients with SSc and controls. As shown in Fig. 3, IL-9-producing $CD4^+$ T cells from patients with SSc were expanded significantly compared to control subjects. Among $CD4^+$ T cells, the percentage of IL-9-producing Th9 (0·7 ± 0·02 *versus* 0·2 ± 0·05) (Fig. 3a,c) and Th17 (1·2 ± 0·3 *versus* 0·33 ± 0·08) (Fig. 3d) cells was significantly higher in SSc patients respect to controls. Conversely, the percentage of IL-9-producing Th2 (0·4 ± 0·09 *versus* 0·2 ± 0·03) cells was not significantly different in the two groups (Fig. 3b). Interestingly, frequencies of Th9 cells were significantly higher in patients with diffuse SSc and the number of IL-9-expressing cells was correlated significantly with the modified Rodnan skin score, but not with pulmonary hypertension (Fig. 3e–g). These findings indicate the presence of a Th9 polarization in the peripheral blood of SSc patients.

Innate lymphoid cells type 2 (ILC2) are expanded in the skin and peripheral blood of SSc

Th9-derived IL-9 has been demonstrated to activate ILC2 in a mast cell-dependent manner [16]. Furthermore, ILC2 have been described to be expanded in SSc patients [17]. Thus, we next evaluated the frequencies of Th2 polarized ILC2 subsets in the skin and peripheral blood of SSc patients (Fig. 4). According to a previous report, ILC2 were expanded significantly in peripheral blood (Fig. 4a,b), and cells resembling the ILC2 phenotype were also increased in the skin (Fig. 4c) of SSc patients compared to controls. A significant and direct correlation was also found between the percentages of circulating ILC2 and Th9 cells (Fig. 4g).

Effect of IL-9 on B cells, mast cells and neutrophils in SSc

Beyond ILC2, IL-9 receptor is also expressed on neutrophils and mast cells (MC), which have been involved in the pathogenesis of SSc [18,19]. We next evaluated, *in vitro*, the role of recombinant IL-9 in the modulation of IL-9R-expressing cells isolated from the peripheral blood of SSc patients. IL-9 stimulation of neutrophils increased the production of IL-8 significantly and neutrophil extracellular trap pathogen-induced cell death (NETosis) strongly, as demonstrated in Fig. 5a where the nuclei of neutrophils lose their shape, and the NETs are released as the cell membrane breaks. Stimulation of MC induces their significant expansion (Fig. 5b) and stimulation of B cells resulted in a significantly increased release of SSc-related autoantibodies (Fig. 5c).

Discussion

In this study we show, for the first time, that the IL-9/IL-9R axis is up-regulated in the inflamed skin of SSc patients and is accompanied by the expansion of pathogenic Th9 cells. We also demonstrated that Th9 polarization occurs in

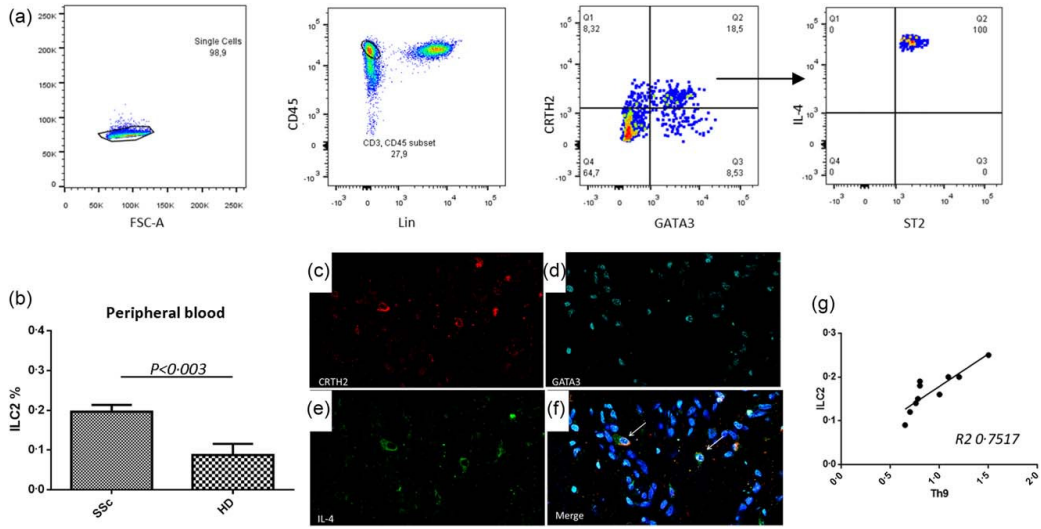

Fig. 4. Innate lymphoid cells (ILC) type 2 are expanded in systemic sclerosis (SSc) patients. Representative dot-plot analysis and gating strategy in a SSc patient (a). Mean percentage of ILC2 cells among peripheral blood mononuclear cells (PBMC) of patients and HD (b). Single staining for chemoattractant receptor Th2 (CRTH2) (c), GATA binding protein 3 (GATA3) (d) and interleukin (IL)-4 (e). (f) Merged triple staining for CRTH2, GATA3 and IL-4. ILC2 are directly correlated with Th9 (g). Data are expressed as mean ± standard error of the mean (s.e.m.); $^{*}P < 0{\cdot}05$.

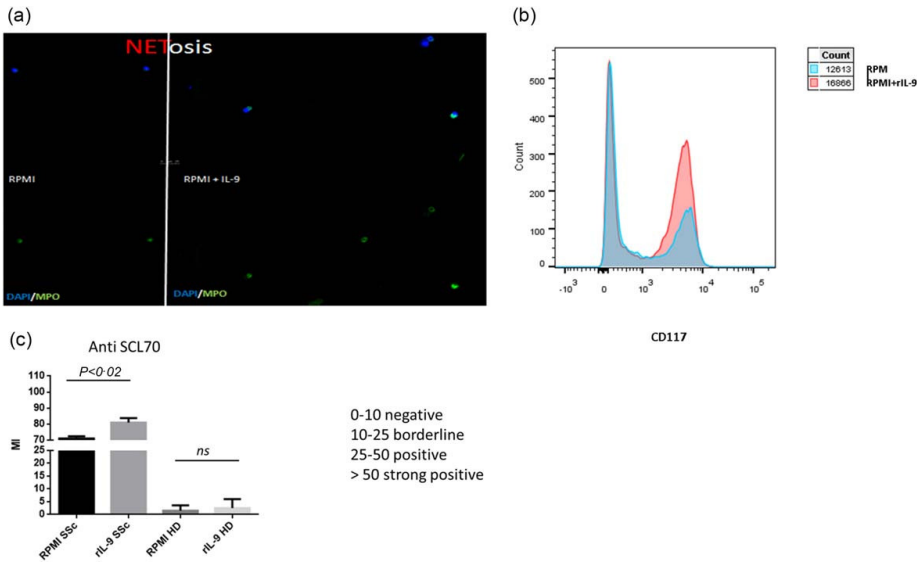

Fig. 5. Effect of recombinant interleukin (IL)-9 on neutrophils, mast cells and B lymphocytes. Immunofluorescences for neutrophil extracellular traps release by dying cells (Netosis): single staining for myeloperoxidase (MPO) in untreated and interleukin (IL)-9 treated neutrophils (a). Expansion of CD117-positive mast cells after *in-vitro* culture with recombinant IL-9 (b). Anti-systemic scleroderma 70 (Scl70) production after *in-vitro* stimulation of B lymphocytes with recombinant IL-9 (c).

inflamed skin and in the peripheral blood of SSc patients, being correlated directly with the modified Rodnan skin score but not with pulmonary hypertension. Similar to the skin, IL-9 expression was also found increased in renal biopsy specimens of SSc patients with renal crisis. Finally, *in-vitro* treatment with rIL-9 was associated with a significant induction of NETosis in neutrophils, significantly increased production of SSc-related autoantibodies by B lymphocytes and the expansion of MC.

IL-9 is a recently described proinflammatory cytokine because of its capacity to support proliferation of B and T cell infiltration [20]. In combination with IL-17, IL-9 increases the accumulation of neutrophils and perpetuates chronic inflammation [21], as observed in several autoimmune disorders such as rheumatoid arthritis (RA), psoriatic arthritis and giant cell arteritis (GCA) [22]. IL-9 production was associated first with the Th2 phenotype, even if other T helper subsets appear to have the potential for IL-9 production such as Th17 cells and regulatory T cells (T_{reg}) [23,24]. More recently, it has been demonstrated that Th0 can differentiate into a specialized subset of IL-9-producing T cells, named Th9. Th9 differentiation and IL-9 expression is enhanced by IL-4, TGF-β and TSLP [5,6,25] and induced by the activation of the ETS-family transcription factor PU.1 [7,26]. Increased serum levels of IL-9 have been found recently in SSc, suggesting that the IL-9 axis might be involved in SSc pathogenesis [10]. However, no studies addressing specifically the nature of IL-9-producing cells and the functional consequences of IL-9 over-expression have yet been published.

In SSc skin, dermal fibroblasts, inflammatory infiltrating cells and endothelial cells produced mainly IL-9. In particular, Th9 cells and, in a lesser manner, Th17 cells were the major sources of IL-9 in SSc skin. Th9 cells seem to be involved in the immunopathology of different systemic rheumatic diseases in which this axis is activated and involved potentially in the triggering and/or maintaining inflammation. The differentiation of Th9 cells requires the activation of IL-2/signal tranducer and activation of transcription-5 (STAT-5) and IL-4/STAT-6 signalling and TGF-β that act by inducing redirection of naive T cells from a Th2 to Th9 cell differentiation pathway [5,27]. Recently, it has been demonstrated that TSLP, IL-25 and IL-33 may also enhance IL-9 production by Th9 cells [6,9]. According to the expansion of Th9 cells, TGF-β, TSLP and IL-4 were up-regulated significantly in SSc skin and correlated directly with the expression of IL-9 and the number of Th9 cells. In addition, the IL-25/IL-17RB pathway was also activated in SSc patients, as indicated by over-expression of IL-25, IL-17RB and of the specific transcription factor TRAF6 in the skin of SSc patients. Taken together, these findings seem to point towards a strong activation of IL-9/Th9 axis in SSc.

A mast cell–ILC2–Th9 pathway has been demonstrated to be functional in human diseases such as cystic fibrosis [28]. Although ILC2 expansion has been demonstrated recently in SSc [17], no studies have been performed on the functional pathways involved in their activation. Accordingly, with IL-9 over-expression, we observed a significant ILC2 expansion in SSc patients that correlated significantly with the percentages of circulating Th9 cells. Interestingly, ILC2 expansion was also accompanied by the increased frequencies of both circulating and tissue mast cells in SSc patients. Finally, isolated mast cells from the peripheral blood were expanded significantly by the addition of IL-9 in *in-vitro* experiments and produced high levels of IL-2, indicating that a mast cell–ILC2–Th9 pathway is also functional in SSc patients.

The biological effects of IL-9 are mediated by IL-9R, a heterodimeric receptor composed by a α-chain (IL-9Rα) and a common γ-chain receptor shared by other cytokines. According to the increased expression of IL-9, IL-9R was also up-regulated significantly in the inflamed skin of SSc patients, essentially among infiltrating lymphocytes, mast cells and neutrophils. In order to study the functional relevance of the increased frequency of IL-9R-expressing cells in SSc, isolated neutrophils and B cells were stimulated with recombinant IL-9. IL-9, through interaction with the cognate IL-9R alpha expressed on human B cells, induces B cell expansion and activation by activating STAT-3 and STAT-5 [29]. In our study, in *in-vitro* studies IL-9 was able to induce the production of SSc-specific autoantibodies in isolated SSc B lymphocytes, indicating an important role of IL-9/IL-9R alpha axis in modulating autoimmunity in SSc patients [24]. Stimulation of neutrophils with IL-9 induces NETosis activation. NETs are chromatin structures loaded with anti-microbial molecules that represent one of the first lines of defence against pathogens. *In vivo*, NETs are released during a form of pathogen-induced cell death, named NETosis. NETosis activation has been demonstrated in autoimmune diseases such as RA and systemic lupus erythematosus but never studied in SSc. Here, we demonstrated that NETosis is also activated in SSc patients and it is possibly induced by IL-9. The functional consequence of NETosis activation in SSc needs to be addressed specifically in further studies.

Taken together, our results suggest that Th9 cells and IL-9 could play an important role in the pathogenesis of SSc by modulating adaptive and innate immune responses and the production of autoantibodies and indicate the IL-9 pathway as a possible therapeutic target.

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Disclosure

The authors have declared no conflicts of interest.

Author contributions

G. G.: study conception and design, data interpretation, literature search, figure creation, writing, paper revision and acceptance; F. C.: study conception and design, data interpretation, literature search, writing, paper revision and acceptance; M. L. P.: data collection, data interpretation, literature search, paper revision and acceptance; D. D. L.: data collection, data interpretation, literature search, paper revision and acceptance; P. C.: data collection, literature search, paper revision and acceptance; A. R.: data collection, data interpretation, literature search, paper revision and acceptance; P. R.: data collection, literature search, paper revision and acceptance; G. C.: data collection, literature search, paper revision and acceptance; C. M. G.: data collection, data interpretation, literature search, paper revision and acceptance; G. S.: data collection, data interpretation, literature search, paper revision and acceptance; F. D.: data collection, data interpretation, literature search, paper revision and acceptance; G. T.: data collection, data interpretation, literature search, paper revision and acceptance; and R. G.: study design, data interpretation, paper revision and acceptance. All authors gave final approval for submitting the manuscript for review and agree to be accountable for all aspects of the work.

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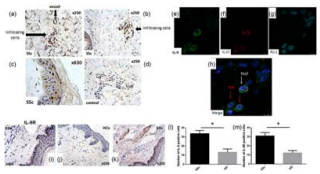
Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. List of antibodies.
Fig. S1. Interleukin (IL)-9 expression in the kidney of systemic sclerosis (SSc) and healthy donors (HD). IL-9 expression in the kidney of SSc patients with renal crisis (a) and controls (b). Quantification of IL-9-producing cells from kidney tissue from SSc patients and controls (c). Original magnification $\times 250$. Data are expressed as mean $\pm$ standard error of the mean (s.e.m.); $^{*}P < 0{\cdot}05$.



- IL-9 and Th9 cells are expanded in the skin, kidney and peripheral blood of SSc patients.
- IL-9 pathway may play an important role in SSc and should be considered as potential therapeutic target in SSc.

Chapter 6

TRIGGERING OF TOLL-LIKE RECEPTORS IN ELDERLY. A PILOT STUDY RELEVANT FOR VACCINATION

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Triggering of Toll-like receptors in the elderly. A pilot study relevant for vaccination

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Abstract

The impaired ability of the elderly to mount an efficient immune response after exposure to microbes or vaccines represents a major challenge in protection against pathogens in ageing. Recently studies have shown that stimulation of Toll-like receptors (TLRs), 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. Since TLR stimulation is a key regulator of the type and magnitude of the immune response, we evaluated cytokine production in dendritic cell populations upon stimula-

tion with two complementary TLR agonists, R848 and MPLA. Our preliminary results demonstrate that TLR activation by this combination of agonists can significantly enhance the activation of dendritic cells in the peripheral blood isolated from healthy elderly donors. This data suggest that the inclusion of appropriate combination of TLR agonists may enhance the efficacy of vaccination in the elderly.

Key Words

Ageing, Cytokines, TLR, Vaccination.

Introduction

Increasing age is accompanied by a progressive decline of both the innate and acquired immune system, noted as "Immunosenescence" [Caruso & Vasto, 2016].

The impact of ageing on the immune system typically includes intrinsic defects within immune cells as well as alterations in number and activity, and possibly defects in the bone marrow and thymic stromal microenvironment. This phenomenon results in a reduction of naive T and B cells, leading to inefficient primary responses of immune effector cells to pathogens, as well as reduced T cell cytotoxicity, proliferation and cytokine production, and defective memory responses in the elderly population [Larbi et al., 2008; Caruso et al., 2009; Nikolich-Zugich & Rudd, 2010]. Consequently, aged individuals exhibit increased incidence of infectious diseases, cancer and autoimmune diseases [McElhaney et al., 2012]. In addition, their aged immune system does not respond to stimuli as efficiently as that of younger adults, therefore current vaccines are less effective in the elderly [Derhovanessian & Pawelec, 2012].

Research in immunological ageing 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 the elderly. One such strategy would be to develop vaccines comprising suitable adjuvants to enhance the impaired cellular immune responses [Wells et al., 2008].

Adjuvants are molecules that stimulate the non-specific, innate immune responses, inducing the activation of antigen presenting cells (APCs) and their recruitment to the site of vaccination. Dendritic cells (DCs) are the most potent APCs, specialized in the uptake, processing, transport and presentation of antigens to T cells [Collin et al., 2013]. 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.

DCs in human blood are defined as Lineage 1 (CD3, CD14, CD16, CD19, CD20, and CD56)-negative and HLA-DR-positive cells. DCs 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). pDCs are characterized by the expression of CD123 marker and possess the capacity to produce high levels of type I

Interferons (IFN-α/β). In contrast, mDCs express the CD11c marker and are divided into two subsets: CD1c+ mDCs and CD141+ mDCs [Collin et al., 2013]. Upon stimulation, mDCs secrete mainly IL-6, IL-12, and TNF-α.

Both pDCs and mDCs express toll-like receptors (TLRs) that recognize conserved molecular patterns on microbes and are key regulators of antimicrobial host defence responses. Recognition of microbial components 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 lead to increased severity of multiple immunological disorders, including sepsis, immunodeficiencies, atherosclerosis and asthma [Cook et al., 2004].

Recently, stimulation of TLRs by adjuvants has been shown to be a promising strategy to enhance vaccine efficacy against both foreign and self, tumour-associated, antigens in aged mice by activating innate immune cells and enhancing production of inflammatory cytokines [Tye et al., 2015].

On the basis of these promising results in mice, we have investigated the ability of combined TLR ligands to induce pro-inflammatory responses in the peripheral blood dendritic cells isolated from healthy donors with evidence of immunosenescence.

Material and Methods

Samples

A total of 23 samples, including five centenarians, five centenarian offspring (CO), six old donors and six young donors used as controls, were processed. All participants were in good health according to their clinical history and none of them had infectious, inflammatory, neoplastic or autoimmune diseases at the time of the study. The University Hospital Ethics Committee approved the study, and written informed consent was obtained from all participants according to Italian law. Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediaminetetraacetic acid. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Lympholyte® (Cedarlane, Canada, United States) and viably cryopreserved according to standard protocols.

DCs stimulation assay

Total PBMCs were cultured at $1x10^6$ cells/well on a 96 U-bottom plate. Cells were plated with a combination of two adjuvants, chosen

based on our preliminary experiments, in the presence of 1x Golgi Plug solution (BD Biosciences, Erembodegem, Belgium). Specifically, we tested the following TLR agonists: the TLR7/8 agonist R848, at 3 μg/ml, and the TLR4 agonist MPLA, at 10 μg/ml (InvivoGen, San Diego, California). For each donor, cells were also left unstimulated and served as controls. After 6 hours of incubation cells were transferred to labelled FACS tubes and stained with antibodies. We chose to study total PBMCs, rather than purified DCs, to minimize manipulations that might result in partial activation of DCs, and because purification procedures would substantially decrease the yield of cells for analysis.

Flow cytometry and cell sorting

Cells were stained with antibodies to human CD3, CD14, CD16, CD19, CD20, CD56 (Lineage 1, BD Biosciences, Erembodegem, Belgium), HLA-DR (clone L243, Biolegend, San Diego, California), CD123 (clone 6H6, Biolegend), CD1c (clone L161, Biolegend), CD11c (clone 3.9,Biolegend), and CD141 (clone 1A4, BD Biosciences) for extracellular staining. For intracellular staining, cells were then washed, fixed with permeabilization/fixation buffer (BD Biosciences) and stained with antibodies to human IL-6 (clone MQ2-13A5, Biolegend), TNF-α (clone Mab11, Biolegend), and the p40 subunit of IL-12/23 (clone C11.5, Biolegend). Samples were analysed by flow cytometry on a LSR Fortessa™ (BD Biosciences). FACS data were analysed and plotted using FlowJo software (Tree Star).

Statistics

Statistical analyses were performed using GraphPad Prism software; the significant level of p value was determined using Student t test. (* $p<0.05$, ** $p\leq0.01$, *** $p\leq0.001$)

Results and Discussion

Given that DC activation is a key regulator of the magnitude and nature of the elicited adaptive immune responses, we evaluated whether TLR ligands could effectively activate naturally occurring, circulating DCs. To this end, we stimulated total PBMCs, derived from young, aged, centenarian and CO participants, with a combination of two TLR ligands. The nature and magnitude of cytokine production was assessed using multi-parameter flow cytometry.

Our preliminary *in vitro* screening experiments suggest that from the various TLR agonists tested, the condition that most effectively activated human leukocytes was the combination of TLR7/TLR8 with TLR4. This TLR agonist combination induces significantly greater cytokine production than that induced by each of the individual agonist. This greater stimulation is probably due to the combined activation of both Myd88 and TRIF-dependent signal transduction pathways.

Figure 1 shows an example of our analysis; after excluding doublets, we gated the Lineage (consisting of a cocktail of antibodies against CD3, CD14, CD16, CD19, and CD56)-negative, HLA-DR-positive population, with mDCs and pDCs identified as CD11c and CD123 positive cells, respectively. Subsequently, using intracellular cytokine staining, we evaluated the selected populations for the production of TNF-α, IL-6 and IL-12p40.

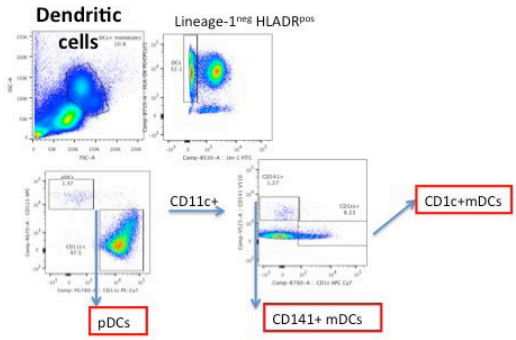

Figure 1. Characterization of human dendritic cells and their subsets. Phenotype of human DCs analysed by flow cytometry. Cells were gated on Lineage-1 negative HLA-DR-positive population by a cocktail of antibodies against CD3, CD14, CD16, CD19, and CD56. Subsequently, mDCs and pDCs were identified using staining against CD11, CD123, CD1c and CD141.

First, we report that the stimulation of cells with the combination of agonists for TLR7/8 and TLR4 is *de facto* an excellent inducer of TNF-α and IL-12/p40 cytokines in the CD141+ mDCs from young, old and CO subjects, with significantly high levels of cytokines compared to unstimulated samples ($p<0.05$, $p\leq0.01$, and $p\leq0.001$; respectively). No significant variation was observed in centenarians (Figure 2A). This, probably, is due to the age-related lower viability and low number of centenarian samples. Nevertheless, we have reported that the difficulties to study centenarians can be overcome by studying CO that are one generation (about 20-30 years) younger than centenarians and are representative of an elderly cohort, characterised by a better functional status and a reduced risk for several age-related pathologies [Balistreri et al., 2014]. Focusing on old people, we observed that both Italian CO and elderly, showed elevated percentage of IL-12/p40 and TNF-α after treatment with the TLR ligands R848 (TLR7/8) and MPLA (TLR4); these differences were highly statistically significant compared to unstimulated cells.

Notably, the combination of R848 and MPLA induce 5–10 fold higher production of IL-12/p40 in CD141+ mDCs isolated from old and CO samples compared with their young counterparts ($p\leq0.01$) (Figure 2A).

In addition, increased amounts of TNF-α, were also observed in CD1c+ mDCs and pDCs from older and CO subjects, in response to R848 and MPLA stimulation. These differences were statistically significant when compared to their unstimulated counterparts (Figure 2B).

Taken together, the data presented suggest that the combination of R848 and MPLA effectively promotes *in vitro* cytokine production in human DCs isolated from elderly, despite their immunosenescent phenotype.

To date, data regarding the influence of ageing on human DCs activity and cytokine production, in response to *in vitro* stimulation, has been inconsistent, showing either comparable or reduced DC function in the elderly [Lung et al., 2000; Pietschmann et al., 2000; Shurin et al., 2007]. Tan et al., [2012] 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., [2013]demonstrated a reduced production of TNF-α by DCs from old people in response to LPS stimulation.

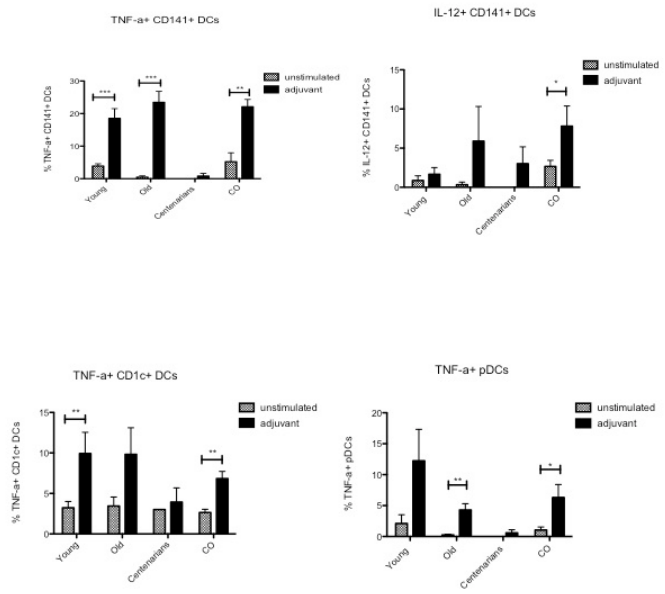

Figure 2. Cytokine secretion by DCs. (A) TNF-α and IL-12 secretion from CD141+ mDCs in response to TLR7/8 and TLR4 stimulation in vitro. PBMCs were cultured in the absence or presence of combined TLR7/8 and TLR4 ligands (unstimulated and adjuvants, respectively). After incubation, CD141+ mDCs were identified by flow cytometry analysis. TNF-α and IL-12 were evaluated using intracellular staining and analysed by FlowJo. The data report the percentage of cytokines produced from young, elderly, CO and centenarian samples after incubation. Statistical significance between the groups has been reported as *p, 0.05, **p, 0.01, ***p, 0.001. (B) TNF-α secretion from pDCs and CD1c+ mDCs in response to TLR7/8 and TLR4 stimulation in vitro. PBMCs were cultured in the absence or presence of combined TLR7/8 and TLR4 ligands (unstimulated and adjuvants, respectively). After incubation, pDCs and CD1c+ mDCs were identified by flow cytometry analysis. The data report the percentage of TNF-α produced from each sample group after incubation. Statistical significance between the groups has been reported as *p, 0.05, **p, 0.01, ***p, 0.001.

In our study, stimulation with the specific combination of TLR agonists, R848 and MPLA, induced significantly higher cytokine secretion by mDCs and pDCs from both elderly and CO subjects. This has potentially important implications, since it has been reported that reduced production of TNF-α by pDCs from old people, caused by defects in TLR signalling pathways, is associated with an ineffective antibody response to influenza vaccination [Panda et al., 2010].

The involvement of TNF-α in DC-induced T cell proliferation is also evident from clinical data of rheumatoid arthritis patients, showing that treatment with anti-TNF-α antibodies cause poor stimulation of T cell activity by DCs [Baldwin et al., 2010; Liu et al., 2012]. Thus, impaired production of TNF-α by older DCs could result in a weak response to vaccination and may contribute to the dysregulation of DC-induced T cell proliferation in the elderly subjects.

Conclusion

Our findings highlight the efficient effect of adjuvant in stimulation of cytokine production, and point towards the potential use of appropriately selected combination of TLR agonists in future vaccination approaches for the elderly.

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Chapter 7

DISCUSSION AND FUTURE DIRECTIONS

My researches during my PhD were mainly focused on two aspects.

The first one, was to study molecular aspects potentially implicated in autoimmunity pathogenesis, in order to identify new potential risk factors useful as therapeutic target. To this end, we focused on two severe and wasting systemic autoimmune diseases: SLE and SSc.

The second aspect was to study potential molecular mechanisms to develop new vaccines or the optimization of immunization in elderly. It is well known, that ageing affects the immune system and its ability to respond pathogens. The current immunological strategies are based on triggering of TLRs on DCs to elicit cytokine production and different immune responses. This aspect is very important because, recently, it has been found out that similar strategy could be used in AIDs treatment by the use of negative/positive ligands of TLRs.

Discussion on chapter 3-5

Molecular aspects in autoimmunity

Because of the incredible vastness symptoms and complexity of AIDs, we decided to detect distinct approach, based on investigation of different genetic and molecular aspects in SLE and SSc, in order to give an overall picture of possible factors involved in AIDs susceptibility.

In **Chapter 3**, we investigated the potential association between KIR gene polymorphisms and SLE risk. The combinations of HLA class I and KIR variants is crucial for the NK cell regulation, participating in the immune response and T cell activation. Recently, it has been suggested that NK not only exert cell-mediated cytotoxicity against infected or cancer cells, but their over activation or dysfunction might be associated with pathogenesis of autoimmune diseases, like SLE (Spada et al., 2015).

These cells are, indeed, able to promote or suppress functions of other immune cells by secretion of cytokines and chemokines (Zhang and Tian, 2017).

According to reviewed data, specific interactions between KIR-HLA ligand can modify the disease susceptibility. To date, several case-control studies investigated the role of KIR in SLE onset, but findings are always inconsistent and population specific. Our results showed that KIR and their HLA class I molecule ligands might to be associated with the onset of the disease. The strongest associations observed in our genetic association study, have been with KIR2DS2 activating receptor gene, as well as with KIR ligand group HLA-C1. Particularly, we observed that patients with SLE, present increased expression of the activating KIR2DS2 gene (OR=3.36, p=0.005), suggesting a role in the susceptibility to disease, as previously reported by Toloza et al., (2008). Similar results have been also obtained by Pedroza et al., (2011), which observed a KIR2DS2+/KIR2DS5+/KIR2DS1+ specific activating KIR profiles in patients with SLE. Our data differ from results reported by Pellet et al., (2007), and Hou et al., (2010), which observed, on the contrary, a significant increase in the frequency of KIR2DS1 in the absence of KIR2DS2 gene in Caucasian and Asian patients with SLE, respectively. So, we decided to perform a meta-analyses, put together our observation and results of previous studies. This analysis failed to find correlations between activating KIR2DS2 genes with susceptibility to SLE ($p > 0.05$).

Regarding HLA-C1 ligands, only a study has actually investigated their role in SLE, so it was not possible to perform a meta-analysis for this group. However, a stepwise logistic regression analysis confirmed the

effect of the HLA-C1 ligands in SLE patients (OR=7.06, p=0.002), while the KIR genes were no longer significant.

To best of our knowledge, since although SLE genetic susceptibility has long been known by some HLA alleles and numerous other genes, it is still not entirely clear how HLA alleles act. On the other hand, attention has also recently been focused on the role of KIR genes, but in literature there are few studies (and even contradictory) about the frequency of these genes in SLE, and only one study evaluated the frequencies of both HLA is KIR in the Iranian population.

To determine which Cw allele in C1 group is involved in the susceptibility, we also determined the frequencies of HLA-Cw alleles in patients and controls. But, no significant difference between SLE patients and healthy controls was observed, suggesting that the increased frequency of HLA-C1 ligands in lupus is probably due to the synergic presence of all alleles belonging to this group showing the same epitope.

Unfortunately, the mechanism by which HLA-C1 is involved in SLE pathogenesis is not yet known. Nevertheless, we can suppose that HLA-C1 induces NK cell activation through its KIR and thereby promote the autoimmune process. Interestingly, the detrimental effects of the activating HLA-C1 ligands were also confirmed by data of oxidative stress. Indeed, we found that SLE patients HLA-C1 positive showed reduced levels of the antioxidant molecule taurine compared to SLE patients HLA-C1 negative.

Summarising, we used a genetic approach to provide the first evidence for a direct association of HLA-C1 ligands with SLE pathogenesis in Sicilian population, suggesting its possible role as a strong risk factor marker of the disease. The binding of HLA-C1 ligands to their receptors might induce NK cells activation and an increase of oxidative stress status

that translate into SLE progression. Our results open new interpretations of SLE pathogenesis, but further studies on the genotyping, expression, and function of KIR receptors and their HLA ligands should be done to fully determine the role of these ligands and receptors in the onset of the disease.

In **Chapter 4**, we primarily demonstrated that a common functional variant in the UCP2 gene as well as HLA genotype is associated with increased oxidative stress in SLE patients.

Interestingly, we observed that the rs659366 UCP2 polymorphism seemingly confers susceptibility for SLE onset. UCP2 −866 G/A is a common functional variant exists in the promoter region of the gene, with the A allele being associated with low levels of UCP2 expression and, consequently, with reduced protection against ROS production. In line with this thesis, some studies reported that the -866 A allele is associated with an increased risk of chronic inflammatory diseases and cardiovascular risk (Hamada et al., 2008; Oberkofler et al., 2005; Dhamrait et al., 2004). Interestingly, we observed that patients with SLE showed less frequencies of the protective -866 G/G UCP2 genotype when compared to healthy age-matched controls ($p = 0.001$). The significant association of the UCP2 –866 G/A polymorphism with SLE was also confirmed by dominant model, with the -866 G/A and A/A genotypes associated with increased risks of diseases as compared with G/G genotype (OR= 0.11; 95% CI 0.03-0.43; $p = 0.0007$). Moreover, allele comparison, showed that the frequency of -866A allele was higher in SLE patients than in control (OR= 0.49; 95% CI, 0.26-0.92; $p = 0.027$) as compared with G allele. Thus, we can suppose that the -866 G/A UCP2 polymorphism is an important genetic risk factor involved in SLE outcome, causing increased ROS levels.

Moreover, we studied the role of oxidative stress in the progression of SLE, by measuring of levels of MDA. In fact, it is well accepted that the increase of ROS production provokes lipid peroxidation, which translate in increased oxidative damage and cardiovascular risk. Particularly, we found higher levels of MDA in SLE patients compared to healthy age-matched controls ($p < 0.0001$).

Previously, Wang et al., (2010) showed that markers of lipid oxidation correlate with worsening disease status in SLE supporting the role of oxidative stress in the pathogenesis of SLE. Our results, similar to the previous report of Perez et al., (2012), indicate that the lipid cell membrane was attacked in SLE confirming the increased oxidative stress in these patients. Thus, MDA can be considered a good marker of oxidative stress of the disease providing a basis for designing appropriate antioxidant interventions to prevent or alleviate SLE clinical manifestations.

Elevated levels of plasma oxidative stress markers associated with UCP2 -866 G/A polymorphism, have been previously reported as predictor of future risk of coronary heart disease events in a cohort of diabetic patients (Palmer et al., 2009). To gain insight into this topics, we investigated the role of UCP2 -866 G/A and oxidative stress in intima media thickness (IMT), a well-established predictor of cardiovascular disease events (Boulos et al., 2016), in SLE group. Stratifying SLE patients according to pathological IMT values, (IMT value > 1 mm) we observed higher plasma MDA levels in patients with pathological IMT value compared to SLE subjects with IMT < 1 mm ($p = 0.05$). Unfortunately, missing data for IMT evaluation has limited the power of this study to investigate the association of UCP2 -866 G/A with atherosclerosis development. Finally, we investigated the implication of

HLA system in oxidative stress in SLE patients, and again observed a significant association between oxidative stress and HLA haplotype. In particular, higher levels of MDA were found significantly increased in SLE patients DR2 negative compared to SLE patients DR2 positive.

In conclusion, we suppose that the -866 G/A UCP2 polymorphism is involved in SLE pathogenesis, causing increased ROS levels resulting, in turn, in a pro-oxidation environment, as confirmed by increased MDA levels. Our observations, firstly, demonstrate that the common functional variant in the UCP2 gene is associated with increased oxidative stress in SLE patients, by regulating ROS generation, and highlight its potential impact upon cardiovascular risk.

Nevertheless, several limitations should be considered in our study. The main limitation is the relatively small sample size and the fact that the participants were from the same geographic area may limit the applicability of our results to the determination of the role of UCP2 and oxidative stress in atherosclerosis risk in SLE. So, the present findings in an Italian population need to be replicated in independent studies to determine whether the 866 G/A UCP2 promoter polymorphism influences MDA levels and whether it is truly implicated in the development of atherosclerosis in individuals at risk.

Lastly, in **Chapter 5**, we showed, for the first time, that patients with SSc display an up-regulation of the IL-9/IL-9R axis. Numerous studies suggest the importance of monitoring plasma levels of cytokines in patients with various AIDs and point out, in particular, the crucial role of cytokines in the pathogenesis of some of them. Cytokines are soluble factors that act as mediators for the differentiation, maturation and activation of the various immune cells. Altered levels of cytokines in the plasma would be a major cause of an immune dysregulation followed by

local inflammatory processes and tissue damage. IL-9 is a recently described pro-inflammatory cytokine because of its capacity to support proliferation of B and T cell infiltration (Lv and Wang, 2013). In combination with IL-17, IL-9 perpetuates chronic inflammation (Neurath and Finotto, 2016), as observed in several autoimmune disorders such as RA (Ciccia et al., 2016). Our investigation highlight that patients with SSc have increased expression of IL-9 both in their inflamed skin than in renal biopsy.

The principle source of IL-9 was represented prevalently by TH9 cells, as demonstrated by the co-localization of IL-9. Accordingly, with IL-9 over-expression, we observed that patients with SSc have significantly expansion of pathogenic TH9 cells compared to normal control subjects, indicating that TH9 pathway is also functional in these patients. In addition, in our study, stimulation *in-vitro* of isolated SSc B lymphocytes with IL-9 induce the production of SSc-specific autoantibodies, indicating an important role of IL-9/IL-9R axis in modulating autoimmunity in these patients. Thus, it is plausible to suppose that TH9 cells and IL-9 could play an important role in the pathogenesis of SSc by modulating the production of autoantibodies and indicate the IL-9 pathway as a possible therapeutic target.

Discussion on chapter 6

Molecular aspects in immunosenescence

In **Chapter 6**, we investigated the efficacy of triggering of TLRs to enhance immune response of elderly. As noted, immunosenescence impacts on the immune system of older people leading to an inefficient reaction to pathogens and reduced ability to develop a powerful immune response after vaccination. Recently, research in immunological ageing

have suggested that stimulation of TLRs is a promising strategy to enhance vaccine efficacy in the elderly by activation of innate immune cells and production of inflammatory cytokines. In this scenario, great importance assume DCs, the most potent APCs, specialized for the uptake, processing, transport and presentation of antigens to T cells (Collin et al, 2013). After their activation in the periphery, DCs migrate to lymphoid tissues where they interact with T and B cells to initiate and shape acquired immune responses.

In our study, we observed that stimulation with the specific combined TLR7/8 and TLR4 agonists, induces significantly increased inflammatory cytokine secretion by pDCs isolated from older subjects. This has potentially important implications, since it has been reported that reduced production of TNF-α by pDCs from old people, caused by defects in TLR pathway, is associated with an ineffective antibody response to influenza vaccination (Panda et al. 2010). The importance of TNF-α in the induction of T cell proliferation by DCs stimulation, is also evident from clinical data of patients with RA. These data report that treatment with anti-TNF-α antibodies causes poor stimulation of T cell activity by DCs (Liu et al. 2012). Impaired production of TNF-α by older DCs could result in a weak response to vaccination and contribute to the dysregulation of DC-induced T cell proliferation in elderly subjects. Thus, regulation of TNF-α secretion might be a useful strategy not only in vaccination development for elderly but also for autoimmunity treatment.

In particular, we observed that the stimulation of cells with TLR7/8 and TLR4 combined ligand, is *de facto* an excellent inducer of TNF-α and IL-12/p40 cytokines in DCs (pDCs and mDCs) from young and older subjects, with significantly high levels of cytokines compared to unstimulated samples. Interestingly, greater cytokines production is

observed after stimulation with both TLR agonist combination, than that induced by each of the individual agonist probably due to the interaction of Myd88 and TRIF-dependent pathway that enhance signal transduction.

Thus, our findings highlight the efficient effect of adjuvants in stimulation of cytokine production in the elderly, and point towards the potential use of TLR agonists in future vaccination approaches for several therapeutic strategies.

There are many possibilities in the continued study of autoimmune diseases. I would like to investigate UCP2 genetic variant as well as KIR polymorphisms and their HLA ligands more in SLE patients. First, to confirm our results, since few and discordant publications so far have replicated this association. Also might it be interesting to see if the association to is only found among SLE patients with increased oxidative stress status, or is associated with SSc too, an autoimmune disorder characterized by increased inflammation.

Moreover we observed, a linear increased of the oxidative stress parameter MDA as well as a reduction of antioxidant marker taurine in SLE patients. Oxidative stress represents an important risk factor in many AIDs. It is particularly crucial for diseases that affect particular organ and/or tissue like skin, cardiovascular tissue, kidney, etc. So, it would be interesting to understand the implication of oxidative stress marker in disease activity, in order to identify patients with specific organ involvement and also to aid in evaluating new preventive therapies before the onset of clinical symptoms or signs.

Last but not least, based on our study on triggering of TLRs in elderly people to enhance cytokine production. I would like to test the role of combined agonist and antagonist peptides on TLRs modulation and their effect in immune cells isolated from patients with AIDs. Indeed, the synergistic regulation of these receptors could be a key mechanism to suppress chronic inflammation and prevent organ damage in autoimmune patients, providing a platform for new drugs.

Mounting evidence indicate that TLRs activation can also promote autoimmune disorders causing uncontrolled auto-inflammation. In particular, TLRs expressed on B and plasmacytoid dendritic cells (pDCs),

are suggested to be respectively responsible to generate anti-nuclear antibodies with the production of IFN-I subtypes (IFN-α and -β). Interestingly, *Zeigler et al*., (2016), revealed a sex-specific differential IFN-I production in response to TLR-7 stimulation, with pDCs from healthy females showing increased IFN-α levels. This phenomenon might explain the higher risk for autoimmunity in women.

Moreover, *Depaolo et al.*, (2008), demonstrated that human DCs polarize into a pro-inflammatory or anti-inflammatory phenotype, according to TLRs activation. Exactly, association of TLR-2 with TLR-1 induced a shift toward IL-12 or IL-17 promoting pro-inflammatory cells differentiation. In contrast, TLR-2 associated with TLR-6 stimulated DCs IL-10 secretion and Tregs activation with suppression of $CD4^+$, $CD8^+$ T cells responses.

These findings shed a new light on how pro- and anti-inflammatory immune responses can be determined *in vivo* by ligands acting on DCs.

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