Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
"Medicina Molecolare e Farmacologia"

CICLO XXXI

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P2X7 receptor (P2X7R) in Systemic Lupus Erythematosus (SLE). Exploring a novel pathogenetic pathway

Settore Scientifico Disciplinare MED/16

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INTRODUCTION

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is traditionally considered the prototypic multisystem autoimmune disorder with a broad spectrum of clinical presentations.

1.Epidemiology

Estimated incidence rates in Europe range from 2 to 8 per 100 000 per year. African Americans and Latin Americans are affected more frequently than Caucasians and have higher disease morbidity. SLE mainly involves female sex, and women are affected nine times more frequently than men.

The age of onset is between 16 and 55 years in 65%, before age 16 in 20%, and after the age of 55 in 15% of patients[1].

In case of elderly onset, SLE tends to be milder with a lower incidence of renal and central nervous systems (CNS), but a greater prevalence of serositis, pulmonary involvement, sicca symptoms, and musculoskeletal manifestations. In male sex, the age of onset tends to be older with a higher mortality compared to woman[2].

2. Etiology

The etiology of SLE is multifactorial including both genetic and environmental factors with female sex strongly influencing pathogenesis.

These factors contribute to the break of immunological tolerance and the development of immune responses against endogenous nuclear antigens[3].

2.1 Genetic factors

Large genome-wide association studies (GWAS) allowed identifying 25 genetic risk loci for SLE[4]. Many genes variant could contribute to lupus development and HLA-DRB1, signal transducer and activator of transcription 4 (STAT4) and interferon regulatory factor 5 (IRF5) are the three most frequently observed alleles. STAT4 is involved in type I interferon (IFN) signaling and IRF5 mediates transcription of type I interferon and pro-inflammatory cytokines. Other rare genetic variances that confers strongest susceptibility to SLE are deficiencies of components of the

classical pathway of complement such as C1q, C2 or C4, with a penetrance rate from 30% (C4 deficiency) to over 90% (C1q deficiency)[5].

2.2 Epigenetic effects

Epigenetics represents an alteration of genetic expression, not due to a direct mutation of DNA, but to a variety of gene expression due to DNA methylation, histone modifications (acetylation and methylation) and micro-RNA (miR) interference [6].

Methylation in specific regulatory regions, can silence gene expression by making DNA inaccessible to transcription factors. Methyltransferases could be defective in T cells of SLE leading to hypo-methylation of the regulatory regions of some genes known to be involved in the pathogenesis of the disease (ITGAL, CD40LG, CD70, and PPP2CA)[7].

2.3 Environmental factors

Several environmental factors have been identified as candidate triggers of SLE: infectious, ultraviolet light, demethylating drugs.

Many reports have identified different mechanism of action of EBV in SLE pathogenesis: latent infection of B lymphocyte, molecular mimicry, impaired T cell responses, promotion of IFNα production by plasmacytoid dendritic cells (pDCs)[8].

Over 100 drugs have been identified as responsible of drug-induced lupus (DIL) such as newer biologics and antiviral agents. The pathogenesis of DIL is not fully understood, some hypothesis regards the alteration of gene expression in CD4+T cells, through DNA methylation inhibition by some drugs as procainamide and hydralazine[9].

3. Pathogenesis and role of adaptive immunity

SLE has traditionally 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[10]. The break of immunological tolerance leads to the immune response against endogenous nuclear antigens and subsequent formation of autoantibodies and immune complexes (ICs) that can be internalized in pDC where, by binding Toll-like receptors (TLR) 7 and 9, stimulate transcription of IFN-α gene[11].

IFN-α contributes to the development of T helper 1 (Th1) cells, and stimulates DCs and myeloid cell leading to a massive production of pro-inflammatory cytokines as interleukin-(IL)-1beta (IL-1ß), IL-6, IL-12, IL-18, Tumor necrosis factor alfa (TNF-α)[12].

3.1 T-cells

T lymphocytes (T-cells) could have a double role in immunity: immunosuppression, pro-inflammatory activity, and promotion of antibody production. In the thymus, precursors of lymphocyte mature into functional T-cells, with negative selection of potentially self-reactive cells. From progenitor cell derived TCRαβ+CD4−CD8− T cells, defined as Double-Negative (DN) T cells, that mature progressively to the CD4+CD8+ double positive (DP) population, which differentiate to mature CD8+CD4- or CD8-CD4+ single positive (SP) cells [13]. (DN) T cells are expanded in SLE and correlate with disease activity. In normal condition, these cells exhibit an immunosuppressive activity, through antigen-competition and T cell killing by Fas-FasL or perforin and granzyme secretion. DN-T cells can activate B cells to produce pathogenic autoantibodies. In vitro study showed that IL-6 and IL-23 can increase the frequency of DN T cells and level of anti-ds-DNA suggests that they could play an important role in SLE pathogenesis[14]. Moreover in lupus glomerulonephritis, it is observed an infiltration of these cells in kidney, where they can directly produce the pro-inflammatory cytokine IL-17 which correlate with the development of a lupus-like nephritis in several mouse models[15]. IL-17 is mainly produced by T helper 17 (Th17) that are increased in SLE for an imbalance of differentiation towards these cells and a down-regulation of the TH1 and regulatory T cell (Treg). TH17 cells and high production of IL-17 are related to SLE disease and correlate with disease activity. In SLE an altered signaling by the T cell receptor (TCR) is one of the main drive of pathogenesis. The TCR recognizes antigenic peptides presented by the major histocompatibility complex (MHC) on antigen presenting cells and is associated with CD3 proteins (CD3δ, ε, γ, and ζ). For TCR signaling, the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) domains, contained in CD3ζ, by Src kinase lymphocyte-specific protein tyrosine kinase (Lck), is fundamental. After being phosphorylated, CD3 ITAMs recruit and activate the spleen tyrosine kinase (Syk) family kinase ζ-associated protein kinase 70 (ZAP-70) that through activation of phospholipase Cγ (PLC-γ), resulting in the calcium flux. In T cells from SLE patients CD3ζ is decreased and substituted by the

homologous Fc receptor common gamma subunit chain (FcRγ), which interact with Syk providing a stronger signal than CD3ζ–ZAP-70 interaction, resulting in the higher calcium influx into T cells[16]. These enhanced calcium signals activate cytoplasmic-nuclear factor of activated T cells (NFAT) that move to the nucleus binding the promoters of CD154 (CD40L) and IL-2 genes, promoting T cell proliferation. CD40-CD40L signaling is also important for the differentiation of Th17 cells. Although augmented transcription of IL-2 genes mediated by NFAT, in SLE patients, IL-2 level are reduced, probably because other transcription factors play a significant role[17]. Low levels of IL-2 in SLE, is associated with reduced activity of cytotoxic T cells (CD8 T cells) that are fundamental in defense against viral infection, neoplasm, and autoreactive cells. Presence of IL-2 can mediate immune tolerance by the development of Tregs which inhibits autoreactive T cells through direct cell-cell interaction or by the release of immunosuppressive factors such as growth factor β (TGFβ) and IL-10. Tregs express transcription factor Foxp3 and high expression levels of the IL-2 receptor alpha chain (CD25) and their role in SLE pathogenesis is still controversial. Some studies demonstrated a decreased number of Tregs in patients with SLE other demonstrate that the number of Tregs is similar for patients with SLE and controls. Some reports 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.

3.2 B-cells

B cells develop from precursor stages during which they rearrange their 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 T-cell 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. Under normal conditions, during this maturation process, B cells go through various stages of development during which they are controlled to ensure immune tolerance. In SLE this process can be defective at several levels leading to the development of autoreactive cells. Enhanced BCR signaling characterized 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.

B cells play also an autoantibody-independent role in SLE pathogenesis mediating different immunological functions such as presenting antigen, co-stimulating T cells that induce immunogenic DCs, production of cytokines and chemokines to promote inflammation[18].

3.3 Cytokine

The imbalance between pro and anti-inflammatory cytokines is involved in SLE pathogenesis. Among the most important cytokines have been reported: interferon type I (IFN-I), IL-6, IL-3, IL-17, B lymphocyte stimulator (BLyS); A proliferation-inducing ligand (APRIL); TNF-α[19]. INF type I, in particular, IFN-α is a fundamental cytokine in SLE pathogenesis. IFN-α is primarily produced by pDCs and promote DCs maturation and expansion of self-reactive T-cells by upregulation of costimulatory molecules (CD80 and CD86)[20] while downregulate function of Tregs[21]. IFN-α enhance BLys/BAFF and consequently promote B cell activation and survival leading the differentiation to antibody-secreting plasma cells that are able to produce auto-antibodies and ICs that enhance INF production through stimulation of TLR-7 and -9 generating a positive loop[22]. BlyS and APRIL are members of TNF superfamily. BlyS induces B cells survival, proliferation, differentiation and immunoglobulin secretion[23]. APRIL is responsible of induction and maintenance of B cells responses[24]. TNF-α promotes expression of other cytokines as IFN-α, IL-6, IL-1 and is produced mainly by monocyte/macrophage but also T cells, natural killer cells (NK), neutrophils, endothelial cells and mast cells[25]. IL-2 is essential for the control of the proliferation of autoreactive T cells and promotes the differentiation of Treg[26.] A deficit of transcription of this cytokine in lupus patients has been demonstrated in both mouse and human models. IL-6 together with transforming growth factor-β promote differentiation of Th17 cell and downregulation of Treg. It is elevated in the serum of SLE patients and can be produced by myeloid cells in response to pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs)[27]. IL-10 has an anti-inflammatory and immunosuppressive action. It is released from B cell and monocytes and is able to downregulate pro-inflammatory cytokine production, inhibit autoreactive T cell function and regulate dendritic cell activity. IL10 is increased in the serum of patients with SLE, nevertheless it appears that the immunosuppressive effect of this cytokine is reduced, partly due to the effect of IFN-α which appears to reduce the ability of IL10 to inhibit the production of IL6 and TNF-α[28].

4. Clinical manifestations

SLE can affect any organ system leading to a broad spectrum of clinical manifestation. Constitutional symptoms such as malaise, fatigue, fever, and weight loss are common but nonspecific.

4.1 Mucocutaneous involvement

Skin involvement can be divided into specific and nonspecific. The specific lesion can be classified in:

1. Acute lesions include acute malar rash: the classic butterfly erythematous lesion often with an acute onset after exposure to sunlight. Malar rash can disappear or remain permanent actively.
2. Subacute lesion includes subacute cutaneous lupus erythematosus (SCLE) that is found in about 10% of SLE patients with skin involvement. It can manifest with annular or psoriasiform skin lesions, usually sparing the face and is typically associated with positivity of antibodies anti-Ro (SS-A) and anti-LA (SS-B).
3. Chronic lesion: discoid lupus erythematosus (DLE) is characterized by an isolated, erythematous, slightly infiltrated plaque, covered by adherent follicular hyperkeratosis and develops in 25% of SLE patients. Is typical located in the face, neck, and scalp

Other cutaneous manifestations included:

1. lupus profundus (lupus panniculitis), a rare event that occurs with fixed nodules with possible skin ulcers, and its rare variant lupus tumidus, characterized by lesions, such as chronic plaque pink or large slow-healing lesions, in sun-exposed areas;
2. alopecia often is a complication of discoid lupus;
3. photosensitivity present in 60-100% of lupus patients, has been defined as a rash that develops after exposure to UV-B

The mucosal involvement is characterized by lesion similar to white and uneven patches and afflicts 25-45% of SLE patients[29]

4.2 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. Among the types of joint involvement arthropathy of Jaccoud is described, which may be present up to 5% of the cases of SLE. It is characterized by reversible joint deformities and the absence of bone erosion at the radiologic exam[30]. In a small proportion of lupus patients, the arthritis is substantially indistinguishable from Rheumatoid arthritis (rhupus)[31]. Muscle pain and stiffness are common and inflammatory myositis is reported in 11.5% of cases. Another musculoskeletal manifestation affecting 6-12% of patients is avascular bone necrosis often associated to antiphospholipid antibody syndrome (APS) and the use of high doses of steroid[32].

4.3 Kidney involvement

Renal involvement occurs in 40-70% of SLE patients and is the leading cause of hospitalization. It varies widely by type and severity but generally represents a potentially serious medical condition. The types of renal involvement vary from asymptomatic forms characterized only by proteinuria and hematuria of glomerular origin, to symptomatic forms such as nephritic syndrome (impaired kidney function, non-nephrotic proteinuria, hematuria with red cell casts, hypertension and edema), nephrotic syndrome (proteinuria> 3 g/day, low serum albumin, edema, high lipids, increased risk of thrombotic events), to renal end stage disease. The main sign of lupus nephritis is the proteinuria and it is usually accompanied by hematuria of glomerular origin. Renal biopsy should be considered in presence of 24-hour proteinuria> 0,5-1g / day, hematuria of dubious origin in particular if you have dysmorphic erythrocytes, presence or absence of cell cylinders. Six immunohistochemical classes have been identified[33]:

1. Class I: glomerulonephritis (GN) mesangial
2. Class II: mesangial proliferative GN.

3. 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.
4. 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.
5. 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.
6. 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.

4.4 Neurological Involvement

According to American College of Rheumatology, neuropsychiatric lupus (SLE-NP) can be defined as: “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”. SLE-NP syndrome could be classified as reported in table 1.

Table 1: Classification of NPSLE manifestations (American College of Rheumatology 1999)[34]

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	

Diagnosis of SLE-NP can be very challenging for rheumatologist due to the presence and is one of the most important cause of morbidity in lupus patients with prevalence reported between 42.5% and 74.7%[34].

4.5 Cardiovascular Involvement

One of the most frequent cardiac manifestation related to SLE is pericarditis that occurs in 25% of SLE patients. It's often moderate, rarely can evolve in cardiac tamponade. SLE patients besides, present higher mortality and morbidity related to cardiovascular disease that comprises principally accelerated atherosclerosis, increased risk of myocardial infarction and heart valve disease. Valvular disease is frequently related to positivity of antiphospholipid antibodies and may be characterized by typical vegetation (Libmann-Sachs vegetation) that are probably formed of fibrin-platelet thrombi which can lead to valve dysfunction[35]. Accelerate atherosclerosis is related to different factors: metabolic syndrome, hypertension, antibodies against lipoprotein and endothelial damage due to inflammation[36]. Finally, also pulmonary hypertension can be a rare and life-threatening complication of SLE.

4.6 Lung involvement

The most common pulmonary manifestation is represented by pleurisy usually bilateral and characterized by pleuritic pain. This symptom can be present in 45-60% of patients also without pleural effusion. Interstitial lung disease (ILD) can be present in 3-13% of cases, but rarely severe[37]. 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 expecially in patients with a longstanding history of SLE, active disease activity and high titer of anti DNA. Another manifestation secondary to muscle dysfunction is the "shrinking lung" syndrome which is characterized by worsening dyspnea and the presence of small lung volumes[38]

4.7 Lymphoid organs involvement and hematological involvement

Lymphadenopathy occurs in about 40% of patients, usually at the onset of the disease or during an exacerbation. Splenomegaly is present in 10-45% of cases, usually, during the active phase of the disease, hyposplenism (autosplenectomy) can be found in SLE patients. The main events haematologic abnormalities include: anemia, leukopenia, thrombocytopenia. Anemia can have different pathogenesis including: anemia of chronic disease, hemolytic anemia (occurred in 10% of cases and can be autoimmune or microangiopathic), iron-deficiency, chronic renal failure,

drug-induced anemia, myelodysplasia, myelofibrosis, aplastic anemia. Leukopenia is very common in SLE, 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. It can be caused by an immune-platelet destruction, but also by microangiopathic hemolysis or for splenic sequestration[39]

4.8 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. Liver disease may include hepatomegaly can be observed in 12-25% of patients, steatosis derived from a primitive involvement of the disease or secondary to steroid medication and finally autoimmune hepatitis that is associated to positivity of ASMA (anti-smooth muscle antibodies) and anti-mitochondrial antibodies (AMA) in 30% of cases and is characterized at histology by periportal hepatitis with segmental necrosis.

5. Laboratory findings

ANA is the most important laboratory parameters and defining autoimmune disease but is non-specific and can be positive up to 5-20% of normal population. Anti-Sm antibodies and anti double-stranded DNA (dsDNA) are more specific and can be positive in 30-40% and 40-60% of patients respectively. Anti-SSA/Ro and anti-SSB/La are associated with subacute cutaneous lupus erythematosus, neonatal lupus, and photosensitivity and can be a suspicious element for secondary Sjögren's syndrome. Antiphospholipid antibodies include anti cardiolipin or to beta-2 glycoprotein 1, and lupus anticoagulant (LA) and can be found in 50% of cases of lupus. The dosage of complement (C3, C4, or total hemolytic complement CH50) can be helpful for diagnosis and monitoring of SLE patients[40].

6.Therapy

Different drugs are currently used in treatment of SLE patients: non-steroidal drugs (NSAIDs), antimalarials, glucocorticoids, immunosuppressants.

Glucocorticoids suppress broadly immune response through depletion of T cells and downregulation of immunoglobulin production by B cells, but are characterized by a lot of side effects such as dyslipidemia, diabetes, hypertension, accelerated atherosclerosis, skin atrophy, susceptibility to infection and osteoporosis. Hydroxychloroquine and anti-malarial control autoimmunity through inhibition of TLR with consequent reduction of disease flares and damage. It's a very effective therapy that has few collateral effects and reduces serum lipids levels. For treatment of more severe manifestations of the disease, immunosuppressive agents are required. Cyclophosphamide is effective in lupus nephritis and is usually administered intravenously and for short therapeutic cycle. This drugs present many side effects such as increase of infection, gonadal failure, bone marrow aplasia and increase risk of neoplasm. Mycophenolate mofetil (MMF) derivates from mycophenolic acid and inhibit the enzyme inosine monophosphate dehydrogenase (IMPDH) resulting in blocking purine synthesis arresting cell proliferation. MMF had fewer side effects than cyclophosphamide and can be a viable therapeutic alternative. Cyclosporine (CYA) inhibits proliferation of T-cells, especially the helper T. Methotrexate (MTX) is effective especially in arthritis or cutaneous manifestations treatment. Belimumab is the only biologic drugs approved for SLE treatment and consists of a monoclonal antibody that blocks the Blys signaling pathway reducing autoantibodies production and disease activity[41].

ATP signaling and Purinergic receptors

Since the 1970s, an increasing number of studies have shown that Adenosine 5'-triphosphate (ATP) has not only to be considered a molecule with an energy storing function, involved in the main biosynthetic processes that promote cell survival, motility, and cell proliferation. ATP, in fact, can be considered a signal macromolecule acting at neurological level (both central and peripheral) and also at a non-neurological level, including the exocrine and endocrine, immune systems and musculoskeletal and endothelial cells[42].

Under physiological conditions, except for the synaptic space where the concentrations may be higher, ATP is poorly represented in the extracellular space, reaching concentrations in the order of nanomoles (nM). ATP can be released in pathological conditions from damaged or dying cells, or be released from cells with an intact plasma membrane and subjected to stimulation, or to oxidative or mechanical stress. This release can be mediated by vesicles or through membrane proteins like ABC cassettes, pannexins, connexins, and some purinergic receptors themselves[43].

Once ATP has reached the extracellular environment, it binds specific receptors that mediate the signal. The purinergic receptors can be distinguished into the three main families:

1. G-protein-coupled adenosine receptor.
2. metabotropic P2Y (G-protein coupled receptors) receptor families.
3. ionotropic P2X (ligand-gated ion channel receptors).

ATP molecules are rapidly degraded in the extracellular space by ectonucleotidase which in turn generate other mediators that bind purinergic receptors such as ADP (target receptor: P2Y1, P2Y12, P2Y13) and adenosine (target receptors (A1, A2A, A2B, A3)[44] .

Nineteen different types of purinergic receptors are encoded by the human genome and each of them is able to bind one or more nucleotides/nucleosides as shown in table 2 [45.]

Table 2: major purinergic receptor agonists [45]

Agonist	**Receptor**
ATP	P2X1-P2X7, P2Y2, P2Y11
ADP	P2Y1, P2Y12, P2Y13
UTP	P2Y2, P2Y4
UDP	P2Y6, P2Y14
UDP-glucose	P2Y14
Adenosine	A1,A2A, A2B, A3

ATP and other nucleotides mediate different activities involving different types of cells and organs such as:

- smooth muscle contraction;
-neurotransmission;
- embryonic development;
- maintenance of ocular homeostasis;
- coronary vasodilation;
-regulation of the immune response;
-platelet activation and thrombosis[42] (table3).

P2X receptor structure and activity

Seven subunits participating in the formation of P2X receptors have been identified in human and animal model (mouse). These subunits can vary in length from 379 to 595 amino acids and all have a similar structure composed by:

1. two transmembrane domains (TM);
2. one large extracellular binding sequence that connects the two transmembrane portions;
3. two intracellular component N and C-terminal end.

The N-terminal end portion consists of a few amino acids (20-30) and contains a residual threonine that is part of the highly conserved protein kinase C consensus site TX (K/R). The activity of this enzyme would be fundamental in slowing the receptor desensitization, thus increasing its activity[46].

The C-terminal end portion ranges from 26 (P2X6) to 239 (P2X7) amino acids sharing only short homogeneity portions, indicating that different subunits confer a specific function in different receptors. This portion is involved in fact, in stabilization and movement of the receptor along the plasma membrane and mediates its interaction with specific proteins. The P2X subunits are able to form a trimeric structure binding three ATP molecules, unlike P2Y receptors that can also bind other nucleotides (table 2 and table 3) [42,47].

More recent evidence has shown that P2X subunits not only bind ATP but also NAD^+, LL37 cathelicidin (cationic peptide synthesized by neutrophils, monocytes, keratinocytes, and macrophages, with antimicrobic activity), polymyxin B and amyloid ß[48].

P2XRs have a wide distribution in multiple organs and tissues, mediating different activities, despite this, is P2X7R the one most studied and for which more evidence is available.

Table 3: nucleotidic receptors[42,47]

P2R subtype	Subunit assembly	Main distribution	Preferred ligand	Signal transduction mechanism
P2X1	P2X1, P2X2, P2X4, P2X5	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	ATP	Na+, K+ and Ca2+-selective ion channel
P2X2	P2X2, P2X3, P2X6	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	ATP	Na+, K+ and Ca2+-selective ion channel
P2X3	P2X3, P2X2	Sensory neurons, NTS, some sympathetic neurons	ATP	Na+, K+ and Ca2+-selective ion channel
P2X4	P2X4, P2X6	CNS, testis, colon	ATP	Na+, K+ and Ca2+-selective ion channel
P2X5	P2X5, P2X1 P2X6,	Proliferating cells in skin, gut, bladder, thymus, spinal cord	ATP	Na+, K+ and Ca2+-selective ion channel
P2X6	P2X6, P2X2, P2X4	CNS, motor neurons in the spinal cord	ATP	Na+, K+ and Ca2+-selective ion channel
P2X7	P2X7	Immune cells, pancreas, skin	ATP	Na+, K+ and Ca2+-selective ion channel and large pore permeable to solutes up to 900 Da
P2Y1		Epithelial and endothelial cells, platelets, immune cells, osteoclasts	ADP	Gq/11(IP3/DAG increase)
P2Y2		Immune cells, epithelial, endothelial cells, kidney tubules, osteoblasts	UTP and ATP	Gq/11 (IP3/DAG increase)
P2Y4		Endothelial cells	UTP	Gq/11 (IP3/DAG increase) and Gi (inhibition of cAMP synthesis)
P2Y6		Some epithelial cells, placenta, T cells, thymus	UDP	Gq/11 (IP3/DAG increase)
P2Y11		Spleen, intestine, granulocytes	ATP	Gq/11 (IP3/DAG increase), Gs (cAMP increase)
P2Y12		Platelets, glial cells	ADP	Gi (inhibition of cAMP synthesis)
P2Y13		Spleen, brain, lymph nodes, bone marrow	ADP	Gi (inhibition of cAMP synthesis)
P2Y14		Placenta, adipose tissue, stomach, intestine, discrete brain regions	UDP-glucose	Gi (inhibition of cAMP synthesis)

P2X7 receptor (P2X7R) signalling

P2X7 subunit (P2X7s) is encoded by the gene located at 12q24.31 near the P2X4 locus. Such proximity and homology of protein sequence with a rate of identity of 41%, enforce the hypothesis of duplication at the origin of the gene.

P2X7s is composed of 595 amino acids that contain:

1. one intracellular N-terminal end portion of 26 amino acids (aa);
2. one extracellular domain of 282 aa;
3. two transmembrane portions of 24 aa;
4. one intracellular C-terminal end portion of 293 aa.

The latter portion contains many binding regions for proteins and lipids and consists of:

-region 441-460 homologous to SRC homology 3 (SH3)-binding domain;

-region 389-405 and 494-508 homologous to a portion of proteins that bind the cytoskeleton;

- region 436-531 homologous to a portion of the tumor necrosis factor receptor 1 (TNFR1);

- 573-590-endotoxin homologous region (LPS) -binding region of serum LPS binging protein[49].

P2X7R, unlike other P2X receptors that can be formed by different subunits, forms a homo-trimer ion channel, and to be activated need higher concentrations of ATP (mMol range) usually reached only in pathological condition such as inflammation or neoplastic disease. In the binding site of P2X7R, there are several hydrophobic aromatic residues and serine residue (normally present in P2X1-P2X5) that are replaced by tyrosine or phenylalanine leading to form a smaller and less polar binding pocket making it less sensitive to ATP[50].

In the experimental models, ATP analogs are therefore used, including synthetic ATP derivative 20 (30)-O-(4-benzoyl) adenosine-50-triphosphate (Bz-ATP). Other P2X7R agonists have been identified: antibacterial peptide cathelicidin LL37, Alzheimer ß-amyloid peptide, polymixin B.

The response to the ATP is characterized by two pathways:

-rapid opening of a non-selective channel to cations that allows the influx of Sodium (Na^+) and Calcium ($Ca2^+$), and the efflux of Potassium (K^+)

- prolonged stimulation with higher concentration of ATP leading to the opening of a non-selective pore that allow the passage of hydrophilic solutes of molecular weight up to 90 kDa leading to cell death[51]. The ability of P2X7R to behave as a

pore was previously thought to be permitted by interaction with additional components such as connexin-43 and pannexin-1. Today it is thought to be an intrinsic capacity of the receptor permitted by the long intracellular portion which to the receptor the ability to became a non-selective pore for organic solute.

The C-terminal end is therefore essential for P2X7R activity and splicing variants of this portion can influence receptor function by the formation of truncated subunits. To date, ten (P2X7A–J) splice variants of human gene of P2RX7 subunits have been described. Four human splice variants (P2X7B, P2X7C, P2X7E, and P2X7G) are C-terminally truncated. Other splice variants are characterized by lack of exons (P2X7C, P2X7D, P2X7E, P2X7F), insertion of an additional exon (P2X7G and P2X7H), point mutation in the first intron (P2X7I). P2X7J variant, truncated after exon 7 is not functional. P2X7B is broadly diffuse in many tissues and when is assembled, its receptor (P2X7RB) is unable to form pore but it preserves the channel function[52].

Several single-nucleotide polymorphisms (SNPs) of P2X7R are reported (up to 1500). Among these only few were related to influence receptor activity including loss of function (LOF) and 3 gain of function (GOF) variants given a susceptibility to various pathologies like osteoporosis[53](table 4).

Table 4: Principal P2X7R gene SNPs associated to Disease.

SNP id	Nucleotide Base change	Amino Acid change	Effect of minor allele	Associated condition
rs3751143	1513A>C	E496A	LOF	Risk of bone fracture; decreased LS BMD; familial CLL; conflicting results on susceptibility to tuberculosis
rs1653624	1729T>A	I568N	LOF	Risk of bone fracture
rs28360457	946G>A	R307Q	LOF	Increased rate of bone loss; protection against MS
rs2230911	1096C>G	T357S	LOF	No association with RA
rs208294	489C>T	H155Y	GOF	No association with MDD and RA; protection against AD
rs1718119	1068G>A	A348T	GOF	Lower vertebral fracture incidence; increased LS BMD values
rs2230912	1405A>G	Q460R	LOF	BD, MDD, osteoporosis
rs28360447	474G>A	G150R	LOF	Increased risk of osteoporosis
rs35933842	151 +1G>T	–	LOF	Increased risk of MM

Legend: -Nucleotide base change: A (adenosine), C (cytosine) , G (guanine) T (thymine). –Aminoacid change: E(Glutamic acid), A (Alanine), I (Isoleucine), N (Asparagine) R(Arginine) Q(Glutamine), T(Threonine), S(Serine), H(Histidine), Y(Thyronine), G(Glycine). –Effector of minor allele: Loss of Function (LOF), Gain of Function (GOF). –Associated condition: Lumbar spine bone mineral density (LS BMD), Chronic lymphocytic leukemia (CLL), Multiple sclerosis (MS), Rheumatoid arthritis (RA), major depressive disorder (MDD), Alzheimer's disease (AD), Bipolar disorder (BD), Multiple myeloma (MM)[130]

1.P2X7R and inflammation

P2X7R is expressed in all immune cells including monocyte/macrophage, T and B lymphocytes, dendritic cells (DC), mast cells and natural killers and its role in immunity is broadly described, especially for the activation in innate immune response. P2X7R was initially described in mast cells and fibroblasts but is in macrophages where its role in inflammasome activation and in intracellular pathogen killing, has been widely described.

In vivo studies have however provided conflicting results regarding the role of P2X7R and the response to intracellular pathogens. In the case of Chlamydia and Leishmania infections, the presence of the receptor seems to facilitate the response against the pathogen by facilitating the fusion of the phagolysosome and the acidification of the vacuole containing the pathogen[54]. While in studies with P2X7R null mice, mycobacteria infection appears to be less severe, probably due to role of the receptor that plays an important action in chronic infection by promoting type IV hypersensitivity and the formation of granuloma, with consequent caseous necrosis and extensive tissue damage[55]. Also in case of viral diseases, the role of P2X7R seems to be not well defined. While in some viral diseases such as Dengue, it plays a role of containment of infection by the release of nitric oxide[56], in other infections such as adenovirus and hepatitis B, the receptor can accelerate the damage caused by the virus. In particular, numerous observations are reported regarding the role of P2X7R and HIV infection, where it could have a facilitating role for the infection as it would mediate the release of the virus from vesicles contained in the macrophages. Moreover, an overexpression of P2X7R on CD34+ progenitor cells would block their differentiation towards T lymphocytes, thus identifying patients with the worst prognosis. Nucleotide reverse transcriptase inhibitors (NRTIs) are an important therapy in HIV infection and have shown both in vitro and in vivo ability to block P2X7R[57].

In DC P2X7R is fundamental in the presentation of the antigen (and the consequent activation of T lymphocytes)[58] and also in the release of microvesicles containing IL-1β and other mediators such as IL-1Receptor antagonist (IL-1Ra), MHC II , caspase- 1 and -3, cathepsin, and tissue factor (TF), leading to the diffusion of inflammatory molecules at distance: between cell and cell and also in the bloodstream[59]. The release of IL-1β mediated by P2X7 has been described by

monocytes, especially during cryopyrinopathies and by neutrophils during bacterial infections[60].

ATP is one of the main danger associated molecular pattern (DAMPS): intracellular molecules, that are released by cells in case of damage or stress. Through the activation of P2X7R, occurs the assembly of NLRP3 inflammasome which determines the activation of procaspase-1 to caspase-1, which mediates the cleavage of pro-IL-1β and pro-IL18 in their active forms (IL-1β and IL18) that are subsequently released on an extracellular space[61].

The link between P2X7R and inflammasome activation is given by K^+ efflux. This modification of intracellular ionic microenvironment is necessary for inflammasome activation although the molecular mechanism is still debated[62]. It has been hypothesized a direct interaction between P2X7R and NLRP3[63] while K^+ efflux would also be involved in enhancing interaction between NLRP3 with the Nima-related kinase 7 (NEK7) which is a promoter of inflammasome activation[64].

2. Inflammasome

Inflammasome is a central cellular processing unit (CPU) that transforms the signal supplied by PAMPs / MAMPs (molecular patterns associated with pathogens / molecular patterns associated with the microbe), DAMPS (molecular patterns associated with damage) to PRRs (receptors of pattern recognition), in an immune system response. DAMPs are molecules that are released in response to secondary cell damage, such as an infection or sterile inflammation[65]. Once activated, inflammasome is responsible for the release of cytokine IL-1β and IL-18, activation of immune cells, and cell death (pyroptosis)[66].

“Canonical inflammasome” stimulates the activation of caspase-1, enzyme responsible of cleavage of pro-IL-1β and pro-IL-18 in their activated isoforms. More recently, "non-canonical" inflammasome, that recruit caspase-4/5 in human and caspase-11 in mouse, have been identified. The exact mechanism of action of "non-canonical inflammasome" is not yet fully understood. The available evidence has shown that procaspase-4/5 can interact with lipopolysaccharide (LPS) in a TLR-4 independent way, activating pyroptosis coupled with the secretion of IL-1β and IL-18. This mechanism could be responsible for LPS-induced septic shock in mice[67].

Canonical inflammasomes are typically composed by:

-the adaptor protein apoptosis-associated speck-like protein containing a CARD domain (ASC): a 22-kDa protein containing an amino-terminal pyrin domain (PYD)

and a carboxyterminal CARD that can be considered a link between PAMPs/MAMPs/DAMPs-sensing to initiation of inflammation. ASC in macrophages is normally located in the nucleus but pathogen's stimulate it's migration in the cytoplasm where form caspase-1 and NLR perinuclear aggregates, that are fundamental for IL-1β maturation[68].

-Procaspase-1: an inactive form of caspase-1, consists a cysteine proteases that cleave their substrates, adjacent to aspartate residues.

-Pattern recognition receptors (PRRs) that can be considered a "sensor of damage" through the recognition of DAMPs and PAMPs.

PRRs associated with inflammasome can be divided into two main families that allow identifying as many different types of inflammasome[69]:

1. nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs).
2. Absent in melanoma 2 (AIM2)-like receptors (ALRs), that include AIM2 and IFI16.

AIM2 contains an N-terminal pyrin domain and a C-terminal HIN200 domain which bind dsDNA from bacteria and virus in the cytosol, so it is fundamental in immunity against intracellular pathogens like Cytomegalovirus, Francisella tularensis and Mycobacterium tuberculosis.

NLRs consist of:

- a C-terminal end domain characterized by the repetition of a classic leucine-rich motif [leucine-rich repeat (LRR)], hypothetical pathogen-sensing (or auto-inhibitory).

-a central sequence named NACHT (NAIP, CIITA, HET-E, and TPI-1) or NOD

- N-terminal end domain consisting in a pyrin domain (PYD), a caspase-recruitment domain (CARD), a baculovirus inhibition of apoptosis protein repeat (BIR), or other less defined motifs[70].

NLR can be divided into five subfamilies: NLRA, NLRB, NRLC, NLRP, and NRLX. The most characterized NRL are NLRP1, NLRP3, NLRP6, and NLRC4 (IPAF).

-NLRP1 inflammasome associates with caspase-1 and caspase-5 and is composed by (from C to N-terminal end) a caspase-recruiting domain (CARD), a FIND (function to find domain), a leucine-rich repeat (LRR), a NACHT, and pyrin (PYD) residue.

CARD residue allows NLRP1 to activate caspase without interaction with ASC while NACHT domain allows ATP-dependent oligomerization and therefore activation, of the inflammasome. The LRR domain folds on the NACHT domain, thus preventing oligomerization and subsequent activation. The main NLRP1 function is immunity against B. anthracis and anthrax LT (letal toxin) that activate NLRP1-inflammasome

through K^+ efflux and cathepsin B release in the cytoplasm. In fact, LT could mediate ATP release which stimulates P2X7R channel opening to allow cationic flow through plasmatic membrane[71].

- NLRP6-inflammasome appears to play a fundamental role in maintaining the normal intestinal microbiota. Studies on mice lacking NLRP6, have shown its dual role: on one side, in fact, by damping the production of cytokines prevents the formation of a too strong inflammatory response, but on the other side can promote the bacterial dissemination and systemic infection mediated by both gram-positive and gram-negative[72],[73].

-NLRC4-inflammasome like NLRP1, is composed by a CARD domain which can interact directly with caspase-1 without the intervention of ASC. Furthermore, as for NLRP1, LRR has an auto-inhibitory activity. It plays a fundamental role in defense against Salmonella, Shigella, Pseudomonas, and Legionella and activation by PAMPs and DAMPs seems to be mediated by K^+ efflux [70],[74].

-NLRP3 protein is composed by a C-terminal LRR domain that is responsible for inhibition to PAMPs/MAMPs/DAMPs sensing, an N-terminal PYD, and a central NACHT that allows ATP-dependent oligomerization. This NRL protein does not have a CARD domain so ASC is fundamental for caspase-1 recruitment. NLRP3 inflammasome is the best characterize and can be activated by large variety of PAMPs and DAMPs like lipopolysaccharide (LPS), bacterial and viral RNA, viral DNA, ATP, monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD), amyloid-ß, cholesterol crystals, the glycosaminoglycan hyaluronan, alum, asbestos, silica. These multiple activation signals appear to activate NLRP3-inflammasome through a common messenger represented by ATP and its P2X7 channel receptor. After P2X7R activation, K^+ efflux from intracellular to extracellular space, determinate a change in the ionic composition of inflammasome microenvironment that is a stimulus for its assembly and procaspase-1 cleavage. Other common messengers that are supposed to be a link between different PAMPs/DAMPs and activation of inflammasome are: reactive oxygen species (ROS) produced by mitochondria, the release of cathepsin B by lysosome and release of HMGB1[75].

3. Pyroptosis

Caspases are a group of aspartate-specific cysteines proteinase that can be divided in two group: 1) inflammatory caspase (that include caspase-1, -4, -5 and -11) and apoptotic caspase (that include caspase-2, -3, -6, -7, -8, -9 and -10)[76].

Pyroptosis is a form of programmed cell death mediated by caspase-1 (and caspase-11 in mice) occurring in immune cells (monocytes, macrophages, and dendritic cells) in response to PAMPs and DAMPs. This form of cellular death plays a fundamental role in the control of intracellular pathogens infections through the osmotic lysis of the host macrophage due to the formation of pores in the plasma membrane of 1-2,4 nm of diameter[77]. This process can facilitate pathogen removal and clearance and further activate the immune system via the release of potent inflammatory molecules. In addition to intracellular pathogens, pyroptosis can also be triggered by damage molecules contributing to the pathogenesis of autoinflammatory and autoimmune diseases[78].

Pyroptosis can be mediated by other caspases (human caspase-4 and-5 and mouse caspase-11) that are activated by non-canonical inflammasome. In fact, these caspases can recognize LPS from gram-negative bacteria in host cytoplasm[79] especially in intestinal tissue. Gasdermin-D (GSDMD) could explain the link between caspase and pyroptosis. In fact GSDMD, after being cleaved by caspase-1, -4, -5 and 11, could bind to phosphoinositides and cardiolipin to form pores in plasmatic membrane[80]. In the course of plasma membrane damage, the fusion of lysosomes is normally carried out for its repairing purpose. During pyroptosis, there is an increase in lysosomal exocytosis mediated by the intracellular entry of Ca^{2+} through the formation of plasmatic pores mediated by caspase-1. Lysosomal exocytosis can occur independently of caspase-1 activation, being secondary to Ca^{2+} influx through the channel formed by P2X7R after stimulation of ATP, as demonstrated by studies on Casp1-/- macrophages. During the exocytosis of the lysosomes, debris of microbes and lysosomal proteins are released. The presence of lysosomal enzyme in extracellular space allows an anti-microbial action aimed to defense against extracellular pathogens[81].

4. P2X7R and cell survival

In addition to the production of cytokines mediated by inflammasome and its role in inducing the inflammatory response, P2X7R is able to regulate cell growth and proliferation. Numerous studies have highlighted in particular the role of P2X7R in the proliferation and activation of TCR-mediated T lymphocytes and L-selectin shedding (CD62L). In particular, the increase of intracellular Ca^{2+} mediated by P2X7R, allows to activation of NFAT (nuclear factor of activated T cell) that activates the transcription of the gene for IL-2 and the proliferation of T lymphocytes[82]. Ca^{2+} influx through P2X7R, mediate also the activation of the nuclear factor kappa B (NF-kB), an important transcriptional factor involved in the production of pro-inflammatory cytokines (IL-1β, IL-18, IL-6, IL-8 and TNF-α)[83], and apoptosis and hypoxia-inducible factor 1a (HIF-1a) consisting in a transcription factor release in case of hypoxia which is typically activated during neoplasm and allow transcription of multiple genes involved in apoptosis resistance, inflammation, angiogenesis, tumor invasions and metastasis diffusion[84]

If prolonged stimulation with ATP of P2X7R can induce apoptosis, a stimulation with low doses of ATP is able to induce cell proliferation and survival[85]. P2X7R, therefore, shows a dual role depending on the degree of activation by ATP (in terms of concentration and duration of stimulation) and based on the expression of the receptor itself, which may vary depending on the type of lymphocyte.

While in fact, Jurkat cells express low levels of P2X7R, thus prevailing the anti-apoptotic effect, other types of lymphocytes such as CD4+ presenting six times higher levels of P2X7R, are more sensitive to P2X7R-mediated apoptosis[82]. So depending on the level of expression of the receptor and the type of cell where it is expressed, pro-proliferative or pro-apoptotic action may prevail. This dual aspect of P2X7R, pro-inflammatory and pro-apoptotic action on one side, and the promotion of cell growth and survival on the other, could be mediated by mitochondria. Studies with HEK293 cells (human embryonic kidney) transfected with P2X7R, have clarified some aspects in this regard. Under basal conditions, there is a release of endogenous ATP that in some portions of the plasma membrane would tend to concentrate up to stimulate in a tonic and sub-maximal way P2X7R. This stimulation would lead to the entry of a weak flow of Ca^{2+} into the cell that would be conveyed, probably through the endoplasmic reticulum, into the mitochondria. The increase of intramitochondrial Ca^{2+} and consequently of the mitochondrial potential (also through the driving of protons) would activate oxidative phosphorylation and the

production of ATP. The accumulation of intra-mitochondrial energy would, therefore, allow the cells to survive, especially in conditions of nutrient deprivation. These aspects are confirmed by the fact that enzymes that inactivate ATP (as apyrase) or that block P2X7R (oxidized ATP) or finally that completely chelate extracellular calcium, block cell survival, and proliferation.

On the contrary, a maximal activation of P2X7R, would determine a massive flow of Ca^{2+} at the intracellular and mitochondrial level, with consequent potential fall. This would lead to damage of the mitochondrial structure and consequently to cell apoptosis[85].

Innate immunity and Systemic Lupus Erythematosus (SLE)

SLE has traditionally considered the prototype of autoimmune disease in which adaptive immunity is the main driver.

More recent evidence emphasize a possible role of innate immunity, and in particular of the purinergic system, in the pathogenesis of SLE.

Several mouse models have allowed studying these aspects:

1. the NZB/W F1 strain, in which especially females are affected by a lupus-like disease;
2. the MLR/lpr strain, characterized by the lpr mutation that is known to impair transcription of the Fas receptor;
3. the BXSB/Yaa strain, in which Yaa is an element termed Y-linked autoimmune accelerator due to a translocation resulting in duplication of at least 16 genes, among which TLR7;
4. pristane-induced lupus model, obtained by intraperitoneal injections of pristane (Tetramethylpentadecan) is characterized by autoantibody production, arthritis, and severe glomerulonephritis [86].

In mouse models of lupus glomerulonephritis, it was possible to highlight the role of IL-1β in the pathogenesis of this complication. In fact, renal concentration of IL-1β correlates with the severity of kidney lesions, while mice that do not express this cytokine are protected. Study with female MRL/lpr mice showed that there is an increased renal expression of P2X7R, NLRP3, ASC and caspase 1, resulting in increased production of IL-1β and IL-18 compared to healthy mice models (MRL/MpJ). In treated MRL/lpr mice with the P2X7R antagonist Brilliant Blue G (BBG), it could be observed a decrease of proteinuria, serum anti-DNA level and, at

renal histology, a reduction in glomerular cellularity, signs of vasculitis, formation of crescent, and IgG and C3 deposition. At splenic level after treatment with BBG, a reduced Th17/T helper ratio has also been shown, highlighting how P2X7R can play a fundamental role in the induction of Th17, which, through the production of IL-17, are fundamental in the lupus pathogenesis [87].

In MRL/lpr mice model mesangial cells, macrophage-like cells resident in the kidney, produce reactive oxygen species (ROS) that regulate renal blow flow and when elevated, enhance tissue damage. Mesangial cells release other mediators as cytokines like IL-6 and TNF-α and eicosanoids. AMP-activated protein kinase (AMPK) is an enzyme that is activated during oxidative stress and in depletion of growth factors and glucose. AMPK inhibits biosynthetic pathway and inflammation and promote catabolic pathway generating ATP. In LPS/IFN-g-stimulated mesangial cells of MRL/lpr mice, treatment with 5-amino- 4-imidazole carboxamide riboside (AICAR), a compound that activates AMPK, inhibits production of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 and IL-6[88]. Other authors have shown that the inhibition, and not the activation of AMPK, is able to improve lupus nephritis. A study conducted on mouse with pristane-induced lupus, showed that piperine (phytochemical present in black pepper) treatment, through blocking AMPK activation, inhibits pyroptosis, activation of NLRP3 inflammasome and IL-1β production and release[89]. These observations from both human and murine models emphasize the role of the P2X7R-NLRP3 axis in the pathogenesis of lupus, representing a possible therapeutic target to be studied for the possible development of new treatments and also targeting AMPK, would be a target to reduce inflammation in SLE.

Another study with murine models of lupus induced by intraperitoneal injection of pristane, confirmed the role of the inflammasome and in particular of caspase-1 in SLE. Comparing wild-type (WT) with casp-1-/- mice after pristane treatment, it was shown that the lack of caspase-1 is associated with a reduction in IL-1β, IL-18 and inflammasome protein (NLRP3) transcripts in the splenic tissue. Moreover, in casp-1-/- mice, the production of autoantibodies (anti-DNA and anti-RNP) and hypergammaglobulinaemia was reduced after 6 months from pristane treatment, demonstrating how the enzyme plays a role in the activation of humoral immunity. Caspase-1 through increased cell death (pyroptosis) and consequent externalization of auto-antigen, promote the formation of ICs and enhance type I IFN response[90].

In humans, a study of renal biopsy samples showed that P2X7R expression was increased in lupus patients compared to controls[91] and that serum levels of IL-1β are increased in SLE patients[92].

Environmental factors like bacterial and virus infection (not yet specifically identified), are fundamental in lupus pathogenesis in a subject genetically susceptible. Cell death may be considered a form of host defense by limit the possibility of the infectious agents to replicate.

In healthy subjects, these processes are normally controlled, so there is an adequate clearance of cellular debris secondary to cell death by cellular and humoral immunity, without an inflammatory response. In the case of SLE, this clearance process is inadequate and leads to the accumulation of nuclear debris, and consequent production of auto-antibodies and patients with genetic deficiency of the C1q protein that is fundamental to promote clearance of apoptotic cells, is highly associated with lupus.

Pyroptosis, as previously described, is a form cells death mediated by activation of the inflammasome, which could be started by with recognition by sensor proteins, including NLR, of PAMPs and DAMPs released during infection. Once activated caspase-1, besides the production and release of IL-1β and IL-18, promote cell death through DNA cleavage, nuclear condensation, lysosome exocytosis, and plasma membrane pore formation[93]. Monocytes and macrophages are the main sources of IL-1β, and pro-IL-1β production is mediated by TLR activation. In studies carried out in vitro, it was shown that the complement could also contribute to the production of IL-1β in monocytes. Stimulation with C3a of monocytes previously stimulated with LPS in fact, induces the release of ATP in the extracellular environment with consequent activation of P2X7R and activation of NLRP3 inflammasome[94].

As previously described, during pyroptosis and during the activation of P2X7R, occurs lysosome exocytosis through the entry of Ca^{2+} into the cell. In addition to lysosomal enzymes and partially processed pathogens, even autoantigens can be released into the extracellular environment during this process becoming a target for the antigen presenting cells, thus stimulating the autoimmune response. It has also been hypothesized that the LE phenomenon, consisting of phagocytosis by a polymorphonuclear cell of a nucleus thanks to the presence of ANAs, may be secondary to a phenomenon of pyroptosis that determines, unlike apoptosis, condensation of the nucleus without compromising its integrity[95].

During the process of pyroptosis, but also apoptosis and necrosis, cells release HMGB1 (high mobility group box 1). This is a non-histone nuclear protein of 215 amino acids long, consisting of two DNA binding regions (A and B boxes) and a C terminal acidic tail that binds histone. Inside the cell, HMGB1, like histone H1, travels freely in the nucleus.

The redox status of three cysteines residues at positions 23, 45 and 103, confers to HMGB1 different activity:

-after binding the CXCL12 chemokine, stimulate chemotaxis via CXCR4, when all three cysteines residues are reduced;

- in presence of disulfide bond between positions 23 and 45 and a thiol at position 103, induce cytokine production, through binding TLR4;

-lose of immune activity in case of complete oxidation[96].

In case of apoptosis the completely oxidated and immunological inactive form prevails, while in case of pyroptosis both totally reduced form and disulfide HMGB1 can be present, thus promoting the chemotaxis and the activation of the immune cells by release of cytokines.

HMGB1 can not only be released cells but can itself induce pyroptosis in macrophages after being endocytosed through binding surface receptor for advanced glycation end products (RAGEs) that induces cathepsin-B activation and lysosome rupture, which is followed by caspase-1 activation and pyroptosis of the cell[97].

NETosis, is another type of cell death firstly associated with neutrophils, causes the extrusion of nuclear DNA, histones and granular antimicrobial proteins entrapped, called neutrophil extracellular traps (NETs). Physiologically, phagocytes ingest and degraded NETs in the lysosomes through C1q and DNAase without inflammatory reaction. In SLE impaired clearance of NETs (like inadequate clearance of apoptotic bodies) is responsible for the accumulation of several autoantigens including self-dsDNA[98]. In macrophages of lupus patients, it has also been shown that NETs are able to activate the inflammasome. NET are found in a particular subset of low-density granulocytes (LDGs) present in the circulation in patients with SLE and it is hypothesized that an imbalance between production and clearance of NET is responsible for loss of tolerance and tissue damage [99].

Evaluating in more detail the activation of caspase-1, it was observed that only the macrophages that were treated with NET, and in particular with LL37, showed the activation of caspase-1 by the assembly of NLRP3-inflammasomes. LL37 is one of

the antimicrobial proteins present in NETs and, as previously described, it would be able to also have a role as a non-nucleotide agonist for the activation of P2X7R in monocytes[100]. If K^+ efflux is inhibited by placing the cells in a medium with a high concentration of potassium, it has also been shown that LL37 is not able to induce the production of IL-1β and IL-18 by macrophages of SLE patients. For the production of these cytokines, it is therefore fundamental the simultaneous presence of P2X7R ion channel that opening and allowing the outflow of K^+ allows the assembly of the inflammasome and the consequent activation of caspase-1. In NETs, self DNA bounded to antimicrobial peptides, becomes more immunogenic and once internalized in pDC, by binding with TLR-9, induces the production of type I interferon essential for differentiation of monocytes into dendritic cells that stimulate autoreactive B and T[101].

TLRs can be considered as PRRs that promptly activate the innate immune response, through action on many inflammatory pathways including the inflammasome promotion[102]. For IL-1β and IL-18 production, in fact, two signals are fundamental: one given by TLR stimulation, which increases pro- IL-1β/pro-IL-18 levels and enhances NLRP3 expression, and the second given by ATP via P2X7R[103].

TLRs can bind different ligands derived from pathogens and endogenous tissue that include nucleic acids, proteins, and lipids.

They can be divided into:

-extracellular like TLR-2, -4, -5;

-intracellular like TLR-3,-7,-9.

TLR-2 and TLR-4 seem to be associated with autoimmune disease both in human and mouse models. In particular, in SLE patients TLR-2 seems to be upregulated in PBMCs[104,] and is increased also in CD8+ T cells, CD19+ B cells, and CD14+ monocyte when compared to healthy control. After isolation of CD4+ T cell from SLE patients, stimulation of these cells with TLR-2 agonist induce production of pro-inflammatory cytokine including IL-6, IL-17A, IL-17F, and TNF-α. Moreover, after TLR-2 activation, it can be observed a decrease of ratio Treg/Th17 contributing to the loss of self-tolerance[105].

Between TLR locate inside the intracellular endosome, TLR-7 and TLR-9 are the most studied and have more evidence about their role in SLE pathogenesis. TLR-7 recognize viral double strand-RNA inducing cytokine and INF type I production. In human and murine SLE models, TLR-7 act as a sensor of endogenous

ribonucleoprotein antigens and active B cell, macrophage e pDC. TLR-9 recognize microbial not methylated 20-deoxyribose (cytidine-phosphate-guanosine) DNA (CpG DNA). Both mediate their signals through Myeloid differentiation primary response 88 (MyD88). Studies from mouse models, suggest a pathogenic role for TLR7 and a protective role for TLR9 in the pathogenesis of SLE**Errore. Il segnalibro non è definito.**.

Many studies compared the differences in the expression of TLR-7 and TLR-9 between SLE patients and healthy controls, with evidence of increased expression of both in PBMCs[104], while other studies confirmed an increased expression of TLR-9 in monocytes, T cells, and B cells, while TLR7 expression was not different between control and SLE groups[106]. Comparing pDC of patients with SLE with those of healthy patients, it was seen that, after stimulation with apoptotic cells derived from polymorphonuclear cells (PMNs), pDC from patients expressed less TLR-9 and were less able to induce the differentiation of T lymphocytes in regulatory T cells[107]. In other observation, TRL9 stimulation seems to implicated in B cell maturation into producing antibodies plasmablast and activation of IL-6 and TNF-a release[108].

Among the molecules that activate IL-1β production, U1-snRNP, an intranuclear molecular complex composed by U1-snRNA, Sm and U1-specific protein, was also identified. In SLE antibodies against U1-snRNP are commonly found, and B cells and pDC can be activated by U1-snRNP through bindings of endosomal TLR-7 and -8. Human monocytes, especially in the presence of U1-snRNP antibodies, increase the production of IL-1β after binding of U1-snRNP with TLR-7 which, by activating NFkb, stimulates pro-IL-1β production.

The K^+ efflux and the presence of ROS however, are fundamental for the maturation for the activation of the NLRP3-inflammasome and the consequent formation of mature IL-1β, as previously indicated. ROS in particular, through separation of thioredoxin-interacting protein from thioredoxin, activate NLRP3 inflammasome allowing binding of thioredoxin interacting protein to NLRP3[109]. Not only NLRP3 inflammasome can be activated during SLE. AIM2 inflammasome, for example, appears to be activated directly by apoptotic DNA as demonstrated in mouse models of SLE nephritis, where it was observed that macrophages are activated directly by DNA through AIM2. In patients' PBMCs, an increased expression of AIM2 was also shown and could correlate with disease activity expressed as SLEDAI[110].

Also, IF6 inflammasome is able to recognize cytoplasmic DNA and consequently to activate caspase-1 and to induce the production of type I INF[111].

Once IL-1β and IL-18 are produced, they play a fundamental role in autoimmune response activation. IL-1β promote Th17 cell differentiation in both human and mice models.

The activity of IL-18 depends on cooperation with other cytokines. For example in association with IL-2 induce IFN-γ-producing Th1 cells and in association with IL-23, enhance IL-17 production from polarized Th17 cells[112].

Genetic polymorphism predisposing to SLE

Many genetic polymorphisms, involving different components of the innate immunity so far described, seem to confer susceptibility to SLE.

For example regarding P2X7R, a study on Chinese population, has shown that rs1718119 (1068G>A) determines a change from threonine to alanine at amino acid 348 in the second transmembrane region of the receptor, conferring an allosteric modification to the structure and stability of the pore structure. In this case carriers of A allele are at lower risk of SLE. G allele and CG/(CG+GG) genotypes of rs2230911 were at a lower frequency among SLE patients with nephritis. In fact rs2230911 (1096C>G) determines a change from serine to threonine at amino acid 357 (Ser357 to Thr) conferring a partial function loss of P2X7R[113].

In a meta-analysis performing to evaluate polymorphism of TLR genes associated to SLE, TLR-7 polymorphisms rs179008 and rs3853839 confer susceptibility in African and Asian patients, while for TLR-9 rs187084 confers susceptibility in the overall population[114] .

As regards the components of the inflammasome, the NLRP1 rs2670660 minor G allele was found to be more frequent in SLE in a study carried out on a Brazilian population[115]. CARD8 rs2043211 variant A allele seems to be associated in SLE susceptibility only in males[116]. As regards IL-1β, allele T at position 3953 of rs1143634 seems to confer protection to the development of SLE, being associated with a reduced production of the cytokine[117]. In a meta-analysis conducted on RA and SLE population IL-18 rs187238 (137G > C) seems to be associated to the risk of SLE development[118].

RESEARCH PROJECT

Rational and aim of the study

The evidence so far described, suggests that innate immunity could be considered a possible therapeutic target in SLE and some attempts in this regard have already been made. For example anakinra, IL-1 receptor antagonist, was shown to be effective and safe in a small study conducted on SLE patients with refractory joint involvement treated for 3 months at the dosage of 100 mg per day[119]. Anti-malarials play an important role in reducing IL-1β, IL-6 and TNF-α production by macrophage and monocyte through their action on TLR[108],[120].

Colchicine and related plant extracts have been used, for more than 2000 years, to treat gout flares and auto-inflammatory disease like familial Mediterranean fever (FMF). Its primary molecular target is β-tubulin, forming a complex that prevents microtubule polymerization and, at high concentrations, causes microtubule depolymerization. The anti-inflammatory properties of colchicine are well characterized in various models of inflammation, including some that involve ATP signaling. There are different reports that P2X7R binds to cytoskeletal elements, including β-tubulin, which are probably involved in channel permeabilization. Several different P2X7R dependent activities are affected by colchicine treatment. Firstly, colchicine diminishes ATP-induced cationic dye uptake in mouse macrophages, witnessing the requirement of microtubules for P2X7R-dependent pore formation. Moreover, the microtubule rearrangement was interpreted as necessary also for IL-1β release, since microtubule demolition with colchicine inhibited the ATP-induced P2X7-dependent release of this cytokine in mouse macrophages[121]. Colchicine is a proposed treatment in SLE serositis, a clinical manifestation that is characterized by increased of inflammation parameters.

The aim of the present study is to explore more deeply the role of the innate immune system in SLE firstly evaluating the P2X7R and NLRP3 expression and activity in lupus patients, and secondly perform a sub-analysis on patients with a history of serositis. The inflammasome is able to directly determine the production of IL-1β and IL-18. With the activation of P2X7R, however, there is also the release of other inflammatory cytokines such as IL-1α, TNF-α and IL-6 but independently of the

inflammasome**Errore. Il segnalibro non è definito.**. During SLE, the altered balance between release and clearance
of cellular death products (secondary to apoptosis, pyroptosis or netosis), leads to the production of a damage signal mediated by different DAMPs, which by binding P2X7R or other PRPs as TLR, activate NF-kb, an important transcription factor for the synthesis of cytokines fundamental in SLE pathogenesis such as TNF-α and IL-6 [122]. To fully investigate the activity of P2X7R, we decided to test both IL-1β and IL-6, as representative of two different pathways by which this purinergic receptor can mediate inflammatory cytokine production.

Materials and methods

1.Patients and study design

Patients satisfying the 1997 revised American College of Rheumatology criteria[123] **(Appendix 1)**, were recruited consecutively from the Rheumatology Unit, S. Anna Hospital, University of Ferrara. Clinical (past or ongoing manifestation), serological demographic characteristics and concomitant therapy were retrospectively recorded.

Disease activity and cumulative damage were assessed by SLE disease activity index-2000 (SLEDAI-2K) (**Appendix 2**)[124] and the Systemic Lupus International Collaborating Clinics (SLICC) (**Appendix 3**) [125]respectively, and recorded in clinical charts and dedicated database.

Sero-immunologic tests included:

1. antinuclear antibodies (ANA) assessed by indirect immunofluorescence using Hep2 cells as substrate (positivity was defined as a titer ≥ 1: 160);
2. C3 and C4 dosage 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);
3. anti-DNA detected by immunofluorescence using Crithidia luciliae (positivity was certified if positivity was checked in at least two separate measurements, with a cut-off titer of 1:40);
4. anti-SSA, anti-SSB, anti-Sm and anti-U1RNP were detected by immunoblot technique;
5. lupus anticoagulant (LA) was measured accordingly with the recommendation of the Scientific and Standardization Committee of the International Society of Thrombosis and Hemostasis and anti-cardiolipin (aCL) and anti beta2 glycoprotein (beta2GPI) were measured by enzyme-linked immunoadsorbent assay (ELISA)[126]. If the result was confirmed in two separate measurements performed 12 weeks apart, positivity for antiphospholipid (aPL) and LA was defined[127];
6. erythrocyte Sedimentation Rate (ESR) and C Reactive Protein (CRP) were performed according to procedures of local laboratory.

Healthy subjects (HC; n=20), matched for similar age and sex ratio, volunteers from Ferrara University Hospital Blood Bank, were considered as control group.

All patients and HC underwent venous blood collection in EDTA tubes after giving written informed consent. The study was approved by the local ethics committee and conducted in accordance with Helsinki Declaration.

2.Separation of plasma and peripheral blood mononuclear cells (PBMCs)

Plasma was obtained by centrifugation of blood samples at 2500 rpm for 15 min at 4°C. Plasma was divided into aliquots, frozen at -80°C and subsequently used in ELISA to measure cytokines concentration. The remaining cellular component was used to separate PBMCs by stratification on a Ficoll (Health Care, Uppsala, Sweden) gradient, followed by centrifugation at 2100 rpm for 20 min at room temperature (RT). The layer of separated cells was collected and subjected to 3 washes with PBS, centrifuging each time for 10 min at RT at decreasing rates: the first at 1900 rpm, the second at 1600 rpm and the third at 1100 rpm. The cells obtained were counted in a Burker's chamber and divided into aliquots each of $2x10^6$ cells, and used for setting up short-term cell cultures, measurement of calcium fluxes in fluorimetry and RNA extraction following resuspension in Trizol (Thermo Fisher Scientific, Waltham, MA, USA).

3.Preparation and stimulation of PBMCs cultures

PBMCs used for short-term culture, were re-suspended at the concentration of $5x10^5$ cells/ml of 10% FBS supplemented RPMI (Gibco, Thermo Scientific, Waltham, MA, USA) without antibiotics and placed into 12-well plates for cell culture, using 4 wells for each patient. After an overnight incubation at 37°, 5% CO_2, non-adherent cells were removed and adherent mononuclear cells (monocytes/macrophages) were treated as follows:

1. culture medium (RPMI + 10% FBS) was added to all wells that were numbered from 1 to 4;
2. lipopolysaccharide (LPS) (Sigma Aldrich, Saint Louis, MO, USA) at 1 μg/ml concentration, was added to culture medium in wells numbered as 2 and 3 and kept for 4 hours at 37°in 5% CO_2;
3. after 4 hours, 500 μM BzATP (Sigma Aldrich) was added to wells 3 and 4 for 1 hour (table 5).

At the end, the supernatants were collected, centrifuged and finally frozen at -80°C until use.

Table 5: Representation of the method of stimulation of PBMCs in the different wells

Well n°1	Well n°2	Well n°3	Well n°4
RPMI	RPMI+LPS	RPMI +LPS+ BzATP	RPMI + BzATP

4.Measurement of IL-1β AND IL-6 concentration by ELISA

IL-1β and IL-6 concentration in both plasma and PBMCs supernatants were measured by the ELISA technique, using the human IL-1β / IL-1F2 and the human IL-6 Quantikine ELISA kits (R&D System, Biotechne, Minneapolis, MN, USA) respectively, following manufacturer's instructions. The standard IL-1β or IL-6 were resuspended in diluent Calibrator to obtain the stock solution of 250 pg/ml, stirred for 15 min before serial dilution up to the final concentration of 3.9 pg/ml. Standard and samples were tested in duplicate. Optical density was detected using a Thermo Scientific Multiskan FC plate reader. Cytokines concentrations were calculated using the calibration curve whore R^2 value ranged from 0.97 to 0.99, and expressed as pg/ml.

5.RNA isolation

RNA was extracted from Trizol-resuspended PBMCs using the PureLink RNA Mini Kit (Thermo Fisher Scientific) following manufacturer's instructions. RNA was resuspended in RNAse free water and its concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

5.1 Real Time PCR (RT-PCR)

RNA retro-transcription was performed using the High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific). RT-PCR for P2X7R, NLRP3 and G3PD as endogenous control was performed using an RT-PCR kit (Thermo Fisher Scientific) in a PCR Biometra UNO- Thermoblock (DASIT, Cornaredo, Milan, Italy) using the following primers: Human p2x7 assay ID: Hs00175721_m1, Human NLRP3 assay ID: Hs00366465_m1; pre-developed taqman endogenous control human GAPDH code 4326317E was used as housekeeping gene.

6.DNA extraction

DNA was extracted from whole blood using the Qiagen DNA extraction kit (Qiagen, Venlo, Paesi Bassi) and resuspended in 200 µL of nuclease free water. DNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific)

6.1 Choice of polymorphisms

In our previous studies we evaluated the role of two polymorphisms that showed a correlation with different diseases and on which our center has a long time experience[128], [129].

One of these polymorphism, the 1513A>C, causes the substitution of a residue of glutamic acid with alanine in position 496, resulting in a loss of functionality of P2X7R or of reduction of activity in case of heterozygosis. On the contrary, the variant 489 C>T determines an increase in functionality through the change from of histidine residue to tyrosine in position 155[130]. A previous evaluation on SLE patients fail to demonstrate a correlation between 1513A>C and the disease[131] , without however considering the clinical data and the disease activity, whereas no studies evaluate the role of 489 C>T in lupus susceptibility. Study of polymorphism was performed using the following TaqMan probes: C 27495274 10 (rs3751143)

1513A/C, C 3019032 1 (rs208294) 489C/T. At the end of the reaction, samples were analyzed by the Applied Biosystem tool StepOne Real-Time PCR System.

7.Measurement of changes in intracellular Ca2 + concentration

Variations of intracellular calcium levels ($\Delta[Ca2]_i$) were measured using the Fura-2/AM (Sigma Aldrich) fluorescent probe. For this $2x10^6$ PBMCs were loaded with 2 μM Fura-2/AM for 20 min at 37°C. After washes, PBMCs were employed for the assay at 37°C in a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Cernuso SN, Milan, Italy) using 500 uM BzATP as stimulus. In some experiments, PBMCs were pre-incubated for 1 hour at 37°C in 5% CO2 with 20-200 μM chloroquine (Sigma Aldrich), washed and tested for variation of $[Ca2]_i$ as above.

8.Statistical analysis and data representation

For descriptive analysis, discrete variables were expressed as absolute and relative frequencies, while continuous variables as mean and standard deviation (SD). Comparison between the groups was performed with the test of chi- squared, t test or Mann-Whitney test for non-parametric variables. To evaluate the relationship between continuous variables, the correlation index for Spearman ranks was used where indicated. The analyses were performed using the Graphpad version 6.

Results

1.Clinical characteristics

A total of 48 SLE patients (45 women and 3 men) were enrolled, 16 (33.3%) had a history of serositis (previous or ongoing at the time of blood collection). The mean age (mean± SD) of SLE population was of 41.9±10.2 years, disease duration 130.6±96.7 months, the mean SLEDAI-2K 4.2± 4.4 and the mean SLICC equal to 0.6±0.8. Demographic, clinical and pharmacologic treatments of all patients were collected (Table 6). No significant difference in clinical characteristics between patients with (SLE-S) or without (SLE-NS) a history of serositis was found, except CRP, which was significantly higher in SLE-S (Table 7).

In addition, 20 HC matched for age and sex ratio, were enrolled.

2.Evaluation of plasmatic levels of IL-1β and IL6

Plasmatic IL-1β levels did not differ significantly between patients (SLE) and healthy controls (HC) (Fig. 1A) and a sub-analysis performed in SLE-S and SLE-NS gave similar results (Fig. 2A, Table 8). On the contrary, plasmatic IL-6 levels resulted higher in SLE patients (3.09±0.57 pg/ml) compared to the HC (1.58±0.67 pg/ml) (Fig. 1B, Table 8), particularly, in SLE-S (4.8±0.97 pg/ml) versus both SLE-NS (2.02±0.37 pg/ml) (p=0.009) and HC (1.58±0.67 pg/ml) (p=0.037) (Fig. 2B, Table 8).

3.Evaluation of IL-1β and IL-6 in cultured monocytes supernatants

Release of IL-1β from monocytes/macrophages cultured in 10% FBS-supplemented RPMI alone or stimulated for 4 h with 1 μg/ml LPS, was not significantly different between SLE and HC (pg/ml; mean±SD) (Fig. 3A, B). Stimulation of monocytes/macrophages with 500 μM BzATP for 1 h following LPS, provoked IL-1β release significantly reduced in SLE-S compared to SLE-NS (988.7±103.6 vs 1237±70.39; p=0.048) (Fig. 3C). The most relevant difference between SLE and HC was visible when monocytes/macrophages were stimulated with BzATP alone (Fig 3D). In this case IL-1β release was significantly lower in SLE patients respect to HC (SLE-NS vs HC= 11.84±4.68 vs 66.19±12.31, p<0.0001; SLE-S vs HC= 11.95±4.99 vs 66.19±12.31, p=0.0002). IL-1β was not different between patients with serositis vs patients without serositis in all different experimental condition (Table 8).

IL-6 was found significantly higher in supernatants of monocytes/macrophages from SLE compared to HC in all different experimental conditions (pg/ml; mean±SD) (Fig.

4, Table 8). IL-6 released from unstimulated monocytes maintained for 5 h in RPMI was significantly higher in SLE patients vs HC (SLE-NS vs HC: 329.0±83.74 vs 11.83±0.51, p= 0.0102; SLE-S vs HC= 29.18±4.85, p= 0.0021) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.014) (Fig. 4A, Table 8). The release of IL-6 from monocytes stimulated for 4 h with 1 μg/ml LPS was significantly higher in SLE patients vs HC (SLE-NS vs HC: 1172±74.25 vs 339.9±41.37, p<0.0001; SLE-S vs HC= 728.8±101.2 vs 339.9±41.37, p=0.002) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.001) (Fig. 4B, Table 8). Stimulation of monocytes for 4 h with 1 μg/ml LPS and for 1 h with 500 μM BzATP, produced a significantly higher IL-6 release in SLE patients vs HC (SLE-NS vs HC: 1159±83.87 vs 330.7±43.46, p<0.0001; SLE-S vs HC= 719.7±112.6 vs 330.7±43.46, p=0.004) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.003) (Fig. 4C, Table 8). Analogously, IL-6 released from monocytes stimulated for 1 h with BzATP was significantly higher in SLE patients vs HC (SLE-NS vs HC: 328±92.1 vs 13.38±0.76, p=0.019; SLE-S vs HC= 43.95±9.49 vs 13.38±0.76, p=0.005) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.028) (Fig. 4D, Table 8).

4.Evaluation of Calcium influx in PBMCs with Fura2/AM after BzATP stimulation.

Variation of intracellular Ca2+ concentration ($\Delta[Ca2+]_i$ (Fura2) nM ±SE) after stimulation with BzATP, a direct measurement of P2X7R activity, was significantly lower in SLE than in HC (SLE vs HC= 64.1±6.84 vs 105.5±12.75, p=0.008; SLE-NS vs HC= 67.69±9.23 vs 105.5±12.75; p=0.0267; SLE-S vs HC= 58.25±10.28 vs 105.5±12.75, p=0.0215), with no difference between patients without serositis vs patients with serositis (SLE-NS vs SLE-S, p=0.674) (Fig. 5A, Table 8).

To evaluate the effect of chloroquine on P2X7R activity, PBMCs from 4 controls and 3 patients underwent an evaluation of Ca2+ influx in normal conditions and after treatment for 1 h with 200 μM chloroquine. The $\Delta[Ca2+]_i$ was higher after chloroquine treatment respect to basal conditions (Wilcoxon matched-pairs signed rank test p=0.016) both in controls (pre vs post treatment: 92.0 ±11.07 vs 132.3± 22.18; p=0.13) and patients (48.67± 15.68 vs 81± 20.65; p=0.25) (Fig. 5B, Table 8).

5.Evaluation of P2X7R and NLRP3 expression with RT-PCR

At RT-PCR, P2X7R resulted significantly less expressed in patients compared to controls (mean±SD) (SLE vs HC= 0.87±0.1 vs 1.29±0.13; p=0.02), particularly in patients with serositis (SLE-S vs HC= 0.724±0.11 vs 1.29±0.13; p=0.019) (Fig. 6A, Table 8). On the contrary, NLRP3 expression resulted significantly higher in patients than in controls (SLE vs HC= 3.80±0.46 vs 1.99±0.30; p=0.018) also considering the two sub-categories separately (SLE-NS vs HC= 3.846±0.633 vs 1.995±0.302, p=0.013; SLE-S vs HC= 3.702±0.552 vs 1.995±0.302, p=0.036) (Fig. 6B, Table 8).

6.Evaluation of P2X7R polymorphisms

To evaluate if the results obtained were due to the presence of polymorphisms of the P2X7R gene, 33 patients who became available for further blood collection, were subjected to analysis of the polymorphisms 1513A> C and 489 C> T. As regard the 1513A>C polymorphism, 20 patients (60.6%) presented the A/A phenotype (normal activity) and 13 patients (39.4%) presented the A/C (reduction of activity), while nobody had phenotype C/C (complete loss of function). Considering the 489 C>T variant, 7 patients (21.2%) presented C/C (normal activity), 12 (36.4%) C/T (intermediate increased activity) and 14 (42.4%) T/T (complete increment of activity) (Table 9). The overall activity of P2X7R was then evaluated considering the combined action of the two polymorphisms, which allowed to divide the patients, according to the overall effect of the phenotype, into three groups: gain of function (GOF), normal function (NF), and loss of function (LOF). Overall, 21/33 patients presented a GOF, 10/33 a normal activity and 2/33 a LOF. Considering patients with NF and LOF in a single group, IL-1β levels produced by stimulated PBMCs and Ca2+ influx after stimulation with BzATP, resulted not correlated with the genotype conferring a GOF (Table 10).

7.Evaluation of the role clinical factor on P2X7R activity evaluated by ΔCa2+ (Fura2)

No significant correlation between disease activity/disease duration and P2X7R activity assessed by $\Delta[Ca2+]_i$ measurement was detected (Table 11). For the evaluation of the effect produced by the therapy, due to the low number of the sample, we have collected all patients taking immunosuppressive therapy in a single

group called "ongoing major immunosuppressive therapy". We also assessed separately the influence of ongoing hydroxychloroquine and of ongoing steroids therapy (Table 11). Finally, the effect of steroid dosage (current or cumulative) was assessed (Table 11). No significant influence of therapy on Ca2+ intake was detected.

Discussion

The aim of our study was to evaluate the expression and the activity of P2X7R and NLRP3 inflammasome in a cohort of patients affected by SLE, analyzing more deeply the cases that presented a history of serositis.

Inflammation parameters [Erythrocyte Sedimentation Rate (ERS) and C-reactive protein (CRP)] are generally altered in the SLE during few conditions like infection or serositis[132]. In addition, this manifestation responds promptly to colchicine, a treatment generally used in diseases considered “inflammasome guided” as the family Mediterranean fever [133],[134].

Evidence in both mouse models and humans has revealed a possible role for P2X7R and inflammation in SLE.

MRL/lpr mice showed increased renal expression of P2X7R, NLRP3, ASC and caspase 1, resulting in increased production of IL-1β and IL-18 and treatment with the P2X7R antagonist Brillian Blue G (BBG) decreased proteinuria, serum anti-DNA antibodies and, at renal histology, reduced glomerular cellularity, signs of vasculitis and IgG and C3 deposition[87]. Also in lupus-like nephritis induced by intraperitoneal injection of pristane, caspase-1-/- mice presented reduction of autoantibodies (anti-DNA and anti-RNP) and hypergammaglobulinemia comparing to pristane-treated wild type mice[88]. In humans, a study on renal biopsies showed increased P2X7R expression in lupus patients compared to controls[91] and increased IL-1β serum levels in SLE patients has been demonstrated[92].

These aspects made us hypothesize that P2X7R-inflammasome axis could be a fundamental actor in the pathogenesis of SLE and especially of this clinical manifestation.

Our study does not seem to confirm the initial hypothesis of a role of P2X7R, as an inducer of the inflammatory response in patients with SLE. Furthermore, from the sub-analysis performed by dividing the patients into two groups based on the presence of a history of serositis, a hypothetical role of the receptor in the pathogenesis of in this clinical manifestation was not confirmed. This finding does not seem to be justified by differences between SLE-NS and SLE-S in the clinical parameters collected, with no significant difference in disease activity, demographic characteristic, and treatment (table 7).

Through the RT-PCR determination, expression of the P2X7 receptor in PBMCs of SLE patients was lower compared to HC, especially in patients with a history of serositis. In contrast, mRNA levels for NLRP3 resulted increased in SLE patients

compared to controls. These data suggest a possible compensatory mechanism between NLRP3 and P2X7R.

In addition to lower expression, a reduction in activity was evidenced by the assessment of variation of $[Ca2+]_i$ with Fura-2 staining, that was reduced in SLE patients, especially in SLE-S group. Evaluation of expression of IL-1β (both plasmatic and produced by isolated PBMC), confirmed this aspect. In detail, only after stimulation with LPS associated with BzATP, there is an increase in the production of IL-1β in SLE (especially for SLE-NS patients), which is however comparable to that of HC. After stimulation with LPS or BzATP alone, IL-1β secretion is reduced in both groups of patients compared to controls, especially after addition of 500 μM BzATP ($p<0,05$) (Table 8). These results suggest a defect in the P2X7 receptor, of which BzATP is indeed a selective and powerful agonist. The augmented release of IL-1β after stimulation with LPS in association with BzATP, suggests that a compensation can be made by NLRP3 which is increased in SLE patients and that would be directly stimulated by LPS.

Since P2X7R activation is also implicated in the production of other inflammatory cytokines such as IL-1α, TNF-α and IL6, independently of the NLRP3 inflammasome[Errore. Il segnalibro non è definito.]. To investigate more extensively the activity of P2X7R we also tested IL-6 levels as representative of a different pathway by which P2X7R can support inflammatory responses.

The behavior of IL-6 was instead very different from that of IL-1β, being more produced by PMBCs of SLE compared to HC both in basal conditions (RPMI) and after stimulation with, LPS, LPS+BzATP, and BzATP, especially in SLE-SN group. Furthermore, the plasma concentration of IL-6 was significantly higher in SLE patients, especially in SLE-S group.

These results lead us to hypothesize that in SLE, a pathogenetic pathway resulting in an increased production of IL-6, would prevail on the IL-1β pathway mediated by P2X7R that appears downregulated. In particular, from the different response after stimulation with LPS and BzATP in the production of IL-1β, and from the results regarding IL-6, it seems that in the patients affected by SLE, the release of these cytokines would be mediated independently from the P2X7R-NLRP3 axis. The inflammasome may indeed be activated by other pathways that stimulate other PRRs, as for example TLR which is able not only to promote pro-IL-1β and pro-IL18, but also to increase NLRP3 and IL-6 production[98],[135].

The evaluation of polymorphisms showed that 66,7% of patients tested (22/33), presented phenotypes whose cumulative effect consists of a gain of function of P2X7R in contrast with other results. This finding could be due to the low number of the sample or may, therefore, be secondary to the presence of other polymorphisms that have not been evaluated in the course of this study.

Cytokine production is not the only activity mediated by P2X7R. In case of pore activation after prolonged stimulation of ATP, P2X7R could mediate indeed a cytotoxic effect promoting cell death. A reduced expression of P2X7R would be implicated in defective apoptosis of different cellular type like Thf in germinative centers with consequent enhanced activation of B lymphocytes[136]. A report by Gualtierrotti et al of a study conducted on 42 lupus patients, show that the enhanced expansion of Tfh cells is correlated with diminished cell death mediated by P2X7R[137]. In accordance with these evidences, reduced expression of P2X7R detected in our study, would be implicated in SLE pathogenesis through a different mechanism mediated by its' role on cellular growth control. Further studies will be needed to clarify the role of P2X7R in SLE, and especially if the reduced expression of the receptor has a major role in SLE pathogenesis or if this finding could be considered as a consequence of the disease itself. In the presence of a persistent inflammatory state as in lupus (consistent with of elevated levels of IL-6 detected in the patients of this study), there is an increase in ATP levels in the extracellular microenvironment able to activate P2X7R. An adaptation mechanism, secondary to the chronic inflammatory state, could, therefore, play a negative feedback effect on P2X7R. On the other side, the increase in IL-6 could represent an activated pathway in response to a primary P2X7R downregulation.

The role of IL-6 in SLE has been evaluated in the pathogenesis of different clinical manifestations. For example it has been detected in serum and urine of SLE patients with lupus nephritis[138] and in and in cerebrospinal fluid in patients with neuropsychiatric manifestations[139], especially in presence of cognitive impairment[140].

Our study has many limitations: first, the patients enrolled had a prolonged disease duration (over 10 years) so have been subjected to a long period of treatment. Consequently, the majority of patients did not present active disease expressed with SLEDAI-2K (4,2 ± 4,4), considering also that 83.3% of patients had both anti-DNA positivity and hypocomplementemia, the probability of the presence of an active clinical manifestation at the enrollment was limited (only 3/16 patients presented

with ongoing serositis) (table 6). Taking these aspects into consideration, we have evaluated the correlation between the main clinical variables and the activity of P2X7R through a direct measure of its mechanism of action (represented by $\Delta[Ca2+]_i$ -Fura2) and no significant correlation with disease duration, SLEDAI-2K and ongoing therapy was detected (table 11).

In particular, in our series, 79% of patients were treated with hydroxychloroquine that has an inhibitory action on TLR and that can be considered a cornerstone in the SLE therapy[108]. Compared to the steroid, hydroxychloroquine accumulates in the body over a long period of time and its effect can be prolonged so we decided to perform a more in-depth assessment of this drug. To evaluate if this drug may have played a role in the detection of the reduced activity of P2X7R, we carried out a subsequent test on few samples in which PMBCs were pre-treated with 200 μM chloroquine and subsequently stimulated with BzATP to evaluate the increase of intracellular Ca2+ levels measured with Fura-2. The results obtained, show that in the same subject, a treatment with chloroquine determines an increase in intracellular Ca2+ levels, although not significant. This data suggests that the reduced activity of P2X7R in SLE patients would not be due to the pharmacological action of the chloroquine. Furthermore, despite the ongoing treatment and the disease activity, an increase of inflammatory cytokine IL-6 was found in patients both in supernatants of stimulated PBMCs and plasma. The limited number of patients (especially those with serositis), and the wide variability among patients in the cytokine expression, did not allow in many cases to reach statistical significance.

Conclusion

P2X7R activity mediated by inflammasome does not seem to have a fundamental role in SLE even in the presence of a complication as serositis, that has been investigated more in-depth given the presence of this clinical manifestation also in auto-inflammatory diseases like FMF.

Compared to the auto-inflammatory diseases where inflammasome has the main role, SLE is a complex condition where adaptive and innate immunity are involved. During different phases of the disease, multiple pathways can be influenced each other and change their activity. A future perspective of the study could be to evaluate patients at onset and naïve from therapy, to evaluate the role of P2X7R as a possible link between environmental risk factors and trigger of autoimmune disease in the early stages of the disease, especially in those categories of patients where a trigger (for example infectious) is recognizable. In this case, P2X7R would be overexpressed during the initial phase of disease and downregulated once the autoimmune process became established. In this phase, other pathways and other cytokine such as IL-6 could be prevalent and should be considered as an important therapeutic target in the treatment of SLE, especially in the presence of clinical manifestations with a more “inflammatory” imprint.

Table of results

Table 6: Clinical. demographic features and pharmacologic treatments of the SLE patients.

	SLE patients (n 48)
Demografic parameters	
No. female/male	45 (93.7%) /3 (6.3%)
Age. mean ± SD years	41.9 ±10.2
Disease duration (months)	130.6 ± 96.7
Past clinical pattern. n° (%)	
Cutaneous (acute/subacute/cronic)	37 (77.1%)
Articular	31 (64.6%)
Serositis	16 (33.3%; 3 ongoing) type of serositis: -4 pericardial - 7 pleural -5 both pleural + pericardial
Renal	12 (25%)→ glomerulonephritis histological class: -1 both II and III class -2 III class -1 both III and V class -5 IV class -1 both IV and V class -2 V class
Neurological	9 (18.8%)→ type of neurological involvement: -6 ischemic lesions -1 headache -1 depression -1 miastenia
Anemia	6 (12.5%)
Leukopenia	17 (35.4%)
Thrombocytopenia	3 (6.25%)
Serological parameters n°(%)	
ANA positivity	48 (100%)
ENA positivity	31 (64.6%)
anti-DNA positivity	40 (83.3%)
hypocomplementemia	40 (83.3%)
aPL (aCL. β2GPI and/or LA) positivity	15 (31.2%)
Ongoing treatment	
Hydroxychloroquine (200 mg/day)	38 (79.2%)

Corticosteroids (2.5 up to 12.5 mg/day)	40 (83.4%)
Mycophenolate mofetil	8 (16.7%)
Cyclosporine A	2 (4.2%)
Azathioprine	4 (8.3%)
Methotrexate (10–15 mg/week)	3 (6.3%)
PEX	1 (2.1%)
Rituximab	1 (2.1%)
Belimumab	1 (2.1%)
Cumulative dosage of steroids gr mean ± SD	19.8±18.0
Ongoing clinical manifestation n° (%)	
Serositis	3 (6.25%)
Arthralgia	6 (12.5%)
Cutaneous manifestation	6 (12.5%)
Renal (proteinuria>500 mg/24h)	5 (10.4%)
Neurological	0
Leukopenia	4 (8.34%)
Current serological parameters	
anti-DNA	40 (83.3%)
hypocomplementemia	40 (83.3%)
ESR (mm/h) mean ± SD years	16.4± 9.3
CRP mean ± SD years	0.7 ±1.6
Current clinimetric parameters	
SLEDAI. mean ± SD	4.2 ± 4.4
SLICC. mean ± SD	0.6±0.8

Legend: SLE disease activity index-2000 (SLEDAI-2K); SLE International Collaborating Clinics Damage Index (SLICC); antiphospholipid antibodies (aPL): including Anti-cardiolipin antibodies/aCL. lupus anticoagulant/LA. Beta-2 Glycoprotein 1 antibodies/ β2GPI); Extractable Nuclear Antigens antibodies (ENA); Plasma Exchange (PEX); Erythrocyte Sedimentation Rate (ESR); C Reactive Protein (CRP)

Table 7: Comparison between patients with a positive history of serositis (SLE-S) vs patients without history of serositis (SLE-NS)

	SLE-NS n° (%)	**SLE-S (n°; %)**	**p (SLE-NS vs SLE-S)**
No. of patients	**32 (66.7%)**	**16 (33.3%)**	
Demografic parameters			
No. female/male	31/1	14/2	0.25
Age. mean ± SD years	40.8± 8.4	44.2± 13.1	0.28
Disease duration (months)	134.7 ± 98.3	122.5± 96.2	0.68
Ongoing treatment			
Hydroxychloroquine (200 mg/day)	25 (78.1%)	13(81.2%)	1
Corticosteroids (2.5 up to 12.5 mg/day)	26 (81.3%)	6 (18.8%)	0.70
Mycophenolate mofetil	7 (21.9%)	1 (6.25%)	0.24
Cyclosporine A	2 (6.3%)	0	0.55
Azathioprine	2 (6.25%)	2(12.5%)	0.59
Methotrexate (10–15 mg/week)	1 (3.3%)	2 (15.38%)	0.21
PEX	0	1 (6.25%)	0.33
Rituximab	1 (3.1%)	0	1
Belimumab	0	1 (6.25%)	0.33
Ongoing dosage of steroids mg mean ± SD	4.3 ±3.6	5.3 ± 4.5	0.42
Cumulative dosage of steroids gr mean ± SD	21.6 ±20.6	18.9 ±16.8	0.64
Current serological parameters			
anti-DNA	20 (62.5%)	11 (68.8%)	1.00
hypocomplementemia	13 (40.63%)	11 (68.8%)	0.125
ESR (mm/h) mean ± SD years	15.4± 1.5	18± 2.7	0.29
CRP mean ± SD years	0.4± 0.02	1.4 ± 0.67	**0.04**
Current clinimetric parameters			
SLEDAI-2K. mean ± SD	3.8 ±4.2	4.9 ±4.6	0.42
SLICC. mean ± SD	0.45 ± 0.8	0.8 ±0.9	0.17

Legend: SLE disease activity index-2000 (SLEDAI-2K); SLE International Collaborating Clinics Damage Index (SLICC); antiphospholipid antibodies (aPL) including: Anti-cardiolipin antibodies/aCL. lupus anticoagulant/LA. Beta-2 Glycoprotein 1 antibodies/ β2GPI); Extractable Nuclear Antigens

antibodies (ENA); Plasma Exchange (PEX); Erythrocyte Sedimentation Rate (ESR); C Reactive Protein (CRP)

Table 8: activity and expression of P2X7R and NLRP3: sub-analysis to evaluate serositis

	HC	**SLE-S**	**SLE-NS**	**SLE-S vs SLE-NS**	**HC vs SLE-NS**	**HC vs SLE-S**
IL-1β plasma levels pg/ml; mean ±SD	2.4±0.12	2,4±0,2	2,8±0,8	0,31	0,37	0,94
IL-6 plasma levels pg/ml; mean ±SD	1.58± 0.67	4.8± 0.97	2.02±0.37	**0.009**	0.54	**0.037**
ΔCa2+ (Fura2) nM±SD	105.5± 12.75	58.25± 10.28	67.69±9.23	0.675	**0.0267**	**0.0215**
Supernatant's dosage of IL-1β pg/ml;mean±SD*						
RPMI	18.05±7.41	14.82±3.76	16.63± 5	0.80	0.88	0.67
LPS	62.28±15.09	55.38±13.47	48.11± 14.53	0.745	0.556	0.736
LPS + BzATP	1246±58.61	988.7±103.6	1237±70.39	**0,0476**	0.936	0.072
BzATP	66.19±12.31	11.95±4.99	11.84±4.68	0.987	**0.0001**	**0.0002**
Supernatant's dosage of IL-6 pg/ml;mean (±SD)*						
RPMI	11.83±0.51	29.18±4.85	329.0±83.74	**0,014**	**0,01**	**0,002**
LPS	339.9±41.37	728.8±101.2	1172±74.25	**0,001**	**0,0001**	**0,002**
LPS + BzATP	330.7±43.46	719.7±112.6	1159±83.87	**0.003**	**0.0001**	**0.004**
BzATP	13.38±0.76	43.95±9.49	328±91.1	**0.028**	**0.019**	**0.005**
RT-PCR P2X7R mean (±SD)	1.29±0.13	0.724±0.11	0.95±0.44	0,31	0,11	**0,02**
RT-PCR NLRP3 mean (±SD)	1.99±0.30	3.702±0.552	3.846±0.633	0.74	**0.013**	**0.036**

***produced by PBMCs (500 000cells)**

Table 9: Distribution of different P2X7R polymorphisms and their role in receptor activity.

	C/C	C/T	T/T
A/A	5 (normal activity)	7 (slightly increased activity)	8 (increased activity)
A/C	2 (slightly reduced activity)	5 (normal activity)	6 (very increased activity)

Legend: adenine (A), cytosine (C), thymine (T).

Table 10: Correlation between P2X7R activity and polymorphisms

	GOF	LOF	p
IL-1β levels in monocytes supernatants pg/ml; mean ±SD			
RPMI	83.2±127.6	25.3±15.9	0.29
LPS	154.2± 121.5	231.3± 235.3	0.71
LPS + BZATP	2262.6 ±810.1	2376.8 ± 557.5	0.71
BZATP	127.3 ± 153.5	165.8 ± 180.54	0.58
ΔCa^{2+} (Fura2) nM±SD	54.8± 40.0	74.2±35.6	0.42

Legend: Gain of function (GOF), Loss of function (LOF)

Table 11: Correlation between disease activity. disease duration and corticosteroids dosage with P2X7R activity.

	SLEDAI-2k Spearmann rho; p	Disease duration rho;p	Ongoing steroids (40 patients) Yes vs No; p	Cumulative dosage of steroids Spearmann rho; p	Current dosage of steroids Spearmann rho; p	Ongoing hydroxychloroquine (38 patients) Yes vs No; p	Ongoing major immunosuppressive therapy (18 patients) Yes vs No; p
ΔCa^{2+} (Fura2) nM (±SD)	-0.3; p=0.2	-0.4; p=0.1	66.3±33.9 vs 61.5±4.9; p=0.84	0.18; p=0.47	-0.13; p=0.61	70.8±30.8 vs 53.6±34.2; p=0.32	53.8±37.7 vs 76.2±22.6; p=0.15

Figures

Figure 1

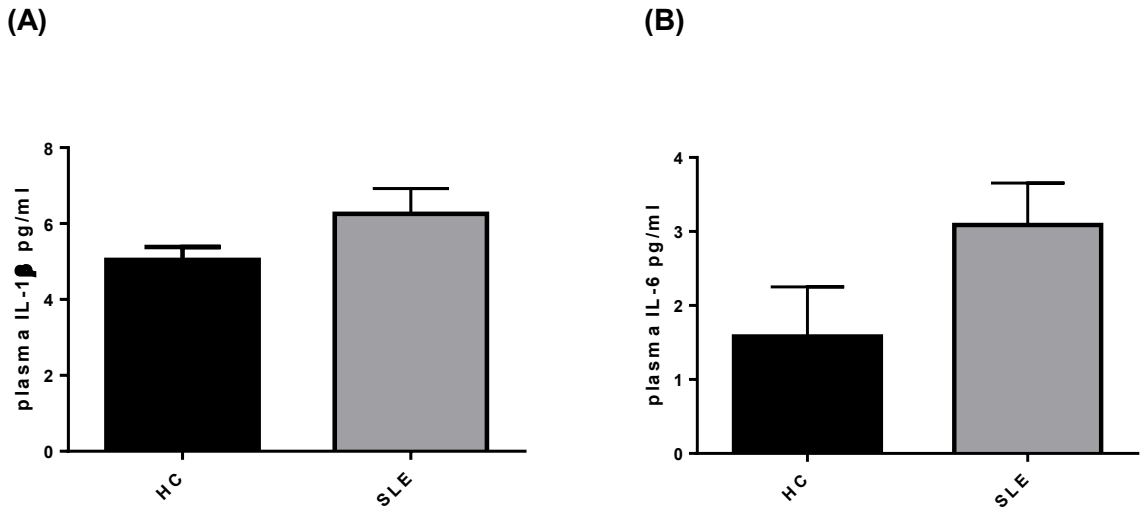

Figure 1. Representation of plasmatic levels of IL-1β and IL-6 in patients (SLE) vs healthy controls (HC). (A) *Plasma IL-1β levels were not statistically different between HC and SLE (SLE vs HC vs = 6.26±0.66 vs 5.05±0.34 p=0.26)* ***Unpaired t-test****. **(B).** Plasma IL-6 levels resulted higher in SLE vs HC (SLE vs HC= 3.09±0.57 vs 1.58±0.67; p=0.191)* ***Unpaired t-test.*** *Data are means ±SD.*

Figure 2

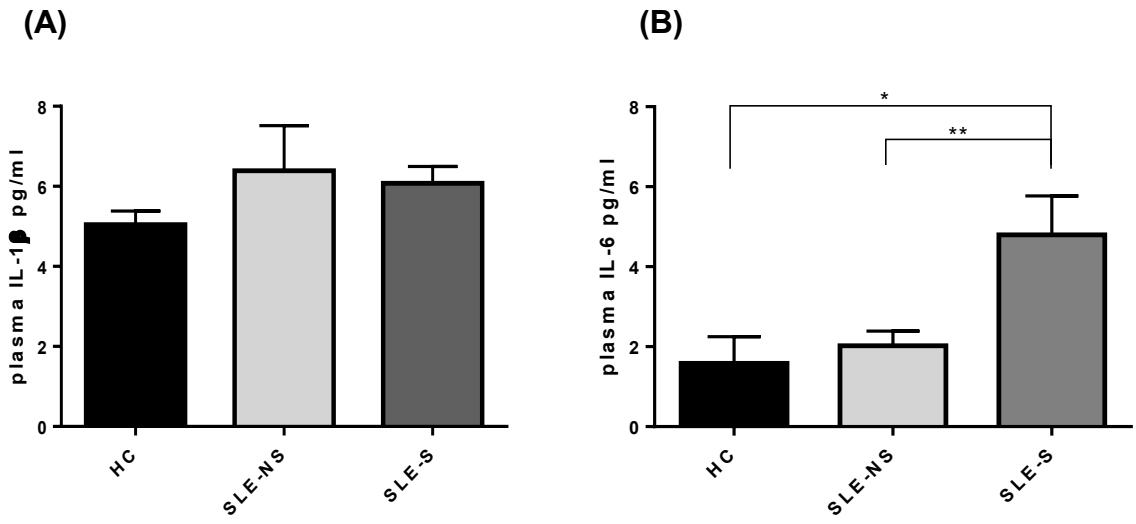

Figure 2. Representation of plasmatic levels of IL-1β and IL-6 in patients with serositis (SLE-S), without serositis (SLE-NS) compared to healthy controls (HC). (A) *Plasmatic IL-1β levels were not statistically significant between HC, SLE-S and SLE-NS (SLE-NS vs HC= 6.39±1.13 vs 5.05±0.34, p=0.34), (SLE-S vs HC= 6.08±0.42 vs 5.05±0.34, p=0.075), (SLE-S vs SLE-NS= 6.08±0.42 vs 6.39±1.13, p=0.82)* ***Unpaired t-test****.* ***(B)*** *Plasmatic IL-6 levels resulted significantly higher in SLE-S compared to both HC and SLE-NS: (SLE-NS vs HC= 2.02±0.37 vs 1.58±0.67, p=0.54), (SLE-S vs HC= 4.8±0.97 vs 1.58±0.67, p=0.037) (SLE-S vs SLE-NS 4.8±0.97 vs 2.02±0.37; p=0.009)* ***Unpaired t-test****. Data are means ±SD.*

Figure 3

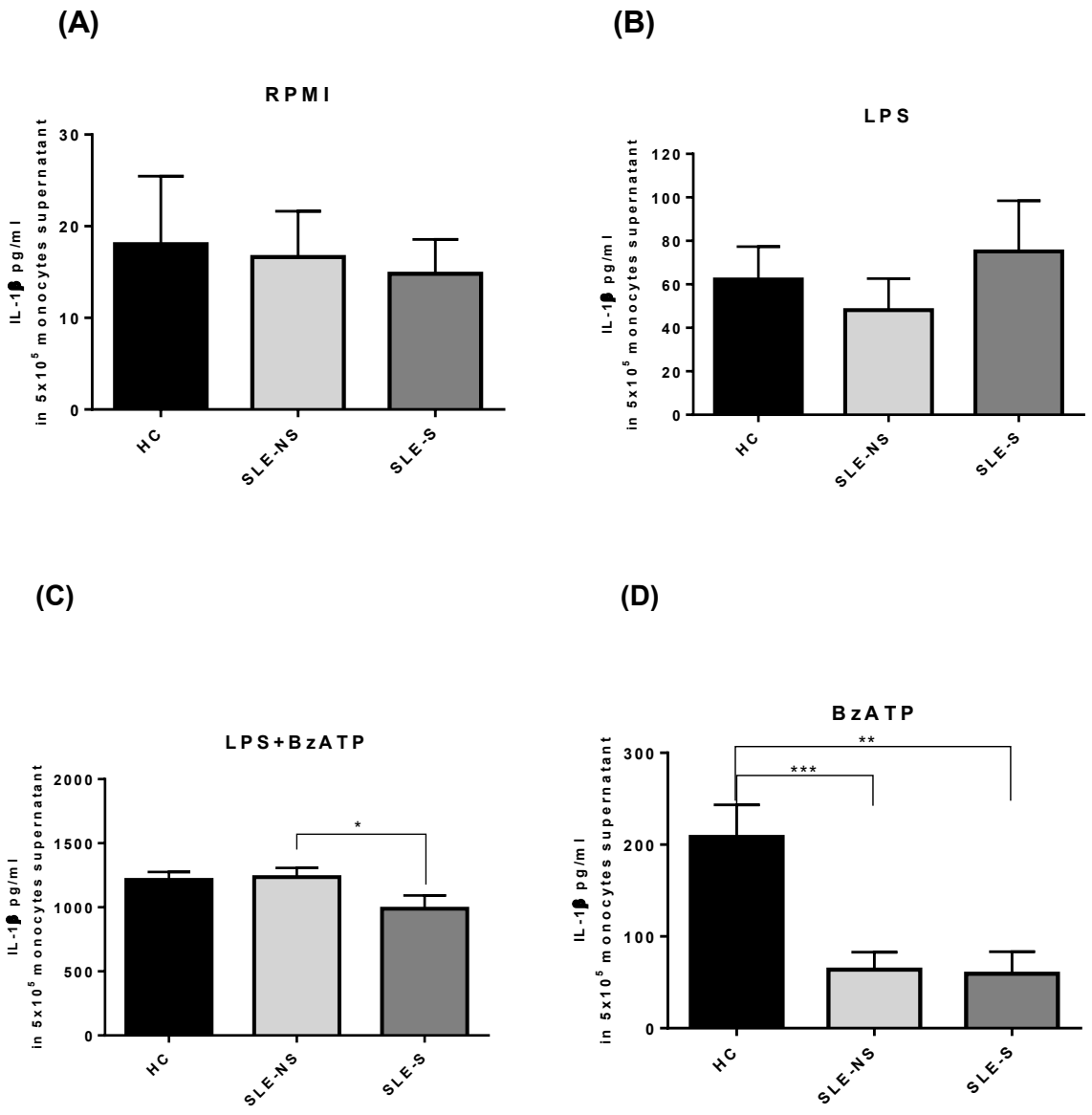

Figure 3. Representation of *IL-1β levels in monocytes supernatants from* patients with serositis (SLE-S), without serositis (SLE-NS) and healthy controls (HC). (A) IL-1β released from unstimulated monocytes maintained for 5 h in 10% FBS supplemented RPMI was not significantly different between HC and SLE patients (SLE-NS vs HC= 16.63±5 vs 18.05±7.41, p=0.88; SLE-S vs HC= 14.82±3.76 vs 18.05±7.41, p= 0.67) and between patients with serositis and patients without serositis (SLE-S vs SLE-NS p= 0.80). **(B)** IL-1β released from monocytes stimulated for 4 h with 1 μg/ml LPS in 10% FBS-supplemented RPMI was not significantly different between HC and SLE patients (SLE-NS vs HC= 48.11±14.53 vs 62.28±15.09, p=0.556; SLE-S vs HC= 55.38±13.47 vs 62.28±15.09, p= 0.736) and between patients with serositis vs patients without serositis (SLE-S vs SLE-NS p= 0.745). **(C**) IL-1β released from monocytes stimulated for 4 h with 1 μg/ml LPS and for 1 h with 500 μM BzATP in 10% FBS-supplemented RPMI was not different between HC and SLE patients (SLE-NS vs HC= 1237±70.39 vs 1246±58.61, p=0.936; SLE-S vs HC= 988.7±103.6 vs

1246±58.61, p= 0.072). IL-1β was significantly lower in SLE-S compared to SLE-NS (p=0.0476). **(D)** IL-1β released from monocytes stimulated for 1 h with 500 μM BzATP in 10% FBS-supplemented RPMI was significantly lower in SLE patients respect to HC (SLE-NS vs HC= 11.84±4.68 vs 66.19±12.31, p<0.0001; SLE-S vs HC= 11.95±4.99 vs 66.19±12.31, p=0.0002). IL-1β was not different between patients with serositis vs patients without serositis (SLE-S vs SLE-NS p= 0.987). ***Unpaired t-test****. Data are means ±SD.*

Figure 4

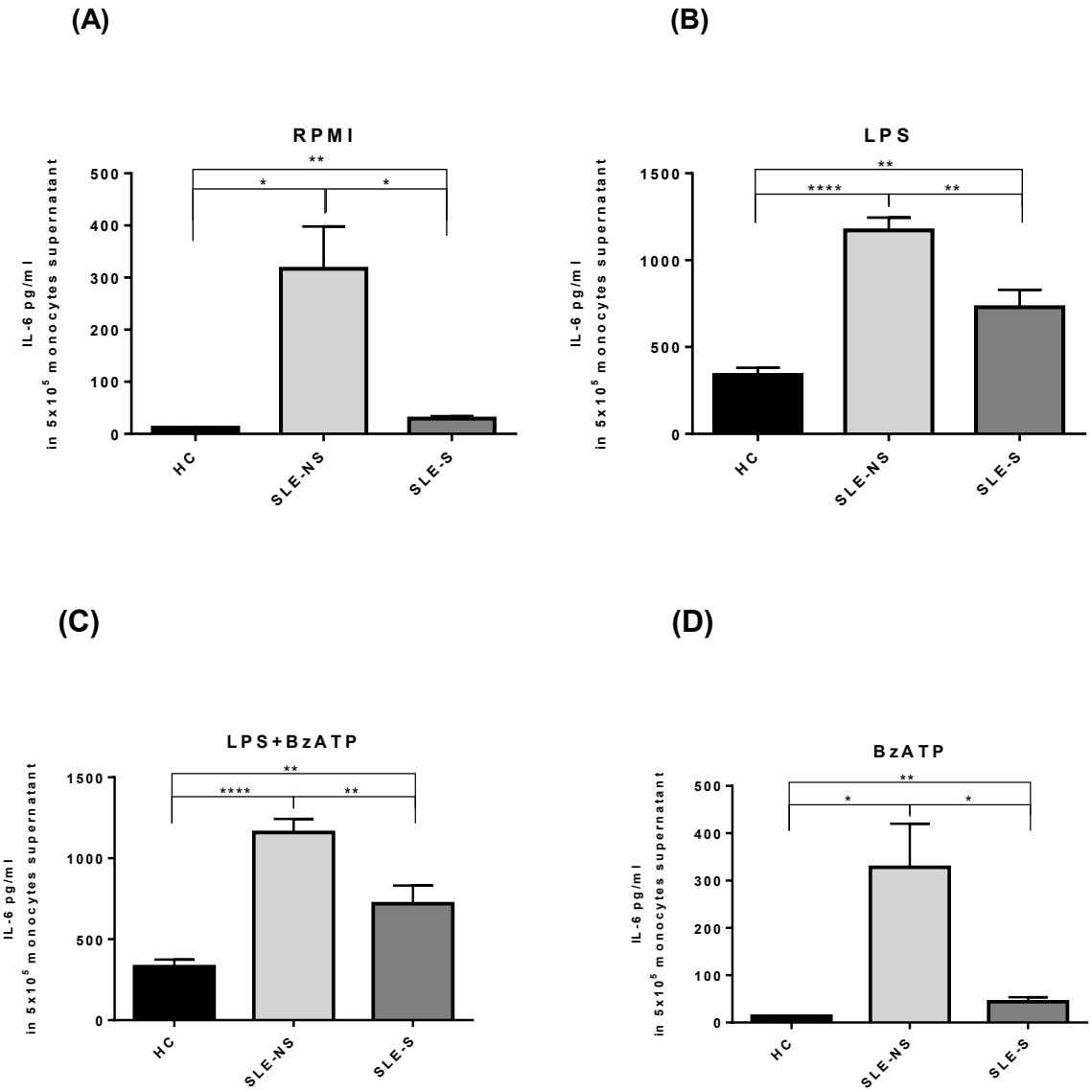

Figure 4. Representation of* IL-6 levels in monocytes supernatants from *patients with serositis (SLE-S), without serositis (SLE-NS) and healthy controls (HC). (A) *IL-6 released from unstimulated monocytes maintained for 5 h in 10% FBS supplemented RPMI was significantly higher in SLE patients vs HC (SLE-NS vs HC: 329.0±83.74 vs 11.83±0.51, p= 0.0102; SLE-S vs HC= 29.18±4.85, p= 0.0021) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.014).* ***(B)*** *IL-6 released from monocytes stimulated for 4 h with 1 μg/ml LPS in 10% FBS-supplemented RPMI was significantly higher in SLE patients vs HC (SLE-NS vs HC: 1172±74.25 vs 339.9±41.37, p<0.0001; SLE-S vs HC= 728.8±101.2 vs 339.9±41.37, p=0.002) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.001).* ***(C)*** *IL-6 levels from monocytes stimulated for 4 h with 1 μg/ml LPS, and for 1 h with 500 μM BzATP in 10% FBS-supplemented RPMI were significantly higher in SLE patients vs HC (SLE-NS vs HC: 1159±83.87*

vs 330.7±43.46, p<0.0001; SLE-S vs HC= 719.7±112.6 vs 330.7±43.46, p=0.004) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.003). ***(D)*** *IL-6 released from monocytes stimulated for 1 h with 500 μM BzATP in 10% FBS-supplemented RPMI was significantly higher in SLE patients vs HC (SLE-NS vs HC: 328±92.1 vs 13.38±0.76, p=0.019; SLE-S vs HC= 43.95±9.49 vs 13.38±0.76, p=0.005) and in patients without serositis vs patients with serositis (SLE-S vs SLE-NS, p=0.028). Data are means ±SD.*

Figure 5

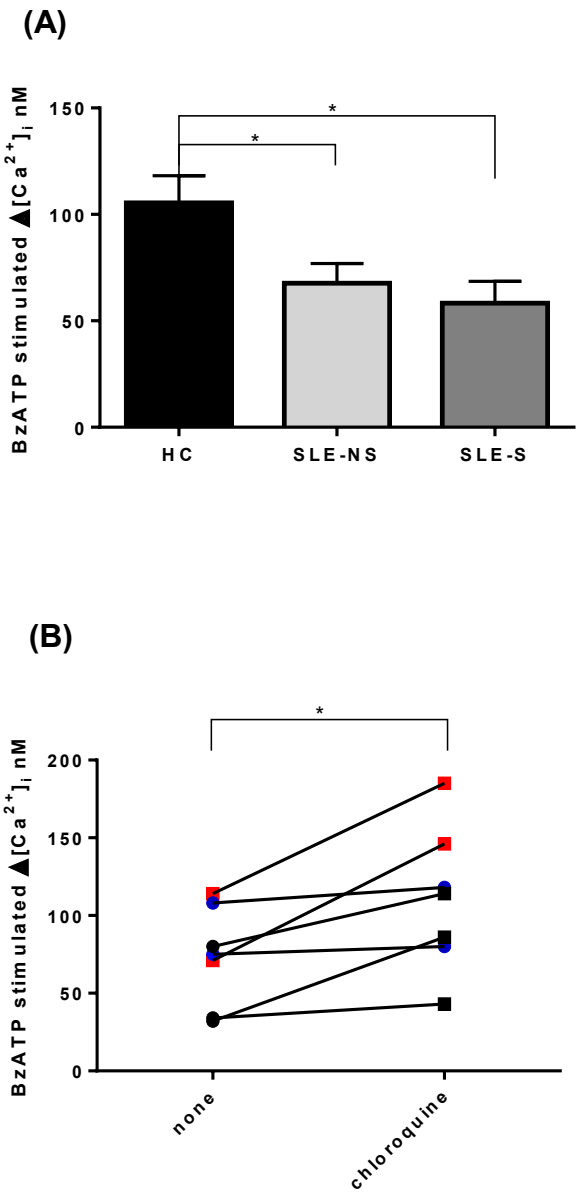

Figure 5. Evaluation of Calcium influx with Fura2 in PBMCs after BzATP stimulation and chloroquine pre-treatment (A) *The increase of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) following stimulation with 500 μM BzATP was significantly lower in PBMCs from patients (SLE) vs controls (HC) (SLE vs HC= 64.1±6.84 vs 105.5±12.75, p=0.008). Kruskal Wallis test p=0.031. Data are means ±SE. Only significant differences are shown.* ***(B)*** *The effect of 200 mM chloroquine on $\Delta[Ca^{2+}]_i$ was evaluated on PBMCs from 4 controls and 3 patients. Each line represents a patients or a control. The $\Delta[Ca^{2+}]_i$ was higher after chloroquine treatment respect to basal conditions (Wilcoxon matched-pairs signed rank test p=0.016) both in controls and patients (pre vs post treatment: 92.0 ±11.07 vs 132.3± 22.18; p=0.13) and patients (48.67± 15.68 vs 81± 20.65; p=0.25).* Data are means ±SD.

Figure 6

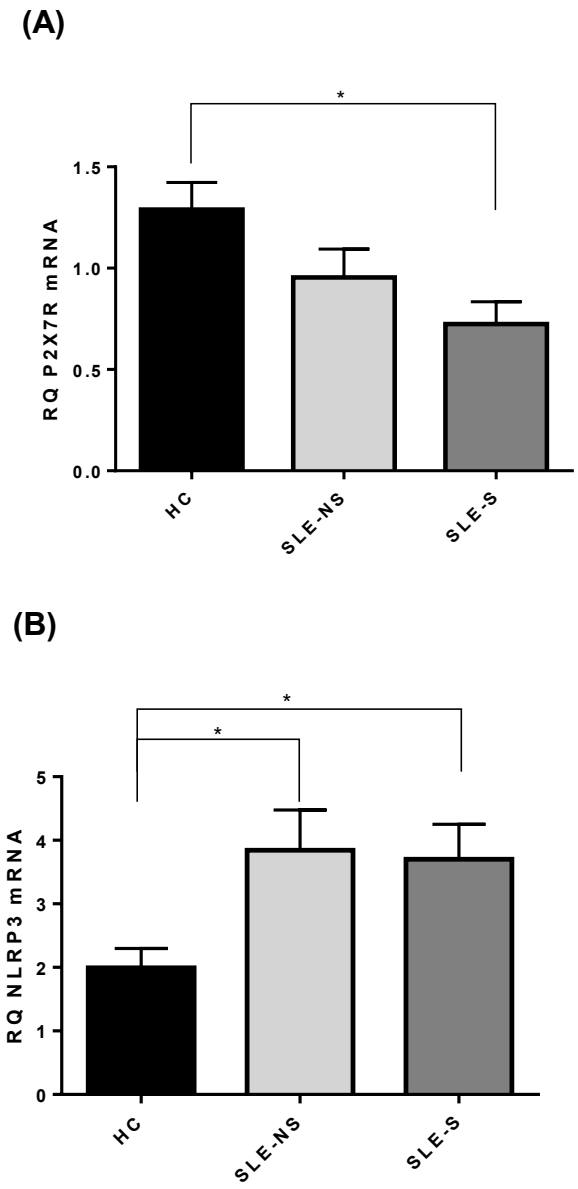

***Figure 6: Expression of P2X7R and NLRP3 mRNA in PMBCs from patients (SLE) vs healthy control (HC)**. **(A)** P2X7R was significantly less expressed in SLE vs HC (SLE vs HC= 0.87±0.10 vs 1.29±0.13; p=0.02), particularly in patients with serositis (SLE-S vs HC= 0.724±0.11 vs 1.29±0.13; p=0.019). **(B)** NLRP3 expression resulted significantly higher in SLE vs HC (SLE vs HC= 3.80±0.46 vs 1.99±0.30; p=0.018). Data are means ±SD.*

Bibliography

1 Jiménez, S., Cervera, R., Font, J., and Ingelmo, M. (2003). The epidemiology of systemic lupus erythematosus. Clin Rev Allergy Immunol 25, 3–12. doi:10.1385/CRIAI:25:1:3.

2 Miller, M. H., Urowitz, M. B., Gladman, D. D., and Killinger, D. W. (1983). Systemic lupus erythematosus in males. Medicine (Baltimore) 62, 327–334.

3 Tsokos, G. C. (2011). Systemic Lupus Erythematosus. The New England Journal of Medicine, 12.

4 Harley, I. T. W., Kaufman, K. M., Langefeld, C. D., Harley, J. B., and Kelly, J. A. (2009). Genetic susceptibility to SLE: new insights from fine mapping and genome-wide association studies. Nat. Rev. Genet. 10, 285–290. doi:10.1038/nrg2571.

5 Manderson, A. P., Botto, M., and Walport, M. J. (2004). The role of complement in the development of systemic lupus erythematosus. Annu. Rev. Immunol. 22, 431–456. doi:10.1146/annurev.immunol.22.012703.104549.

6 Hedrich, C. M., and Tsokos, G. C. (2011). Epigenetic mechanisms in systemic lupus erythematosus and other autoimmune diseases. Trends Mol Med 17, 714–724. doi:10.1016/j.molmed.2011.07.005

7 Verjans, E., Ohl, K., Reiss, L. K., van Wijk, F., Toncheva, A. A., Wiener, A., et al. (2015). The cAMP response element modulator (CREM) regulates TH2 mediated inflammation. Oncotarget 6, 38538–38551

8 James, J. A., Harley, J. B., and Scofield, R. H. (2006). Epstein–Barr virus and systemic lupus erythematosus: Current Opinion in Rheumatology 18, 462–467. doi:10.1097/01.bor.0000240355.37927.94.

9 Rubin, R. L. (2015). Drug-induced lupus. Expert Opin Drug Saf 14, 361–378. doi:10.1517/14740338.2015.995089.

10 Shlomchik, M. J., Craft, J. E., and Mamula, M. J. (2001). From T to B and back again: positive feedback in systemic autoimmune disease. Nat. Rev. Immunol. 1, 147–153. doi:10.1038/35100573.

11 Obermoser, G., and Pascual, V. (2010). The interferon-α signature of systemic lupus erythematosus. Lupus 19, 1012–1019. doi:10.1177/0961203310371161.

12 Iwata, Y., Furuichi, K., Kaneko, S., and Wada, T. (2011). The Role of Cytokine in the Lupus Nephritis. J Biomed Biotechnol 2011. doi:10.1155/2011/594809.

13 D'Acquisto, F., and Crompton, T. (2011). CD3+CD4−CD8− (double negative) T cells: Saviours or villains of the immune response? Biochemical Pharmacology 82, 333–340. doi:10.1016/j.bcp.2011.05.019.

14 Shaltout, A. S., Sayed, D., Badary, M. S., Nafee, A. M., El Zohri, M. H., Bakry, R., et al. (2016). Effect of IL6 and IL23 on double negative T cells and anti-ds-DNA in systemic lupus erythematosus patients. Human Immunology 77, 937–943. doi:10.1016/j.humimm.2016.06.007.

15 Crispin, J. C., Oukka, M., Bayliss, G., Cohen, R. A., Van Beek, C. A., Stillman, I. E., et al. (2008). Expanded Double Negative T Cells in Patients with Systemic Lupus Erythematosus Produce IL-17 and Infiltrate the Kidneys. The Journal of Immunology 181, 8761–8766. doi:10.4049/jimmunol.181.12.8761.

16 Katsuyama, T., Tsokos, G. C., and Moulton, V. R. (2018). Aberrant T Cell Signaling and Subsets in Systemic Lupus Erythematosus. Frontiers in immunology 9, 1088. doi:10.3389/fimmu.2018.01088.

17 Kyttaris, V. C., Wang, Y., Juang, Y.-T., Weinstein, A., and Tsokos, G. C. (2007). Increased levels of NFAT differentially regulate CD154 and IL-2 genes in T cells from patients with systemic lupus erythematosus. The Journal of immunology 178, 1960–6. doi:10.4049/jimmunol.178.3.1960.

18 Wardemann, H., and Nussenzweig, M. C. (2007). B-cell self-tolerance in humans. Adv. Immunol. 95, 83–110. doi:10.1016/S0065-2776(07)95003-8.

19 Lourenço, E. V., and La Cava, A. (2009). Cytokines in systemic lupus erythematosus. Curr. Mol. Med. 9, 242–254.

20 Gilliet, M., Cao, W., and Liu, Y.-J. (2008). Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat. Rev. Immunol. 8, 594–606. doi:10.1038/nri2358

21 Yan, B., Ye, S., Chen, G., Kuang, M., Shen, N., and Chen, S. (2008). Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. Arthritis Rheum. 58, 801–812. doi:10.1002/art.23268

22 Jego, G., Palucka, A. K., Blanck, J.-P., Chalouni, C., Pascual, V., and Banchereau, J. (2003). Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity 19, 225–234.

23 Mackay, F., Woodcock, S. A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., et al. (1999). Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J. Exp. Med. 190, 1697–1710.

24 Yu, G., Boone, T., Delaney, J., Hawkins, N., Kelley, M., Ramakrishnan, M., et al. (2000). APRIL and TALL-I and receptors BCMA and TACI: system for regulating humoral immunity. Nat. Immunol. 1, 252–256. doi:10.1038/79802.

25 Vassalli, P. (1992). The pathophysiology of tumor necrosis factors. Annu. Rev. Immunol. 10, 411–452. doi:10.1146/annurev.iy.10.040192.002211.

26 Yu, A., Zhu, L., Altman, N. H., and Malek, T. R. (2009). A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. Immunity 30, 204–217. doi:10.1016/j.immuni.2008.11.014.

27 Kishimoto, T. (2006). Interleukin-6: discovery of a pleiotropic cytokine. Arthritis Res. Ther. 8 Suppl 2, S2. doi:10.1186/ar1916.

28 Peng, X., Yang, T., Liu, G., Liu, H., Peng, Y., and He, L. (2018). Piperine ameliorated lupus nephritis by targeting AMPK-mediated activation of the NLRP3 inflammasome. International Immunopharmacology 65, 448–457. doi:10.1016/j.intimp.2018.10.025.

29 Kuhn, A., and Landmann, A. (2014). The classification and diagnosis of cutaneous lupus erythematosus. J. Autoimmun. 48–49, 14–19. doi:10.1016/j.jaut.2014.01.021.

30 Santiago, M. B., and Galvão, V. (2008). Jaccoud Arthropathy in Systemic Lupus Erythematosus. Medicine 87, 37–44. doi:10.1097/MD.0b013e3181632d18.

31 Fernández, A., Quintana, G., Matteson, E. L., Restrepo, J. F., Rondón, F., Sánchez, A., et al. (2004). Lupus arthropathy: historical evolution from deforming arthritis to rhupus. Clin. Rheumatol. 23, 523–526. doi:10.1007/s10067-004-0951-2.

32 Gontero, R. P., Bedoya, M. E., Benavente, E., Roverano, S. G., and Paira, S. O. (2015). Osteonecrosis in systemic lupus erythematosus. Reumatol Clin 11, 151–155.

33 Weening, J. J., D'Agati, V. D., Schwartz, M. M., Seshan, S. V., Alpers, C. E., Appel, G. B., et al. (2004). The classification of glomerulonephritis in systemic lupus erythematosus revisited. Kidney Int. 65, 521–530. doi:10.1111/j.1523-1755.2004.00443.x.

34 Hanly, J. G. (2004). ACR classification criteria for systemic lupus erythematosus: limitations and revisions to neuropsychiatric variables. Lupus 13, 861–864. doi:10.1191/0961203304lu2024oa.

35 Tincani, A., Rebaioli, C. B., Taglietti, M., and Shoenfeld, Y. (2006). Heart involvement in systemic lupus erythematosus, anti-phospholipid syndrome and neonatal lupus. Rheumatology (Oxford) 45 Suppl 4, iv8-13. doi:10.1093/rheumatology/kel308.

36 Frieri, M., and Stampfl, H. (2016). Systemic lupus erythematosus and atherosclerosis: Review of the literature. Autoimmun Rev 15, 16–21. doi:10.1016/j.autrev.2015.08.007.

37 Gutsche, M., Rosen, G. D., and Swigris, J. J. (2012). Connective Tissue Disease-associated Interstitial Lung Disease: A review. Curr Respir Care Rep 1, 224–232. doi:10.1007/s13665-012-0028-7.

38 Borrell, H., Narváez, J., Alegre, J. J., Castellví, I., Mitjavila, F., Aparicio, M., et al. (2016). Shrinking lung syndrome in systemic lupus erythematosus: A case series and review of the literature. Medicine (Baltimore) 95, e4626. doi:10.1097/MD.0000000000004626.

39 Bashal, F. (2013). Hematological disorders in patients with systemic lupus erythematosus. Open Rheumatol J 7, 87–95. doi:10.2174/1874312901307010087.

40 Lam, G. K. W., and Petri, M. (2005). Assessment of systemic lupus erythematosus. Clin. Exp. Rheumatol. 23, S120-132.

41 Xiong, W., and Lahita, R. G. (2014). Pragmatic approaches to therapy for systemic lupus erythematosus. Nat Rev Rheumatol 10, 97–107. doi:10.1038/nrrheum.2013.157.

42 Burnstock, G. (2006). Pathophysiology and Therapeutic Potential of Purinergic Signaling. Pharmacological Reviews 58, 58–86. doi:10.1124/pr.58.1.5.

43 Pellegatti, P., Falzoni, S., Pinton, P., Rizzuto, R., and Di Virgilio, F. (2005). A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. Molecular biology of the cell 16, 3659–3665. doi:10.1091/mbc.e05-03-0222

44 Yegutkin, G. G. (2014). Enzymes involved in metabolism of extracellular nucleotides and nucleosides: Functional implications and measurement of activities. Critical Reviews in Biochemistry and Molecular Biology 49, 473–497. doi:10.3109/10409238.2014.953627.

45 Lazarowski, E. R. (2012). Vesicular and conductive mechanisms of nucleotide release. Purinergic Signalling 8, 359–373. doi:10.1007/s11302-012-9304-9.

46 Boué-Grabot, É., Archambault, V., and Séguéla, P. (2000). A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of P2X2 ATP-gated channels. Journal of Biological Chemistry 275, 10190–10195. doi:10.1074/jbc.275.14.10190.

47 Di Virgilio, F., and Adinolfi, E. (2017). Extracellular purines, purinergic receptors and tumor growth. Oncogene 36, 293–303. doi:10.1038/onc.2016.206.

48 Kahlenberg, J. M., Carmona-Rivera, C., Smith, C. K., and Kaplan, M. J. (2013). Neutrophil Extracellular Trap-Associated Protein Activation of the NLRP3 Inflammasome Is Enhanced in Lupus Macrophages. The Journal of Immunology 190, 1217–1226. doi:10.4049/jimmunol.1202388.

49 Denlinger, L. C., Fisette, P. L., Sommer, J. A., Watters, J. J., Prabhu, U., Dubyak, G. R., et al. (2001). Cutting Edge: The Nucleotide Receptor P2X7 Contains Multiple Protein- and Lipid-Interaction Motifs Including a Potential Binding Site for Bacterial Lipopolysaccharide. The Journal of Immunology 167, 1871–1876. doi:10.4049/jimmunol.167.4.1871.

50 Dal Ben, D., Buccioni, M., Lambertucci, C., Marucci, G., Thomas, A., and Volpini, R. (2015). Purinergic P2X receptors: structural models and analysis of ligand-target interaction. Eur J Med Chem 89, 561–580. doi:10.1016/j.ejmech.2014.10.071.

51 Gilbert, S. M., Oliphant, C. J., Hassan, S., Peille, A. L., Bronsert, P., Falzoni, S., et al. (2018). ATP in the tumour microenvironment drives expression of nfP2X7, a key mediator of cancer cell survival. Oncogene, 1. doi:10.1038/s41388-018-0426-6.

52 Adinolfi, E., Cirillo, M., Woltersdorf, R., Falzoni, S., Chiozzi, P., Pellegatti, P., et al. (2010). Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 24, 3393–3404. doi:10.1096/fj.09-153601.

53 Sluyter, R. (2017). The P2X7 Receptor. Adv. Exp. Med. Biol. 1051, 17–53. doi:10.1007/5584_2017_59.

54 Morandini, A. C., Savio, L. E. B., and Coutinho-Silva, R. (2014). The role of P2X7 receptor in infectious inflammatory diseases and the influence of ectonucleotidases. Biomed J 37, 169–177. doi:10.4103/2319-4170.127803.

55 Amaral, E. P., Ribeiro, S. C. M., Lanes, V. R., Almeida, F. M., de Andrade, M. R. M., Bomfim, C. C. B., et al. (2014). Pulmonary infection with hypervirulent Mycobacteria reveals a crucial role for the P2X7 receptor in aggressive forms of tuberculosis. PLoS Pathog. 10, e1004188. doi:10.1371/journal.ppat.1004188

56 Corrêa, G., de A Lindenberg, C., Fernandes-Santos, C., Gandini, M., Petitinga Paiva, F., Coutinho-Silva, R., et al. (2016). The purinergic receptor P2X7 role in control of Dengue virus-2 infection and cytokine/chemokine production in infected human monocytes. Immunobiology 221, 794–802. doi:10.1016/j.imbio.2016.02.003.

57 Fowler, B. J., Gelfand, B. D., Kim, Y., Kerur, N., Tarallo, V., Hirano, Y., et al. (2014). Nucleoside reverse transcriptase inhibitors possess intrinsic anti-inflammatory activity. Science 346, 1000–1003. doi:10.1126/science.1261754

58 Mutini, C., Falzoni, S., Ferrari, D., Chiozzi, P., Morelli, A., Baricordi, O. R., et al. (1999). Mouse dendritic cells express the P2X7 purinergic receptor: characterization and possible participation in antigen presentation. Journal of immunology (Baltimore, Md. : 1950) 163, 1958–1965.

59 Pizzirani, C., Ferrari, D., Chiozzi, P., Adinolfi, E., Sandona, D., Savaglio, E., et al. (2007). Stimulation of P2 receptors causes release of IL-1beta-loaded microvesicles from human dendritic cells. Blood 109, 3856–3864. doi:10.1182/blood-2005-06-031377.

60 Di Virgilio, F., Sarti, A. C., and Grassi, F. (2018). Modulation of innate and adaptive immunity by P2X ion channels. Current Opinion in Immunology 52, 51–59. doi:10.1016/j.coi.2018.03.026.

61 Piccini, A., Carta, S., Tassi, S., Lasiglie, D., Fossati, G., and Rubartelli, A. (2008). ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1β and IL-18 secretion in an autocrine way. Proceedings of the National Academy of Sciences 105, 8067–8072. doi:10.1073/pnas.0709684105.

62 Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., and Núñez, G. (2013). K+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity 38, 1142–1153. doi:10.1016/j.immuni.2013.05.016.

63 Franceschini, A., Capece, M., Chiozzi, P., Falzoni, S., Sanz, J. M., Sarti, A. C., et al. (2015). The P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein. FASEB Journal 29, 2450–2461. doi:10.1096/fj.14-268714.

64 He, Y., Zeng, M. Y., Yang, D., Motro, B., and Núñez, G. (2016). NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. Nature 530, 354–357. doi:10.1038/nature16959.

65 Schroder, K., Zhou, R., and Tschopp, J. (2010). The NLRP3 inflammasome: a sensor for metabolic danger? Science 327, 296–300. doi:10.1126/science.1184003.

66 Dinarello, C. A. (2009). Immunological and Inflammatory Functions of the Interleukin-1 Family. Annual Review of Immunology 27, 519–550. doi:10.1146/annurev.immunol.021908.132612.

67 Yang, J., Zhao, Y., and Shao, F. (2015). Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. Current Opinion in Immunology 32, 78–83. doi:10.1016/j.coi.2015.01.007.

68 Bryan, N. B., Dorfleutner, A., Rojanasakul, Y., and Stehlik, C. (2009). Activation of inflammasomes requires intracellular redistribution of the apoptotic speck-like protein containing a caspase recruitment domain. J. Immunol. 182, 3173–3182. doi:10.4049/jimmunol.0802367

69 Caneparo, V., Landolfo, S., Gariglio, M., and De Andrea, M. (2018). The Absent in Melanoma 2-Like Receptor IFN-Inducible Protein 16 as an Inflammasome Regulator in Systemic Lupus Erythematosus: The Dark Side of Sensing Microbes. Front Immunol 9. doi:10.3389/fimmu.2018.01180.

70 Di Virgilio, F. (2013). The Therapeutic Potential of Modifying Inflammasomes and NOD-Like Receptors. Pharmacological Reviews 65, 872–905. doi:10.1124/pr.112.006171.

71 Ali, S. R., Timmer, A. M., Bilgrami, S., Park, E. J., Eckmann, L., Nizet, V., et al. (2011). Anthrax toxin induces macrophage death by p38 MAPK inhibition but leads to inflammasome activation via ATP leakage. Immunity 35, 34–44. doi:10.1016/j.immuni.2011.04.015.

72 Anand, P. K., Malireddi, R. K. S., Lukens, J. R., Vogel, P., Bertin, J., Lamkanfi, M., et al. (2012). NLRP6 Negatively Regulates Innate Immunity and Host Defense Against Bacterial Pathogens. Nature 488, 389–393. doi:10.1038/nature11250.

73 Ghimire, L., Paudel, S., Jin, L., Baral, P., Cai, S., and Jeyaseelan, S. (2018). NLRP6 negatively regulates pulmonary host defense in Gram-positive bacterial infection through modulating neutrophil recruitment and function. PLoS Pathog 14. doi:10.1371/journal.ppat.1007308.

74 Arlehamn, C. S. L., Pétrilli, V., Gross, O., Tschopp, J., and Evans, T. J. (2010). The role of potassium in inflammasome activation by bacteria. J. Biol. Chem. 285, 10508–10518. doi:10.1074/jbc.M109.067298.

75 Davis, B. K., Wen, H., and Ting, J. P.-Y. (2011). The Inflammasome NLRs in Immunity, Inflammation, and Associated Diseases. Annu Rev Immunol 29, 707–735. doi:10.1146/annurev-immunol-031210-101405.

76 Siegel, R. M. (2006). Caspases at the crossroads of immune-cell life and death. Nature Reviews Immunology 6, 308.

77 Fink, S. L., and Cookson, B. T. (2006). Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cellular Microbiology 8, 1812–1825. doi:10.1111/j.1462-5822.2006.00751.x.

78 Yuan, Y.-Y., Xie, K.-X., Wang, S.-L., and Yuan, L.-W. (2018). Inflammatory caspase-related pyroptosis: mechanism, regulation and therapeutic potential for inflammatory bowel disease. Gastroenterol Rep (Oxf) 6, 167–176. doi:10.1093/gastro/goy011.

79 Knodler, L. A., Crowley, S. M., Sham, H. P., Yang, H., Wrande, M., Ma, C., et al. (2014). Non-canonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. Cell Host Microbe 16, 249–256. doi:10.1016/j.chom.2014.07.002.

80 Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., et al. (2016). Inflammasome - activated gasdermin D causes pyroptosis by forming membrane pores. Nature 535, 153–158. doi:10.1038/nature18629.

81 Bergsbaken, T., Fink, S. L., den Hartigh, A. B., Loomis, W. P., and Cookson, B. T. (2011). Coordinated host responses during pyroptosis: caspase-1-dependent lysosome exocytosis and inflammatory cytokine maturation. J Immunol 187, 2748–2754. doi:10.4049/jimmunol.1100477

82 Yip, L., Woehrle, T., Corriden, R., Hirsh, M., Chen, Y., Inoue, Y., et al. (2009). Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. The FASEB Journal 23, 1685–1693. doi:10.1096/fj.08-126458.

83 Liu, Y., Xiao, Y., and Li, Z. (2011). P2X7 receptor positively regulates MyD88-dependent NF-κB activation. Cytokine 55, 229–236. doi:10.1016/j.cyto.2011.05.003.

84 Tafani, M., De Santis, E., Coppola, L., Perrone, G. A., Carnevale, I., Russo, A., et al. (2014). Bridging hypoxia, inflammation and estrogen receptors in thyroid cancer progression. Biomedicine & Pharmacotherapy 68, 1–5. doi:10.1016/j.biopha.2013.10.013.

85 Adinolfi, E., Callegari, M. G., Cirillo, M., Pinton, P., Giorgi, C., Cavagna, D., et al. (2009). Expression of the P2X7 receptor increases the Ca2+ content of the endoplasmic reticulum, activates NFATc1, and protects from apoptosis. The Journal of biological chemistry 284, 10120–10128. doi:10.1074/jbc.M805805200.

86 Xu, Y., Zeumer, L., Reeves, W. H., and Morel, L. (2014). Induced murine models of systemic lupus erythematosus. Methods in Molecular Biology 1134, 103–130. doi:10.1007/978-1-4939-0326-9_9.

87 Zhao, J., Wang, H., Dai, C., Wang, H., Zhang, H., Huang, Y., et al. (2013). P2X 7 Blockade Attenuates Murine Lupus Nephritis by Inhibiting Activation of the NLRP3/ASC/Caspase 1 Pathway: Attenuation of Lupus Nephritis by P2X 7 Blockade. Arthritis & Rheumatism 65, 3176–3185. doi:10.1002/art.38174.

88 Peairs, A., Radjavi, A., Davis, S., Li, L., Ahmed, A., Giri, S., et al. (2009). Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. Clin Exp Immunol 156, 542–551. doi:10.1111/j.1365-2249.2009.03924.x.

89 Peng, X., Yang, T., Liu, G., Liu, H., Peng, Y., and He, L. (2018). Piperine ameliorated lupus nephritis by targeting AMPK-mediated activation of NLRP3 inflammasome. International Immunopharmacology 65, 448–457. doi:10.1016/j.intimp.2018.10.025

90 Kahlenberg, J. M., Yalavarthi, S., Zhao, W., Hodgin, J. B., Reed, T. J., Tsuji, N. M., et al. (2014). An essential role of caspase 1 in the induction of murine lupus and its associated vascular damage. Arthritis & Rheumatology (Hoboken, N.J.) 66, 152–162. doi:10.1002/art.38225.

91 Turner, C. M., Tam, F. W. K., Lai, P.-C., Tarzi, R. M., Burnstock, G., Pusey, C. D., et al. (2006). Increased expression of the pro-apoptotic ATP-sensitive P2X7 receptor in experimental and human glomerulonephritis. Nephrology Dialysis Transplantation 22, 386–395. doi:10.1093/ndt/gfl589

92 Cigni, A., Pileri, P. V., Faedda, R., Gallo, P., Sini, A., Satta, A. E., et al. (2015). Interleukin 1, Interleukin 6, Interleukin 10, and Tumor Necrosis Factor α in Active and Quiescent Systemic Lupus Erythematosus. Journal of Investigative Medicine 62, 825–829. doi:10.2310/jim.0000000000000085.

93 Magna, M., and Pisetsky, D. S. (2015). The Role of Cell Death in the Pathogenesis of SLE: Is Pyroptosis the Missing Link? Scandinavian Journal of Immunology 82, 218–224. doi:10.1111/sji.12335.

94 Asgari, E., Le Friec, G., Yamamoto, H., Perucha, E., Sacks, S. S., Kohl, J., et al. (2013). C3a modulates IL-1 secretion in human monocytes by regulating ATP efflux and subsequent NLRP3 inflammasome activation. Blood 122, 3473–3481. doi:10.1182/blood-2013-05-502229.

95 Bergsbaken, T., Fink, S. L., den Hartigh, A. B., Loomis, W. P., and Cookson, B. T. (2011). Coordinated host responses during pyroptosis: caspase-1-dependent lysosome exocytosis and inflammatory cytokine maturation. Journal of immunology (Baltimore, Md. : 1950) 187, 2748–54. doi:10.4049/jimmunol.1100477.

96 Pisetsky, D. S. (2014). The complex role of DNA, histones and HMGB1 in the pathogenesis of SLE. Autoimmunity 47, 487–493. doi:10.3109/08916934.2014.921811.

97 Xu, J., Jiang, Y., Wang, J., Shi, X., Liu, Q., Liu, Z., et al. (2014). Macrophage endocytosis of high-mobility group box 1 triggers pyroptosis. Cell Death Differ 21, 1229–1239. doi:10.1038/cdd.2014.40.

98 Villanueva, E., Yalavarthi, S., Berthier, C. C., Hodgin, J. B., Khandpur, R., Lin, A. M., et al. (2011). Netting Neutrophils Induce Endothelial Damage, Infiltrate Tissues, and Expose Immunostimulatory Molecules in Systemic Lupus Erythematosus. The Journal of Immunology 187, 538–552. doi:10.4049/jimmunol.1100450.

99 Khandpur, R., Lin, A. M., Rubin, C. J., Zhao, W., Olsen, S. H., Kretzler, M., et al. (2012). Netting neutrophils induce endothelial damage, infiltrate tissues and expose immunostimulatory molecules in systemic lupus erythematosus . 187, 734–763. doi:10.4049/jimmunol.1100450.Netting.

100 Di Virgilio, F., Giuliani, A. L., Vultaggio-Poma, V., Falzoni, S., and Sarti, A. C. (2018). Non-nucleotide agonists triggering P2X7 receptor activation and pore formation. Frontiers in Pharmacology 9, 1–10. doi:10.3389/fphar.2018.00039.

101 Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C., Gregorio, J., et al. (2012). Neutrophils Activate Plasmacytoid Dendritic Cells by Releasing Self-DNA–Peptide Complexes in Systemic Lupus Erythematosus . Science translational medicine 3, 1–20. doi:10.1126/scitranslmed.3001180.Neutrophils.

102 Anders, H.-J., and Schaefer, L. (2014). Beyond tissue injury-damage-associated molecular patterns, toll-like receptors, and inflammasomes also drive regeneration and fibrosis. J. Am. Soc. Nephrol. 25, 1387–1400. doi:10.1681/ASN.2014010117

103 Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A. B., Opitz, B., van der Meer, J. H. M., et al. (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood 113, 2324–2335. doi:10.1182/blood-2008-03-146720.

104 Komatsuda, A., Wakui, H., Iwamoto, K., Ozawa, M., Togashi, M., Masai, R., et al. (2008). Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin. Exp. Immunol. 152, 482–487. doi:10.1111/j.1365-2249.2008.03646.x.

105 Liu, Y., Liao, J., Zhao, M., Wu, H., Yung, S., Chan, T. M., et al. (2015). Increased expression of TLR2 in CD4+ T cells from SLE patients enhances immune reactivity and promotes IL - 17 expression through histone modifications. European Journal of Immunology 45, 2683–2693. doi:10.1002/eji.201445219.

106 Wong, C. K., Wong, P. T. Y., Tam, L. S., Li, E. K., Chen, D. P., and Lam, C. W. K. (2010). Activation profile of Toll-like receptors of peripheral blood lymphocytes in patients with systemic lupus erythematosus. Clin Exp Immunol 159, 11–22. doi:10.1111/j.1365-2249.2009.04036.x.

107 Jin, O., Kavikondala, S., Mok, M.-Y., Sun, L., Gu, J., Fu, R., et al. (2010). Abnormalities in circulating plasmacytoid dendritic cells in patients with systemic lupus erythematosus. Arthritis Res Ther 12, R137. doi:10.1186/ar3075.

108 Torigoe, M., Sakata, K., Ishii, A., Iwata, S., Nakayamada, S., and Tanaka, Y. (2018). Hydroxychloroquine efficiently suppresses inflammatory responses of human class-switched memory B cells via Toll-like receptor 9 inhibition. Clinical Immunology 195, 1–7. doi:10.1016/j.clim.2018.07.003.

109 Shin, M. S., Kang, Y., Lee, N., Kim, S. H., Kang, K. S., Lazova, R., et al. (2012). U1-snRNP activates the NLRP3 inflammasome in human monocytes. J Immunol 188, 4769–4775. doi:10.4049/jimmunol.1103355.

110 Zhang, W., Cai, Y., Xu, W., Yin, Z., Gao, X., and Xiong, S. (2013). AIM2 Facilitates the Apoptotic DNA-induced Systemic Lupus Erythematosus via Arbitrating Macrophage Functional Maturation. Journal of Clinical Immunology 33, 925–937. doi:10.1007/s10875-013-9881-6.

111Choubey, D. (2012). DNA-responsive inflammasomes and their regulators in autoimmunity. Clinical Immunology 142, 223–231. doi:10.1016/j.clim.2011.12.007.

112 Davis, B. K., Wen, H., and Ting, J. P.-Y. (2011). The Inflammasome NLRs in Immunity, Inflammation, and Associated Diseases. Annu Rev Immunol 29, 707–735. doi:10.1146/annurev-immunol-031210-101405.

113 Chen, G.-M., Feng, C.-C., Ye, Q.-L., Tao, J., Li, R., Peng, H., et al. (2013). Association of P2X7R gene polymorphisms with systemic lupus erythematosus in a Chinese population. Mutagenesis 28, 351–355. doi:10.1093/mutage/get007.

114 Lee, Y. H., Choi, S. J., Ji, J. D., and Song, G. G. (2016). Association between toll-like receptor polymorphisms and systemic lupus erythematosus: a meta-analysis update. Lupus 25, 593–601. doi:10.1177/0961203315622823.

115 Pontillo, A., Girardelli, M., Kamada, A. J., Pancotto, J. A. t., Donadi, E. A., Crovella, S., et al. (2012). Polimorphisms in Inflammasome Genes Are Involved in the Predisposition to Systemic Lupus Erythematosus. Autoimmunity 45, 271–278. doi:10.3109/08916934.2011.637532.

116 Yang, C.-A., Huang, S.-T., and Chiang, B.-L. (2015a). Sex-dependent differential activation of NLRP3 and AIM2 inflammasomes in SLE macrophages. Rheumatology 54, 324–331. doi:10.1093/rheumatology/keu318.

117 Camargo, J. F., Correa, P. A., Castiblanco, J., and Anaya, J.-M. (2004). Interleukin-1β polymorphisms in Colombian patients with autoimmune rheumatic diseases. Genes & Immunity 5, 609–614. doi:10.1038/sj.gene.6364133.

118 Wen, D., Liu, J., Du, X., Dong, J.-Z., and Ma, C.-S. (2014). Association of Interleukin-18 (-137G/C) Polymorphism with Rheumatoid Arthritis and Systemic Lupus Erythematosus: A Meta-Analysis. International Reviews of Immunology 33, 34–44. doi:10.3109/08830185.2013.816699.

119 Ostendorf, B., Iking-Konert, C., Kurz, K., Jung, G., Sander, O., and Schneider, M. (2005). Preliminary results of safety and efficacy of the interleukin 1 receptor antagonist anakinra in patients with severe lupus arthritis. Ann Rheum Dis 64, 630–633. doi:10.1136/ard.2004.025858.

120 Wozniacka, A., Lesiak, A., Narbutt, J., McCauliffe, D. P., and Sysa-Jedrzejowska, A. (2006). Chloroquine treatment influences proinflammatory cytokine levels in systemic lupus erythematosus patients. Lupus 15, 268–275. doi:10.1191/0961203306lu2299oa

121 Marques-da-Silva, C., Chaves, M., Castro, N., Coutinho-Silva, R., and Guimaraes, M. (2011). Colchicine inhibits cationic dye uptake induced by ATP in P2X2 and P2X7 receptor-expressing cells: implications for its therapeutic action. Br J Pharmacol 163, 912–926. doi:10.1111/j.1476-5381.2011.01254.x.

122 Park, J. S., Svetkauskaite, D., He, Q., Kim, J.-Y., Strassheim, D., Ishizaka, A., et al. (2004). Involvement of Toll-like Receptors 2 and 4 in Cellular Activation by High Mobility Group Box 1 Protein. J. Biol. Chem. 279, 7370–7377. doi:10.1074/jbc.M306793200

123 Hochberg, M. C. (1997). Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 40, 1725. doi:10.1002/1529-0131(199709)40:9<1725::AID-ART29>3.0.CO;2-Y

124 Gladman, D. D., Ibañez, D., and Urowitz, M. B. (2002). Systemic lupus erythematosus disease activity index 2000. J. Rheumatol. 29, 288–291.

125 Gladman, D. D., Goldsmith, C. H., Urowitz, M. B., Bacon, P., Fortin, P., Ginzler, E., et al. (2000). The Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index for Systemic Lupus Erythematosus International Comparison. J. Rheumatol. 27, 373–376.

126 Harris, E. N., Gharavi, A. E., Patel, S. P., and Hughes, G. R. (1987). Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986. Clin Exp Immunol 68, 215–222.

127 Horbach, D. A., van Oort, E., Donders, R. C., Derksen, R. H., and de Groot, P. G. (1996). Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for the detection of antiphospholipid antibodies. Thromb. Haemost. 76, 916–924.

128 Sanz, J. M., Falzoni, S., Rizzo, R., Cipollone, F., Zuliani, G., and Di Virgilio, F. (2014). Possible protective role of the 489C>T P2X7R polymorphism in Alzheimer's disease. Experimental gerontology 60, 117–119. doi:10.1016/j.exger.2014.10.009.

129 Dardano, A., Falzoni, S., Caraccio, N., Polini, A., Tognini, S., Solini, A., et al. (2009). 1513A>C polymorphism in the P2X7 receptor gene in patients with papillary thyroid cancer: correlation with histological variants and clinical parameters. The Journal of clinical endocrinology and metabolism 94, 695–698. doi:10.1210/jc.2008-1322.

130 De Marchi, E., Orioli, E., Dal Ben, D., and Adinolfi, E. (2016). P2X7 Receptor as a Therapeutic Target. 1st ed. Elsevier Inc. doi:10.1016/bs.apcsb.2015.11.004.

131 Forchap, S. L., Anandacoomarasamy, A., Wicks, J., Di Virgilio, F., Baricordi, O. R., Rubini, M., et al. (2008). P2X7 gene polymorphisms do not appear to be a susceptibility gene locus in sporadic cases of systemic lupus erythematosus. Tissue antigens 72, 487–490. doi:10.1111/j.1399-0039.2008.01136.x.

132 Ryu, S., Fu, W., and Petri, M. A. (2017). Associates and predictors of pleurisy or pericarditis in SLE. Lupus Sci Med 4. doi:10.1136/lupus-2017-000221.

133 Morel, N., Bonjour, M., Le Guern, V., Le Jeunne, C., Mouthon, L., Piette, J.-C., et al. (2015). Colchicine: a simple and effective treatment for pericarditis in systemic lupus erythematosus? A report of 10 cases. Lupus 24, 1479–1485. doi:10.1177/0961203315593169.

134 Padeh, S., and Berkun, Y. (2016). Familial Mediterranean fever. Curr Opin Rheumatol 28, 523–529. doi:10.1097/BOR.0000000000000315.

135 Klonowska-Szymczyk, A., Kulczycka-Siennicka, L., Robak, T., Smolewski, P., Cebula-Obrzut, B., and Robak, E. (2017). The impact of agonists and antagonists of TLR3 and TLR9 on concentrations of IL-6, IL10 and IL-2R in culture supernatants of peripheral blood mononuclear cells derived from patients with systemic lupus erythematosus. Postepy Hig Med Dosw (Online) 71, 867–875. doi:10.5604/01.3001.0010.5266.

136 Perruzza, L., Gargari, G., Proietti, M., Fosso, B., D'Erchia, A. M., Faliti, C. E., et al. (2017). T Follicular Helper Cells Promote a Beneficial Gut Ecosystem for Host Metabolic Homeostasis by Sensing Microbiota-Derived Extracellular ATP. Cell Rep 18, 2566–2575. doi:10.1016/j.celrep.2017.02.061.

137 Gualtierotti, R., Faliti, C. E., Gerosa, M., Grassi, F., and Meroni, P. L. (2017). THU0240 Defective regulation by ATP-GATED ionotropic P2X7 receptor drives T follicular helper cell expansion in systemic lupus erythematosus. Annals of the Rheumatic Diseases 76, 294–295. doi:10.1136/annrheumdis-2017-eular.3914.

138 Peterson, E., Robertson, A., and Emlen, W. (1996). Serum and urinary interleukin-6 in systemic lupus erythematosus. Lupus 5, 571–575. doi:10.1177/096120339600500603.

139 Yoshio, T., Okamoto, H., Kurasawa, K., Dei, Y., Hirohata, S., and Minota, S. (2016). IL-6, IL-8, IP-10, MCP-1 and G-CSF are significantly increased in cerebrospinal fluid but not in sera of patients with central neuropsychiatric lupus erythematosus. Lupus 25, 997–1003. doi:10.1177/0961203316629556.

140 Wiseman, S. J., Bastin, M. E., Hamilton, I. F., Hunt, D., Ritchie, S. J., Amft, E. N., et al. (2017). Fatigue and cognitive function in systemic lupus erythematosus: associations with white matter microstructural damage. A diffusion tensor MRI study and meta-analysis. Lupus 26, 588–597. doi:10.1177/0961203316668417.

APPENDIX

APPENDIX 1: 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, characterized 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, hemoglobin, granular tubular, or mixed
Neurological disorder	a. Seizures: in the absence of offending drugs or known metabolic derangements (eg, uremia, acidosis, or electrolyte imbalance) or b. Psychosis: in the absence of offending drugs or known metabolic derangements (eg, uremia, acidosis, or electrolyte imbalance)
Haematologic disorder	a. Hemolytic anemia with reticulocytosis, or b. Leukopenia: <4000/mm3, or c. Lymphopenia: <1500/mm3, ord. Thrombocytopenia: <100 000/mm3 in the absence of offending drug
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

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

APPENDIX 2: 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 behavior. 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, infarction, splinter haemorrhages confirmed by 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 a 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	2
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
Increase 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 3: 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)