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INVESTIGATION OF PATHOGENESIS, CLINICAL COURSE, PROGNOSIS AND RESPONSE TO THERAPY OF SYSTEMIC LUPUS ERYTHEMATOSUS:

PERSPECTIVE AND COMPARATIVE STUDY USING DNA MICROARRAYS IN BONE MARROW AND PERIPHERAL BLOOD SAMPLES

PhD THESIS

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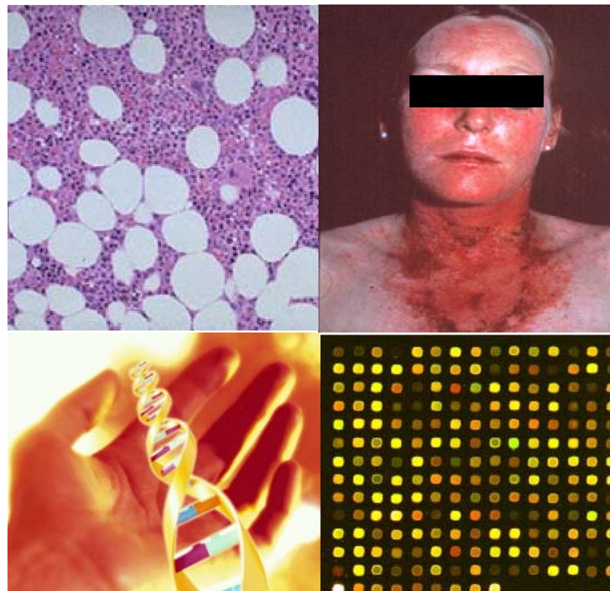

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ABSTRACT

Purpose The cells of the immune system originate from the bone marrow (BM), where many of them also mature. To better understand the aberrant immune response in systemic lupus erythematosus (SLE), we examined the BM in lupus patients using DNA microarrays and compared it to the peripheral blood.

Patients and Methods**:** Bone marrow mononuclear cells (BMMCs) from 20 SLE patients (12 with active disease, including nephritis and neuropsychiatric disease; 8 with inactive disease) and peripheral blood mononuclear cells (PBMCs) from 27 patients (16 active/ 11 inactive); BMMCs and PBMCs from 7 healthy individuals and 3 osteoarthritis patients served as controls. Samples were analyzed on genome-scale microarrays with 21,329 genes represented. Multiplex cytokine assay was also performed evaluating the levels of 22 cytokines in the sera of lupus patients relative to controls. These cytokines were IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12 (p40), IL-13, IL-15, IL-17, IL-1Ra, IP-10 interferon (IFN)-α and IFN-γ, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF), CCL2 [monocyte chemoattractant protein (MCP)-1]/(MCAF), CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β) and CCL11 (Eotaxin)

Results**:** We found 102 differentially expressed genes between patient's and control's bone marrow samples (unpaired student t-test). These genes represent several important biologic processes; 54 out of 100 differentially expressed genes are involved in major networks including cell death, cell growth and differentiation cell signaling and cellular growth and proliferation. Comparative analysis of the bone marrow with the peripheral blood of SLE patients identified 88 genes differentially expressed between the two immune compartments; 61 out of 88 participate in various processes that include cell growth and differentiation, cellular movement and morphology, immune response and other hematopoietic cell functions.

Unsurpervised clustering of highly expressed genes revealed two major SLE patient clusters in bone marrow, but not in peripheral blood. The first cluster was comprised of patients with active disease and the second patients in remission. The upregulated genes in the bone marrow of active patients included genes involved in cell death and

granulopoiesis. These genes represent components of activated neutrophils as well as positive or negative regulators of cell viability and apoptosis. Linear regression analysis showed that granulopoiesis signature was significantly correlated with SLEDAI in the bone marrow ($r = 0.55$, $p = 0.0259$), and the total score was higher in active group of patients versus the inactive ($p = 0.041$).

Serum levels of 22 cytokines were compared between SLE patients and controls. Among the "cellular cytokines", TFNα (p=0.0486) was significantly elevated in SLE patients compared to controls, while IL-15 (p= 0.0097) was also increased in patients. We did not observe any changes in serum "humoral cytokine" levels. Serum chemokine IP10, IL8 and MCP1 levels were also higher in SLE patients. Serum IP10 and IL12 concentration showed a negative association with arthritis. The chemokine MIP1α showed a negative association with neuropsychiatric manifestations of lupus while serum IL2 concentration positively associated with lupus nephritis.

***Conclusion*:** Compared to the peripheral blood, microarray analysis of the bone marrow better differentiated active from inactive lupus patients and patients from controls. These data corroborate previous findings regarding the importance of apoptosis and granulocytes in the pathogenesis of the disease. Furthermore, two cellular cytokines, TNF-alpha and IL15, characteristic of the Th1-mediated immune response, were elevated in patients' sera relative to controls. Although lupus is considered by many as a Th2 cytokine disease none of the cytokines associated with humoral immunity were found elevated in the patients' cohort relative to the controls.

ΠΕΡΙΛΗΨΗ

Σκοπός: Στην παρούσα ερευνητική μελέτη διερευνήθηκε η δυνατότητα καθορισμού της αιτιοπαθογένειας, πρόγνωσης, της πορείας της νόσου και της ανταπόκρισης στην θεραπεία κατά την έναρξή της επί τη βάσει της έκφρασης γονιδίων σε ιστούς όπως ο μυελός των οστών και το περιφερικό αίμα. Οι ιστοί αυτοί εμπεριέχουν κύτταρα τα οποία συμμετέχουν στην παθογένεια της νόσου (μονοπύρηνα/μακροφάγα, Β και Τ λεμφοκύτταρα, NK κύτταρα και ουδετερόφιλα). Η συλλογή δειγμάτων αποτελείται από ασθενείς με ΣΕΛ και υγιείς μάρτυρες από τους οποίους έγινε λήψη περιφερικού αίματος και μυελού των οστών.

Ασθενείς και Μέθοδοι Κατά τη διάρκεια των εργασιών χρησιμοποίηθηκαν 27 ασθενείς με Συστηματικό Ερυθηματώδη Λύκο και φυσιολογικοί μάρτυρες. όπου έγινε η απομόνωση RNA από τα κύτταρα του περιφερικού αίματος και του μυελού των οστών. Μετά τον έλεγχο του RNA ο αριθμός των δειγμάτων που χρησιμοποιήθηκαν στις μικροσυστοιχίες είναι 27 δείγματα περιφερικού αίματος και 22 μυελού των οστών από ασθενείς με ΣΕΛ και 6 δείγματα περιφερικού αίματος και 10 μυελού των οστών από φυσιολογικούς μάρτυρες.Εν συνεχεία χρησιμοποιήθηκαν 2μg ολικού RNA για την σύνθεση cDNA και την περαιτέρω σήμανση του με Cy χρώση. Τα δείγματα υβριδοποιήθηκαν σε πίνακες (glass slides) που αντιπροσωπεύουν 21,329 γονίδια και η ανάλυσή τους έγινε μετά από σάρωση σε Agilent DNA microarray scanner.

Για την στατιστική ανάλυση των αποτελεσμάτων χρησιμοποιήθηκαν 4 ομάδες για τον καθορισμό της έκφρασης των γονιδίων, κρίνοντας στατιστικά σημαντικές τις διαφορές της έκφρασης των γονιδίων μεγαλύτερες από 2-fold. Στην πρώτη ανάλυση πραγματοποιήθηκε σύγκριση των εκφραζομενων γονιδίων μεταξύ των δειγμάτων του φυσιολογικού μυελού των οστών και των ασθενών με ΣΕΛ. Στη δεύτερη ανάλυση έγινε σύγκριση μεταξύ των δειγμάτων του περιφερικού αίματος των μαρτύρων και των ασθενών με λύκο. Η τρίτη ανάλυση αφορά στην ομάδα των ασθενών και πραγματοποιήθηκε συγκριτική ανάλυση μεταξύ του περιφερικού αίματος και του μυελού των οστών.

Επίσης έγινε η ανάλυση 22 κυτταροκινών στον ορό ασθενών με ΣΕΛ και η σύγκρισή τους με υγιείς. Οι κυτταροκίνες που εκτιμήθηκαν ήταν IL-1β, IL-2, IL-4, IL-5, IL-6, IL-

7, CXCL8 (IL-8), IL-10, IL-12 (p40), IL-13, IL-15, IL-17, IL-1Ra, IP-10 interferon (IFN)-α και IFN-γ, TNF-α, GM-CSF, CCL2, MCP-1/(MCAF), CCL3 , MIP-1α, CCL4 (MIP-1β) και CCL11

Αποτελέσματα Μετά από την στατιστική ανάλυση (unpaired student's t-test, 5% false discovery rate) προέκυψαν 103 διαφορετικά εκφραζόμενα γονίδια στον μυελό των οστών μεταξύ των ασθενών και των υγιών μαρτύρων τα οποία αντιπροσωπεύουν διάφορους μηχανισμούς δράσης. 54 εκ των 103 γονιδίων ενέχονται σε 4 μεγάλα δίκτυα που περιλαμβάνουν κυτταρικό θάνατο, καρκίνο, κυτταρική ανάπτυξη και πολλαπλασιασμό καθώς και διάφορα σηματοδοτικά μονοπάτια.

Ακολούθησε ιεραρχική ομαδοποίηση (hierarchical clustering) των δειγμάτων του μυελού των οστών –και του περιφερικού αίματος- βάσει της ομοιότητας του προτύπου της γονιδιακής έκφρασης και παρατηρήθηκε ότι οι ανενεργοί ασθενείς στον μυελό ομαδοποιήθηκαν διαφορετικά από τους ενεργούς βάσει 2652 γονιδίων με υψηλή έκφραση. Στον μυελό των οστών των ενεργών ασθενών παρατηρήθηκε υψηλή έκφραση γονιδίων σχετιζόμενα με τον κυτταρικό θάνατο και με την κοκκιοποίηση. Επίσης τα γονίδια που εμπλέκονται στην κοκκιοποίηση παρουσίασαν υψηλή συσχέτιση με την ενεργότητα του λύκου (SLEDAI) στο μυελό των οστων ($r = 0.55$, $p = 0.0259$).

Στη συγκριτική ανάλυση του μυελού των οστών με το περιφερικό αίμα προέκυψαν 88 γονίδια, 61 εκ των οποίων εμπλέκονται σε μηχανισμούς όπως ο καρκίνος, κυτταρική κίνηση και μορφολογία, ανοσολογική απάντηση καθώς και σε μηχανισμούς λειτουργίας του αιμοποιητικού συστήματος.

Στον ορό των ασθενών με ΣΕΛ , έξι κυτταροκίνες ήταν αυξημένες σε σχέση με τους υγιείς μάρτυρες : IL-1Ra, IP-10, IL-8, TNF-α, IL-15, και MCP-1. Καμια από τις κυτταροκίνες της χυμικής ανοσίας δεν ήταν ιδιαιτερα αυξημένη στον ορό των ασθενών.

Συμπέρασμα Η ανάλυση των δειγματων μυελού των οστών ασθενών με ΣΕΛ, με μικροσυστοιχίες διαχώρισε καλύτερα τους ασθενείς με ΣΕΛ βάσει της ενεργότητας της νόσου και αποκάλυψε ομάδες γονιδίων σχετιζόμενα με την απόπτωση και την κοκκιοποίηση, υπογραμμίζοντας την σπουδαιότητά τους στην παθογένεια της νόσου. Επίσης η αυξημένη έκφραση κυτταροκινών TNF-α και IL15, στον ορό ασθενών με ΣΕΛ, συνιστούν την επικράτηση της Th1-εξαρτώμενης ανοσολογικής απόκρισης.

CONTENTS

I.INTRODUCTION **11**

1.1 Systemic Lupus Erythematosus **11**

1.1.1 The disease 11

1.2.2 Immunopathology of SLE 15

B lymphocytes 15

T lymphocytes 17

The role of innate immunity in SLE 18

The role of adaptive immunity in SLE 21

1.2.3. Apoptosis 23

The role of apoptosis in SLE 23

Phagocytosis of apoptotic cells 26

1.2 Bone Marrow **29**

1.2.1 Components of BM 29

General 29

Haemopoietic cells 31

Lymphopoiesis 32

Granulopoiesis 33

1.2.2 Role of BM in SLE 34

1.3 Microarrays **38**

1.3.1 The technology 38

Limitations and pitfalls 40

1.3.2 Use of microarrays in autoimmune diseases 42

1.3.3 Microarrays in SLE 43

1.4 Hypothesis. Biological question **45**

II. PATIENTS. MATERIALS AND METHODS **47**

2.1 Patients and controls 47

2.2 Materials and methods 49

2.2.1 Processing of peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) 49

2.2.2 RNA extraction 50

2.2.3 RNA integrity 51

2.2.4 cDNA synthesis 52

2.2.5 Microarrays 54

2.2.6 Real-time PCR validation 56

2.2.7 Statistical analysis 58

2.2.8 Multiplex cytokine assay 59

III. RESULTS **62**

3.1 Differentially expressed genes in the bone marrow of SLE patients vs controls **62**

3.2 Bone marrow genes associated with SLE activity **65**

3.3 Genes differentially expressed in BMMCs relative to PBMCs of SLE patients **67**

3.4 Differentially expressed genes in the peripheral blood of SLE patients vs controls **69**

3.5 Hierarchical clustering reveals patient subgroups in the bone marrow **71**

3.6 Granulopoiesis and apoptosis signature in the bone marrow of active patients **74**

3.7 Granulopoiesis signature in the bone marrow as a marker for SLE activity **78**

3.8 Cytokine levels in SLE patients and healthy individuals 80

3.8.1 Serum levels of chemokines and Th1/Th2 cytokines 81

3.8.2 Associations between disease manifestations and serum concentrations of chemokines and cytokines. 82

IV. DISCUSSION **83**

4.1 Microarray studies in SLE 83

4.2 Granulopoiesis signature: a specific finding or an epiphenomemon 84

4.3 Neutrophil products and tissue injury 85

4.4 Integrins and their role in inflammation 86

4.5 Neutrophils and tissue injury in SLE 87

4.5.1 Apoptosis signatures in SLE 88

4.6 Neutrophil circulation model 89

4.7 Association of genes with disease activity 91

4.8 Cytokine balance in SLE patients 91

V. REFERENCES **93**

I. INTRODUCTION

1.1 Systemic Lupus Erythematosus

1.1.1. The disease

Systemic lupus erythematosus (SLE) is the prototypic systemic autoimmune disease characterized by the production of autoantibodies to components of the cell nucleus in association with diverse clinical manifestations encompassing almost all organ systems (**Figure 1.1**). SLE is a complex disease with variable course characterized by remissions and flares. Although its etiology is not established, much is known about the pathogenic pathways that lead to tissue injury: abnormally activated T-cells, overactive B-cells, monocytes and B cells with enhanced ability for antigen presentation, activated macrophages and neutrophils with increased capacity for tissue injury, all contribute to the initiation and amplification of the autoimmune response and the resultant tissue damage [1, 2]

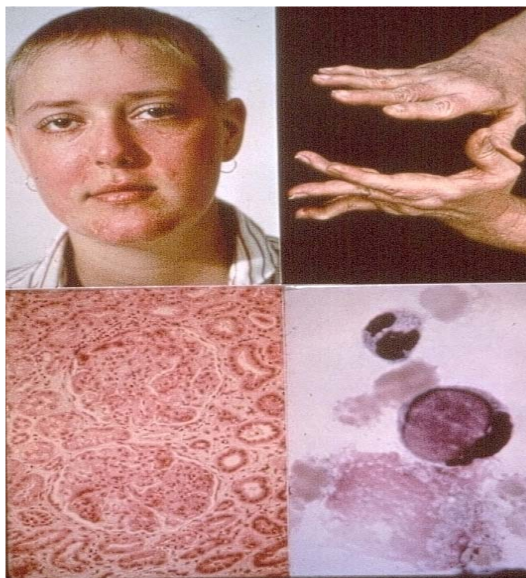

Figure 1.1 SLE is characterized by diverse clinical manifestations. Photosensitivity with "butterfly rash", glomerulonephritis, arthritis and immune complex disposition leading to tissue injury are manifestations of SLE

SLE affects predominantly women, with an incidence of 1 in 700 among women between the ages of 20 and 60 years (about 1 in 250 among black women) and a female to malio ratio 10:1. The principal clinical manifestations are rashes, arthritis and glomerulonephritis, but haemolytic anaemia, thrombocytopenia and central nervous system involvement are also common. According to the Diagnostic and Therapeutic Criteria Committee of the American College of Rheumatology (ACR) published revised criteria for the classification of SLE in 1982, patients must fulfil any combination of four (4) or more of the eleven (11) criteria. **(Table 1.1).** The activity of the disease is defined by the SLE disease activity index (SLEDAI). Based on the SLEDAI assessment system (**Table 1.2**) each clinical or laboratory manifestation accounts for a specific score and their sum defines the SLE activity.

Table 1.1. ACR criteria for classification of SLE

	Diagnostic and Therapeutic Criteria according to the American College of Rheumatology	Decsription
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 1.2. SLE Disease activity Index (SLEDAI)

Score	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.

In more detail, the pathophysiologic mechanism that leads to disease onset involves recognition of autoantigens from autoreactive lymphocytes and their activation and differentiation to effector cells (autoantibody producing B cells, autoreactive T cells providing activation signals to B cells and cytotoxic T cells) and finally disposition of immune complexes on tissues, triggering inflammation mechanisms that lead to tissue injury. Therefore SLE is a disease where both cellular and humoral immunity are involved.

1.1.2 Immunopathology of SLE

B lymphocytes

Hyperactivity of B lymphocytes is a well established manifestation of immune deregulation in SLE. Peripheral blood of SLE patients has increased number of activated B lymphocytes that produce immunoglobulins. These cells can produce autoantibodies against many soluble and cellular autoantigens. The most characteristic and common autoantigens in SLE are the ones directed against components of the cell nucleus which are called anti-nuclear antibodies (ANA). Among ANA specificities in SLE, two appear unique to this disease. Antibodies to double-stranded (ds) DNA and an RNA-protein complex termed Sm are found essentially only in SLE patients. Perhaps the most remarkable feature of the anti-DNA response is its association with immunopathologic events in SLE- in particular glomerulonephritis.

SLE is considered as a T cell dependent and autoantibody dependent immune response. However, B cells recognize autoantigens and produce autoantibodies in a T cell dependent or independent process. Peng et al. have shown that lupus-prone MRL mice congenitally deficient in alpha beta T cells could produce autoantibodies and develop lupus-like phenomena [3] Based on this observation and taking into consideration that one of the mechanisms in self tolerance is lack of T cell help, one possible explanation for activation of autoreactive B cells may be their intrinsic capacity to function independently and promote autoimmunity.

Hyperactivity of B cells in SLE can be also documented by the increased expression of activation markers on their surface. Lupus patients with active disease have 8 times more increased expression of the CD40 Ligand (CD40L), a costimulatory molecule through which B cells and T helper cells interact. Deactivation of CD40L with monoclonal antibody leads to reduction of anti-ds DNA antibodies in SLE patients [4]. Apart of CD40L, B lymphocytes of lupus patients also exhibit increased expression of CD80, CD86 and and CD21 molecules.

Besides their ability to produce autoantibodies, B cells of SLE patients can function as antigen presenting cells. The importance of B cell action was shown in MRL-lpr animal model where B cell deficient mice had a complete absence of T cell infiltrates and by comparing B cell-deficient and control mice, they demonstrated that the expansion of activated and memory T cells in the MRL-lpr/lpr mouse is indeed highly dependent on B cells. Therefore B cells can activate helper cells as well as cytotoxic cells by acting as antigen presenting cells[5]. This is achieved through the CD40-CD40L complex with cell contact as well as with molecules of the major histocompatibility

complex (MHCII or HLA-DR). Finally B cells can produce a series of cytokines and chemokines (IL-2, IL-6, IL-10, IFNγ, TNF-α and LTβ) and they can also take part in the polarization of immune response (Th1/Th2) which is of particular importance in SLE.

T lymphocytes

T lymphocytes take part in the initiation of SLE as well as in disease progression through various mechanisms. All three types of T cells, helper T cells (CD4+ cells), cytotoxic T cells (CD8+ cells) and regulatory T cells (CD4+/CD8+CD25++) have abnormal function in SLE patients.

Helper T cells promote B cell activation and autoantibody production. These autoreactive T cells are activated through stimuli that promote autoantigen presentation in the immune system as well as from foreign antigens. It has been shown that helper T cells specific for an autoantigen are capable of helping autoreactive B cells and shift immunity from homeostasis to autoimmunity. The expression of CD40L on activated T cells is one of the strongest signals that permit the continuous stimulation of autoreactive B cells thus prolonging autoimmunity.

In SLE there is impaired anti-CD3 driven cytolytic activity since cytotoxic T cells cannot target cell death which leads to the impaired clearance of autoreactive CD4 and B cells, overlooking immunological homeostasis and contributing to the onset of autoimmunity.

In the last few years, there is increasing evidence for the role of regulatory T cells in the pathogenesis of autoimmune diseases. These cells, also called Th3 cells, under physiological conditions they are able of suppressing the activation and expansion of

autoreactive T lymphocytes by secreting cytokines, like IL-10 and TGF-β. It has been documented that SLE patients have decreased number of regulatory T cells which function abnormally.

Individuals who develop SLE do so in a series of steps. There is a long period of predisposition to autoimmunity, then in a small proportion of those predisposed development of autoantibodies, which usually precede clinical symptoms by months to years [6] A proportion of individuals with autoantibodies develop a clinical prodrome with non specific symptoms that do not meet the criteria for SLE classification, and then among those a proportion develop full-blown SLE with various symptoms, autoantibodies and laboratory abnormalities that make the diagnosis clear. Individuals with clinical SLE usually experience over a period of many years intermittent disease flares and improvements and compile organ damage and cormorbidities related to chronic inflammation, to therapies, and to aging.

Generation of autoreactive T cells and autoantibodies in SLE patients involves two major networks; the innate immune network and the adaptive network.

The role of innate immunity in SLE

External and internal danger signals including infections and self antigens, activate innate immunity primarily via dendritic cells (DC) located in tissues that sample the environment (lungs, intestines, skin, peripheral lymphoid tissues). Pathogen-associated molecular patterns (PAMPs) shared by many bacteria and viruses are recognized by toll-like receptors in DC. In SLE it is important that TLR9 in DC and B lymphocytes can bind CpG DNA sequences [7-9] Such sequences are common in

bacterial DNA and uncommon in mammalian DNA but increased in SLE patients. Therefore, lupus DC can be activated by CpG nucleotides in tissue or circulation. Furthermore, DNA in DNA/anti-DNA immune complexes binds TLR9, and the anti-DNA binds FcRγIIA receptors on DC, thus further activating innate immunity. Other TLRs (TLR3, 7, 8) recognize viral single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA); RNA/protein complexes characteristic of SLE can also bind TLR7 [10]. Binding of any of these TLR, particularly in plasmacytoid DC (pDC) results in release of IFNα and other cytokines from the immature pDC; interferon –α (IFN-α) promotes maturation of DC and of monocytes / macrophages. Both cell types then express peptides in their major histocompatibility complex (MHC) surface molecules, becoming potent antigen-presenting cells (APCs) for activation of T lymphocytes. Additionally, the activated macrophages release multiple cytokines that promote maturation and activation of T lymphocytes (particularly toward Th1 phenotype) and of B cells which increase in immunoglobulin production and in switching to pathogenic IgG subclasses. This series of events leads to the activation of T and B cells (some of which are autoreactive) ready to participate in adaptive immune responses. The difference in these processes in SLE patients compared to healthy individuals is that lupus patients have greater amounts of hypomethylated DNA with CpG sequences [11], circulating DNA/anti-DNA complexes and possibly recent or reactivated infection with EBV [12-14] Additionally, certain polymorphisms in various TLR and in FcγR may predispose these individuals to abnormal innate responses. Therefore it is likely that innate immune responses are upregulated in patients with SLE (**Figure 1.2**), promoting antigen processing/

presentation and the activated state of T and B lymphocytes participating in and promoting adaptive immunity against self.

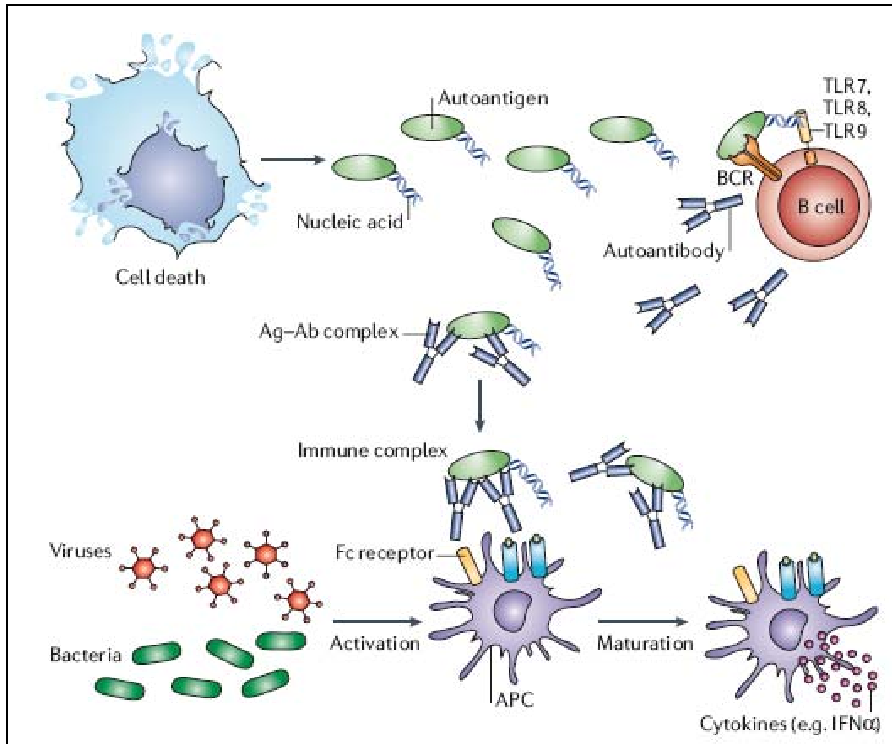

Figure 1.2 Model of innate immune responses in SLE. Innate immunity can be triggered by both endogenous and exogenous ligands, in cooperation with adaptive immune mechanisms (specific antibodies). Dying cells release particulate autoantigens containing protein and nucleic-acid fragments. Circulating B cells that have specificity for these antigens can be activated as a result of stimulation through the B-cell receptor (BCR) along with Toll-like receptors (TLR7, TLR8 and TLR9). This leads to further production of autoantibodies and immune complexes. These in turn might stimulate plasmacytoid dendritic cells, or other antigen-presenting cells (APCs) to produce type 1 interferons, such as interferon-α (IFNα). Similar triggering can occur directly through TLRs by microbial agents. These pathways are involved in the pathogenesis of human systemic lupus erythematosus. *(Peter K. Gregersen & Timothy W. Behrens,* Nature Reviews Genetics ***7****, 917-928 (December 2006)*

The role of adaptive immunity in SLE

Adaptive immunity is triggered when a pathogen evades the innate immune system and generates a threshold level of antigen. Antigens, both external (foreign) and internal (auto), encounter activated APCs which internalize them and process them into peptides, subsequently presenting the peptides in surface MHC molecules. B lymphocytes and monocyte/macrophages are the main APCs in adaptive immunity, whereas DC are prominent APC in innate immunity. Some non-immune cell, when activated such as mesangial cells, also express MHC class II on their surfaces and serve as APC for local T cell activation. T lymphocytes with antigen-specific TCR receive first activating signals from MHC/peptide complexes and the second signals from molecules such as CD86, which are also expressed on activated APC. In the case of helper T cells (mostly CD4+, CD8+ also included in SLE patients), cells are activated to secrete cytokines (IFNγ, IL6, IL10) that help B lymphocytes to produce autoantibodies (**Figure 1.3**). Some antibodies fix directly to target organs , such as platelet surface molecules or α-actinin in glomeruli [15] Other autoantibodies form pathogenic immune complexes of correct size, charge, conformation and antigen reactivity to bind to tissue (platelet, glomeruli, skin, blood vessels are frequent targets). Antibody-dependent cell cytotoxicity, or complement activation with subsequent inflammation can result, leading to clinical disease and to tissue damage. While this process of autoantibody production is quite active in SLE patients, many of the regulatory networks designed to shut off antibody production are defective. This includes decreased phagocytosis of apoptotic cells and of immune complexes [16]. Additionally, generation of regulatory

CD4+CD25+ T cells and suppressive CD8+ T cells is probably defective [17] Defects in function of these cells have been well documented in several models of murine lupus and evidence that they occur in human disease is accumulating . It has been demonstrated that SLE patients compared to normals produce abnormally small quantities of transforming growth factor beta (TGFβ) from peripheral blood cells [18]. TGFβ along with IL2 (production of which is also reduced in T cells of many SLE patients), is required for generation of CD4+CD25+ Treg and some CD8+ T cells, one group has shown that such cells from SLE patients cannot suppress proliferation of CD4+ T cells and autoantibody production in the normal manner, particularly when disease is clinically active [19]

In summary the immune abnormalities characteristic of SLE include: 1. brisk production of autoantibodies and immune complexes that contain pathogenic subsets. 2. enhanced upregulating mechanisms and 3. defective downregulating mechanisms.

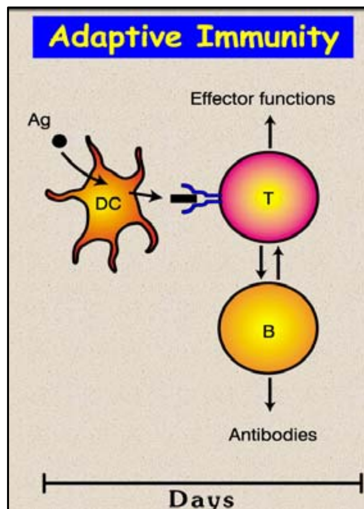

Figure 1.3 Schematic representation of adaptive immunity

1.1.3 Apoptosis

General

One of the most well documented events in SLE is the defective clearing mechanism of necrotic and apoptotic cells that result in a pool of autoantigens for autoreactive B and T cells for prolonged periods of time. Deficiencies of classical pathway complement proteins especially C2 or C4, are seen in about 10% of patients with SLE and also result in defective clearance of immune complexes. Apart from complement deficiencies, other protein deficiencies of non specific immunity such as MBL, DNAse and C-reactive protein that also take part in clearance of apoptotic bodies are also involved in SLE [20, 21] Furthermore, other factors that increase apoptosis, such as ultraviolet light can lead to deterioration of the disease.

The role of apoptosis

Apoptosis or programmed cell death is recognized as being fundamental to maturation and homeostasis of the immune system. During maturation of the immune system apoptosis of autoreactive lymphocytes in the central lymphoid organs underlies the development of tolerance. Furthermore, due to apoptosis the size of the peripheral lymphoid and myeloid compartments is limited. Activated lymphocytes are deleted through apoptosis following an immune response [22] Furthermore, alterations of cellular constituents during apoptotic degradation, occurring via posttranslational modifications such as oxidation, phosphorylation, or citrullination, can induce immunogenicity [23]

These findings made it understandable why in autoimmunity an immune response can develop against intracellular, cryptic epitopes, and stresses the important role apoptotic cells might play in the pathogenesis of autoimmune disorders. Whenever apoptotic cells do accumulate by an increased rate of apoptosis, decreased elimination, or a combination of both, it can be imagined that, in the proper environment, tolerance can be broken. For the initiation of the apoptotic process ligation of the cell death receptors is mandatory.
Cell death receptors belong to the tumor necrosis factor (TNF) receptor superfamily and share a similar, cysteine rich extracellular domain. In addition, the death receptors contain a homologous cytoplasmic sequence called the death domain. The best characterized death receptors are Fas and TNFR1. The Fas-mediated apoptosis pathway has been extensively studied and is crucial for the development of immune tolerance [24] **(Figure 1.4.)**

Studies in human SLE have demonstrated increased levels of apoptotic cells even in the presence of factors that can inhibit apoptosis induction like elevated levels of sFas and increased expression of Bcl-2. The increased levels of apoptotic cells found might be due to increased in vivo activation and Fas-mediated apoptosis of lymphocytes. Taken together, data from lupus patients supply evidence that the increased induction of apoptosis via Fas or via other pathways can trigger the development of autoimmunity.

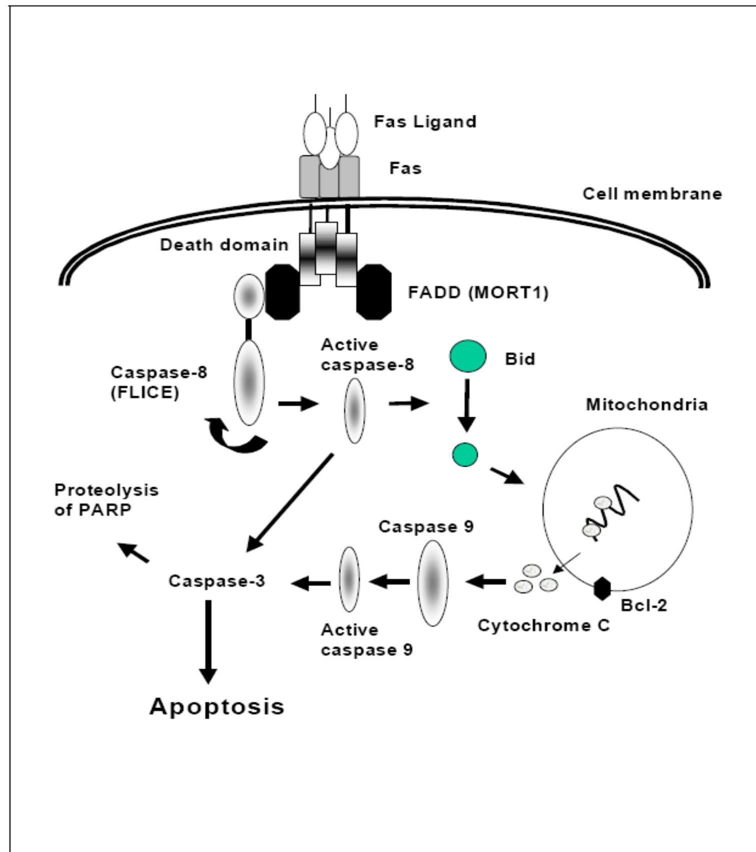

Figure 1.4 : Apoptotic signal transduction induced by binding of Fas ligand (FasL) to its receptor Fas (CD95). FasL is a homotrimeric molecule. Binding to Fas results in trimerization of Fas. The Fas cytoplasmatic region carries a death domain. Trimerization of this death domain recruits caspase 8 via an adaptor called FADD (Fas associated death domain)/MORT1. Upon recruitment by FADD, caspase-8 (also called FLICE) drives its activation through self-cleavage. Activated caspase-8 then degrades poly (ADP-ribose) polymerase (PARP), an enzyme that is thought to be involved in DNA repair, and activates downstream effector caspases committing the cell to apoptosis with the characteristic degradation of chromosomal DNA and morphological changes.

Furthermore, caspase-8 cleaves Bid, a pro-apoptotic member of the BH3 subfamily. Cleaved Bid stimulates the release of cytochrome C from mitochondria which activates caspase-9, which subsequently activates caspase-3. The release of cytochrome C into the cytoplasm can be inhibited by bcl-2 which resides on the cytoplasmic face of the mitochondrial outer membrane.

Phagocytosis of apoptotic cells

The increased presence of apoptotic cells as demonstrated in the peripheral blood of SLE patients can be accounted for by an increased level of activation induced cell death. However, apoptosis is a physiological mechanism and occurs continuously in impressive amounts. Removal of apoptotic cells occurs very effectively via phagocytosis by bystander (semi-professional) or professional phagocytes like monocytes and macrophages. Rapid elimination of apoptotic cells is important as it prevents the release of (toxic) cell constituents like cytolytic enzymes. Furthermore, during the process of apoptosis antigens are newly exposed in the cell membrane of the apoptotic cell [25] . Adequate removal of apoptotic cells therefore also seems important for the prevention of (excessive) autoantigenic exposure. The interaction between apoptotic cells and other cells, necessary for their elimination, is very complex . Monocytes and macrophages constitutively express several receptors like CD14, CD36 and scavenger receptors, all involved in the recognition, binding and internalisation of apoptotic cells. Next to binding of apoptotic cells to these receptors several serum proteins play a role in this process of elimination **(figure 1.5**). Already early in the apoptotic cascade the cell membrane changes. For example, phosphatidylserine is exposed at the outer surface of the cell membrane. Due to these changes several serum constituents like complement C1q, C3 and C4, C-reactive protein (CRP), serum amyloid protein P (SAP) and phospholipase A2 can bind to the apoptotic cell and facilitate, the interaction with phagocytes Furthermore, phagocytosis itself modulates phagocyte behaviour. The ingestion of apoptotic cells in vitro promotes the secretion of anti-inflammatory cytokines in human macrophages [26]

Furthermore, uptake of apoptotic neutrophils by macrophages reduced the uptake of the former cells when a rechallenge was performed after 48 hours [27].In this context, it can be speculated that an increased rate of apoptosis could lead to an overflow of the phagocytic system with apoptotic cells through this negative feedback loop.

Thus, next to increased induction of apoptotic cells an intrinsic decreased clearance capacity of the phagocytic system, possibly in combination with defects in the production of anti-inflammatory mediators by macrophages, might be an important pathogenic factor in the development of SLE.

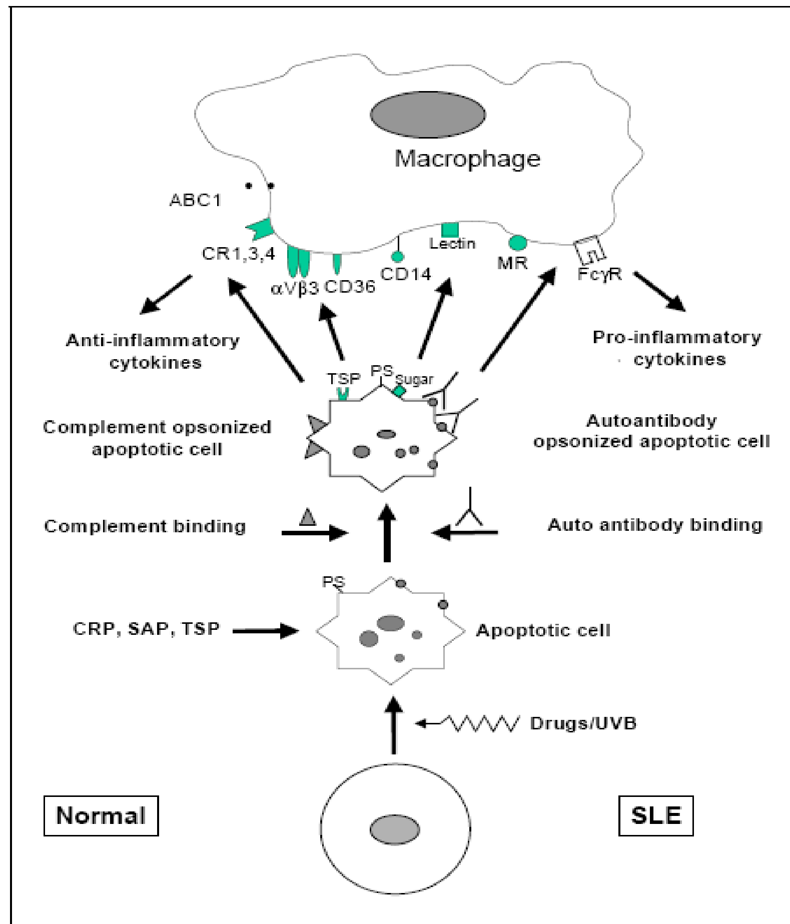

Figure 1.5 Phagocytosis of apoptotic cells by macrophages. Cells die by apoptosis during tissue turnover or at the end of an immune response. In SLE patients additive stimuli like UVB or certain drugs can increase the number of apoptotic cells generated. In SLE patients autoantibodies can bind to the resulting antigens whenever exposed. This will enable FcγR-bearing cells to interact with antibody opsonized apoptotic cells via the FcγR resulting in the release of pro-inflammatory cytokines.

1.2. BONE MARROW

1.2.1 Components of the bone marrow

General

Bone marrow (BM) is a central lymphoid organ consisting of various types of hemopoietic (neutrophils, lymphocytes, macrophages, osteoclasts) and non- hemopoietic cells (endothelial cells, osteoblasts, adipocytes) and the “stroma” that supports their growth, differentiation and function, collectively called as “the BM micro-environment” [28] (**Figure 2.1)**

The stroma is composed of fat cells and a meshwork of blood vessels. Branching fibroblasts, macrophages, some myelinated and non-myelinated nerve fibers and a small amount of reticular cells. There is a close interaction between haemopoietic cells and their microenvironment, with each modifying the other.

In humans normal haemopoiesis, with the exception of some thrombopoiesis at extramedullary sites, is confined to the intertitium. In pathological conditions haemopoiesis can occur within sinusoids. Mature haemopoietic cells enter the circulation by passing transcellularly, through sinusoidal endothelial cells.

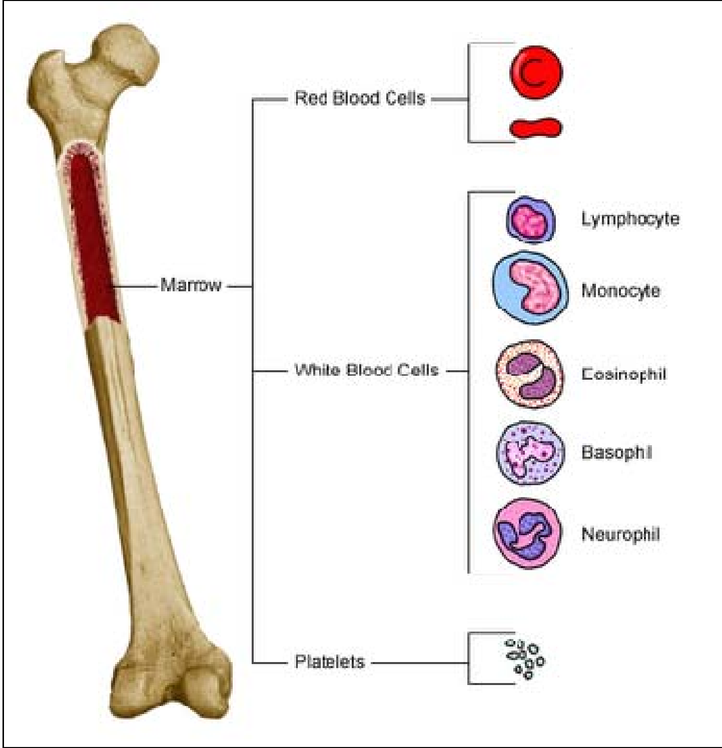

Figure 2.1 Cells of the bone marrow

Haemopoietic cells

A multipotent stem cell gives rise to all types of myeloid cell: (i) erythrocytes and their precursors; (ii) granulocytes and their precursors; (iii) macrophages, monocytes and their precursors; (iv) mast cells; and (v) megakaryotes and their precursors (**Figure 2.2.)**

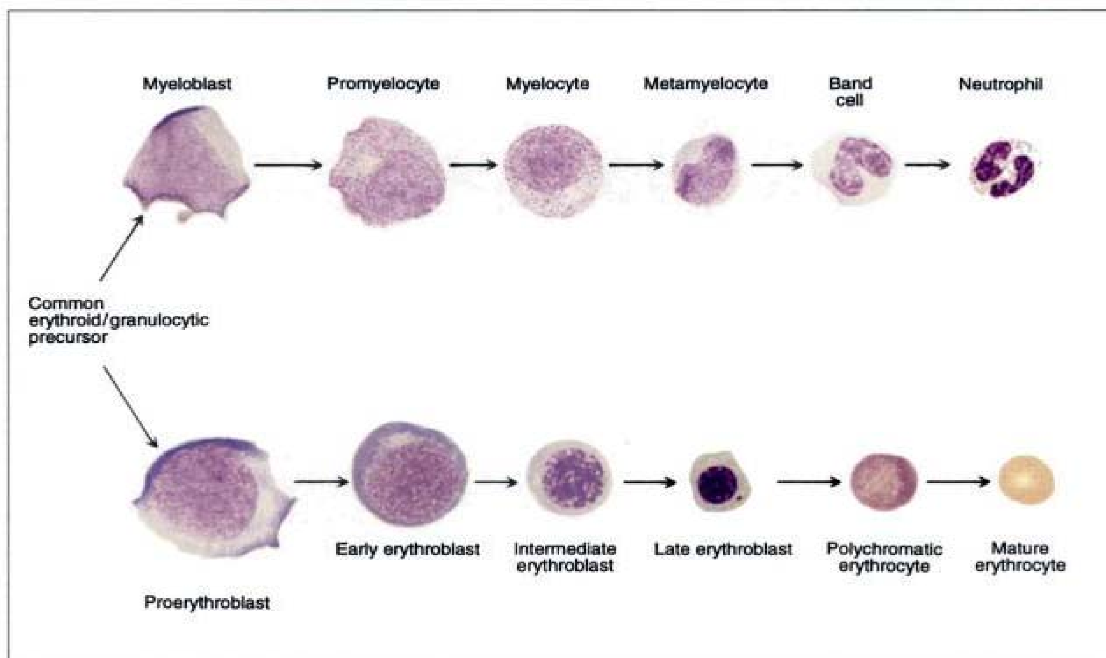

Figure 2.2 Semi-diagrammatic representation of granulopoiesis and erythropoiesis. Cell division occurs up to the myelocyte and intermediate erythroblast stages

The term “myeloid” can be used with two rather different meanings. It is used to indicate all cells derived from the common myeloid stem cell and also to indicate only the granulocytic and monocytic lineages, as in the expression “myeloid:erythroid ratio” (M:E). The common myeloid stem cell and stem cells committed to the specific myeloid

lineages cannot be identified morphologically but it is likely that they are cells of similar size and appearance to a lymphocyte. The various myeloid lineages differ both morphologically and in their disposition in the bone marrow. The normal bone marrow contains in addition to myeloid cells, smaller numbers of lymphoid cells (including plasma cells) and the stromal cells.

Lympopoiesis

Both B and T lymphocytes share a common origin with myeloid cells, all of these lineages being derived from a pluripotent stem cell. The bone marrow contains mature cells and precursor cells of both T- and B-lymphoid lineages. T cells are more numerous among mature cells whereas, among precursor cells those of B lineage are more frequent. Bone marrow lymphocytes are small cells with high nucleocytoplasmic ratio and weakly basophilic cytoplasm. The nuclei show some chromatin condensation but the chromatin often appears more diffuse than that of peripheral blood lymphocytes. Lymphocytes are not very numerous in the marrow in the first few days of life but otherwise during infancy they constitute a third to a half of bone marrow nucleated cells. Numbers decline during childhood and in adults they do not generally comprise more than 15-25% of nucleated cells, unless the marrow aspirate has been considerably diluted with peripheral blood . If there is no haemodilution , they usually account for approximately 10% of nucleated cells. The majority of lymphocytes in normal bone marrow are CD8 positive T lymphocytes.

Normal bone marrow contains scattered interitial lymphocytes and sometimes small lymphoid nodules or follicles . Estimates of lymphocyte numbers based on histological sections are considerably lower than those based on aspirates. In one study approximately 10% of bone marrow cells were lymphocytes with the ratio of T to B cells being 6:1. Lymphocytes appear to concentrate about arterial vessels near the centre of the haemopoietic cords. Lymphoid follicles of normal marrow have small blood vessels at their centre and may contain a few macrophages, peripheral mast cells or plasma cells.

Plasma cells

Plasma cells are infrequent in normal bone barrow in which they rarely constitute more than 1% of nucleated cells. They are distinctive cells with a diameter of 15-20µm and an eccentric nucleus, moderately basophilic cytoplasm and a prominent paranuclear Golgi zone.

Granulopoiesis

There are at least four generations of cells between the morphologically unrecognizable committed granulocyte-monocyte precursor and the mature granulocyte but cell division does not necessarily occur at the same point as maturation from one stage to another. The first recognizable granulopoietic cell is the myeloblast which is capable of cell division and matures to promyelocyte which in turns matures to a myelocyte. Myelocytes are smaller than promyelocytes and are quite variable in size- from 10 µm to 20µm. Their nuclei show partial chromatin condensation and lack nucleoli. Their cytoplasm is less basophilic than that of promyelocytes and specific neutrophilic, eosinophilic and basophilic granules can be discerned. Late myelocytes

mature to metamyelocytes which in turn matures to a polymorphonuclear granulocyte with a segmented nucleus and specific neutrophilic, eosiniphilic or basophilic granules. The bone marrow is a major reservoir for mature neutrophils.

1.2.2 The role of Bone Marrow in SLE

Bone marrow (BM) is the site of production and maturation of B cells as well as T cell production in adults. Furthermore it is the site where cells of the monocytoid lineage are produced and among them are the myeloid dendritic cells (mDCs) which are the professional antigen presenting cells of the immune system. Apart of production and maturation of the immune system cells, bone marrow is the site where antigen presentation takes place; both foreign antigens and autoantigens are presented in the site. Therefore BM has a central role in regulating the immune response and this underlies its importance as a significant organ in SLE pathogenesis.

Bone marrow is an important site for the biology of B cells. It is the site where B lymphocytes are produced and expand after positive selection. After their encounter with antigens, IgM and IgG receptors are expressed leading to activation of B cells. In this stage activated immature B cells leave the bone marrow and migrate to secondary lymphoid organs (spleen, lymph nodes) where they form germinal centers. Inside germinal centers and in the presence of T helper cells, B cells undergo T-cell –dependent antibody production with affinity maturation. During this process B cells produce antibodies with increased affinity for antigen during the course of an immune response. With repeated exposures to the same antigen, a host will produce antibodies of

successively greater affinities. A secondary response can elicit antibodies with several log fold greater affinity than in a primary response. The process is thought to involve two interrelated processes, occurring in the germinal centers of the secondary lymphoid organs: somatic hypermutation and clonal expansion. In somatic hypermutation (SHM), polymorphisms in the variable, antigen-binding coding sequences (known as complementarity-determining regions) of the immunoglobulin genes clonally accumulate with repeated stimuli, by a process mediated by Activation-Induced (Cytidine) Deaminase. These polymorphisms stochastically alter the binding specifity and binding affinities of the resultant antibodies produced by progeny. In clonal selection, B cells that have undergone SHM must compete for limiting growth resources, including the availability of antigen. The follicular dendritic cells (FCDs) of the germinal centers present antigen to the B cells, and only the B cell progeny with the highest affinities for antigen will be selected to survive. B cell progeny that have undergone SHM, but bind antigen with lower affinity will be outcompeted, and be deleted. Over several rounds of selection, the resultant secreted antibodies produced will have effectively increased affinities for antigen

Immunoglobulin secreting cells, ISCs can be distinguished in short lived plasmablasts that produce antibodies for a short time period and long-lived plasma cells. Long-lived plasma cells, normally migrate to the bone marrow where they survive for a long periods of time (> 6 months) and are able to produce antibodies independently of T cell help or the presence of antigens in survival niches. (**Figure 2.3)**.

Thus, BM is crucial for the development of central B cell tolerance with as many as 70% of B cells dying within the BM. Homing, survival and antibody production by

long-lived plasma blasts as well as production of antibodies in an antigen-, T-cell-independent manner are additional features of BM which may be relevant to the pathogenesis of SLE [29]

Hagraves et al. was the first to demonstrate the “lupus cell phenomenon” in the bone barrow, suggesting a possible implication of BM in SLE pathogenesis. In their study they describe lupus cells as polymorphonuclear granulocytes that rearrange their morphology (nucleus is shifted to the cell periphery) after they phagocytose nuclear content (lupus autoantigens) [30]

In recent years it is well established that bone marrow in lupus patients presents hematologic abnormalities and exhibits a variety of histopathologic findings including necrosis, stromal alterations and distortions of the architecture characterized by abnormal localization of immature precursors [28, 31]

In some tissues such as the BM and spleen of mice with SLE, up to 40% of plasma cells are long-lived and resistant to the current immunosuppressive /cytotoxic therapies, in contrast to short-lived cells. This maybe explains their inefficiency to induce prolonged disease remission. It is possible that the BM in SLE creates a microenvironment that favors the prolonged survival and activation of plasma cells. Therefore these long-lived, autoreactive plasma cells can be a target for therapies.

The heterogeneity of SLE extends to the type of clinical manifestations at disease onset and to the time it may take for these manifestations to evolve and make the diagnosis possible. As the understanding of the pathogenesis of SLE is improving more advanced tests are available.

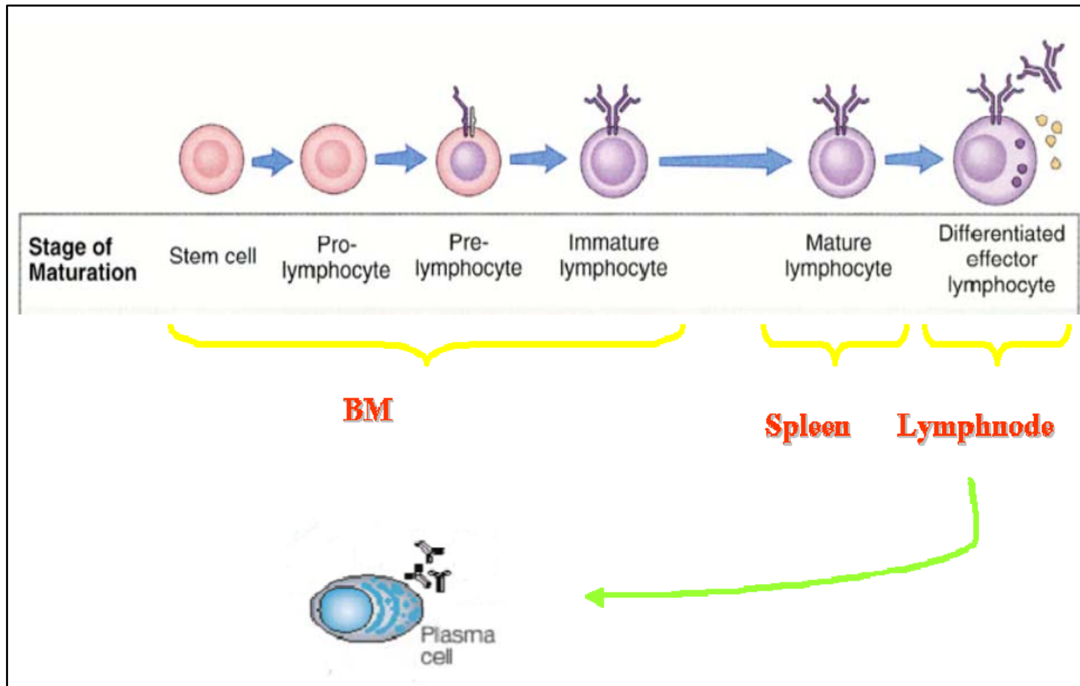

Figure 2.3 B cell biology. Schematic representation of B cell maturation stages in the bone marrow and peripheral lymphoid organs until their differentiation to plasma cells and homing to BM

1.3 MICROARRAYS

1.3.1 The technology

Microarray analysis is a broad-based profiling method that permits the concomitant comparison of gene expression profiles among different study groups revealing active networks of interrelated genes within subpopulations under study. Through the clustering of subgroups of patients sharing common or close gene expression profiles, it delineates associations of active genes/gene networks with distinct disease manifestations, providing important clues about pathogenesis, diagnosis, prognosis and potential therapeutic targets of the disease.

The first step in the construction of a microarray is to identify and collect clones (cDNAs) or short oligonucleotides that encode genes important for research purposes. cDNA arrays can be designed and constructed with a number of different goals in mind. Such arrays may be focused on a particular tissue, chromosome, developmental stage, gene family, disease, or functional characteristic (e.g., signaling molecules, cytokines, apoptotic-mediators), or may be unfocused. Oligonucleotide microarrays are manufactured by *in situ* synthesis on glass using a combination of photolithography and oligonucleotide chemistry. The result is a panel of short oligonucleotides that, depending on the particular array, identify up to about 33,000 discrete human genes. Recently, other manufacturers have begun to produce what are being called “spotted” oligonucleotide arrays. Rather than the oligonucleotide being directly synthesized on the array substrate,

these arrays are constructed using a robotic pin-based microarrayer to spot conventionally synthesized 40- to 80-bp oligonucleotides onto glass slides or nylon filters.

The process of DNA hybridization involves the reassociation of single-stranded DNA to form double-stranded DNA with one strand originating from a cell or tissue under study and the other strand with the target sequence that has been printed or synthesized on the microarray. A crucial factor for successful hybridization is the purity and quality of the RNA extracted from the cells or tissue of interest. Contamination of this RNA with genomic DNA, proteins or detergent residues, or its degradation by ubiquitous ribonucleases may cause serious problems during the RT-PCR steps of the procedure. The method of labeling probe RNA depends on the particular type of microarray being used for the study. With microarrays printed on glass slides, it is customary to label during reverse transcription one sample with the dye cyanine-3 (Cy3) that, when excited by light, yields green fluorescence and, the other sample with cyanine-5 (Cy5) that yields red fluorescence. Posthybridization, the arrays are washed and quantity of signal incorporated in each spot is measured using either a specialized slide reader or an imaging system

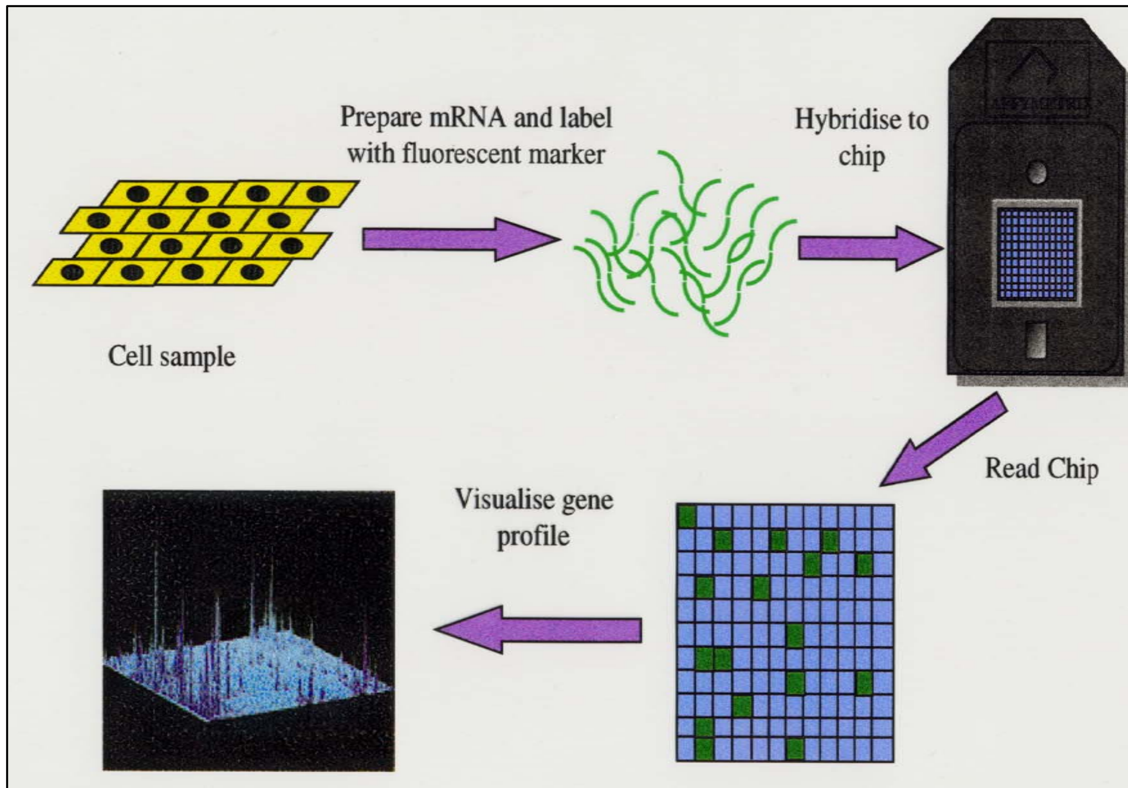

Figure 3.1 Basic microarray steps. Clones or oligonucleotides are selected and robotically spotted onto a glass slide or nylon filter. RNA is extracted from cells or tissues of interest and labeled either with cy3 or cy5 (glass slides) or with P33 (nylon filters) and hybridized. Images are analyzed and data is clustered and information extracted using bioinformatics.

Limitations and pitfalls

Genome-scale assessment of gene expression have already proved to be a valuable technique Based on these findings, gene-expression profiling is largely a research tool, although it allows for future classification and the prospect of providing us with prognostic data [32] However there are pitfalls and limitations with the existing methods that cannot be neglected.

The methods used for studying gene expression are seldom directly comparable [33]. First, in some studies filters and not glass support have been used, requiring different techniques for printing, probing and hybridization of the arrays and visualization of the signals. Secondly, the statistical methods used are not always optimal, or the mathematical algorithms are not always appropriate [34]. Thirdly, some arrays encompass genes from the whole genome, while others have a reduced number of genes printed. This makes comparison difficult. Even if a gene is not mentioned as differentially expressed by a research group, it may still be differentially expressed. It may be that a particular gene is not printed on an array, therefore is not examined, and consequently not reported. Fourthly, handling of the biopsies or patient samples may differ a lot. At what stage in the disease development is the biopsy taken? Where in the organ is it taken from? Is there a standardized sample preparation? Fifthly, the biggest challenge is probably the biological variability, which underscores the importance of replicate data and a proper experimental design [35]. To account for the biological variations, further validations with post-analysis follow up are crucial, such as for instance to confirm the microarray results with RT-PCR, in situ hybridization or Northern blot [35, 36]. Finally, one should be aware that the mRNA expression is not a mirror image of the protein expression. Not all transcribed mRNA are translated into functional proteins. Therefore gene-expression profiling will not provide biochemical information on functional activities of proteins. Genomic and proteomic technologies will therefore be complementary in their application [37].

1.3.2 Microarrays in autoimmunity and other diseases

Microarrays have enabled the molecular classification and prediction of clinical outcome of human malignancies such as diffuse large B cell lymphoma, leukemia, breast, colon and prostate carcinoma, and identified the genes and pathways whose altered expression is implicated in diseased human tissues [38, 39].

In multiple sclerosis (MS), DNA microarrays have provided insights into disease pathogenesis and patient classification as well as biomarkers that can guide the use of patient-tailored therapies [40, 41] Gene-expression profile of peripheral blood CD3+ T cells isolated from MS and healthy control subjects, identified distinct subgroups of Japanese MS patients associated with differential disease activity and therapeutic response to IFN-b treatment [42] They suggested that microarray-based classification of MS is useful to predict therapeutic response to IFN-b. T cell gene-expression profiling identified aberrant expression of key regulators for drug metabolism

In inflammatory diseases such as RA, expression patterns of diverse cell types contribute to the pathology. Microarray technology has been used to define the differences in the gene expression profiles in synovial tissue of RA in comparison with osteoarthritis [43, 44]. There is growing evidence that B cells contribute to the pathogenesis of RA. As the pathological regulation of these functions may involve various sets of genes controlling several aspects of cellular homeostasis, our group used microarray technology to define the geneexpression profile of peripheral blood B cells in RA [45] Peripheral blood B cells in RA overexpress or repress specific clusters of genes that regulate cell cycle, programmed cell death, proliferation and innate immunity, furthermore control important developmental stages of B cells

1.3.3 Microarrays and SLE

The gene-expression profile of peripheral blood from patients with SLE shows that active SLE can be distinguished by a remarkably homogeneous gene-expression pattern with overexpression of granulopoiesis-related and IFN-induced genes [46] In patients with active lupus, the level of circulating IFN-a is increased. Striking results support the idea that a so-called IFN signature regulates disease activity in lupus. A significant increase was found in the expression of genes induced by IFN in SLE subjects. Type I IFN (including IFN-a, IFN-b, IFN-s, IFN-x and IFN-j) is extremely important in the gene-expression pattern that distinguishes between PBMCs of SLE patients and healthy controls [47] Other findings also support the idea that in patients with SLE, the IFN gene signature plays a crucial role, both in PBMC [48, 49], and in the target organ of SLE, as shown by Peterson et al. by gene-expression profiling of the glomeruli from kidney biopsies from SLE patients [50] The gene-expression profile of PBMC from SLE patients exhibited more than 2.5-fold difference in expression level compared with controls, while 20 genes were significantly different between patients and controls [48] . These genes belong to different families like tumour necrosis factor (TNF)/death receptor, interleukin (IL)-1 cytokine family and IL-8 and its receptors. These results emphasize the potential use of the microarray in identifying genes associated with SLE [34].

Another study on PBMC from SLE patients with active and inactive disease revealed that genes belonging to a variety of functional groups, such as adhesion molecules, proteases, members of the TNF superfamily and neurotrophic factors have

key roles in the disease development and flares [51] .Bennett et al. reported that several IFN-induced genes highly correlate with disease activity in lupus (SLEDAI) [46]. It is difficult to measure the effect of an ongoing therapy on lupus activity, because there are often several months passing until objective findings can reflect the improvement of the patients. Yet, the need to possess objective and fast measurements to control medication efficacy is critical. Kirou et al. could differentiate between active and inactive lupus patients by identifying type I IFN pathway [52]. Accordingly, IFN-a-induced gene expression has been described in SLE patients with high-dose glucocorticoid treatment [46] By using microarray technology, the beneficial effect of a given therapy could be measured in a few days, even in hours. Based on these findings we assume that type I IFN pathway could be an excellent target of therapeutical approaches. Gene-expression profiling by microarray in SLE has been reviewed by Qing and Putterman [53]

1.4 Hypothesis. Biological question

Systemic lupus erythematosus (SLE) is a chronic, inflammatory autoimmune disease characterized by the production of antibodies with specificity for a wide range of self-antigens The organs commonly targeted in SLE include the skin, kidneys, joints, lungs, various blood elements, and the central nervous system (CNS). The severity of disease, the spectrum of clinical involvement, and the response to therapy vary widely between patients, and this leads to significant challenges in the diagnosis and management of lupus. Therefore there is a growing need for identification of novel targets for lupus treatment.

Although peripheral blood is an easily accessible and disease appropriate tissue to study the immune response in a variety of autoimmune diseases, it is limited by the fact that the relevant cells and molecules may be "diluted" by a whole host of less relevant counterparts. On the other hand, sampling of lymphoid organs such as peripheral lymph nodes, while lacking these limitations, is invasive and thus not feasible for the examination of a large number of patients. Bone marrow, a central lymphoid organ, offers a less invasive and thus more practical site to study immune responses in SLE.

DNA microarrays on the other hand is a powerful emerging technology which provide us a genome-wide gene expression profiling and allows the simultaneous measurement of thousands of mRNA transcripts in a biologic sample therefore a very attractive tool for the elucidation of genes involved in lupus pathogenesis.

To this end, we decided to explore gene expression profile using the microarrays and cytokine assays in lupus patients and healthy individuals and compare it to the peripheral blood and answer the following questions:

1. Is there a correlation between the expression of genes in peripheral blood and the BM?
2. Which are the genes that are expressed differently in these tissues and which is their contribution in the pathogenesis and course of SLE
3. Is there any correlation between known biological functions of the BM (production of blood cells , maturation and activation of B cells) and the expression of particular genes or group of genes?
4. Can microarrays facilitate the characterization of patients' subgroups with different pathogenesis, clinical manifestations and prognosis?
5. Can the evaluation of gene expression improve disease prognosis, independently of known clinical and laboratory findings?
6. Is there a cytokine signature in lupus patients that correlates with disease manifestations?

II. PATIENTS AND METHODS

2.1 Patients and controls

Twenty seven patients with SLE - followed by the Rheumatology Department of the University Hospital of Crete, a tertiary referral center- were studied following written informed consent. All bone marrow samples were obtained from patients that provided peripheral blood. All patients met the 1982 American College of Rheumatology revised criteria for the classification of SLE [54]. In order to capture patients with higher disease activity, we used a SLE Disease Activity Index score cut-off of (SLEDAI) ≥8.

Clinical and laboratory characteristics of the patients included in the study are summarized in **Table 2.1**. Seven patients had active proliferative and/ or membranous nephritis, while six had active neuropsychiatric lupus with manifestations such as psychosis, major depression, myelitis and polyneuropathy.

Patients had not received steroids for at least 24 hours before blood (20ml) and bone marrow (20ml) was obtained for study. Controls enrolled in the study included seven healthy individuals and three osteoarthritis patients (5 males and 5 females, age ranging from 35-55 years) from the Department of Transfusion Medicine, Hematology Clinic and Rheumatology Clinic of University Hospital of Crete.

Table 2.1. **Clinical and demographic characteristics of SLE patients ***

Sex, female/male	26 /1
Age, mean ± SD	47.28 ± 17.2
Active / Inactive	16 (59%) / 11 (41%)
SLE duration, mean ± SD	6.7 ±5.6
SLEDAI (mean ± SD)	
Active SLE	13.86 ± 4.99
Inactive SLE	4.22 ± 1.56
Nephritis	
Active SLE	7 / 16
Inactive SLE	0 /11
Total	7 / 27
CNS	
Active SLE	6 / 16
Inactive SLE	0 /11
Total	6 / 27
Cytotoxic therapy	
Active SLE	4 / 16
Inactive SLE	1 / 11
Total	5 / 27
Steroids	
Active SLE	9 / 16
Inactive SLE	3 / 11
Total	12 / 27
Hydroxychloroquine	
Active SLE	10 / 16
Inactive SLE	3 / 11
Total	13 / 27

2.2 Materials and methods

2.2.1 Processing of peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) .

Reagents-Materials

Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO)
Phospate Buffer Saline (Gibco, UK)
Heamocytometer glass slide (Neubauer)
Optical microscope (Olympus, Japan)
Trypan Blue dye
Trizol (Invitrogen, Carlsbad, CA, USA)
RNeasy kit (Qiagen)
capillary gel electrophoresis (Agilent 2100 BioAnalyzer, Agilent Technologies, Santa Clara CA)

Methods

PBMCs and BMMCs from lupus patients or healthy volunteers were isolated by Ficoll-Histopaque density-gradient centrifugation of heparinized venous blood and bone marrow aspirates immediately after peripheral blood and bone marrow draw. In more detail, samples were diluted 1: 1 with phosphate buffer saline (PBS) followed by layering of 2/3 of the sample on 1/3 of Ficoll in a 15ml falcon tube. Samples were centrifuged in 1800 rpm/min for 30min at 20° C. Cells were then collected with a Pasteur pipette and transferred in a 50ml falcon tube where 5 volumes of PBS were also added. Tubes were centrifuged in 1500 rpm/ min for 10min at 20° C and followed by another washing step. After centrifugation supernatant was discarded without disturbing the pellet and

resuspended in 5ml of PBS. Equal volumes of cells and Trypan Blue were mixed on a Neubauer heamocytometer and cells were measured under an optical microscope.

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2.2.2 RNA extraction

Cells after centrifugation were immediately placed in 1ml of Trizol in 1.5ml eppendorf tube and processed for RNA extraction using the RNeasy kit according to the manufacturer's instructions. A volume of 200µl of chloroform for every 1 ml of trizol used was added in the eppendorf followed by shaking for 15 seconds by inversion. Tubes were then incubated at room temperature for 3 minutes and centrifuged at 14,000 rpm at 4° C for 20 min. Supernatant was collected and transferred to a new 1.5ml microfuge tube and 0.53 volumes of 100% ethanol were added. Up to 700µl of sample were transferred into the RNeasy column placed in a 2 ml collection tube and centrifuged for 15sec at 14,000 rpm. Supernatant was collected from the collection tube and placed back into the RNeasy column. Centrifugation was repeated and the flow-through supernatant was discarded. A volume of 700 µl of RW1 buffer was added into RNeasy column and the column with the collection tube was centrifuged for 15 sec at 14,000 rpm. RNeasy column was then transferred to a new collection tube and 500µl of RPE buffer were added. Column was then centrifuged for 15sec at 14,00rpm and the flow –through supernatant was discarded. Another 500µl of RPE buffer were added in the column which was then centrifuged for 2 min at 14,000rpm. Flow-through was discarded and column was centrifuged for an additional minute to ensure removal of excess buffers. Collection tube was discarded and the RNeasy column was transferred into a RNA collection tube. A volume of 55 µl of RNase –free water was added dropwise into the center of the membrane and the samples were incubated at room temperature for 10 min

and then centrifuged for 1 min at 14,000 rpm. The flow-through (RNA) was collected and stored at -80° C.

2.2.3 RNA integrity

Principle of method

The electrophoretic assays are based on traditional gel electrophoresis principles that have been transferred to a chip format. The chip format dramatically reduces separation time as well as sample and reagent consumption. The system provides automated sizing and quantitation information in a digital format. On-chip gel electrophoresis is performed for the analysis of DNA, RNA and proteins. The chip accommodates sample wells, gel wells and a well for an external standard (ladder). Micro-channels are fabricated in glass to create interconnected networks among these wells. During chip preparation, the micro-channels are filled with a sieving polymer and fluorescence dye. Once the wells and channels are filled, the chip becomes an integrated electrical circuit. The 16-pin electrodes of the cartridge are arranged so that they fit into the wells of the chip. Each electrode is connected to an independent power supply that provides maximum control and flexibility. Charged biomolecules like DNA, RNA, or protein/LDS micells are electrophoretically driven by a voltage gradient—similar to slab gel electrophoresis. Because of a constant mass-to-charge ratio and the presence of a sieving polymer matrix, the molecules are separated by size. Smaller fragments are migrating faster than larger ones. Dye molecules intercalate into DNA or RNA strands or protein/LDS micells. These complexes are detected by laser-induced fluorescence. Data is translated into gel-like images (bands) and electropherograms (peaks). With the help of

a ladder that contains components of known sizes, a standard curve of migration time versus fragments size is plotted. From the migration times measured for each fragment in the sample, the size is calculated. Two markers (for RNA only one marker) are run with each of the samples bracketing the overall sizing range. The “lower” and “upper” markers are internal standards used to align the ladder data with data from the sample wells. This is necessary to compensate for drift effects that may occur during the course of a chip run.

For RNA assays, quantitation is done with the help of the ladder area. The area under the ladder is compared with the sum of the sample peak areas. The area under the “lower” marker is not taken into consideration. For total RNA assays, the ribosomal ratio is determined, giving an indication on the integrity of the RNA sample. Additionally, the RNA integrity number (RIN) can be utilized to estimate the integrity of total RNA samples based on the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products.

RNA integrity was assessed using capillary gel electrophoresis (Agilent 2100 BioAnalyzer) to determine the ratio of 28s:18s rRNA in each sample. A threshold of 1.0 was used to define samples of sufficient quality and only samples above this limit were used for microarray studies.

2.2.4 cDNA synthesis

Materials

Omniscript RT™ Kit (Qiagen #205113)

Cy3-dUTP (Amersham #PA53032)

2M HEPES (Ambion)

Montage PCR 96-well Cleanup (Millipore #LSKC09604)
10x dNTP mixture (made from 100 mM dNTP commercial stocks
2.5 N NaOH
Cy-3-labeled anchored oligo-dT(20)VN primer (Operon, custom synthesis)
Poly d(A)40-60 (Amersham # 27-7988-01)
Yeast tRNA (10 mg/ml) (Ambion # 7119)
Cot-1 (1 mg/ml) (Invitrogen # 15279-011 [Human])
50X Denhardts Solution (Sigma # D-2532)
Deionized Formamide (Sigma # F-9037)
ChipHybeTM buffer (Ventana Medical # 760-103)
20X SSC (Ambion # 9763)

Method

cDNA was synthesized using Omniscript reverse transcriptase with direct incorporation of Cy3-dUTP from 2 μg of RNA. In more detail, in a PCR RNase free tube we used 2 mg total RNA and added nuclease-free water to bring the volume to 12.5 ml and 1 ml of anchored oligo dT primer (500 ng/ml stock concentration). The final [oligo-dT] was 0.5 mM in 20 ml volume. The mix was heated to 65°C for 5 minutes. A reaction mix containing: 2 ml 10X RT buffer, 2 ml 10x dNTP (containing 2.5 mM dATP, dCTP, dGTP and 1.5 mM TTP in water), 0.5 ml Cy3 (0.5 nmole), 1 ml 40 U RNAse Inhibitor, 1 ml 4 U Omniscript RT was added to the tube containing the RNA and the primer. The mix was then incubated 37°C for 2 hours followed by another incubation of 37°C for 15 min after addition of 2ml 2.5 N NaOH. At the final step 10ml 2M HEPES was added and the mix was centrifuged briefly.

Labeled cDNA was purified using a Montage 96-well vacuum system (Millipore). The cDNA was then processed for hybridization. **Hybridization steps included preparation of cDNA for hybridization after labeling, placement of the microarray and cDNA on the Discovery machine, automated steps carried out by the Discovery machine, and final manual washing steps of the microarrays prior to scanning.**

The cDNA was added to hybridization buffer containing human CoT-1 DNA (0.5 mg/ml final concentration), yeast tRNA (0.2 mg/ml), and poly(dA)$_{40-60}$ (0.4 mg/ml) and stored at -20° until use.

2.2.5 Microarrays

Principle

The principle of DNA arrays is based on the hybridization technique. Oligonucleotides or PCR probes are immobilized on a solid support (the matrix) and due to their specificity to a target gene they detect complementary sequences present in a mix which is to be analyzed.

The hybridization signals are detected, depending upon the type of labeling, by either radiography or fluorescence and then quantified. The microarray technology consists of spotting PCR products or long oligonucleotides (50mer-70mer) on glass slides at densities of up to 6000 spots / cm^2. These slides are hybridised using fluorescent targets (cDNAs or genomic DNAs). The fluorescent molecules most commonly used are members of the cyanine family, Cy3 et Cy5. After hybridization, the signals are detected using a fluorescence scanner. The use of two different fluorochromes allows the

determination of hybridization signals from two distinct strains in one single experiment. Once the fluorescent intensities have been obtained, the data are analyzed in order to extract the biological information

Reagents

Corning UltraGAPS™ amino-silane coated slides
succinic anhydride (Sigma/Aldrich)
1-methyl-2-pyrolidinone (Sigma/Aldrich)
sodium borate (Sigma/Aldrich)
0.1X SSC (Gibco BRL)
scanner (Agilent Technologies)

Method

A commercially available genome-scale oligonucleotide library containing gene-specific 70 mer oligonucleotides representing 21,329 human genes was used for microarray production. The library includes 16 replicate spots of 12 random negative controls that are designed to have no significant homology to known human DNA sequences (Qiagen Inc., Valencia, CA, USA). Oligonucleotides were spotted onto Corning UltraGAPS™ amino-silane coated slides, which were then rehydrated with water vapor and then, snap dried at 90°C. Oligonucleotide DNAs were covalently fixed to the surface of the glass using 300 mJ of ultraviolet radiation at a 254nm wavelength. Unbound, free amines on the glass surface were blocked for 15 min with moderate agitation in a solution of 143mM succinic anhydride dissolved in 1-methyl-2-pyrolidinone, 20mM sodium borate, pH 8.0. Slides were rinsed for 2 min in distilled water, immersed for 1 min in 95% ethanol and dried with a stream of nitrogen gas.

Hybridization was performed in an automated liquid delivery, air-vortexed, hybridization station for 9 h at 58°C under an oil based cover slip (Ventana Medical Systems Inc., Tucson, AZ, USA). Microarrays were washed at a final stringency of 0.1X SSC. Microarrays were scanned using a simultaneous dual-colour, 48-slide scanner (Agilent Technologies). Background substracted luorescent intensity values were determined using Kaodarray software (Koada Technology, Stirling, UK)

2.2.6. Real-time PCR validation

Reagents

OmniScript Reverse Transcriptase (Qiagen, Valencia, CA)
Montage PCR Cleanup kit (Millipore, Billerica, MA)

Principle of method

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (qPCR) is based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-stranded DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA. Frequently, real-time polymerase chain reaction is combined with reverse transcription

polymerase chain reaction to quantify low abundance messenger RNA (mRNA), enabling a researcher to quantify relative gene expression at a particular time, or in a particular cell or tissue type.

Nine genes were selected out of the set related to granulopoiesis for confirmation by PCR: S100A8, DEFA4, S100A12, ELA2, S100P, CD24, CD63, LYZ, NCF4. Eight patients were selected, four that represented patients in the "active" group and four that represented patients in the "inactive" group according to SLEDAI.

Reverse Transcription: cDNA was generated from 1.0 μg of total RNA per sample according to the OmniScript Reverse Transcriptase (Qiagen, Valencia, CA) manual, with the replacement of the RT primer mix with for 500ng anchored oligo dT(dT20VN). cDNA was purified with the Montage PCR Cleanup kit (Millipore, Billerica, MA) according to manufacturer's instructions. cDNA was diluted 1:20 in water and stored at -20° C.

Quantitative PCR: Gene-specific primers for the human genes S100A8, DEFA4, S100A12, ELA2, S100P, CD24, CD63, LYZ, NCF4 were designed with a melting temperature close to 60°C length of 19-25 bp for PCR products with a length of 110-150 bp, using Applied Biosystems Inc.(ABI) Primer Express 1.5 software. PCR was run with 2 ul cDNA template in 15ul reactions in triplicate on an ABI SDS 7700 using the ABI SYBR Green I Master Mix and gene specific primers at a concentration of 1μM each. The temperature profile consisted of an initial 95° C step for 10 minutes (for Taq activation), followed by 40 cycles of 95° C for 15 sec, 60° C for 1 min, and then a final melting curve analysis with a ramp from 60° C to 95° C over 20 min. Gene-specific

amplification was confirmed by a single peak in the ABI Dissociation Curve software. No template controls were run for each primer pair and no RT controls were run for each sample to detect nonspecific amplification or primer dimers. Average Ct values for B-Actin (run in parallel reactions to the gene of interest) were used to normalize average Ct values of the gene of interest. These values were used to calculate the average group (active vs inactive) and the relative change in Ct was used to calculate fold change between the two groups.

2.2.7 Statistical analysis

a)Normalization: The R/Bioconductor Package "Affy" was used to perform quantile normalization to adjust the marginal distribution of each sample. b) Filtering: Genes that had an average background adjusted fluorescent intensity value > 50 across all arrays were retained in the analysis. Additionally, the variance across all genes was calculated. Genes that have a variance below the median variance are unlikely to be differentially expressed and are therefore removed from further analysis.c) Class Comparison: Genes that are differentially expressed between two classes were identified through an unpaired Student's t test. A 10% false discovery rate p-value multiplicity adjustment was used. The false discovery rate is the proportion of the list of genes claimed to be differentially expressed that are false positives. Only statistically significant differentially expressed genes with greater than a 2- fold change in expression between groups were retained. d) SLEDAI Modeling: The SLEDAI score was modelled as a continuous variable according to the Generalized Linear Model (GLM) equation: Log_2 (Expression) = B1*SLEDAI + B2*Disease_State + B3*SLEDAI * Disease_State + Intercept, where Disease_State is a

categorical indicator variable for Active or Inactive Disease, and B# are beta coefficients. A 10% false discovery rate was used on the SLEDAI term to determine statistical significnce. All analysis was performed in JMP Genomics 6.0.3 (Cary, NC). e) SLEDAI Modeling (PBMC): The SLEDAI score was modeled as a continuous variable according to the GLM equation: SLEDAI = B1*Gene X + B2 * Gene Y + … + B**N** * Gene **N,** where B1 through BN are beta coefficients and Gene X through Gene N are log base 2 normalized expression values. Multivariate models were created using only genes identified to be significantly associated to SLEDAI in the BM comparison f) Granulopoiesis Score: The score was created by taking genes known to be associated with granulopoiesis from the literature (LYZ, CD63, DEFA4, ELA2, S100A8, S100A12,S100P, CD24, NCF4) and adding their log expression values together. This resulted in a score that was used to correlate this plurality of genes vs SLEDAI through a GLM.

2.2.8 Multiplex cytokine assay

Serum levels of cytokines and chemokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12 (p40), IL-13, IL-15, IL-17, IL-1Ra, IP-10 interferon (IFN)-α and IFN-γ, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF), CCL2 [monocyte chemoattractant protein (MCP)-1]/(MCAF), CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β) and CCL11 (Eotaxin) were measured using a bead-based immunofluorescence assay (Luminex Inc. Austin, TX, USA) using multiplex cytokine reagents supplied by Biosource International, Camarillo, CA, USA

Validation of the multiplex assays was performed using single protein ELISAs (Biosource International). Values obtained from multiplex assay analytes were highly correlative (Spearman's rank correlation coefficient, 0.97_0.03) when compared with individual ELISAs for particular cytokines.

Serum levels of 23 cytokines were compared among 51 SLE patients and 47 unaffected control individuals. The cytokines assayed included modulators of several key aspects of disease pathology including regulation of inflammation, cellular and humoral immunity, leukocyte trafficking, cell growth and angiogenesis.

Principle of method

Briefly, a sandwich immunoassay-based protein array system (Biosource International), which contains dyed microspheres conjugated with a monoclonal antibody specific for a target protein was used in this assay. Serum samples were thawed and run in duplicates. Antibody-coupled beads were incubated with the serum sample (antigen) after which they were incubated with biotinylated detection antibody before finally being incubated with streptavidin–phycoerythrin. A broad sensitivity range of standards (Biosource International), ranging between 0.13 and 28,000 pg/ml were used to help enable the quantitation of a dynamic wide range of cytokine concentrations and provide the greatest sensitivity. This captured bead-complexes were then read by the Luminex 100TM (Luminex Corporation Austin, TX, USA), which uses Luminex fluorescent bead-based technology (Luminex Corporation Austin, TX, USA) with a flow-based

dual laser detector with real-time digital signal processing to facilitate the analysis of up to 100 different families of colour-coded polystyrene beads and allow multiple measurements of the sample ensuring in the effective quantification of cytokines.

Statistical analysis

The concentrations of analytes in these assays were quantified using a calibration or standard curve. A 5-parameter logistic regression analysis was performed to regress a known serial dilution of analyte vs median fluorescent intensity read from a flow cytometer. The resulting equation was then used to predict the concentration of the unknown samples Statistical differences in measured values were analysed using a Mann–Whitney U-test. P-values <0.05 were considered statistically significant. All P-values reported are nominal due to the exploratory nature of this analysis. Matlab R13 (Natick, MA, USA) and SAS v9.1.3 (Cary, NC) were used to perform all statistical analyses.

III. RESULTS

3.1 Differentially expressed genes in the bone marrow of SLE patients vs controls

A total of 102 genes were found to have differential levels of expression between the SLE patients and the control subjects using unpaired student t-test. Of the 102 differentially expressed genes, 53 genes are involved in major networks including cell death, differentiation, cell signaling and cellular growth and proliferation **(Table 3.1)**.

Data mining was performed to identify genes that were expressed in various subpopulations of BMMCs including B and T cells, monocytes and neutrophils. Of the 102 differentially expressed genes, 37 were up-regulated in the bone marrow of patients relative to controls including: *TNFR17* (Tumor necrosis factor receptor superfamily, member 17) usually expressed in mature B lymphocytes and may be important for B cell development and autoimmune response. B cell involvement was highlighted by the presence of genes involved in the antigen presentation pathway such as, *HLA-F* (major histocompatibility complex, class I, F), and *IGHG3* (Immunoglobulin heavy constant gamma 3). We found *ITPR1*, belonging to the family of Inositol 1,4,5-trisphosphate receptors (IP3Rs) which are expressed in most hematopoietic cells, including B cells, upregulated in the bone marrow of patients as well as a transcriptional co-activator *BCL3* (B-cell CLL/lymphoma 3) reported to be upregulated in polyclonal plasmablastic cells[55]. In mouse bone marrow, transgenic human BCL3 protein increases accumulation of mature B lymphocytes [56].

Sixty five (65 genes) were expressed at lower levels in patients than controls including the chemokine receptors *CX3CR1* and *CCR5*, the latter is normally expressed by T cells and macrophages. Also downregulated were *CDH2* (Cadherin 2), *CTNNAL1* (Catenin,cadherin-associated protein), *CDKN* (Cyclin-dependent kinase inhibitor 3) and *KALI* (Activating NK receptor) whose protein is expressed on Natural Killer, T and B lymphocytes and lung. CD276 (B7-H3) a costimulatory molecule for T cell activation and IFN-gamma production was also found downregulated in the bone marrow of patients.

Name	Description	Genbank	Normalized ratio	Location	Family
	upregulated genes in BM				
BCL3	B-cell CLL/lymphoma 3	NM_005178	4,24	Nucleus	transcription regulator
ITPR1	inositol 1,4,5-triphosphate receptor, type 1	NM_002222	3,388	Cytoplasm	ion channel
RPL32	ribosomal protein L32	NM_000994	2,883	Cytoplasm	other
PHACTR1	phosphatase and actin regulator 1	AB051520	2,311	Cytoplasm	other
RPS2	ribosomal protein S2	NM_002952	2,258	Cytoplasm	other
RPS13	ribosomal protein S13	NM_001017	2,094	Cytoplasm	other
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	NM_004827	2,062	Plasma Membrane	transporter
EPS15L1	epidermal growth factor receptor pathway substrate 15-like 1	AK023744	2,056	Plasma Membrane	other
ABLIM1	actin binding LIM protein 1	NM_002313	1,987	Cytoplasm	other
HLA-F	major histocompatibility complex, class I, F	NM_018950	1,904	Plasma Membrane	transmembrane receptor
NPTX1	neuronal pentraxin I	NM_002522	1,85	Extracellular Space	other
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	NM_001192	1,837	Plasma Membrane	other
ADD3	adducin 3 (gamma)	U92992	1,781	Cytoplasm	other
TEAD2	TEA domain family member 2	BC007556	1,735	Nucleus	transcription regulator
BAIAP3	BAI1-associated protein 3	NM_003933	1,726	Unknown	other
PRKAA2	protein kinase, AMP-activated, alpha 2 catalytic subunit	NM_006252	1,641	Cytoplasm	kinase
GMFG	glia maturation factor, gamma	NM_004877	1,613	Cytoplasm	growth factor
BACE1	beta-site APP-cleaving enzyme 1	NM_012104	1,505	Cytoplasm	peptidase
TMEPAI	transmembrane, prostate androgen induced RNA	AF305616	1,481	Plasma Membrane	other
K-ALPHA-1	alpha tubulin	NM_006082	1,165	Cytoplasm	other
	downregulated in BM				
PRKD1	protein kinase D1	NM_002742	4,724	Cytoplasm	kinase
CCR5	chemokine (C-C motif) receptor 5	NM_000579	3,877	Plasma Membrane	G-protein coupled receptor
CRHR1	corticotropin releasing hormone receptor 1	X72304	3,387	Plasma Membrane	G-protein coupled receptor
GJB3	gap junction protein, beta 3, 31kDa (connexin 31)	NM_024009	3,376	Plasma Membrane	transporter
GUCY2D	guanylate cyclase 2D, membrane (retina-specific)	NM_000180	3,32	Plasma Membrane	kinase
MPHOSPH1	M-phase phosphoprotein 1	NM_016195	3,023	Nucleus	enzyme
GAP43	growth associated protein 43	NM_002045	2,938	Plasma Membrane	other
MBP	myelin basic protein	NM_002385	2,901	Extracellular Space	other
PCLO	piccolo (presynaptic cytomatrix protein)	AB011131	2,895	Cytoplasm	transporter
MYH10	myosin, heavy polypeptide 10, non-muscle	AK026977	2,786	Cytoplasm	other
AEBP2	AE binding protein 2	BC015624	2,7	Nucleus	transcription regulator
PAX6	paired box gene 6 (aniridia, keratitis)	NM_001604	2,646	Nucleus	transcription regulator
MAG	myelin associated glycoprotein	NM_002361	2,541	Plasma Membrane	other
USP33	ubiquitin specific peptidase 33	AB029020	2,407	Cytoplasm	peptidase
ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	NM_000789	2,385	Plasma Membrane	peptidase
CX3CR1	chemokine (C-X3-C motif) receptor 1	U20350	2,334	Plasma Membrane	G-protein coupled receptor
OTP	orthopedia homolog (Drosophila)	NM_032109	2,27	Nucleus	transcription regulator
SRGAP1	SLIT-ROBO Rho GTPase activating protein 1	AB037725	2,227	Unknown	other
RGS11	regulator of G-protein signalling 11	NM_003834	2,176	Plasma Membrane	enzyme
HOXB3	homeobox B3	U59298	2,171	Nucleus	transcription regulator
SLAMF6	SLAM family member 6	NM_052931	2,164	Plasma Membrane	transmembrane receptor
SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein	NM_014021	2,076	Unknown	other
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	NM_001792	2,064	Plasma Membrane	other
DUSP4	dual specificity phosphatase 4	NM_057158	2,015	Nucleus	phosphatase
GRB2	growth factor receptor-bound protein 2	NM_002086	1,938	Plasma Membrane	other
EIF4EBP2	eukaryotic translation initiation factor 4E binding protein 2	AK057643	1,82	Cytoplasm	other
CD276	CD276 molecule	NM_025240	1,813	Plasma Membrane	other
PRKCG	protein kinase C, gamma	NM_002739	1,768	Cytoplasm	kinase
C10ORF10	chromosome 10 open reading frame 10	NM_007021	1,662	Unknown	other
CTNNAL1	catenin (cadherin-associated protein), alpha-like 1	NM_003798	1,65	Plasma Membrane	other
MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	AK023358	1,635	Plasma Membrane	kinase
CDKN3	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	NM_005192	1,426	Nucleus	phosphatase
SNX2	sorting nexin 2	NM_003100	1,306	Cytoplasm	transporter

Table 3.1. Selected up- and down-regulated genes in the bone marrow of SLE patients relative to controls.

3.2 Bone marrow genes associated with SLE disease activity

By the use of multiple regression analysis, as outlined in Materials and Methods, seven genes were statistically associated with SLEDAI. KIAA1674 (GenBank AB051461) (r^2 =0.82) , NY-REN-25 antigen, an ankyrin repeat domain (GenBank AF155103) (r^2=0.81), cDNA FLJ32586 fis (GenBank AK057148) (r^2 = 0.79), the hypothetical protein FLJ10254 (GenBank NM_018041) (r^2 = 0.83) and the coiled coil domain CCDC91 (GenBank NM_018318) (r^2 = 0.84), *CENPH* (Centromere protein H, GenBank NM_022909) (r^2 =0.79) and *EBI3* (Epstein-Barr virus induced gene 3, GenBank NM_005755)) (r^2 = 0.77) a subunit of IL-27 which may play an important role in initiation of Th1 responses. The expression of these genes was highly correlated with one another producing Pearson correlation coefficients of 0.89 to 0.97. Due to co-linearity concerns we were not able to combine these terms into a single multivariate model.

In order to test if these 7 SLEDAI-associated bone marrow expressed genes were also associated with SLEDAI in the peripheral blood, we re-analyzed the data and used the genes selected in the BM and refitted the terms to the PBMC data to predict SLEDAI. Only 2 genes, NY-REN-25 antigen, an ankyrin repeat domain (GenBank AF155103) and coiled coil domain CCDC91 (GenBank NM_018318) associated with SLEDAI in the active PBMC patients (r^2=0.37, p=0.0108) **(Figure 3.1).**

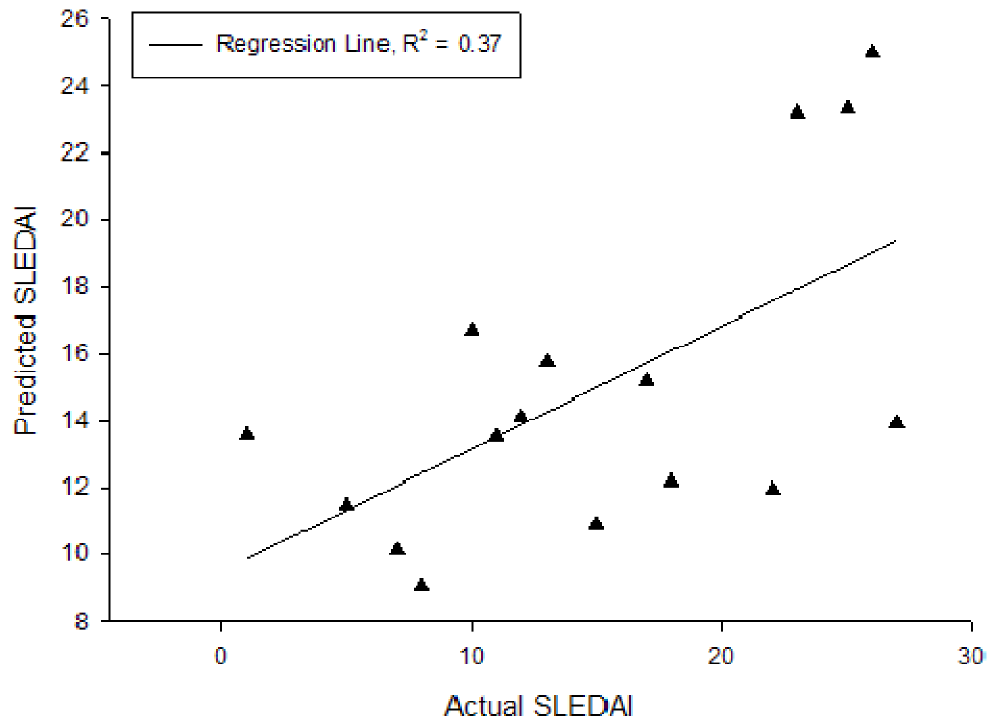


Figure 3.1 *SLEDAI-associated bone marrow expressed genes were also associated with SLEDAI in the periphery.* Two genes, NY-REN-25 antigen, an ankyrin repeat domain (GenBank AF155103) and coiled coil domain CCDC91 (GenBank NM_018318) associated with SLEDAI in active PBMC patients thorugh a GLM (r^2=0.37, P=0.0108). The Y Axis represents the predicted SLEDAI from our model of 2 genes and the X axis is the observed SLEDAI from the patient's medical records.

3.3 Genes differentially expressed in BMMCs relative to PBMCs of SLE patients

We next compared bone marrow derived mononuclear cells with peripheral blood mononuclear cells in the lupus cohort. Eighty eight (88) genes were differentially expressed, 41 out of 88 were up-regulated in the bone marrow of lupus patients relative to the peripheral blood **(Table 3.2)** while the remaining 47 were up-regulated in the peripheral blood. Among the lupus bone marrow up-regulated genes, the highest overexpression was found in granulopoiesis- related genes. These genes include major components of neutrophils such as myeloperoxidase *MPO*, *ELA2* (elastase 2) responsible for hydrolyzing proteins within granules, *CTSG* (cathepsin G), *DEFA4* (defensin), *LTF* (lactotransferrin) and *CD24* found on mature granulocytes. Three small abundant proteins found in human neutrophil cytosol *S100A9*, *S100A12* and *S100P* were upregulated in the bone marrow of SLE patients. Finally, in the peripheral blood of lupus patients we identified a number of chemokines such as *CCR5*, *CXCL3L1*, *CXCL2* and *CXCL3* that are overexpressed relative to the bone marrow. These genes participate in processes such as chemotaxis and migration of leukocytes. Comparison of bone marrow versus peripheral blood in the control cohort revealed that most of the neutrophil related genes found in the previous comparison, were also overexpressed in the bone marrow of control subjects relative to the peripheral blood with minor differences in gene expression level. For example, cathepsin *CTSG* was overexpressed by 7.3-fold in bone marrow of controls relative to peripheral blood and by 6.6-fold in the bone marrow of lupus patients when

compared to the peripheral blood. Thus many of these genes are tissue associated differences rather than disease-associated differences.

Symbol	Short Description	Normalized ratio	Location	Family
CTSG	Cathepsin G	6,65	cytoplasm	peptidase
HBD	Hemoglobin, delta	5,84	cytoplasm	transporter
ELA2	Elastase 2, neutrophil	4,49	extracellular space	peptidase
MPO	Myeloperoxidase	3,3	cytoplasm	enzyme
S100A9	S100 calcium binding protein A9 (calgranulin B)	2,83	cytoplasm	other
NR2C2	Nuclear receptor subfamily 2, group C, member 2	2,58	nucleus	ligand-dependent nuclear receptor
PRDX2	Peroxiredoxin 2	2,41	cytoplasm	enzyme
S100A12	S100 calcium binding protein A12 (calgranulin C)	2,41	cytoplasm	other
PPIB	Peptidylprolyl isomerase B (cyclophilin B)	2,34	cytoplasm	enzyme
ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	2,23	cytoplasm	ion channel
STAG3	Stromal antigen 3	2,2	nucleus	other
CRMP1	Collapsin response mediator protein 1	2,18	cytoplasm	enzyme
HP	Haptoglobin	2,15	extracellular space	peptidase
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	2,14	cytoplasm	enzyme
PSCD1	Pleckstrin homology, Sec7 and coiled/coil domains 1(cytohesin 1)	2,02	cytoplasm	other
K-ALPHA-1	Tubulin, alpha, ubiquitous	2,01	cytoplasm	other
PGLYRP	Peptidoglycan recognition protein	1,96	plasma membrane	transmembrane receptor
S100P	S100 calcium binding protein P	1,95	cytoplasm	other
LTF	Lactotransferrin	1,91	extracellular space	peptidase
ATP7A	ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)	1,86	plasma membrane	transporter
LCN2	Lipocalin 2 (oncogene 24p3)	1,78	extracellular space	transporter
SNCA	Synuclein, alpha (non A4 component of amyloid precursor)	1,77	cytoplasm	other
DUSP4	Dual specificity phosphatase 4	1,74	nucleus	phosphatase
CSPG4	Chondroitin sulfate proteoglycan 4 (melanoma-associated)	1,7	plasma membrane	other
PHKA1	Phosphorylase kinase, alpha 1 (muscle)	1,64	cytoplasm	kinase
DLC1	Deleted in liver cancer 1	1,56	cytoplasm	other
UQCRC2	Ubiquinol-cytochrome c reductase core protein II	1,54	cytoplasm	enzyme
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	1,41	plasma membrane	other
CDKN3	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	1,34	nucleus	phosphatase

Table 3.2. Selected upregulated genes in BMMCs relative to PBMCs of lupus patients

3.4 Differentially expressed genes in the peripheral blood of SLE patients vs controls

Among the SLE upregulated genes in the PBMCs two IFN-inducible genes were overexpressed in lupus patients: *IL6R* whose expression is regulated by IFNα and *PRKCG* (protein kinase C, gamma) that is involved in antiviral response of IFNγ and its signaling **(table 3.3)**. In total 35 genes were upregulated in SLE peripheral blood including a number of regulatory molecules such as: *TCF7* (transcription factor 7, T-cell specific), *CYC1* (cytochrome c-1), *UBTF* (upstream binding transcription factor), *HDAC10* (histone deacetylase 10) involved in the acetylation status of histone tails, a ubiquitin-conjugating enzyme *UBE2D3* and *U2AF1* (U2(RNU2) small nuclear RNA auxillary factor 1) belonging to the splicing factor SR family of genes. The expression of 18 genes was downregulated in the PBMCs of SLE patients compared to controls including: BACE (beta-site APP-cleaving enzyme), HOXD13 (homeo box D13), K-ALPHA-1 (tubulin), PHKA1 (phosphorylase kinase,alpha) and PRKAA2 (protein kinase , AMP-activated).

Symbol	Short Description	
	upregulated in patients	**fold change**
PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein	7,62
FUCA1	Fucosidase, alpha-L- 1, tissue	4,03
PRDM1	PR domain containing 1, with ZNF domain	4,02
UBE2D3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	3,92
DBI	Diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	3,89
ARF1GAP	ADP-ribosylation factor 1 GTPase activating protein	3,24
TOP1MT	Mitochondrial topoisomerase I	2,74
EMP3	Epithelial membrane protein 3	2,65
MGC4549	Hypothetical protein MGC4549	2,59
TCF7	Transcription factor 7 (T-cell specific, HMG-box)	2,42
HKR3	GLI-Kruppel family member HKR3	2,40
UBTF	Upstream binding transcription factor, RNA polymerase I	2,39
HDAC10	Histone deacetylase 10	2,25
KIAA1575	KIAA1575 protein	2,25
CBF2	CCAAT-box-binding transcription factor	2,22
IL6R	Interleukin 6 receptor	2,21
C2orf9	Chromosome 2 open reading frame 9	2,19
CYC1	Cytochrome c-1	2,18
PRKCG	Protein kinase C, gamma	2,16
FBI1	HIV-1 inducer of short transcripts binding protein- lymphoma related factor	2,15
RPS9	Ribosomal protein S9	2,11
U2AF1	U2(RNU2) small nuclear RNA auxillary factor 1	2,11
MRPL12	Mitochondrial ribosomal protein L12	2,10
FLJ22233	Hypothetical protein FLJ22233	2,08
MGC12904	Hypothetical protein MGC12904	2,04
HSPC023	HSPC023 protein	2,04
KATNA1	Katanin p60 (ATPase-containing) subunit A 1	2,03
	downregulated in patients	
DCTD	DCMP deaminase	3,25
LOC51112	CGI-87 protein	2,94
NXPH2	Neurexophilin 2	2,82
NR2C2	Nuclear receptor subfamily 2, group C, member 2	2,67
OMD	Osteomodulin	2,54
LHFP	Lipoma HMGIC fusion partner	2,38
HOXD13	Homeo box D13	2,37
CSPG4	Chondroitin sulfate proteoglycan 4 (melanoma-associated)	2,11
EMAP-2	Microtubule-associated protein like echinoderm EMAP	2,09
KIAA1881	KIAA1881 protein	2,07
	Homo sapiens clone 24527 mRNA sequence	2,06
C2F	C2f protein	2,01
SFRP2	Secreted frizzled-related protein 2	1,96
TMEPAI	Transmembrane, prostate androgen induced RNA	1,89
PHKA1	Phosphorylase kinase, alpha 1 (muscle)	1,80
KIAA1661	KIAA1661 protein	1,79
PRKAA2	Protein kinase, AMP-activated, alpha 2 catalytic subunit	1,72
BACE	Beta-site APP-cleaving enzyme	1,64
K-ALPHA-1	Tubulin, alpha, ubiquitous	1,16

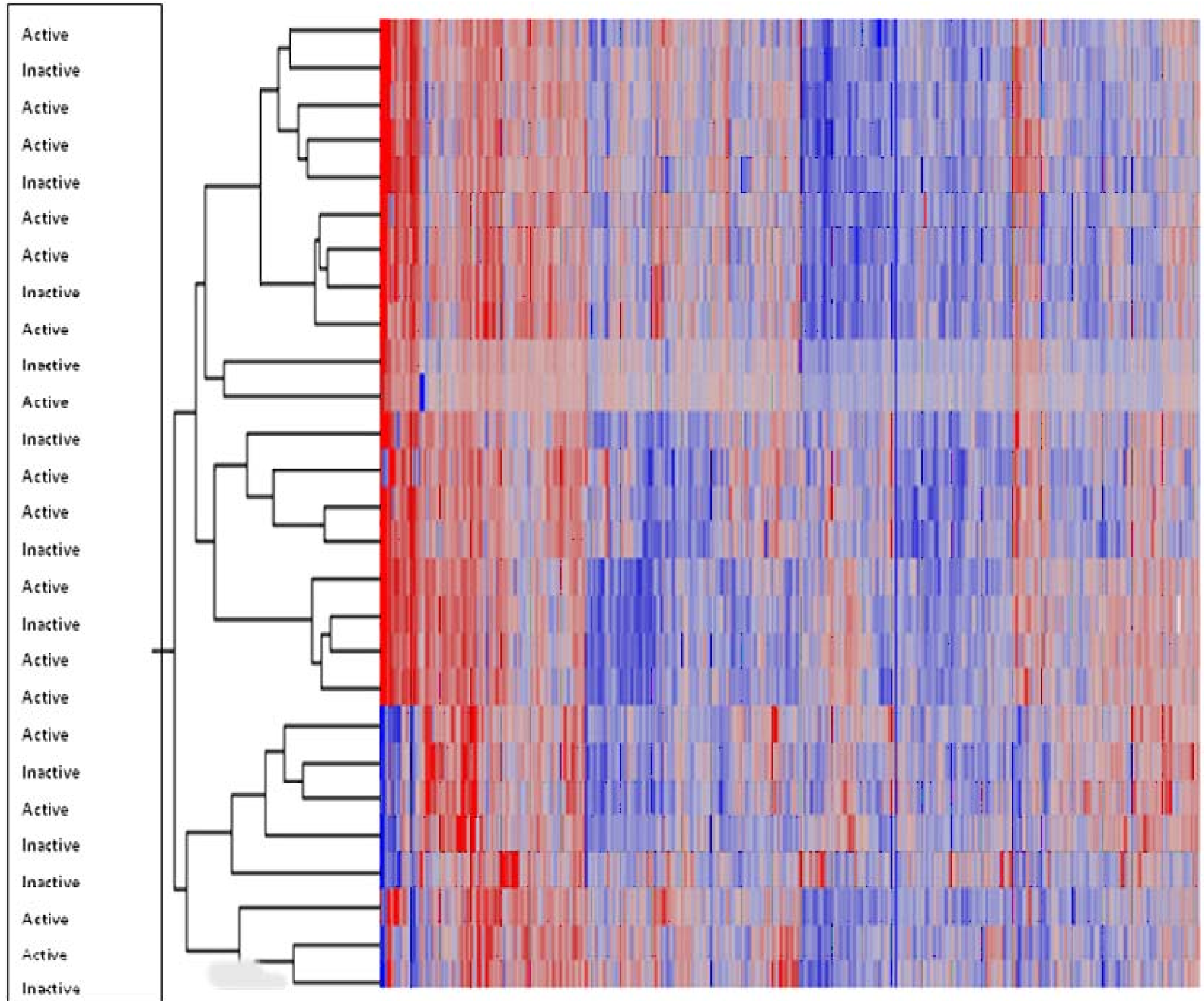
Table 3.3. Upregulated and downregulated genes in the peripheral blood of SLE patients relative to controls

3.5 Hierarchical clustering reveals patient subgroups in the bone marrow

In order to group individuals with similar expression profiles in their peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs), we used unsupervised hierarchical clustering of the top 25% of expressed genes (n= 2652) in each group separately. Lupus samples derived from peripheral blood did not form distinct, well-characterized clusters but scattered across the graph regardless of disease activity **(Figure 3.2A)**. In contrast, hierarchical clustering in the bone marrow demonstrated that lupus patients fell into two groups that displayed different pattern of expression for these 2652 genes **(Figure 3.2B)** and the controls formed another distinct cluster. Of interest, these two groups were primarily active patients in one cluster and inactive patients in the other with 20% and 30% misclassification respectively (p=0.07, Fisher's Exact test). These clusters were not associated with disease manifestations or drug treatment. Taken together, these findings suggest that bone marrow in SLE provides supplemental information to that obtained from peripheral blood in the context of disease activity.

Figure 3.2

A



B

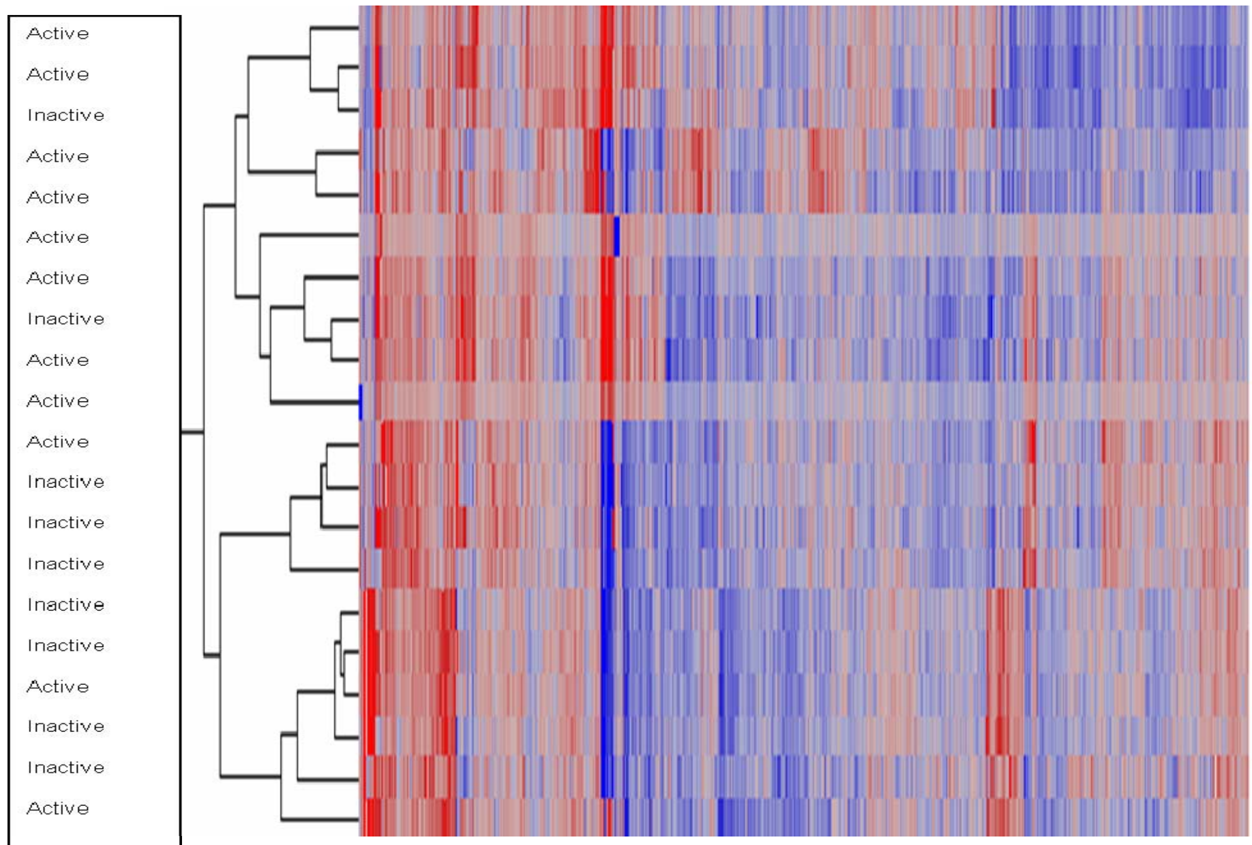


Figure 3.2. *Hierarchical clustering of the top 25% (2652) of highly expressed genes in the peripheral blood and bone marrow of SLE patients.* Each column represents a gene and each row shows the expression for the top 25% genes expressed by each individual. (A). Peripheral blood samples from SLE patients scattered across the graph regardless of disease activity. (B). Hierarchical clustering distinguish SLE active patients from inactive in the bone marrow. The first big cluster corresponds to the active patients and the second one to the inactive

3.6 Granulopoiesis and apoptosis signature in the bone marrow of active patients

To explore any gene signatures that characterize the two patient subgroups in the bone marrow, we further analyzed on a whole-genome scale the differentially expressed genes between patients in these two clusters. We found 245 differentially expressed genes that were up-regulated in the active patient cluster as compared to the inactive **(Table 3.4)**. No genes were identified as significantly repressed in the active relative to inactive group. Genes involved in antigen presentation such as *HLA-A*, *HLA-C* and *CD74* were expressed in higher levels in the bone marrow of active SLE patients. Among the up-regulated genes we noticed granulopoiesis-related genes such as *ELA2* (elastase) which is usually transcribed within the earliest granulocytes, *LYZ* (lysozyme) a component of azurophil and specific granules which was overexpressed in active patients, *DEFA* (defensin), CD63 a marker of azurophil granules and *CD24* expressed on mature granulocytes. Three family members of S100 calcium-binding proteins, *S100A6*, *S100A8* and *S100P* were also upregulated in the active group. Among these three genes, *S100A8* a leukocyte chemoattractant protein was overexpressed 10-fold compared to the inactive group. All genes that were upregulated on the microarrays were also upregulated when analyzed by quantitative real-time PCR. The expression values of these granulopoiesis genes correlated highly with each other. Additional up-regulated genes included genes involved in processes such as cell death, immune response and cellular movement. The genes implicated in the cell death of leukocytes include *ANXA1*, *CD24*, *CST3*, *CXCR4*, *ELA2*, *FOS*, *FOXO3A*, *IER3*, *ITGB2*, *LCN2*, *LYN*, and *PCBP2*. We also identified the genes involved in the apoptosis of granulocytes which include *ANXA1* (annexin 1), chemokine *CXCR4*, *FOXO3A*, *ITGB2* (integrin) and *LYN*.

name	description	normalized ratio	location	family
HSPB1	heat shock 27kDa protein 1	19,969	Cytoplasm	other
RAP1B	RAP1B, member of RAS oncogene family	17,784	Cytoplasm	enzyme
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	16,509	Plasma Membrane	other
H3F3A	H3 histone, family 3A	14,347	Nucleus	other
G0S2	G0/G1switch 2	14,192	Unknown	other
MRLC2	myosin regulatory light chain MRLC2	14,025	Cytoplasm	other
MRCL3	myosin regulatory light chain MRCL3	11,738	Unknown	other
S100A8	S100 calcium binding protein A8 (calgranulin A)	10,686	Cytoplasm	other
GRN	granulin	10,351	Extracellular Space	growth factor
LSP1	lymphocyte-specific protein 1	8,902	Cytoplasm	other
PRG1 (includes EG:5552)	proteoglycan 1, secretory granule	8,773	Extracellular Space	other
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	7,637	Plasma Membrane	transmembrane receptor
DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	7,573	Nucleus	enzyme
TMSB10	thymosin, beta 10	6,974	Cytoplasm	other
AMPH	amphiphysin (Stiff-Man syndrome with breast cancer 128kDa autoantigen)	6,745	Plasma Membrane	other
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	6,67	Nucleus	other
TPT1	tumor protein, translationally-controlled 1	6,586	Cytoplasm	other
S100A6	S100 calcium binding protein A6 (calcyclin)	6,251	Cytoplasm	other
ATP5G2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subu	6,065	Cytoplasm	transporter
UBE2D3	ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	5,866	Cytoplasm	enzyme
TMC6	transmembrane channel-like 6	5,324	Unknown	transporter
SF3A3	splicing factor 3a, subunit 3, 60kDa	5,26	Nucleus	other
SFRS2	splicing factor, arginine/serine-rich 2	5,252	Nucleus	other
PAPOLA	poly(A) polymerase alpha	5,208	Nucleus	enzyme
ELA2	elastase 2, neutrophil	5,171	Extracellular Space	peptidase
PFN1	profilin 1	5,146	Cytoplasm	other
PMPCB	peptidase (mitochondrial processing) beta	5,119	Cytoplasm	peptidase
SRP14	signal recognition particle 14kDa (homologous Alu RNA binding protein)	5,034	Cytoplasm	other
COTL1	coactosin-like 1 (Dictyostelium)	4,898	Cytoplasm	other
ERAF	erythroid associated factor	4,881	Cytoplasm	other
CST7	cystatin F (leukocystatin)	4,701	Extracellular Space	other
HLA-A	major histocompatibility complex, class I, A	4,612	Plasma Membrane	transmembrane receptor
PCBP2	poly(rC) binding protein 2	4,557	Nucleus	other
PKM2	pyruvate kinase, muscle	4,47	Cytoplasm	kinase
ATP7A	ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)	4,343	Plasma Membrane	transporter
ANXA1	annexin A1	4,273	Plasma Membrane	other
TBCA	tubulin-specific chaperone a	4,088	Cytoplasm	other
ALOX5	arachidonate 5-lipoxygenase	4,046	Cytoplasm	enzyme
HP	haptoglobin	4,03	Extracellular Space	peptidase
LCN2	lipocalin 2 (oncogene 24p3)	4,029	Extracellular Space	transporter
C6ORF115	chromosome 6 open reading frame 115	4,004	Unknown	other
ARD1A	ARD1 homolog A, N-acetyltransferase (S. cerevisiae)	3,983	Nucleus	enzyme
TMSB4X	thymosin, beta 4, X-linked	3,942	Cytoplasm	other
PPAP2B	phosphatidic acid phosphatase type 2B	3,872	Plasma Membrane	phosphatase

CD63	CD63 molecule	3,861	Plasma Membrane	other
YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,	3,841	Cytoplasm	other
EXOSC6	exosome component 6	3,784	Nucleus	other
HLA-C	major histocompatibility complex, class I, C	3,754	Plasma Membrane	transmembrane receptor
SNCA	synuclein, alpha (non A4 component of amyloid precursor)	3,689	Cytoplasm	other
PDHA1	pyruvate dehydrogenase (lipoamide) alpha 1	3,652	Cytoplasm	enzyme
HSPE1	heat shock 10kDa protein 1 (chaperonin 10)	3,605	Cytoplasm	other
SNRPD2	small nuclear ribonucleoprotein D2 polypeptide 16.5kDa	3,57	Nucleus	other
ACTG1	actin, gamma 1	3,52	Cytoplasm	other
SYNGR2	synaptogyrin 2	3,493	Plasma Membrane	other
NDUFA13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	3,449	Cytoplasm	enzyme
CXCR4	chemokine (C-X-C motif) receptor 4	3,411	Plasma Membrane	G-protein coupled receptor
AURKAIP1	aurora kinase A interacting protein 1	3,407	Nucleus	other
CYC1	cytochrome c-1	3,386	Cytoplasm	enzyme
MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian)	3,38	Nucleus	transcription regulator
BLOC1S1	biogenesis of lysosome-related organelles complex-1, subunit 1	3,375	Cytoplasm	other
BPI	bactericidal/permeability-increasing protein	3,368	Plasma Membrane	other
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	3,295	Nucleus	transcription regulator
S100P	S100 calcium binding protein P	3,204	Cytoplasm	other
PSMB1	proteasome (prosome, macropain) subunit, beta type, 1	3,174	Cytoplasm	peptidase
APOB	apolipoprotein B (including Ag(x) antigen)	3,131	Extracellular Space	transporter
GTF3C5	general transcription factor IIIC, polypeptide 5, 63kDa	3,117	Nucleus	transcription regulator
SF3A2	splicing factor 3a, subunit 2, 66kDa	3,11	Nucleus	other
MAP3K11	mitogen-activated protein kinase kinase kinase 11	3,078	Cytoplasm	kinase
NRG1	neuregulin 1	3,069	Extracellular Space	growth factor
CST3	cystatin C (amyloid angiopathy and cerebral hemorrhage)	3,046	Extracellular Space	other
PSCD1	pleckstrin homology, Sec7 and coiled-coil domains 1(cytohesin 1)	3,038	Cytoplasm	other
COPE	coatomer protein complex, subunit epsilon	3,014	Cytoplasm	transporter
TALDO1	transaldolase 1	3,003	Cytoplasm	enzyme
YBX1	Y box binding protein 1	3	Nucleus	transcription regulator
RNASE3	ribonuclease, RNase A family, 3 (eosinophil cationic protein)	2,889	Extracellular Space	enzyme
SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	2,886	Cytoplasm	other
SAT	spermidine/spermine N1-acetyltransferase 1	2,881	Cytoplasm	enzyme
ARHGAP4	Rho GTPase activating protein 4	2,863	Cytoplasm	other
LAPTM5	lysosomal associated multispanning membrane protein 5	2,861	Plasma Membrane	other
ABCA7	ATP-binding cassette, sub-family A (ABC1), member 7	2,859	Plasma Membrane	transporter
CALCR	calcitonin receptor	2,844	Plasma Membrane	G-protein coupled receptor
HNRPM	heterogeneous nuclear ribonucleoprotein M	2,794	Plasma Membrane	transmembrane receptor
CD24	CD24 molecule	2,77	Plasma Membrane	other
TM7SF3	transmembrane 7 superfamily member 3	2,734	Plasma Membrane	other
GYPC	glycophorin C (Gerbich blood group)	2,705	Plasma Membrane	other
ATP6V1F	ATPase, H+ transporting, lysosomal 14kDa, V1 subunit F	2,702	Cytoplasm	transporter
HMGB2	high-mobility group box 2	2,661	Nucleus	transcription regulator
EIF4B	eukaryotic translation initiation factor 4B	2,569	Cytoplasm	translation regulator
NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	2,568	Cytoplasm	enzyme
WBSCR1	Williams-Beuren syndrome chromosome region 1	2,562	Cytoplasm	translation regulator
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme	2,498	Cytoplasm	enzyme
BCCIP	BRCA2 and CDKN1A interacting protein	2,459	Nucleus	other
SNIP1	Smad nuclear interacting protein 1	2,456	Nucleus	other
UBA52	ubiquitin A-52 residue ribosomal protein fusion product 1	2,376	Cytoplasm	transcription regulator

USP7	ubiquitin specific peptidase 7 (herpes virus-associated)	2,374	Nucleus	peptidase
NR6A1	nuclear receptor subfamily 6, group A, member 1	2,36	Nucleus	ligand-dependent nuclear rece
ZNF9	CCHC-type zinc finger, nucleic acid binding protein	2,334	Nucleus	transcription regulator
GPSM3	G-protein signalling modulator 3 (AGS3-like, C. elegans)	2,332	Unknown	other
IDI1	isopentenyl-diphosphate delta isomerase 1	2,324	Cytoplasm	enzyme
KLF6	Kruppel-like factor 6	2,311	Nucleus	transcription regulator
UQCRC2	ubiquinol-cytochrome c reductase core protein II	2,308	Cytoplasm	enzyme
RAN	RAN, member RAS oncogene family	2,289	Nucleus	enzyme
SUB1	SUB1 homolog (S. cerevisiae)	2,286	Nucleus	transcription regulator
TGOLN2	trans-golgi network protein 2	2,282	Cytoplasm	other
BZRP	translocator protein (18kDa)	2,273	Cytoplasm	transmembrane receptor
SCPEP1	serine carboxypeptidase 1	2,249	Cytoplasm	peptidase
FOXO3A	forkhead box O3A	2,241	Nucleus	transcription regulator
BLVRB	biliverdin reductase B (flavin reductase (NADPH))	2,233	Cytoplasm	enzyme
C1D	nuclear DNA-binding protein	2,212	Nucleus	transcription regulator
TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor	2,177	Nucleus	transcription regulator
RAD23A	RAD23 homolog A (S. cerevisiae)	2,175	Nucleus	other
UGCG	UDP-glucose ceramide glucosyltransferase	2,165	Cytoplasm	enzyme
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, z	2,157	Nucleus	transcription regulator
ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2	2,155	Cytoplasm	transporter
CTSW	cathepsin W (lymphopain)	2,153	Cytoplasm	peptidase
CCNI	cyclin I	2,138	Unknown	other
UBE1	ubiquitin-activating enzyme E1 (A1S9T and BN75 temperature sensitivity cor	2,136	Cytoplasm	enzyme
ARPC3	actin related protein 2/3 complex, subunit 3, 21kDa	2,13	Cytoplasm	other
DEK	DEK oncogene (DNA binding)	2,129	Nucleus	transcription regulator
SSBP3	single stranded DNA binding protein 3	2,107	Nucleus	transcription regulator
CAMLG	calcium modulating ligand	2,102	Cytoplasm	other
CHAF1A	chromatin assembly factor 1, subunit A (p150)	2,102	Nucleus	other
F11R	F11 receptor	2,093	Plasma Membrane	other
NCF4	neutrophil cytosolic factor 4, 40kDa	2,092	Cytoplasm	enzyme
COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2 (liver)	2,089	Cytoplasm	enzyme
H1FX	H1 histone family, member X	2,083	Nucleus	other
FOSL1	FOS-like antigen 1	2,077	Nucleus	transcription regulator
ACTB	actin, beta	2,075	Cytoplasm	other
PNN	pinin, desmosome associated protein	2,068	Plasma Membrane	other
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	2,054	Cytoplasm	kinase
TNC	tenascin C (hexabrachion)	2,049	Extracellular Space	other
SUMO2	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae)	2,045	Nucleus	other
CKAP4	cytoskeleton-associated protein 4	2,043	Cytoplasm	other
SLC44A2	solute carrier family 44, member 2	2,036	Extracellular Space	other

Table 3.4. Selected up-regulated genes in the bone marrow of active patients relative to inactive patients after unsupervised hierarchical clustering. Shown highlighted are selected genes involved in granulopoiesis, apoptosis and antigen presentation.

3.7 Granulopoiesis signature in the bone marrow as a marker for SLE activity

Nine genes selected a priori were used to investigate associations between a granulopoeisis signature and SLEDAI. To facilitate these comparisons we created a granulopoeisis "score" for both PBMCs and BMMCs, as described above in Materials and Methods and found a correlation between granulopoiesis score and SLEDAI (r = 0.33) in the periphery of the SLE patient subset that had also provided bone marrow **(Figure 3.3A)**. Linear regression analysis showed that granulopoiesis signature significantly correlated with SLEDAI in the bone marrow (r = 0.55, p = 0.013), **(Figure 3.3B)** and the granulopoiesis score from bone marrow active group of patients was higher versus the inactive (p =0.004), **(Figure 3.3C)**

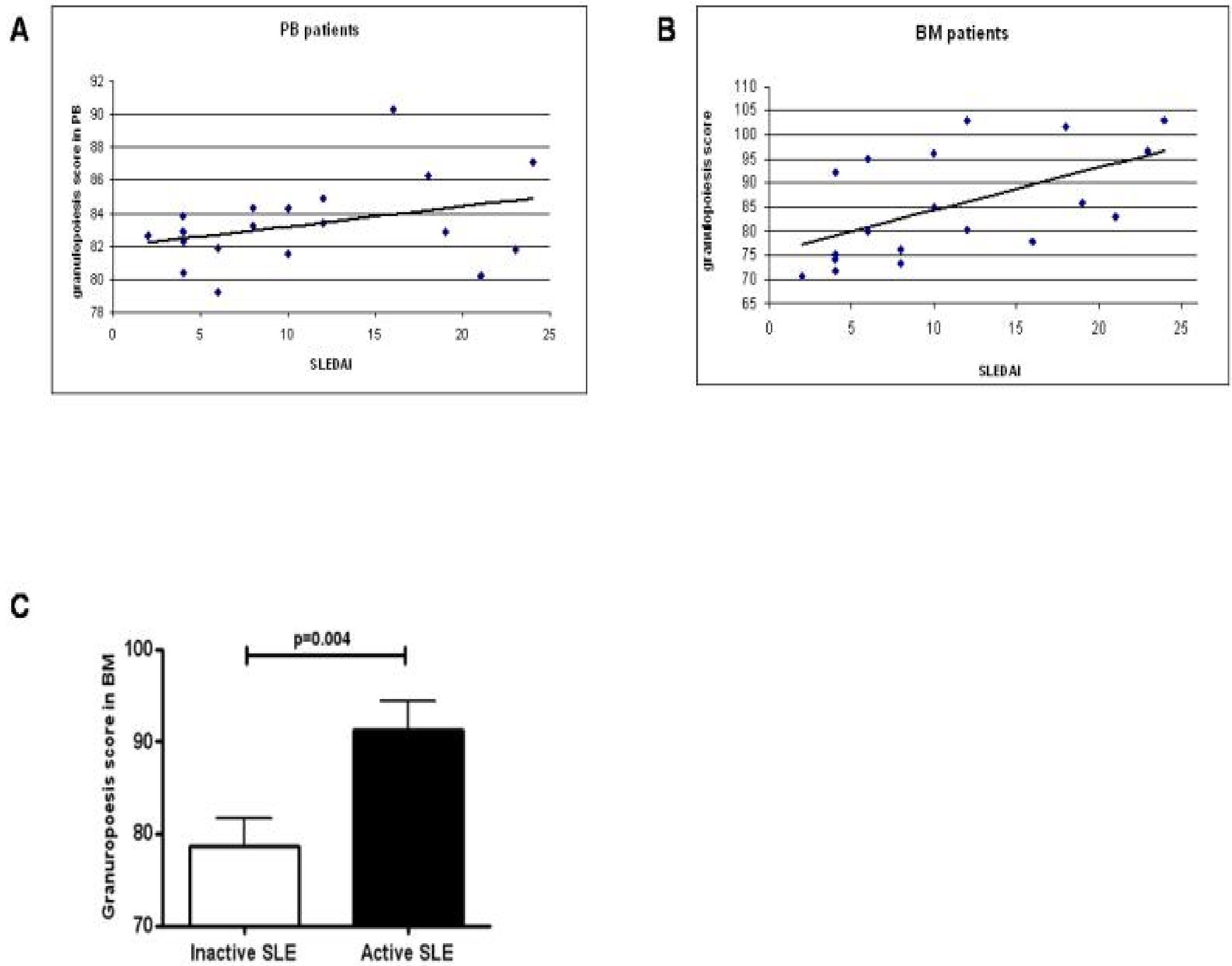


Figure 3.3 *Granulopoiesis signature in the bone marrow as a marker for SLE activity.* Nine genes selected a priori were used to investigate associations between a granulopoeisis signature and SLEDAI. These genes resulted in a statistically significant model of SLEDAI. (A) A numerical score was calculated by using the normalized expression levels of 9 granulopoiesis-related genes that comprise the granulopoiesis signature. Linear regression analysis demonstrates a correlation between granulopoiesis score and SLEDAI (r = 0.33) in the peripheral blood of patient subset that have also provided BM. (B) Granulopoiesis signature of the patients analyzed on Panel A, correlates stronger with SLEDAI in the bone marrow (r = 0.55, p = 0.013). C**.** The granulopoiesis score from bone marrow was higher in the active group of SLE patients vs the inactive (p =0.004)

3.8 Cytokine levels in SLE patients and healthy individuals

3.8.1 *Serum levels of chemokines and Th1/Th2 cytokines*

Serum levels of 22 cytokines were compared between SLE patients and healthy controls. The cytokines assayed included key modulators of inflammation, humoral and cellular immunity and leukocyte trafficking. This includes IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12 (p40), IL-13, IL-15, IL-17, IL-1Ra, IP-10 interferon (IFN)-α and IFN-γ, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF), CCL2 [monocyte chemoattractant protein (MCP)-1]/(MCAF), CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β) and CCL11 (Eotaxin).

Six cytokines were elevated in patients' serum relative to controls. These were: IL-1Ra, IP-10, IL-8, TNF-α, IL-15, and MCP-1 and were identified by Mann-Whitney U test (**Figure 3.4)**.

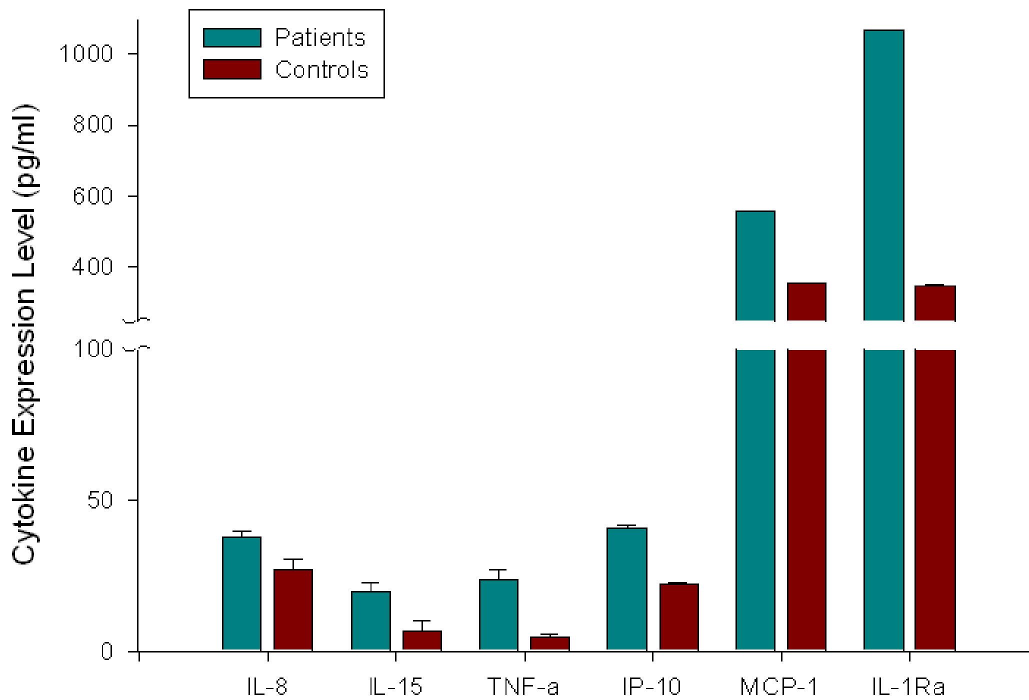


Figure 3.4 Elevated cytokines in SLE patients' serum relative to healthy controls

Among the "cellular cytokines", TFNα (*p*=0.0486) was significantly elevated in SLE patients when compared with levels of healthy individuals, while IL-15 (*p*= 0.0097) was also increased in patients. We did not observe any changes in serum "humoral cytokine" levels. IL1R antagonist was also elevated (*p*=0.0046) in the patient group compared with controls. Serum chemokine IP10, IL8 and MCP1 levels were also higher in SLE patients (**Table 3.5**), with the IP10 levels being significantly elevated (*p*=0.03) in lupus patients compared with unaffected individuals.

Cytokines	P value
IL-1Ra	**0.0046**
IP-10	**0.030295**
IL-8	**0.0026**
TNF-α	**0.0486**
IL-15	**0.0097**
MCP-1	**0.0034**

Table 3.5 : A two sided Mann-Whitney U test was used to test if the median expression was equal between patients and healthy controls. A p-value less than 0.05 was considered statistically significant.

3.8.2 Associations between disease manifestations and serum concentrations of chemokines and cytokines.

We also tried to identify associations between disease parameters and serum cytokines and chemokines. Disease parameters included SLEDAI scores, age, nephritis, arthritis, CNS, hematological disorders and skin manifestations. Serum IP10 concentration showed a negative association with arthritis (predictive ability 0.86364) meaning that the lower the levels of IP10 in patient's serum the higher the risk of developing arthritis as age progresses. Furthermore arthritis associated negatively with IL12 therefore there is probably a higher risk of developing arthritis as serum IL12 levels decrease. The chemokine MIP1α showed a negative association with neuropsychiatric manifestations of lupus while serum IL2 concentration positively associated with lupus nephritis indicating that increase in IL2 levels increases the risk of nephritis.

IV. DISCUSSION

Systemic lupus erythematosus (SLE) is the prototype systemic autoimmune disease characterized by multiple defects in nearly all aspects of immune response culminating in autoantibody production, immune complex deposition and tissue injury. This study sought to shed additional light on the pathogenesis of SLE by analyzing gene expression in the bone marrow, an organ with a central role not only in hemopoiesis but also in the immune response. Herein, we report that bone marrow better discriminates active from inactive lupus and that it displays apoptosis and granulopoiesis signatures. Unsupervised hierarchical clustering did not reveal any sub-clusters of patients with nephritis or CNS lupus and patients with these manifestations scattered in the active disease cluster. These findings expand and corroborate data from animals and humans obtained by the use of both cellular and molecular biology techniques that implicate both apoptosis and neutrophils in the diseases process.

4.1 Microarray analysis studies in SLE

Our microarray analysis has several strong points. First, we only selected the statistically significant genes with a change in expression of at least 2-fold when comparing the means of the two groups; the increased stringency increases the specificity of the results. Second, hierarchical clustering revealed two overlapping groups: active and inactive patients and we determined the differentially expressed genes between these two clusters. By doing so, the analysis was not biased by any arbitrary clustering. Third,

our clinical data on organ involvement were obtained at the time on the blood drawn and were not based on historical data on involvement of a particular organ at some time in the course of the disease. Lastly, in addition to the peripheral blood our analysis included the bone marrow an important organ in the biology of lymphocytes and neutrophils. Using this analysis, we were able to duplicate-only in part- data suggesting an interferon signature in SLE by finding up-regulation of only two IFN regulated genes: *IL6R* whose expression is regulated by IFNα and *PRKCG* which is involved in IFNγ signaling. This may be due to differences in the analysis between our study and those of Baechler et al [47] and Bennet et al [46] or-more likely-to genetic differences in the populations studied (North American vs Meditteranean). The later is supported by data both in murine models [57] and in humans whereby the relative contribution of IFN in the pathogenesis of the disease may depend upon the genetic background[58].

4.2 Granulopoiesis signature: a specific finding or an epiphenomemon

Although we have used a Ficoll-based protocol for the separation of bone marrow and peripheral blood mononuclear cells which do not normally contain granulocytes, we found increased expression of early neutrophil genes. Ficol-Histopaque density gradient preparations of peripheral blood mononuclear cells of patients with lupus contain high numbers of low buoyant density activated neutrophils [59]. We found, a higher expression of neutrophil related genes not only in the bone marrow of lupus patients compared to peripheral blood but, more importantly, in the active group of patients.

Most of these genes encode products of immature granulocytes and their expression is regulated during myeloid cell differentiation. Two of these genes include

defensin A4 and cathepsin G which are found in the granules of neutrophils and exhibit an antimicrobial activity. It has been suggested that defensins and cathepsin G may play important roles in the activation of immune cells and initiate immune responses including the production of autoantibodies like ANCA in autoimmune disorders [60]

4.3 Neutrophil products and tissue injury

Autoantibodies directed against constituents of neutrophil cytoplasm were first described in patients with idiopathic necrotizing glomerulonephritis [61]. Anti neutrophil cytoplasm autoantibodies (ANCA) were soon identified in patients with Wegener's granulomatosis [62] microscopic polyangitis (mPA) and Churg-Strauss syndrome and were also linked to their pathophysiology [63] The most common antigens recognized by ANCA in vasculitides are proteinase 3 (PR-3) and myeloperoxidase (MPO) but other antineutrophil antibodies such as elastase-ANCA and cathepsin G-ANCA have also been detected in the serum of patients with other inflammatory disorders [64, 65] Systemic lupus erythematosus is characterized by the presence of autoantibodies usually targeted against nuclear antigens, nevertheless autoantibodies against neutrophilic cytosplasmic antigens have been described in lupus patients [66, 67] Defensin and cathepsinG-ANCA have been detected in the sera of lupus patients and it has been reported that the value in active SLE was significantly higher than inactive [60]

Myeloperoxidase (MPO) is another major component of the neutrophil granules, synthesized during myeloid differentiation and displaying antimicrobial activity and was overexpressed in the bone marrow of lupus patients relative to the peripheral blood, in this study. Furthermore lactoferrin (LTF) was upregulated in the bone marrow of lupus patients; the encoded protein has an antimicrobial activity and is stored in secondary

granules of PMN from where it is released after cell activation [68, 69]. Anti-LF antibodies are usually present in the sera of SLE patients but its pathogenic role is not well elucidated [70] Another interesting finding of our study was elastase 2 (ELA2), a serine protease that hydrolyses proteins within the azurophil granules of neutrophils as well as proteins of the extracellular matrix following the protein's release from activated neutrophils. This gene was expressed in the bone marrow of active patients and it has been reported as one of the antigens targeted by ANCAs as previously described.

4.4 Integrins and their role in inflammation

It is of particular interest that we found a significant increase in the levels of β2-integrin and other genes involved in the integrin signaling pathway such as MAP3K11, ACTB, ACTG1, ARPC3, MRCL2 and MRCL3 in the bone marrow of active patients relative to inactive. The integrin beta 2, LFA-1 (leukocyte function-associated antigen-1) and its ligand ICAM-1, has a key role in tissue injury in lupus [71]. Integrin signaling in neutrophils and macrophages plays a crucial role in functions such as chemotaxis, cell spreading and adhesion as well as the production of reactive oxygen intermediates (ROI) and different cytokines during inflammation. Corticosteroid therapy decreases the expression of β2 integrin [72]. In Csk (C-terminal Src kinase) deficient animals, that hyperinduction of integrins leads to exaggerated inflammatory responses [73]. Neutrophil activation and secretion, in the absence of proinflammatory stimuli, can be initiated by engagement of β2 integrins, and lead to increased endothelial cell permeability [74]. A humanized anti-LFA-1 antibody for the treatment of multiple inflammatory diseases such as psoriasis exhibited increased clinical efficacy [75].

4.5 Neutrophils and tissue injury in SLE

Neutrophils are important effector cells in a variety of acute and chronic inflammatory states including lupus. Recent animal data suggest that direct hemopoietic FcR bearing effector cells are central to the induction of immune complex–triggered nephritis. Excessive reactive oxygen species (ROS) production can induce oxidant-induced injury and organ dysfunction. The uncontrolled production of neutrophil metabolites into the surrounding environment of the bone marrow could partially account for tissue injury in lupus patients. This is further supported by the fact that the release of neutrophil proteolytic enzymes has been implicated in the most severe manifestations of SLE such as vasculitis, renal failure and CNS [76].

Our data corroborate and expand those from Bennet at al [46]. Although we did not observe the granulopoiesis signature in the peripheral blood we found it in the BM when comparing active vs inactive and BM vs peripheral blood. Common genes overexpressed in our and their study include myeloperoxidase, elastase, cathepsin, CD24, S100P and defensin.

We also observed the expression of early neutrophil genes although we also have used a Ficoll-based protocol for the separation of bone marrow and peripheral blood mononuclear cells which do not normally contain granulocytes. Low density neutrophils have been previously reported in the blood of SLE patients [59] However the analysis of flow cytometry data, in Pascual's paper, showed that all the patients expressing granulopoiesis-related genes had a population of highly granular cells and Giemsa staining of the sorted granular cells revealed the presence of cells at all stages of

granulocyte development; only the presence of immature granulocytes correlated with the granulopoiesis signature [46].

These results suggest that the granulopoiesis signatures reported by others in the SLE peripheral blood may originate from the bone marrow cells, and either persist or expand in the blood of certain SLE patients.

4.5.1 Apoptosis signatures in SLE

In our study we also identified genes involved in the apoptosis of granulocytes in the bone marrow of active SLE, such as FOXO3A which has been described as one of the genes induced after phagocytosis of pathogens [77] as well as annexin 1 reported to have a pro-apoptotic role in human neutrophils [78] and CXCR4 usually expressed on senescent neutrophils. Accelerated apoptosis of lymphocytes in the peripheral blood of lupus patients has a pathogenic role in SLE [79-84]. A number of these studies have also described that the rate of lymphocyte apoptosis correlated positively with disease activity. Courtney et al found increased neutrophil apoptosis which correlated with disease activity and autoantibodies to dsDNA [79]. Under physiological conditions, apoptotic cells are recognized and ingested by macrophages and other phagocytes, which ensure the clearance of apoptotic bodies in vivo. In SLE, there is an impaired clearance of apoptotic cells due to the decreased phagocytic ability of macrophages, monocytes and neutrophils[85, 86]; increased peripheral blood cell apoptosis and ineffective clearance by macrophages correlates with increased disease activity in SLE and suggests that this is maybe due to the lack or presence of specific serum factors [87]. In addition to the necrotic changes in the bone marrow reported in SLE by Voulgarelis et al. [28],

apoptotic bodies were also recently observed in the bone marrow of 8 of 10 SLE patients, many of whom had cytopenias. Both alive and apoptotic neutrophils have been implicated in tissue injury in patients with Wegener granulomatosis [88]. We speculate that neutrophil apoptosis is probably a result of the excessive activation of polymorphonuclear cells.

4.6 Neutrophil circulation model

Neutrophils are polymorphonuclear leukocytes that have a constitutively short life span which is limited by an apoptotic cell death program. The molecular regulation of neutrophil apoptosis is unclear, but in different contexts, neutrophils are sensitive to the death receptor –initiated apoptosis induced by TNF and FasL. It has been described that CXCR4 ligation by SDF-1 (stromal cell derived factor 1) or other CXCR4 agonists significantly increases the expression of both TNF-related apoptosis inducing ligand (TRAIL) and of the death inducing TRAIL receptors on neytrophils and results in TRAIL-dependent apoptosis [89]. Based on Lum et al. model of neutrophil chemotaxis and survival, immature neutrophils are recruited into the circulation by a variety of mediators where they can either enter tissues in response to IL2, IL15 or acquire a relative resistance to apoptosis [89]. Thereafter they die by apoptosis due to the effects of bacteria, bacterial products or reactive oxygen species (ROS). Once in the circulation, they may age where upon they acquire CXCR4 and become responsive to the chemotactic effects of SDF-1, which are produced in the bone marrow. SDF-1 also induced TRAIL and TRAIL receptor expression which causes apoptosis of senescent neutrophils (**Figure 4.1**).

Based on this model and our findings we assume that lupus patients have a rapid activation of neutrophils with uncontrolled production of neutrophil metabolites which accounts for tissue injury. Furthermore our findings on overexpression of specific receptors, such as CXCR4, indicate that probably the rapid activation of neutrophils in SLE leads to their rapid ageing and homing to the bone marrow where they undergo apoptosis.

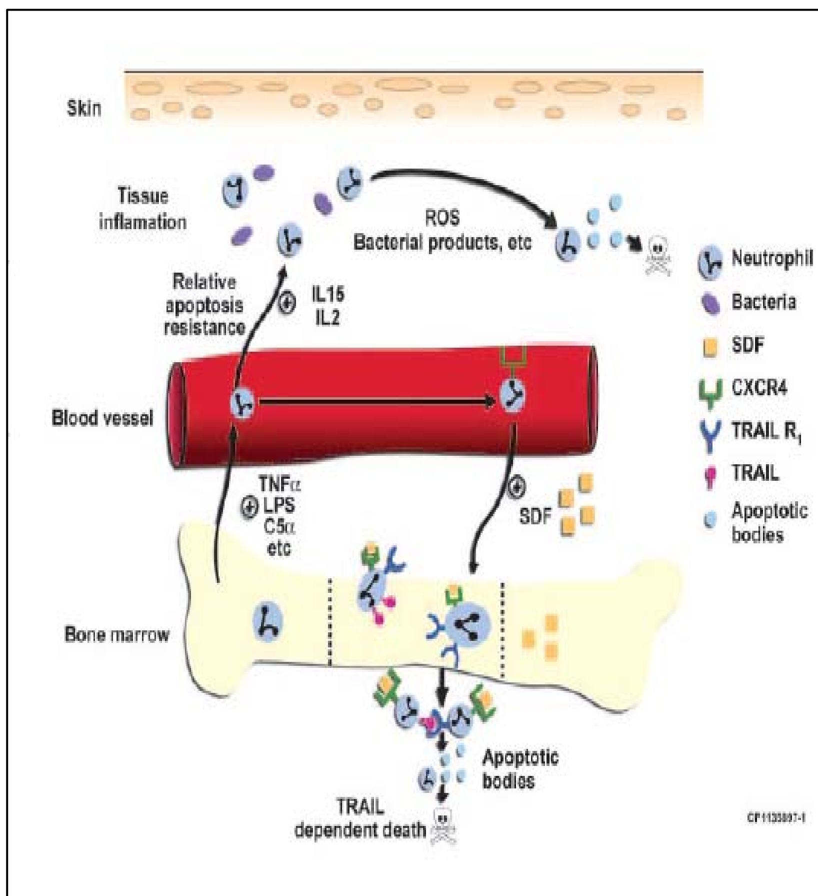


Figure 4.1 Model of neutrophil chemotaxis and survival. Immature neutrophils are recruited into the circulation and enter tissues in response to specific factors. Apoptotic death occurs due to the effects of bacteria, bacteria products or ROS. Once in circulation neutrophils may age and acquire CXCR4 and become responsive to chemotactic effects that lead to apoptotic death of senescent neutrophils in the bone marrow.

4.7 Assocuations of genes with disease activity

In our analysis, we found seven genes correlating with SLEDAI in the bone marrow. Although these genes are highly correlated, they share no known functional relationships. Out of these 7 genes associated with SLEDAI in the bone marrow, only two genes, NY-REN-25 antigen and the coiled-coil domain CCDC91 associated with SLEDAI in active PBMC patients. For both genes there is considerable variation between predicted and actual SLEDAI probably because these genes were selected within the bone marrow comparison.

4.8 Cytokine balance in SLE patients

Infiltrating leukocytes play an important role in tissue injury is lupus and the chemokines that are involved in leukocyte chemotaxis have been implicated in the pathogenesis of SLE.

We found 2 cellular cytokines, TNF-alpha and IL15, characteristic of the Th1-mediated immune response, elevated in patients' sera relative to controls. IL15 is a pro-inflammatory cytokine that inhibits T cell apoptosis and induces other cytokine production and chemotaxis [90, 91] It has been implicated in several autoimmune diseases with the most characteristic being rheumatoid arthritis in which it acts upstream of TNF-alpha and can induce its production through activation of synovial T cells [92]. IL15 has been reported to be elevated in SLE patients [93] We found that not only serum IL15 levels were significantly elevated in patients relative to controls but it also correlates with TNF-alpha levels in patients. We assume that in SLE, like RA, the

regulation of TNFα production is likely to be related to IL15, underlying its role as a significant cytokine in lupus.

Although lupus is considered by many as a Th2 cytokine disease none of the humoral cytokines were found elevated in the patients' cohort relative to the controls. However recent reports in animal models and human lupus have stressed that Th1/Th2 cytokine balance is shifted towards Th1 cytokines like IFN that appears to be dominant over Th2 cytokines such as IL4 and IL10 [94]

IL1Ra was increased in lupus patients when compared with unaffected individuals. It has been reported that a polymorphism of the IL1 receptor antagonist (IL1RN*2) associates with higher risk of lupus in Swedish and Chinese SLE patients [95, 96] Several studies have reported the role of chemokines in the pathogenesis of lupus and three of them, IP10, IL8 and MCP1 were found elevated in patients' serum.

In summary, these data support the use of microarray analysis to uncover novel immunopathologic pathways in the disease and have shown that the bone marrow, distinguishes active from inactive lupus patients. Moreover, we have found evidence for up-regulation of apoptosis and granulopoiesis-related genes in active lupus, two biologic processes intimately linked with the diseases and tissue injury. More importantly, our data suggest up-regulation of genes such as β2-integrin which are of crucial importance in the effector function of neutrophils and monocytes and tissue injury in animal models of lupus. These data provide additional credence to the role of bone marrow and neutrophils in the pathogenesis of the disease and suggest additional pathways for potential therapeutic modulation targeted at the effector cells to minimize tissue injury.

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