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PHARMACOLOGICAL ROLE OF A2A ADENOSINE RECEPTORS IN SYSTEMIC LUPUS ERYTHEMATOSUS, CLINICAL AND LABORATORY CORRELATIONS

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Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is the prototypic multisystem autoimmune disorder with a broad spectrum of clinical presentations.

1. Epidemiology

The incidence of lupus has nearly tripled in the last 40 years, mainly due to improved diagnosis of mild disease. Estimated incidence rates in Europe range from 2 to 8 per 100 000 per year. Women are affected nine times more frequently than men and African Americans and Latin Americans are affected more frequently than Caucasians, and have higher disease morbidity (1) (2).
Sixty-five % of patients with SLE have disease onset between 16 and 55 years, 20% present before age 16, and 15% after the age of 55. Men with lupus tend to have more serositis, an older age at diagnosis, and a higher mortality rate compared to women. SLE tends to be milder in the elderly with a lower incidence of malar rash, photosensitivity, purpura, alopecia, Raynaud's phenomenon, renal and central nervous system (CNS) involvement, but greater prevalence of serositis, pulmonary involvement, sicca symptoms, and musculoskeletal manifestations (3).

2. Aetiology

The aetiology of SLE includes both genetic and environmental components with female sex strongly influencing pathogenesis. These factors lead to an irreversible break in immunological tolerance manifested by immune responses against endogenous nuclear antigens (4).

2.1 Genetic factors

The rate of gene discovery in SLE has increased in the past few years thanks to large genome-wide association studies (GWAS) and more than 25 genetic risk loci have been identified (5).

The genetic risk for lupus is likely derived from variation in many genes: HLA-DRB1, signal transducer and activator of transcription 4 (STAT4) and interferon regulatory factor 5 (IRF5) are the three most frequently observed alleles each accounting for a 1% of the variance in genome-wide association analysis. IRF5 is involved in the transcription of type I interferon and pro-inflammatory cytokines and STAT4 plays a key role in type I interferon (IFN) signalling. Moreover deficiencies of early components of the classical pathway of complement such as C1q, C2 or C4 are rare, but they confer the strongest genetic susceptibility to an SLE-like disease in humans, with a penetrance rate from 30% (C4 deficiency) to over 90% (C1q deficiency) (6). A study reported that C1q inhibits immune-complex triggered interferon α (IFNα) production by plasmacytoid dendritic cells (pDC), providing a link between complement deficiency and the activation of the type I IFN pathway in SLE (7).

2.2 Epigenetic effects

Epigenetics represents a new aspect in the pathogenesis of SLE and refers to changes in gene expression, sensitive to external stimuli, which do not involve changes in DNA sequences. Epigenetic effects may represent the missing link between genetic and environmental risk factors. The risk for SLE may be influenced by epigenetic effects such as DNA methylation, histone modifications and microRNA (miR) interference (8).

DNA methylation is triggered by DNA methyltransferases and usually occurs in

specific regulatory regions of genes making DNA inaccessible to transcription factors and causing gene silencing. The activity of the methyltransferases is reduced in T cells of SLE patients as well as in mouse models of the disease causing DNA hypomethylation and over-expression of methylation-sensitive genes, that may lead to autoimmunity (9).

2.3 Environmental factors

Candidate environmental triggers of SLE include ultraviolet light, demethylating drugs, and infectious or endogenous viruses or viral-like elements. Sunlight is the most obvious environmental factor that may exacerbate SLE. Epstein Barr virus (EBV) has been identified as a possible factor in the development of lupus. EBV may reside in and interact with B cells and promotes IFNα production by pDCs, suggesting that elevated IFNα in lupus may be at least in part due to aberrantly controlled chronic viral infection (10).
It is well established that certain drugs induce autoantibodies in a significant number of patients, most of whom do not develop signs of an autoantibody associated disease. Over 100 drugs have been reported to cause drug-induced lupus (DIL), including a number of the newer biologics and antiviral agents. Although the pathogenesis of DIL is not well understood, a genetic predisposition may play a role in the case of certain drugs, particularly those agents that are metabolised by acetylation such as procainamide and hydralazine. These drugs may alter gene expression in CD4+ T cells by inhibiting DNA methylation and thus promoting auto reactivity (11).

3. Pathogenesis and pathophysiology

A key feature of SLE is a breakdown in innate and adaptive immune responses that leads to a loss of tolerance (12). The irreversible break in immunological tolerance is manifested by immune responses against endogenous nuclear antigens and the subsequent formation of autoantibodies and immunecomplexes (ICs). SLE has classically been considered an autoimmune disease with a predominant adaptive immune system component, since T and B cells have been considered the most important pathogenetic players (4). During the early inflammatory phase, pDCs are able to internalize nucleic acids containing ICs that reach the endosomes and stimulate Toll-like receptors (TLR) 7 or 9, leading to IFNα gene transcription (13)(14). IFN-α contributes to the maturation of myeloid DCs that can activate autoreactive T cells through antigen presentation and costimulation. This favours the development of T helper 1 (Th1) cells responsible for high-level production of proinflammatory cytokines, and again IFN, which in turn, stimulates DCs and myeloid cell production of interleukin-(IL)-1β, IL-6, IL-12, IL-18, TNF-α, and BAFF creating a perpetual proinflammatory loop (15).

3.1 Adaptive Immunity in SLE

T cells

Aberrant TCR Signaling in Patients with SLE

T cells are mainly activated via the T cell receptor (TCR), a heterodimer formed by the α and β chains, which are responsible for the recognition of antigen. In patients with SLE, an aberrant TCR signalling has been reported. This leads to hyperresponsiveness of T cells. Changes have been found in the expression of TCR and in the activation of intracellular spleen tyrosine kinase (Syk), calcium signalling, and various other kinase pathways. Normal interaction of both TCR and CD3 is needed for proper signal transduction once an antigen binds to the TCR. When antigen binds to TCR, CD3ζ recruits zeta-chain-associated protein 70 (ZAP-70), which then initiates multiple signalling cascades. In SLE, the CD3ζ chain levels are decreased and are replaced by an analogous protein, the common γ chain of the Fc receptor that recruits Syk (16). Syk is much more potent than ZAP-70 in phosphorylating its targets, which could explain the hyperresponsiveness of T cells in SLE. Indeed, Syk expression is massively increased in T cells of patients

with SLE compared to healthy individuals, moreover inhibition of Syk resulted in decreased phosphorylation and calcium responses in T cells of SLE, and ameliorated skin and kidney disease in a mouse model of lupus (17).

T Cell Subsets in the Pathogenesis of SLE

T cells can be divided into multiple subsets according to their phenotype and function. Tregs are important CD4-positive T cells that function in peripheral T cell tolerance by inhibition of autoreactive T cells. Tregs can exert their tolerogenic functions via direct cell–cell contact or by the release of immunosuppressive factors, such as transforming growth factor β (TGFβ) and IL-10. The majority of Tregs cells are characterized by the expression of the transcription factor Foxp3 and high expression levels of the IL-2 receptor alpha chain (CD25). The development of Tregs depends on the presence of IL-2 and TGFβ. The role of aberrancies in the number of Tregs and/or function of Tregs in the pathogenesis of SLE remains controversial (18). Some studies demonstrated a decreased number of Tregs in patients with SLE (19) other demonstrate that the number of Tregs is similar for patients with SLE and controls (20). Some report demonstrate that the suppressive function of Tregs in SLE is impaired, other that autoreactive effector T cells in SLE are less susceptible to suppression by Tregs (21).

In addition to Tregs also Th17 cells and double negative T cells, lacking both CD4 and CD8 expression, appear to play a role in the pathogenesis of SLE. Th17 cells constitute a subset of CD4-positive T cells that have been identified a decade ago. Development of Th17 cells requires TGFβ and IL-6, and they are identified by the specific transcription factor RORγt, and their characteristic production of IL-17 (22). IL-17 and Th17 cells are increasingly present in mice that show a lupus-like autoimmune phenotype and have been implicated to contribute to the formation of germinal centres (GCs) and subsequent production of pathogenic antibodies (23). In addition to Th17 cells, double negative (CD4−, CD8−) T cells can produce IL-17 in SLE. Notably, the number of double negative T cells is increased in patients with SLE compared to healthy individuals. Taken together, an increased level of IL-17 in patients with SLE establishes an autoreactive and inflammatory environment that can lead to tissue damage (24).

B cells

Under normal conditions B cells follow a tightly regulated life cycle with a large number of check points at indicated stages to prevent the development of autoimmunity (25). In the bone marrow, B cells develop from stem cells from precursor stages during which they rearrange their variable immunoglobulin (Ig) genes. Immature transitional B cells expressing surface IgM/IgD emigrate from the bone marrow into the peripheral blood and then mature into naïve B cells. After encountering antigen and Tcell in follicles (Tfh) of secondary lymphoid organs, mature naïve B cells undergo GC reactions leading to their clonal expansion, somatic hypermutation of Ig gene rearrangements, and Ig heavy-chain class-switch recombination. During this maturation process central B cell tolerance occurs because immature B cells are highly sensitive to signalling through the B cell receptor (BCR) and the receptor engagement results in cell death (13).

B cells are commonly affected in SLE patients and are responsible for the production of autoantibodies against soluble and nuclear antigens (10). In active SLE a reduction in the number of naive B cells is observed, and the number of plasmacells is increased in the peripheral blood (11). B cell tolerance is defective at several levels, including both abnormalities in central and peripheral selection responsible for removal of self-reactive immature B cells (12) (26). B cells also play an autoantibody-independent role in SLE pathogenesis. Actually, B cells exert multipotent immunological functions such as presenting antigen, co-stimulating T cells that induce immunogenic DCs. They also produce cytokines and chemokines to promote inflammation, affect immune regulation and lymphogenesis (27).

Intracellular signal transduction in B cells

Abnormal BCR down-stream signalling as indicated by augmented calcium influx and increased phosphorylation of protein tyrosine residues is observed in SLE patients and this leads to defective self-limitation of cell activation and breakdown of self-tolerance. Enhanced BCR signalling can lead to auto-reactivity and signals from several molecules, including CD19, intracellular TLRs and the BAFF (B cell activating factor) receptor (BAFF-R) interacts with BCR signals to enhance B cell activation and survival at this stage, and an excess of any of these molecules may predispose to SLE. B cell tolerance can also be broken during antigen activation. SLE is characterized by a loss of B cell tolerance with an impaired capability to delete autoreactive B cells. Compromise of tolerance checkpoints, along with other factors, may lead to increased production of autoantibodies. Germline variants of

sialic acid acetylesterase, an enzyme that limits signalling of B-lymphocyte antigen receptors, are linked to SLE and other autoimmune diseases. These variants have reduced activity and thus may contribute to increased B-cell signalling (28).

B cell subsets in Systemic Lupus Erythematosus

SLE B cells can be divided into pathogenetic autoreactive B cells involved in autoimmune response, protective B cells involved in defence against microorganisms and regulatory B cells (Breg) that mantain self-tolerance (29). Differences among healthy subjects suggest that mechanisms that control pre-immune B-cell maturation, negative selection at defined maturational checkpoints, receptor editing, somatic hypermutation and effector B-cell generation, are diffusely abnormal in subjects with SLE (30).

With regard to early defects of selection against autoreactive B cells, it has been demonstrated that patients with SLE fail to remove B naïve cells expressing self-reactive BCRs. Consistent with this, earlier studies demonstrated that self-reactivity or loss of proper selection during early B-cell development from immature (CD10+CD27−IgM+ B cells) to mature (CD10−CD27−IgM+ B cells) naïve B cells is a feature of SLE (31).

The stage from immature pre-B to naive B in bone marrow is an important central checkpoint to eliminate harmful autoreactive B cells. At the stage of pre-BCR, surrogate light chain pairs with Ig heavy chain to form pre-BCR and preclinical studies have shown that surrogate light chain-deficient mice produced antinuclear antibodies in serum (32).

Regarding disturbances of memory B cells, a central finding in phenotyping peripheral blood B cells in adult SLE is the substantial increase of antigen experienced CD27+IgD− post-switched memory B cells, which are less susceptible to immunosuppressive therapy. The peripheral memory BCR repertoire in SLE is shaped by abnormal selection, exaggerated somatic hypermutation, and increased receptor editing. Moreover, autologous stem cell transplantation in SLE provides that the disease can undergo successful remission along with the emergence of predominating naïve T and B cells after transplantation (33). This is consistent with the conclusion that abnormalities in the shaping of the Ig repertoire after antigenic stimulation and differentiation of memory B cells, rather than molecular disturbances in generating the initial BCR repertoire of pre-immune B cells, may be more important in lupus pathogenesis (30).

An area of intense interest is the clonal expansion of B cells at the GC where B lymphocytes undergo somatic mutation, class switching and differentiation to long-lived effector cells. GC selection is clearly defective in SLE, allowing autoreactive B cells to differentiate into pathogenic memory and plasma cells. The loss of germinal centre tolerance in SLE could be either the result of a defect in B cell signalling or B cell death or an excess of self- antigens resulting from inadequate clearance of apoptotic cells (34).

The currently available data indicate that active SLE is characterized by increased numbers of CD27+ plasmablasts/plasma cells in active disease in children as well as adults, indicating dysregulation of the homeostasis of these cells. Notably, the plasma cells were oligoclonal and included cells that were actively secreting anti-dsDNA antibodies (35).

Finally, though not definitely proven to play an important immunoregulatory role in humans, regulatory B cells (B reg) appear to be induced after antigen-dependent activation in mice and, therefore, are also considered to be part of the memory subset. Their precise role in human lupus, however, remains to be confirmed. B reg are capable of secreting IL-10 and controlling T cell proliferation. In human SLE patients Breg produces less IL-10 and has compromised suppressive capacity (36).

4. Cytokines: Functions and Contributions to the Pathogenesis of SLE

A lot of studies show that expression disturbances of certain cytokines are significant for SLE pathogenesis. Imbalance between pro and anti-inflammatory cytokines is critical for development of clinical manifestations and organ damage in SLE patients. Among the most important cytokines in disease development are: interferon type I (IFN-I); interleukin-6 (IL-6); interleukin-2 (IL-2), interleukin-17 (IL-17); B lymphocyte stimulator (BLyS); A proliferation-inducing ligand (APRIL); tumor necrosis factor-α (TNF-α) (37).

4.1 Interferon type I

IFN type I, in particular IFN-α, is a central player in SLE pathogenesis. IFN-α was found to be elevated in lupus sera SLE (73, 74) and the ability of DNA containing immune complexes from lupus sera to induce IFN-α production by a novel cell type later identified as the pDCs was described in the late 1990s (38) (39). IFN-α is primarily produced by pDCs upon recognition of nucleic acids by toll-like receptor 7 and 9 (75). This leads to induction of type I IFN gene transcription. DNA structures traffic through early endosomes into endolysosomes where TLR7/9 activation recruits a different set of signalling molecules (i.e. NF-kB) leading to transcription of inflammatory cytokines (IL-6, TNF-a) and costimulatory molecules (CD80, CD86 and CD40) (40). IFN-α promotes DCs maturation disrupting peripheral immune tolerance and through upregulation of costimulatory molecules, such as CD80 and CD86, contributes to the survival, expansion and differentiation of self-reactive T cells.

IFN-α impairs the suppressive function of Tregs in SLE. Importantly, in the presence of IFN-α producing DCs, Tregs of SLE patients as well as Tregs of healthy controls were not capable of suppressing T effector cells (41) (42).

IFN-α has myelosuppressive effects explaining in part the lymphopenia observed in the peripheral blood of lupus patients. At the same time IFN-α induces BLyS/BAFF and contributes to the survival of mature, peripheral B cells promoting the differentiation of activated B cells into plasmablasts. IFN-α together with IL-6 permit plasmablasts to develop into antibody-secreting plasma cells (43). Autoreactive B cells generate autoantibodies and provide a positive feedback loop

for enhanced TLR7/9 activation and even more increased IFN-α production (44) through the formation of nucleic-acid-containing immune complexes..
Overall interferon is dysregulated in lupus and that overexpression of IFNα can result from the autoantibodies present in lupus. Many components of the molecular pathways through which IFNα and TLRs drive immune activation include genetic risk factors for lupus, further implicating IFN in lupus etiology and pathogenesis.

4.2 B lymphocyte stimulator (BLyS) and A proliferation-inducing ligand (APRIL)

Strong evidence implicates the TNF superfamily ligands BLyS and APRIL in SLE. BLyS is one of the most important cytokines governing the functioning of B cells. It induces the survival, proliferation, differentiation and immunoglobulin secretion (45) of B cells. Its over expression increases the number of hyperactive effector T and mature B cells promoting the development of autoimmune-like manifestations in mice (46). Its concentration in serum is higher in SLE patients than in healthy controls and generally correlates with anti-dsDNA antibodies, although the changes in serum BLyS level do not correlate with disease activity in the individual patient (47).
APRIL is responsible for induction and maintenance of B cell responses, it increases Ig production and upregulates surface expression of B cell effector molecules (48). Its level is increased in cerebrospinal fluid of patients with neuropsychiatric involvement and with renal disease activity in lupus nephritis (49) (50).

4.3 Tumor necrosis factor alpha (TNF-α)

TNF (tumor necrosis factor) is mainly produced by activated monocytes/macrophages, but T cells, natural killer (NK) cells, neutrophils, mast cells and endothelial cells can also secrete this cytokine (51). TNF has multiple effects in the development and activation of the immune system. TNF induces the expression of other proinflammatory cytokines, such as IL-1 and IL-6, but can also down regulate production of IFN-α by DCs, the latter effect may be one reason why SLE can occasionally develop in patients receiving anti-TNF therapy (52).

In SLE, high serum levels of TNF and soluble TNF receptors have been reported to correlate with disease activity (53). However in experimental models of lupus TNF have been shown to have both positive and deleterious effects probably because of its immunoregulatory and pro inflammatory roles (54).

4.4 Interleukin-2

There is a significant defect in the production of IL-2 from in vitro-activated T cells both in murine lupus models and humans with SLE. IL-2 is a key T cell-derived cytokine that is mainly produced by antigen-activated T cells (55). IL-2 is implicated in the differentiation of effector cytolitic T cells and has a unique role in promoting activation-induced cell death (AICD), an important apoptotic process that is responsible for the elimination of repeatedly activated, and potentially autoreactive, T cells (56). Both in vitro and in vivo studies have provided evidence that IL-2 plays an important role in the development and survival of regulatory T cells (Tregs) (57). The discovery that IL-2 may restrict the differentiation of naïve CD4+ T cells into IL-17 secreting cells (Th17 cells) in vitro (58) is of particular interest. The molecular mechanisms responsible for the IL-2 defect in lupus T cells is the result of defective transcriptional regulation

4.5 Interleukin-6

IL-6 is a proinflammatory cytokine that is secreted at high levels by myeloid cells in response to a broad array of pathogen-associated molecular patterns; this cytokine can also be produced by activated lymphocytes. In addition to its activation of myeloid cells and B lymphocytes, IL-6 together with transforming growth factor-β (TGF-β) promotes the differentiation of Th17 cells and suppresses the transcription factor forkhead box P3 (FoXP3), leading to a reduction in regulatory t cells (59). IL‑6 is elevated in the serum of SLE patients. It is necessary for B-cell stimulation and promotes immunoglobulin secretion by plasma cells. It also inhibits the differentiation of regulatory T cells (59).

4.6 Interleukin-10

IL-10 is an immunoregulator that inhibits T cells function and suppresses proinflammatory cytokines. IL-10 levels are elevated in SLE and might have a role in disease pathogenesis, despite their potent immunosuppressive and antiinflammatory activity. Extensive researches in humans have shown that serum levels of IL-10 are markedly increased in patients with SLE and have correlated with disease activity (SLEDAI), or production of antibodies (anti-dsDNA) (60).

The accumulated data suggest that in SLE patients the overproduced IL-10 may be ascribed to B cells and monocytes (61). B cell secretion of IL-10 could regulate DCs and T cell function. Additionally, the enhanced excretion of IL-10 might also contribute to an increasing number of the earlier peripheral B cell abnormalities observed in SLE, including plasma cell expansion (35). Moreover in SLE patients, the capacity of IL-10 to suppress production of inflammatory cytokines such as TNF-α and IL-6 by myeloid lineage cells is attenuated. A likely implicated mechanism is related to INF-α which decreases the capacity of IL-10 to suppress inflammation.

5. Clinical manifestations

SLE can affect any organ system. Constitutional symptoms such as malaise, fatigue, fever, and weight loss are common presenting symptoms. These symptoms are not specific and it is important to distinguish other aetiologies such as fibromyalgia, depression, infection, malignancy, endocrinopathy, or other connective tissue diseases.

5.1 Mucocutaneous involvement

Skin involvement includes lupus-specific and non-specific lesions (62). The specific lesions may be classified as acute, subacute and chronic.

Acute-malar rash: the classic butterfly rash occurs acutely as an erythematous lesion and commonly the trigger factor is represented by the exposure to sunlight. The rash may disappear in days or weeks. The malar rash may be temporary and disappear without scarring, although permanently active rash may result in a permanent injury (62).

Subacute rash: subacute cutaneous lupus erythematosus (SCLE) is found in about 10% of SLE patients with skin involvement. It can manifest with annular or psoriasiform skin lesions. These lesions are strongly associated with the expression of serological antibodies anti-Ro (SS-A) and anti-LA (SS-B). In rare cases, the lesions may be similar to a multiform erythema (Rowell's syndrome). These lesions are typically erythematous, marginalized, sometimes crusted. The parts involved are shoulder, forearm, neck and upper portion of the trunk, usually the face is spared (62).

Chronic rash: discoid lupus erythematosus (DLE) develops in about 25% of SLE patients. The discoid lesion is characterized by the presence of an isolated, erythematous, slightly infiltrated plaque, covered by adherent follicular hyperkeratosis. Most common sites involved are the face, neck, scalp, and less frequently ears and upper trunk (62).

Other cutaneous manifestations are lupus profundus (lupus panniculitis), a rare event that occurs with fixed nodules with possible skin ulcers. The painful symptoms associated with nodules, derives from the presence of macrophage infiltration in the perivascular and panniculitis, which looks like a hyaline-fat necrosis with infiltration of monocytes and lymphocytic vasculitis in adipose

lobules. The sites involved could be scalp, face, arms, back, chest, thighs, buttocks (63).

Another variant is the rare lupus tumidus, characterized by lesions, such as chronic plaque pink or large slow-healing lesions, in sun-exposed areas (64).

The diagnosis of a skin lesion lupus related is supported by biopsy that shows the presence of immune complexes at the level of the dermal-epidermal junction. The detection of these complexes is defined "lupus band test" (65).

Alopecia is defined as excessive loss of hair. The scalp, eyebrows and beard may be involved. Alopecia with scarring is a complication of discoid lupus and usually occurs on the scalp. "Lupus hair" is, instead, the phenomenon characterized by extremely dry and brittle hair. This phenomenon is related to the degree of disease activity.

Photosensitivity is defined as the development of a rash against exposure to UV-B resulting from sunlight or lamps. This phenomenon is present in approximately 60-100% of patients. The photosensitivity is one of the entries in the classification criteria for SLE.

The mucosal involvement afflicts 25-45% of SLE patients. The most common manifestations are similar to white and uneven patches (62).

5.2 Musculoskeletal Involvement

Skeletal muscle involvement occurs in 53-95% of patients with SLE.

Joint involvement is usually described as non-erosive, non-deforming, arthritis involving the small joints of the hands, wrists, knees. Synovitis may be temporary, migrant and reversible. In a small proportion of lupus patients the arthritis is substantially indistinguishable from RA. The term "rhupus" has been coined for this patient. Other events include tenosynovitis and subcutaneous nodules (66). Myositis with muscle pain and muscle stiffness are common during flares of the disease. Inflammatory myositis involving proximal muscles was found in 11.5% of cases and can develop at any time during the course of the disease. Avascular bone necrosis is a major cause of morbidity and disability in SLE and can affect 6-12% of patients. The factors related to bone ischemia are Raynaud's phenomenon, vasculitic phenomena, emboli-fat content, the antiphospholipid antibody syndrome (APS) and the use of high doses of steroid (67).

5.3 Kidney involvement

Renal involvement occurs in 40-70% of SLE patients and is the leading cause of hospitalization. It varies widely in type and severity, but generally represents a potentially serious medical condition. The main sign of lupus nephritis is the proteinuria and it is usually accompanied by hematuria of glomerular origin. The urine test is the most important method of identification and monitoring the renal disease. Hematuria (usually microscopic) may indicate a glomerular or tubulointerstitial inflammation. In presence of a severe disease with a widespread proliferation, the urinary sediment can be of any type as a result of severe glomerular and tubular damage. Renal biopsy should be considered in any patient with SLE who has clinical or laboratory evidence of active nephritis, especially upon the first episode of nephritis. Renal biopsy is the best way to document the renal damage. The current indications for renal biopsy include: 24-hour proteinuria> 0,5-1g / day, hematuria of dubious origin in particular if you have dysmorphic erythrocytes, presence or absence of cell cylinders Based on the biopsy and subsequent pathologic analysis, six immunohistochemical classes have been identified (68):

- Class I: glomerulonephritis (GN) mesangial,
- Class II: mesangial proliferative GN.
- Class III: focal proliferative GN. It is a focal proliferative glomerulonephritis (involvement of at least 50% of the glomeruli); segmental (III-S; involving less than 50% of the surface of the glomerulus) or global (III-G; > 50% of glomeruli involved in more than 50% of their surface), typically with subendothelial deposits, with or without mesangial alterations.
- Class IV: diffuse proliferative GN. In this class more than 50% of glomeruli are involved with diffuse subendothelial immune deposits, with or without mesangial alterations.
- Class V: membranous lupus nephritis. It is characterized by subepithelial immune deposits with a segmental or global distribution. It is usually associated with lesions of III or IV or with sclerotic lesions. Clinically there is a constant proteinuria often associated with hematuria and nephrotic syndrome.
- Class VI: advanced sclerosis lupus nephritis. It is characterized by sclerosis of more than 90% of glomeruli and can be associated with interstitial fibrosis and

tubular atrophy. The clinical picture is presented with renal failure, hypertension and proteinuria.

5.4 Neurological Involvement

SLE can affect both the central nervous system (CNS) and the peripheral nervous system (PNS). Data from a meta-analysis have suggested that the prevalence of NP syndromes in SLE patients is between 42.5% and 74.7%. The involvement of the nervous system represents a major cause of morbidity and one of the most important challenges for the rheumatologist. In terms of frequency, the central involvement is reported in 93.6% of cases, while de PNS involvement in 6% of manifestations. To try to classify the clinical picture of CNS involvement, in 1999 a multi-disciplinary committee of international experts from the American College of Rheumatology drew up a classification system also giving the following definition of neuropsychiatric SLE (SLE-NP) (69):
"Neuropsychiatric lupus erythematosus includes the neurologic syndromes of the central, peripheral and autonomic nervous system and the psychiatric syndromes observed in patients with SLE in which other causes have been excluded". The headache is a syndrome frequently observed in SLE, which occurs with a frequency that varies between 3 and 56%. To date there have been no clinical-pathogenetic evidence to determine whether the headache is a primitive expression of the disease or a syndrome associated with SLE. One also should consider that disabling and chronic diseases, such as connective tissue diseases, can arouse anxiety and depression from which tension headache may be a possible and well-known manifestation. Cognitive dysfunction has been reported in 20-30% of SLE patients. The psychosis has been reported in 3.5% of cases and it is characterized by disillusions or hallucinations, the latter of auditory nature. Focal or generalized seizures are reported in 7-10% of patients and are usually due either to a generalized systemic multi involvement of SLE or to a localized neurologic injury. Seizures are also associated with the presence of aPL antibodies. Demyelination, transverse myelopathy and chorea are rare events that occur in <1% of cases. Clinical and instrumental evidence (neuroimaging) of demyelination may be indistinguishable from a framework of multiple sclerosis. Myelitis may manifest with signs of grey matter (upper motor neuron syndrome, spasticity, hyperreflexia) or signs of white matter (syndrome of the second moto

neuron with flaccid paralysis and reduced reflexes). Sensorimotor peripheral neuropathy has been observed in only 1% of SLE patients and may occur independently of other events (70). See a list of NP manifestations reported in SLE in Table 1.

Table 1. Classification of NPSLE manifestations (American College of Rheumatology 1999)

Central NPSLE	**Peripheral NPSLE**
Aseptic meningitis	Guillain Barré syndrome
Cerebrovascular disease	Autonomic neuropathy
Demyelinating syndrome	Mononeuropathy
Headache	Myasthenia gravis
Movement disorder	Cranial neuropathy
Myelopathy	Plexopathy
Seizure disorders	Polyneuropathy
Acute confusional state	
Anxiety disorder	
Cognitive dysfunction	
Mood disorder	
Psychosis	

5.5 Cardiovascular Involvement

Pericarditis is an event that occurs in 25% of SLE patients. Pericardial effusion may be asymptomatic and often moderate; cardiac tamponade is however very rare. The myocardial involvement is rare and typically occurs in the presence of a generalized lupus activity. Lupus is associated to an increased mortality and morbidity related to cardiovascular disease. These include premature and accelerated atherosclerosis and heart valve disease. In addition, some studies have reported an increased risk of myocardial infarction compared to healthy population only partly explained by traditional cardiovascular disease risk factors. The presence of valvular heart disease is frequently found in SLE patients and correlates with the presence of antiphospholipid antibodies. The most common abnormalities include the thickening of the aortic and mitral valve due to the presence of vegetation, valvular insufficiency, and valvular stenosis. Studies on SLE patients have shown the presence on the valves of vegetation (called

Libmann-Sachs vegetations) probably caused by the formation of fibrin-platelet thrombi, which are organized and lead to fibrosis and valve dysfunction (71).
Atherosclerosis is significantly more frequent in lupus patients compared to the healthy population. A contribution to this increased rate can be given by different factors, such as antibodies against lipoproteins, hypertension and metabolic syndrome. The endothelial cells are damaged due to the presence of immune and inflammatory molecules. These molecules attract immune cells that infiltrate the sub endothelial space as well as creating endothelium dysfunction (72).

5.6 Lung involvement

The most common pulmonary manifestation is represented by pleurisy. Pleuritic pain is present in 45-60% of patients and may present with or without pleural effusion. The pleural effusion is usually bilateral and equally distributed between the two chest walls.
The interstitial lung disease (ILD) is a complication present in 3-13% of cases, but rarely severe. Acute lupus pneumonia presents with cough, dyspnea, pleural pain, hypoxemia, fever can be observed in 1% of cases. Hemorrhagic alveolitis is a rare complication of lupus; the characteristic clinical and radiographic picture shows diffuse alveolar infiltrates, hypoxemia, dyspnea, anaemia, hemoptysis. The alveolar hemorrhage occurs in patients with a known history and longstanding SLE, high anti-dsDNA titles and with an active extrapulmonary disease. Bronchoscopy, bronchial lavage (BAL) and transpulmonary biopsy are often necessary to confirm the diagnosis.
The "shrinking lung" syndrome is characterized by worsening dyspnea, the presence of small lung volumes secondary to muscle dysfunction. Pulmonary hypertension is a rare and life-threatening complication. The clinical picture is manifested by rapidly progressive dyspnoea and Raynaud's phenomenon (58%) (73).

5.7 Lymphoid organs involvement

Lymphadenopathy occurs in about 40% of patients, usually at the onset of the disease or during an exacerbation. Patients with current lymphadenopathy is most likely to present constitutional symptoms. Splenomegaly is present in 10-45% of

cases, usually during the active phase of the disease, and is not necessarily associated with cytopenia. The splenic atrophy and functional hyposplenism (autosplenectomia) can be found in the LES and it exposes the patient to a higher risk of septic complications (3).

5.8 Haematological involvement

Hematologic abnormalities are common. The main events include: anaemia, leukopenia, thrombocytopenia.

Anaemia is common in SLE patients and correlate with disease activity. Its pathogenesis includes anaemia of chronic disease, haemolytic anaemia (autoimmune or microangiopathic), iron-deficiency, chronic renal failure, drug-induced anaemia, myelodysplasia, myelofibrosis, aplastic anaemia. It is commonly the consequence of the suppression of erythropoiesis because of the chronic inflammatory state. Hemolytic anaemia occurs in over 10% of patients; to note that SLE patients can have a positive Coombs test without having haemolysis. Leukopenia is very common in SLE, it can be a sign of onset of the disease and usually correlates with disease activity. A count of leukocytes (GB <4500 / mm3) has been reported in 30-40% of cases, especially in the presence of active disease. Severe leucopenia (neutrophils <500mm3) is a fairly rare event. Lymphopenia (lymphocytes <1500 / mm3) occurs in 20% of SLE patients. A mild thrombocytopenia (100000-150000 / mm3) has been reported in about 25-50% of patients; instead a platelet count <50,000 / mm3 in only in 10% of patients. The cause of thrombocytopenia in due to an immune-platelet destruction, but a reduced platelet count can occur for microangiopathic haemolysis or for splenic sequestration (74).

5.9 Gastrointestinal and liver involvement.

Gastrointestinal tract manifestations (GI) has been reported in 25-40% of SLE patients and may represent either an intestinal involvement of the disease or a side effect of certain medications. Dyspepsia is reported in 11-50% of cases and peptic ulcer disease (gastric usually) in 4-21% (75).

Abdominal pain accompanied by nausea and vomiting occurs in about 30% of cases. Special care should be made in case of peritonitis, mesenteric vasculitis

with intestinal infarction, pancreatitis and inflammatory bowel diseases (IBD). The symptoms are usually very insidious, since they can be intermittent for several months prior to evolving into an acute abdomen. Vasculitis usually affects the small arteries, thus angiography may be negative.
Liver involvement is more common than has been believed in the past. Hepatomegaly can be observed in 12-25% of patients. Steatosis is a common finding and can be derived from a primitive or involvement of the disease or secondary to steroid medication. The term "lupus hepatitis" was coined to describe a form of autoimmune hepatitis. ASMA (anti-smooth muscle antibodies) and anti-mitochondrial antibodies (AMA) are infrequent in SLE (≤30%) and if present, they have a low titer. The histology of hepatitis associated with SLE rarely found periportal hepatitis with segmental necrosis, typical of autoimmune hepatitis. Also liver enzymes tend to be lower in SLE autoimmune hepatitis. The ascites is uncommon in SLE and, when found, must be excluded by paracentesis, the hypothesis of an infection or perforation. Other possible causes of ascites are congestive heart failure, and nephrotic syndrome (75).
The enteropathy with loss of protein has been described in different patients and can occur as the first manifestation of the disease. The clinical picture of this enteropathy is characterized by the presence of a deep hypoalbuminemia and oedema.

6. Laboratory findings

A positive ANA is perhaps the most important finding to establish if initially there is a spy of autoimmunity. However, a positive ANA is non-specific and can be found in 5-20% of the normal population. Anti-Sm antibodies and antibodies to double-stranded DNA (dsDNA) are diagnostic and are found with a frequency of 30-40% and 40-60% of SLE patients (76).
Antiphospholipid antibodies may also be found in lupus (50%) and can cause venous and arterial thromboses, as well as recurrent foetal loss. They include antibodies to cardiolipin or to beta-2 glycoprotein 1, or the presence of a lupus anticoagulant. Anti-SSA/Ro and anti-SSB/La are associated with secondary Sjögren’s syndrome, subacute cutaneous lupus erythematosus, neonatal lupus, and photosensitivity (76).
The dosage of complement (C3, C4, or total haemolytic complement CH50) may be helpful in the diagnosis of lupus, as well as in the routine monitoring of SLE patients. However, hypocomplementemia is not specific to SLE and can be found in any disease in which there is a large antigen-antibody production (77).

7. Classification criteria

Criteria for SLE classification were developed in 1971, revised in 1982, and revised again in 1997 (Table 2). These criteria are not diagnostic and allow for classifying a patient as having SLE only if at least 4 among the clinical or serological manifestations listed in table are present simultaneously or in succession (78).

In 2012 the Systemic Lupus International Collaborating Clinics (SLICC), an international group dedicated to SLE clinical research, realized a revision of the SLE classification criteria to address multiple concerns that had arisen since the 1982 criteria were developed. Concerns about the clinical criteria in the ACR classification were related to the possible duplication of cutaneous lupus terms (such as malar rash and photosensitivity) and the absence of many other lupus cutaneous manifestations; omission of many SLE neurologic manifestations; the need to utilize new standards in the quantification of urine protein. Concerns about the immunologic criterion included the omission of low complement, and the need to include new knowledge on antiphospholipid antibodies (79).

The new SLICC clinical criteria have introduced several innovations (Table 3). Malar rash and photosensitivity were grouped in one cutaneous criterion including both acute and subacute cutaneous lupus, whereas a separate cutaneous criterion including discoid rash and other different types of chronic cutaneous lupus was added. The arthritis criterion has been substantially redefined. The renal criterion was updated including measurement of proteinuria by the urine protein/creatinine ratio without the requirement of a time frame for collection, recognizing, however, the urine protein/creatinine ratio done on a 24-hour urine collection as “a gold standard”. The neurologic criterion has been substantially re-written to include a greater number of SLE neurologic manifestations than the original ACR definition of seizures or psychosis. The hematologic criteria have been split into three parts: hemolytic anemia, leukopenia/ lymphopenia and thrombocytopenia.

Therefore, to simplify assessment, the SLICC criteria require only one abnormal assessment. Many improvements have been made in the immunologic criteria to reflect new knowledge about serologic tests in SLE. The ANA criterion remains unchanged. In the old immunologic criterion, anti-dsDNA antibodies, antiSm antibodies, lupus anticoagulant, false-positive test for syphilis, and anticardiolipin antibodies were combined. The new SLICC classification criteria have split these

features into separate criteria, so that each may contribute to classification. Low complement (defined by C3, C4 or total hemolytic complement) was added reflecting thecontribution of complement to disease pathogenesis and also the direct Coombs test was included.

Finally the biopsy confirmed nephritis compatible with SLE according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 Classification of Lupus Nephritis (68) in the presence of ANA or anti-dsDNA antibodies was deemed sufficient criterion to identify a case of lupus. The SLICC classification criteria performs better than the revised ACR criteria in terms of sensitivity, but not specificity. These criteria are meant to be clinically more relevant, allowing the inclusion of more patients with clinically-defined lupus than using ACR criteria.

Table 2 1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of Systemic Lupus Erythematosus

Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring occurs in older lesions
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterised by tenderness, swelling or effusion
Serositis	a. Pleuritis: convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion or b. Pericarditis: documented by ECG or rub or evidence of pericardial effusion
Renal disorder	a. Persistent proteinuria >0.5 g per day or >3+ if quantitation is not performed or b. Cellular casts: may be red cell, haemoglobin, granular tubular, or mixed
Neurological disorder	a. Seizures: in the absence of off ending drugs or known metabolic derangements (eg, uraemia, acidosis, or electrolyte imbalance) or b. Psychosis: in the absence of off ending drugs or known metabolic derangements (eg, uraemia, acidosis, or electrolyte imbalance)
Haematologic disorder	a. Haemolytic anaemia with reticulocytosis, or b. Leukopenia: <4000/mm3, or c. Lymphopenia: <1500/mm3, or d. Thrombocytopenia: <100 000/mm3 in the absence of off ending drugs
Immunologic disorder	a. Anti-DNA: antibody to native DNA in abnormal titre, or b. Anti-Sm: presence of antibody to Sm nuclear antigen, or c. Positive finding of antiphospholipid antibodies based on: (1) an abnormal serum concentration of IgG or IgM anticardiolipin antibodies, (2) a positive test result for lupus anticoagulant using a standard method, or (3) a false positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilisation or fluorescent treponemal antibody absorption test
Antinuclear antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with 'drug-induced lupus' syndrome

Table 3 Clinical and Immunologic Criteria Used in the SLICC Classification Criteria

CLINICAL CRITERIA

(1) Acute Cutaneous Lupus OR Subacute Cutaneous Lupus

Acute cutaneous lupus: lupus malar rash (do not count if malar discoid), bullous lupus, toxic epidermal necrolysis variant of SLE, maculopapular lupus rash, photosensitive lupus rash (in the absence of dermatomyositis)

Subacute cutaneous lupus: nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with postinflammatory dyspigmentation or telangiectasias)

(2) Chronic Cutaneous Lupus

Classic discoid rash localized (above the neck) or generalized (above and below the neck), hypertrophic (verrucous) lupus, lupus panniculitis (profundus), mucosal lupus, lupus erythematosus tumidus, chillblains lupus, discoid lupus/lichen planus overlap

(3) Oral Ulcers OR Nasal Ulcers

Oral: palate, buccal, tongue

Nasal ulcers

In the absence of other causes, such as vasculitis, Behcet’s disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis, and acidic foods

(4) Nonscarring alopecia

Diffuse thinning or hair fragility with visible broken hairs, in the absence of other causes such as alopecia areata, drugs, iron deficiency, and androgenic alopecia

(5) Synovitis involving 2 or more joints

Characterized by swelling or effusion

OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness

(6) Serositis

Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub

Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day OR pericardial effusion OR pericardial rub OR pericarditis by electrocardiography

In the absence of other causes, such as infection, uremia, and Dressler’s pericarditis

(7) Renal

Urine protein–to-creatinine ratio (or 24-hour urine protein) representing 500 mg protein/24 hours OR red blood cell casts

(8) Neurologic

Seizures, psychosis, mononeuritis multiplex(in the absence of other known causes such as primary vasculitis), myelitis, peripheral or cranial neuropathy (in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus), acute confusional state (in the absence of other causes, including toxic/metabolic, uremia, drugs)

(9) Hemolytic anemia

(10) Leukopenia (<4000/mm3) OR Lymphopenia (<1000/mm3)

Leucopenia at least once: In the absence of other known causes such as Felty’s syndrome, drugs, and portal hypertension.

Lymphopenia at least once: in the absence of other known causes such as corticosteroids, drugs, and infection

(11) Thrombocytopenia (<100,000/mm3)

At least once in the absence of other known causes such as drugs, portal hypertension, and thrombotic thrombocytopenic purpura

IMMUNOLOGIC CRITERIA

(1) ANA level above laboratory reference range

(2) Anti-dsDNA antibody level above laboratory reference range (or 2-fold the reference range if tested by ELISA)

(3) Anti-Sm: presence of antibody to Sm nuclear antigen

(4) Antiphospholipid antibody positivity, as determined by

Positive test for lupus anticoagulant

False-positive test result for rapid plasma reagin

Medium- or high-titer anticardiolipin antibody level (IgA, IgG, or IgM)

Positive test result for anti–2-glycoprotein I (IgA, IgG, or IgM)

(5) Low complement (C3, C4, or CH50)

(6) Direct Coombs' test (in the absence of hemolytic anaemia)

8. Clinimetrics of SLE

8.1 Disease activity

Lupus activity represents the sum of all abnormalities due to ongoing immune inflammatory pathways involved in SLE, which are mostly reversible. Lupus activity may be distinguished into clinical and serological disease activity. The assessment of disease activity in lupus is based on the application standardized indices. Different instruments to measure disease activity have been elaborated and validated for SLE since the mid 80ies (80).

The SLEDAI is perhaps the easiest assessment tool to use and includes twenty-four features that are attributed to lupus (81). The more serious manifestations (such as renal, neurologic, and vasculitis) are weighed more than others (such as cutaneous manifestations). The maximum possible score is 105. The index was revised in 2000 to reflect persistent, active disease in those descriptors that had previously only considered new or recurrent occurrences (SLEDAI-2000 index, **Appendix 1**) including persistent proteinuria, rash, alopecia or mucocutaneous lesions (82).

8.2 Patterns of SLE disease activity

Variations in disease activity over time are commonly recognized in SLE regardless of therapy. Longitudinal studies identified four major disease activity patterns in SLE calculating by excluding serology: clinically quiescent disease (CQD; SLEDAI-2K= 0 in three annual visits); CAD (SLEDAI-2K ≥ 2 in at least two out of three annual visits); RRD (SLEDAI-2K ≥ 2 in one out of three annual visits); minimal persistent disease activity (MDA), being defined as SLEDAI-2K = 1 in one or more annual visits (80).

8.3 Damage

In 1996, a damage index for SLE was developed by the Systemic Lupus International Collaborating Clinics (SLICC) and endorsed by the ACR: the SLICC/ACR Damage Index (**Appendix 2**).

It is the best instrument to measure organ damage after the diagnosis of lupus. It covers 12 systems assessed by 41 items for damage. This is defined as a non-reversible change that is not related to active inflammation and that has occurred since the onset of lupus and ascertained by clinical assessment and present for at least 6 months. If evidence of damage is noted for a particular item, it is given a score of 1. Some items may score 2 points if they occur more than once, so that the maximum possible score is 47. Scores can only increase with time, but scores rarely reach over 12 (83). Higher early damage index scores have been associated with a poor prognosis and with increased mortality (84). It is an important outcome measure and it is usually completed yearly.

9. Hints of treatment

Different drugs are currently used in treatment of SLE patients: non-steroidal drugs (NSAIDs), antimalarials, glucocorticoids, immunosuppressants (e.g cyclosporine, cyclophosphamide, mycophenolate mofetil).

Glucocorticoids: The broad, generally suppressive effects of corticosteroids on the immune response render them useful for the management of most organ system manifestations of lupus (85). Their administration in rapid depletion of circulating T-cells due to a combination of effects including induction of apoptosis, inhibition of T-cell growth factors, and impaired release of cells from lymphoid tissues. Corticosteroid effects on B-cell function and immunoglobulin production are more delayed. Long-term treatment with glucocorticoids leads to a number of well-known side effects, such as dyslipidemia, diabetes, hypertension, accelerated atherosclerosis, skin atrophy, susceptibility to infection and osteoporosis (85).

Hydroxychloroquine and anti-malarial constitute a valid therapeutic solution with limited toxicity. This drug works by inhibiting the function of Toll-like-Receptors (TLR), which contributes to maintaining autoimmunity. Anti-malarials are drugs widely used for their anti-inflammatory properties and their role as steroid-sparing agents; these drugs reduce serum lipids and the number of disease flares. The use of hydroxychloroquine is associated with a lower damage.

Cytotoxic / immunosuppressive agents. More severe manifestations of the disease require treatment with immunosuppressive agents that include cyclophosphamide, azathioprine, mycophenolate mofetil, and methotrexate. The majority of data with these agents are in the area of SLE nephritis. Some of these agents have also shown promise in neuropsychiatric symptoms as well as in severe cutaneous manifestations of lupus (86).

The intravenous route for administration of cyclophosphamide is generally preferred due to fewer side effects compared with oral administration. Intravenous administration can be given with mercapto-ethanesulphonic acid to decrease the risk of bladder damage that occurs with cyclophosphamide administration. Cyclophosphamide is a drug usually used in cycles and according to well-defined schemes. Cycles of cyclophosphamide (low-dose parenteral infusions every month or twice a month) are effective in the treatment of lupus nephritis. Possible side effects are bone marrow aplasia, increased susceptibility to infections and gonadal failure.

Mycophenolate mofetil (MMF) is a semi-synthetic derivative of mycophenolic acid. This acid is a reversible inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH). The inhibition of this enzyme results in a blocking of purine synthesis and therefore of the DNA, with arrest of cell proliferation. Mycophenolate mofetil is therefore a viable therapeutic alternative with few side effects; the long-term effects of this therapy on the preservation of renal function are still not proven.

Cyclosporine (CYA) acts by inhibiting the proliferation of T-cells, especially the helper T, preventing transcription of DNA in activated T cells and the production of messenger RNA for the synthesis of various cytokines including IL-2.

Methotrexate (MTX) is effective in reducing SLE disease activity and confers a steroid-sparing effect. MTX appears to be most useful in patients with active arthritis or cutaneous manifestations (87).

Nowadays the only monoclonal antibody approved in the treatment of SLE is represented by Belimumab. This drug consists of a monoclonal antibody that targets the Blys signaling pathway. The blocking of BLyS with the antibody anti-BLyS improved the clinical status significantly within the first year of treatment in patients with mild to moderate SLE, moreover Belimubab inhibits production of autoantibodies and reduces disease activity (88).

Of the above therapeutic strategies unfortunately none have proven to be capable to resolve the pathology of SLE in a definitive way. The choice of the type of medication is mainly determined on the basis of the severity of the disease and the organ involved functionality. The continuous development that is being presented in terms of biological and immunological therapy could open new scenarios in the approach to the patient.

Adenosine and adenosine receptors: expression and function

Adenosine is an endogenous nucleoside identified as a major local regulator of tissue function released from almost all cell types. It is produced primarily from the metabolism of adenosine triphosphate (ATP) and exerts pleiotropic functions. Intracellular adenosine is produced from its immediate precursor, 5'-adenosine monophosphate (5'-AMP), by the action of the enzyme 5'-nucleotidase. Adenosine can then follow several metabolic/synthetic pathways. It can be metabolized to inosine and hypoxanthine by adenosine deaminase and to uric acid by xanthine oxidase. Adenosine can also be transported out of the cell to the extracellular space by specific bi-directional nucleoside transporters (89).

Adenosine can also be converted back to 5'-AMP by the action of the enzyme adenosine kinase and subsequently to ADP and ATP. (90).

Adenosine accumulates in the extracellular space in response to metabolic stress and cellular damage (91). As it is a key metabolite, there is always a finite intracellular concentration of adenosine and a finite level of adenosine in the extracellular space, even under the most basal conditions and this is in the range of 30-200 nM (92). From this baseline level adenosine can increase substantially via several mechanisms: 1) formation intracellularly and export via transporters, and 2) formation in the extracellular space from adenine nucleotides released from cells. Adenosine is formed intracellularly whenever there is a discrepancy between the rates of ATP synthesis and ATP utilization; e.g. when work load is markedly enhanced or when the supply of oxygen and glucose is limiting as in ischemia.

Elevations in extracellular adenosine are found in conditions of ischaemia, hypoxia, trauma asthma, neurodegenerative disorders, chronic inflammatory diseases and cancer (93)(94)(95). Adenosine effects are widespread and pleiotropic.

Adenosine mediates its effects through activation of a family of four G-protein-coupled adenosine receptors (ARs), named A_1, A_{2A}AR, A_{2B}, and A_3. These receptors differ in their affinity for adenosine, in the type of G proteins that they recruit, and finally in the downstream signalling pathways that are activated in the target cells. (96) (97). These receptors are seven transmembrane glycoproteins widely distributed throughout the body. The cellular response to adenosine is dependent on the adenosine concentration at the cell surface and several other

factors, such as receptor density and its functionality, are key determinants in generating the effect of adenosine on the cell.

Traditionally, adenosine receptor signalling is thought to occur through the inhibition or stimulation of adenylyl cyclase with a concomitant decrease or increase in intracellular cyclic AMP concentrations. Based on their ability to decrease or increase cAMP accumulation, adenosine receptors were initially classified as A_1 or A_2 receptors, respectively (98). Subsequent studies have refined the classification of adenosine receptors and cAMP-increasing A_2 receptors have been divided into two groups: high-affinity A_{2A} receptors and low-affinity A_{2B} receptors (99). More recently a fourth type of receptor has been identified, the A_3 receptors acting by decreasing cAMP concentrations (100).

This early picture of adenosine receptor signalling through the adenylyl cyclise-cAMP system has been substantially expanded, and it is now established that adenosine receptors can be linked to various other pathways.

1. A_1 adenosine receptor

A_1 receptor activation had traditionally been linked to Gi-mediated inhibition of adenylyl cyclase (101). The primary changes in second messengers associated with A_1 adenosine receptor activation are decreased cAMP production or increased calcium levels, depending on the effector system (102).

A_1 adenosine receptors are widely distributed in the CNS and in peripheral tissues. Adenosine, by A_1 adenosine receptors activation, produces inhibition of neurotransmitter release and induces neuronal hyperpolarization mediating sedative, anticonvulsant, anxiolitic and locomotor depressant effects (93). Literature evidence has indicated the involvement of A_1 adenosine receptors in controlling pain transmission, producing antinociceptive effects in various animal models (103). In the cardiovascular system, A_1 adenosine receptors mediate negative chronotropic, dromotropic and ionotropic effects, suggesting the potential use of A_1 adenosine receptors agonists as cardioprotective agents and in the treatment of arrhythmias and atrial fibrillation (104). The role of adenosine in regulating the respiratory system is well known and A_1 adenosine receptors antagonists could also be used in asthma and in COPD since adenosine induces acute bronchoconstriction via stimulation of A_1 adenosine receptors (105).

A_1 adenosine receptors play an anti-inflammatory effect on osteoclast function exerting a stimulatory effect on these cells. The antagonist of this receptor may be able to prevent bone loss associated with inflammatory diseases (106).

2. A_{2A} Adenosine Receptors

A lot of evidence indicates that A_{2A} adenosine receptors represent the major immunoregulatory arm of the adenosine system, and there is also general agreement that this receptor promotes marked anti-inflammatory activities in specific cells and various models of inflammation (107). The A_{2A} adenosine receptor gene consists of multiple exons, the expression of which is driven by at least four independent promoters. Interestingly A_{2A} receptor gene expression is highly responsive to alterations in the extracellular environment and A_{2A} receptor expression is sensitive to changes in the concentrations of exogenous and endogenous factors involved in inflammation. A_{2A} receptors exert their effect through activation of adenylate cyclase, generation of intracellular cAMP, and activation of protein kinase A (PKA), which can phosphorylate and thereby activate the transcription factor CREB (nuclear protein that binds to cAMP-response element-binding protein) directly affecting gene expression by direct binding to gene promoters or indirectly, by competing with NF-κB for an important cofactor (CREB-binding protein) (108). The predominant anti-inflammatory effect of A_{2A} receptor activation is a result of the expression of A_{2A} receptors on monocytes/macrophages, dendritic cells, mast cells, neutrophils, endothelial cells, eosinophils, epithelial cells, as well as lymphocytes, NK cells. A_{2A} receptor activation inhibits early and late events occurring during an immune response, which include antigen presentation, costimulation, immune cell trafficking, immune cell proliferation, proinflammatory cytokine production, and cytotoxicity. (109). This aspect is discussed in the section dedicated to adenosine and inflammation. Adenosine has been reported to reduce inflammation in several in vivo models. In synoviocytes obtained from osteoarthritis patients, the activation of A_{2A}AR inhibited NF-κB pathways, as well as the production of TNF-α and IL-8 (110). In preclinical studies of murine collagen induced arthritis A_{2A} receptor stimulation reduced the plasma levels of several proinflammatory cytokines, such as TNF, IL-1β and IL-6 (111).

Several preclinical studies have shown the involvement of A_{2A} receptors in several kidney disorders. Activation of the A_{2A} adenosine receptors during reperfusion of various tissues has been found to markedly reduce ischemia-reperfusion injury. In particular, in a model of ischemia-reperfusion kidney injury, A_{2A}AR stimulation with the selective agonist was effective in decreasing kidney damage as well as in

counteracting the progression of fibrosis occurring in presence of chronic kidney inflammation (112).

Another area where A_{2A}ARs signalling has received attention as a potential therapeutic target is the GI tract. Studies have highlighted the protective effects of A_{2A} receptor activation in various animal models of colitis, and these protective effects can be ascribed to two major mechanisms: decrease of inflammatory-cell infiltration and function in the mucosa, and increased activity of Treg cells (113). In murine model of intestinal inflammation activation of A_{2A} receptors suppressed pro-inflammatory cytokines and induced IL-10 and TGF-beta, moreover the pharmacological stimulation of A_{2A} receptors was associated with a beneficial effect in a mouse model of toxin-A-induced enteritis (114).

Adenosine levels are increased in the lungs of individuals with asthma or COPD, and ARs are known to be expressed on most inflammatory and stromal cell types involved in the pathogenesis of these diseases (115).In addition, pharmacological treatment of allergic rats with an A_{2A}AR agonist resulted in diminished pulmonary inflammation (116). A study performed in peripheral lung parenchyma demonstrated that affinity and/or density of adenosine receptors are altered in patients with COPD compared with control smokers with normal lung function. Moreover, there was a significant correlation between the density and affinity of adenosine receptors and the forced expiratory volume/forced vital capacity ratio, an established index of airflow obstruction. In particular A_{2A}, as well as A_3ARs, was found to be upregulated in COPD patients (117). This alteration may represent a compensatory response mechanism and may contribute to the anti-inflammatory effects mediated by the stimulation of these receptors. Given the central role of inflammation in asthma and COPD, substantial preclinical research targeted at understanding the function of A_{2A}AR in models of airway inflammation has been performed (118).

Activation of the A_{2A}ARs during reperfusion of various tissues has been found to markedly reduce ischemia-reperfusion injury. Hypoxia-induced accumulation of adenosine may represent one of the most fundamental and immediate tissue-protecting mechanisms in which oxygen deprivation and extracellular adenosine accumulation serve as 'reporters', while A_{2A}As serve as 'sensors' of excessive tissue damage (119). In ischemic brain damage the pharmacological or genetic blockade of A_{2A} receptors reduced the infarcted area and the neurological score after ischemic insult (120). The administration of a selective A_{2A} receptor agonist

(CGS21680) to rats four hours after ischemia protected from neurological deficits through a reduction of microgliosis, astrogliosis and maintenance of cytoarchitecture in the ischemic cortex and striatum (121).

It has also been demonstrated that A_{2A} receptors play an important role in the promotion of wound healing and angiogenesis. A_{2A} and A_{2B} receptors stimulation increases angiogenesis through direct stimulation of microvascular endothelial cell-proliferation as well as increasing autocrine production of vascular endothelial growth factor (VEGF) and inhibiting the production of thrombospondin 1, an anti angiogenic matrix protein (122) (123) (124).

3. A_{2B} adenosine receptors

The A_{2B} ARs are linked with Gs protein and stimulate the activity of the adenylate cyclase mediating an increase of cAMP accumulation. Moreover, A_{2B} ARs has been shown to be coupled with Gq (a G protein that stimulates the activity of enzymes such as phospholipase C) thereby regulating intracellular calcium levels (56).

The role of A_{2B}ARs has been clarified in several diseases. A_{2B} receptor activation on human lung fibroblasts promotes their differentiation into myofibroblasts that are capable of overproducing extracellular matrix suggesting their role in fibrosis and remodelling of the lung during asthma (125). In mice model of asthma, A_{2B} receptors have been identified as the main contributors for the activation of mast cell (126). In addition to mast cells, pro-inflammatory effects of A_{2B} stimulation have also been observed with human smooth-muscle cells and human bronchial epithelia cells which respond to adenosine by increased release of IL-6 (127). On intestinal epithelial cells A_{2B} receptors increase IL-6 production with a consequent neutrophil activation. In epithelial cells isolated from human or mouse colitis A_{2B} receptors are overexpressed and the administration of a selective A_{2B} antagonist to mice suffering from colitis markedly decrease IL-6 production, neutrophil infiltration and reduce the extent of mucosal damage ameliorating the course of the disease (128).

4. A_3 Adenosine Receptors

A_3ARs are coupled to Gi and Gq proteins. Activation of A_3ARs results in an inhibition of adenylate cyclase or stimulation of phospholypase C and D (129).
The anti-inflammatory effect of adenosine is also mediated by the activation of A_3ARs that are present in immune cells and involved in the physiopathologic regulation of inflammatory and immune processes. Several results from in vitro and in vivo studies suggest that the activation of the A_3ARs can be both pro- or anti-inflammatory depending on the cell type examined or on the animal species considered. Binding and functional studies have shown that human neutrophils expressed A_3ARs primarily coupled to the inhibition of adenylate cyclase and calcium signalling, mediating the inhibition, are responsible for the inhibition of superoxide production and chemotaxis of mouse bone marrow neutrophils (130). A_3ARs directly control histamine release by antigen-stimulated mouse mast cells and the stimulatory effect of exogenous adenosine noted in wild-type mast cells is not observed in A_3AR-knockout mast cells (131). Literature data support a role for adenosine in dictating dendritic cell function, promoting the recruitment of immature dendritic cells to sites of inflammation and injury via A_3AR (132). The possibility that A_3ARs plays a role in the development of cancer has aroused considerable interest in recent years. In particular, A_3ARs were found to be highly expressed in tumour cells and tissues but not in normal cells or adjacent tissue. Interestingly, high A_3AR expression levels were found in peripheral blood mononuclear cells derived from tumour-bearing animals and cancer patients, reflecting receptor status in the tumours (133).
Considerable interest has been given to the study of A_3 receptors in arthritis. Experiments on knockout animals have shown evidence that adenosine acting A_3ARs mediates the anti-inflammatory effects of low-dose Methotrexate (MTX). In adjuvant-induced arthritis in rats and in peripheral blood mononuclear cells from Rheumatoid Arthritis (RA) patients, MTX treatment has been shown to enhance the anti-inflammatory effects of typical A_3AR agonists via an upregulation of A_3AR expression (134). In RA patients, the overexpression of A_3ARs has been directly correlated with high levels of pro-inflammatory cytokines acting via upregulation of NF-κB (135). The anti-inflammatory effect of A_3AR was also shown in fibroblast-like synoviocytes derived from synovial fluid of RA patients (136). In particular, the effect of a novel A_3AR agonist (CF502) induces a dose-dependent inhibitory effect

on the proliferation of fibroblast-like synoviocytes via deregulation of the NF-kB signalling pathway and suppresses the clinical and pathological manifestations of adjuvant-induced arthritis in a rat experimental model. Other data have shown that the use of $A_{2A}AR$ and A_3AR agonists significantly reduces NF-κB levels and inhibits IL-1β, IL-6 and TNF-α release in mononuclear cells from peripheral blood samples of RA patients, suggesting the involvement of these ARs in the modulation of inflammatory response (134). It has also been found that the production of metalloproteinase (MMP) 1 and 3 was inhibited by $A_{2A}AR$ or A_3AR agonists in RA patients more than in healthy controls, demonstrating the direct involvement of the adenosine receptor subtypes in the mechanism regulating joint damage in RA (137)

5. Adenosine and immune cells

Adenosine is implicated in the regulation of the innate and adaptive immune systems through every cell type involved in immune/inflammatory response and ARs are able to mediate the majority of anti-inflammatory effects of endogenous adenosine (134). In particular, the ability of $A_{2A}AR$ activation to suppress cytokine and chemokine expression by immune cells is probably the dominant mechanism involved (138). In neutrophils, adenosine, acting at $A_{2A}AR$, regulates the production of different cytokines. In mice lacking the A_{2A} receptor, after LPS stimulation, granulocytes expressed higher mRNA levels of TNF-alpha, MIP-1alpha, and MIP-1beta than neutrophils from wild-type mice (139). Moreover adenosine modulates the phagocytic activity of neutrophils as well as the generation of reactive oxygen species (ROS) developing a stimulatory or inhibitory effect on these different neutrophil functions through the stimulation of A_1 receptors or A_{2A} and A_3 receptors, respectively (140). In inflammatory processes involving the endothelium, neutrophils are recruited to inflammatory sites by the post-capillary venular endothelium. Adenosine inhibits the adhesion of neutrophils, via A_{2A} receptors, decreasing the expression of their adhesion molecules (139). By contrast, A_1 receptors promote neutrophil adhesion to different adhesive molecules on the endothelium and A_1 and A_3 receptors promote directed migration of neutrophils (141).

A variable expression of adenosine receptors has been observed on monocytes and macrophages based in part on their maturation phase. A_1, A_{2A} and A_3 receptors are scarcely expressed on quiescent monocytes and increase their density during the differentiation of monocytes into macrophages (142). The activation of A_{2A}, A_{2B} and A_3 receptors decreases the production of several pro-inflammatory cytokines TNF-α, interleukin IL-6, IL-12, macrophage inflammatory protein (MIP)-1α and increases the production of the anti-inflammatory cytokine IL-10 favouring the expression of an anti-inflammatory phenotype on monocytes and macrophages (143).

Adenosine modulate dendritic cell functions and also in this case the modulation depends on the expression of different types of adenosine receptors related to the maturation status of the cells. Immature human dendritic cells express mainly A_1 and A_3 receptors while dendritic cell maturation is associated with the increase of

A_{2A}AR-mediated signalling responses, culminating in a decrease in pro-inflammatory cytokine release (144).

A_{2A} receptors are the dominant players in dictating lymphocyte responses. Studies using A_{2A}-knockout models have shown that A_{2A}AR activation inhibits IL-2 secretion by naive CD4+ T cells thereby reducing their proliferation, confirming the immunosuppressive effects of A_{2A}AR stimulation (113). A_{2A} receptor inhibits IL-2 secretion by naïve CD4+ T cells reducing their proliferation. Moreover A_{2A} receptor activation suppresses IL-4 and IFN-γ secretion from naive CD4+ cells (145), whereas human and murine CD4+-stimulated lymphocytes and CD8+ cells express more A_3ARs. Moreover A_{2A}ARs are found on Natural Killer (NK) T cells, a subset of important cells for adaptative immunity that triggers fast host response to infection. Whereas the strongly activated NKT cells play a beneficial role in combating environmental pathogens, they could play a deleterious role in autoimmunity by producing disease-promoting cytokines. A_{2A}ARs are found on NK cells and strongly suppress the release of pro-inflammatory cytokines (146) (147).

Figure 1. Schematic representation of the main actions mediated by adenosine receptors in human immune cells. Abbreviations: IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; ↑, increases; ↓, decreases.

Neutrophils		Macrophages		Dendritic cells		Lymphocytes	
+ A_{2A}AR + A_{2B}R + A_3R	+ A_1R	+ A_{2A}AR + A_{2B}R + A_3R	+ A_{2A}AR + A_{2B}AR	+ A_{2A}AR + A_3R	+ A_1R + A3R	+ A_{2A}AR + A_{2B}R + A_3R	+ A_3R
↓ adhesion to endothelial cells ↓ superoxide anion production	↑ expression of adhesion molecules	↓TNF, IL-6, IL-12, MIP-1α release↓ iNOS expression	↑ IL-10		↑ chemotaxis and maturation of dendritic cells	↓ IL-2 and IFN-γ	↓ T cell proliferatio n

Pharmacological role of A_{2A} adenosine receptors in Systemic Lupus Erythematosus, clinical and laboratory correlations

1. Aim of the study

Systemic lupus erythematosus (SLE) is the prototypic multisystem autoimmune disorder with a broad spectrum of clinical presentations encompassing almost all organs and tissues (3). The irreversible break in immunological tolerance is manifested by immune responses against endogenous nuclear antigens and the subsequent formation of autoantibodies and immune complexes. SLE has classically been considered an autoimmune disease with a predominant adaptive immune system component, since T and B cells have been considered the most important pathogenetic players (4).

During the early inflammatory phase plasmacytoid dendritic cells (pDCs) are able to internalize nucleic acids containing interferogenic immune complexes (ICs) that reach the endosomes and stimulate Toll-like receptors (TLR) 7 or 9, leading to interferon (IFN)-α gene transcription (13)(148)(14). IFN-α contributes to the maturation of myeloid DCs that can activate autoreactive T cells through antigen presentation and costimulation. This favours the development of T helper 1 cells responsible for high-level production of proinflammatory cytokines (15)(149)(8) and enhances B cell maturation and differentiation, antibody production, and IC formation. In SLE the IC and IFN-α secreting monocytes modulate interleukin (IL)-10 function (150). The capability of IL-10 to suppress production of inflammatory cytokines such as tumour necrosis factor (TNF)-α and IL-6, implicated in promoting autoimmunity and tissue inflammation in SLE, is attenuated(150).

Growing evidence emphasizes that the purine nucleoside adenosine plays an active role as a local regulator of inflammation in different pathologies. Adenosine is an ubiquitous nucleoside involved in various physiological and pathological functions by stimulating the G protein-coupled A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (ARs) (151) (152) (153) (101). The role of ARs is well known in physiological conditions and in a variety of pathologies including inflammatory damage, neurodegenerative disorders and cancer (154)(109)(155). In particular, A_{2A}AR stimulation mediates inhibition of TNF-α, IL-1β, IL-2, IL-6, IFN-α (156)(157)(158) and increases the production of the anti-inflammatory cytokine IL-10 (159).

From this background, the aim of the present study was to explore arrangement and functionality of ARs in SLE and to evaluate their relationship with clinical phenotype and disease activity.

2. Methods

2.1 Patient Study Design

SLE patients, regularly attending our Lupus clinic satisfying the 1997 revised American College of Rheumatology criteria (160), were consecutively recruited from the Rheumatology Unit, S. Anna Hospital, University of Ferrara, Italy. Clinical, demographic, serological data as well as data regarding therapy, including corticosteroids (measured as prednisone equivalent), antimalarials and immunosuppressants were recorded.

Disease activity routinely assessed by SLE disease activity index-2000 (SLEDAI-2K) (82) and cumulative damage assessed by the Systemic Lupus International Collaborating Clinics (SLICC) index were extracted by retrieving information from clinical records and dedicated database. Moreover disease activity and progress through time was considered according to 4 different patterns defined using SLEDAI-2K excluding serological descriptors (hypocomplementaemia and anti-dsDNA antibodies) in order to focus on clinical activity: chronic active disease (CAD), relapsing-remitting disease (RRD), clinical quiescent disease (CQD), minimal disease activity (MDA) (161).

Sero-immunologic tests included C3 and C4 dosage, antinuclear antibody (ANA), anti-dsDNA, anti-SSA (Ro), anti-SSB (La), anti-Sm, anti-RNP, anticardiolipin (aCL), anti-beta2 glycoprotein I (anti-beta2GPI), and lupus anticoagulant (LA). C3 and C4 (g/l) were measured by nephelometry and hypocomplementaemia was defined by local lab reference values (e.g. C3<0.8 and C4<0.11 g/l detected on at least two separated occasions); ANA were detected by indirect immunofluorescence using Hep2 cells as substrate (positivity was defined as a titre $\geq$ 1: 160). Anti-dsDNA were detected by indirect immunofluorescence using *Crithidia luciliae* with a cut-off titre of 1:40; (positivity was certified if positivity was checked in at least two separate *measurements).* Anti-SSA, anti-SSB, anti-Sm, and anti-RNP were detected by immunoblot technique. aCL antibodies and anti-beta2GPI were measured by enzyme-linked immunosorbent assay (ELISA) (162). LA was measured accordingly with the recommendation of the Scientific and Standardization Committee of the International Society of Thrombosis and Hemostasis). Positivity for aPL and LA was defined if confirmed in two separate measurements performed 12 weeks apart (163).

As control group, healthy subjects (n=80), matched for similar age and sex ratio, were volunteers from Ferrara University Hospital Blood Bank. The study was approved by the local Ethic Committee of the University Hospital of Ferrara (Italy) and informed written consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

2.2 Sample collection and human lymphocyte preparation

Lymphocytes were isolated and prepared as previously described from the peripheral blood of control subjects and SLE patients (137) (117) (164). Leukocytes were separated from erythrocytes with a solution of 6% Dextran T500 (Sigma-Aldrich, St Louis, MO, USA), suspended in Krebs-Ringer phosphate buffer and layered onto 10 ml of Fycoll-Hypaque (GE Healthcare, Little Chalfont, UK). To obtain membrane suspensions, cell fractions were centrifuged in hypotonic buffer at 20,000 x g for 10 min. The resulting pellet was incubated in Tris HCl 50 mM buffer pH 7.4 with 2 IU/ml adenosine deaminase (Sigma-Aldrich) for 30 min at 37°C. After a centrifugation at 40,000 x g for 10 min, the final pellet was used for radioligand binding assays. The protein concentration was determined by a Bio-Rad method with bovine albumin as reference standard (137).

2.3 Real-Time RT-PCR experiments

Total cytoplasmic RNA was obtained from human lymphocytes by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay (137)(117)(164)(165) of A_1, A_{2A}, A_{2B} and A_3ARs mRNAs was performed using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR for A_1, A_{2A}, A_{2B} and A_3ARs was carried out with the assays-on-demand TM Gene expression Products NM 000674, NM 000675, NM 000676 and NM 000677, respectively. For the real-time RT-PCR of the reference gene the endogenous control human β-actin was used, and the probe was fluorescently-labeled with VICTM (Applied Biosystems).

2.4 Western blotting analysis

Human lymphocytes were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing protease inhibitors and 1 mM sodium orthovanadate. Proteins were eluted in Laemmli buffer, resolved by SDS-PAGE and transferred to polyvinyliden fluoride membranes. Next, the membranes were incubated with specific antibodies for ARs (Alpha Diagnostic, Owings Mills, MD, USA) followed by washing and incubation with horseradish peroxidase-conjugated secondary antibodies. After stripping, the blots were reprobed with anti β-actin antibody (clone EPR1123Y, Millipore).

2.5 Saturation binding experiments to ARs

Since ARs mRNA and protein expression experiments in SLE patients have shown an upregulation of A_{2A}ARs compared to controls, saturation binding experiments to this receptor subtype were carried out. For these assays different concentrations of ^{3}H-ZM 241385 (0.01-30 nM) as radioligand and cell membranes (60 µg of protein/assay) were incubated for 60 min at 4°C (164). The radioligand ^{3}H-4-(2-(7-amino-2-(2-furyl)(1,2,4)-triazolo(2,3-a)(1,3,5) triazin-5-ylamino) ethyl) phenol (specific activity, 27 Ci/mmol) was purchased from Biotrend (Cologne, Germany). Non-specific binding was determined in the presence of ZM 241385 1 µM. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters by using a Brandel cell harvester (165). The filter bound radioactivity was counted in a 2810 TR liquid scintillation counter Packard (Perkin Elmer Life and Analytical Sciences, USA).

2.6 Pro- and anti-inflammatory cytokines release in cultured lymphocytes

Isolated lymphocytes from healthy subjects or SLE patients were suspended at a density of 10^6 cells/ml in RPMI 1640 medium supplemented with 2% fetal bovine serum (Euroclone, Milan, Italy) and seeded into 24-well plates. Lymphocytes were incubated for 24 h in the absence or in the presence of an A_{2A}AR agonist, CGS 21680 (100 nM and 1 µM). A selective A_{2A}AR antagonist SCH 442416 (1 µM) was also used to verify the specific involvement of these receptors in cytokine release. CGS 21680, (2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido

adenosine) was obtained from Sigma-Aldrich and SCH 442416 (2-(2-Furanyl)-7-(3- (4-methoxyphenyl) propyl)- 7H- pyrazolo (4,3-e) (1,2,4) triazolo (1,5-c)pyrimidin-5-amine) was purchased from Tocris (Bristol, UK). At the end of incubation, the cell suspension was collected and centrifuged at 1000 x g for 10 min at 4°C. IFN-α, TNF-α, IL-2, IL-6, IL-1β and IL-10 levels were determined with specific quantitative sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer instructions (137).

2.7 NF-kB activation in human cultured lymphocytes

Nuclear extracts from human cultured lymphocytes of the examined patients were obtained by using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) following the manufacturer instructions. The NF-kB subunit p65 activation was evaluated in lymphocyte nuclear extracts by using the TransAM NF-kB kit (Active Motif). The primary antibody against NF-kB recognized an epitope in the subunits that is accessible only when it is activated and bound to its DNA target. The reaction was developed with streptavidin-horseradish peroxidase and optical density was read by spectrophotometry at 450 nm wavelength (164).

2.8 Data and statistical analysis

Dissociation equilibrium constants for saturation binding, affinity or K_D values, as well as the maximum densities of specific binding sites, Bmax were calculated for a system of one or two-binding site populations by non-linear curve fitting (165). All experimental data are reported as mean ± SEM of independent experiments as indicated in Figure legends. Statistical analysis of the data was performed by Student's t-test or one way analysis of variance (ANOVA) followed by Dunnett's test. Analysis were carried out using GraphPad Prism 5.0 statistical software package and differences were considered statistically significant with a p value less than 0.01 (Graph Pad Software, San Diego, CA, USA).

3. Results

3.1 Clinical characteristics

A total of 80 SLE patients (71 women and 9 men) with a mean ± SD age of 44 ± 11.9 years, disease duration of 139 ± 100 months, SLEDAI 4 ± 4.3 were studied. In addition, 80 healthy subjects matched for age and sex ratio, were enrolled. Demographic, clinical and pharmacologic treatments of the study subjects are reported in Table 1.

3.2 A_{2A}AR mRNA and protein expression are upregulated in lymphocytes from SLE patients

Adenosine receptors mRNA and protein expression were evaluated in lymphocytes from SLE patients in comparison with healthy subjects by means of quantitative RT-PCR assay and Western blot analysis, respectively. Figure 1A reports the relative A_1, A_{2A}, A_{2B}, and A_3AR mRNA levels determined by RT-PCR in human lymphocytes from healthy subjects and SLE patients. Among these receptors, only A_{2A}AR mRNA expression had significantly increased in patients with SLE respect to control subjects. Western blotting and densitometric analysis indicated a significant increase in A_{2A}AR protein expression in lymphocytes from SLE patients compared with those from healthy subjects, whilst no differences were found in A_1, A_{2B}, and A_3ARs (Figure 1B, C).

3.3 Alteration of A_{2A}AR affinity and density in lymphocytes from SLE patients

Figure 2 (panels A and B) shows the saturation curves and Scatchard plots of [^{3}H]-ZM 241385 in human lymphocytes confirming the upregulation of A_{2A}ARs in SLE patients with respect to healthy subjects. The affinity of the radioligand [^{3}H]-ZM 241385 for A_{2A}AR (expressed as K_D, nM) had decreased in lymphocytes from SLE patients if compared with that of the control group. Interestingly, the A_{2A}AR density expressed as Bmax value has significantly increased in SLE patients with respect to healthy subjects reaching a 2.3-fold increment (Figure 2A, B).

3.4 Clinical correlations

An inverse correlation was found between $A_{2A}AR$ density expressed as B_{max} values in fmol/mg protein, SLEDAI measured at the time of blood sampling (Figure 2C) and disease activity through time evaluated accordingly to the course patterns (chronic quiescent disease versus chronic active disease; $p < 0.0001$). In addition, $A_{2A}ARs$ density inversely correlated with serositis (p 0.0043), hypocomplementemia (p 0.0005) and with anti-dsDNA positivity (Table 2). Regarding treatments, a modulation of the $A_{2A}AR$ density was found among corticosteroid users (p 0.0078). About $A_{2A}AR$ affinity only one significant correlation was found with anti-dsDNA positive patients (p 0.008, Table 2).

3.5 $A_{2A}AR$ activation reduce pro-inflammatory cytokine production from lymphocytes

To investigate the potential anti-inflammatory role of $A_{2A}AR$ stimulation in SLE, we evaluated the effect of CGS 21680 on the release of some of the most relevant proinflammatory cytokines involved in the pathogenesis of SLE such as IFN-α, TNF-α, IL-6, IL-1β and IL-2. In cultured lymphocytes from SLE patients a marked release of IFN-α was observed following the incubation of the cells with 0.1 mg/ml LPS for 24 h (figure 3A). Interestingly, CGS 21680 at the 100 nM and 1 μM was able to inhibit the LPS-induced IFN-α release both in lymphocytes from SLE patients and in lymphocytes from healthy subjects. However, the effect of CGS 21680 was significantly greater in lymphocytes obtained from SLE patients in comparison to healthy subject ($p<0.0001$), most likely due to the upregulation of $A_{2A}ARs$ (see online supplementary table S1). The inhibitory effect of the $A_{2A}AR$ agonist was counteracted by the selective antagonist SCH 442416 (1 μM) demonstrating the specific $A_{2A}AR$-mediated response (Figure 3A). Similar results were obtained evaluating the capability of CGS 21680 to inhibit the release of TNF-α induced by LPS (Figure 3B). Again, the inhibitory effect of the $A_{2A}AR$ agonist was more evident in lymphocytes from SLE patients than from healthy subjects ($p<0.0001$, see Table 3). The anti-inflammatory effect of $A_{2A}AR$ activation induced by CGS 21680 was also confirmed when analyzing the production of other different pro-inflammatory interleukins such as IL-6 (Figure 3C), IL-1β (Figure 3D) and IL-2 (Figure 4A). As reported in the table 3, a greater inhibitory

effect was obtained in lymphocytes from SLE patients. Moreover, the use of the selective $A_{2A}AR$ antagonist SCH 442416 (1 μM) demonstrated that the effect was mediated by $A_{2A}ARs$.

3.6 CGS 21680 increases the production of the anti-inflammatory cytokine IL-10 in lymphocytes

The incubation of lymphocytes with the $A_{2A}AR$ agonist CGS 21680 (1 μM) increased basal IL-10 release in lymphocytes from healthy subject and from SLE patients (Figure 4B). A more pronounced effect of CGS 21680 was obtained when cells were stimulated with LPS (0.1 mg/ml), although LPS alone did not alter IL-10 production. In the presence of LPS, the effect of CGS 21680 was significantly greater ($p<0.01$) in lymphocytes from SLE patients than from healthy subjects (Table 3).

3.7 $A_{2A}AR$ activation inhibits LPS-induced NF-κB activation in lymphocytes

Many of the anti-inflammatory effects of $A_{2A}AR$ stimulation are mediated by the inhibition of NF-kB activation (137). To verify if CGS 21680 was able to inhibit NF-kB in lymphocytes from SLE patients in comparison with healthy subjects, the activation of p65 subunits following LPS treatment was investigated. As shown in Figure 4C, the $A_{2A}AR$ agonist determined a marked reduction of LPS-stimulated NF-kB p65 subunit activation in nuclear extract from lymphocytes obtained from SLE patients and healthy subjects, with a significantly greater effect in the former (see Table 3). The inhibitory effect of CGS 21680 was completely counteracted by the selective $A_{2A}AR$ antagonist SCH 442416 (1 μM).

4. Discussion

The primary aim of this study was to investigate the role of ARs in SLE pathogenesis and to assess potential relationships between these receptors and clinical data. Within the complexity of the pathogenic mechanisms of lupus, innate immune responses play a significant role contributing either to tissue injury via release of inflammatory cytokines or to the aberrant hyperactivation of T and B cells, qualified as the most important players leading to autoreactive autoantibody production and resultant end-organ injury (13)(14)(6)(12)(166).

The role of adenosinergic system is attractive for its multifunction in this wide spectrum of inflammatory-related processes (152)(154)(109) and for its potential engagement in SLE.

AR mRNA and protein analysis supported a higher A_{2A}ARs expression in lymphocytes from SLE patients with respect to control subjects while no changes in A_1, A_{2B} or A_3 ARs were found, suggesting a specific A_{2A}AR alteration. Moreover, saturation binding experiments confirmed the upregulation of A_{2A}ARs in lymphocytes from SLE patients.

To note, the highest levels of A_{2A}AR density were tightly correlated with the lowest levels of clinical - namely clinimetric indexes - and serological parameters (anti-DNA, C3 and C4) of disease activity suggesting that the endogenous activation of these receptors could lead to a mitigation of the disease. This aspect has been further supported by the finding of an inverse correlation between the CAD progression of the disease and the receptor density, suggesting that the mutual modulation of A_{2A}AR expression well identifies a persistent and stable regulation of the inflammatory status.

The hypothesis that A_{2A}AR upregulation could represent a compensatory mechanism to better counteract the inflammatory background in SLE is supported by a preclinical study in a MRL/lpr mouse model of lupus nephritis (167) in which the A_{2A}AR mRNA expression in kidneys of MRL/lpr mice was significantly increased compared with controls mice. In this study the treatment with A_{2A}AR agonist ameliorated the severity of nephritis and renal vasculitis and reduced leukocytic infiltration.

Since IFN-α plays a central role in SLE pathogenesis (5), in cultured lymphocytes, the anti-inflammatory effect of A_{2A}AR activation on this cytokine was investigated. The effects of the interferon signature on lupus lymphocytes have been studied

mainly in the regulatory T subpopulation where the action of IFN-α diminished their activity (42), while, on B-cells, it stimulated antibodies production (168). We demonstrated that the $A_{2A}AR$ agonist CGS 21680 inhibited IFN-α release in cultured lymphocytes with a greater effect in SLE patients than healthy subjects. This observation further supports the competence of $A_{2A}AR$ signaling, as suggested by previous study (169), to promote peripheral tolerance by generation of regulatory T cells. The reduction of inflammatory response by $A_{2A}AR$ activation was also confirmed when studying the release of the typical pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β and IL-2. Furthermore we found that CGS 21680 mediated a significant increase of anti-inflammatory IL-10, which is an important immunoregulator that supports T cells differentiation and suppresses pro-inflammatory cytokines (170).

It is well known that activation of NF-kB pathways leads to enhanced B-cell survival and T-cell activation and maturation (171). Moreover NF-kB positively regulates gene encoding cytokines and other inflammatory factors, suggesting that this transcription factor could be one of the master regulators of inflammatory responses. Thus, the inhibition of NF-kB by CGS 21680 could explain the reduction of LPS-stimulated pro-inflammatory cytokines in cultured lymphocytes from SLE patients.

Taken together our data demonstrate the presence of an $A_{2A}AR$ upregulation in SLE patients and a significant inverse correlation of $A_{2A}AR$ density with SLEDAI and CAD. The anti-inflammatory response of $A_{2A}ARs$ open up a new prospective on the translational role of the $A_{2A}AR$ agonists in the pharmacological treatment of SLE highlighting their therapeutic potential in the management of this disorder.

5. Conclusion

This study suggests the involvement of A_{2A}ARs in the complex pathogenetic network of SLE, acting as a modulatory system of the inflammatory process. It could represent a compensatory pathway to better counteract the disease activity. The A_{2A}AR activation significantly reduces the release of pro-inflammatory cytokines while enhancing those with anti-inflammatory activity suggesting a potential translational use of A_{2A}AR agonists in SLE pharmacological treatment

6. Tables

Table 1 - Clinical and demographic features of the study subjects, as well as pharmacologic treatments in the SLE patients

	SLE patients (n 80) n° (%)
Clinical parameters	
No. female/male	71/9
Age, mean ± SD years	44 ± 11.9
Disease duration (months)*	139 ± 100
SLEDAI, mean ± SD	4 ± 4.3
SDI, mean ± SD	0.8 ± 1.2
Disease activity patterns	
CQD	46 (57.5%)
CAD	18 (22.5%)
MDA	14 (17.5%)
RRD	2 (2.5%)
Serological parameters	
aPL (aCL, β2GPI and/or LAC)	39 (48.75%)
ENA	48 (60%)
hypocomplementemia	53 (62.2%)
anti-dsDNA ab ongoing/previous	41 (51.2%)/17 (21.1%)
Treatments	
Corticosteroids (2.5 up to 12.5 mg/day)	67 (83.7%)
Hydroxychloroquine (200 mg/day)	48 (60%)
Immunosuppresants ongoing therapy	25 (31.2%)
Mycophenolate mofetil	11 (13.7%)
Cyclosporine A	4 (5%)
Azathioprine	5 (6.2%)
Methotrexate (10–15 mg/week)	3 (3.7%)
Thalidomide	1 (1.2%)
IVIg	1 (1.2%)
PEX	1 (1.2%)
Anticoagulants	11 (13.7%)
Antiaggregant	33 (41.25%)

SLEDAI-2K, SLE disease activity index-2000, SDI; SLE International Collaborating Clinics Damage Index; CQD, clinical quiescent disease; MDA, minimal disease activity; CAD, chronic active disease; RRD, relapsing-remitting disease aPL, antiphospholipid antibodies (including Anti-cardiolipin antibodies/ACA, lupus anticoagulant/LA, Beta-2 Glycoprotein 1 antibodiesβ2GPI); ENA, Extractable Nuclear Antigens antibodie; PEX, Plasma Exchange; IVIg, Intravenous immunoglobulin; Disease duration at the time of sample collection*

Table 2 - Clinical, serological and pharmacologic treatments in the SLE patients: correlation with A_{2A}AR affinity and density (unpaired *t test*).

	N° of pt	K_D (nM)		Bmax (fmol/mgp)	
Disease activity patterns					
CQD/CAD	46/18	1.96± 1.19/1.77±0.89	NS	141.6 ± 48.9/76 ± 28.6	< 0.0001
Organ involvement					
Renal (yes/no)	13/67	1.33± 0.59/1.94±1.15	0.06	114 ± 45.05/120.8±52.3	NS
Neuropsychiatric (yes/no)	20/60	1.73± 0.81/1.88±1.18	NS	103.85 ± 48.2/125±51.2	NS
Articular (yes/no)	46/34	1.79± 1.22/1.9±0.9	NS	110.85 ± 49.3/131.8±51.5	0.06
Cutaneous (yes/no)	25/55	1.46± 0.71/1.89±1.2	NS	105.60 ± 43.9/123.8±53.7	NS
Haematological (yes/no)	41/39	1.70± 0.89/1.98±1.28	NS	116.85 ± 47.8/123.6±54.5	NS
Serositis (yes/no)	21/59	1.60± 1.3/1.9±0.9	NS	92.86 ± 38.7/129.2±51.6	0.0043
Serological parameters					
aCL (yes/no)	22/58	1.91± 1.01/1.81±1.14	NS	134.04±52.55/114.27±47.3	NS
β2GPI (yes/no)	9/71	1.59± 0.46/1.87±1.15	NS	116.11 ± 53.15/120.2±51.1	NS
LAC (yes/no)	28/52	1.92± 1.20/1.8±1.05	NS	109.03 ± 51.60/125.5±50.2	NS
ENA (yes/no)	58/22	1.92± 1.15/1.77±1.07	NS	114,5±47.34/123.1±53.52	NS
Hypocomplementemia (yes/no)	53/27	1.57± 0.78/2.15±1.32	NS	97.88±39.25/145.08±51.78	< 0.0001
anti-dsDNA ab (yes/no)	41/39	1.65± 1.01/2.31±1.18	0.008	106.26±48.42/153.04±41.76	< 0.0001
Treatments					
Corticosteroids (2.5 up to 12.5 mg/day) (yes/no)	67/13	1.83± 1.17/1.88±0.63	NS	113.12 ± 50.30/153.69±41.47	0.0078
Hydroxychloroquine (200 mg/day) (yes/no)	48/32	1.93± 1.07/1.69±1.13	NS	126.89 ± 51.09/108.93±49.72	NS
Immunosuppresants or induction therapy (yes/no)	25/55	1.98± 1.19/1.54±0.8	NS	124.31 ± 48.04/110.15±56.46	NS
Anticoagulants (yes/no)	11/69	2.01± 1.47/1.81±1.04	NS	128.91 ± 64.01/128.91±64	NS
Antiaggregant (yes/no)	33/47	2.03± 1.24/1.7±0.97	NS	123.39 ± 50.61117.13±51.66	NS

Table 3. Effect of CGS 21680 in lymphocytes from healthy subjects (n=20) or SLE patients (n=20) on different inflammatory mediators.

	Healthy subjects			SLE patients		
	Cellular stimulation[a]	A_{2A} stimulation[b]	% of reduction[c]/ fold of increase[d]	Cellular stimulation[a]	A_{2A} stimulation[b]	% of reduction[c]/ fold of increase[d]
IFN-α	67±4	37±3	44.39±1.21	74±4	32±2	56.89±1.52*
TNF-α	225±12	102±10	54.66±1.14	227±10	79±5	64.95±2.33*
IL-6	305±15	167±12	45.38±1.18	322±13	105±6	67.71±1.25*
IL-1β	294±14	198±13	32.64±1.02	310±11	140±7	54.20±2.35*
IL-2	36±3	21±2	41.59±1.08	42±3	19±2	54.59±1.76*
IL-10	2832±112	4802±189	1.70±0.02	2973±104	5329±199	1.79±0.02[#]
NF-kB	245±14	161±13	34.07±1.08	266±10	150±6	43.69±1.47*

Note:

[a] Cellular stimulation with LPS for IFN-α, TNF-α, IL-6, IL-1β, IL-10 and NF-kB or with PMA + ionomycin for IL-2

[b] A_{2A} stimulation with CGS 21680 (1μM)

[c], % of reduction of CGS 21680 (1μM) on LPS (0.1 mg/ml)-stimulated IFN-α, TNF-α, IL-6, IL-1β, IL-2 and NF-kB levels

[d], fold of increase of CGS 21680 (1μM) on LPS (0.1 mg/ml)-stimulated IL-10 production

**, p<0.0001 vs healthy subjects; #, p<0.01 vs healthy subjects.*

7. Figure Legends

Figure Legends

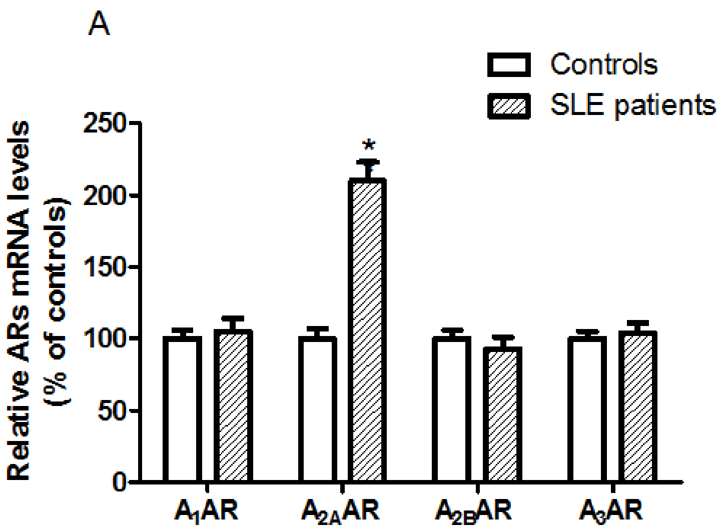
Figure 1- mRNA and protein expression of ARs in human lymphocytes from SLE patients and healthy subjects. (A) Relative AR mRNA levels were determined by RT-PCR. Experiments were performed in duplicate with lymphocytes obtained from individual SLE patients (n=80) and healthy subjects (n=80) and data are shown as mean ± SEM. (B) Western blotting analysis showing immunoblot signals of ARs in one SLE patient and one healthy subject, representative of blots obtained with lymphocytes from 80 SLE patients and 80 healthy controls. β-actin was used as a loading control. (C) Densitometric analysis of AR expression in human lymphocytes from SLE patients (n=80) and healthy subjects (n=80) indicated as a ratio of β-actin (loading control). Data are expressed as the mean ± SEM of densitometric analysis results obtained from the indicated number of subjects. *, $p < 0.01$ versus control group by one way ANOVA with Dunnett's test.

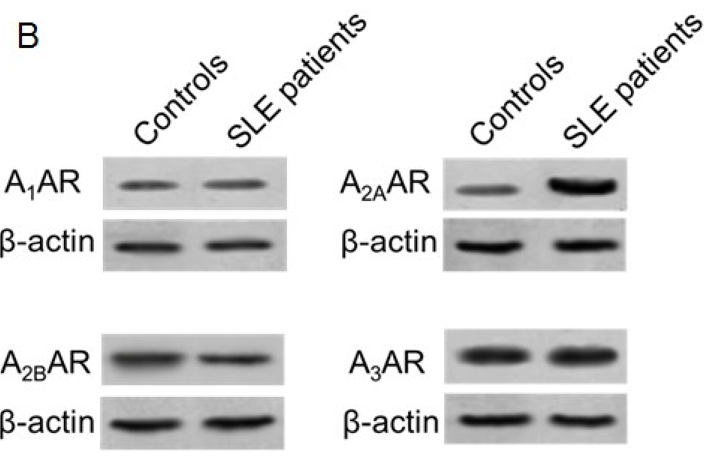
Figure 2- A_{2A}ARs are upregulated in lymphocytes from SLE patient. (A) Saturation curves and (B) Scatchard plot showing the binding of ^{3}H-ZM 241385 to A_{2A}ARs in lymphocyte membranes derived from 80 healthy controls (•) and 80 SLE patients (■) are also shown. Saturation binding experiments were performed as described in Materials and Methods. Data in the saturation curves are expressed as the mean ± SEM of results pooled from one experiment performed in duplicate for the indicated number of subjects. (C) Linear regression analysis between SLEDAI score and maximum number of A_{2A}ARs (Bmax) in lymphocytes (n=80, r=0.68, $P<0.0001$ by using Pearson or Spearman correlation (Pearson r=-0.68 or Spearman r=-0.75).

Figure 3- A_{2A}AR stimulation inhibits pro-inflammatory cytokine release. The effect of a well-known A_{2A}AR agonist (CGS 21680, 100 nM and 1 μM) and antagonist (SCH 442416, 1 μM) on (A) IFN-α, (B) TNF-α, (C) IL-6 and (D) IL-1β release in cultured lymphocytes from SLE patients (n=20) and healthy subjects (n=20) stimulated by LPS 0.1 mg/ml was determined by ELISA kit. Data are expressed as the mean ± SEM of three independent experiments performed in triplicate *, $p < 0.01$ versus LPS-treated cells by one way ANOVA with Dunnett's test.

Figure 4- CGS 21680 effect on IL-2 and IL-10 release and on NF-kB activation. Effect of CGS 21680 (100 nM and 1 μM) and SCH 442416 (1 μM) in cultured lymphocytes of SLE patients (n=20) and healthy subjects (n=20) stimulated by LPS (0.1 mg/ml) or PMA (2 ng/ml) and ionomycin (0.2 μM) on (A) IL-2 and (B) IL-10 release and on (C) NF-kB activation. Data are expressed as the mean ± SEM of three independent experiments performed in triplicate *, $p < 0.01$ versus LPS-treated cells by one way ANOVA with Dunnett's test.

Figure 1





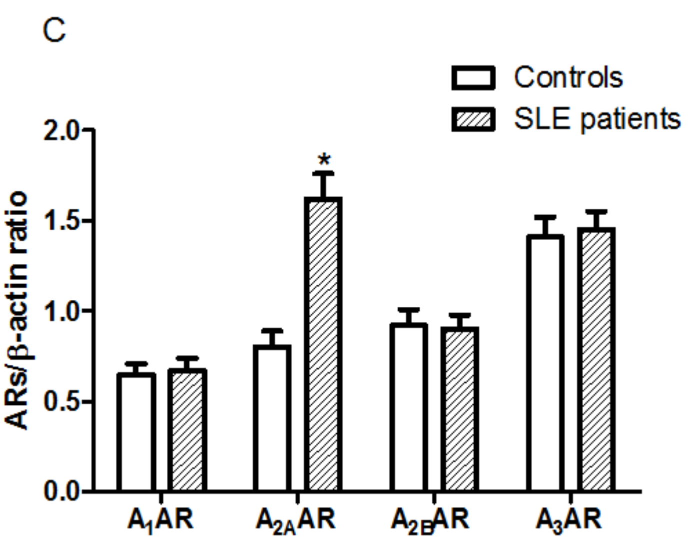
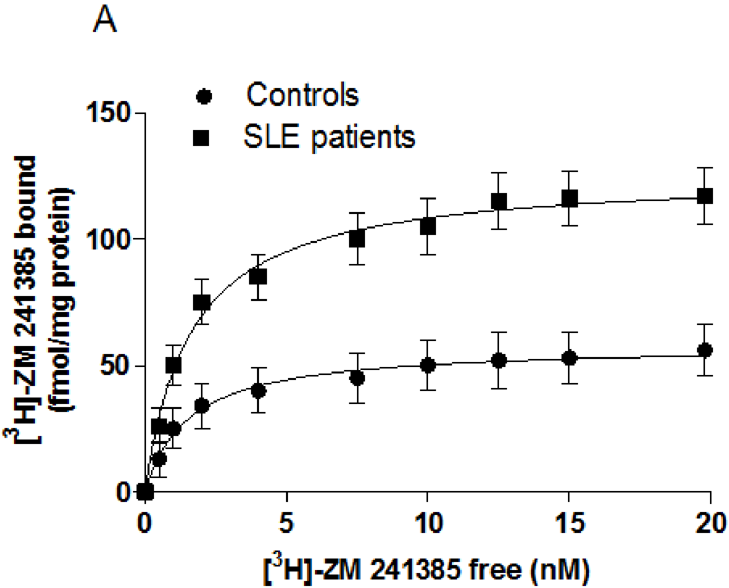
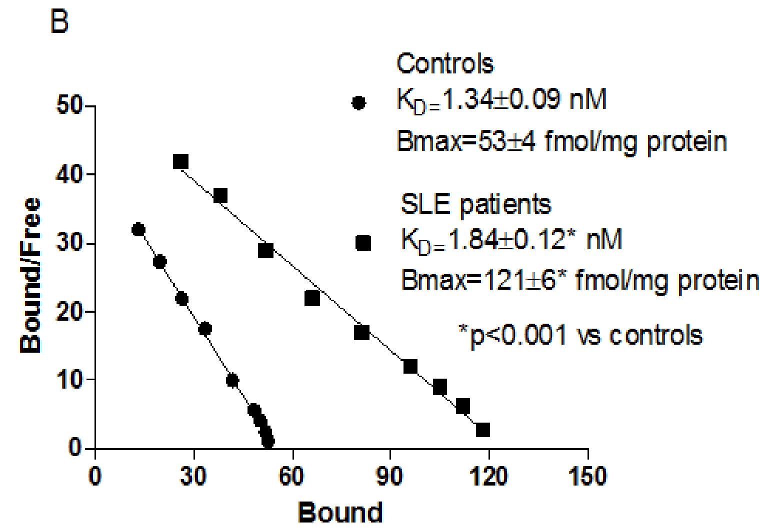


Figure 2





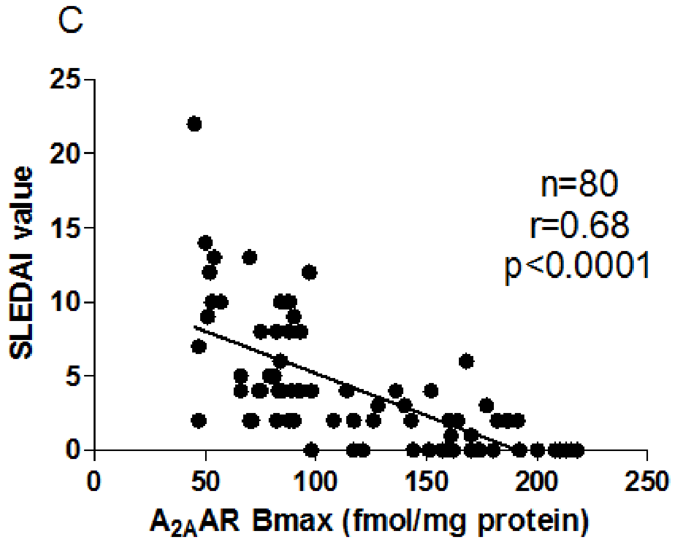
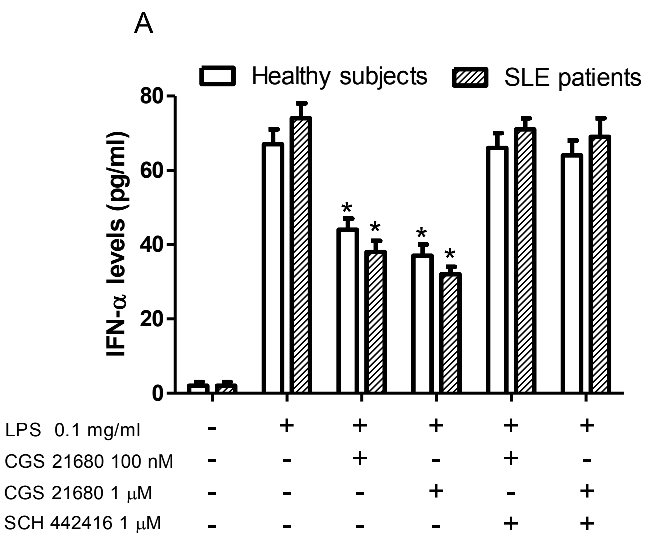
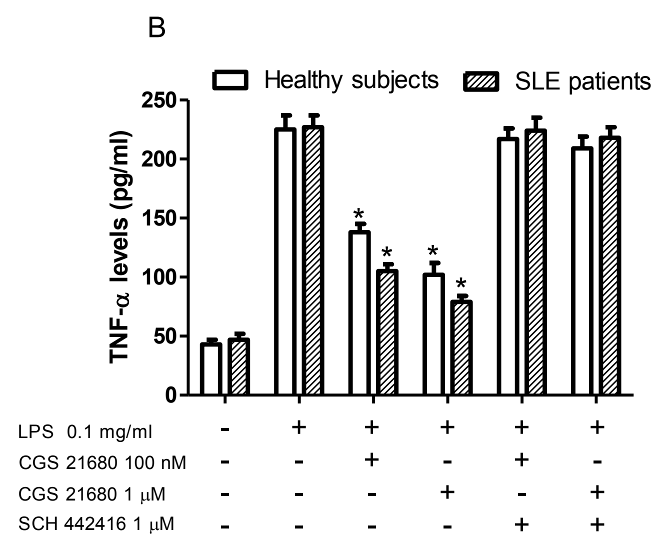
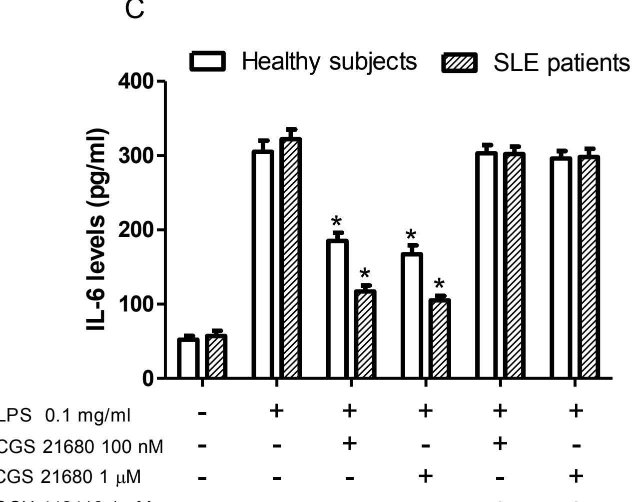


Figure 3







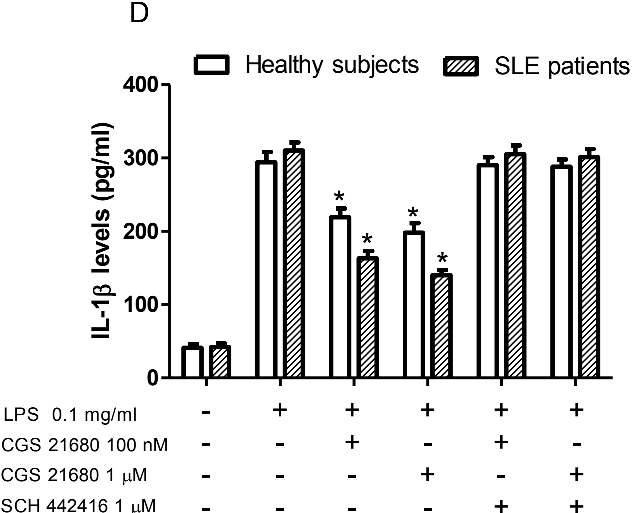
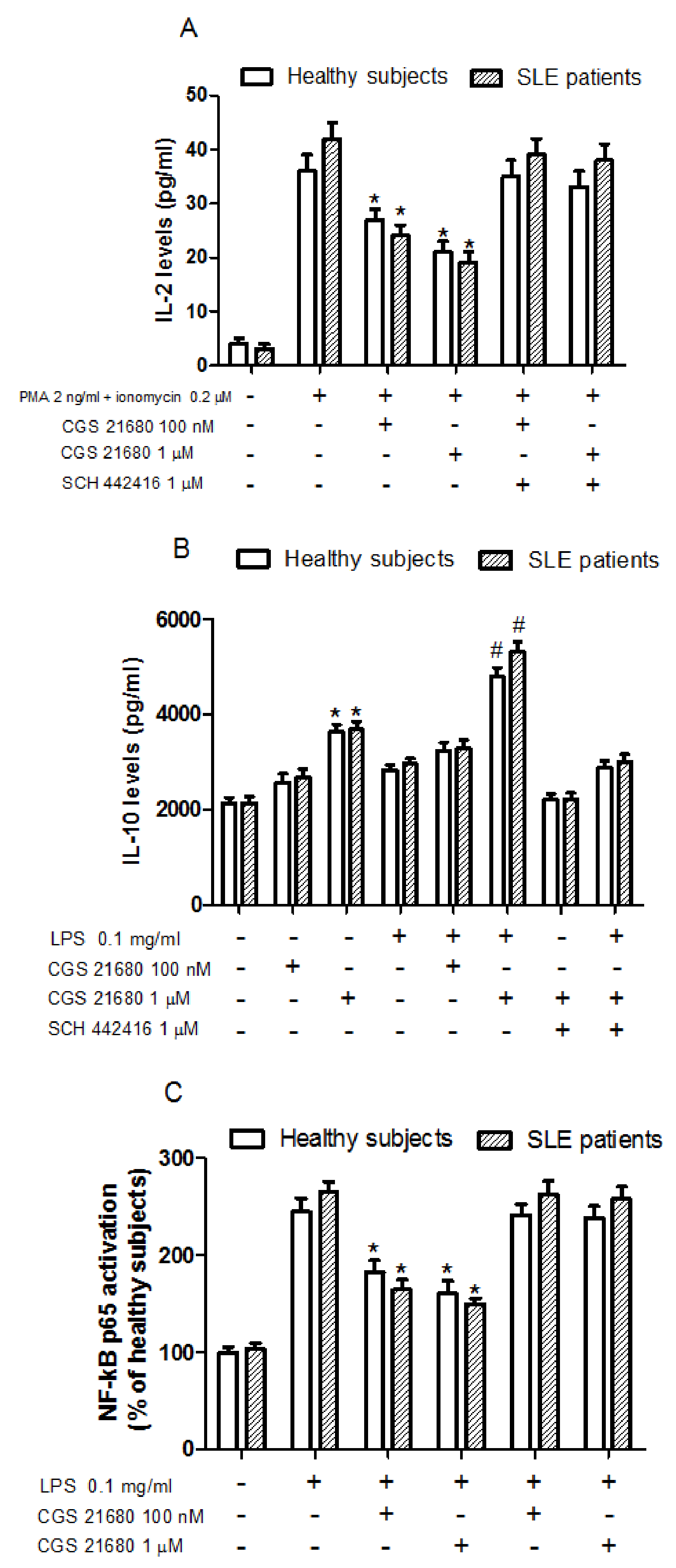


Figure 4



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Appendix

Appendix 1. SLEDAI (Systemic Lupus Erythematosus Disease Activity Index)-2K

Seizure	Recent onset. Exclude metabolic, infectious or drug-related causes	8
Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Includes hallucinations; incoherence; marked loose associations; impoverished thought content; marked illogical thinking; bizarre disorganised or catatonic behaviour. Exclude the presence of uraemia and off ending drugs	8
Organic brain syndrome	Altered mental function with impaired orientation or impaired memory or other intellectual function, with rapid onset and fluctuating clinical features. Includes a clouding of consciousness with a reduced capacity to focus and an inability to sustain attention on environment and at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, increased or decreased psychomotor activity. Exclude metabolic infectious and drug-related causes	8
Visual	Retinal changes from systemic lupus erythematosus cytoid bodies, retinal haemorrhages, serous exudate or haemorrhage in the choroid, optic neuritis (not due to hypertension, drugs or infection)	8
Cranial nerve	New onset of a sensory or motor neuropathy involving a cranial nerve	8
Lupus headache	Severe, persistent headache; may be migrainous	8

Cerebrovascular	New event. Exclude arteriosclerosis	8
Vasculitis	Ulceration, gangrene, tender finger nodules, periungal infarction, splinter haemorrhages. Vasculitis confirmed by biopsy or angiogram	8
Arthritis	More than two joints with pain and signs of inflammation	4
Myositis	Proximal muscle aching or weakness associated with elevated creatine phosphokinase/aldolase levels, electromyographic changes, or a biopsy showing myositis	4
Casts	Heme, granular or erythrocyte	4
Haematuria	More than 5 erythrocytes per high power field. Exclude other causes	4
Proteinuria	More than 0.5 g of urinary protein excreted per 24 h. New onset or recent increase of more than 0.5 g per 24 h	4
Pyuria	More than 5 leucocytes per high power field. Exclude infection	4
New malar rash	New onset or recurrence of an inflammatory type of rash	2
Alopecia	New or recurrent. A patch of abnormal, diffuse hair loss	2
Mucous membrane	New onset or recurrence of oral or nasal ulceration	
Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening	2
Low complement	A decrease in CH50, C3 or C4 levels (to less than the lower limit of the laboratory determined normal range)	2
Increased DNA binding	More than 25% binding by Farr assay (to more than the upper limit of the laboratory determined normal range, eg, 25%)	2
Fever	More than 38oC after the exclusion of infection	1
Thrombocytopenia	Fewer than 100000 platelets	1
Leucopenia	Leucocyte count <3000/mm3 (not due to drugs)	1

Appendix 2. The Systemic Lupus International Collaborating Clinics (SLICC) and endorsed by the ACR: the SLICC/ACR Damage Index

Ocular (either eye by clinical assessment)	
Cataract	1
Retinal change or optic atrophy	1
Neuropsychiatric	
Cognitive impairment (ex. memory deficit, difficulty with calculation, poor concentration, difficulty in spoken or written language, impaired performance level) or major psychosis	1
Seizures requiring therapy for 6 months	1
Cerebrovascular accident (score 2 if >1)	1 (2)
Cranial or peripheral neuropathy (excluding optic)	1
Transverse myelitis	1
Renal	
Estimated or measured glomerular filtration rate <50%	1
Proteinuria >3.5 g/24 h or end-stage renal disease (regardless of dialysis or transplantation)	1
Pulmonary	
Pulmonary hypertension (right ventricular prominence, or loud P2)	1
Pulmonary fibrosis (physical and radiographical)	1
Shrinking lung (radiograph)	1
Pleural fibrosis (radiograph)	1
Pulmonary infarction (radiograph)	1
Cardiovascular	
Angina or coronary artery bypass	1
Myocardial infarction ever (score 2 if >1)	1 (2)
Cardiomyopathy (ventricular dysfunction)	1
Valvular disease (diastolic murmur, or systolic murmur >3/6)	1
Pericarditis for 6 months or pericardiectomy	1
Peripheral vascular	
Claudication for 6 months	1
Minor tissue loss (pulp space)	1
Significant tissue loss ever (ex. loss of digit or limb) (score 2 if >1 site)	1 (2)
Venous thrombosis with swelling, ulceration or venous stasis	1
Gastrointestinal	
Infarction or resection of bowel below duodenum, spleen, liver or gallbladder, for any cause (score 2 if >1 site)	1 (2)
Mesenteric insufficiency	1
Chronic peritonitis	1
Stricture or upper gastrointestinal tract surgery	1
Chronic pancreatitis	1

Musculoskeletal	
Muscle atrophy or weakness	1
Deforming or erosive arthritis (including reversible deformities, excluding avascular necrosis)	1
Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)	1
Avascular necrosis (score 2 if >1)	1 (2)
Osteomyelitis	1
Tendon rupture	1
Skin	
Scarring chronic alopecia	1
Extensive scarring of panniculum other than scalp and pulp space	1
Skin ulceration (excluding thrombosis for >6 months)	1
Premature gonadal failure	1
Diabetes (regardless of treatment)	1
Malignancy (exclude dysplasia) (score 2 if >1 site)	1 (2)