ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ
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Ο ΡΟΛΟΣ ΤΟΥ TLR9 ΚΑΙ ΤΗΣ IL21 ΣΤΗΝ ΠΑΘΟΓΕΝΕΙΑ ΤΟΥ ΣΥΣΤΗΜΑΤΙΚΟΥ ΕΡΥΘΗΜΑΤΩΔΟΥΣ ΛΥΚΟΥ

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

ΜΑΡΙΑ ΜΟΥΤΑΦΗ

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ΠΡΟΛΟΓΟΣ

Με την ολοκλήρωση της μεταπτυχιακής μου εργασίας θα ήθελα να ευχαριστήσω κάποιους ανθρώπους που βρέθηκαν κοντά μου τα τελευταία δύο χρόνια. Πρώτα από όλους θα ήθελα να ευχαριστήσω τους καθηγητές μου, που μου μετέδωσαν τις γνώσεις τους, και κυρίως τον καθηγητή μου κύριο Δημήτριο Μπούμπα που με εμπιστεύτηκε και με φιλοξένησε στο εργαστήριο του. Δεν θα μπορούσα να μην αναφερθώ στα μέλη των εργαστηρίων Ρευματολογίας και Παθολογίας, πρώτα από όλους στη Μάγδα που με βοήθησε σημαντικά, την Κα Χουλάκη, τον Κο Γουλιέλμο, τον Κο Σιδηρόπουλο, τον Πάνο, τη Μελίνα, τον Ηλία, τη Μαριάννα, την Ελένη, την Δέσποινα, το Γιώργο, την Ειρήνη, την Ελένη, την Εύα, την Melanie, τον Αντώνη. Ένα ιδιαίτερα μεγάλο ευχαριστώ θα ήθελα να πω στη Κα Κουταλά η οποία είχε την υπομονή να με διδάξει και να με συμβουλεύσει στις δυσκολίες που αντιμετώπισα. Θα ήθελα ακόμη να ευχαριστήσω όλα τα παιδιά του μεταπτυχιακού για τις όμορφες ώρες που περάσαμε μαζί. Ειρήνη και Δήμητρα ευχαριστώ κυρίως εσάς γιατί με στηρίξατε όταν σας είχα πραγματικά ανάγκη.

Δεν θα μπορούσα να μην αναφερθώ στα μέλη της οικογένειάς μου που 6 χρόνια που είμαι μακριά κάνουν υπομονή και με υποστηρίζουν σθεναρά. Τέλος, θέλω να ευχαριστήσω θερμά τον Ανδρέα που ήταν πάντα δίπλα μου στα ευχάριστα και τα δύσκολα.

Ο πιο μεγάλος έπαινος πιστεύω ότι ταιριάζει στην Κα Μαίρη Αδαμάκη που είναι υπεύθυνη για τα πρακτικά θέματα που αφορούν την οργάνωση του μεταπτυχιακού μας προγράμματος και είναι πάντα γλυκιά, χαμογελαστή και πρόθυμη να μας βοηθήσει και να μας συμβουλεύσει για πάσης φύσεως θέματα.

Μαρία Μουτάφη

ΠΕΡΙΛΗΨΗ

Ο Συστηματικός ερυθηματώδης λύκος (ΣΕΛ) είναι ένα πρότυπο αυτοάνοσο νόσημα με πολλαπλές κλινικές εκδηλώσεις και μη πλήρως διευκρινισμένη αιτιολογία. Δεκαετίες κλινικών ερευνών και εργαστηριακών μελετών με ζώα έχουν αποκαλύψει την εμπλοκή της φυσικής μα και της επίκτητης ανοσίας στην παθογένεια του ΣΕΛ. Ένα από τα κύρια χαρακτηριστικά της νόσου είναι η επαγόμενη από Τ λεμφοκύτταρα και μεσολαβούμενη από Β λεμφοκύτταρα παραγωγή αυτοαντισωμάτων έναντι πυρηνικών αυτοαντιγόνων. Σε αυτή τη μελέτη διερευνήθηκε, η συμβολή του ενδοκυττάριου toll- like υποδοχέα TLR9 ο οποίος αναγνωρίζει αλληλουχίες δίκλωνου DNA, και της πρόσφατα ταυτοποιημένη ιντερλευκίνης 21(IL21), στην παθογένεια της νόσου. Πρόσφατες γενετικές μελέτες συσχετίζουν πολυμορφισμούς των δύο αυτών παραγόντων με τάση για την ανάπτυξη αυτοανοσίας του ΣΕΛ. Επιπροσθέτως, η αυξημένη παραγωγή της IL21 και η εκτεταμένη ενεργοποίηση του TLR9 εμπλέκονται σε αύξηση της διαφοροποίησης των Β κυττάρων και της παραγωγής αντισωμάτων, που συσχετίζονται με την παθογένεια του ΣΕΛ σύμφωνα με έρευνες.

Με αυτή τη μελέτη προτείνεται ότι σε ασθενείς με ενεργή νόσο υπάρχει αυξημένη έκφραση της IL21. Η αυξημένη έκφραση αυτής της κυτταροκίνης, σε συνδυασμό με τον αυξημένο αριθμό Β κυττάρων και μονοκυττάρων που εκφράζουν TLR9 ενδεχομένως να έχουν λειτουργικές επιδράσεις στον ΣΕΛ. Βρέθηκε ότι η IL21 σε συνδυασμό με τη διέγερση του TLR9 επάγουν την εκτεταμένη διαφοροποίηση των ανώριμων Β κυττάρων σε πλασμακυτταροειδή Β κύτταρα. Επίσης, αυξάνουν την έκφραση του CD86 συν- διεγερτικού μορίου στην επιφάνεια των Β κυττάρων, η οποία σχετίζεται με την αντιγονοπαρουσιαστική ικανότητα των κυττάρων. Ο αυξημένος αριθμός πλασμακυτταροειδών κυττάρων που προέκυψε παρουσία συνδυασμού IL21 και TLR9 διεγερτών ενδεχομένως να αντικατοπτρίζει την συμβολή τους στην απορύθμιση της λειτουργίας των Β κυττάρων και στην αυξημένη παραγωγή αυτοαντισωμάτων σε ασθενείς με ΣΕΛ. Επιπροσθέτως, ίσως η αυξημένη έκφραση του CD86 να υποδηλώνει την ενισχυμένη ικανότητα των Β κυττάρων να ενεργοποιούν ανώριμα Τ κύτταρα. Η επίδραση της διέγερσης του TLR9 στην αντιγονοπαρουσιαστική ικανότητα μελετήθηκε και σε μονοκύτταρα χωρίς σημαντικές διαφορές μεταξύ ασθενών ΣΕΛ και υγιών του δείγματος ελέγχου. Επίσης δείχθηκε ότι δενδριτικά κύτταρα που διαφοροποιήθηκαν από μονοκύτταρα στο εργαστήριο δεν εκφράζουν τον υποδοχέα TLR9 ούτε στην περίπτωση υγιών αλλά ούτε και ασθενείς με ΣΕΛ.

Συνοψίζοντας, η υπερπαραγωγή της IL21 και η ενισχυμένη ενεργοποίηση του TLR9 πιθανότατα λειτουργούν συνεργατικά στην παθογένεια του ΣΕΛ επάγοντας την διαφοροποίηση των

ανώριμων Β κυττάρων σε πλασμακυτταροειδή και ενισχύοντας την αντιγονοπαρουσιαστική τους ικανότητα. Η αυξημένη διαφοροποίηση των Β κυττάρων ίσως συσχετίζεται με την ενισχυμένη παραγωγή αυτοαντισωμάτων, που είναι ένα κοινό χαρακτηριστικό του ΣΕΛ. Ακόμη, η επαυξημένη αντιγονοπαρουσιαστική δυνατότητα των Β κυττάρων μπορεί να ευθύνεται για την μη φυσιολογική ενεργοποίηση των Τ κυττάρων σε αυτή την αυτοάνοση νόσο.

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The role of IL21 and TLR9 in the pathogenesis of Systemic Lupus Erythematosus

MASTER THESIS

MARIA MOUTAFI

Heraklion 2008

SUMMARY

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease with multiple clinical manifestations and unclear etiology. Decades of clinical research and animal studies have indicated that both the innate and the adaptive immune system are involved in the pathogenesis of SLE. The main characteristic of the disease is the T cell induced– B cell mediated autoantibody production, against nuclear autoantigens. In this study, the contribution of the intracellular toll- like receptor 9 (TLR9) that recognizes dsDNA and the recently discovered interleukin 21 (IL21) is assessed in the disease pathogenesis. Genetic studies have associated polymorphisms of these two factors with susceptibility to SLE development. In addition IL21 overproduction and TLR9 activation are implicated in enhanced differentiation of naïve B cells and antibody responses, which correlate with SLE pathogenesis.

Here, it is shown that IL21 in combination with TLR9 stimulation promote the robust differentiation of patient naïve B cells into plasma cells and induce the expression of CD86, a co-stimulatory molecule that is related to antigen presenting capacity. The increased numbers of plasma cells that occurred in the presence of IL21 and TLR9 agonist may reflect their contribution to the aberrant function of B cells and the great numbers of auto- antibodies detected in SLE patients. In addition, the increase in the expression of CD86 may indicate the enhanced ability of B cells to activate naïve T cells. The effect of TLR9 stimulation was assessed on the antigen presenting capacity of monocytes, which also have increased TLR9 expression in SLE, with no significant results. In addition, TLR9 was found not to be expressed in monocyte derived dendritic cells from SLE patients, as in healthy controls.

Presumably, the overproduction of IL21 and the enhanced TLR9 activation may act synergistically in the pathogenesis of SLE by inducing the differentiation of naïve B cells into plasma cells and enhancing B cell antigen presenting capacity. The accelerated plasma cell differentiation may be related to the augmented production of autoantibodies, a common hallmark of SLE. In addition, the enhanced capacity of B cells to present antigens may account for the aberrant activation of naïve T cells in this autoimmune disease.

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A. INTRODUCTION

A.1. Innate and Adaptive Immunity

The term “immunity” originates from the Latin word “*immunis*” for “exempt from public service or charge” (Oxford dictionary) and describes the state of being protected from something and the ability to avoid or not be affected by infection and disease. The mammalian immune system consists of many types of proteins, cells, tissues and organs which interact in an elaborate and dynamic network. The main function of this system is to defend against a wide variety of infectious agents, such as bacteria, viruses, fungi and parasites, that tend to change the homeostatic balance.

The first line of defense, that the invading pathogens encounter, consists of cells and mechanisms of the *innate immune system*. This system confers an immediate immune response but unlike the *adaptive immune system*, this response is not specific and does not create memory. The adaptive immune system consists of highly specialized cells that upon recognition of threatening agents trigger an immense immune response, antigen specific, with the ability to memorize and act immediately at a second invasion (*immunological memory*). The innate and the adaptive “branch” of the immune system are linked via *antigen presentation*. This is a process upon which *antigen presenting cells* (APCs) capture antigens, process them and present them on their surface, linked with major histocompatibility complex (MHC) molecules. This procedure is of great importance for the discrimination between self and non- self. The MHC- antigen complex is recognized by cells with highly specific receptors that decide whether to start an adaptive immune response or not (*immunity* or *immunological tolerance*) [23].

A.1.1. The cells of the immune system

Most of the cells of the immune system originate from progenitor stem cells in the bone marrow. Some of them patrol or reside in the periphery (blood stream and most of the tissues) in order to detect possible dangers and other cells reside in specialized lymphoid tissues. The immune system cells are equipped with a large array of extracellular or intracellular receptors. These receptors let them sense environmental signals and interact with other cell types or identify material that is foreign to the body. In response to these signals, cells have the ability to change their gene expression profile and further differentiate, or mature, express receptors and secrete cytokines in order to alert other cell types [23].

The antigen presenting cells

Most cells of the body express MHC class I molecules and they are able to present antigens to CD8+ T cells. However there are three types that can present MHC Class II linked-antigens, to naïve CD4+ T cells (Th cells). These are the dendritic cells, the macrophages/ monocytes, and the B cells, also called *professional antigen presenting cells*. The professional APCs are very efficient in internalizing antigens and exposing them on their surface, as well as upregulating co- stimulatory signals that lead to the activation of naïve T cells. The internalization of antigen is performed by phagocytosis or via specific membrane receptors [23].

Dendritic cells *(DCs)* are the most potent, professional APCs and their role is of great importance in both the innate and the adaptive immunity [53, 54]. Morphologically, they are characterized by large projections and they are found in almost every tissue that forms an interface with the environment, in the lymphoid organs and in most other organs of the body. The great diversity of DC subsets is related with the wide spectrum of responses against different pathogens and the different requirements for T and B cell priming. In addition, the polarization into distinct T cell types (e.g. Th1, Th2, Tregs, Th17) might be due to their interaction with specific DC subtypes. The type of the T cell response (Tolerance Vs Immunity, Th1 Vs Th2, Tregs Vs Th17) that will occur after the antigen presentation to T cells is related to the type and the maturation stage of the dendritic cells, and the environmental signals that they receive [53, 54].

The monocytes that circulate in the blood stream have two roles in the immune system. First of all, they replenish the macrophages and the dendritic cells under normal conditions. Secondly, in response to inflammatory signals, they migrate to the site of inflammation and they differentiate into macrophages or dendritic cells. Monocytes have the ability to phagocytose foreign material either via opsonization or by binding directly to it via *pattern recognition receptors (PRRs)* that recognize molecular patterns of the pathogen. After a few days in the blood stream, monocytes migrate to various tissues and they differentiate into tissue resident macrophages or DCs with distinct morphologies and names, depending on the tissue. ***Macrophages*** are phagocytes that develop from *monocytes* and their role is to phagocytose cell debris and pathogens. They are able to present antigens to lymphocytes and secrete cytokines, chemokines and complement proteins [23].

B cells apart from their antigen presenting capacity have one unique function. They have the ability to recognize antigens and secrete soluble immunoglobulins -also called *antibodies*- against them. Antibodies are the secreted form of the membrane bound B cell receptor (BcR) that recognizes exactly the same antigen. Apart from the recognition of an antigen by BcR receptor, B cells need additional signals by T helper cells that recognize the antigen bound to MHC Class II. Hence, B cells can further differentiate into one of the two different types, *plasma B cells* or *memory B cells*. Plasma cells are the "antibody producing factories" of the body. They are large with great endoplasmic reticulum (ER) and they are short- lived. On the other hand, memory B cells are long- lived and can respond quickly upon second exposure to the same antigen. The differentiation into these two B cell types may include changes in the B cell DNA that modify the antibodies produced. *Somatic hyper- mutation* results in changes in the antigen- binding region of the antibody and alters its antigen binding capacity. *Immunoglobulin class switch* is a change in the antibody class (which is initially IgM and IgD) into other immunoglobulin classes (IgG, IgA or IgE) that trigger different immune reactions. Antibodies and B cells, in contrast to other cell types recognize antigens in their native form, which means not processed by other cells.

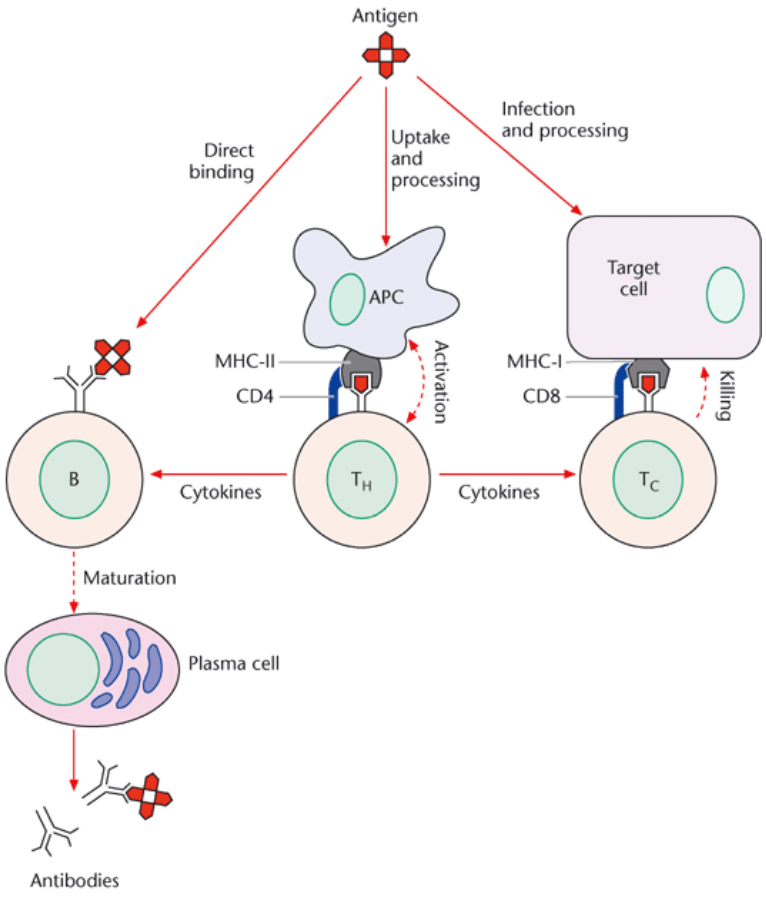


Figure a.1: The interaction between innate and adaptive immunity. Antigen presenting cells recognize pathogen associated molecular patterns and upregulate the expression of surface molecules that facilitate antigen presentation to T cells. B cells after recognition of the pathogen differentiate into antibody secreting plasma cells or memory B cells. This interplay of cells and cytokines leads to the complete eradication of the pathogen.

A.1.2. The cytokines

Cytokines are small proteins or glycoproteins that are secreted by hematopoietic or non-hematopoietic cells in response to immune stimuli. Cytokines play crucial role in the regulation of the innate and the adaptive immune system. They are usually produced by immune system cells after identification of danger signals and they travel around the body to recruit more leukocytes. Each cytokine binds on a specific cell surface receptor on the target cells. This results in the activation of an intracellular cascade that modifies the gene transcription "program" of the cell. Consequently, the target cell may produce other cytokines and/or upregulate or down-regulate the expression of cell surface receptors and change the way it responds to the environment. Cytokines are classified according to their structure or their function in the immune system. One important classification divides the cytokines in type I (IFN-γ, TGF-β) that favor cytokine responses and type II (IL4, IL10, IL13) that favor antibody responses. The type I and type II responses seem to be mutually exclusive in clinical and experimental practice.

A.1.3. Toll- Like Receptors

Toll- like receptors (TLRs) are a family of pattern recognition receptors that identify *pathogen associated molecular patterns* (PAMPs). TLRs appeared early in the development of animal species, before the development of adaptive immunity. These receptors reflect a conserved signalling system that has homologous components in a wide range of organisms. Mammalian TLRs share structural similarities with the *Drosophila* Toll protein that has both developmental role in the generation of dorsal-ventral axis and immunological role in the defense against fungi.

The primary function of TLRs is to alert the innate immune system by identifying structural components of pathogens. Consequently, they trigger activation cascades that lead to the production of proinflammatory cytokines and antimicrobial peptides. In addition, TLR ligation can stimulate macrophages, dendritic cells and other APCs, to secrete soluble factors or upregulate an array of co-stimulatory molecules that allow a more efficient presentation to the cells of the adaptive immune system (T and B cells). TLRs are also expressed in T and B cells and upon additional stimulatory signals can promote their survival, proliferation and antibody production by B cells (Reviewed in [60]).

There are at least 11 different mammalian Toll- like receptors and they are differentially expressed in cells of the immune system, in epithelial and endothelial cells. The cytoplasmic part of TLRs show high similarity to that of the interleukin-1 receptor (IL-1R) family and is called the Toll/IL-1 receptor (TIR) domain. A TIR domain is required to initiate intracellular signaling. The extracellular regions of TLRs contain leucine-rich repeats. Each TLR has its own intrinsic signaling pathway and induces specific biological responses against microorganisms. Most TLRs are expressed on the cell surface. A subset of TLRs, including TLR3, TLR7, TLR8, and TLR9, are expressed intracellularly within one or more endosomal compartments.

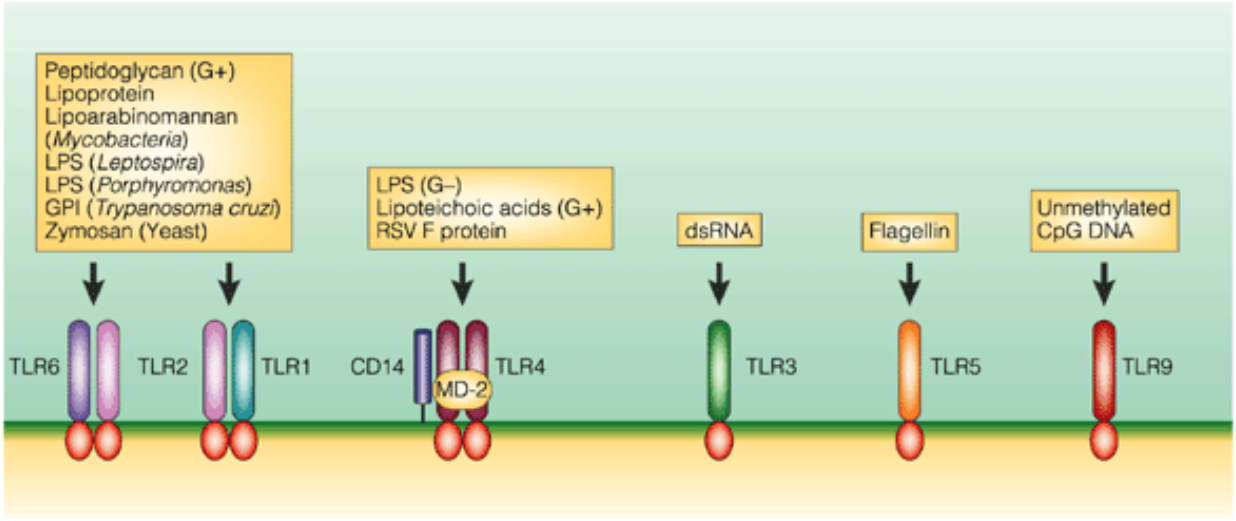


Figure a.2: The mammalian Toll– like receptors and their ligands.

TLR ligands act as adjuvant to an immune response. They are conserved molecular patterns of pathogenic micro- organisms that are not produced by mammalian cells under normal circumstances. Genetic studies and knock-out mice experiments have revealed the specific targets of each TLR. The gram negative lipopolysacharide (LPS) is identified by TLR4, TLR2 that can form heterodimers with TLR1 and TLR6 identifies gram positive bacterial counterparts and TLR5 recognises the fungal protein, flagelin. The intracellular TLRs usually recognise nucleic acids from endocytosed pathogens. TLR7 and TLR8 recognize single- stranded RNA (ssRNA) and imidazoquinoline, TLR3 recognizes double- stranded RNA (ds RNA) and TLR9 recognizes hypomethylated CpG containing DNA [44].

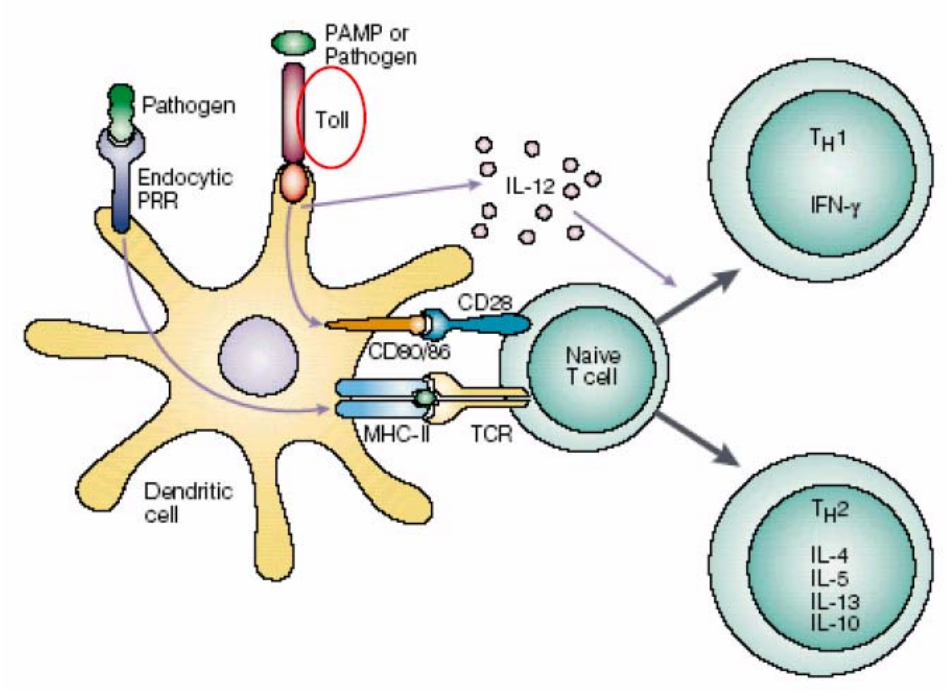


Figure a.3: TLR stimulation promotes the activation of the adaptive immune system. Upon pathogen–associated pattern recognition by TLRs, the expression of co– stimulatory and MHC molecules on APC surface is upregulated. In addition, TLR stimulation triggers the secretion of cytokines and chemokines. These processes, lead to the activation of naïve T cells and their polarization into Th1 or Th2 T helper cells.

A.2. Self tolerance and autoimmunity

One of the most important traits of healthy immune system is the ability to discriminate self- from non- self material and not to trigger immune response against its own components. This is called *self tolerance*. The sophisticated mechanisms of tolerance induction can be divided in two categories; the *central* and *peripheral tolerance mechanisms*. Under normal conditions, the autoreactive T and B cells are eliminated before they exit the primary lymphoid organs (thymus and the bone marrow respectively), where they "see" for the first time self- antigens (*auto- antigens*). A fraction of auto- reactive cells manages to escape elimination and exits the primary lymphoid organs. These cells are exposed to the mechanisms of peripheral tolerance [23]. These mechanisms include peripheral anergy, ignorance, or deletion or suppression by inhibitory cytokines and regulatory T cells (Table:1).

Mechanisms of tolerance	*Mechanism*	*Site of action*
Central tolerance	*Deletion* *Editing*	*Thymus* *Bone marrow*
Antigen segregation	*Physical barrier to self- antigen access to lymphoid system*	*Peripheral organs*
Peripheral anergy	*Cellular inactivation by weak signaling without co- stimulus*	*Secondary lymphoid tissue*
Clonally exhaustion	*Apoptosis post- activation*	*Secondary lymphoid tissues and sites of inflammation*
Clonally ignorance	*Certain auto- antigens are undetected*	*Periphery*
Regulatory cells	*Suppression by cytokines, intercellular signals*	*Secondary lymphoid tissues and sites of inflammation*
Cytokine deviation	*Differentiation into Th2 cells. Limiting inflammatory cytokine secretion*	*Secondary lymphoid tissues and sites of inflammation*

Table 1: Mechanisms of central and peripheral tolerance.

Autoimmunity is the inappropriate response of the immune system against self components. When this results in tissue damage and deregulation of the immune system the suitable term is *autoimmune disease*. These diseases can be divided into two distinct categories, the *organ specific* and the *systemic autoimmune diseases*. In organ specific autoimmune diseases, such as type I diabetes mellitus, the immune system attack is restricted to one organ or organ system. In contrast, systemic autoimmune disease- targets are found all over the body. Prototypic systemic autoimmune diseases are rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

Autoimmune diseases result from the interaction of multiple factors which either determine susceptibility to disease or trigger autoimmune responses. Clinical studies, as well as animal model experiments suggest that autoimmune diseases should be regarded as polygenic diseases, of which the penetration is strongly influenced by environmental factors. The most important environmental factors that affect their development are infectious agents, dietary causes, toxic substances or drugs and stress. These factors may have a robust impact on the regulation of immune tolerance.

The pathogenesis of autoimmune diseases can be divided in three phases. In the first phase, there is enhanced antigen presentation by APC, followed, in a second phase, by aberrant activation of autoreactive B and T cells. The last phase of the pathogenesis development includes the effects of autoreactive lymphocytes against the target tissues. Apart from the involvement of the adaptive immune system in the breakage of tolerance, there are indications for the innate immune system participation. Toll- like receptors that normally detect pathogens and trigger innate and sometimes adaptive immune responses, are one possible suspect for the pathogenesis of autoimmune diseases.

A.3. Systemic Lupus Erythematosus

A.3.1. Pathophysiology of the disease

Systemic Lupus Erythematosus (SLE) is a highly variable in severity, systemic autoimmune disease with multiple clinical manifestations. Typical features are arthritis, photosensitivity, malar rash, discoid skin lesions, vasculitis, myocarditis, pleuritis, renal disorders and central nervous system lesions. SLE is more prevalent in females (9:1 ratio over males) and the overall prevalence has notable geographical variation (1.9 per 100.000 inhabitants in northwestern Greece) [1]. It can occur at any age but its typical onset is during the late adolescence or early adulthood. The serological hallmark of SLE is the presence of high titer of IgG auto- antibodies that recognize intracellular or intra- nuclear antigens such as double stranded DNA (dsDNA) and nucleoproteins. These auto-antibodies form immune complexes (ICs) that result in tissue injury.

In 1982, the Diagnostic and Therapeutic Criteria Committee of the American College of Rheumatology (ACR) published revised criteria for the classification of SLE [57] (Table:2). Any combination of four or more of the eleven criteria, well-documented at any time during a patient's history makes it likely that the patient has SLE. The activity of the disease is defined by the SLE disease activity index (SLEDAI) [4]. There are other disease activity indices for SLE, but SLEDAI is commonly used and has been validated in several clinical trials. The SLEDAI assessment system is presented in Table 3. The sum of the weights of each clinical or laboratory manifestation is the total score that defines the SLE activity.

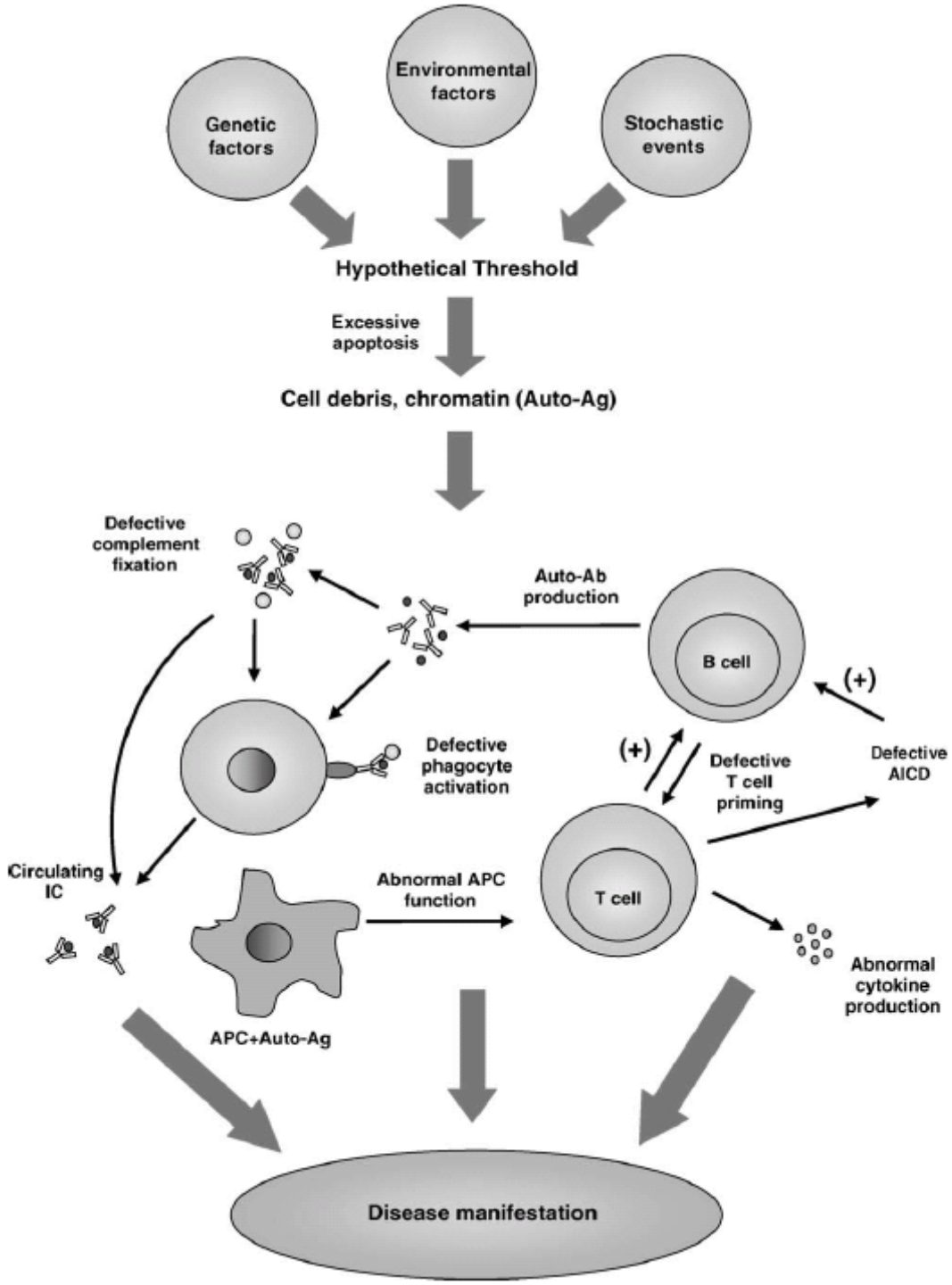


Figure a.4.: Schematic representation of the possible mechanisms that lead to SLE pathogenesis. A number of genetic or environmental factors may lead to increased apoptosis, which results in abundance of autoantigens. Defects in the action of phagocytic cells contribute to the prolonged availability of cell debris that can be phagocytosed and interpreted as foreign by the antigen presenting cells. Dysfunction of the innate and the adaptive immune system forms the basis for autoimmunity. The unsuccessful clearance of autoreactive lymphocytes may lead to strong adaptive immune responses. All these alterations result in tissue damage mediated by deposited immune complexes, immune cells and abnormal production of inflammatory cytokines.

	Diagnostic and Therapeutic Criteria according to the American College of Rheumatology	*Description*
1	*Malar rash*	*Fixed erythema, flat or raised, over the malar eminences*
2	*Discoid rash*	*Erythematous circular raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur*
3	*Photosensitivity*	*Exposure to ultraviolet light causes rash*
4	*Oral ulcers*	*Includes oral and nasopharyngeal ulcers, observed by physician*
5	*Arthritis*	*Nonerosive arthritis of two or more peripheral joints, with tenderness, swelling, or effusion*
6	*Serositis*	*Pleuritis or pericarditis documented by ECG or rub or evidence of effusion*
7	*Renal disorder*	*Proteinuria >0.5 g/d or 3+, or cellular casts*
8	*Neurologic disorder*	*Seizures or psychosis without other causes*
9	*Hematologic disorder*	*Hemolytic anemia or leukopenia (<4000/L) or lymphopenia (<1500/L) or thrombocytopenia (<100,000/L) in the absence of offending drugs*
10	*Immunologic disorder*	*Anti-dsDNA, anti-Sm, and/or anti-phospholipid*
11	*Antinuclear antibodies*	*An abnormal titer of ANA by immunofluorescence or an equivalent assay at any point in time in the absence of drugs known to induce ANAs*

Table 2: Diagnostic criteria according to the American College of Rheumatology

SLE Disease activity Index (SLEDAI)		
Weight	*Description*	*Definition*
8	*Seizure*	*Recent onset (last 10 days). Exclude metabolic, infectious or drug case, or seizure due to past irreversible CNS damage.*
8	*Psychosis*	*Altered ability to function in normal activity due to severe disturbance in perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behaviour. Exclude uremia and drug case.*
8	*Organic Brain Syndrome*	*Altered mental function with impaired orientation, memory or other intelligent function, with rapid onset fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious or drug causes.*
8	*Visual Disturbance*	*Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serious exodate or hemorrhages in the choroids, or optic neuritis. Exclude hypertension, infection, or drug causes.*
8	*Cranial Nerve Disorder*	*New onset of sensory or motor neuropathy involving cranial nerves.*
8	*Lupus Headache*	*Severe persistent headache: may be migrainous, but must be nonresponsive to narcotic analgesia.*
8	*CVA*	*New onset of cerebrovascular accident(s). Exclude arteriosclerosis*
8	*Vasculitis*	*Ulceration, gangrene, tender finger nodules, periungual, infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.*
4	*Arthritis*	*More than 2 joints with pain and signs of inflammation (i.e. tenderness, swelling, or effusion).*
4	*Myositis*	*Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/adolase or electromyogram changes or a biopsy showing myositis.*
4	*Urinary Casts*	*Heme-granular or red blood cell casts*
4	*Hematuria*	*>5 red blood cells/high power field. Exclude stone, infection or other cause.*
4	*Proteinuria*	*>0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.*
4	*Pyuria*	*>5 white blood cells/high power field. Exclude infection.*

2	*Rash*	*New onset or recurrence of inflammatory type rash.*
2	*Alopecia*	*New onset or recurrence of abnormal, patchy or diffuse loss of hair.*
2	*Mucosal Ulcers*	*New onset or recurrence of oral or nasal ulcerations.*
2	*Pleurisy*	*Pleuritic chest pain with pleural rub or effusion, or pleural thickening.*
2	*Pericarditis*	*Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram confirmation.*
2	*Low complement*	*Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.*
2	*Increased DNA Binding*	*>25% binding by Farr assay or above normal range for testing laboratory.*
1	*Fever*	*>38°C. Exclude infectious cause.*
1	*Thrombocytopenia*	*<100,000 platelets/mm3*
1	*Leukopenia*	*<3,000 White blood cell/mm3. Exclude drug causes.*

Table 3: The SLE disease activity index for assessment of the disease activity

Although many different scenarios for the development of SLE have been reported (Figure 4), the exact mechanism that results in SLE pathogenesis remains elusive [29]. One possible explanation is that intracellular autoantigens become 'visible' to the immune system during apoptotic cell death caused by environmental factors. The defective clearance of apoptotic cell debris may be a reason of the great abundance of autoantigens. [15, 16, 17, 18, 38, 59]. Subsequent uptake and processing by activated APCs can leads to presentation of autoantigens. The presented autoantigens can be recognised by autoreactive lymphocytes, which may have escaped the mechanisms of tolerance. The action of T cells and B cells is also deregulated in SLE. B cells overproduce antibodies against nuclear antigens, which form immune complexes that promote tissue damage. T cells from SLE patients promote the activation of B cells to produce IgG in the absence of any antigen or mitogen. In addition T cells overproduce CD40L that binds on B cell CD40 surface molecule and promotes B cell activation [29]. The function of regulatory T cells that normally prevent autoimmunity seems to be impaired in SLE. Recent study provides evidence that the reduced numbers of Tregs and their failure to exert their suppressive function is related to IFNα that is overproduced in SLE [63]. All these mechanisms that have been described contribute to the perpetuation of autoimmune responses and tissue damage in SLE.

A. 3.2. The role of TLR9 in the pathogenesis of SLE

TLR9 is an intracellular receptor of the PRR family that recognizes hypomethylated CpG motifs of DNA [19]. It is localized in the endoplasmic reticulum (ER) and co-localizes with CpG DNA in the lysosolmal compartments of several immune cells. Hypomethylated CpG motifs are more abundant in microbial DNA than in the mammalian hence, TLR9 is able to distinguish between self- and non- self DNA. TLR9 ligation triggers an NFκB mediated pathway that leads to the expression of proinflammatory cytokines, such as IL1β, IL6, IFN-a, and TNF-a [36]. MyD88 (myeloid differentiation primary- response gene 88) is an adaptor protein, required for TLR signaling (except TLR3), that also accumulates in these compartments. According to Ruprecht and colleagues, TLR9 activation upon BcR stimulation can promote proliferation, isotypic switch and differentiation into antibody secreting cells [48]. This mechanism, under normal circumstances, provides a "safety valve" to the immune system since it permits activation by "innate" signals only upon "adaptive" recognition of antigens. In addition, TLR9 stimulation in the absence of BcR signaling supports naïve B cell proliferation and enhanced antigen presenting function [24].

A plethora of scientific studies provide evidence for TLR9 signaling involvement in SLE pathogenesis [25] (Figure A.5.). Whether TLR9 signaling has positive or negative effect in the production of pathogenic autoantibodies and the development of clinical features is a matter of controversy. Recent study of our laboratory indicates that an increased proportion of B cells and monocytes express TLR9 among patients with active SLE compared to patients with inactive disease and healthy individuals. Moreover, the increased numbers of TLR9 expressing B cells positively correlate with high titers of anti- dsDNA autoantibodies [41]. On the other hand, recent genetic study describes two alleles which down-regulate TLR9 expression in a reporter assay and are associated with increased SLE susceptibility, thus implying that the normal TLR9 expression prevents SLE development [58].

Different murine models that mimic one or more features of SLE have been described. Defects in either apoptosis or clearance of immuno-stimulatory cell debris, or BCR signaling are the pathogenic reason for *lupus- like disease* in experimental models [28]. Double knock- out mice for the *lpr* mutation do not express a functional form of the FAS death receptor. TLR9 deficient *lpr/lpr* mice fail to produce anti- dsDNA antibodies, in contrast to their TLR9- efficient littermates [67]. This implies that the production of anti- dsDNA antibodies is TLR9 dependent. The lupus- prone C57BL/6 mice fail to express the inhibitory receptor FcγRIIb. In the case of

their TLR9 deficient littermates, B cells fail to induce class switching to the pathogenic IgG2a and IgG2b isotypes. This study confirms that TLR9 has a crucial role in the production of antibodies against DNA and nucleoproteins [11]. In addition, administration of inhibitory oligonucleotides (ODNs) to MRL- *lpr* lupus prone mice ameliorates the severity of clinical features [45, 2]. According to Leadbetter and colleagues, both BcR and TLR9 stimulation of B cells is involved in increased production of autoantibodies (*"two receptor paradigm"*). Now it is admitted that immune cells can respond to DNA in a TLR9 dependent or independent way [5, 63].

	Active SLE	Inactive SLE	Controls
CD3+ cells			
n	20	10	11
% expression	19.4 ± 14.0	12.4 ± 8.2	16.1 ± 13.5
MFI	13 ± 4	13 ± 5	12 ± 3
CD19+ cells			
n	19	15	12
% expression	49.5 ± 24.4†	22.8 ± 19.6	35.2 ± 16.0
MFI	19 ± 23	11 ± 2	12 ± 4
CD14+ cells			
n	25	12	11
% expression	30.7 ± 24.1‡	14.3 ± 8.4	13.4 ± 9.5
MFI	30 ± 21	47 ± 47	39 ± 23

* Values are the mean ± SD. TLR-9 = Toll-like receptor 9; PBMCs = peripheral blood mononuclear cells; MFI = mean fluorescence intensity.

Table 4: Expression of TLR9 in PBMCs from patients with active and inactive SLE and healthy individuals.

In contrast to the studies that suggest a negative role of TLR9 in murine lupus- like pathogenesis, there are studies that propose the opposite [65]. It has been demonstrated that genetic loss of TLR9 signaling results in accelerated disease progress and high mortality of *lpr* mice [62, 31]. This means that TLR9 protects against murine lupus-like syndrome. Presumably, TLR9 signals differentially affect B cell tolerance breakdown, antibody responses, and development of disease pathology, depending of the genetic background of the experimental mice [12]. Recent studies provide evidence that TLR9 promotes the induction of Tregs by pDCs [36]. This may explain in part the induction of tolerance and the contradictory results. However, TLR9 stimulation on B cells promotes their activation and tolerance breakdown.

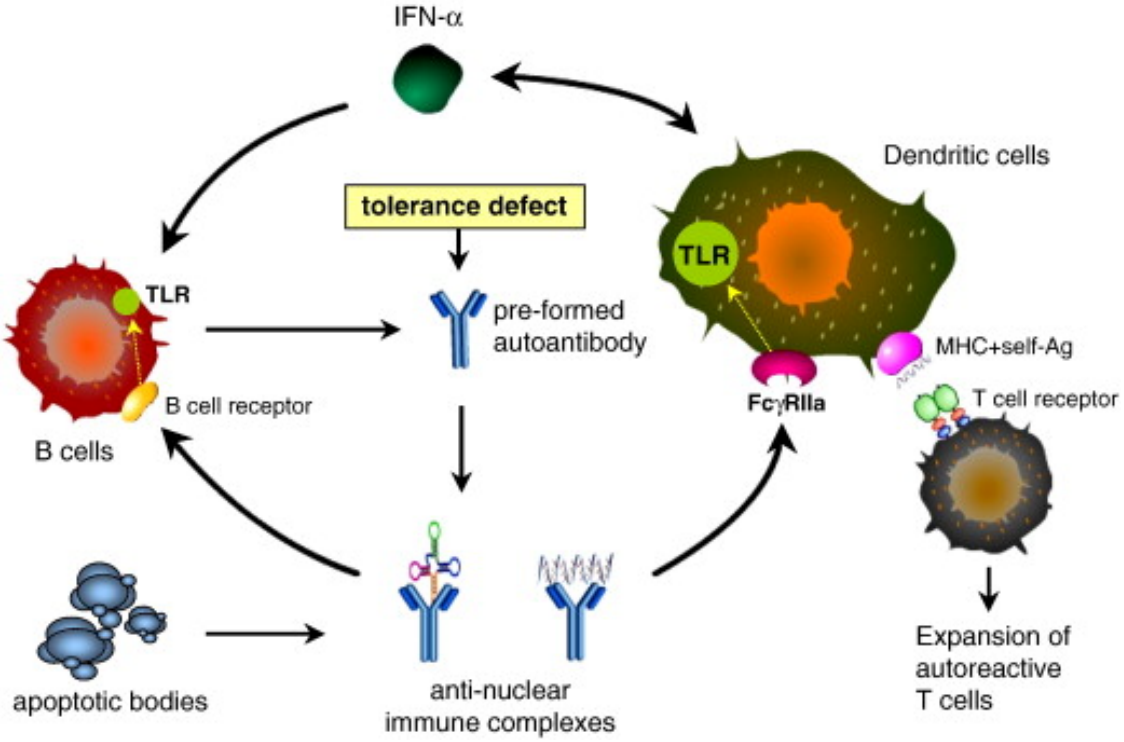


Figure a.5.: Schematic representation of the TLR9 hypothesis in SLE. A preceding defect in tolerance induction and clearance of apoptotic bodies results in the formation of DNA containing immune complexes (ICs). These ICs are phagocytosed antigen presenting cells and are transported into the endosomal compartments where DNA interacts with TLR9. In response, plasmacytoid dendritic cells produce IFNα, which induces the expression of MHC and costimulatory molecules that increase the antigen presenting capacity of professional APCs. DNA containing ICs also, directly stimulate autoreactive B cells by dual engagement on BcR and TLR9. Stimulation of TLR9 leads to B cell proliferation, differentiation and Ig class switch. The antibodies that are produce perpetuate the IC formation and create a vicious circle in SLE pathogenesis.

Among the cells that express TLR9 are the monocytes and the macrophages. These cells have multiple roles in the pathogenesis of SLE. They act as APCs, as phagocytes that uptake apoptotic cell debris and as effector cells that upon stimulation produce and secrete proinflammatory cytokines, such as IL1, TNFα, IL10, IL6 and IFNα. The phagocytic activity of macrophages has been found to be impaired in SLE. SLE macrophages have reduced capacity to engulf apoptotic cells, which may contribute to the pathogenesis of lupus since newly presented intracellular antigens become apparent [59]. Recent study of our laboratory have shown that there is increased expression of TLR9 in CD14+ macrophages in active lupus patients compared to inactive patients and healthy controls [41]. The effect of TLR9 on human CD14+ macrophages should be further examined.

Patients with SLE have a 50–100-fold decrease in the number of DCs circulating in the blood [8, 3, 40]. This decrease appears to be caused by cell migration into peripheral lymphoid tissues and sites of inflammation. Due to the small number in the peripheral blood and other accessible tissues and the high variability of DC population, their manipulation is difficult. Usually, the dendritic cells that are used in these studies have developed by monocytes *in vitro* that differentiate in the presence of GM- CSF and IL4 and mature in the presence of several stimuli such as lipopolysacharide (LPS) and TNFa. It was recently suggested that human moDCs express TLR9 and they can mature in response to TLR9 stimulation, using CpG ODN D19 [21]. The study of DCs in autoimmunity is of great importance since they have central roles in both the generation of immunity and tolerance. The inability of DCs to delete autoreactive T cells, to induce regulatory T cells and their ability to present autoantigens and overproduce inflammatory cytokines, is correlated with the development of autoimmunity.

A. 3.3. The role of IL21 in the pathogenesis of SLE.

Similarly to TLR9, IL21 has been implicated to the pathogenesis of SLE by promoting autoantibody production by B cells. IL21 is a recently discovered type I cytokine with immunomodulatoty action [6]. It is produced by T, NKT and Th17 cells and it has multiple effects on several immune cell populations (Figure A.6). Its receptor, IL21R consists of the specific IL21aR chain and the common γ chain (γ_c) that is shared with IL2, IL4, IL7, IL9 and IL15. IL21R is expressed on T cells, B cells, NK cells and DCs. IL21 acts on Th17 cells in an autocrine manner, since it is critical for their development. Its action is stimulatory or inhibitory with respect to the cell population [34]. IL21 promotes the proliferation of lymphoid cells and it increases the cytotoxicity of CD8+ T cells and NK cells. Moreover, regarding the co- existing stimuli (CD40L, IL2, TLR4), IL21 can cause proliferation, differentiation or apoptosis of B cells [14, 27, 39]. Apoptosis dominates upon non- specific stimulation- for example by TLRs (LPS, CpG ODNs). When there is BcR stimulation and costimulatory signals (e.g CD40L ligation), IL21 promotes B cell proliferation. Animal model experiments have revealed that this cytokine promotes the polarization towards the Th17 pathway instead of Tregs.

Recent genetic studies describe polymorphisms of the IL21 gene that are genetically associated with SLE [49, 55, 56]. The involvement of IL21 in SLE pathogenesis is supported by animal model experiments, as well. The initial observation was that the lupus prone BXSB.B6-*Yaa*+ mice express high levels of IL21 [39]. The high levels of IL21 is consistent with the increased IgG levels detected in these mice, and the fact that IL21 promotes B cell differentiation

into antibody- secreting plasma cells [39]. Additional evidence for IL21 involvement in the SLE pathogenesis was given by the *sanroque* mutant mouse. These mice have amplified levels of a follicular T helper cell population that produce increased amounts of IL21. Interestingly, they display increased levels of anti-nuclear antibodies and hallmarks of lupus pathogenesis [20, 61].

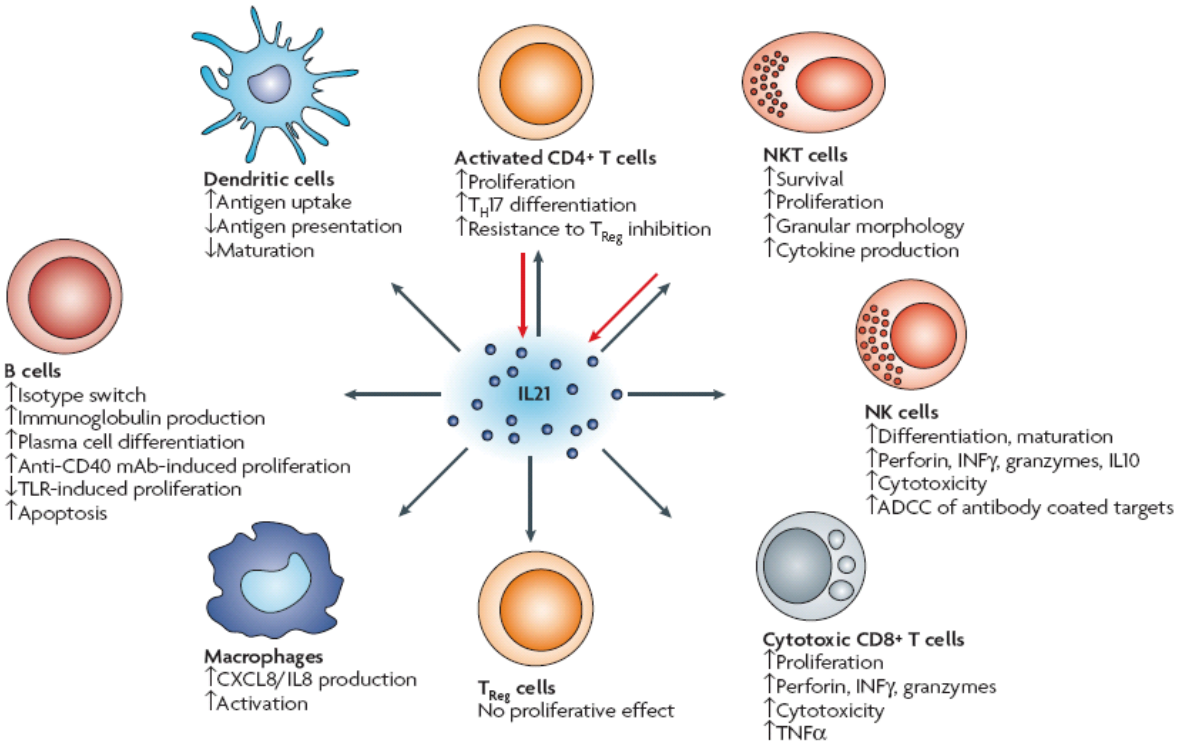


Figure a.6: Pleiotropic immune modulation by IL21.

A.4 The biological question and study hypothesis

Systemic lupus erythematosus (SLE) is a prototype systemic autoimmune disease with contribution of both the innate and the adaptive immune system. Although a plethora of mechanisms have been proposed, the exact cause of tolerance breakdown and disease pathogenesis remains unknown. Undoubtedly, environmental factors play a major role in the initiation and the development of SLE in a susceptible genetic background.

A possible contributor to the disease pathogenesis is the intracellular Toll- like receptor 9 (TLR9). The fact that TLR9 identifies dsDNA, and that one common feature of SLE is the abnormally high titer of anti-nuclear antibodies, make the study of this receptor interesting. Indeed, according to a number of animal- model and human studies TLR9 is implicated in SLE pathogenesis. Recent study of our laboratory [41], gives evidence that there is expansion of TLR9 expressing B cells and monocytes in active SLE patients, compared to patients in remission and healthy controls. The high expression in B cells correlates with the elevated titers of anti-dsDNA antibodies [41]. Recent studies indicate that TLR9 activation promotes B cell survival, proliferation or even differentiation into antibody secreting cells or memory cells, depending on the additional stimuli, under normal circumstances.

TLR9 is also expressed in monocytes and dendritic cells. Although many studies indicate that TLR9 signaling affects the phagocytic activity of monocytes it is not yet known whether it affects their antigen presenting capacity. In addition, according to one study, monocyte derived DCs express TLR9 and they are able to respond to ODNs. This finding should be verified, since it is contrary to a number of other studies according, to which these cells do not express TLR9 receptor.

In addition to TLR9 activation, high levels of IL21 seem to be involved in the production of autoantibodies in SLE. IL21 promotes isotype switch, immunoglobulin production and differentiation into plasma cells, depending on additional stimuli. The possible involvement of IL21 in SLE pathogenesis was first revealed by mice that expressed high levels of this cytokine and had hallmarks of lupus pathogenesis. Additionally, human genetic study has revealed association of IL21 polymorphisms with SLE susceptibility. However, the exact functional role of IL21 in the pathogenesis of SLE in humans has not been extensively examined.

The examination of TLR9 and IL21 contribution in the pathogenesis of SLE was the aim of this study. The increased numbers of TLR9 expressing B cells and monocytes, as well as the increased IL21 levels in active SLE patients, found during this study, gave rise to many questions. Based on these observations, it was decided to examine the potential synergistic effect of IL21 and TLR9 on several aspects of the biology of B cells, monocytes and dendritic cells, all antigen presenting cell types, in SLE patients. These cell types were chosen because they play central roles in SLE pathogenesis. The levels of IL21 expression in SLE patients and healthy controls were first measured. In addition, the effect of IL21 and TLR9 on the antigen presenting capacity and the differentiation of B cells into plasma cells and memory cells was assessed. The effect of TLR9 stimulation on monocytes was also evaluated and the expression of TLR9 was examined in SLE patient and healthy control moDCs. In this context, the following experiments were performed:

1. Isolation of human peripheral blood mononuclear cells (PBMCs) and measurement of IL21 and IL21R mRNA levels, from SLE patients and healthy individuals.
2. Comparative analysis of the expression B cell differentiation markers (CD19, CD27 and CD38) to naïve, memory and plasma cells, after stimulation with combinations of IL21 and TLR9 ligand (ODN2006).
3. Investigation of the antigen presenting capacity of B cells from SLE patients and healthy individuals after culturing with combinations of IL21 and ODN2006 and additional stimuli.
4. Examination of the effect of TLR9 and other stimuli on the antigen presenting capacity of monocytes.
5. Analysis of the intracellular expression of TLR9 receptor in monocyte- derived dendritic cells from SLE patients and healthy individuals

B. PATIENTS, MATERIALS AND METHODS

B.1 Patients

Forty-two (42) patients with SLE- followed by the Rheumatology Department of the University Hospital of Crete were studied following the written informed consent. All patients met the 1982 American College of Rheumatology revised criteria for the classification of Systemic Lupus Erythematosus. Eighteen (18) patients had active disease (defined by the Systemic Lupus Erythematosus Activity Index –SLEDAI – score ≥8) and twenty-four (24) had inactive disease (SLEDAI≤8). Patients have not received steroids for at least 24 hours before the peripheral blood was obtained. Blood samples from age- and sex- matched healthy blood donors (n=17) from the Department of Transfusion Medicine of the University Hospital of Crete were used as control samples. All the blood samples were obtained by heparinized 20mL syringe.

B.2 Materials and Methods

B.2.1 Cell isolation methods and cell cultures

B.2.1.1. Peripheral blood mononuclear cell isolation

Reagents and instrument requirements

- Ficoll- Histopaque (Sigma Aldrich,St Louis, MO, USA)
- Fetal Bovine Srum (FBS) (GIBCO- Invitrogen GmbH)
- PBS (Phosphate Buffered Saline)
- Pasteur pipettes
- Trypan Blue (Invitrogen, Garlsbad, CA,USA)
- Neubauer haemocytometer
- Light microscope (Olympus, Japan)

Method

Peripheral blood mononuclear cells are isolated from heparinized venous blood aspirates from healthy volunteers or SLE patients, by Ficoll- Histopaque density gradient centrifugation. Specifically, 20mL of heparinized venous blood were diluted in PBS (1:1) and then added in

15mL- falcon tubes on the top of Ficoll- Histopaque in 1:2 (blood: Ficoll- Histopaque) dilution. The samples were centrifuged for 30 minutes in 20°C, 1800 rpm, with no brake. The cellular interphase was collected by a Pasteur pipette in a 50mL falcon tube and washed two times with sterile BPS/FCS solution (1500 rpm for 10 minutes centrifugation). The pellette was resuspended in 5mL of PBS. A volume of cells were mixed with Trypan Blue and placed on a Neubauer haemocytometer to be counted by light microscopy.

B.2.1.2. B cell magnetic isolation

Reagents and instrument requirements

- MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with autoMACS™ Rinsing Solution)
- Biotin antibody cocktail that contains monoclonal antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a (CD19 B cell isolation kit) (Miltenyi Biotech, Bergisch Gladbach, Germany)
- Magnetic beads against biotin (Miltenyi Biotech, Bergisch Gladbach, Germany)
- MACS MS columns (Miltenyi Biotech, Bergisch Gladbach, Germany)

Method

For the isolation of B cells, PBMCs were incubated in MACS buffer with a biotin antibody cocktail that contains monoclonal antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a conjugated with biotin, in 4°C for 15 minutes. Subsequently, the cells were incubated with addition of MACS buffer, with anti- biotin magnetic beads for 15 minutes in 4°C and then washed with MACS buffer (1-2 mL per 10^7 cells). The cells were resuspended in 500μL of MACS buffer and the suspension was placed in MS columns for negative selection of CD19+ B cells using a high gradient magnetic cell separation system MACS. The flow-through is the cells of interest. After one wash with MACS buffer, the cells were resuspended in PBS and counted with Neubauer haemocytometer. The purity was assessed by flow cytometry and was defined by the proportion of B cells over the non- B cells acquired after the magnetic isolation.

B.2.1.3. B cell cultures

Reagents and instrument requirements:

- RPMI 1640 medium (GIBCO-Invitrogen GmbH,)
- Fetal Bovine Serum (FBS) (GIBCO- Invitrogen GmbH)
- CpG ODN2006 (0.50μM) (Cooley- Pharmaceuticals, USA)
- Recombinant Human sCD40L (Peprotech, GmbH, Hamburg)
- Poly I:C (100ng/mL) (Peprotech, GmbH, Hamburg)
- Penicillin and streptomycin (GIBCO- Invitrogen GmbH)
- Trypan Blue (Invitrogen Garlsbad, CA,USA)
- Neubauer haemocytometer
- Light microscope (Olympus, Japan)

Method:

B cells were cultured at $2x10^5$ cells/mL in RPMI, supplemented with heat inactivated FCS(10%), 100 IU/mL penicillin and 100μg/mL streptomycin, in 37^oC and $5\%CO_2$. The cultures were set in 96- well plates U bottom, with IL21 (10μg/mL), CpG ODN2006 (0.50μM), poly I:C (100ng/mL) and CD40L (50μg/mL), in different combinations. To assess the expression of CD19, CD27, CD38 and IdD the cells were cultured for 7 days whereas, to assess the expression of CD80, CD86, HLA-DR and BAFF-R the cells were cultured for 24 hours. Poly I:C is a TLR3 ligand and ODN 2006 is TLR9 ligand with positive effect on its activation.

B.2.1.4. Monocyte magnetic isolation

Reagents and instrument requirements

- MACS buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution 1:20 with autoMACS™ Rinsing Solution)
- CD14 magnetic MicroBeads, human conjugated to monoclonal anti-human CD14 antibodies (Miltenyi Biotech, Bergisch Gladbach, Germany)
- MACS MS columns (Miltenyi Biotech, Bergisch Gladbach, Germany)

Method

For the isolation of CD14+ monocytes, PBMCs were incubated in MACS buffer with CD14 magnetic MicroBeads, human conjugated to monoclonal anti-human CD14 antibodies, in 4°C for 15 minutes. Subsequently, the cells were washed with MACS buffer (1-2 mL per 10^7 cells) and resuspended in 500μL of MACS buffer. The suspension was placed in MS columns for positive selection of CD14+ monocytes using a high gradient magnetic cell separation system (MACS). The cells of interest remained bound on the MS column. The column was removed from the separator and placed on a 15mL falcon- collection tube. 1mL of MACS buffer was added in the column and the magnetically labeled cells were immediately flushed out by firmly pushing the plunger into the column. The cells were washed one time with MACS buffer (1400rpm, 10 minutes), resuspended in PBS and counted with Neubauer haemocytometer. The purity was assessed by flow cytometry and was defined by the proportion of B cells over the non- B cells acquired after the magnetic isolation.

B.2.1.5. Monocyte cultures

Reagents and instrument requirements

- RPMI 1640 medium (GIBCO-Invitrogen GmbH,)
- Fetal Bovine Serum (FBS) (GIBCO- Invitrogen GmbH)
- CpG ODN2006 (0.50μM) (Cooley- Pharmaceuticals, USA)
- Recombinant Human sCD40L (Peprotech, GmbH, Hamburg)
- IFNγ (100ng/mL) (Peprotech, GmbH, Hamburg)
- Penicillin and streptomycin (GIBCO- Invitrogen GmbH)
- Trypan Blue (Invitrogen Garlsbad, CA,USA)
- Neubauer haemocytometer
- Light microscope (Olympus, Japan)

Method

Isolated CD14+ monocytes were cultured at $2x10^5$ cells/mL in RPMI, supplemented with heat inactivated FCS(10%), 100 IU/mL penicillin and 100μg/mL streptomycin, in 37^oC and 5% CO_2. The cultures were set in 96- well plates U bottom, with CpG ODN2006 (0.50μM), IFNγ (100ng/mL) and CD40L (50μg/mL), in different combinations. To assess the expression of CD80, CD86 and HLA-DR the cells were cultured for 24 hours. CD40L and IFNγ are Th1 stimuli and ODN 2006 is TLR9 stimulus with positive effect.

B.2.1.6. Cultures for differentiation of monocytes into dendritic cells

Reagents and instrument requirements

- RPMI 1640 medium (GIBCO-Invitrogen GmbH,)
- Fetal Bovine Serum (FBS) (GIBCO- Invitrogen GmbH)
- GM-CSF (50ng/mL) (Peprotech, GmbH, Hamburg)
- IL4 (50ng/mL) (Peprotech, GmbH, Hamburg)
- Penicillin and streptomycin (GIBCO- Invitrogen GmbH)
- Trypan Blue (Invitrogen, Garlsbad, CA,USA)
- Neubauer haemocytometer
- Light microscope (Olympus, Japan)

Method

Isolated CD14+ monocytes were cultured for 7 days at $1x10^6$ cells/mL in RPMI, supplemented with heat inactivated FCS(10%), 100 IU/mL penicillin and 100μg/mL streptomycin, in 37^oC and 5% CO_2. The cultures were set in 6- well plates, with addition of GM-CSF (50ng/mL) and IL4 (50ng/mL). There was change of half the medium every third day and addition of GM-CSF and IL4 (50ng/mL each). At the 7^{th} day the cells were harvested by mild pipetting and counted with Trypan Blue on a Neubauer haemocytometer.

B.2.2. Flow cytometric analysis

Reagents and instrument requirements

- Anti-human antibodies:
  - TLR-9, HLA-DR, CD11c, CD80, CD86, CD1a e-Bioscience (San Diego, CA, USA)
  - CD14 Beckman-Coulter (Miami, Florida)
  - CD19, CD27, CD38 Immunotech (Marseilles, France)
- FACS permeabilizing solution (BD Biosciences, Belgium)
- Paraformaldehyde (PFA) (Sigma)
- Flow Cytometer: Epics Elite model flow cytometer (Coulter, Miami FL)

Method

After harvesting from the culture well plates and one wash with PBS (centrifuge at 1 400 rpm for 4 minutes), $2x10^5$ cells were stained for 30 minutes in 4°C, in the dark, with combinations of the appropriate mouse anti- human monoclonal antibodies. All the antibodies are conjugated to fluoredcein isothiocyanate (FITC) or phycoerythrin (PE) or phycoerythrin- Cy5 (PE-Cy5). For intracellular staining of the TLR9 receptor, the cells were first permeabilized with FACS permeabilizing solution (500 μL of solution in 1/10 proportion) for 10 minutes, in room temperature, in the dark and then stained for 30 minutes with anti TLR9 antibody. The appropriate IgG control antibodies were used in each case. The cells were subsequently washed with PBS/FCS (1%) and stained with the appropriate monoclonal antibodies against extracellular markers, for 30 minutes, in 4°C, in the dark. After one wash with PBS/FCS the cells were fixed with PFA 2%. Monocytes were washed after 5 minutes in PFA whereas dendritic cells were washed after 15 minutes. B cells are not susceptible to the PFA toxicity and can be kept in this solution for 24-48 hours. The cell populations that were subject to flow cytometry, according to the expression of specific surface markers are listed below:

- CD19+ cells: B-cells
- CD19+CD27- cells: naïve B cells
- CD19+CD27+ cells: memory B cells
- $CD19^{low}CD27^{high}$ cells: plasmacytoid B cells
- CD14+ cells: monocytes
- $CD11c+CD14^{low}$ cells: monocyte derived dendritic cells.

Stained cells were analysed with an Epics Elite model flow cytometer and the results were analysed with WinMDI 2.8 Version.

B.2.3. RNA isolation from peripheral blood mononuclear cells

Reagents and instrument requirements

- Trizol (Invitrogen, Carlsbad, CA,USA)
- RNeasy kit (Qiagen) (Contains RNeasy mini spin columns, collection tubes of 1.5mL and 2.0mL, RW1 buffer, RPE buffer and RNase free water)
- 100% pure ethanol
- Chloroform (Sigma, USA)
- Quant- iT RNA assay kit (GIBCO- Invitrogen GmbH)
- Qbit fluorometer (GIBCO- Invitrogen GmbH)
- Microcentrifuge

Method

PBMCs were stored in Trizol (1mL per $2x10^7$ cells) in 1.5mL eppendorf tubes after their isolation from whole blood. At this point the samples could be freeze at -80^{o}C. After thawing (for 10 minutes) 200μL of chloroform were added per 1mL of Trizol. The tubes were shaken for 15 seconds by inversion and incubated at room temperature for 3 minutes. Consequently, the samples were centrifuged at 14.000rpm for 20 minutes in 4°C. The centrifugation results in a gradient of three different phases. The lower phase (red colour) contains the proteins, the middle phase contains the DNA and the upper phase contains the RNA. The upper phase (about 500μL) was transferred to a new 1.5mL microcentrifuge tube. Ethanol 100% (0.53 volumes) was then added and mixed. Up to 700μL of the solution were added into an RNeasy column adjusted on a collection tube. Ethanol creates conditions that promote selective binding of RNA to the RNeasy membrane. The tube is centrifuged for 15 seconds at 14.000rpm and the flow- through is placed into the collection tube and centrifuged again (15 seconds at 14.000rpm). Next, the flow- through was discarded and 700μL of RW1 buffer were added in the RNeasy column. The collection tube was centrifuged in 14.000rpm for 15 seconds and then the RNeasy column was placed on a new collection tube. Subsequently, there was addition of 500μL of RPE buffer and centrifugation in 14.000rpm two times (for 15 seconds and 2 minutes, respectively). The tubes were immediately centrifuged again for 1 minute at 14.000rpm. As a result, total RNA was bound to the membrane, and the contaminants were efficiently washed away. 55μL of RNase- free water were added in the column and there is incubation for 5-10 minutes at room temperature. Finally, the RNA was

eluted by centrifugation (1 minute, 14.000rpm) and the RNA sample was kept at -80°C. The quantization and the purity control of the RNA is done by the Quant- iT RNA assay kit and the Qbit fluorometer according to the instructions of the manufacturer.

B.2.4. Reverse Transcription- Polymerase Chain Reaction (RT- PCR)

Reagents and instrument requirements

- Thermoscript RT kit (Invitrogen) that contains:
  - Reverse transcriptase Thermoscript RT (15 U/μL)
  - 5x cDNA synthesis buffer
  - Dithiothreitol (DTT) (0.1M)
  - dNTP mix (10 mM)
  - RNAse OUT (40 U/μL)
  - Random hexamers (50ng/μL)
  - Diethyl Pyrocarbonate (DEPC) treated water
  - E Coli RNAse H (2U/μL)
- PCR machine: Thermal Cycler: DNA endine (MJ Research)

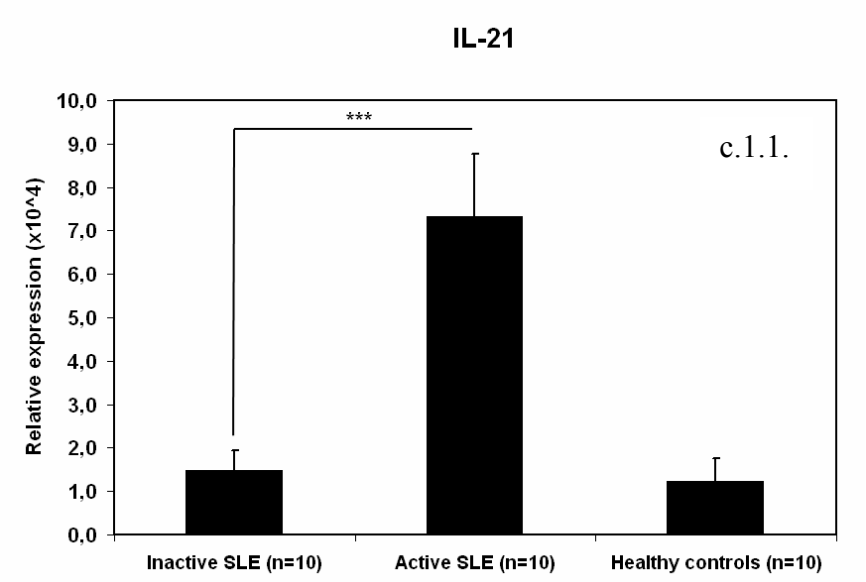
Method

The reaction mix that contains the RNA was prepared on ice. 2μg of RNA is the total RNA quantity that can be included in the reaction and it must be diluted in DECP water, in a total volume equal to 9μL (final concentration of RNA should be 0.22μg/μL). 0.2mL reaction tubes were used. After the appropriate dilutions of RNA, the mix that contains the primers was prepared with 1μL of primer and 2μL of dNTPs for each reaction. Usually, a master mix is prepared for more than one tube, and 3μL are added to each reaction tube. The reaction tubes were placed in the PCR machine for 5 minutes in 65°C and immediately after, out on ice. Simultaneously, the master mix that contains the reverse transcriptase was prepared on ice. This includes 5x cDNA synthesis buffer -after vortex-(5μL), 0.1M (1 μL), RNase out (40U/ μL) (1 μL), DEPC- treated water (1 μL) and reverse transcriptase (1 μL) for each reaction tube. After addition of the master- mix, the reaction tubes were placed in the PCR machine for 50 minutes in 55°C, when there is hybridization of the primer with the complementary sequences of the RNA and reverse transcription initiation. The reaction was terminated with 5 minutes in 85°C and the PCR program was finished in 4°C. The RNA samples were placed directly on ice and they were stored at -20°C.

RESULTS

C.1. Measurement of IL21 and IL21R mRNA levels expressed by SLE patients

In order to assess the expression of IL21 and IL21R, total mRNA from PBMCs was isolated and RT-PCR was performed. Subsequent Real- Time PCR showed that the expression of IL21 in mRNA level is significantly higher among active SLE patients, compared to SLE patients with inactive disease (p=0.002) and healthy controls (p=0.003) (Figure c.1.1). In contrast, no significant difference in the expression of IL21R was evident among SLE patients and healthy controls as shown in Figure c.1.2.



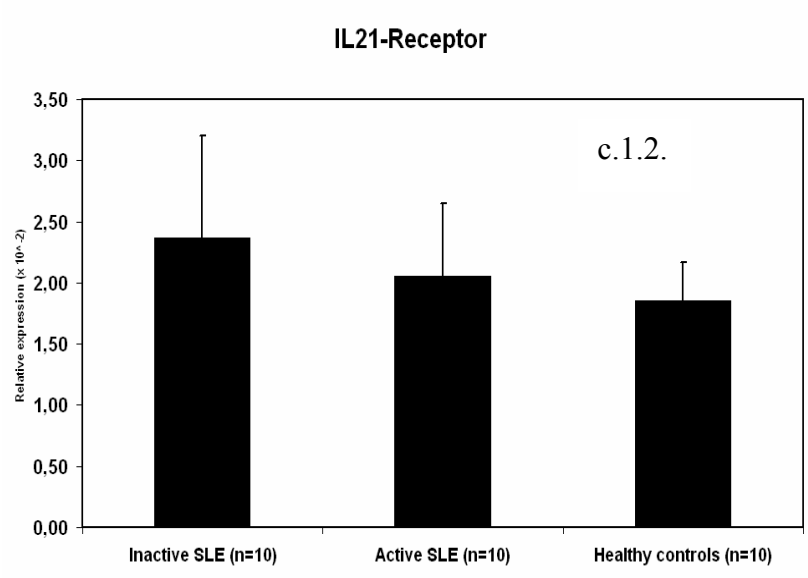


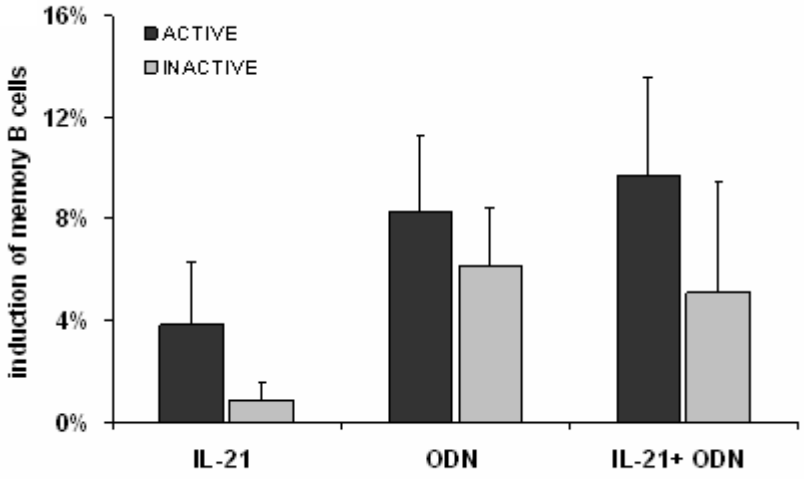
Figure c.1: Relative mRNA expression of IL21 (c.1.1.) and IL21R (c.1.2.) in SLE patients with active and inactive disease and healthy controls. Active SLE patients overexpress IL21.

C2. Active SLE patients display enhanced differentiation of naïve B cells into plasma cells upon stimulation with IL21 and TLR9 ligand.

Given that IL21 and TLR9 promote B cell differentiation into plasma cells ($CD19^{+}CD38^{high}IgD^{-}$) and memory B cells ($CD19^{+}CD27^{+}$), in cells derived from healthy individuals, and that these two factors are over- expressed in SLE, their effect was assessed on B cells from SLE patients. Isolated B cells from SLE patients with active and inactive disease were cultured for 7 days with IL21, TLR9 and TLR3 ligands and their combinations and the phenotype of B cells was finally assessed by flow cytometry.

The differentiation of naive B cells from active SLE patients into plasma cells is remarkable (Figure c.2.2.) compared to the almost null induction of plasma cells by inactive SLE patients. The combination of IL21 and TLR9 stimulation promotes plasma cell differentiation (16%) more than each stimulus alone (5% with IL21 and 7% with ODN2006). Interestingly, none of the stimuli could promote the differentiation of inactive patient B cells into plasma cells. IL21 in combination with TLR9 stimulation promotes the differentiation into plasma cells more than IL21 or TLR9 alone (16% with both IL21 and TLR9, 5% with IL21, 7% with ODN2006). Differences seem to result in the case of memory B cells (Figure c.2.1.). With any of the stimuli, the percentages of memory B cells generated are higher in SLE patients with active disease. It is notable that the combination of both TLR9 and IL21 stimulation results in importantly higher induction memory B cells from active SLE patients (10%) compared to cells from inactive patients (5%). This induction might be greatly supported by TLR9 stimulation that seems to elevate the percentages of plasma cells at comparable levels (8% in active SLE patients), alone. IL21 alone seems to promote plasma cell generation in active SLE patients (4%) compared to inactive (1%), and might be the additional "help" that accounts for the difference between active and inactive SLE patients upon stimulation with both TLR9 and IL21 stimuli.

c.2.1



c.2.2

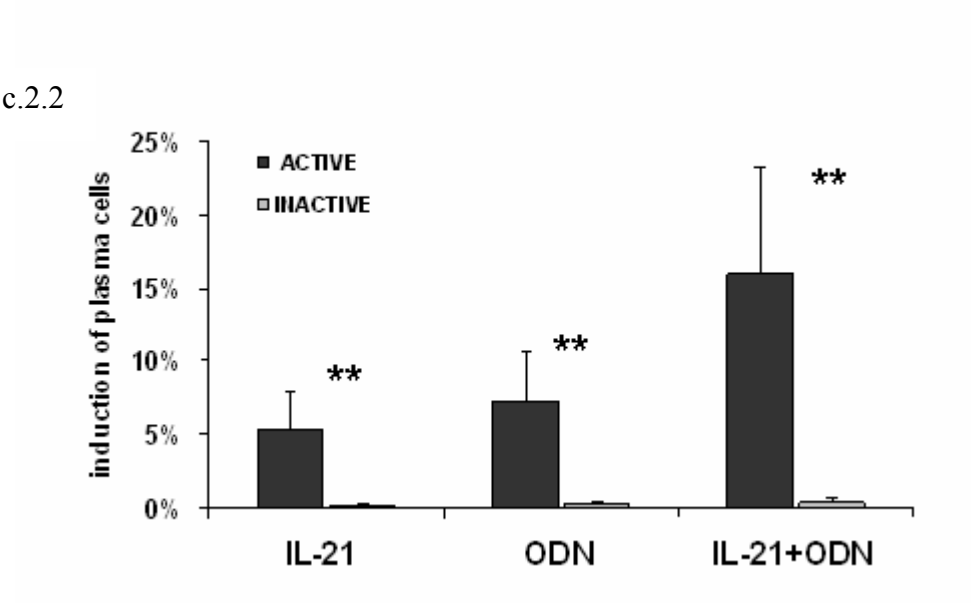


Figure c.2.: Induction of memory (c1.1.) and plasma cells (c.1.2.) by active and inactive SLE patients after stimulation with IL21, ODN2006 (TLR9 agonist) and a combination of both. The combination of the two stimuli promotes enhanced induction of plasma cells by naïve B cells from active SLE patients. The black bars represent the induction of memory or plasma cells by active patients and the grey bars represent the induction by inactive patients.

C.3. IL21 and TLR9 stimulation promote the expression of co-stimulatory molecules on B cells.

Apart from antibody secretion, another function of B cells is their ability to present antigens to T cells. In order to assess the effect of IL21 and TLR9 on the antigen presenting capacity of B cells in active SLE patients, flow cytometric analysis was performed after 48h-stimulation with these two stimuli alone or in combination. TLR3 stimulus (poly I:C) was also used. The expression of CD86 seems to be dramatically increased upon IL21 and TLR9 stimulation, more than with other stimuli or combinations of stimuli, on B cells from active SLE patients. Treatment of the cells with CpG ODN seems to augment the expression of CD80, whereas IL21 does not induce high levels of expression alone. Treatment of the cells with poly I: C or poly I: C and IL21 together resulted in similar levels of CD80 and CD86 induction.

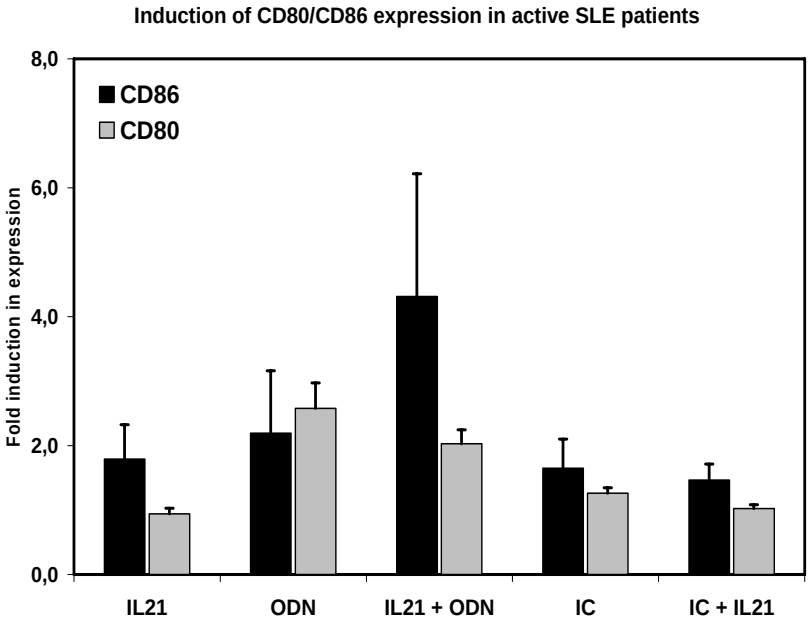


Figure c.3.1.: Fold induction of the expression of CD80 and CD86 co-stimulatory molecules on B cells from active SLE patients. IL21 and TLR9 synergistically promote the expression of antigen presenting capacity markers on B cells from active SLE patients. The black bars represent the induction of CD86 and the grey bars the induction of CD80.

C.4. TLR9 stimulation of monocytes

Given that TLR9 expression is elevated in monocytes from active SLE patients, the effect of TLR9 stimulation on the antigen presenting capacity of these cells was assessed. Monocytes from SLE patients and healthy controls were magnetically isolated from PBMCs and flow cytometry was performed after 24 hours of cultures. Apart from TLR9 ligand, IFNγ and CD40L were also used as additional stimuli. The expression of CD80, CD86 and HLA-DR surface markers was assessed. No significant differences were found in the expression of these molecules among patients and healthy individuals. The results should be interpreted with caution due to the great lethality of cells in the culture.

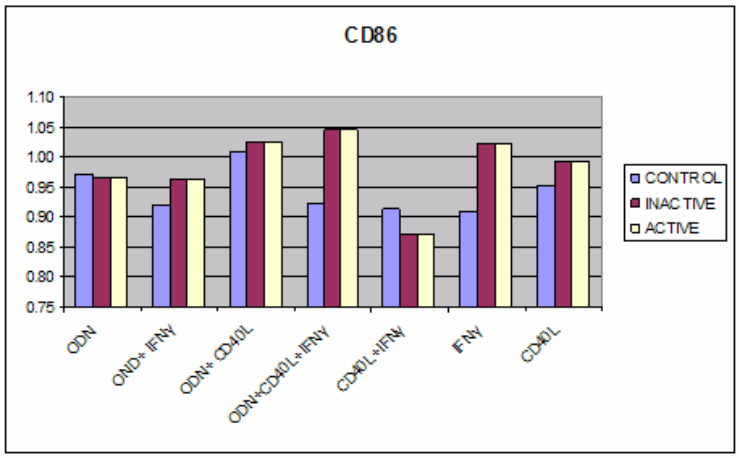


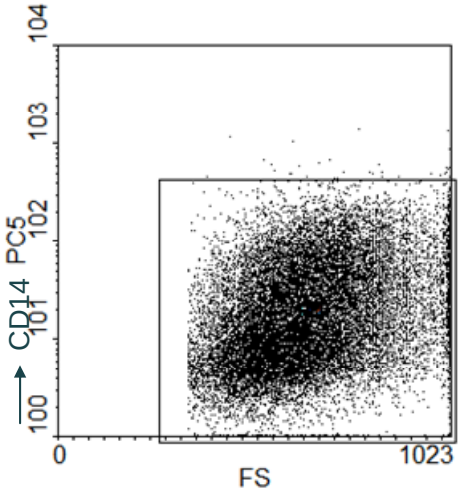
Figure c.4.: Fold induction of the expression of CD86 in SLE patients and healthy controls. No significant differences in CD86 expression were identified after treatment with the stimuli.

C.5. TLR9 is not expressed in monocyte derived dendritic cells both in SLE patients and healthy controls

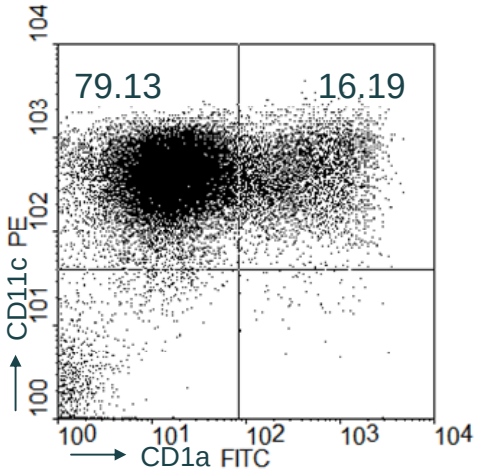
In order to assess the expression of TLR9 in moDCs, monocytes from SLE patients and healthy controls were isolated and differentiated into dendritic cells after 7 days in cultures with GM- CSF and IL4. The differentiation into DCs was verified by assessment of CD11c, CD14 and CD1a surface markers at flow cytometric analysis (Figure: c.4.1., c.4.2.). The expression of CD14, which is a monocyte marker was significantly reduced after differentiation (Figure c4.1.), whereas, there was an increased percentage of CD11c positive cells, about 95% (Figure c.4.2.). CD11c is a differentiation marker for myeloid dendritic cells. There was a small number of double positive $CD11c^{+}CD1a^{+}$ cells (16%), which represents one distinct DC population.

In order to assess the expression of TLR9 in moDCs, intracellular staining for TLR9 was performed. Neither healthy controls, nor SLE patients express the intracellular receptor that recognizes dsDNA. The histogram of figure c.4.3 depicts the fact that TLR9 is not expressed in moDCs from active SLE patient.

c.4.1.



c.4.2.



c.4.3.

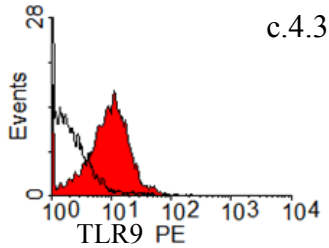


Figure c.4.: moDCs do not express TLR9. Monocytes did differentiate into dendritic cells (moDCs) that express characteristic differentiation markers (dotplots: c.4.1 and c.4.2). TLR9 is not expressed in moDCs neither from SLE patients nor from healthy individuals. Representative histogram displays the expression of TLR9 on day 7 moDCs from SLE patients. The black line represents the expression of TLR9 and the red area the isotype control (c.4.3.).

D. DISCUSSION

Systemic lupus erythematosus is a complex autoimmune disease. Although there is much information at clinical and molecular level, the exact pathogenic mechanism remains elusive. The disease is characterized by aberrant action of autoreactive lymphocytes and production of autoantibodies against DNA. Recent study of our laboratory revealed that there is increased number of B cells and monocytes expressing the TLR9 receptor that recognises DNA, in SLE patients with active disease [41]. Clinical, as well as animal studies have indicated that TLR signalling is implicated in the increased production of autoantibodies, by acting as a third signal - in addition to BcR and T cell help- that activates naïve B cells and induces their differentiation into plasma cells [48, 44]. IL21 is a newly identified cytokine that has similar effects on naïve B cells [14, 55, 56] and may act as an additional signal that leads to aberrant B cell function and increased production of autoantibodies in SLE.

In this study, the relative contribution of the IL21 and the TLR9 pathways on SLE pathogenesis was assessed, first by measuring the expression levels of IL21 and IL21R in patients and second by comparing the effect of these two cytokines on B cells from SLE patients and healthy controls. It was found that the expression of IL21 in mRNA level is dramatically increased in SLE patients with active disease compared to patients in remission and healthy controls. The expression of IL21R is similar amongst all individuals. In addition, TLR9 in combination with IL21 seems to promote the differentiation of active patient- naïve B cells into plasma cells, more than each one alone, thus implying that IL21 and TLR9 may act synergistically on plasma cell differentiation. In contrast, plasma cell differentiation was not feasible either with IL21 and TLR9 alone or with combination of both stimuli in SLE patients in remission. The antigen presenting capacity of B cells has also been assessed. TLR9 in combination with IL21 promote the expression of CD86 in patients with active disease more that any other stimuli.

Different antigen presenting cell types were examined as well. TLR9 is not expressed in monocyte derived dendritic cells from SLE patients or healthy controls. In addition, no significant differences between SLE patients and controls in the antigen presenting capacity after stimulation with TLR9 and other stimuli were identified. In the future it would be interesting to investigate the effect of IL21 on several aspects of the biology of dendritic cells, the most potent antigen- presenting cell type, that seem to be deregulated in SLE patients.

To sum up, based on the findings of this study IL21 expression is increased in SLE patients with active disease. It is proposed that the elevated expression of TLR9 and IL21 in SLE patients with active disease have functional consequences. IL21 and TLR9 may have synergistic stimulatory effect on plasma cell differentiation of naïve B cells into plasma cells in SLE patients with active disease. The increased differentiation into plasma cells in the presence of IL21 and TLR9 agonist may account for the aberrant function of B cells and the great numbers of auto-antibodies detected in SLE patients. In addition, the increase in the expression of CD86 co-stimulatory molecule indicates the enhanced ability of these cells to activate naïve T cells.

D.1. IL21 expression in SLE patients.

The expression of IL21 in SLE patients is measured in this study. IL21 mRNA levels are significantly elevated in active SLE patients. Unpublished studies of Ettinger and colleagues (reported by Ettinger at al., [13]) suggest that a cohort of SLE patients have higher IL21 levels in their plasma but there were no correlations between IL21 levels and disease activity index. IL21 is a recently identified, immunomodulatory cytokine with pleiotropic action. It can induce proliferation of lymphoid cells and differentiation of naïve B cells into plasma cells or induce apoptosis of B cells and NK cells and cause impaired antigen presentation and T cell- activating capacity by DCs. The fact that IL21 expression is significantly elevated in SLE patients with active disease may reflect a causal role of this cytokine on the aberrant function of several cell types.

D.2. IL21 and TLR9 stimulation promote enhanced differentiation into plasma cells in active SLE patients.

It was recently proposed that proliferation and differentiation human naïve B cells require a combination of three signals: 1) BcR signaling, 2) T cell help and 3) TLR stimulation [48], contrary to the previous paradigm, proposing that BcR stimulation and T cell help are necessary and sufficient [42]. It was also shown that TLR9 stimulation triggers naïve B cell proliferation and differentiation into IgM secreting cells with enhanced antigen presenting capacity [24]. Experiments with transgenic mice have indicated that IL21 also triggers differentiation into plasma cells, by inducing the B lymphocyte- induced maturation protein-1 (Blimp-1), a transcription factor that establishes the terminal differentiation into plasma cells. IL21 also promotes the production of B cell lymphoma-6 (Bcl-6) transcription factor, which promotes memory B cell differentiation. Human studies have shown that IL21 in combination with BcR or

CD40-CD40L signals can trigger differentiation of human naïve B cells and memory B cells into antibody secreting plasma cells [14]. Using an IL-21R fusion protein for IL21 neutralization, Kuchen and colleagues [30] indicated that IL21 is essential for T cell- induced B cell activation, proliferation, plasma cell differentiation and antibody production. In this study, it is proposed that IL21 can act as a stimulus that in combination with TLR9 signaling promotes B cell differentiation into plasma cells and memory B cells in healthy individuals.

Given that IL21 and TLR9 promote differentiation of naïve B cells into memory B cells and plasma cells in healthy individuals, the effect of these two stimuli on B cells from SLE patients was assessed. The increased proportions of TLR9 expressing B cells from SLE patients correlates to the presence of anti- dsDNA antibodies identified, according to Papadimitraki and colleagues [41]. This may reflect a deregulated system were naïve B cells are abnormally activated by TLR9 ligands and exhibit accelerated differentiation into antibody secreting plasma cells. Indeed, the numbers of peripheral $CD19^{low}CD27^{high}$ plasma cells in SLE correlate with disease activity and are reduced upon remission, caused by immunosuppressive drugs [37, 22]. SLE patients have higher levels of hypo- methylated CpG containing DNA [47] which acts as a stimulus for TLR9. The great abundance of these sequences that can act as autoantigens in SLE, may be due to increased apoptosis and defective clearance of cell debris. DNA containing immune complexes that form can activate B cells by dual engagement of BcR and TLR9 and promote their proliferation and antibody secretion [32].

Here it is shown that IL21, in combination with TLR9 stimulation can lead to robust differentiation of B cells into plasma cells in active SLE patients. Of interest, none of the stimuli could promote differentiation into plasma cells in patients with inactive disease. This may mean that the breakage of tolerance and the alterations in the immune system of active SLE patients make naïve B cells more vulnerable to the effect of IL21 and TLR9 on plasma cell differentiation.

D.3. IL21 and TLR9 affect the antigen presenting capacity of B cells from active SLE patients.

The antigen presenting capacity of B cells from SLE patients after stimulation with TLR9 and IL21 was assessed. The expression of CD86 co-stimulatory molecule is more increased after stimulation with both IL21 and TLR9 than with each one alone. This means that these cells may have enhanced antigen presenting function, which may reflect an enhanced ability of these cells

to activate T cells. The fact that IL21 and TLR9 treated B cells from SLE patients have increased capacity to activate and induce proliferation of T cells may explain the enhanced activation of T cells in this autoimmune condition.

D.4. TLR9 stimulation of monocytes.

The fact that there is increased number of monocytes in the peripheral blood expressing TLR9 is of great interest due to the role of these cells in tissue injury. Monocytes can present antigens and activate T helper cells but also they secrete soluble factors that activate or recruit more cells to the site of inflammation. TLR9 stimulation leads to upregulation of MHC and co-stimulatory molecules that facilitate the interaction between APCs and T cells [51]. The fact that there were no significant differences between healthy controls and SLE patients after stimulation of TLR9 should be interpreted with caution, due to the great lethality of cells in the cultures.

D.5. TLR9 expression in moDCs.

Several studies propose that unmethylated CpG motifs are able to activate human pDCs and promote their maturation [52, 64, 66]. It was recently proposed that synthetic CpG oligodeoxynucleotides (ODNs) are able to promote human pDCs to prime allogenic naïve T cells to differentiate into CD4+CD25+ Tregs. These Tregs express Foxp3 (forkhead transcription factor) and secrete IL-10, TGF-β and IL6, IFN-γ. Furthermore, they are able to strongly repress the proliferation of allogenic or autologous naïve T cells [36]. Means TK et al. (25) demonstrated that DNA containing ICs isolated from SLE patients were able to induce pDC activation in a TLR9 dependent manner. DNA containing ICs seem to be more potent in activating murine DCs in cell cultures than ICs that contain a foreign protein [5]. In contrast to previous studies that propose that moDCs do not express TLR9, it was recently suggested that human monocyte derived dendritic cells express TLR9 and they can mature in response to TLR9 stimulation, using CpG ODN D19. [21]. Monocytes act as precursors of mDCs and the differentiation of monocytes into DCs can be recapitulated in vitro via GM-CSF and IL4. Thus, it would be possible that there is altered expression of TLR9 in moDCs from SLE patients based on the fact that an increased proportion of monocytes from SLE patients express TLR9, compared to healthy controls. According to the results of this study neither SLE patient nor healthy control moDCs express TLR9 based on intracellular staining of the receptor. Hoene and colleagues [21] measured the RNA levels of expression of the receptor which may mean that the RNA levels do not correspond to protein levels of this intracellular receptor.

D.6. Conclusions- Future plans

Systemic lupus erythematosus is an autoimmune disease that concerns millions of people worldwide. Many theories have tried to explain the abnormal lymphocyte function and the aberrant production of autoantibodies that play a critical role in tissue destruction. Undoubtedly, B cells are of major importance in the initiation and maintenance of the disease, first by their ability to uptake and present antigens and second by producing antibodies against self components. TLR9, that is upregulated upon BcR engagement, is increased in SLE patients probably due to the great abundance of autoantigens. IL21 expression is also increased in SLE patients according to this study. IL21 and TLR9 have similar effect on the differentiation process of naïve B cells. Here it is shown that IL21 in combination with TLR9 have a great effect on B cells depending on the disease activity. This effect includes the promotion of differentiation of naïve B cells into plasma cells and the enhancement of the antigen presenting capacity of B cells that is related to their ability to activate T cells.

Future plans should focus on different cell populations that seem to be involved in the pathogenesis of SLE. Dendritic cells play a major role in the regulation of the immune system. The latest studies propose that the differentiation and maturation profile of SLE dendritic cells is altered [50 ,33, 10, 9]. According to Dacheng Ding et al. [10] monocyte derived dendritic cells (moDCs) and freshly isolated DCs from SLE patient exhibit accelerated differentiation, maturation and cytokine secretion based on specific markers. The expression of CD1a differentiation marker, CD86, CD80 and HLA-DR costimulatory molecules, and IL-8 were significantly higher compared to healthy controls. Moreover, these DCs from SLE patients were more potent in inducing the proliferation and activation of allogenic T cells in mixed lymphocyte reactions (MLR). Decker et al. [9] show that moDCs express high levels of CD86 upon stimulation. IL21 has an inhibitory effect on DCs from healthy controls. This might not happen in SLE patients since several reports suggest that SLE moDCs display accelerated differentiation, maturation and cytokine expression. Thus, in SLE patients, the increased expression of IL21 is not concomitant with the effect that we would anticipate. This increase might even be a compensatory effect as a result of non- responsive dendritic cells. The effect of this recently identified cytokine should be further investigated in the context of SLE since it seems to be involved in the regulation of the immune responses of several cell types.

REFERENCES

1. Alamanos, Y. et al. Epidemiology of systemic lupus erythematosus in northwest Greece 1982-2001. *J Rheumatol* **30**, 731-5 (2003).
2. Barrat, F.J. et al. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* **202**, 1131-9 (2005).
3. Blanco, P., Palucka, A.K., Gill, M., Pascual, V. & Banchereau, J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* **294**, 1540-3 (2001).
4. Bombardier, C., Gladman, D.D., Urowitz, M.B., Caron, D. & Chang, C.H. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum* **35**, 630-40 (1992).
5. Boule, M.W. et al. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J Exp Med* **199**, 1631-40 (2004).
6. Brandt, K., Singh, P.B., Bulfone-Paus, S. & Ruckert, R. Interleukin-21: a new modulator of immunity, infection, and cancer. *Cytokine Growth Factor Rev* **18**, 223-32 (2007).
7. Busconi, L. et al. Functional outcome of B cell activation by chromatin immune complex engagement of the B cell receptor and TLR9. *J Immunol* **179**, 7397-405 (2007).
8. Cederblad, B. et al. Patients with systemic lupus erythematosus have reduced numbers of circulating natural interferon-alpha- producing cells. *J Autoimmun* **11**, 465-70 (1998).
9. Decker, P., Kotter, I., Klein, R., Berner, B. & Rammensee, H.G. Monocyte-derived dendritic cells over-express CD86 in patients with systemic lupus erythematosus. *Rheumatology (Oxford)* **45**, 1087-95 (2006).
10. Ding, D., Mehta, H., McCune, W.J. & Kaplan, M.J. Aberrant phenotype and function of myeloid dendritic cells in systemic lupus erythematosus. *J Immunol* **177**, 5878-89 (2006).
11. Ehlers, M., Fukuyama, H., McGaha, T.L., Aderem, A. & Ravetch, J.V. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J Exp Med* **203**, 553-61 (2006).
12. Ehlers, M. & Ravetch, J.V. Opposing effects of Toll-like receptor stimulation induce autoimmunity or tolerance. *Trends Immunol* **28**, 74-9 (2007).
13. Ettinger, R., Kuchen, S. & Lipsky, P.E. The role of IL-21 in regulating B-cell function in health and disease. *Immunol Rev* **223**, 60-86 (2008).
14. Ettinger, R. et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol* **175**, 7867-79 (2005).

15. Gaipl, U.S. et al. Defects in the disposal of dying cells lead to autoimmunity. *Curr Rheumatol Rep* **6**, 401-7 (2004).
16. Gaipl, U.S. et al. Clearance deficiency and systemic lupus erythematosus (SLE). *J Autoimmun* **28**, 114-21 (2007).
17. Gaipl, U.S. et al. Impaired clearance of dying cells in systemic lupus erythematosus. *Autoimmun Rev* **4**, 189-94 (2005).
18. Grossmayer, G.E. et al. IgG autoantibodies bound to surfaces of necrotic cells and complement C4 comprise the phagocytosis promoting activity for necrotic cells of systemic lupus erythaematosus sera. *Ann Rheum Dis* **67**, 1626-32 (2008).
19. Hemmi, H. A Toll-like receptor recognizes bacterial DNA. *letters to nature* **408** (2002).
20. Herber, D. et al. IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R.Fc reduces disease progression. *J Immunol* **178**, 3822-30 (2007).
21. Hoene, V., Peiser, M. & Wanner, R. Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. *J Leukoc Biol* **80**, 1328-36 (2006).
22. Jacobi, A.M. et al. Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* **48**, 1332-42 (2003).
23. Janeway. Immunobiology. *Garland Science* (2001).
24. Jiang, W. et al. TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. *Eur J Immunol* **37**, 2205-13 (2007).
25. Kim W.U., S.A., Bucala R. Toll- like receptros in systemic lupus erythematosus prospects for therapeutic intervention. *Autoimmunity Reviews* (2008).
26. Koller, M., Zwolfer, B., Steiner, G., Smolen, J.S. & Scheinecker, C. Phenotypic and functional deficiencies of monocyte-derived dendritic cells in systemic lupus erythematosus (SLE) patients. *Int Immunol* **16**, 1595-604 (2004).
27. Konforte, D. & Paige, C.J. Identification of cellular intermediates and molecular pathways induced by IL-21 in human B cells. *J Immunol* **177**, 8381-92 (2006).
28. Kono, D.H. & Theofilopoulos, A.N. Genetics of SLE in mice. *Springer Semin Immunopathol* **28**, 83-96 (2006).
29. Krishnan S., C.B., Tsokos G.C. Autoimmunity in systemic lupus erythematosus: Integrating genes and biology. *Seminars in Immunology* **18** 230-243 (2006).
30. Kuchen, S. et al. Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. *J Immunol* **179**, 5886-96 (2007).

31. Lartigue, A. et al. Role of TLR9 in anti-nucleosome and anti-DNA antibody production in lpr mutation-induced murine lupus. *J Immunol* **177**, 1349-54 (2006).
32. Leadbetter, E.A. et al. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* **416**, 603-7 (2002).
33. Lenert, P.S. Targeting Toll-like receptor signaling in plasmacytoid dendritic cells and autoreactive B cells as a therapy for lupus. *Arthritis Res Ther* **8**, 203 (2006).
34. Leonard, W.J., Zeng, R. & Spolski, R. Interleukin 21: a cytokine/cytokine receptor system that has come of age. *J Leukoc Biol* **84**, 348-56 (2008).
35. Marshak-Rothstein, A. Toll-like receptors in systemic autoimmune disease. *Nature Reviews Immunology* **6**, 823- 835 (2006).
36. Moseman, E.A. et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol* **173**, 4433-42 (2004).
37. Odendahl, M. et al. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J Immunol* **165**, 5970-9 (2000).
38. Ogawa, Y., Yoshinaga, T., Yasuda, K., Nishikawa, M. & Takakura, Y. The uptake and degradation of DNA is impaired in macrophages and dendritic cells from NZB/W F(1) mice. *Immunol Lett* **101**, 32-40 (2005).
39. Ozaki, K. et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol* **173**, 5361-71 (2004).
40. Palucka, A.K., Banchereau, J., Blanco, P. & Pascual, V. The interplay of dendritic cell subsets in systemic lupus erythematosus. *Immunol Cell Biol* **80**, 484-8 (2002).
41. Papadimitraki, E.D. et al. Expansion of toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: implications for the induction and maintenance of the autoimmune process. *Arthritis Rheum* **54**, 3601-11 (2006).
42. Parker, D.C. T cell-dependent B cell activation. *Annu Rev Immunol* **11**, 331-60 (1993).
43. Pasare, C. & Medzhitov, R. Control of B-cell responses by Toll-like receptors. *Nature* **438**, 364-8 (2005).
44. Pasare, C. & Medzhitov, R. Toll-like receptors: linking innate and adaptive immunity. *Adv Exp Med Biol* **560**, 11-8 (2005).
45. Patole, P.S. et al. Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells. *J Am Soc Nephrol* **16**, 1326-38 (2005).
46. Pelletier, M., Bouchard, A. & Girard, D. In vivo and in vitro roles of IL-21 in inflammation. *J Immunol* **173**, 7521-30 (2004).

47. Richardson, B. et al. Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Rheum* **33**, 1665-73 (1990).
48. Ruprecht, C.R. & Lanzavecchia, A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur J Immunol* **36**, 810-6 (2006).
49. Sawalha, A.H. et al. Genetic association of interleukin-21 polymorphisms with systemic lupus erythematosus. *Ann Rheum Dis* **67**, 458-61 (2008).
50. Scheinecker, C., Zwolfer, B., Koller, M., Manner, G. & Smolen, J.S. Alterations of dendritic cells in systemic lupus erythematosus: phenotypic and functional deficiencies. *Arthritis Rheum* **44**, 856-65 (2001).
51. Schnare, M. et al. Toll-like receptors control activation of adaptive immune responses. *Nat Immunol* **2**, 947-50 (2001).
52. Kadowaki, N. et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* **194**, 863-9 (2001).
53. Shortman, K. & Liu, Y.J. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* **2**, 151-61 (2002).
54. Shortman, K. & Naik, S.H. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* **7**, 19-30 (2007).
55. Spolski, R. & Leonard, W.J. The Yin and Yang of interleukin-21 in allergy, autoimmunity and cancer. *Curr Opin Immunol* **20**, 295-301 (2008).
56. Spolski, R. & Leonard, W.J. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol* **26**, 57-79 (2008).
57. Tan, E.M. et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* **25**, 1271-7 (1982).
58. Tao, K. et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. *Ann Rheum Dis* **66**, 905-9 (2007).
59. Tas, S.W., Quartier, P., Botto, M. & Fossati-Jimack, L. Macrophages from patients with SLE and rheumatoid arthritis have defective adhesion in vitro, while only SLE macrophages have impaired uptake of apoptotic cells. *Ann Rheum Dis* **65**, 216-21 (2006).
60. Uematsu, S. & Akira, S. Toll-like receptors and Type I interferons. *J Biol Chem* **282**, 15319-23 (2007).
61. Vinuesa, C.G. et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* **435**, 452-8 (2005).
62. Wu, X. & Peng, S.L. Toll-like receptor 9 signaling protects against murine lupus. *Arthritis Rheum* **54**, 336-42 (2006).

63. Yan B., Y.S., Chen G., Kuang M., Shen N., Chen S. Dysfunctional CD4+CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon- α producing antigen presenting cells. *Arthritis and Rheumatism* **58**, 801- 812 (2008).
64. Yasuda, K. et al. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. *J Immunol* **174**, 6129-36 (2005).
65. Yu, P., Musette, P. & Peng, S.L. Toll-like receptor 9 in murine lupus: more friend than foe! *Immunobiology* **213**, 151-7 (2008).
66. Bauer, M. et al. Bacterial CpG-DNA triggers activation and maturation of human CD11c, CD123+ dendritic cells. *J Immunol* **166**, 5000-7 (2001).
67. Cristensen SR, K.M., Alexopoulou L, Flavell RA, Shlomchik MJ Toll- like receptor 9controls anti- DNA autoantibody production in murine lupus. *J Exp Med* **202**, 321-31 (2005).