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Systems biology of lupus: Mapping the impact of genomic and environmental factors on gene expression signatures, cellular signaling, metabolic pathways, hormonal and cytokine imbalance, and selecting targets for treatment

ANDRAS PERL

Division of Rheumatology, Departments of Medicine and Microbiology and Immunology, College of Medicine, State University of New York, Upstate Medical University, Syracuse, NY 13210, USA

Abstract

Systemic lupus erythematosus (SLE) is characterized by the dysfunction of T cells, B cells, and dendritic cells, the release of pro-inflammatory nuclear materials from necrotic cells, and the formation of antinuclear antibodies (ANA) and immune complexes of ANA with DNA, RNA, and nuclear proteins. Activation of the mammalian target of rapamycin (mTOR) has recently emerged as a key factor in abnormal activation of T and B cells in SLE. In T cells, increased production of nitric oxide and mitochondrial hyperpolarization (MHP) were identified as metabolic checkpoints upstream of mTOR activation. mTOR controls the expression T-cell receptor-associated signaling proteins CD4 and CD3 ζ through increased expression of the endosome recycling regulator Rab5 and HRES-1/Rab4 genes, enhances Ca²⁺ fluxing and skews the expression of tyrosine kinases both in T and B cells, and blocks the expression of Foxp3 and the generation of regulatory T cells. MHP, increased activity of mTOR, Rab GTPases, and Syk kinases, and enhanced Ca²⁺ flux have emerged as common T and B cell biomarkers and targets for treatment in SLE.

Keywords

Lupus; mitochondria; nitric oxide; glutathione; mammalian target of rapamycin; Syk

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease of unknown etiology where the pathogenesis is attributed to genetic and environmental factors causing the dysfunction of T and B lymphocytes [1,2] and dendritic cells (DCs) [3,4], and the formation of antinuclear antibodies (ANA) and immune complexes of ANA with DNA, RNA, and nuclear proteins. Aberrant activation and death signaling in T lymphocytes underlie the pathology of SLE (Table I) [5]. Mitochondria, the organelle that controls death

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Correspondence: A. Perl, College of Medicine, State University of New York, 750 East Adams Street, Syracuse, NY 13210, USA. Tel: 1 315 464 4192. Fax: 1 315 464 4176. perla@upstate.edu.

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signal processing, is dysfunctional in lupus T cells. They are exhibiting a persistent elevation of the mitochondrial transmembrane potential (ψ_m) or mitochondrial hyperpolarization (MHP) [6] and ATP depletion, which predispose to cell death by necrosis that is highly proinflammatory [7]. The increased release of necrotic materials from T cells could drive disease pathogenesis by activating macrophages and DCs and enhancing their capacity to produce nitric oxide (NO) and interferon α (IFN- α) in SLE [7]. Indeed, DCs exposed to necrotic, but not apoptotic, cells induce lupus-like disease in MRL mice and accelerate the disease of MRL/lpr mice [8].

Dysregulated signaling through TCR ζ is a critical determinant of abnormal T cell activation in SLE [9,10]. Activation of the mammalian target of rapamycin (mTOR) contributes to abnormal signaling through the TCR by enhancing the expression of the small GTPase HRES-1/Rab4 that targets CD4 [11] and TCR ζ for lysosomal degradation [12]. Lupus B cells also exhibit abnormal signaling through the B cell receptor (BCR) [2], mTOR activation [13], and enhanced Ca^{2+} flux [14]. The release of necrotic materials from T cells promotes the activation of B cells and DCs [7].

T cell dysfunction in-systemic lupus erythematosus (SLE)

Impact of mitochondrial dysfunction and oxidative stress on T cell activation

With MHP and extrusion of H^+ ions from the mitochondrial matrix, the cytochromes within the electron transport chain become more reduced which promotes ROI production and generates oxidative stress [15]. ROI modulate T cell activation, cytokine production, and proliferation at multiple levels [7]. The antigen-binding $\alpha\beta$ or $\gamma\delta$ TCR is associated with a multimeric receptor module comprising the CD3 $\gamma\delta\epsilon$ and ζ chains (Figure 1). The cytoplasmic domain of CD3 ζ chain contains an immunoglobulin receptor family tyrosine-based activation motif (ITAM) which is crucial for coupling of intracellular tyrosine kinases [16]. Expression of CD3 ζ is suppressed by ROI [17]. Binding of p56^{lck} to CD4 or CD8 attracts this kinase to the TCR-CD3 complex, leading to phosphorylation of ITAM. Phosphorylation of both tyrosines of each ITAM is required for SH-2-mediated binding by zeta-associated protein-70 (ZAP-70) or the related SYK. ZAP-70 is activated through phosphorylation by p56^{lck}. Activated ZAP-70 and SYK target two key adaptor proteins, LAT and SLP-76 [18].

Phosphorylated LAT binds directly to phospholipase C- γ 1 (PLC γ 1) that controls hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Phosphorylation of inositol lipid second messengers is mediated by phosphatidylinositol 3'-hydroxyl kinase (PI3K). The stimulatory effect of the TCR alone on PI3K activity is small. Concurrent triggering of the CD28 co-stimulatory molecule by its ligands CD80 or CD86 is required for optimal PI3K activation. IP3 binds to its receptors in the endoplasmic reticulum (ER), opening Ca^{2+} channels that release Ca^{2+} to the cytosol. Decreased ER Ca^{2+} concentration activates the Ca^{2+} release-activated Ca^{2+} channel (CRAC) in the cell membrane. Ca^{2+} depletion in the ER initiates the redistribution of STIM1 toward ER-plasma membrane junctions and direct binding of Stim1 to Orai1 that opens the CRAC channel. The resultant Ca^{2+} influx activates the phosphatase calcineurin, which dephosphorylates the transcription factor, NFAT.

Dephosphorylated NFAT can translocate to the nucleus where it promotes transcription of IL-2 in concert with AP-1, NF- κ B, and Oct-1. While activities of AP-1 and NF- κ B are increased by oxidative stress [19], both thiol insufficiency and H₂O₂ treatment suppress calcineurin-mediated activation of NFAT [20]. Lupus T cells have decreased amounts of DNA-binding 98 kDa form of the Elf-1 transcription factor that reduces the expression of TCR ζ and augments the expression of Fc ϵ RI γ . Further upstream, the expression of protein phosphatase 2A (PP2A), that dephosphorylates Elf-1 at Thr-231 is enhanced in lupus T cells. Dephosphorylation results in limited expression and binding of the 98 kDa Elf-1 form to the TCR ζ and Fc ϵ RI γ promoters. Suppression of the expression of the PP2A leads to increased expression of TCR ζ and decreased expression of Fc ϵ RI γ and correction of the early signaling response [21].

PP2A expression is increased through demethylation of its promoter [22], a process triggered by oxidative stress [23]. Thus, expression of T-cell signal transduction molecules (TCR ζ and Fc ϵ RI γ) and cytokines, i.e. IL-2 (with AP-1 and NFAT motif-containing promoter) and IL-4 (with AP-1-less NFAT enhancer), can be selectively regulated by oxidative stress depending on the relative expression level of transcription factors involved (Figure 1). Expression of Stat3, which is also driven by oxidative stress [24], is increased in both lupus T [25] and B cells [13]. Stat3-dependent signals play a key role in the differentiation of Th17 cells [26], which appear to mediate nephritis in patients with SLE [27,28].

Activation of the-mammalian target of rapamycin (mTOR) in lupus T cells

Rapamycin normalized T-cell mitogen-stimulated splenocyte proliferation and IL-2 production, prevented the typical rise in anti-double-stranded DNA antibody and urinary albumin levels and glomerulonephritis (GN), and prolonged survival of lupus-prone MRL/lpr mice [29]. mTOR is associated with the outer mitochondrial membrane and senses mitochondrial dysfunction and changes in the ψ_m of T cells [30]. With a focus on mitochondrial dysfunction, we began to utilize rapamycin for treatment of SLE patients resistant or intolerant to conventional medications. Rapamycin markedly improved disease activity [31]. This occurred with the normalization of baseline Ca²⁺ levels in the cytosol and mitochondria and of CD3/CD28-induced Ca²⁺ fluxing while MHP persisted, indicating that increased Ca²⁺ fluxing is downstream or independent of MHP in the pathogenesis of T cell dysfunction in SLE [31].

The immunosuppressive properties of rapamycin have been attributed to the blocking of TOR complex 1 that is required for transducing T cell activation initiated by cytokines [32]. TORC2 is required for organization of the actin cytoskeleton and it is rapamycin insensitive. While mTOR expression was similar in lupus and control T cells, phosphorylation levels of two key mTOR substrates S6K1 and 4E-BP1 were dramatically increased in lupus T cells and such changes were reversed in rapamycin-treated patients [12]. These findings suggest that mTOR kinase activity is increased in lupus T cells and it is reversed by rapamycin treatment. Overexpression of the cellular receptor of rapamycin, FKBP12, was identified as a component of the mitochondrial gene expression signature in lupus T cells.

In accordance with the persistence of increased mitochondrial mass and MHP in rapamycin-treated patients [31], elevated expression of FKBP12 was unaffected by rapamycin treatment [12], suggesting that mTOR activation may be a consequence of genetic factors underlying MHP and increased mitochondrial biogenesis in lupus T cells [33]. Such genetic factors may also operate in lupus B cells [13,34]. Autophagy mediates the bulk degradation of cytoplasmic contents, proteins, and organelles including mitochondria in T cells [35] and in lysosomes. This process is induced by rapamycin through inactivating mTOR [36]. Interestingly, rapamycin treatment *in vivo* did not affect MHP or mitochondrial mass of lupus T cells [31]. However, rapamycin treatment normalized baseline and T-cell activation-induced Ca^{2+} fluxing. Such outcome may result from modulating the expression of small GTPases Rab5 and HRES-1/Rab4 that control endocytic traffic and degradation of key molecules of T-cell signal transduction including CD4 [11] and TCR/CD3 ζ [12].

Akt, a kinase that phosphorylates multiple targets in T cells, may be a key link in the chain of events that activate mTOR [33]. Upstream, phosphatidylinositol accumulation by phosphoinositide 3-kinases (PI3K) induces the localization of 3-phosphatidylinositol-dependent protein kinase 1 (PDK1) to the plasma membrane that in turn phosphorylates and activates Akt [37]. Activation of class IA-PI3K in T cells extends CD4⁺ memory cell survival, triggering an invasive lymphoproliferative disorder and systemic lupus [38]. In turn, the lipid phosphatase phosphatase and tensin (PTEN) homolog deleted in chromosome 10) counteracts phosphatidylinositol accumulation by PI3K and mice with PTEN haploinsufficiency also develop systemic autoimmunity [39].

Importantly, the rictor–mTOR complex directly phosphorylates Akt/PKB on Ser473 *in vitro* and facilitates Thr308 phosphorylation by PDK1 [40]. Thus, activation of mTOR may also account for elevated Akt activity and provide a positive feed-back loop of T cell activation in SLE. mTOR controls the expression of Foxp3 and development of regulatory T cells [41,42] which are deficient in patients with SLE [43,44]. The effectiveness of rapamycin in murine and human SLE suggests that mTOR is a key mediator of autoimmunity in SLE. Therefore, understanding the mechanisms of persistent MHP that leads to mTOR activation and enhanced Ca^{2+} fluxing may be fundamental to the pathogenesis of lupus (Figure 2).

Activation of endocytic recycling pathway is characterized by partially nitric oxide (NO) inducible and mTOR dependent overexpression of Rab5 and HRES-1/Rab4

The overexpression of Rab5A and HRES-1/Rab4, which control internalization and recycling of surface receptors via early endosomes, respectively, [45,46], are markers of an activated recycling gene expression signature in lupus T cells [12]. In accordance with a dominant impact of HRES-1/Rab4 on the endocytic recycling of CD4 [11], there is an inverse correlation between enhanced HRES-1/Rab4 expression and diminished CD4 expression in negatively isolated CD4 T cells.

Overexpression of HRES-1/Rab4 is also inversely correlated with TCR ζ protein levels. These changes in gene expression are associated with enhanced constitutive recycling of CD3e and CD4 in lupus T cells relative to healthy and rheumatoid arthritis (RA) disease controls. mTOR emerged as a critical checkpoint between the enhanced mitochondrial and receptor recycling gene expression signatures [12]. Such gatekeeper function of mTOR is

consistent with its role in (1) sensing mitochondrial dysfunction and changes of the ψ_m in T cells [30] and (2) modulating the traffic of GLUT4 [47,48] and transferrin receptor (TFR) [49] which are associated with Rab4-positive endosomes in adipocytes [50] and epithelial cells [46], respectively.

Rapamycin treatment *in vivo* not only reduced the expression of HRES-1/Rab4 and Rab5A but also reversed the loss of CD4, Lck, and TCR ζ chain and the overexpression of Fc ϵ RI γ and Syk in lupus T cells. GST pull-down studies revealed a direct interaction of HRES-1/Rab4 with TFR, CD4, CD2AP, and TCR ζ . Both the knockdown of HRES-1/Rab4 expression by siRNA and the inhibition of lysosomal function increased TCR ζ levels in lupus T cells. These observations identified HRES-1/Rab4-dependent lysosomal degradation as a novel mechanism contributing to the critical loss of TCR ζ in lupus T cells [51]. The HRES-1 endogenous retrovirus was earlier mapped to 1q42 [52] and polymorphism of its long terminal repeat (LTR) region has been associated with the development of SLE [53,54]. Although HRES-1/Rab4 overexpression was moderated in rapamycin-treated patients, it remained elevated relative to healthy controls [12]. Increased expression of HRES-1/Rab4 may also be influenced by polymorphic haplotypes of the LTR [55] which harbors an enhancer of HRES-1/Rab4 gene transcription [11].

Checkpoints of Ca²⁺ fluxing

The Ca²⁺ signal is dysregulated in SLE T cells in a number of ways. SLE T cells have elevated intracellular and mitochondrial Ca²⁺ at baseline [56], while the amount of Ca²⁺ present in the ER is normal [57]. Although the T-cell activation-induced Ca²⁺ flux is elevated initially, the plateau phase is reduced relative to activated control T cells [56]. There are at least three potentially interrelated mechanisms that account for altered Ca²⁺ flux in SLE: (1) abnormal formation of the immunological synapse in T cells [58,59] and possibly also in B cells [60,61], (2) increased mitochondrial biogenesis and Ca²⁺ storage in mitochondria [56], and (3) mTOR activation enhances IP3R-mediated Ca²⁺ release from the ER [62].

Dysregulation of NO production may play a role in all three mechanisms since normal T cells pretreated with NO donors recapitulate the Ca²⁺ fluxing abnormalities observed in SLE T cells [56]. NO induces (1) the expression of HRES-1/Rab4 that regulates receptor recycling and targets CD4 [11] and TCR ζ for lysosomal degradation [12] and (2) promotes mitochondrial biogenesis and mitochondrial storage of Ca²⁺ [56] and enhances mTOR activation [12]. Considering that Ca²⁺ is required for activation of PKC, calcineurin, NFAT, and production of IL-2, the altered Ca²⁺ homeostasis may account for the inappropriate activation of T cells in SLE [5].

The importance of T-cell synapse formation and Ca²⁺ homeostasis in lupus has been highlighted by the recent discovery that a nonsense mutation of the filamentous actin inhibiting Coronin1A gene inhibits T-cell synapse formation and T-cell activation-induced Ca²⁺ fluxing [63]. This mutation suppressed the development of lupus on the MRL/lpr background, which was associated with functional alterations in T cells, reduced T-dependent humoral responses, and no detectable intrinsic B cell defects. By transfer of T

cells, it was shown that suppression of autoimmunity could be accounted for by the presence of the Coronin1A mutation in T cells [63].

B cell dysfunction in SLE

Skewing of B lymphocyte subpopulations promotes autoantibody production in the peripheral blood of patients with SLE

SLE is characterized by abnormal B cell activation and differentiation to memory or plasma effector cells [2]. In addition to secretion of autoantibodies, B cells take up and present autoantigens via specific cell surface immunoglobulins to T cells, release a variety of cytokines, and help to regulate and organize inflammatory responses [64]. B cell hyperreactivity may either reflect intrinsic abnormalities or result from immunoregulatory defects in other cell types. The number of B cells is increased in the peripheral blood of patients with active disease [64]. The frequency and absolute number of plasmablasts and plasma cells (PCs) in the peripheral blood of patients with SLE correlated with overall disease activity as measured by the SLEDAI and with anti-dsDNA antibody titers [64].

Plasma effector cells secrete pathogenic antibodies: among others anti-DNA antibodies associated with GN, anti-RoSSA, or anti-La SSB involved in subacute cutaneous lupus erythematosus and congenital heart block, and anti-phospholipid antibodies causing thrombotic events. Autoantibody production usually precedes disease onset, suggesting that the breakdown of B cell tolerance occurs very early in SLE [65]. Early infection by Epstein–Barr virus may represent an environmental trigger contributing to polyclonal B cell activation [66] and promoting the production of antibodies that cross react with autoantigens in SLE [67].

Active SLE is linked to B cell lymphopenia and alterations in peripheral blood B cell subsets that appear integral to the pathogenesis of disease [68]. Naive (CD19+/IgD+/CD27–) peripheral blood B cells are more highly depleted than memory B cells (CD19+/IgD–/CD27+) leading to a predominance of CD27 + memory B cells [2]. This selective depletion of naive B cells could be resolved using SLE IgG VH4.34 antibodies that target a developmentally regulated B220-specific glycoform of CD45 that is an *N*-linked *N*-acetylglucosamine determinant expressed on naive B cells and sterically masked on memory B cells. VH4.34-encoded antibodies are cytotoxic potentially accounting for the depletion of naive B cells in active disease.

The majority of SLE patients show an increase in VH4.34 antibodies during disease activity [69,70]. SLE patients can have expanded populations of both switched memory B cells and CD27 – memory cells as well as increased frequencies of transitional cells and dramatically reduced numbers of unswitched IgD+CD27+ memory cells. The presence of CD19hi B cells correlates with long-term adverse outcomes. These B cells do not appear anergic, as they exhibit high basal levels of phosphorylated Syk and ERK1/2 in response to BCR cross linking, and can become PCs *in vitro*. Autoreactive anti-Sm B cells are enriched in this population and the degree of enrichment correlates with the log of the serum anti-Sm titer, arguing that they undergo clonal expansion before PC differentiation. PC differentiation may occur at sites of inflammation, as CD19hi B cells have elevated CXCR3 levels and

chemotax in response to its ligand CXCL9. Thus, CD19^{hi} B cells are precursors to anti-self PCs, and identify an SLE patient subset likely to experience poor clinical outcomes [71]. B cell subpopulations vary in their magnitude and duration of anti-BCR induced signaling responses.

Abnormal signaling through the B cell receptor (BCR) in SLE

The BCR is a hetero oligomer comprising membrane immunoglobulin (mIg), which confers antigen specificity and the disulfide linked CD79 α/β heterodimer which is non-covalently linked to mIg. Crosslinking of the BCR is rapidly followed by phosphorylation of ITAMs in the intracellular domains of CD79 α/β [72]. The ITAMs on the cytoplasmic domains of CD79 α/β associate non-covalently with Src family protein tyrosine kinases Lyn, Fyn, Lck, and Blk via their SH2 domains [68].

A prerequisite for this binding is the phosphorylation of CD79 α/β by Lyn. Phosphorylation of two precisely spaced tyrosine residues within the ITAM allows recruitment and activation of Syk, a gatekeeper of downstream signaling through PKC, MAPK, or phospholipase C γ 2 (PLC γ 2). PLC γ 2 cleaves the membrane phosphoinositide, PIP2 generating DAG and inositol 1,4,5-triphosphate (IP3). DAG activates protein kinase C β (PKC β) while IP3 binds to specific receptors on the ER inducing the Ca²⁺ release from intracellular stores [24].

Both *Lyn* and *Lyn* deficient mice develop mild lupus-like disease. *Lyn* deficiency was associated with high levels of autoantibody production and GN [73]. B cells from young *Lyn*^{-/-} mice are hyperresponsive to antiIgM induced proliferation, suggesting involvement of Lyn in negative regulation of BCR signaling. Tyrosine phosphorylation of Fc γ RIIB and CD22 coreceptors, which are important for feedback suppression of BCR induced signaling, is severely impaired in *Lyn*^{-/-} B cells. Hypophosphorylation on tyrosine residues of these molecules resulted in failure of recruiting the tyrosine phosphatase SHP1 and inositol phosphatase SHIP, SH2 containing potent inhibitors of BCR induced B cell activation. Consequently, *Lyn*^{-/-} B cells exhibit defects in suppressing BCR induced Ca²⁺ influx and proliferation [74].

Interestingly, mice expressing a constitutively activated form of Lyn due to a single point mutation (Y508F) that negatively regulates Lyn activity, also display heightened Ca²⁺ flux in response to BCR stimulation, develop circulating autoantibodies and lethal GN [75]. Thus, both overexpression and underexpression of Lyn results in lupus-like auto-immunity, suggesting that signaling molecules such as Lyn are tightly regulated [68].

Fc γ RIIB is an important downstream target of Lyn and a negative regulator of signaling through the BCR [68]. Inhibition through Fc γ RIIB is mediated primarily by the activation of SHIP which hydrolyzes PI(3,4,5)P3, an essential element of BCR signaling. SHIP protein levels are lower in SLE B cells, suggesting that SHIP is less available to connect with Fc γ RIIB and inhibit BCR signaling [76]. B cells of lupus-prone mice exhibit diminished SHIP and stress-activated protein kinase (SAPK) activity [77]. An I232T variant of Fc γ RIIB, which has been genetically linked to lupus, was found less effectively distributed to detergent-insoluble lipid rafts at baseline and after BCR ligation [78]. Membrane fractionation revealed that Fc γ RIIB readily segregated into the lipid rafts, while the

Fc γ RIIBI232T isoform was excluded [79]. Although the mechanism by which this transmembrane domain polymorphism prevents partitioning into the lipid raft fraction is unclear, this study suggests that defective signaling through Fc γ RIIB enhances the B cell reactivity in SLE.

The potential importance of B cell synapse formation has been revealed by the observation that a decrease of Lyn is accompanied by concomitant increase of CD45 in lipid raft microdomains [60]. CD45 is a protein tyrosine phosphatase, involved in regulating B lymphocyte selection and modulating signal thresholds. CD45 couples BCR signaling to both cell proliferation and differentiation. Knock-in mice with a single E613R point mutation in the inhibitory wedge of CD45 develop a lymphoproliferative syndrome, with polyclonal T and B lymphocyte activation, anti DNA antibody production, and severe autoimmune nephritis [80]. CD45 can dephosphorylate both the activating pY-396 and negative regulatory pY-507 tyrosines in Lyn. CD45 dephosphorylates the fraction of Lyn that is localized in lipid rafts.

Thus, the Lyn that is in proximity to relevant substrates would be inactive, static, and unable to down regulate BCR signaling. Such uninhibited B cell activation appears to drive autoimmunity in mice with the CD45 E613R wedge mutation and Fas deficiency [81]. The CD45 E613R wedge mutation also promotes autoimmunity in concert with the deficiency of the PEST domain enriched tyrosine phosphatase (Pep $-/-$) [82]. Here, the Pep deficiency serves as a surrogate for lupus-linked inactivating R620W mutation in the analogous human gene PTPN22 [83]. While the CD45 E613R wedge mutation appears to drive B cell autoreactivity, Pep deficiency promotes T cell autoreactivity [82].

Activation of mTOR has recently been demonstrated in B cells isolated from genetically distinct mouse models of lupus and rapamycin derivative ameliorated clinical disease [13]. Surprisingly, mTOR blockade was associated with the crippling of several other signaling axes, suggesting that activation of this pathway plays a central role in human [12,31] and murine lupus [13,29].

Enhanced Ca²⁺ fluxing is linked to altered composition of lipid rafts in lupus B cells

Following BCR stimulation, the activation of PLC C- γ 1 and PLC C- γ 2 enzymes leads to the hydrolysis of PIP₂ to IP₃ and DAGI. IP₃ binds to its receptor on the ER and releases Ca²⁺ to the cytosol by opening Ca²⁺ channels, followed by capacitative Ca²⁺ entry through the plasma membrane. Lupus B cells have higher intracytoplasmic Ca²⁺ responses than controls when stimulated with anti-sIgM or anti-IgD mAb (Table III) [14]. The magnitude of anti-sIgM-mediated protein tyrosine phosphorylation and Ca²⁺ release from intracellular stores are increased in SLE patients' B cells [14]. Fc γ RIIB-dependent inhibition of sIgM-mediated Ca²⁺ flux is deficient in lupus B cells irrespective of disease activity [76].

The accelerated Ca²⁺ flux has been associated with an altered composition of lipid rafts in membrane domains of B cells from patients with SLE [60]. B lymphocytes from SLE patients, but not those from healthy controls, expressed a low molecular weight isoform of CD45 in lipid raft signaling microdomains. SLE patients had increased numbers of B lymphocytes (20–60%) with CD45 in the lipid rafts. Importantly, there was an inverse

relationship between CD45 in the lipid rafts and Lyn levels in these microdomains. This inverse correlation was not significantly associated with disease activity, nor was it influenced by medication use [60,61,76]. Thus, activation of mTOR, altered lipid raft composition, and enhanced Ca^{2+} flux represents common metabolic pathways underlying T and B cell dysfunction in patients with SLE (Tables II and III).

Dendritic cells

DCs are highly potent antigen-presenting cells and stimulators of B and T lymphocytes and; therefore, they are also key controllers of immunity and tolerance [84]. DCs play important roles as sensors of necrotic debris [7] and producers of type I IFN [3].

In particular, plasmacytoid DCs (pDCs) that infiltrate lupus skin lesions are potent producers of IFN- α [3] that in turn induces myeloid DC (mDC) maturation. pDCs are also important sources of increased IL-10 [85] and promote PC differentiation through production of IL-6 [86]. IFN-matured mDCs activate autoreactive T cells [87]. Clinical trials with type I IFN-neutralizing antibodies are currently ongoing in patients with SLE, which will ultimately determine the safety of IFN blockade and further clarify the relative importance of type I IFN and DC activation in SLE.

Gene expression signatures of type I IFN activation, mitochondrial dysfunction, oxidative stress, and receptor recycling underlie the importance of a systems biology approach to signaling abnormalities in SLE

Microarray analysis of gene expression in whole blood or PBMC has been used to identify a variety of genetic signatures in SLE [88]. Several groups described an interferon gene expression signature associated with disease activity in patients with SLE in this subset of cells [89–91]. These previous studies had failed to provide subsets of genes linked to mitochondrial dysfunction of T cells. Without elimination of previously described signatures, microarrays on negatively isolated “untouched” T cells identified two novel transcriptional programs termed mitochondrial and endocytic recycling signatures.

The functional relevance of these gene expression signatures was validated on the protein level by western blot analysis and functional studies. Increased expression of the mitochondrial proteins voltage-dependent anion channel 1 and superoxide dismutase 2 (SOD2) is consistent with increased mitochondrial mass and oxidative stress in lupus T cells [56]. Of note, increased expression of SOD2 was previously reported [90] but remained unidentified as a component of the mitochondrial/metabolic signature. Over-expression of TAL enhanced MHP in Jurkat cells [92] and may also act upstream of MHP of lupus T cells. Reduced expression of NOSIP, which inhibits activity of eNOS and may act upstream of elevated NO production in lupus T cells, was not affected by rapamycin treatment. Thus, TAL and NOSIP control metabolic pathways that regulate ψ_m and may be regarded as markers of the mitochondrial signature in lupus T cells.

Overexpression of Rab5A and Rab4, which control internalization and recycling, respectively, of surface receptors via early endosomes [45,46], were considered markers of an activated recycling gene expression signature. These changes in gene expression were associated with enhanced constitutive recycling of CD3e and CD4 in lupus T cells relative to healthy and RA disease controls [12].

Cytokine imbalance in SLE

Interferon- α

Most patients with SLE have increased IFN serum levels and increased transcription of IFN- α -regulated genes that correlate with disease activity [89–91]. Detection of nucleic acids and induction of type I IFNs are principal elements of antiviral defense and, potentially, could lead to autoimmunity. Mutations in the human 3' repair exonuclease 1 (Trex1) gene cause AicardiGoutieres Syndrome and SLE [93]. Trex1 is an essential negative regulator of the interferon stimulatory DNA (ISD) response. Trex1 can metabolize reverse transcribed DNA and single stranded DNA derived from endogenous retroelements accumulates in Trex1 deficient cells [94]. These findings provide new evidence for the contribution of endogenous retroelements to autoimmunity [95–97].

Interleukin 10

Production of Interleukin 10 (IL-10) is enhanced in patients with SLE [98–100]. pDCs are important sources of increased IL-10 production due to exposure HMGB1 from necrotic debris [85]. Serum IL-10 concentrations are higher in patients with SLE and correlate with clinical and serologic disease activity and anti-DNA antibody titers [99]. IL-10 promotes ROI production and T cell apoptosis [99]; however, it is not responsible for MHP in T cells of patients with SLE. On the other hand, IL-10 acts as a potent stimulator of B cell proliferation and differentiation in SLE [101].

Tumor necrosis factor- α

Tumor necrosis factor- α (TNF- α) is a key mediator of activation-induced cell death; its production is reduced in lupus T cells that exhibit diminished activation-induced apoptosis [102]. TNF- α production is also reduced in lupus-prone mice and exogenous TNF delays the development of nephritis in these mice [103]. TNF- α inhibitors revolutionized the treatment of RA. The use of TNF- α antagonists has been advocated in patients with SLE [104,105]. However, *de novo* development of ANA and drug-induced lupus have also been recently been reported in RA patients treated with TNF- α blockers [106], suggesting that further studies are needed to clarify the usefulness of TNF- α blockade in SLE. As described below, TNF- α blockade could be particularly relevant for the pathogenesis of lupus nephritis.

Interleukin 17

As noted above, a protective role for TNF- α administration in lupusprone (New Zealand Black (NZB) $\times$ New Zealand White (NZW))F1 mice has been established; it remains uncertain whether this effect segregates at the levels of individual TNF- α receptors (TNFR). Interestingly, SLE prone New Zealand Mixed 2328 mice genetically deficient in TNF

receptors 1 and 2 developed accelerated pathological and clinical nephritis with elevated levels of circulating IgG anti-dsDNA autoantibodies and increased numbers of CD4+T lymphocytes, especially activated memory (CD44 highCD62L low) CD4+T cells [27].

These latter cells expressed a Th17 gene profile, were positive for IL-17 intracellular staining by FACS, and produced exogenous IL-17 in culture. In contrast, immunological, pathological, and clinical profiles of mice deficient in either TNFR alone did not differ from those in each other or from those in wildtype controls. Thus, total ablation of TNF α signaling was highly deleterious in the New Zealand Mixed 2328 SLE model. Double negative (DN) T cells are expanded in patients with SLE and produce significant amounts of interleukin 17 (IL-17) and IFN γ [28]. Furthermore, IL17+ and DN T cells are found in kidney biopsies of patients with lupus nephritis. These new findings indicate that the IL-17-producing Th17 T cells may play a critical role in triggering GN both in murine and human SLE.

Cytokines involved in B-cell activation

B-lymphocyte stimulator (BLyS, also known as BAFF) is a member of the TNF ligand superfamily of cytokines that is expressed and secreted by monocytes, macrophages, DCs, and granulocyte colony stimulating factor activated neutrophils [107]. The biologically active, soluble form of BLyS is enzymatically cleaved from the cell membrane-bound form and that in turn can bind to any of three receptors: transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI); B-cell maturation antigen (BCMA); and B-cell lymphocyte activating factor receptor (BAFFR)/BLyS receptor 3, localized primarily on B lymphocytes. Constitutive overexpression of BLyS in transgenic mice results in the development of an autoimmune-like disease that is characterized by hypergammaglobulinemia, autoantibody production, and GN [108].

Treatment with a BLyS antagonist TACI significantly reduces the occurrence of proteinuria and prolongs survival in MRL/lpr mice [109]. BLyS levels are elevated in patients with SLE [110]. The level of BAFF-R occupancy is increased in SLE patients regardless of disease activity [111]. The increased BAFF-R occupancy may contribute to the pathogenesis of SLE since excess BAFF can rescue self-reactive B cells from peripheral deletion [112].

Targeting biomarkers of T cell dysfunction for treatment of SLE

Depletion of intracellular glutathione

Reduced glutathione (GSH) is profoundly depleted in lymphocytes of SLE patients [6]. Low GSH in T cells overexpressing transaldolase predispose to MHP [92]. GSH depletion is robust trigger of MHP via S-nitrosylation of complex I upon exposure to NO [113]. Thus, the effect of NO on MHP is tightly related to GSH levels. Diminished production of GSH in face of MHP and increased ROI production is suggestive of a metabolic defect in *de novo* GSH synthesis or maintenance of its reduced state due to deficiency of NADPH [114]. Recent studies showed diminished GSH/GSSG ratios in the kidneys of 8-month-old versus 4-month-old (NZB $\times$ NZW) F1 mice; treatment with *N*-acetylcysteine (NAC), a precursor of GSH and stimulator of its *de novo* biosynthesis, prevented the decline of GSH/GSSG ratios,

reduced autoantibody production and development of GN, and prolonged the survival of (NZB $\times$ NZW) F1 mice [115].

Oral NAC has been used to treat oxidative stress in patients with idiopathic pulmonary fibrosis (IPF) [116]. In a one-year study of IPF patients treated with prednisone and azathioprine, addition of NAC (3 $\times$ 600 mg/d) improved vital capacity and reduced myelotoxicity in comparison to placebo. Therefore, prospective clinical studies appear justified to assess whether NAC treatment can reverse GSH depletion, correct T-cell signaling defects, and provide clinical benefit to patients with lupus.

Inhibiting NO production

NO induces MHP and mitochondrial biogenesis, increases Ca^{2+} in the cytosol and mitochondria of normal T cells, and recapitulates the enhanced CD3/CD28-induced Ca^{2+} fluxing of lupus T cells [56]. As recently reported, eNOS is recruited to the site of T-cell receptor engagement, locally increasing NO at the immunological synapse in a Ca^{2+} and PI3K-dependent manner, resulting in reduced IL-2 production [117], which is characteristic of SLE [5]. NO contributes to the development of GN in the MRL/*lpr* lupus mouse model [118]. Inactivation of iNOS does not block the development of lupus [119], suggesting a role for eNOS and nNOS isoforms expressed in T cells. However, given the widespread expression of these isoforms in vascular smooth muscle and brain, it will be necessary to develop T-cell-specific approaches for inhibiting NOS to avoid potentially deleterious side effects.

Rapamycin

With a focus on mitochondrial dysfunction, we began to utilize rapamycin for treatment of SLE patients resistant or intolerant to conventional medications. We observed normalization of baseline Ca^{2+} levels in the cytosol and mitochondria and of CD3/CD28-induced Ca^{2+} fluxing as well as persistence of MHP [31], indicating that increased Ca^{2+} fluxing is downstream or independent of MHP in the pathogenesis of T cell dysfunction in SLE. The effectiveness of rapamycin in murine [29] and human SLE suggests that mTOR is a key mediator of autoimmunity in SLE. Rapamycin can selectively expand CD4 $^{+}$ /CD25 $^{+}$ /Foxp3 $^{+}$ regulatory T cells [120,121], which appear to be deficient in patients with SLE [43]. Therefore, understanding the mechanism of persistent MHP that leads to mTOR activation and enhanced Ca^{2+} fluxing may be fundamental to the pathogenesis of SLE.

Syk inhibition

TCR ζ depletion and its functional replacement by Fc ϵ RI γ is a hallmark of altered TCR signaling in lupus T cells [122]. The abnormal expression of the spleen tyrosine kinase Syk and its preferential binding to Fc ϵ RI γ greatly contributes to enhanced Ca^{2+} fluxing after TCR cross-linking [123]. Lupus B cells also have high basal levels of phosphorylated Syk and ERK1/2 in response to BCR cross-linking that promotes differentiation into PCs *in vitro* [71]. R788, an orally bioavailable Syk inhibitor was recently shown to prevent the development of renal disease and to treat established nephritis in NZB/W mice [124]. R788 minimally affected autoantibody titers, while its dose dependence reduced the numbers of

CD4 + activated T cells, suggesting that T cells might be the effective targets of Syk inhibition [124].

B cell-directed therapies

B-cell depletion with rituximab appears to be safe and holds significant therapeutic promise, at least for the majority of SLE patients experiencing profound B cell depletion [125]. Approximately, a third of the patients developed human anti-chimeric antibody titers, which correlated with poor B cell depletion [126]. Rituximab also offers long term, 1 year or longer after treatment, improvement in B cell homeostasis characterized by naive lymphopenia, expansion of a CD27⁺/IgD⁺ (DN) B cells, plasmablasts, and autoreactive VH4.34 memory B cells [127]. The combination of rituximab and mycophenolate mofetil (MMF) was found to be safe and to improve the outcome of relapsing proliferative lupus nephritis [128]. Controlled trials to define the role of rituximab/MMF in this condition are warranted. Preliminary evidence suggests that B-cell-depletion therapy with rituximab is also safe and efficacious for treatment of autoimmune thrombocytopenia and autoimmune hemolytic anemia in pediatric SLE [129].

Belimumab (LymphoStatB; Human Genome Sciences, Inc., Rockville, MA, USA) is a recombinant, fully human, IgG1 mAb that binds to soluble BLyS with high affinity. The antibody exerts its biologic activity by preventing the binding of BLyS to its receptors. Belimumab potently inhibits BLyS-induced proliferation of B cells *in vitro* and prevents human BLyS-induced increases in splenic B cell numbers and serum IgA titers in mice. In cynomolgus monkeys treated with belimumab, reductions as great as 75% were observed in the number of lymphoid tissues and peripheral blood CD20⁺ B cells and CD21⁺ plasmacytoid cells. Importantly, intravenous doses of up to 50 mg/kg delivered every 2 weeks over 6 months were well tolerated in cynomolgus monkeys. On discontinuation of belimumab in cynomolgus monkeys, the numbers of peripheral blood CD20⁺B cells recovered to normal levels within 3–5 months [130]. A phase I study of belimumab in SLE patients with stable, mild to moderate disease activity demonstrated its safety and biologic activity [131].

Intercepting T and B cell interaction: Promising new therapies in lupus

CD40 ligand: CD40 binding to CD40 ligand (CD154) is one of the most robust co-stimulatory signals for T cells. CD154 a member of the TNF family is inducibly expressed on the surface of CD4 T cells, while CD40, is constitutively expressed on B lymphocytes. CD154–CD40 interactions are essential in the formation of germinal centers (GC) and the differentiation of memory and PCs. CD154-expressing T cells engage CD40-expressing B cells inducing them to express activation markers (CD154 and CD69) and differentiation markers (CD38, CD5, and CD27). CD154 have been found to be hyper-expressed on T lymphocytes from patients with active SLE [132]. CD40–CD154 and B7/CD28 co-stimulation blockade effectively treated lupus in mice [133]. Anti-CD154 treatment also appeared initially well tolerated and potentially effective in patients with SLE [134]; however, it has been discontinued due to thrombotic events [135]. **CTLA4Ig:** Costimulatory blockade with CTLA4Ig is an effective treatment of nephritis in lupus-prone mice [136].

Clinical trials with CTLA4Ig (abatacept), which is FDA-approved to treat RA, are underway in patients with lupus nephritis.

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Figure 1.

Schematic outline of the metabolic pathways controlling (PPP, GSH, and NO) and sensing (mTOR) MHP in lupus T cells. In normal T cells, MHP and mitochondrial biogenesis is mediated via production of NO by eNOS or nNOS [140] and up-regulation of transcription factors PGC-1 α , Tfam, and ALAS [147]. NO production by eNOS may be compartmentalized to the T cell synapse [117]. NO causes transient MHP via reversible inhibition of complex IV/cytochrome c oxidase [148] and persistent MHP via S-nitrosylation of complex I of the ETC in a state of GSH depletion [113]. The PPP regulates the ψ_m by producing (1) NADPH that serves as a reducing equivalent for GSH regeneration from its oxidized form GSSG and for the production of NO by NOS and (2) ribose 5-phosphate for biosynthesis of nucleotides, ADP, ATP, NAD, NAADP, (c)ADP-ribose, and cGMP, the latter is a second messenger of NO. NAADP and (c)ADP-ribose induce Ca^{2+} release from the ER via ryanodine receptors (RyR). mTOR senses ψ_m and regulates IP3R-mediated Ca^{2+} release [62]. The intracellular rapamycin receptor FKBP12 directly binds the RyR. The Bcl-2 family proteins control permeability of the outer mitochondrial membrane and release of apoptosis-inducing factors. Necrosis-prone T cells release oxidized DNA and HMGB1 which stimulate B cells, macrophages, and DCs. In turn, B cells produce IL-10 and macrophages and DCs produce NO which stimulate MHP of T cells.

Figure 2. Schematic cascade of signaling pathways controlling MHP and enhanced receptor recycling in lupus T cells. Broken line demarcates checkpoints affected by rapamycin.

Table I

Biomarkers of abnormal T cell death in patients with SLE.

Signal	Effect	Reference
Spontaneous apoptosis ↑	Compartmentalized autoantigen release, disease activity ↓	[6,137]
AICD ↓	Persistence of autoreactive cells	[100,102]
FasL ↑	Spontaneous apoptosis ↑	[99]
ψ_m ↑	ROI ↑, ATP ↓	[6]
GSH ↓	ROI ↑, spontaneous apoptosis ↑	[6,138]
ATP ↓	AICD ↓, predisposes for necrosis ↑	[6,139]
NO ↑	ψ_m ↑, mitochondrial biogenesis ↑	[56,140]
ROI ↑	Spontaneous apoptosis ↑	[6,100]
IL-10 ↑	Spontaneous apoptosis ↓, ROI ↓	[99,100]
IL-12 ↓	Spontaneous apoptosis ↓, ROI ↓	[100]

↑, increase; ↓, decrease.

Table II

Biomarkers of abnormal T cell activation in patients with SLE.

Signal	Effect	Reference
CD3-induced Ca^{2+} flux $\uparrow$	Altered T- cell activation	[57]
Baseline $[\text{Ca}^{2+}]_c$ $\uparrow$	Ca^{2+} influx $\uparrow$	[31,56]
Baseline $[\text{Ca}^{2+}]_m$ $\uparrow$	Ca^{2+} influx $\uparrow$	[31,56]
TCR/CD3 ζ $\downarrow$	Ca^{2+} influx $\uparrow$, IL-2 production $\downarrow$	[9]
Fc ϵ RI γ $\uparrow$	Ca^{2+} influx $\uparrow$	[122]
PP2A $\uparrow$	Elf-1 $\downarrow$	[21]
Lck $\downarrow$	Ca^{2+} influx $\uparrow$	[59,122]
Syk $\uparrow$	Ca^{2+} influx $\uparrow$	[59,141]
Lipid raft formation $\uparrow$	TCR ζ , $\downarrow$, Fc ϵ RI γ $\uparrow$, Syk $\uparrow$	[58,59,141]
Receptor recycling $\uparrow$	CD4 $\downarrow$, TCR ζ $\downarrow$	[12]
Rab5 $\uparrow$, HRES-1/Rab4 $\uparrow$	Receptor recycling $\uparrow$	[12]
mTOR $\uparrow$	Ca^{2+} influx $\uparrow$	[31]
mTOR $\uparrow$	Rab5 $\uparrow$, HRES-1/Rab4 $\uparrow$	[12]
ψ_m $\uparrow$	ROI $\uparrow$, ATP $\downarrow$	[6]
NO $\uparrow$	ψ_m $\uparrow$, mTOR $\uparrow$, Ca^{2+} flux $\uparrow$	[12,56,140]
ROI $\uparrow$	IL-10 $\uparrow$	[6,100]
ROI $\uparrow$	DNMT1 $\downarrow$	[23]
ROI $\uparrow$	TCR ζ $\downarrow$	[17,142]
DNMT1 $\downarrow$	PP2A $\uparrow$, Elf-1 $\downarrow$, TCR ζ $\downarrow$, Fc ϵ RI γ $\uparrow$	[22]
PKC δ $\downarrow$	ERK $\downarrow$, DNMT1 $\downarrow$	[143]
IBP $\downarrow$	Rho GTPase $\downarrow$, ERK $\downarrow$	[144]
Stat3 $\uparrow$	Chemokine-induced T-cell migration $\downarrow$	[25]
CD4 $^-$ /CD8 $^-$ /Th17 $^+$ $\uparrow$	Nephritis	[27,28]

 $\uparrow$, increase; $\downarrow$, decrease.

Table III

Biomarkers of abnormal B cell activation in patients with SLE.

Signal	Effect	Reference
Anti-IgM	Ca ²⁺ flux ↑	[14]
Altered B-cell Synapse	Sustained BCR signaling	[60,61]
Lyn ↓	Sustained BCR signaling	[61,73]
CD45 ↑	Sustained BCR signaling	[60]
SAPK ↓, SHIP ↓	Sustained BCR signaling	[77]
FCγRIIB ↓	Sustained BCR signaling	[76,145]
CD19 ^{high} ↑	Syk ↑, ERK ↑	[71]
Blys ↑	B-cell activation ↑	[146]
Akt/mTOR/PKCα/STAT3 ↑	B-cell activation ↑	[13]

↑, increase; ↓, decrease.