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NON-CLASSICAL MONOCYTES IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE): MOLECULAR AND FUNCTIONAL CHARACTERIZATION

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DOCTORAL THESIS

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Όρκος του Ιπποκράτη

Ὄμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγείαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, ἵστορας ποιεύμενος, ἐπιτελέα ποιήσειν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὅρκον τόνδε καὶ ξυγγραφὴν τήνδε.

Ἡγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτῃσιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ ωὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄῤῥεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἁπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοισί τε καὶ ὡρκισμένοις νόμῳ ἰητρικῷ, ἄλλῳ δὲ οὐδενί.

Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν.

Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑφηγήσομαι ξυμβουλίην τοιήνδε. Ὁμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἁγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν.

Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτῃσιν ἀνδράσι πρήξιος τῆσδε.

Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἑκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπί τε γυναικείων σωμάτων καὶ ἀνδρῴων, ἐλευθέρων τε καὶ δούλων.

Ἃ δ' ἂν ἐν θεραπείῃ ἢ ἴδω, ἢ ἀκούσω, ἢ καὶ ἄνευ θεραπηίης κατὰ βίον ἀνθρώπων, ἃ μὴ χρή ποτε ἐκλαλέεσθαι ἔξω, σιγήσομαι, ἄῤῥητα ἡγεύμενος εἶναι τὰ τοιαῦτα.

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2. **E.M. Stergioti** et al. Antiviral Innate Immune Responses in Autoimmunity: Receptors, Pathways, and Therapeutic Targeting, **Biomedicines**, 10 (2022) 2820. https://doi.org/10.3390/biomedicines10112820.

PUBLICATIONS IN CONFERENCES *(posters & oral presentations)*

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   Poster: "In SLE patients treated with Belimumab, type I IFN and BAFF gene signatures predict clinical response: Evidence for reversal of molecular pathways linked to activity and severity upon treatment" (G. S. Moysidou, P. Garantziotis, M. Grigoriou, **E. Stergioti** et al)

2. **09-11.03.2023:** ***42nd European Workshop for Rheumatology Research, EWRR, Dublin, Ireland.***

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3. **25-26.11.2022:** ***5th Lupus School, Athens, Greece.***

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4. **26-27.11.2021:** ***4th Lupus School, Athens, Greece.***

Oral: "Non-classical monocytes in Systemic Lupus Erythematosus (SLE): Molecular and Functional characterization" (**E. Stergioti** et al)

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Contents

Abstract

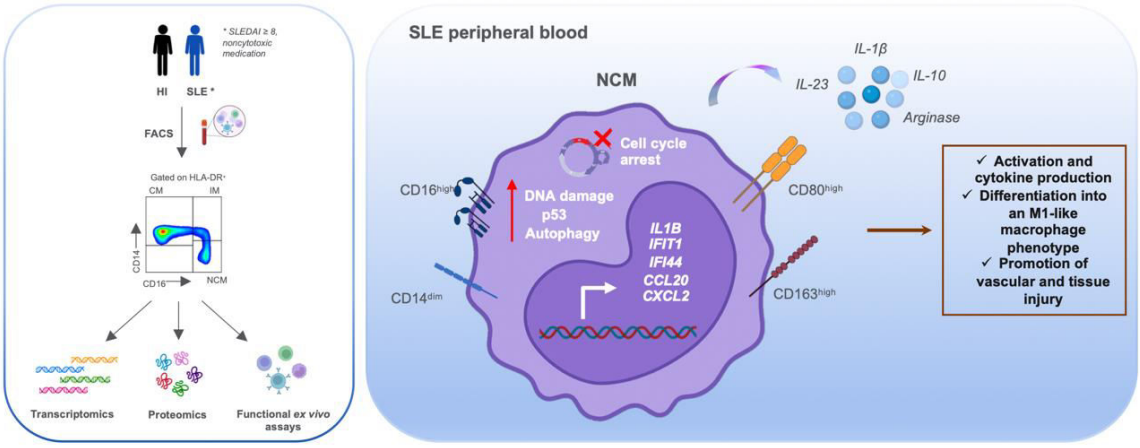
Systemic Lupus Erythematosus (SLE) is a chronic, multisystem autoimmune disease that manifests diverse clinical and molecular abnormalities attributed to the loss of self-tolerance to nucleic acids and endogenous antigens and sustained autoantibody production. Excessive and irreversible tissue damage caused by autoantibodies and immune-complex deposition affects multiple organs leading to significant morbidity and increased mortality. Peripheral blood monocytes propagate inflammation and the development of end-organ damage in SLE. Three major populations of monocytes have been recognized namely classical (CM), intermediate (IM) and non-classical monocytes (NCM). Aberrations in monocytic pathophysiology underlie the SLE pathology and lead to perpetuation of inflammation and tissue injury. Recent studies emphasize the involvement of terminally differentiated immune cell subsets on tissue damage and severity of the disease. Despite the compelling evidence that NCM substantially contribute to disease progression in the target tissues, they remain the least explored subtype. The aim of this study is to further investigate the role and function of NCM in SLE. Understanding the molecular pathways involved in NCM activation and function in the periphery of patients with active SLE, before migrating into the tissues can guide the development of targeted therapies to modulate their effects. To this end, we performed a comprehensive transcriptomic, proteomic and functional characterization of the three peripheral monocytic subsets from active SLE patients and healthy individuals. Our data demonstrate extensive molecular disruptions in circulating SLE NCM, characterized by enhanced inflammatory features such as deregulated DNA repair and cell cycle and heightened IFN signaling combined with differentiation and developmental cues. Enhanced DNA damage, elevated expression of p53, G_0 arrest of cell cycle and increased autophagy stress the differentiation potential of NCM in SLE. This immunogenic profile is associated with an activated macrophage phenotype of NCM exhibiting M1 features in the circulation, fueling the inflammatory response. Together, these findings identify circulating SLE NCM as a pathogenic cell type in the disease that could represent an additional therapeutic target.

Περίληψη

Ο Συστηματικός Ερυθηματώδης Λύκος (ΣΕΛ) είναι μια χρόνια, συστημική αυτοάνοση νόσος που εκδηλώνει ποικίλες κλινικές και μοριακές διαταραχές που αποδίδονται στην απώλεια αυτοανοχής στα νουκλεϊκά οξέα και άλλα ενδογενή αντιγόνα, με συνέπεια την παραγωγή αυτοαντισωμάτων. Η υπερβολική και μη αναστρέψιμη βλάβη των ιστών που προκαλείται από τα αυτοαντισώματα και την εναπόθεση των ανοσουμπλεγμάτων επηρεάζει τα υποκείμενα όργανα οδηγώντας σε σημαντική νοσηρότητα και αυξημένη θνησιμότητα. Τα μονοκύτταρα του περιφερικού αίματος επιδεινώνουν τη φλεγμονή και την ανάπτυξη βλάβης τελικών οργάνων στον ΣΕΛ. Τρεις κύριοι υποπληθυσμοί μονοκυττάρων έχουν αναγνωριστεί και συγκεκριμένα τα κλασικά, τα ενδιάμεσα και τα μη κλασικά μονοκύτταρα. Οι διαταραχές στην φυσιολογία των μονοκυττάρων αποτελούν βασικό πυλώνα της παθογένειας του ΣΕΛ και οδηγούν στη διαιώνιση της φλεγμονής και στη βλάβη των ιστών. Πρόσφατες μελέτες τονίζουν τη συμμετοχή τελικώς διαφοροποιημένων υποσυνόλων ανοσοκυττάρων στη βλάβη των ιστών και τη σοβαρότητα της νόσου. Πάρα τα σημαντικά ευρήματα ότι τα μη κλασικά μονοκύτταρα συμβάλλουν στην εξέλιξη της νόσου στους ιστούς στόχους, παραμένουν ο λιγότερο μελετημένος υπότυπος. Στόχος της παρούσας διατριβής είναι η περαιτέρω διερεύνηση του ρόλου και της λειτουργίας των μη κλασικών μονοκυττάρων στον ΣΕΛ. Η κατανόηση των μοριακών οδών που εμπλέκονται στην ενεργοποίηση και λειτουργία των μη κλασικών μονοκυττάρων στην περιφέρεια των ασθενών με ενεργό νόσο, πριν από τη μετανάστευση τους στους ιστούς στόχους, μπορεί να οδηγήσει στην ανάπτυξη στοχευμένων θεραπειών για τη ρύθμιση των αποτελεσμάτων τους. Για τον λόγο αυτό, στη παρούσα διατριβή πραγματοποιήσαμε έναν ολοκληρωμένο μεταγραφικό, πρωτεομικό και λειτουργικό χαρακτηρισμό των τριών περιφερικών μονοκυτταρικών υποπληθυσμών από ασθενείς με ενεργό ΣΕΛ και υγιή άτομα. Τα αποτελέσματά μας καταδεικνύουν εκτεταμένες μοριακές διαταραχές στα μη κλασικά μονοκύτταρα του περιφερικού αίματος, που χαρακτηρίζονται από ενισχυμένα φλεγμονώδη χαρακτηριστικά όπως η απορυθμισμένη επιδιόρθωση του DNA, ο κυτταρικός κύκλος και η αυξημένη σηματοδότηση ιντερφερόνης σε συνδυασμό με στοιχεία διαφοροποίησης και αναπτυξιακές διαδικασίες. Η ενισχυμένη βλάβη του DNA, η

αυξημένη έκφραση του p53, η διακοπή του κυτταρικού κύκλου στην G0 φάση και η αυξημένη αυτοφαγία, τονίζουν τη δυνατότητα διαφοροποίησης των μη κλασικών μονοκυττάρων στον ΣΕΛ. Αυτό το παθογονικό προφίλ σχετίζεται με έναν ενεργοποιημένο φαινότυπο των μη κλασικών μονοκυττάρων που εμφανίζει χαρακτηριστικά Μ1 στην κυκλοφορία, τροφοδοτώντας τη φλεγμονώδη απόκριση. Συμπερασματικά, τα ευρήματα αυτά υπογραμμίζουν τα μη κλασικά μονοκύτταρα ως ένα παθογόνο κυτταρικό τύπο στη νόσο που θα μπορούσε να χρησιμοποιηθεί ως ένας επιπλέον θεραπευτικός στόχος για την νόσο.

GRAPHICAL ABSTRACT



Outline

Herein, we performed a comprehensive transcriptomic, proteomic and functional characterization of the three peripheral monocytic subsets from active SLE patients and healthy individuals. Our data demonstrate extensive molecular and proteomic perturbations in peripheral SLE NCM, characterized by enhanced inflammatory features, increased DNA damage response, cell cycle arrest and enhanced autophagy. This immunogenic profile is associated with an activated macrophage phenotype of NCM exhibiting M1 characteristics in the circulation, fueling the inflammatory response. NCM differentiation in SLE may contribute to the vascular inflammation and tissue injury. Together, these findings identify circulating SLE NCM as a pathogenic cell type in the disease that might represent an additional target for therapeutic intervention.

Introduction

Autoimmune diseases

Autoimmune diseases (AD), with the exception of rheumatoid arthritis and autoimmune thyroiditis, are individually rare, but collectively they affect approximately one in ten individuals [1]. These disorders can involve essentially any organ system and affect individuals of any age, with a much greater prevalence among women [2–4]. They are chronic pathologies characterized by the loss of immunological tolerance to self-antigens. The manifestations range from acute, life-threatening organ failure to subtle laboratory abnormalities that can easily escape notice. Clinically, autoimmune diseases can be restricted in the pattern of organ involvement (organ-specific) or be generalized (systemic or non-organ-specific). An interplay of environmental, genetic and epigenetic factors leading to perturbation of complex biological networks has been well documented with a steady increase in prevalence after WWII that cannot be merely attributed to genetic factors [5].

Systemic Lupus Erythematosus

Understanding the Complex Landscape of SLE: Prognosis, Diagnosis and Management of the Disease

Systemic lupus erythematosus (SLE) is a prototypic, chronic, multisystem autoimmune disease that manifests diverse clinical and molecular abnormalities attributed to the loss of self-tolerance to nucleic acids and endogenous antigens and sustained autoantibody production [6,7]. Excessive and irreversible tissue damage caused by autoantibodies or immune-complex deposition affects multiple organs such as skin, kidneys, blood, brain and heart, leading to significant morbidity and increased mortality [5,8]. SLE is a worldwide disease, with an annual occurrence rate ranging from 1.5 to 7.4 cases per 100,000 individuals in Europe [9]. While SLE can affect individuals across all age groups, the most significant risk factor is the female sex, with a female to male ratio of 9 to 1 [10,11]. SLE is defined by clinical

heterogeneity, with patients encountering a range of symptoms such as fatigue, skin rashes, fever, and painful or swollen joints **(Figure 1)**. In some individuals, SLE symptoms can manifest periodically, referred as "flares", with intermittent occurrences that may span years, followed by symptom-free periods known as "remission". Moreover, symptoms can differ from one individual to another and may fluctuate depending on the affected part of the body, ranging in severity from mild to moderate or severe [5,12].

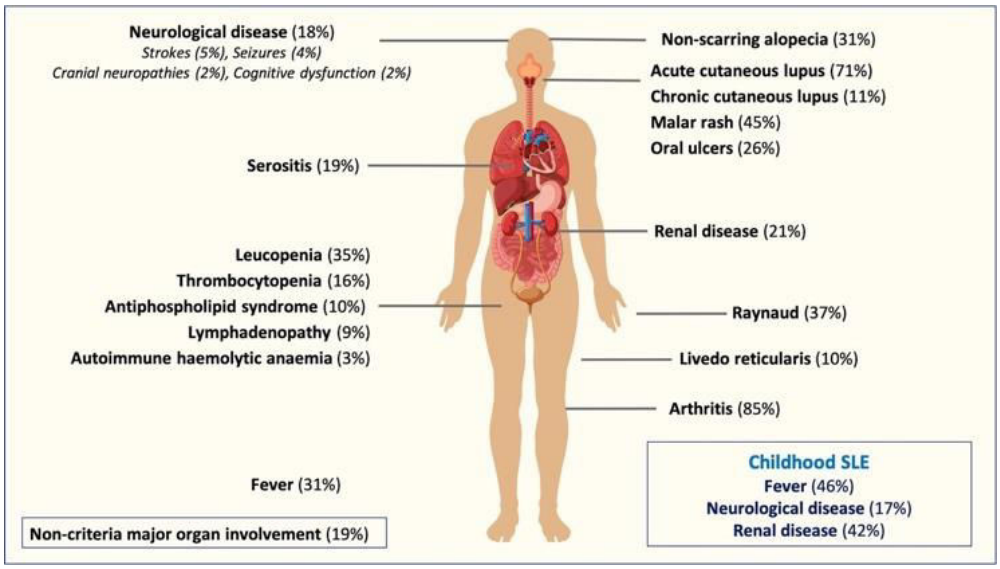


Figure 1. The multifaceted nature of SLE. SLE is a chronic, autoimmune inflammatory condition that can impact various organs, with each organ displaying distinct manifestations. Adopted by Fanouriakis et al [5].

Despite advances in the pathogenesis and treatment, several unmet needs exist in SLE. Flares are common, with an average of around 0.3 flares per year even in well controlled patients, elevating the risk of additional damage and increasing morbidity and mortality. Achieving remission is associated with a reduced likelihood of long-term damage accumulation in SLE. Remission rates can vary significantly across different studies. Unfavorable prognostic factors associated with persistent disease activity include hematological manifestations and glomerulonephritis [13,14].

Diagnosing lupus can be challenging due to its ability to mimic other medical conditions, necessitating the use of clinical and serologic criteria. Precise evaluation of disease activity and the establishment of well-defined criteria for measuring treatment response and disease states are pivotal, as they largely influence clinical decision-making in the management of SLE. A diagnostic approach to patients with SLE includes a combination of the ACR-1997, SLUCC-2012 and EULAR/ACR-2019 classification criteria [5]. SLE activity indices or assessment tools help in monitoring disease progression, guiding treatment decisions, and evaluating the response to therapy. Several activity indices are available, and the choice of which one to use may depend on clinical practice preferences and the specific need of the patient. Among them, SLEDAI is one of the most widely used indices as it assesses disease activity based on a set of 24 clinical and laboratory features. These parameters cover a wide range of SLE manifestations including skin rashes, joint involvement, kidney function, as well as laboratory tests and immunological features. Blood tests are a crucial part of the diagnostic process for SLE and include (i) the evaluation of serum complement levels (C3, C4) which may be low in active SLE, (ii) the measurement of complete blood count (CBC) to assess for anemia, leukopenia, or thrombocytopenia, (iii) the antinuclear antibodies (ANA) test, (iv) the autoantibody testing for specific autoantibodies such as anti-double-stranded DNA (anti-dsDNA) and anti-Smith (anti-Sm) antibodies, (v) the assessment of erythrocyte sedimentation rate (ESR) and C-Reactive Protein (CRP) and (vi) finally the urinalysis to check for proteinuria, hematuria, or cellular casts, which may indicate kidney damage. Each parameter is assigned a numerical score based on its severity or presence. Moreover, the Lupus Low Disease Activity State (LLDAS) is a state rather than an index and represents a desirable treatment goal. It signifies a state of low disease activity or quiescence based on specific criteria related to clinical and serological parameters. Of note, the current activity indices exhibit limitations affecting the success of clinical trials and the emergence of new therapeutic strategies to manage SLE pathogenesis [15,16].

Managing SLE remains a formidable task due to the unpredictable progression of the disease and its impact on multiple organs **(Figure 2)** [17]. Current therapeutic schemes in SLE include antimalarial drugs such as chloroquine (CQ), and

hydroxychloroquine (HCQ) and are commonly prescribed to manage skin rashes, joint pain, and fatigue and reduce the risk of disease flares. Antimalarial agents primarily exert their effects by suppressing lysosomal activity and autophagy, as well as by inhibiting the secretion of pro-inflammatory cytokines and signaling cascades of the immune system [18]. Also, non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen or naproxen, are used to relieve mild to moderate pain, joint pain, and inflammation associated with SLE. They exert their effects primarily through inhibition of enzymes known as cyclooxygenases (COX) which are responsible for converting a fatty acid called arachidonic acid into prostaglandins. Prostaglandins are signaling molecules that promote inflammation and pain when released in response to tissue injury or inflammation. By inhibiting COX enzymes, NSAIDs reduce the production of prostaglandins, which in turn leads to a decrease in the inflammatory response and a reduction in pain and fever [19]. Moreover, glucocorticosteroids (GCs) like prednisone are often prescribed to reduce inflammation and manage symptoms during SLE flares. In contrast, GCs can effectively manage disease activity by diminishing the secretion of cytokines, adhesion molecules and inflammatory mediators, as well as by inhibiting leukocyte activity and trafficking [20,21]. They can also inhibit the function of immune cells involved in the immune response, including T cells by modulating gene expression induced upon TCR (T Cell Receptor Signaling) triggering [22]. Moreover, a recent study unveiled that GCs orchestrate the metabolic processes of macrophages by suppressing glycolysis while enhancing the flow of the tricarboxylic acid (TCA) cycle, fostering an anti-inflammatory response [23]. Due to the remarkable effectiveness of GCs in dampening disease flares, they have been used as basic treatment strategy for SLE over the years, however they are typically used at the lowest possible dose for the shortest duration in order to minimize side effects [24]. Furthermore, non-corticosteroid immunosuppressants are a group of medications that directly target the immune system and include cyclophosphamide (CYC), mycophenolate mofetil (MMF), azathioprine (AZA), and methotrexate (MTX). These medications are used to prevent or manage severe organ involvement, and control immune system activity and more aggressive forms of SLE. These agents are employed to target activated T cells and B cells [25–27]. In addition, another medication for SLE includes biologic agents such as belimumab,

anifrolumab, and rituximab, that especially target components of the immune system. Monoclonal antibodies, used in the treatment of patients with SLE, directly or indirectly influence the survival and activation of B-cells, leading to either B-cell depletion or the inhibition of their functional repertoire [28–30]. Currently, the treatment strategy for SLE centers around the "treat-to-target" principle, aiming to achieve low disease activity or, ideally, remission of systemic symptoms and organ manifestations in patients with SLE [31]. There exists a substantial demand for advancements in both the diagnosis and treatment of SLE. The inherently heterogeneous nature of SLE pose significant challenges in evaluating the effectiveness of pharmaceutical interventions in clinical trials.

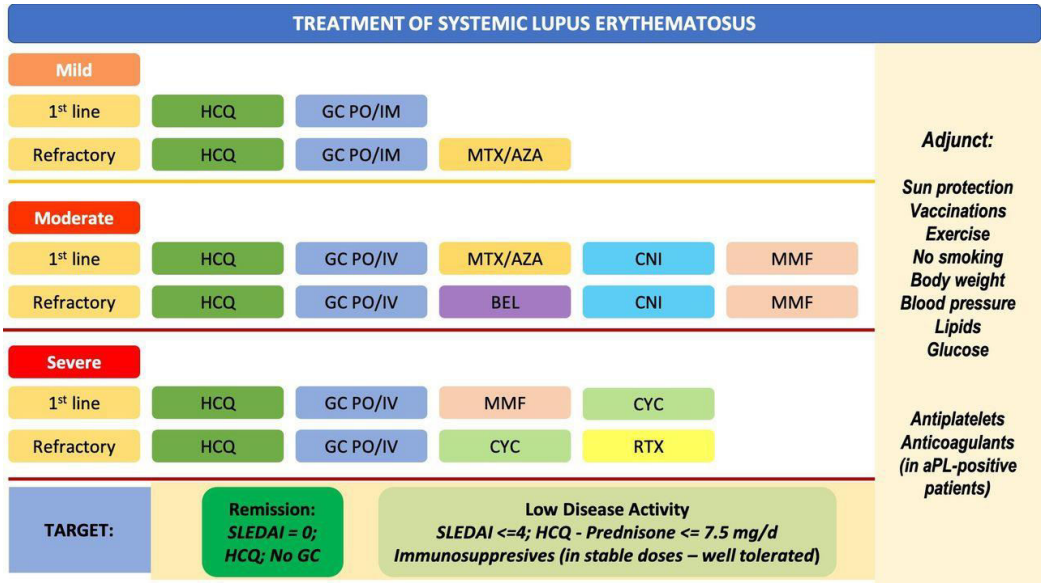


Figure 2. Treatment strategy for the management of SLE. Assessing the severity of SLE relies on (i) the presence of major organ involvement or conditions that pose a threat to organs; (ii) concurrent activity affecting several non-major organs; and (iii) the necessity for administering elevated doses of GCs and/or immunosuppressive treatment. aPL, antiphospholipid antibody; AZA, azathioprine; BEL, belimumab; CNI, calcineurin inhibitors; CYC, pulse cyclophosphamide; EULAR, European League Against Rheumatism; GC, glucocorticoids; HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; RTX, rituximab; SLEDAI, SLE Disease Activity Index. Adopted by Fanouriakis et al [5].

The Etiology of SLE: An In-Depth Examination of Causative Factors

Immunological tolerance is an active, tightly regulated, fine-tuned response of the immune system to autoantigens or against various environmental entities that prevent the immune system to mount possibly harmful responses. The discrimination between self- and nonself antigens is pivotal for the proper functioning of the immune system. Failure of immunological tolerance leading to an aberrant immune response against host antigens is critical for the development of autoimmunity [32]. The etiology remains elusive although the emergence of SLE is associated with genetic vulnerability, and a variety of factors such as environmental stresses, sex, stochastic events that can trigger disease onset in genetically susceptible individuals. Viral infections have been proposed as potential triggers or contributors to the pathogenesis of SLE, although their precise mechanism of action is still unclear **(Figure 3)**.

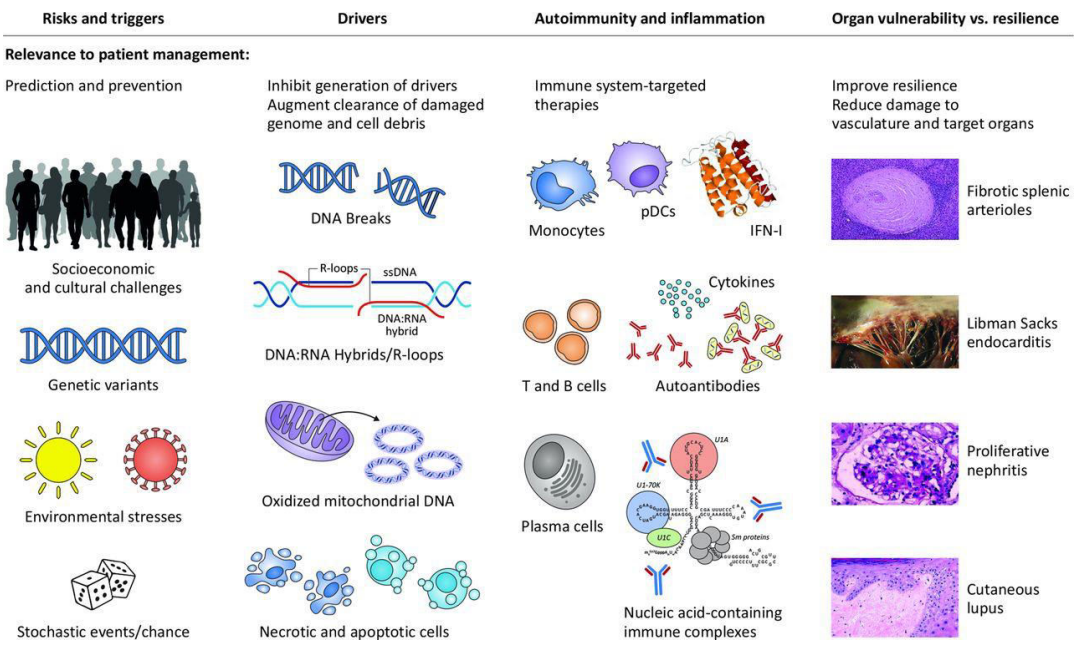


Figure 3. The pathogenetic landscape of SLE. Genetic, environmental and hormonal factors, and stochastic events affect both innate and adaptive immune responses in various ways and culminate in organ failure. Additionally, identification and characterization of the molecular drivers of immune system activation may lead to

interventions that prevent autoimmunity or at least prevent progression to clinical disease. Adopted by Crow [33].

Environmental factors

Environmental factors play a crucial role in the development of SLE. One important environmental factor is ultraviolet (UV) light exposure [34]. Sunlight, specifically UVB radiation, is known to trigger or exacerbate SLE symptoms in susceptible individuals. UV radiation can induce reactive oxygen species (ROS) and DNA damage, production of self-antigens and autoreactive T cells and alter the expression of genes involved in immune regulation [35–37]. Additionally, UV exposure can trigger the secretion of pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 1 alpha/beta (IL-1α/β), that contribute to cutaneous inflammation [38–40]. As a result, these cytokines can stimulate the generation of inflammatory chemokines like chemokine (C-C motif) ligand 5 (CCL5), chemokine (C-C motif) ligand 20 (CCL20), chemokine (C-C motif) ligand 22 (CCL22), and chemokine (C-X-C motif) ligand 8 (CXCL8) by epidermal keratinocytes, leading to increased recruitment of leukocytes into the skin [41]. In addition, monocytes have been identified as a source of type I interferon (IFN) production following UV exposure [42]. In the MRL-Fas^{lpr} lupus mouse model, UVB irradiation was found to elevate keratinocyte production of colony stimulating factor 1 (CSF-1), which is an essential factor for macrophage infiltration and the development of Cutaneous Lupus Erythematosus (CLE) - like skin lesions [43]. These findings underscore the significant role of monocytes in promoting skin inflammation in UV-induced injury in mice. Human studies have further substantiated the key role of monocytes in promoting inflammation in cutaneous lupus lesions [44]. Additionally, there is a correlation between type I IFN-stimulated gene expression and the infiltration of monocytes in the skin exposed to UV of lupus patients [45].

Another environmental factor linked to SLE is hormonal fluctuations, particularly in women. Hormones, such as estrogen, have been shown to influence the immune

system and may contribute to the development of SLE. For example, the female predominance of SLE suggests a hormonal role in disease susceptibility. During pregnancy or when taking oral contraceptives, which affect hormonal levels, some women with a genetic predisposition to SLE may experience disease flares [46–48]. The relationship between hormones and SLE is complex and various mechanisms are involved, including the modulation of immune cell activity and the production of autoantibodies. These highlight the importance of understanding the interplay between hormonal factors and genetic susceptibility in SLE pathogenesis.

Viral infections

Viral infection can alter immunological tolerance against self-antigens and has been associated with the initiating or flaring of several autoimmune and inflammatory phenomena in individuals with genetic susceptibilities [49–52]. These infections trigger the antiviral immune response mechanisms resulting in the activation of signaling pathways and the induction of cytokine and chemokine secretion, the production of autoantibodies, and the deposition of immune complexes in tissues some of which could overwhelm the immune regulatory mechanisms **(Figure 4)** [53]. Several mechanisms can explain how viruses might trigger a series of actions leading to the development of an autoimmune disease [49,54]. One proposed mechanism is molecular mimicry, where viral proteins or nucleic acids share structural similarities with self-antigens. In this concept, the immune system, while targeting the virus, may also mistakenly attack host tissues, leading to autoimmunity. Next, epitope spreading is another mechanism by which the immune response may be directed against specific viral antigens, however, as the infection progresses, the immune system can spread its response to include self-antigens that are structurally related to the viral antigens, leading to autoimmune reactions. Another proposed mechanism is bystander activation, where immune cells may become activated in a nonspecific manner and mistakenly attack healthy host tissues, promoting the autoimmune phenomena.

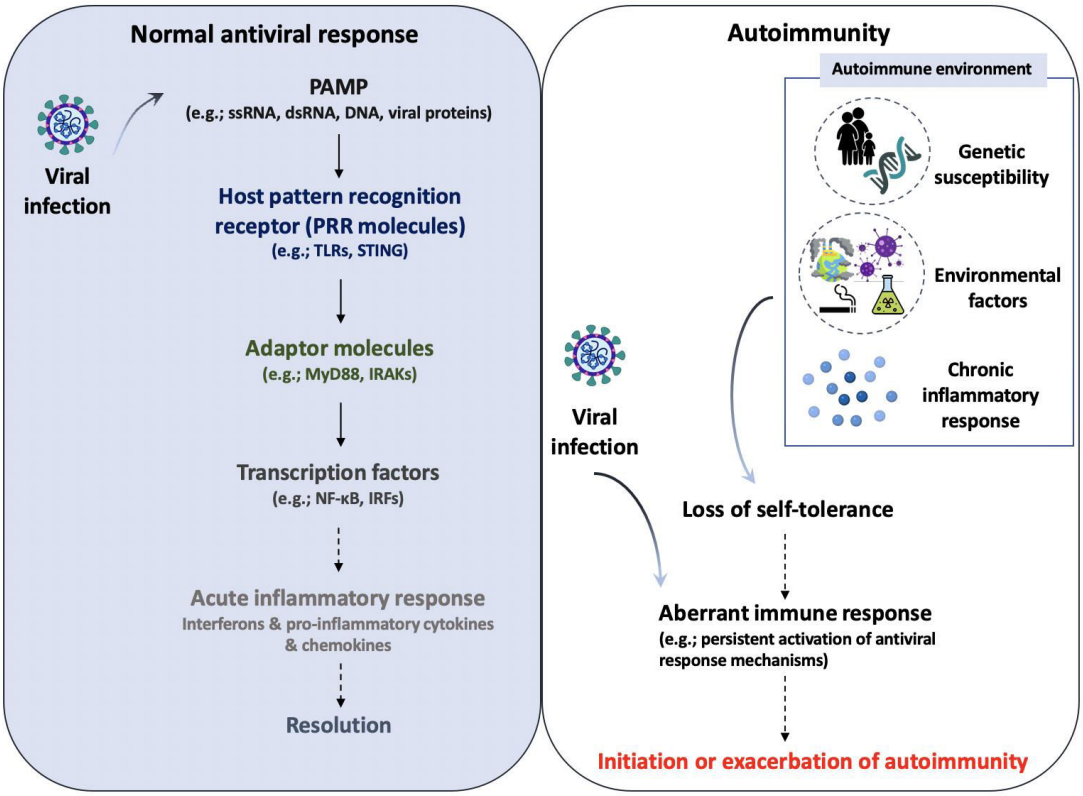


Figure 4. PRR signaling upon viral infection may either lead to acute inflammatory response and resolution or trigger autoimmune responses. Innate immunity is activated upon viral exposure, and the response is mediated through pattern recognition receptor (PRR) molecules that recognize the viral nucleic acids, the adaptor molecules that mediate the signal to downstream components, and the transcription factors that are responsible for the outcome. An acute inflammatory response is orchestrated by the release of antiviral molecules, such as interferons, proinflammatory cytokines, and chemokines at sites of infection. In autoimmune diseases, a combination of genetic susceptibility, such as gene copy variations and single-nucleotide polymorphisms (SNPs) and environmental as well as hormonal factors including UV light, toxic chemicals, and genes defined by the X chromosome, leads to loss of self-tolerance and chronic inflammation. In this environment, when a viral infection occurs, the host defense mechanisms are exposed and may promote an exaggerated immune response, which can lead to initiation or exacerbation of autoimmunity. Adopted by Stergioti et al [53].

Viral infections have long been implicated in the complex pathogenesis of SLE, with several notable examples underscoring their potential role as triggers of the disease. One compelling instance involves the Epstein-Barr Virus (EBV), a member of the herpesvirus family. Numerous studies have shown a strong association between EBV and SLE [55]. In fact, individuals with a history of EBV infection are more likely to develop SLE. Several studies have demonstrated an increased viral load of EBV DNA in patients with SLE compared to healthy individuals [56,57]. Furthermore, a serologic association to EBV infection has been reported with high titers of anti-early antigen (EA) IgG and IgA in lupus patients compared to healthy controls [55,58]. In addition, several reports have revealed the molecular similarity between the EBV nuclear antigen-1 (EBNA-1) and the common lupus autoantigen Ro, as well as the inability of $CD8^+$ T cells to control EBV-infected B cells, suggesting that viruses may influence the development of SLE pathogenesis [59–63]. In addition, EBV can persistently infect B cells and may induce abnormal activation of these cells, leading to the production of autoantibodies that are characteristic of SLE, such as ANAs and anti-dsDNA antibodies [64,65]. This viral-induced immune response may contribute to the breakdown of self-tolerance and the onset of autoimmune reactions seen in patients with SLE. Another illustrative example involves parvovirus B19, which has been linked to SLE pathogenesis [66]. Parvovirus B19 infection can trigger an immune response that leads to an overproduction of type I IFNs. In SLE, there is often a dysregulated type I IFN response, and viral infections like parvovirus B19 or EBV can exacerbate this response [67]. The resulting excessive production of type I IFNs can further fuel the autoimmune phenomena in patients with SLE. These viral-induced immune disruptions demonstrate how infections can contribute to the development and progression of SLE by perturbing the delicate balance of the immune system and promoting autoimmune responses.

Genetics

The pathogenesis of SLE is complex and multifactorial, with a strong genetic component. Genetic factors play a significant role in an individual's susceptibility to developing SLE and contribute to the dysregulation of the immune system [68]. Several key genetic factors and risk loci have been identified in SLE. The Major Histocompatibility Complex (MHC) was the first region reported as associated with SLE. The MHC region comprises the Human Leukocyte Antigen (HLA) region, which is one of the most well-established genetic risk factors for SLE. Specific HLA alleles, such as HLA-DR2, HLA-DR3, and HLA-DRB1*15:01, are associated with an increased risk for developing SLE [69]. These genes are involved in antigen presentation and immune regulation, and their variants can contribute to the development of autoimmunity. Interestingly, HLA-DR and HLA-DQ alleles exhibit a strong connection with SLE autoantibodies [70,71]. Moreover, genetic variants in complement pathway genes, such as C1q, C2, and C4, have been linked to SLE susceptibility [72–74]. Deficiencies or abnormalities in complement components can impair the body's ability to clear immune complexes and apoptotic cells, potentially leading to increased inflammation and tissue damage. Moreover, the IFN signaling pathway plays a crucial role in SLE pathogenesis [75]. Genetic variants in genes involved in this signaling cascade, such as interferon regulatory factor 5 (IRF5), interferon regulatory factor 7 (IRF7), non-receptor tyrosine-protein kinase TYK2, and signal transducer and activator of transcription 4 (STAT4), have been associated with SLE risk [76–79]. These variants can lead to enhanced levels of IFN and excessive activation of the innate and adaptive immune responses. In addition, genetic variations in TLR genes, particularly TLR7 and TLR9, predispose to SLE pathogenesis since overactivation of these receptors can contribute to the formation of autoantibodies and inflammation [80,81]. A recently published study revealed that a TLR7 gain-of-function genetic variation cause human SLE [82]. Finally, variants in Fcγ receptor genes, such as Fcγ receptor IIB (FcγRIIB) and Fcγ receptor IIIA (FcγRIIIA), which play a crucial role in the clearance of immune complexes and regulation of immune signaling pathways, have been linked to the development of SLE [83]. Understanding the genetic basis of SLE has not only shed light on the disease's pathogenesis but also has important

implications for personalized medicine and targeted therapies. It is important to highlight that while genetic factors predispose to SLE pathology, they interact with environmental factors and epigenetic modifications in order to determine an individual's overall risk for developing an autoimmune syndrome.

Epigenetics

Epigenetics also play a vital role in the development and progression of SLE [84–86]. DNA methylation abnormalities is a key epigenetic mechanism involved in SLE pathogenesis. DNA methylation inhibits the expression of genes by methylating the deoxycytosine base at the 5' position in order to form deoxymethylcytosine [87]. DNA hypomethylation, an epigenetic modification, can influence gene expression and has been linked with the development of SLE. This can result in reduced or silenced gene expression by the methylation of C-G dinucleotides (CpG) [88,89]. In patients with SLE, there is often aberrant DNA methylation in specific genes that regulate immune responses such as CD11a and CD70 [90,91]. Furthermore, in patients with active lupus, CD11a and KIR genes exhibited elevated expression levels, and the corresponding sequences demethylated in proportion to disease activity and heightened overexpression of these genes [92]. In addition, hypomethylation has also been noted to disrupt the regulation of interferon-stimulated genes (ISGs) in naïve T cells from SLE patients. These genes include interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), interferon-induced GTP-binding protein (MX1), signal transducer and activator of transcription 1 (STAT1), interferon-induced protein 44 like (IFI44L), ubiquitin specific peptidase 18 (USP18), tripartite motif-containing 22 (TRIM22), and bone marrow stromal cell antigen 2 (BST2), indicating an epigenetic transcriptional accessibility in these genetic loci [93]. This epigenetic change can help explain the increased levels of STAT1 and other ISGs in lupus immune cells. Also, hypomethylation of CpG sites in genes involved in several pathways has also been correlated with the production of autoantibodies in patients with SLE [94]. Additionally, epigenetic modifications can affect the expression of genes involved in the IFN pathway, such as IRF5 and IRF7, which are known to be involved in SLE

pathogenesis [95]. Abnormal DNA methylation in these genes can exacerbate the production of interferons and promote inflammation in patients with SLE.

Histone modifications are another epigenetic mechanism implicated in the pathogenetic landscape of SLE. Histones constitute a group of proteins that envelop DNA, creating the structural unit known as the nucleosome. Post-translational modifications of histone proteins are recognized as crucial epigenetic mechanisms that have a role in governing chromatin remodeling and gene expression. These modifications include processes such as phosphorylation, acetylation, and methylation [96]. Epigenetic codes exhibit a high degree of conservation and are responsible for shaping the phenotype and function of cells and tissues. One of the modifications within the histone code takes place at lysine residues 9 and 27 of histone H3, which can initiate chromatin compacting and gene silencing. In $CD4^+$ T cells of patients with SLE, there is evidence of reduced histone acetylation and diminished H3K9 methylation. The promoter region of hematopoietic progenitor kinase 1 (HPK1) exhibits trimethylation at lysine 27 of histone H3, which results in inhibition of HKP1 expression, contributing to the inflammatory response in SLE [97,98]. There is often increased acetylation and decreased methylation of histones in SLE, leading to a more permissive chromatin structure in immune cells. This chromatin structure allows for the enhanced expression of pro-inflammatory genes like TNF-α [99]. These cytokines can contribute to the inflammation and tissue damage observed in SLE. Additionally, it has been reported that histone modification revealed that histones H3 and H4 are hypoacetylated in lupus B cells [100]. Moreover, the overexpression of immune-related genes that drive $CD4^+$ T cells autoreactivity in SLE is facilitated by histone acetylation and methylation [101]. In addition, gene expression perturbations in peripheral blood immune cells in SLE may be attributed to altered epigenetic profiles and chromatin accessibility. A recent study by Ntasis et al. [102] unveiled that the genomes of patients with SLE demonstrate more fragmented and less organized co-expression patterns, a trend that correlated with the severity of the disease. The defined Domains of Co-ordinated Expression (DCEs) demonstrate intricate dynamics, that are linked with the molecular signatures and clinical aspects of SLE. For instance, high disease

activity genomes display extensive redistribution of co-expression domains, featuring the expansion and emergence of new DCEs. This study sheds light on the connection between the SLE phenotype and the underlying genome structure, emphasizing the pivotal role of genome organization in influencing gene expression patterns in SLE. Overall, epigenetic modification in DNA methylation and histone marks are integral to the dysregulated immune responses seen in SLE and provide potential targets for therapeutic interventions.

Immunological mechanisms leading to SLE pathogenesis

A complex interplay between innate and adaptive immunity lies at the core of the autoimmune phenomena. This interplay is not static, since initial inflammatory cascades might change as organ damage accumulates. The loss of tolerance to self and the subsequent elevation in serum antinuclear antibody (ANA) levels is proposed to be a crucial first step in the development of SLE [103–105]. SLE progression unfolds through three interlinked phases. One phase comprises the breakdown of adaptive immune tolerance resulting in an elevated presence of autoreactive B cells. Various signals from self-antigens, Toll-like receptor (TLR) ligands, B-cell activating factor (BAFF)/a proliferation-inducing ligand (APRIL), and T-cell-derived cytokines contribute to the formation of germinal centers and the production of autoantibodies. Another phase involves deficiencies in innate immunity, including heightened NETosis, impaired clearance of apoptotic debris, and diminished phagocytosis, resulting in elevated levels of self-antigens. The interaction between self-antigens from immune complexes (ICs) with autoantibodies enables their internalization through FcRγ receptors, subsequently triggering multiple downstream cascades. The final phase involves the release of mediators by recruited inflammatory cells and complement activation induced by ICs, ultimately leading to inflammation and tissue damage **(Figure 5)**.

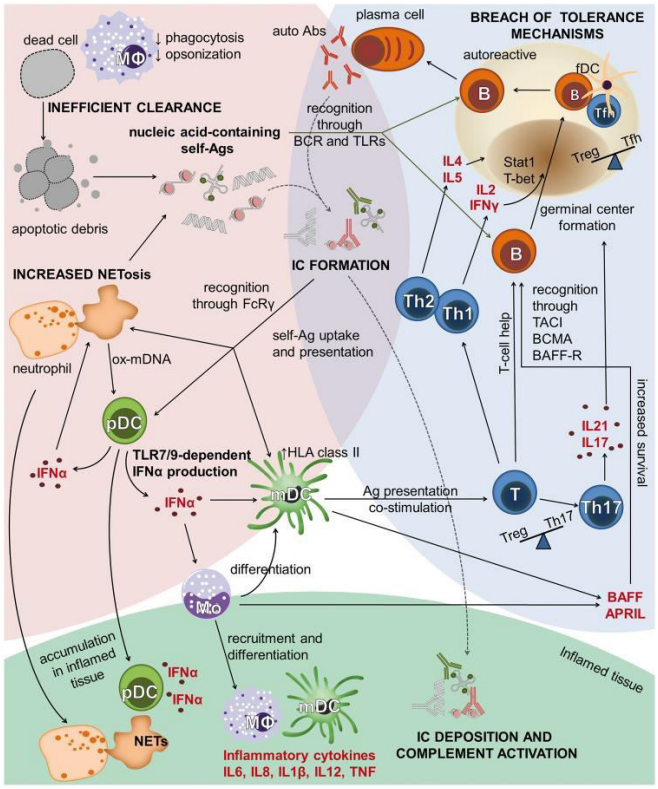


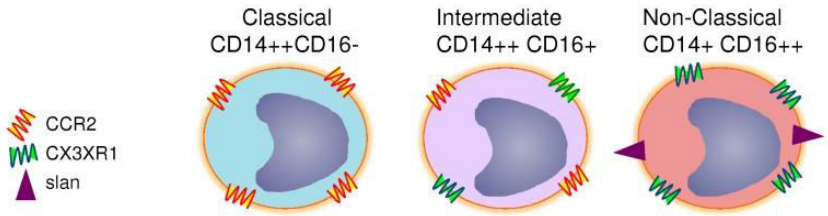
Figure 5. Overview of the immunological mechanisms leading to SLE pathogenesis. The development of the disease unfolds in three interlinked phases underscoring the interplay of innate and adaptive immunity and the complexity of the autoimmune phenomena. Adopted by Zharkova et al [106].

Monocytes: Key Regulators of the Innate Immune System

Exploring the Three Monocytic Subsets

Monocytes are blood mononuclear cells that arise from bone marrow phagocyte progenitors, circulate in the blood and then are recruited into tissues [107]. They represent about 10% and 4% of leukocytes in the human and mice peripheral blood, respectively. Three major populations of monocytes are identified both in mice and humans. Based on CD14 (lipopolysaccharide (LPS) coreceptor) and CD16 (FcγRIII) expression patterns on HLA-DR^{+} cells, human monocytes are divided into three phenotypically and functionally distinct subsets: the CD14^{++}CD16^{-} classical monocytes that make up to ~85% of the total circulating monocyte pool, whereas

the remaining ~15% consists of the $CD14^{++}CD16^{+}$ intermediate monocytes, and the $CD14^{dim}CD16^{++}$ non-classical monocytes. In mice, classical monocytes are defined by the surface marker expression $Ly6C^{high}CX3CR1^{int}CCR2^{+}CD62L^{+}CD43^{low}$, whereas non-classical monocytes are characterized as $Ly6C^{low}CX3CR1^{high}CCR2^{low}CD62L^{-}CD43^{+}$ [108,109]. The ratio of circulating classical and non-classical monocytes fluctuates depending on factors such as monopoiesis, tissue infiltration and their emergence from either the bone marrow or peripheral reservoirs. Additionally, the number of non-classical monocytes is closely tied to the overall health of the organism, making it a potential valuable diagnostic indicator **(Figure 6)**.



Chemotaxis	CCR2/CCL2	CCR2/CCL2 CX3CR1/CX3CL1	CX3CR1/CX3CL1
Surface markers	**CCR2**, CD62L, CD11b, TLR-4	CCR2, CX3CR1, HLA-DR, CD74, CD163,CLEC10A, GFRA2	**CX3CR1**, **slan**, CXCR4, HLA-DR, LFA-1
Cytokines	IL-10, IL-1	TNF-α, IL-10	⇧TNF-α
Frequency in Peripheral Blood	~80-90%	<15%	<15%
Mice equivalent	$Ly6C^{++}CD43^{+}$ $CX3CR1^{-}$	$Ly6C^{++}CD43^{++}$ $CX3CR1^{+}$	$Ly6C^{+}CD43^{++}$ $CX3CR1^{++}$

Figure 6. The three monocyte subsets. Human monocyte subsets are defined as follows: Classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{dim}CD16^{++}$). The corresponding murine markers and specific characteristics for each subset are presented. Adopted by Wacleche et al [110].

Developmental Trajectories of the Three Monocytic Subsets

In monocyte differentiation, there is a linear trajectory starting with the classical monocytes (CM) that exit the bone marrow and give rise to intermediate monocytes (IM) and sequentially to non-classical monocytes (NCM) in peripheral blood circulation with NCM representing the most mature monocytic subpopulation [107,111–114]. The three monocytic subsets exist in a state of dynamic equilibrium within the circulation, where the classical monocytes exhibit a circulating lifespan of approximately one day, while the intermediate and non-classical monocytes have longer circulating lifespans of four and seven days, respectively [107] (Figure 5). On the molecular level, the transition from classical to non-classical monocytes involves the increased expression of C/EBPβ, NR4A1, and KLF2. The lack of NR4A1 results in diminished $Ly6C^{low}$ monocyte survival and numbers [113]. Epigenetic studies have pinpointed a specific Nr4a1 enhancer (E2) specific to monocytes and deletion of this enhancer results in the absence of $Ly6C^{low}$ monocytes [115]. In addition, KLF2 and C/EBPβ bind to the E2 enhancer in order to promote the expression of Nr4a1, and actively participate in the generation of $Ly6C^{low}$ monocytes [113,115–117]. Moreover, computational transcriptional network analysis strongly suggests that C/EBPβ plays a pivotal role in human monocyte biology [118]. Furthermore, single-cell ATAC sequencing reveals clear enrichment of C/EBPβ and NR4A1 motifs in human monocytes [119].

Monocyte Functions During Physiological Conditions

Monocytes are integral components of the immune system, actively engaging in the defense against both bacterial and viral infections. They also act as a bridge, linking mononuclear phagocyte precursors in the bone marrow (BM) with terminally differentiated immune cells [120,121]. Monocytes can give rise to either dendritic cells (DCs), tissue resident macrophages (Mϕ) or osteoclasts. This differentiation process is initiated when monocytes cross the endothelium. Plasticity and heterogeneity are monocyte hallmarks, allowing them to quickly adapt their functional characteristics in response to diverse immunological signals [122,123]. It is

believed that the local environment, especially the cytokines milieu, has a pivotal role in influencing their differentiation process [124]. The regulation of this differentiation process of monocytes to terminally differentiated immune cells remains ill-defined. Emerging literature implicates autophagy as a key regulatory pathway in preventing monocytes from apoptosis and inducing their differentiation towards macrophages [125].

Monocyte subsets possess also unique functional characteristics, influenced in part by variations in the methylation status of genes associated with the immune system [126]. More specifically, CM play a crucial role in phagocytosis and innate immune response, and they also display migratory capabilities. Furthermore, $CD14^+$ human monocytes elevated expression levels of chemokine receptors, including CCR1, CCR2, CCR5, CXCR1, and CXCR2, underscoring their capacity to respond to signals from damaged or inflamed tissues [127,128]. In addition, they secrete pro-inflammatory molecules, such as IL-6, IL-8, CCL2, CCL3, and CCL5 [127,129] (Figure 5).

On the other hand, IM play a crucial role as effector cells in antigen presentation, and have been shown to release molecules such as TNF-α, IL-1β, IL-6, and CCL3 when stimulated by TLR ligands [129–131]. They also express elevated levels of CCR5 compared to CM. Another study has revealed that IM are the main producers of IL-10 upon TLR activation [132] (Figure 5). Further investigation is needed to determine whether these cells can concurrently generate both pro-inflammatory and anti-inflammatory mediators, or if there are distinct patterns of expression for these factors over time.

However, when comparing $CD16^+$ and $CD16^-$ monocytes, it becomes evident that despite their striking resemblances, indicating a shared developmental origin, $CD16^+$ cells exhibit a more advanced phenotype, as apparent by transcriptome profiling where they are associated with pathways such as cell-to-cell adhesion, cell trafficking, proliferation, and differentiation [133]. Furthermore, the elevated expression of CX3CR1 in $CD16^+$ cells explains the fact they migrate and adhere more than $CD16^-$ monocytes to fractalkine-secreting endothelium [134].

In contrast, NCM specialize in functions related to complement and Fc gamma-mediated phagocytosis, as well as cellular adhesion [127,135]. Similar to IM, they possess antigen presenting processing capabilities, but they differ from CM due to their involvement in wound healing processes [136]. NCM also exhibit patrolling behavior when adoptively transferred into immune-compromised mice [129]. This crawling behavior enables non-classical monocytes to effectively scavenge the endothelium for signs of damage **(Figure 7)**.

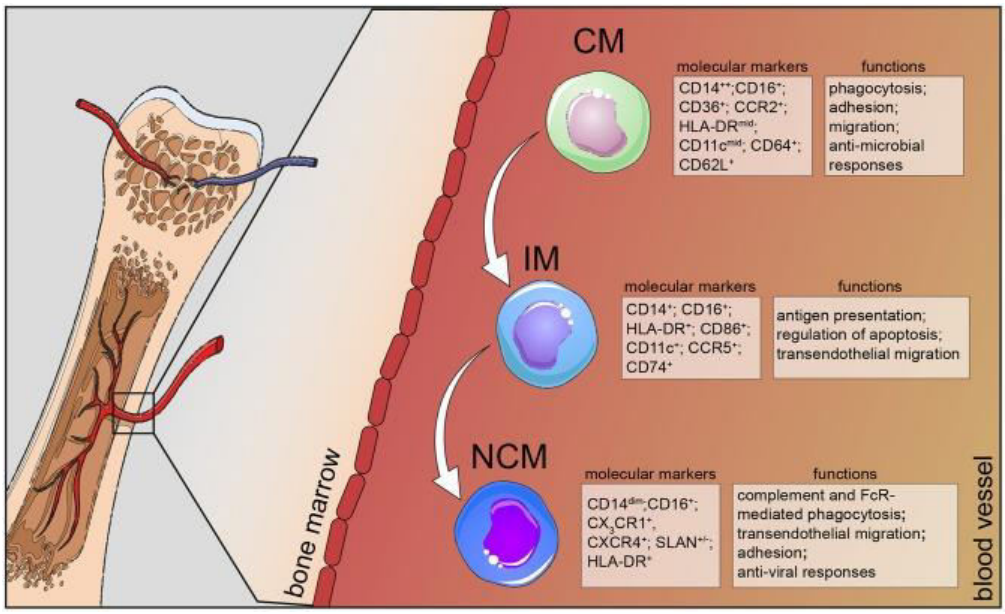


Figure 7. Molecular characteristics and functions of the three human monocytic subsets in homeostasis. Human monocytes undergo maturation within the bone marrow and are subsequently released into the bloodstream as CM. Over time, CM differentiate into NCM via an intermediate stage involving IM. In humans, CM can be distinguished from the other two subsets with additional markers such as CD36, CCR2, and CD64. They play a crucial role in the host's antimicrobial responses, including functions like adhesion to the endothelium, migration, and phagocytosis. IM are characterized by their high expression of CCR5 and HLA-DR molecules and are primarily involved in processes related to antigen presentation and transendothelial migration. On the other hand, NCM are divided into $SLAN^{+}$ and $SLAN^{-}$ populations, express elevated levels of CX3CR1, and play a crucial role in complement and FcR-

mediated phagocytosis, transendothelial migration, and anti-viral responses. Adopted by Kapellos et al [137].

Non-classical Monocytes Abnormalities in SLE Pathogenesis

The equilibrium among monocyte subsets is frequently disrupted in several disease conditions. Recent findings underscore the pivotal role of innate immune cell subsets in the initiation and propagation of the systemic autoimmune response and the development of end-organ damage in SLE. Aberrations of monocytes underlie the SLE pathology and lead to perpetuation of inflammation and tissue injury, which positions monocytes at the core of SLE etiology. An interesting study using expression quantitative trait loci (eQTLs) analysis pinpointed an aberrant activation of both adaptive and innate immune cells, including monocytes, in the development of SLE [138]. The significance of the innate immune system in the development of SLE pathogenesis is becoming increasingly evident, as convincing susceptibility genes are now linked to not only T and B cell signaling but also TLRs and type I IFN signaling [139].

Notably, a growing body of evidence indicates that the concept of aberrant activation, rather than impaired function, of monocytes may better capture the dynamic role they play on the onset and development of the disease [140]. The specific monocytic subsets contributing to the severity of the disease may vary across studies; nevertheless, numerous reports indicate the pivotal role of NCM in the development of SLE [141–144]. Recent studies further underscore the involvement of terminally differentiated immune cell subsets on tissue damage and severity of the disease. For instance, it has been reported that $CD16^{+}$ DCs arise from NCM in lupus skin contributing to the development of cutaneous lupus erythematosus (CLE) [145]. In addition, circulating $Ly6C^{low}$ NCM undergo differentiation into M1 macrophages and are recruited to the joint, playing a pivotal role in coordinating the progression of autoimmune joint inflammation [146]. Furthermore, TLR-activated NCM infiltrate the glomerulus, representing important regulators in glomerular inflammation and kidney injury in SLE [147]. Moreover, a

significant correlation between serum levels of anti-dsDNA antibodies and the proportion of NCM in circulation has been reported [144]. Additionally, another study highlighted that NCM secrete increased levels of IL-1β upon TLR stimulation [129]. Also, the contributions of NCM to antigen presentation, as well as the activation of T and B cells, have been documented in SLE patients [141,142]. These findings align with observations made in lupus models associated with the *Yaa* locus [147–149].

Despite the compelling evidence that NCM substantially contribute to disease progression in the target tissues, their precise role in the development and progression of SLE remains inadequately explored. Moreover, understanding the molecular pathways involved in NCM activation and function in the periphery of patients with active SLE, before migrating into the tissues can guide the development of targeted therapies to modulate their effects. Current non-specific immunosuppressive treatments for SLE sometimes cause serious side effects [150–152]. Thus, advances in monocyte immunology may pave the way for the development of novel, safer and more effective therapeutic schemes that target specific pathogenic features and pathways of monocyte biology. A better understanding of the core immune responses of NCM, could provide further insights for the design of more targeted therapies.

Multiomics

Omics technologies represent a groundbreaking approach to biological research that allows scientists to comprehensively study various biological molecules on a large scale. These technologies encompass fields such as genomics, proteomics, transcriptomics, metabolomics, and more. By analyzing the complete sets of genes, proteins, RNA molecules, metabolites, and other biomolecules within a biological system, omics technologies offer profound insights into the complexity of living organisms, their functions, and their responses to various conditions. These powerful tools have revolutionized our understanding of biology, medicine, and biotechnology, paving the way for personalized medicine, disease diagnostics, and the discovery of novel therapeutic targets **(Figure 8)** [153,154].

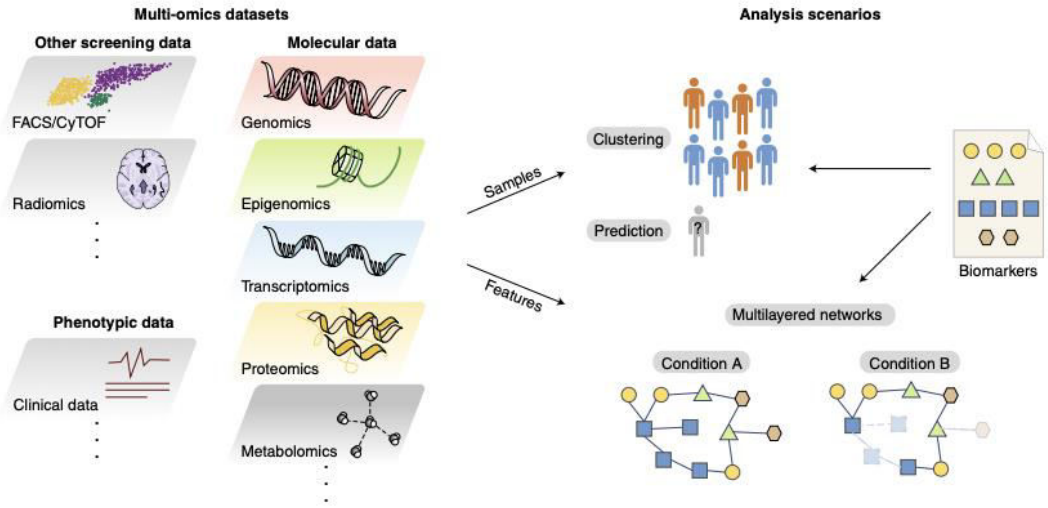


Figure 8. Illustrative depiction of the objectives in multi-omics investigations. Multi-omics datasets encompass a wide array of molecular profiling data modalities, including genomics, epigenomics, transcriptomics, proteomics, or metabolomics, along with high-throughput data types like FACS/CyTOF or radiomics measurements, and may also incorporate phenotypic or clinical variables. The categorization of multi-omics studies hinges on their primary analytical goals. When the emphasis is on the samples, these samples can undergo unsupervised clustering to unveil the underlying dataset structure or supervised analysis for predicting the classification of

new samples. The attributes involved in these groupings may be extracted and utilized as biomarkers. Conversely, when the emphasis is on the attributes, the analysis aims to discern significant relationships among omic variables originating from different omics types, which can be visualized as a network. Adopted by Tarazona et al [153].

Transcriptomics

Transcriptomics is a field of molecular biology that focuses on studying the entire set of RNA molecules such as mRNA, non-coding RNA that are present in a cell or tissue at a given moment, providing valuable insights into gene expression patterns and regulation. Transcriptomics aids to understand the quantity, diversity, regulation, and function of these RNA molecules. The cornerstone of transcriptomics is RNA-sequencing (RNA-seq), a powerful technique that enables the comprehensive analysis of an organism's transcriptome [155,156]. RNA-seq is a cutting-edge molecular biology technique used to determine the sequence of RNA molecules. It involves high-throughput sequencing of cDNA molecules, which are derived through reverse transcription of RNA from a biological sample. This effort aims to unveil the primary sequence and quantify the relative abundance of each RNA molecule within the sample [157].

The RNA-seq workflow involved several fundamental steps, starting with the isolation of RNA from the biological sample of interest. The extracted RNA represents the pool of RNA molecules present in the sample at the time of extraction. Subsequently, this RNA is converted into a sequencing library. This involves several steps, including RNA fragmentation, cDNA synthesis, adapter ligation, and PCR amplification. The RNA library is loaded onto a high-throughput sequencer in order to generate vast amounts of short sequence reads from the RNA fragments. The generated sequence reads are processed computationally. This includes aligning the reads to a reference genome or transcriptome, quantifying gene expression levels, identifying splice variants, and performing various downstream analyses, such as differential gene expression analysis. RNA-seq has

revolutionized our understanding of gene expression dynamics and has become an indispensable tool for diverse biological and medical research applications **(Figure 9)** [158]. The results of RNA-seq analysis can be used to understand gene expression patterns, regulatory mechanisms, and functional insights into the biological processes under investigation. It can also help in identifying biomarkers, studying disease mechanisms, and uncovering novel therapeutic targets. Notably, RNA-seq enables the identification of comprehensive transcriptome signatures that extent beyond gene expression patterns, providing a higher-resolution molecular characterization of the disease. In this context, a recent study by Panousis et al [159] has made a remarkable advancement in the field. They conducted a comprehensive RNA-seq analysis on whole-blood samples from 142 patients with SLE and 58 healthy individuals, unveiling a signature associated with disease susceptibility, a signature linked with disease activity and also a signature associated with disease severity. This discovery has the potential to enhance personalized care for lupus patients.

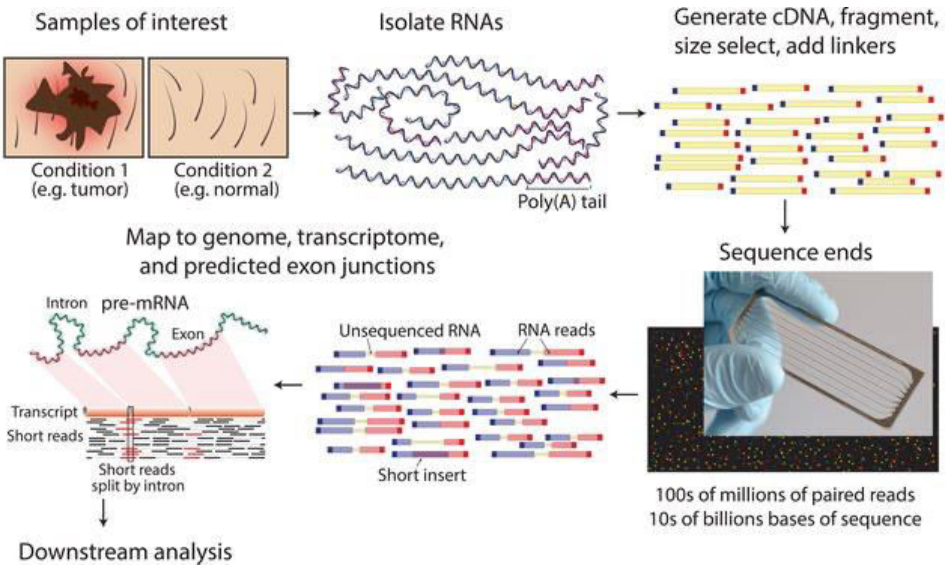


Figure 9. RNA-sequencing workflow. The standard procedure for RNA-sequencing experiments generally includes several key steps: first, the extraction of RNA from the selected samples, followed by the creation of sequencing libraries. Next, a high-throughput sequencer is employed to generate numerous short paired-end reads.

These reads are then aligned to a reference genome or transcriptome, and subsequent analysis is conducted. Adopted by Griffith et al [158].

Proteomics

The proteome encompasses all the proteins found within a cell, tissue, or organism at a specific moment, and this composition can vary with time, growth conditions, and between cell types due to disparities in gene expression. Proteomics investigates the functions, modifications, and interactions within protein complexes, unveiling their roles. Proteins are large, complex biomolecules composed of amino acid chains that play crucial roles in the structure, and regulation of cells and organisms. They exhibit a wide variety of functions based on their unique three-dimensional structures, which are determined by the sequence of amino acids in their chains. Proteomics is a field of biological research for the comprehensive study of proteins, the workhorses of cellular function. It seeks to understand the full complement of proteins within a biological system, including their structures, functions, interactions, modifications, and abundance levels.

The workflow of proteomics typically involves several key steps. First, proteins are extracted from the biological sample of interest, which can be cells, tissues, or fluids and digested using the single-pot, solid phase-enhanced sample-preparation (SP3) technology [160]. Next, these proteins are separated using liquid chromatography tandem mass spectrometry (LC-MS/MS). In the first part of the process, a liquid sample is injected into a chromatography system. This system separates the peptides of the sample based on their chemical properties as they pass through a chromatographic column. After separation by the LC, the individual components of the sample are directed into a mass spectrometer. The compounds are ionized and converted into charged particles. These fragment ions are then analyzed in the mass spectrometer. This step provides information about the structure and composition of the selected compound. Data generated from mass spectrometry are then analyzed using specialized software to identify proteins, quantify their abundance, and characterize post-translational modifications **(Figure 10)**. Proteomics is a cutting-edge technology that have significantly advanced our ability to comprehensively

analyze complex protein profiles within biological systems, allowing for the identification of novel biomarkers and therapeutic targets in diseases. These technological breakthroughs have also enabled researcher to gain deeper insights into the cellular signaling pathways, protein-protein interactions, and post-translational modifications, revolutionizing our understanding of cellular functions and molecular mechanisms.

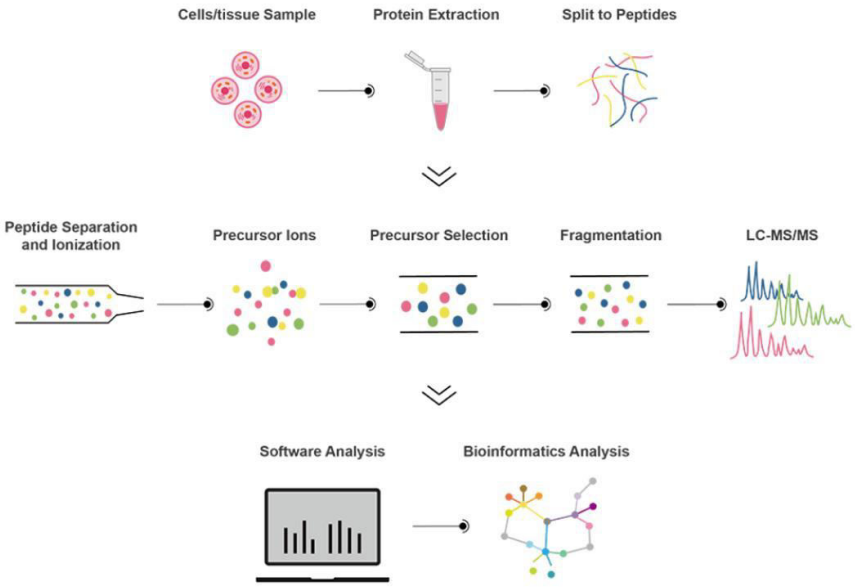


Figure 10. The technical workflow of proteomics by LC-MS/MS. Adopted by https://www.creative-proteomics.com/services/dia-quantitative-proteomics-service.htm.

Aim of the study

Autoimmune conditions arise from the interplay of environmental, genetic and epigenetic elements, leading to downstream perturbations of complex and interactive biological networks. The aberrant immune response occurring in the inflammatory environment can potentially result in impaired antiviral responses, predispose to autoimmunity, or precipitate a flare of an existing autoimmune condition. For this reason, we review the literature on the crosstalk between antiviral immune responses within the innate immune branch, which includes monocytes, macrophages, neutrophils and DCs, along with autoimmune responses. Additionally, we explore the pitfalls and challenges associated with the therapeutic targeting of the mechanisms involved.

Moreover, the equilibrium among monocyte subsets is frequently disrupted in several disease conditions. Aberrations of monocytes underlie the SLE pathology and lead to perpetuation of inflammation and tissue injury, which positions monocytes at the core of SLE etiology. Three major populations of monocytes have been recognized namely classical (CM), intermediate (IM) and non-classical monocytes (NCM). The specific monocytic subsets contributing to the severity of the disease may vary across studies; nevertheless, numerous reports indicate the pivotal role of NCM in the development of SLE. Understanding the molecular pathways involved in NCM activation and function in the periphery of patients with active SLE, before migrating into the tissues can guide the development of targeted therapies to modulate their effects. To this end, monocyte-based immunosuppressive approaches are being explored as potential strategies to modulate the immune response in patients with SLE. A better understanding of the core immune responses of NCM, could provide further insights for the design of more targeted therapeutic schemes.

Herein, by the use of transcriptomic and proteomic analyses as well as flow cytometry, microscopy and *ex vivo* studies, we sought to investigate disease-specific signatures of the three monocytic subsets in SLE. The main goals of this study are: i)

to delineate the transcriptome profile of the three monocytic subsets and identify the signaling cascades involved in the pathogenic SLE landscape; ii) to investigate the proteomic disparities of the three monocytic subsets in SLE; iii) to identify specific signaling pathways involved in NCM function and determine the pathogenic aspect of SLE NCM.

Results & Publications

Antiviral Innate Immune Responses in Autoimmunity: Receptors, Pathways, and Therapeutic Targeting

biomedicines

MDPI

Review

Antiviral Innate Immune Responses in Autoimmunity: Receptors, Pathways, and Therapeutic Targeting

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Abstract: Innate immune receptors sense nucleic acids derived from viral pathogens or self-constituents and initiate an immune response, which involves, among other things, the secretion of cytokines including interferon (IFN) and the activation of *IFN-stimulated genes* (*ISGs*). This robust and well-coordinated immune response is mediated by the innate immune cells and is critical to preserving and restoring homeostasis. Like an antiviral response, during an autoimmune disease, aberrations of immune tolerance promote inflammatory responses to self-components, such as nucleic acids and immune complexes (ICs), leading to the secretion of cytokines, inflammation, and tissue damage. The aberrant immune response within the inflammatory milieu of the autoimmune diseases may lead to defective viral responses, predispose to autoimmunity, or precipitate a flare of an existing autoimmune disease. Herein, we review the literature on the crosstalk between innate antiviral immune responses and autoimmune responses and discuss the pitfalls and challenges regarding the therapeutic targeting of the mechanisms involved.

Keywords: viral infection; autoimmunity; innate immunity; antiviral response; monocytes; macrophages; dendritic cells; NK cells; neutrophils; therapeutic opportunities

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1. Introduction

The innate immune system provides an immediate defense mechanism by recognizing molecular structures produced by microbial pathogens and allows the adaptive immune responses to mediate an antigen-specific response. The initial sensing of a virus infection depends on the detection of molecules derived from pathogens by cellular receptors of innate immune cells that are encoded by inherited genes (germline-encoded host sensors) and called pattern recognition receptors (PRRs) [1,2], having a critical role in the host defense against viral particles. PRRs detect two classes of molecules: a. pathogen-associated molecular patterns (PAMPs), which are small molecular nonself motifs, such as viral nucleic acids, DNA, or RNA, and b. damage-associated molecular patterns (DAMPs), which are produced by or released from damaged or dying cells. PRRs that recognize viral PAMPs consist of the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), C-type lectin-like receptors (CLRs), and DNA sensors, such as IFI16 and the cGAS–STING signaling pathway [3–5]. The binding of PAMPs to PRRs triggers the activation of several signaling cascades in the host immune cells that lead to the expression of proinflammatory cytokines, type I interferons (type I IFNs), and *interferon-stimulated genes* (*ISGs*), to orchestrate the antiviral response and promote inflammation. The infection activates a robust and fine-tuned immune response that is crucial for the clearance of the virus. Short-term activation of the innate immune system is beneficial for host defense mechanisms, while overactivation of PRRs or downstream components may lead to a sustained immune system response and irreversible changes in organ structure and function [6–10]. The breakdown of immune

regulatory mechanisms may culminate in the loss of self-tolerance, leading to an immune-mediated attack directed against both viral and self-antigens.

In autoimmune diseases, the imbalance between innate and adaptive immune responses may lead to hyperinflammation. Moreover, the exaggerated response of the immune cells with a hyperproduction of cytokines called a "cytokine storm" plays an important role in the manifestation and progression of autoimmunity. Cytokine storms occur in various autoimmune diseases, though the presence of a viral infection often serves as a trigger [11]. Cytokine storms are characterized by the hyperproduction of proinflammatory cytokines in response to various triggering stimuli (e.g., viral infection) leading the immune system to cause tissue damage. This aberrant immune response in autoimmunity in combination with a viral infection may lead to defective antiviral immunity or precipitate a disease flare. Herein, we review the literature on how antiviral mechanisms may drive autoimmune disease pathogenesis. Specifically, we report PRR-driven responses of innate immune cells that are involved in autoimmunity and discuss the pitfalls and challenges regarding the therapeutic targeting of the mechanisms involved.

1.1. Innate Detection of Viral Infection by PRRs

1.1.1. Toll-like Receptors (TLRs)

TLRs serve as the first-line defense mechanism of the host in order to trigger the innate immune response and then orchestrate the initiation of the adaptive immune response [12]. The TLRs are type I integral membrane glycoproteins that contain leucine-rich repeats (LRRs) flanked by characteristic cysteine-rich motifs (involved in ligand binding) in their extracellular domain (ECD), a middle transmembrane domain (TM), and a C-terminus cytoplasmic Toll/IL-1 receptor (TIR) homology domain, which is essential for signaling. The formation of an M-shaped dimer or multimer is essential for the activation of all TLRs, so that the C-terminus regions of the two TLR ECDs are in proximity. The next step is the multimerization of cytoplasmic tails, which will then recruit the downstream adaptors Toll-interleukin-1 receptor (TIR) domain-containing adapter-inducing *IFN-β* (TRIF) or myeloid differentiation primary response 88 (MyD88) through homotypic interaction leading to the activation of specific transcription factors and inducing an antiviral type I interferon response and cytokine production.

The cellular localization of TLRs correlates with their functions in sensing invading pathogens [13]. To date, 10 human TLRs (TLR 1–10) and 12 mouse TLRs (TLR 1–9, 11–13) have been identified, each one of them having a unique ligand specificity [14]. They collectively sense a wide range of bacteria, viruses, fungi, and endogenous ligands. In the current review, we focus on the TLRs that detect viral PAMPs. TLRs, based on their cellular localization, respond to different types of molecules. TLRs 3, 7, 8, and 9 are mainly expressed inside cells on the endoplasmic reticulum (ER) and endosomal membranes where they detect different viral nucleic acids [15]. TLRs found on the cell surface, such as TLR1, TLR2, TLR4, and TLR6, are able to mediate innate immune responses to viral pathogens or more specifically viral envelope and capsid proteins (Figure 1).

TLR2 is expressed in various immune cells including neutrophils, monocytes/macrophages (MΦs), and dendritic cells (DCs). TLR2 detects a variety of microbial components, such as lipoproteins, peptidoglycan, and lipoteichoic acids, derived from Gram-positive bacteria, viruses, and parasites. Recognition of specific ligands by the host immune cells and the downstream signaling from TLR2 occurs with the heterodimerization with either TLR1 or TLR6. TLR1 and TLR6 contain highly homologous structures to TLR2 and are also expressed on the plasma membrane of all innate immune cells, such as monocytes, macrophages, and DCs [16]. TLR2/1 heterodimers mainly recognize bacterial triacylated lipopeptides, while TLR2/6 heterodimers tend to interact with mycoplasmal diacylated lipopeptides [17]. This ligand-mediated dimerization is important for the recruitment of the adaptor proteins, which are crucial for transmitting the signal to downstream effector molecules leading to proinflammatory cytokine production and the activation of an innate immune response. Although these heterodimers are best known for recognizing bacterial

components, studies in mice have revealed that TLR2 contributes to the antiviral response in murine cytomegalovirus (MCMV), respiratory syncytial virus (RSV), and vaccinia virus infections [18]. More specifically, in mice with an RSV infection, the association of TLR2 with TLR6 in leukocytes mediates an immune response with the secretion of various costimulatory molecules, such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), chemokine (C—C motif) ligand 2 (CCL2), and chemokine (C–C motif) ligand 5 (CCL5) [19]. Moreover, TLR2 senses virus envelope proteins and glycoproteins in order to mediate antiviral immunity [20–22]. For example, the glycoproteins B and H from the cytomegalovirus (CMV) are detected by TLR2, resulting in nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) activation in activated B cells and the production of proinflammatory cytokines [23]. In addition, the activation of TLR2 in Ly6C^{high} "inflammatory" mouse monocytes leads to the production of type I IFNs and the blocking of viral replication [24].

TLR3 is expressed in myeloid DCs and macrophages and is not found in neutrophils and plasmacytoid dendritic cells (pDCs) and is localized in the endosomes [3]. TLR3 is a sensor of viral double-stranded RNA (dsRNA) and its synthetic analogue, polyinosinic:polycytidylic acid (poly I:C). TLR3 can also sense the presence of viral genomes derived from damaged host cells and viral particles, such as ssRNA and DNA viruses [22,25–27]. TLR3, in contrast to all other known TLRs, upon activation with synthetic or viral dsRNAs, does not recruit the adaptor molecule MyD88 and instead associates with TRIF [28]. TRIF binds to TNF receptor-associated factor 6 (TRAF6) and the receptor-interacting protein-1 (RIP-1) in order to favor the induction of NF-κB and MAPKs via TAK1 in a similar manner to MyD88. In addition, TRIF associates with TNF receptor-associated factor 3 (TRAF3) and binds TANK-binding kinase-1 (TBK1) and IκB kinase ε (IKKε) to favor the production of type I interferon by phosphorylating the interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7). This allows their dimerization and entrance to the nucleus where they associate with NF-κB and activator protein 1 (AP-1) in order to transcriptionally activate inflammatory genes [29].

TLR4-expressing cells are mainly myeloid cells, such as monocytes, macrophages, and dendritic cells. TLR4 is an important PRR for Gram-negative bacterial components, such as lipopolysaccharide (LPS). Most myeloid cells express high levels of CD14, which facilitates the activation of the TLR4/MD2 complex by LPS. TLR4 association with myeloid differentiation 2 (MD2) on the cell surface is crucial for the activation of downstream adaptor proteins MyD88 and TRIF resulting in the expression of proinflammatory cytokines. CD14 also controls the immunoreceptor tyrosine-based activation motif (ITAM)-mediated tyrosine kinase Syk and its effector molecule phospholipase C gamma 2 (PLCγ2) to promote endocytosis and favor TLR4 internalization into endosomes for the activation of the TRIF-dependent signaling cascade [30]. It has also been revealed that TLR4 is an important sensor in the detection of endogenous molecules, such as DAMPs, released by inflamed tissues and necrotic cells [31]. In addition, TLR4 can also detect several viral glycoproteins that are found on the surface of an enveloped virus and mediate attachment with the target host cell by interacting with a cellular receptor and then fusion with the host membrane through the hydrophobic peptide. TLR4 mediates the production of IL-6 through the F protein upon RSV infection [32–34]. Moreover, TLR4 senses the envelope proteins of mouse mammary tumor virus (MMTV) and promotes the maturation of bone marrow-derived dendritic cells (BMDCs) by increasing the production of inflammatory cytokines, such as TNF-α, IL-6, and interleukin-12 subunit p40 (IL-12p40). Contrarily, in bone marrow-derived dendritic cells (BMDCs) upon MMTV infection, TLR4 enhances the expression of the MMTV entry receptor CD71 on these cells thus promoting viral infection [35,36]. Like viral glycoproteins, cellular glycoproteins are also detected by the immune system, potentially leading to autoimmune disorders. For instance, YKL-40, also known as chitinase 3-like 1 glycoprotein, is a member of chitinase-like glycoproteins and is produced in inflammatory conditions by neutrophils and macrophages. In joint tissues of rheumatoid arthritis (RA) patients, this glycoprotein is recognized as a potential biomarker of disease activity [37]. Moreover, dickkopf-related protein 1 (DKK-1) is another glycoprotein that plays a significant role

in the inhibition of Wnt/b-catenin signaling by binding to the low-density lipoprotein receptor-related protein 5/6 (LRP-5/6) complex and favoring its degradation. DKK-1 is considered as a potential target for diseases associated with enhanced Wnt signaling activity. For instance, DKK-1 is elevated in the sera and urine samples of systemic lupus erythematosus (SLE) patients, and it is used as a positive biomarker for the identification of active lupus nephritis patients [38].

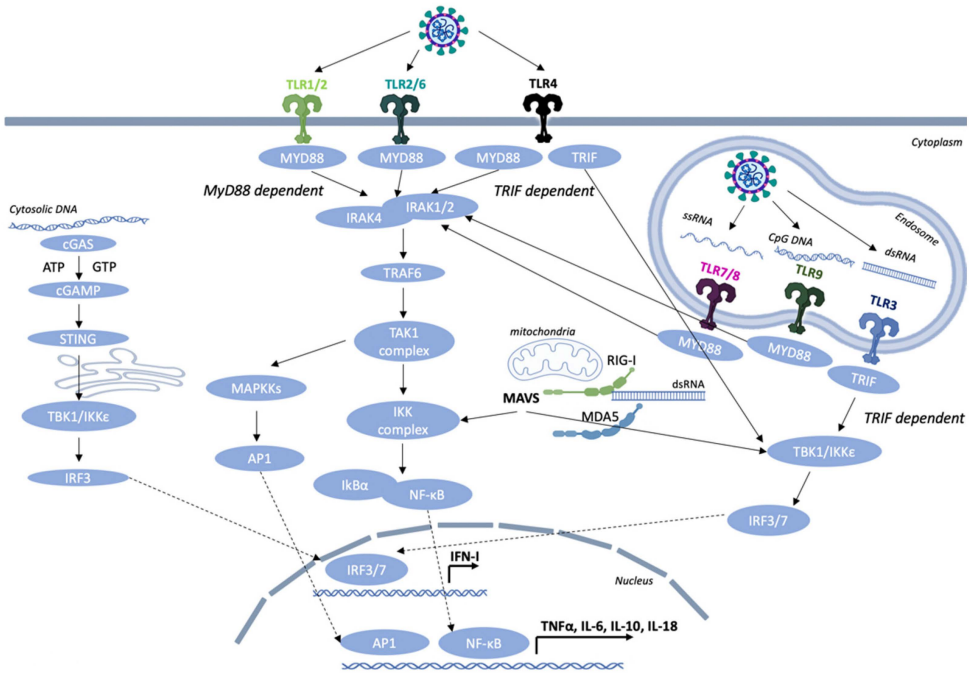

Figure 1. Innate immune cells sense viruses using distinct pattern recognition receptors (PRRs). Surface Toll-like receptors (TLRs) and TLRs located in endosomes, cytosolic nucleic acid sensors, RLRs, and DNA sensors detect viral nucleic acids or viral proteins. Most viral proteins are either components of the capsid or the envelope of the virus. Viral envelope glycoproteins are sensed via surface TLRs, such as TLR1/2, TLR2/6, and TLR4. Homo- or heterodimer formation initiates signaling to the two major downstream adaptor molecules, myeloid differentiation primary response 88 (MyD88) and Toll-interleukin-1 receptor (TIR) domain-containing adapter-inducing *IFN-β* (TRIF). Downstream signaling from surface TLRs requires the MyD88-dependent pathway. In endosomes, TLR3 detects double-stranded RNA (dsRNA); TLR7/8 detects single-stranded RNA (ssRNA), while TLR9 detects CpG DNA. TLR7/8 and TLR9 recruit the signaling adaptor molecule MyD88 to activate the IκB kinase (IKK) complex, resulting in the activation of nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) and interferon regulatory factor (IRF) family members. In contrast, TLR3 binds with TRIF in order to activate downstream signaling, resulting in IRF3/7 translocation to the nucleus. The cytosolic sensors, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5), sense viral dsRNA, and signal transduction occurs through the adaptor molecule mitochondrial antiviral-signaling protein (MAVS) located at the mitochondria that activates the TANK-binding kinase-1 (TBK1) and IκB kinase ε (IKKε) complex resulting in activation of NF-κB and IRFs. Cytoplasmic DNA is sensed through cytosolic DNA sensor cyclic GMP–AMP synthase (cGAS), which synthesizes cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) in order to induce the ER-resident stimulator of interferon genes (STING) and leads to the activation of downstream molecules through the TBK1/IKKε complex. When NF-κB and IRFs are activated, they translocate to the nucleus and trigger the expression of proinflammatory cytokines and type I interferon (IFN) production. Secretion of these proteins promotes *interferon-stimulated genes* (*ISGs*) production, which results in the establishment of the "antiviral state", recruitment of innate immune cells to sites of infection, and activation of the adaptive immunity to shape the overall antiviral immune response.

After ligand recognition by TLR4, the activation of two distinct signaling pathways are triggered, the MyD88-dependent and the MyD88-independent/TRIF-dependent pathways. TIRAP, which is a Toll-interleukin-1 receptor domain-containing adapter protein, mediates the signal from TLR4 to MyD88, whereas TRIF-related adaptor molecule (TRAM) mediates the signal from TLR4 to TRIF. A balanced activation between the MyD88- and TRIF-dependent pathways is crucial in order to elicit specific antiviral responses for controlling tumor cell growth and autoimmune diseases. A recent study by Mlcochova et al. [39] revealed that upon HIV-1 infection in macrophages, TLR4 binds to TRIF and induces G0 arrest and SAMHD1 antiretroviral activity by a MyD88/NF-κB-independent pathway.

TLR7 and TLR8 recognize single-stranded RNA (ssRNA) molecules. TLR7 is predominantly expressed in plasmacytoid dendritic cells (pDCs) and monocytes and is involved in the induction of IFN-α gene transcription. On the other hand, human TLR8 is expressed in monocytes, macrophages, and conventional dendritic cells (cDCs) and in low-levels in B cells and pDCs [40–42]. Both receptor genes are located on the X chromosome, encode proteins that recognize self-RNA-containing autoantigens, and induce the production of IFN-α. The *TLR7* gene escapes X chromosome inactivation, and that may contribute to stronger female antiviral immunity and the female predisposition to SLE pathogenesis since IFN stimulation by TLR7 is a fundamental driver of SLE pathogenesis [43,44]. TLR7 and TLR8 also sense synthetic oligoribonucleotides (ORNs), such as imiquimod (R837), resiquimod (R848), and guanine analogue. TLR7/8 agonists have also been used as vaccine adjuvants due to their beneficial properties in host defense [39,40]. Upon ligand activation of TLR7 and TLR8, the dimer conformation changes, and the cytoplasmic TIR domains multimerize in order to recruit the downstream adaptor molecule MyD88 through homotypic interaction. MyD88 contains a C-terminus TIR (Toll IL-1R) domain for association with other receptors or adaptor molecules that contain a TIR domain and a N-terminus death domain (DD) for interacting with the interleukin-1 receptor-associated kinase (IRAK) family members. The association of MyD88 and TLR through their TIR domains results in the activation of the IRAKs, IRAK-1, and IRAK-4. In turn, IRAK-4 phosphorylates IRAK-1, which allows the binding to the C-portion of TRAF6 and enables the dissociation from the TLR complex. Upon activation, TRAF6 performs K63-linked polyubiquitination of the tumor growth factor beta (TGF-β)-activated kinase 1 (TAK1) and IκB kinase gamma (IKKγ), also known as NEMO (NF-κB essential modulator). IKKγ then interacts with the TAK1-binding protein 1 (TAB1), TAB2, and TAB3, resulting in IKK-mediated phosphorylation and the degradation of IκB. NF-κB is now able to translocate to the nucleus and induce gene transcription. Moreover, TAK1 forms a complex with TAB1, TAB2, and TAB3 that triggers the MAPK pathway leading to the formation of the activator protein 1 (AP-1) and its translocation to the nucleus. AP-1 and NF-κB are key modulators to orchestrate the expression of many proinflammatory genes. The formation of a complex by IRAK4, IRAK1, TRAF6, TRAF3, and the downstream transcription factors NF-κB and IRF7 leads to the activation and induction of proinflammatory cytokines and IFNs [44,45]. The complex of IRAKs and TRAF6 also associates with IRF5 and IRF7, leading to the subsequent IRAK1-dependent phosphorylation and nuclear translocation of both transcription factors. IRF5 is a key transcription factor in the induction of proinflammatory cytokines, such as IL-6 and IL-12p40, while IRF7 is primarily involved in type I IFN production [46,47]. A recent study by Marcken et al. [48] in human blood $CD14^+$ classical monocytes infected with different RNA viruses, such as coxsackie (CV), encephalomyocarditis (EMCV), influenza A (IAV), measles (MV), Sendai (SV), or vesicular stomatitis virus (VSV), revealed that the RNA virus infection triggered distinct responses in the human monocytes, and the engagement of TLR7 or TLR8 is virus-specific. In detail, TLR7 favors the production of cytokines involved in $CD4^+$ T helper 17 (Th17) cell polarization (IL-1β, IL-6, and IL-23) after virus infection with MV and VSV, whereas TLR8 promotes the T_H1-promoting cytokine response and type I IFN production after viral infection with ECMV. On the contrary, the influenza A virus promoted the secretion of both types of cytokines. They also revealed that FOS-like 1 (FOSL1), which is an AP-1 transcription factor subunit, was increased upon

TLR7 stimulation resulting in a reduced secretion of T_H1-type cytokines, such as IL-27 and TNF-α in monocytes [48]. Moreover, an enhanced Ca^{2+} flux was induced by TLR7 rather than TLR8 signaling leading to a blockade of type I IFN production suggesting a contradictory role between these two receptors. Overall, these studies suggest that TLR7 and TLR8 activate different signaling cascades in human monocytes during RNA virus infection with different phenotypes in antiviral immunity.

TLR9 is the only known endosomal ssDNA sensor. This receptor preferentially binds single- or double-stranded DNA and unmethylated CpG motif-containing viral DNA, such as herpes simplex virus (HSV) type 1 and type 2, leading to the production of type I IFNs and an antiviral immune response. TLR9 along with TLR7 is highly expressed in pDCs, also known as type I IFN-producing DCs and B cells among the rest of the immune cells [12,18,49]. CpG oligodeoxynucleotides (ODNs) are short synthetic DNA molecules containing cytosine ("C") and guanine ("G") motifs linked with a phosphodiester bond. They are naturally occurring analogs derived from viral and bacterial DNA. In addition, CpG ODNs are used as vaccine adjuvants in order to enhance the function of professional antigen-presenting cells (APCs) and favor the generation of vaccine-specific immune responses. There are three structurally distinct categories of CpG ODN: CpG-A, CpG-B, and CpG-C. The sequence of CpG ODNs, the secondary domains, and the effect on other immune cells play an important role in this separation. CpG-A preferentially induces the production of type I IFNs from pDCs and the maturation of APCs, but low B-cell stimulation. CpG-B favors strong activation of the B cells, induction of the TLR9-dependent NF-κB signaling cascade, and weakly stimulates type I IFNs and maturation of APCs. CpG-C combines functions of both classes as they strongly activate the secretion of IFN-α from pDCs, and they also stimulate B cells. TLR9 is located in the ER, and upon activation with CpG-DNA, it interacts with Unc93b and translocates to the endosomal compartments resulting in optimal TLR9 signaling [50]. In the endosome, ligand binding induces conformational change and the dimerization of TLR9, which results in the recruitment of the signaling adaptor molecule MyD88 [51]. The interaction between the TIR domains of MyD88 and TLR9 activates the IRAK4 and IRAK1. IRAK-4 is an essential modulator for the gene transcription of proinflammatory cytokines upon TLR9-induced activation of the signaling cascade. The activation of IRAK4 results in the recruitment of TRAF6, which in turn leads to the activation of TAK1. The phosphorylation of the IκB kinase (IKK) complex by TAK1 leads to the activation of NF-κB, MAPKs, and AP-1. The key transcription molecules NF-κB and AP-1 are then responsible for the induction of cytokines, such as IL-1, IL-12, and TNF, and the upregulation of costimulatory molecules, such as CD80 and CD86 [52]. Depending on the functional and morphological differences, endosomes can be classified as early or late. More specifically in pDCs, in the early endosomes, a signaling complex including IRAK4, IRAK1, TRAF6, and TRAF3 is formed, resulting in IRF7 activation and type I IFN production. In contrast, in the late endosomes of pDCs, the signaling complex includes IRAK4, TRAF6, TAK1, NF-κB, MAPKs, and IRF5, leading to the production of proinflammatory cytokines [53].

Failure to restore homeostasis by the uncontrolled expression of inflammatory mediators may predispose the host to autoimmune diseases, such as SLE and RA. To this end, fine-tuning of the TLR signaling cascades is pivotal in order to obtain a balance between pro- and anti-inflammatory immune responses for eliminating invading pathogens without damaging the host. Several regulatory checkpoints in the TLR pathways are developed in order to tightly regulate the immune system's response including (i) removal of receptors from the cell surface, (ii) expression of negative regulators of the signaling cascades, (iii) adaptor complex destabilization, (iv) phosphorylation and ubiquitin–proteasome-mediated control of the signaling molecules, (v) manipulation of the expression of the other receptors and downstream components, and (vi) transcriptional control [54,55]. Potentially harmful TLR signaling pathways can be regulated by negative feedback mechanisms and by anti-inflammatory factors, such as interleukin (IL)-10 and steroids [56,57]. A study by Curtale et al. [57] revealed an increased expression of miR-146b upon LPS stimulation

through an IL-10-dependent loop. They also highlighted that this miRNA exhibited anti-inflammatory features in human monocytes by targeting TLR4 and several other components of the TLR4 signaling cascade, such as MyD88, IRAK-1, and TRAF6, thus suggesting that miR-146b is a negative modulator of the TLR-induced inflammatory response. In addition, many combinations of TLR-TLR and TLR-NOD modulate inflammatory responses. For instance, the NOD-like receptor family CARD domain containing 3 (NLRC3) regulates the activation of the transcription factor NF-κB upon TLR stimulation by inhibiting the TRAF6 activation. In a study by Schneider et al. [58], the expression levels of NLRC3 were reduced upon LPS stimulation, and mice lacking the NLRC3 developed enhanced secretion of proinflammatory mediators, proposing a negative role of NLRC3 in the TLR signaling cascade.

1.1.2. RIG-like Receptors (RLRs)

RIG-like receptors (RLRs) are cytoplasmic sensors of viral infection and key players in the recognition of viral nucleic acids by inducing the secretion of type I IFNs and chemokines. RLRs can sense double-stranded RNA and DNA/RNA heteroduplex oligonucleotides, including regions of the genome of RNA viruses and RNA transcripts of RNA and DNA viruses. The two best-characterized RLRs are the retinoic acid-inducible gene I, **RIG-I**, and the melanoma differentiation-associated gene 5, **MDA5**. RLRs are characterized by a conserved domain of a central DExD/H-box helicase region and a C-terminus domain (CTD), both of which are implicated in the recognition of viral RNA. In addition, both RLRs contain two N-terminus caspase activation and recruitment domains (CARDs), and upon sensing of viral components, they induce the activation of downstream signaling molecules resulting in type I IFN production [59]. More specifically, RIG-I and MDA5 detect distinct types of viral dsRNAs. RIG-I senses short dsRNA (<1000 bp) and a 5′ triphosphate (5′ ppp) moiety, found in the genomic RNA of several viruses, in association with short blunt-end double-stranded RNA (dsRNA), such as "panhandle" domains, that are important for RIG-I's ability to discriminate viral from self-RNA. Host cell RNA evades recognition by RIG-I due to post-transcriptional modifications, such as 5′ppp capping with 7-methyl guanosine (m7G) and 2′-0-methylation of 5′-end nucleotides [60,61]. MDA5 recognizes long-chain dsRNA fragments (at least 2 kbp) organized in web-like structures [62]. Mitochondrial antiviral-signaling protein (MAVS) acts as a central hub for signal transduction initiated by RIG-I and MDA5 via TBK1 and IKKε in order to activate NF-κB and IRFs, leading to the expression of proinflammatory cytokines and type I interferons [63]. RIG-like receptors are expressed in a wide variety of cell types, including bone marrow-derived leukocytes and various tissue cells, and enable them to participate in innate immune responses to these viruses.

1.1.3. The cGAS–STING Pathway

Stimulator of Interferon Genes (STING) is a protein consisting of four transmembrane regions (TMs) and a CTD and is located in the ER. Human STING contains a transmembrane domain in the N-terminus for the regulation of its cellular localization and homodimerization and an intracellular soluble portion in the C-terminus for interacting with downstream molecules, including TBK1/IKKε and IRF3/IRF7. Upon recognition of cytoplasmic DNA from DNA viruses and abnormal endogenous DNA, the **cytosolic DNA sensor cyclic GMP–AMP synthase (cGAS)** synthesizes cyclic guanosine monophosphate-adenosine monophosphate (2′,3′-cGAMP) in order to induce translocation of the ER-resident adaptor protein STING to the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and then the Golgi apparatus and the endosomes for degradation in lysosomes. The activated STING dimer recruits TBK1 to form the translocation complex from the ER to the perinuclear lysosomal compartments through an autophagy-like process. The STING–TBK1 complex phosphorylates IRF3 and NF-κB to promote entry into the nucleus. Then, IRF3 and NF-κB induce the production of *type I IFN* genes and other

proinflammatory cytokines through the TBK1–IRF3 axis and NF-κB signaling pathway, establishing an antiviral state [64–66].

2. Viral Infection and Autoimmunity

Immunological tolerance is an active, tightly regulated, fine-tuned response of the immune system to self-antigens or against various environmental entities that prevent the immune system to mount possibly harmful responses. There are two types of immune tolerance, central and peripheral tolerance, and both provide and maintain self-tolerance. The discrimination between self- and nonself antigens is pivotal for the proper functioning of the immune system. Failure of immunological tolerance leading to an aberrant immune response against host antigens is critical for the development of autoimmunity [67]. Several triggering factors have been linked to autoimmune responses, such as genetics, environment, age, and viral infections. Viral infection can alter immunological tolerance against self-antigens and has been associated with the initiating or flaring of several autoimmune and inflammatory phenomena in individuals with genetic susceptibilities [68–71]. These infections trigger the antiviral immune response mechanisms resulting in the activation of signaling pathways and the induction of cytokine and chemokine production, the production of autoantibodies, and the deposition of immune complexes (ICs) in tissues some of which could overwhelm the immune regulatory mechanisms. Several mechanisms, such as molecular mimicry, bystander activation of dendritic cells and T-cells, and epitope spreading can explain how viruses might trigger a series of actions leading to the development of an autoimmune disease [68,72]. For instance, the possibility that Epstein–Barr virus (EBV) may trigger several autoimmune diseases, such as SLE and multiple sclerosis (MS), has been reported during the past decades [73,74]. Several studies have demonstrated an increased viral load of EBV DNA in SLE patients compared to healthy individuals [75,76]. Furthermore, a serologic association to EBV infection has been reported with high titers of anti-early antigen (EA) IgG and IgA in SLE patients compared to healthy controls [73,77]. In addition, several reports have revealed the molecular similarity between the EBV nuclear antigen-1 (EBNA-1) and the common lupus autoantigen Ro, as well as the inability of $CD8^+$ T cells to control EBV-infected B cells suggesting that viruses may influence the development of SLE pathogenesis [78–82]. Therefore, environmental factors, such as viral infections, may influence the function of PRRs and the expression of downstream molecules involved in the signaling cascades. In innate immune cells, PRR signaling upon viral infection may exaggerate immune responses in autoimmunity (Figure 2). How risk alleles in PRR signaling pathways and genetics contribute to autoimmunity and how viral-induced autoimmunity can be carried out through the above-mentioned mechanisms, is outside the scope of the current review and is discussed elsewhere [68,72,83–87]. However, in the current review, we focus on the crosstalk between innate antiviral immune responses and autoimmune responses mediated by PRR molecules and downstream components and discuss the potential therapeutic targeting of the mechanisms involved.

2.1. The Role of Innate Immune Cells in Antiviral Responses in an Autoimmune Background

2.1.1. Linking Plasmacytoid Dendritic Cell (pDC) Antiviral Response with Autoimmunity

Plasmacytoid dendritic cells (pDCs), members of the dendritic cell (DC) family, are key players in antiviral immunity and are known to secrete large amounts of type I IFNs in response to viremia. They represent a heterogeneous cell population with 0.2–0.8% of peripheral blood mononuclear cells (PBMCs) that links innate and adaptive immune responses [88]. The main function of these cells is to detect viral nucleic acids through TLR7 and TLR9; capture, process, and present antigens to adaptive immune cells; and mediate their polarization into effector cells orchestrating a proper immune response [89,90]. TLR7- and TLR9-mediated PRR signaling in pDCs has been reported in various autoimmune conditions suggesting their role in the aberrant immune response, such as cytokine storm and excessive activation of the innate and adaptive immune system observed in many autoimmune and inflammatory phenomena. The emerging literature indicates how type I

IFN produced by pDCs in antiviral immunity may contribute to autoimmune pathology and how similar pathways are triggered and may drive disease pathogenesis [91–94].

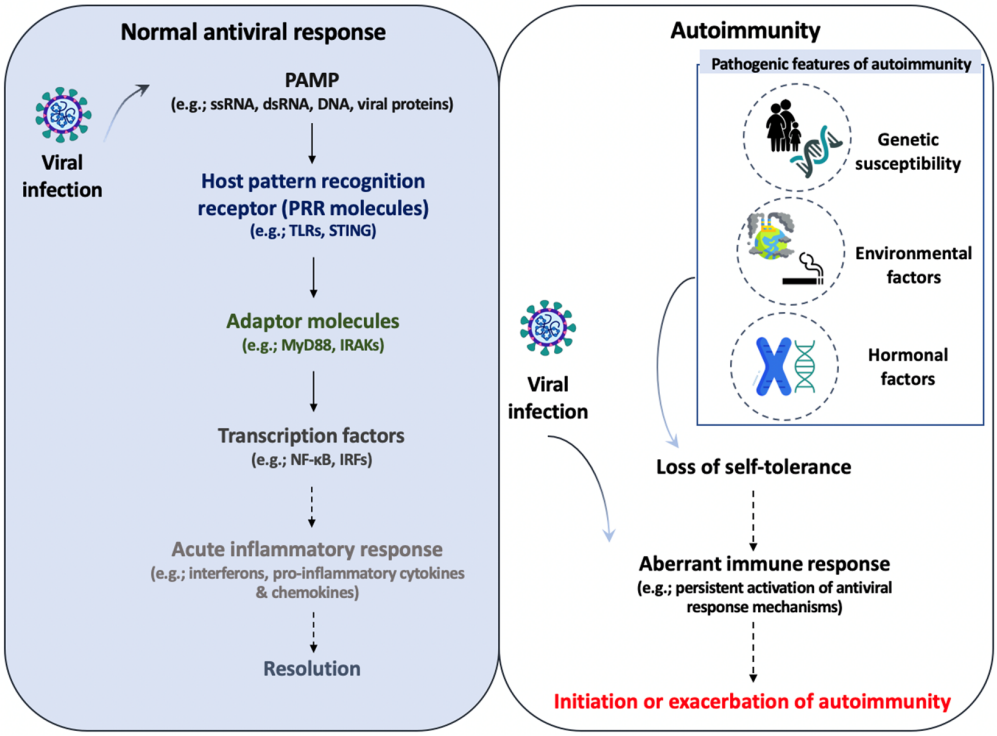

Figure 2. PRR signaling upon viral infection may either lead to acute inflammatory response and resolution or trigger autoimmune responses. Innate immunity is activated upon viral exposure, and the response is mediated through pattern recognition receptor (PRR) molecules that recognize the viral nucleic acids, the adaptor molecules that mediate the signal to downstream components, and the transcription factors that are responsible for the outcome. An acute inflammatory response is orchestrated by the release of antiviral molecules, such as interferons, proinflammatory cytokines, and chemokines at sites of infection. In autoimmune diseases, a combination of genetic susceptibility, such as gene copy variations and single-nucleotide polymorphisms (SNPs) and environmental as well as hormonal factors including UV light, toxic chemicals, and genes defined by the X chromosome, leads to loss of self-tolerance and chronic inflammation. In this environment, when a viral infection occurs, the host defense mechanisms are exposed and may promote an exaggerated immune response, which can lead to initiation or exacerbation of autoimmunity.

Hillen et al. [95] demonstrated that in primary Sjögren's Syndrome (pSS), which is a systemic autoimmune disease characterized by salivary and lacrimal gland dysfunction, circulating pDCs from pSS patients display an activated transcriptional profile and are primed for enhanced proinflammatory cytokine secretion compared to healthy donor (HD) pDCs. More specifically, they exhibit high levels of proinflammatory cytokines upon stimulation with endosomal TLR ligands as the activation of TLR7 triggered more type I IFN production in pSS pDCs compared to HD, similar to an antiviral response. Moreover, in a study by Mavragani et al. [96], endogenous virus-like genomic repeat elements in pSS patients triggered the IFN-I pathway through the activation of TLR7/8 signaling cascade in pDCs further influencing the initiation or amplification of SS. Thus, such studies provide insights into the role of viral infection in the initiation or propagation of several autoimmune diseases. Another recent study by Wang et al. [97] revealed that TLR7 signaling influences the development of Sjögren's syndrome (SS) since TLR8-deficient mice that develop SLE due to enhanced TLR7 signaling by DCs also develop a secondary pathology similar to SS. This highlights that the development of the SS phenotype is dependent on TLR7 signaling. In light of this, they also revealed an increased TLR7 expression and enhanced inflammatory cytokine and chemokine secretion, such as TNF, LT-α, CXCL13, and CXCR5 in pDCs of pSS

patients. These data substantiate the role of TLR signaling in mediating the inflammatory features of pDCs in pSS immunopathology supporting their contribution in the initiation or progression of autoimmunity. This enhanced signaling in pSS pathology through TLR7/8, often triggered by viral entities, suggests that PRRs promote and influence the progression of autoimmune diseases by favoring a sustained inflammatory response.

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease that manifests a wide range of clinical and molecular abnormalities and is characterized by the loss of self-tolerance to nuclear antigens [98]. An elevated expression of type I IFNs and *type I IFN*-regulated genes termed as the IFN signature has been reported in the majority of SLE patients [99–102]. The IFN gene signature observed in SLE patients is characterized by the increased expression of *IFN-regulated* genes, such as *ISG15*, *IFI16*, and *FcgRI* (*CD64*), and is mainly reflected by the circulating type I IFNs [101,103]. The disease activity correlates with *IFN-α* expression levels and the strength of the IFN signature [104–106]. In lupus, IFN-α-driven immunologic alterations culminate into persistent self-directed immune responses against autologous nucleic acids, mimicking a sustained antiviral response. In detail, an initial viral infection that can be sensed by different PRR molecules and mediated by several signaling cascades as discussed previously, can promote type I IFN secretion and release of cellular material from dying or apoptotic cells. Then, apoptotic cells release DNA- or RNA-containing autoantigens as well as neutrophil extracellular traps (NETs), which triggers B cells to produce autoantibodies against CpG-rich DNA or ss-RNA and associated proteins. The formation of interferogenic ICs will act as an endogenous adjuvant for triggering type I IFNs, leading to a prolonged activation of the immune system to produce IFNs. This will result in the chronic activation of the IFN system, which will enable the development of autoimmune phenomena, chronic inflammatory processes, and tissue damage in a vicious circle [107].

IFN-α has multiple immunostimulatory properties that include the expression of several pivotal molecules in the response of the immune system, such as MHC II, CXCL10, CXCR3, CD40, CD80, and CD86. Depending on the cell type exposed to IFN, the effects will vary and may be detrimental. For example, type I IFNs influence the function of B cells through several mechanisms, such as the production of B-cell activating factor (BAFF) from monocytes and neutrophils, thus leading to prolonged survival and activation of B cells and enhanced T-cell-independent and -dependent antibody production [108,109]. Genetic, epigenetic, environmental, and immunoregulatory factors influence the outcome of SLE pathogenesis (Figure 2) [110,111]. It is also well established that SLE is a female-predominant disease, although the causes of sex bias are ill-defined. Moreover, mounting evidence suggests that PRRs promote and influence the progression of autoimmune diseases by favoring a sustained inflammatory response to self-components [112,113]. In addition, enhanced TLR7-mediated IFN-α production was also demonstrated in pDCs from SLE patients in a study by Murayama et al. [114]. It was also reported that pDCs stimulated with the TLR7 agonist promoted an autoimmune Th17 phenotype and increased levels of type I IFNs were correlated with high amounts of Th17 cytokines in the serum of SLE patients [115,116]. In addition, a distinctive feature of SLE immunopathology is the increased numbers of ICs in the serum of SLE patients correlating with disease severity [117]. It was revealed that these nucleic acid-containing immune complexes are internalized by pDCs via FcγRIIa into endosomes where they stimulate TLR7 and TLR9 leading to type I IFN secretion. Also, type I IFN production by pDCs stimulated with ICs is robustly enhanced in the presence of activated T cells [118,119]. Furthermore, it is well established that EBV RNA and DNA enhance the secretion of IFN-α through TLR7 and TLR9 in pDCs and SLE patients demonstrate high titers of EBV and increased *latent membrane protein 1* (*LMP1*) expression levels, which is a well-known oncoprotein of the EBV latent gene products [114,120]. Thus, these studies highlight that EBV might be linked with the initiation and progression of SLE since the type I IFN pathway is activated. In addition to this, it has also been reported that IFN-α and several other proinflammatory cytokines are able to induce LMP-1 expression in B cells infected with EBV thus supporting the notion that

EBV and its products are involved in key pathways modulating SLE activity and severity and may exacerbate the autoimmune phenotype observed via promoting IFN production through PRR pathways [121]. Interestingly, it seems that viruses can trigger autoimmunity through several mechanisms, one of them being the stimulation of intracellular PRR inflammatory cascades thus leading to the production of IFNs and cytokines that may excessively activate the immunoregulatory mechanisms and predispose to autoimmunity or exacerbate the pathology. Another study by Dominique et al. [122] on systemic sclerosis (SSc), which is a multisystem, fibrosing autoimmune disorder characterized by immune dysregulation, revealed that SSc pDCs demonstrated enhanced expression of TLR8 leading to the promotion of IFN-α production. This study underlines the key role of pDCs in the sensing of RNA and the subsequent activation of TLR inflammatory cascades in the establishment of fibrosis (Table 1). Moreover, a possible pathogenic association of viral infection and the development of SSc has been proposed, with the human parvovirus B19 (B19) and human cytomegalovirus (HCMV) as the important triggering agents in SSc pathology. Further studies are needed in order to expand our knowledge on the crosstalk between SSc viral products, PRR inflammatory pathways, and SSc immunopathology.

In summary, pDCs are important players in the mechanisms underlying several autoimmune diseases and demonstrate enriched inflammatory responses through the activation of TLR7/8 and TLR9 signaling pathways and the IFN system. Frequently, this activation is either triggered by environmental agents, such as viruses or self-components, both resulting in the excessive stimulation of the immune response. It is of great importance to evaluate how these cells can orchestrate an effective antiviral immune response with type I IFN production and cytokine secretion in autoimmunity.

2.1.2. Linking Monocyte Antiviral Response with Autoimmunity

Monocytes are blood mononuclear cells that arise from bone marrow progenitors. Based on CD14 (lipopolysaccharide (LPS) coreceptor) and CD16 (FcγRIII) expression levels, human monocytes are divided into three phenotypically and functionally distinct populations: the $CD14^{++}CD16^{-}$ classical, $CD14^{++}CD16^{+}$ intermediate, and $CD14^{low}CD16^{++}$ nonclassical [123,124]. They are key players in recognizing pathogen-associated molecular patterns via PRRs and eliciting an immune response via the secretion of proinflammatory cytokines. By sensing the inflammatory environment, circulating monocytes can replenish the pool of tissue monocyte-derived macrophages (moMφs) and inflammatory monocyte-derived dendritic cells (moDCs) [125,126]. Several lines of evidence showed that monocyte activation is associated with the disease progression and severity in several autoimmune diseases [127]. Nucleic acid sensing leads to the activation of IFN-α immunity, which in combination with clearance pathways, orchestrates the antiviral response. However, how can the aberrant activation of the PRR signaling pathways in monocytes through the sensing of nucleic acids or other triggering factors be implicated in the development of autoimmunity?

A recent study by Kyogoku et al. [128] revealed that monocytes from SLE patients demonstrated a pathogenic IFN signature (activation of IRFs, GTPases, and kinases) [102] observed in autoimmune conditions augmented by the expression of cytokines, such as IL-9, IL-10, and IL-15 and mediated by the JAK/STAT signaling pathway. This can be compared to monocytes from HDs immunized with the yellow fever vaccine (YFV), which express more normal cell-specific and virus-induced signatures. A common IFN signature is a pure virus-induced signature detected in healthy donors immunized with the influenza vaccine and has a composition of mainly type I IFNs, which are the major antiviral cytokines. In contrast, type I IFNs are also dominant in SLE pathogenesis and govern the immune responses of $CD4^{+}$ T cells and monocytes, but at the same time, these responses are influenced by additional immunoregulatory events. Such events might be responsible for the difference observed between "common" and "autoimmune-specific" IFN signatures, reflecting the sustained IFN response in SLE patients. Moreover, the IFN signature observed in SLE patients leads circulating monocytes to differentiate into

potent antigen-presenting dendritic cells (DCs) with an increased capacity to orchestrate T and B cell responses [129]. In a study by Gkritzimanaki et al. [130], mitochondrial DNA (mtDNA) was accumulated in the cytosol of $CD14^+$ monocytes due to a deregulation of the mitochondrial metabolism caused by IFN-α and the dysfunction of autophagic digestion and was sensed via STING to favor the differentiation to autoinflammatory DCs and enhance the autoimmune response. Specifically, autoinflammatory DCs activate T cells and then contribute to the expansion and survival of autoantibody-producing cells by T–B cell aberrant communication [131]. STING and one of its ligands, mtDNA, cause an immune stimulatory output through NF-κB and/or TBK1/IRF3 similar to an antiviral response and have a critical involvement in disease pathogenesis. Moreover, the overexpression of *TLR7* due to the duplication of the Yαα (Y-chromosome-linked autoimmune acceleration) locus leads to the exacerbation of autoimmunity in murine lupus [132]. In addition, a recent study by Brown et al. [133] showed that a *TLR7* gain of function gene variant may cause human SLE. In addition, another recent study by Murakami et al. [134] demonstrated that TLR7 drives autoantibody production and lupus-associated monocytosis in NZBWF1 mice. The antiTLR7 mAb, but not antiTLR9, ameliorated nephritis in lupus-prone mice by inhibiting IgG deposition in glomeruli and autoantibody production in B cells and monocytes. They also revealed that $Ly6C^{low}$ patrolling monocytes were enhanced in the circulation, spleen, and glomeruli of NZBWF1 mice and displayed an overexpression of genes linked with lupus pathogenesis, such as *TLR7*, *IL-10*, *CD115*, *CD31*, and *TNFSF15*. This evidence suggests the importance of TLR7 in the progression of the disease as the hyperactivation of TLR7 may cause the uncontrolled sensing of several triggering components and an aberrant inflammatory response.

In addition, germline mutations in the human *SAMHD1* gene represent the main cause of the progression of the autoinflammatory Aicardi–Goutières Syndrome (AGS). SAMHD1 protein is known to restrict HIV-1 infection in cells of the myeloid lineage, such as monocytes/macrophages and DCs, by inhibiting the synthesis of viral DNA. AGS mimics congenital viral infection as defective nucleases in AGS are involved in the deficient removal of endogenous nucleic acids resulting in the accumulation of ssDNA and the chronic activation of the innate immune response and the DNA damage response network [135–137]. The phenotypic overlap of AGS with congenital infection and some traits of SLE pathogenesis highlights the IFN-α-mediated immune response upon viral and host nucleic acids triggering the PRR signaling cascades [138]. A recent study [139] revealed that SAMHD1 KO human monocytic THP-1 cells displayed nucleic acid deposition in the cytosol and spontaneous expressions of type I IFNs and *ISGs*, replicating the phenotype observed in patients with AGS. The inhibition of the TBK1–IRF3 pathway, through BX795, which is an inhibitor of the catalytic activity of TBK1/IKKε thus attenuating the phosphorylation of IRF3 and blocking its activation, overruled the secretion of type I IFNs observed in the SAMHD1 KO cells. Therefore, in AGS patients, monocytes exhibit a failure to process nucleic acids leading to the activation of autoimmune responses.

Rheumatoid arthritis (RA) is an autoimmune disease in which many cells from the innate and adaptive immune branch take part in the development of inflammation in synovial joints. Focusing on monocytes, these cells display a significant role in the progression of synovial inflammation since they are recruited at sites of infection by interacting with chemotactic ligands that are present in fibroblasts, such as synoviocytes (FLS), and other autoimmune cells, and sustain the perpetuation of autoimmunity [140]. The interplay between genetic and environmental factors as well as a defective immune response is of great importance in the development and progression of RA pathogenesis [141]. Furthermore, infection by viral particles, such as EBV, has been linked with multiple malfunctions in RA, increasing the prevalence of flares of disease activity [72]. It is well established that human B cells are the main targets of EBV, although several reports indicate that EBV can also infect other cells including monocytes [79,142,143]. In a study by Lacerte et al. [144], active RA patients demonstrated an overexpression of TLR2 and TLR9 in blood and synovial monocytic subsets and an increased secretion of a wide range of proinflammatory cytokines upon

induction with synthetic and viral ligands for TLR2 and TLR9. They also demonstrated that the EBV genome was present in monocytes and neutrophils, strengthening its role in the exaggeration of the disease [144,145]. Another study revealed that the expression of TLR2 in $CD16^+$ blood monocytes contributed to the production of TNF-α in RA patients [146]. The above-mentioned findings suggest that both classical and intermediate monocytes are key players in the development of inflammation in the tissues of active RA patients through the PRR-mediated activation of inflammatory cascades. In addition, classical monocytes produce costimulatory molecules and chemoattracting factors and regulate the progression of inflammation. Viral components, such as EBV, virions can induce the activation of TLR2 and TLR9 in the synovial compartment and sustain the inflammatory response, causing the exacerbation of the disease in susceptible RA patients. Moreover, the removal of pathogenic components through phagocytosis could also trigger TLR activation. Neutrophils and macrophages are the main phagocytes of the immune system as they engulf dead cells and then elicit an inflammatory response. Activation of the innate immune response may also occur, for instance, when the genetic material of dead cells is not effectively degraded, leading to B cell activation through B cell receptor (BCR) and TLR stimulation [147]. Complement deficiencies is another way of promoting autoimmune features in SLE through insufficient clearance [148]. This insufficient clearance may lead to the break of self-tolerance and then to autoimmunity [149].

Another study by Farina et al. [150] revealed that newly lytic EBV mRNA enhanced TLR8 expression in infected SSc and HD monocytes. MyD88 and IRF7 expression was also induced in infected EBV monocytes from SSc patients and HDs. This increase was associated with a robust induction of *IFN-regulated genes* and chemokines, such as CXCL9, OAS3, Siglec1, CCL2, IL-6, and TNFα, in SSc- and EBV-infected monocytes, which are markers associated with the activation state of monocytes. Studies with THP-1 cells indicated that EBV was influencing the immune system in a TLR8-dependent manner. Moreover, viral mRNA and proteins were detected in freshly isolated SSc monocytes where a microarray analysis demonstrated an increased IFN proinflammatory response and an altered level of TLR8 expression in EBV-infected SSc monocytes compared to HD monocytes. Overall, through the EBV paradigm, this study highlights that TLR8 possesses a central role in the activation of SSc monocytes by mediating a robust increase in the IFN signature. In addition, the activation of monocytes in SSc through TLR8 might be attributed to an EBV infection, thus supporting the notion that viral infection promotes autoimmunity through PRR signaling cascades, which affects the IFN innate immune responses.

Primary Sjögren's Syndrome is also associated with the altered immune response of monocytes. Additional research by Lopes et al. [151] demonstrates that the transcriptome of pSS monocytes is enriched for gene expression profiles associated with intermediate and nonclassical monocytes. Monocytes from pSS patients exhibit an activating profile with dysregulation in gene expression for translation, IFN signaling, and TLR signaling pathways, such as TLR4, TLR5, and TLR7/8. Serum from pSS patients primed monocytes to an increased secretion of TNF-α upon activation with TLR ligands compared to HD monocytes, and that may promote inflammation and the activation of other immune cells and contribute to pSS immunopathology. As it has been previously shown [152], monocytes from pSS patients exhibit an impaired phagocytic capacity of apoptotic cells and fail to promote an immunosuppressant cytokine profile to resolve inflammation and tissue injury. The interferon-α/β receptor (IFNAR) is a cell surface receptor that binds type I IFNs leading to the activation of the JAK–STAT signaling and the MAPK, PI3K, and Akt signaling cascades and therefore favoring the production of *ISGs*. IFNAR blockade partially abrogated the alterations observed, suggesting that the transcriptome profile of pSS monocytes can be characterized as IFN-dependent and independent (Table 1). However, it remains to be established whether monocytes in pSS pathology are functional in order to mediate antiviral immune responses. In this direction, characterizing the mechanisms of actions mediating the sustained activation of monocytes in pSS seems to be of great importance.

Collectively, these studies highlight the activating profile of monocytes with aberrant PRR response in various autoimmune diseases. This abnormal PRR response is often triggered by viral components culminating in the initiation or exacerbation of autoimmunity. The exact causes for the observed PRR manifestations and the differential roles of PRR signaling in autoimmunity but also how the antiviral response is affected remain to be defined in more detail.

2.1.3. Linking Macrophage Antiviral Response with Autoimmunity

Macrophages are tissue-resident or infiltrated immune cells with critical immunoregulatory, antimicrobial, and tissue-repairing roles to decrease immune reactions and promote tissue regeneration. They either originate from yolk sac (YS) progenitors during embryonic development and are maintained in postnatal life in certain adult tissues, or they derive from bone marrow (BM) hematopoietic stem cell (HSC) progenitors and circulating monocytes [125,153–155]. M1-like (classically activated) and M2-like (alternatively activated or wound-healing) macrophages are the two major subsets of activated macrophages with distinct cellular and molecular functions. Both subsets are involved in inflammatory responses with the difference that M1 macrophages have an important role in pro-inflammatory response, whereas M2 macrophages are mostly involved in tissue repair and anti-inflammatory responses [156,157]. Recent data have demonstrated that the activation of macrophages cannot be fully described using the M1/M2 paradigm. Macrophage phenotype alternates in response to various stimuli. For example, an increased number of macrophages in the circulation expresses both M1 surface markers, such as CD80, CD86, and TLR4 and M2 molecules, such as CD204 and CD163. In addition, macrophages express various TLRs, such as TLR2, TLR3, TLR4, and TLR7/8, and have many functions in restoring cell homeostasis. Some of their roles include the recognition and elimination of invading pathogens through phagocytosis and the subsequent presentation of antigens to T cells in order to orchestrate an inflammatory response [158]. There is also growing evidence supporting a causal link between the presence or activation of macrophages and the development of autoimmune diseases [159]. Their contribution to autoimmunity and inflammation comes through their ability to present autoantigens and their potent effector mechanisms, unleashed during innate and adaptive immune responses [159]. However, the emerging literature highlights a causal link between antiviral response mechanisms in macrophages and autoimmune phenomena [159–162].

IRF3-induced type I interferon production has an important role in antiviral responses and SLE. Serine/threonine kinase AKT2 regulates the type I IFN production by phosphorylating IRF3 at Thr207 to attenuate IRF3 nuclear translocation, resulting in diminished type I IFN production. To this end, a recent study by Zheng et al. [163] demonstrated that in viral-infected macrophages or monocytes and samples from SLE patients and mouse models, AKT2 expression is decreased, and *Akt2* deficiency promotes IFNβ1 and the production of *ISGs* to enhance an antiviral defense while heightening SLE in mouse models. These findings indicate that cells in autoimmune diseases, such as lupus, may be already prone to PRR defects, and the infection of viral pathogens further exacerbates the pre-existing deregulated PRR signaling [81,82,164–167].

Macrophages also play an important role in the progression of rheumatoid arthritis (RA). A study by Quero et al. [168] revealed that differentiated M2 macrophages from monocytes of HD or RA patients depicted an impeded anti-inflammatory profile due to the production of proinflammatory cytokines, such as IL-6 and IL-8, upon TLR2 stimulation. The critical role of TLR signaling in the pathogenesis of RA has been already stated by various studies in murine arthritis models. Abdollahi et al. [169] demonstrated that the development of streptococcal cell wall (SCW)-induced arthritis in mice was dependent on TLR2 during the acute phase, and this effect was changed to TLR4 dependency during the chronic joint inflammation phase. They have also previously reported that the inhibition of TLR4 by a TLR4 antagonist in a collagen-induced RA mouse model (CIA) repressed the clinical manifestations and the severity of arthritis [170]. Moreover, TLR7 displays

high levels in synovial tissue (ST) lining and sublining macrophages ($CD68^+$ macrophages identified in synovial biopsies) from RA patients. In a study by Kim et al. [171], it was revealed that miR-let-7b is a TLR7 endogenous ligand and is found in RA synovial fluid macrophages. The activation of TLR7 by miR-let-7b favors the differentiation of anti-inflammatory into inflammatory M1 macrophages and promotes the progression of arthritis. It was also shown [172] that ligands present in the inflamed joint of RA patients, stimulate TLR3 and TLR7 leading to a proinflammatory cytokine production in an IRF5-dependent manner. Therefore, since TLR signaling is enhanced in the macrophages of RA patients in a similar manner to an antiviral response, it would be intriguing to extend the investigation beyond the systemic autoimmune phenotype and delineate how nucleic acids and viral infection mediated by these TLRs would be affected.

Another recent study by Witas et al. [173] demonstrated that when C57BL/6 (B6) and Sjögren's syndrome-susceptible (SS^S) bone marrow-derived macrophages (BMDMs) were incubated with apoptotic cells (ACs), an inflammatory profile was induced in SS^S BMDMs. This profile was characterized by the overexpression of genes involved in the IFN signaling pathway, costimulatory molecules, and myeloid activation genes as well as inflammatory cytokines, such as IL-6, IL-12b, IL-1β, and IL-10. Witas et al. also demonstrated an increased *TLR7* and *TLR9* expression in PBMCs of pSS patients and increased secretion of IL-1β and TNF. By inhibiting TLR7 and TLR9, they showed a decreased inflammatory response of SS^S BMDMs to ACs. A separate study by Wang et al. [97] also indicated that TLR7 expression is enhanced in SS patient salivary gland tissue leading to the secretion of inflammatory cytokines and chemokines, such as TNF, CXCL12, and CXCR5. This group also reported that TLR8 KO mice developed SS pathology that was driven by enhanced TLR7 signaling. After the inhibition of *TLR7* and *TLR9*, a diminished AC-induced secretion of inflammatory genes in SS^S BMDMs was observed. This underlines that the inflammatory response observed upon AC activation in SS^S BMDMs is mediated by the stimulation of *TLR7* and *TLR9*. In addition, monocytes from SS patients that are IFN positive exhibit a high expression of *TLR7* and downstream effector molecules *MyD88*, *RSAD2*, *IRF7*, *RIG-I*, and *MDA5* [174]. To conclude, the AC stimulation of TLR9 resembles the inflammatory milieu in SS autoimmune disease as in many other autoimmune diseases, and the enhanced inflammatory cytokine and costimulatory response in SS^S BMDMs may contribute to the initiation of an autoimmune environment. In general, macrophages, as well as DCs, are efferocytic cells that can elicit a rapid and efficient clearance of apoptotic cells and debris in order to maintain cell homeostasis and sustain self-tolerance. Through efferocytosis, macrophages ensure the well-organized elimination of apoptotic cells without antigen presentation and inflammation. When efferocytosis is disturbed, apoptotic cells can rupture, secreting cellular components and inducing a hyperactivation of the innate and adaptive immunity resulting in autoimmunity. These findings indicate an expanded role of macrophages in SS pathology and autoimmunity, with apoptotic cells stimulating an inflammatory response in a similar way to an antiviral response through different TLRs (Table 1).

To summarize, macrophages are key modulators in the host defense mechanisms, such as the PRR signaling pathways, that are often exposed in an autoimmune context. Since these cells are essential components of the innate immune response, it is of interest to determine whether pathogenic components might trigger the autoimmune phenotype observed through PRR molecules and the impact in the antiviral response mechanisms.

2.1.4. Linking Neutrophil Antiviral Response with Autoimmunity

Neutrophils are polymorphonuclear (PMN) leukocytes of the innate immune system that play a significant role in the defense against invading pathogens. They patrol the organism for signs of tissue damage or infection and participate in mediating inflammation through phagocytosis and intracellular degradation, release of granules, and NET formation after the detection of pathogenic invaders [175,176]. NETs are extracellular fibers that are composed of nuclear chromatin associated with proteins released upon neutrophil lysis.

Released NETs display antimicrobial functions as they are able to capture and kill pathogens. Neutrophils may also be a source of the DNA, through NET formation, that triggers PRRs and leads to hyper-responses [177]. In lupus, DNA-containing ICs released upon NETosis are able to activate TLR9 in pDCs and induce the production of IFN-α [178]. Moreover, NET formation can trigger complement activation leading to the exacerbation of SLE pathogenesis [179]. NETs also activate neutrophils as reported by recent data through the TLR8 and TLR9 signaling cascades, although further studies need to elucidate the exact receptors and molecules involved [177,180,181]. Neutrophils are also important producers of cytokines and chemokines in order to boost the inflammatory response and recruit other immune cells at the sites of infection during viral invasion and autoimmunity [182,183].

During viral infection, neutrophils engulf virions and apoptotic cells through phagocytosis in order to eliminate viral replication and favor the clearance of the virus. NET formation can also promote viral elimination by capturing the viral particles and inactivating the virus [184,185]. Within lupus serum, ICs induce NETs and the production of type I IFNs through the activation of neutrophil Fcg receptors and TLR7 receptors [186,187]. Lood et al. [188] revealed that the TLR7/8-mediated shedding of Fc gamma receptor II A (FcgRIIA), which is the most widely expressed FcgR of the human leukocytes, shifts neutrophil function from the phagocytosis of nucleic acid-containing ICs to NETosis, a programmed form of necrosis, thus favoring their inflammatory potential while also impairing the phagocytic capacity of other immune cells, such as monocytes and DCs. They also reported that in SLE patients, FcgRIIA shedding in monocytes and neutrophils is present and correlated with the activation of neutrophils. Therefore, neutrophils seem to play an important role in regulating inflammation and autoimmunity through TLR7/8 activation and in influencing other immune cell effector functions. However, another study revealed that SLE-derived ICs activate neutrophils to release ROS and chemokines in a FcgRIIA-dependent and TLR7- and TLR9-independent manner contributing to local tissue inflammation and injury [189]. To this end, in SLE, a switch in neutrophil activity with a diminished phagocytic capacity but an increased NET formation is observed. Therefore, it remains unexplored how efficiently neutrophils will facilitate antiviral responses in an autoimmune setting (Table 1).

Taken together, these studies support the complex role of neutrophils in mediating immune responses during viral infections and autoimmune diseases. However, a direct causative link of antiviral response mechanisms of neutrophils with flares in autoimmunity remains to be established.

2.1.5. Linking Natural Killer (NK) Cell Antiviral Response with Autoimmunity

Innate lymphoid cells (ILCs) are a recently discovered group of innate immune cells that originate from common lymphoid progenitors (CLPs). There are five subsets of ILCs based on the differences observed in development, phenotype, and function: NK cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue-inducer (LTi) cells [190]. Natural killer (NK) cells, which belong to the family of ILCs, represent 5–20% of all human circulating lymphocyte subsets. There are two subsets of human NK cells: the $CD3^{-}CD56^{bright}CD16^{-}$ and the $CD3^{-}CD56^{dim}CD16^{+}$ subsets. $CD56^{dim}$ display cytotoxic functions, whereas $CD56^{bright}$ are important players in cytokine secretion [191]. Altered functional and regulatory profiles of NK cells could influence the outcome of several autoimmune diseases [192,193]. Upon TLR activation, NK cells produce cytokines, such as IL6, TNF-α, and (MIP)-1α, and crosstalk with other immune cells, thus having an important role in the development of inflammation and the severity of the disease. Moreover, an important role of NK cells in antiviral innate immunity has been demonstrated [194]. Upon viral infection, type I IFNs have the ability to directly regulate the activation of NK cells by promoting their proliferation and cytotoxic properties for efficient clearance of the virus [195,196].

A study by Cossu et al. [197] reported that an increase in activated $CD56^{bright}$ NK cells with SSc progression from early to definite SSc was shown upon TLR1/2 stimulation. This increase was further combined with an enhanced secretion of IL6, TNF-α, and

MIP-1α/CCL3 underlying the role of NK cells in SSc onset. $CD56^+$ cells from patients at different stages of SSc respond in a different manner to TLR activation, highlighting the role of immunity in the developmental and prefibrotic SSc (Table 1). The interplay of NK cells with other immune cells, such as DCs, following TLR activation is an important homeostatic control that balances the efficiency of innate and adaptive immune responses with the risk to develop autoimmune events. Interestingly, in a study by Schuster et al. [198], it was revealed that a $TRAIL^+$ NK cell subset controls immune responses during chronic murine cytomegalovirus (MCMV) infection by eliminating the effector $CD4^+$ T cells thus reducing the antiviral response and hindering the clearance of the virus. In addition, they demonstrated that upon MCMV infection, mice that lack the $TRAIL^+$ NK cell subset develop an autoimmune disorder that has the clinical and histopathological characteristics of SS. It is apparent that in this setting, NK cells act in a protective manner and limit autoimmune responses. This well-coordinated balance between homeostasis and chronic viral infection underlines the mechanisms of action that may culminate in systemic autoimmune phenomena with a viral etiology.

Although NK cells seem to have a significant role in the fine-tuning of antiviral response mechanisms and adaptive immune responses that may lead to autoimmune features, further studies are needed to establish their functions in the pathophysiology of autoimmune diseases and the mechanisms of action.

Table 1. Key findings in studies addressing PRR manifestations in innate immune cells in autoimmunity.

Cell Type	Autoimmune Context	Affected Molecule	Type of Defect/Triggering	Reference
pDCs	pSS	TLR7	Activation	[95–97]
pDCs	SLE	TLR7	Activation	[114–116]
pDCs	SLE	TLR7, TLR9	Activation (ICs stimulation, EBV genome)	[118,119]
pDCs	SSc	TLR8	Activation	[122]
Monocytes	SLE	STING	Activation (mtDNA)	[130]
Monocytes	SLE	TLR7	Activation (inflamm [43])	
Monocytes	AGS	SAMHD1	SAMHD1 deficiency	[139]
Monocytes	RA	TLR2, TLR9	Activation (inflammation and EBV virions)	[144]
Monocytes	RA	TLR2	Activation (inflammation)	[146]
Monocytes	SSc	TLR8	Activation (viral EBV mRNA and proteins and inflammation)	[150]
Monocytes	SSc	MyD88, IRF7	Activation (viral EBV mRNA and proteins and inflammation)	[150]
Monocytes	pSS	TLR4, TLR5, TLR7/8	Dysregulation (inflammation)	[151]
Macrophages	SLE	AKT2	Decreased AKT2 expression (viral infection or inflammation)	[163]
Macrophages	RA	TLR2	Activation (inflammation)	[168]
Macrophages	RA	TLR2, TLR4	Activation (inflammation)	[169,170]
Macrophages	RA	TLR7	Activation (inflammation)	[171]
Macrophages	RA	TLR3, TLR7, IRF5	Activation (inflammation)	[172]
Macrophages	SS	TLR7, TLR9	Activation (inflammation)	[97,173]
Macrophages	SS	TLR7, MyD88, RSAD2, IRF7, RIG-I, MDA5	Activation (ACs and inflammation)	[174]
Neutrophils	SLE	TLR7	Activation (ICs and inflammation)	[186,187,197]
Neutrophils	SLE	TLR7/8	Activation (ICs and inflammation)	[186–188]
NK cells	SSc	TLR1/2	Activation (inflammation)	[188,197]

3. Therapeutic Manipulation of PRR Signaling in Autoimmunity

PRRs are essential elements in innate immunity and play a significant role in the host defense mechanism against viral microbes. The overactivation of PRRs or downstream molecules disrupts the homeostasis of the immune system resulting in excessive inflammatory cytokine secretion. The involvement of nucleic acid-sensing mechanisms in the immune response against infections and in autoimmune diseases makes these pathways

interesting targets for drug design. The exaggerated response of the immune cells with a hyperproduction of cytokines called a "cytokine storm" plays an important role in the manifestation and progression of autoimmunity [11]. In this context, targeting the PRR signaling cascades in autoimmunity could be accomplished within the following frames: (a) blocking the binding of TLR ligands or the dimerization of the receptors and (b) interfering and inhibiting the signal transduction downstream of the PRRs [5,199].

The development of therapeutic agents for inhibiting PRR signaling, such as small molecule inhibitors, antibodies, oligonucleotides, lipid-A analogs, microRNAs, new emerging nano-inhibitors, and drugs, may help to control the hyperinflammation observed in autoimmune diseases induced by various factors, such as the uncontrolled recognition of self-nucleic acids and autoantibodies. Antimalarial drugs, such as chloroquine (CQ), hydroxychloroquine sulfate (HQ), and quinacrine, serve as antagonists for TLR7, 8, and 9 and have been used for the treatment of autoimmune diseases, such as SLE and RA. More specifically, hydroxychloroquine is a widely used antimalarial to treat both RA and SLE by modulating neutrophil function. It has recently been reported that the inhibition of TLR9 can lead to the inhibition of NET formation [200]. Antimalarial drugs are also lysosomotropic agents that target the lysosomal compartment leading to the permeabilization of the lysosomal membrane and secretion of enzymes along with signaling for apoptosis. These lysosomotropic agents could affect the PRR signaling cascades and dampen the hyper-responses to self-nucleic acids. For instance, HCQ and CQ can inhibit the uptake of nucleic acids and therefore the activation of nucleic acid sensors, such as TLR3, TLR8, TLR9, and cyclic cGAS, leading to the inhibition of the PRR-mediated activation of downstream molecules and the subsequent secretion of type I IFNs and inflammatory cytokines [201]. Another TLR7/8 inhibitor, M5049, inhibits TLR7/8 activation in pDCs, neutrophils, and monocytes, thus reducing IFN and inflammatory cytokine production. In addition, it was reported that M5049 may also block adaptive inflammation by inhibiting autoreactive B cells and seems to be a promising drug candidate [202]. Moreover, there is no evidence of inhibitors targeting CLRs and RLRs for the treatment of autoimmune diseases in clinical trials [199]. However, ARL5B, which is an MDA5-binding protein, has been reported to block the interaction of MDA5 with dsRNA [203]. In addition, DNAJB1 binds to MDA5 and prevents multimer formation attenuating type I IFN production and innate immune response [204]. In addition, another good therapeutic target could be the UNC93B1, as it has recently been demonstrated to block the cGAS–STING signaling cascade by attenuating IRF3 nuclear translocation and decreasing STING stability by promoting its intracellular degradation via the autophagy pathway [205]. Various therapeutic agents are in clinical trials targeting TLRs in order to control inflammation and hyper-responses in autoimmune diseases. This observation highlights the importance of TLRs in the initiation and progression of autoimmunity and places their agonists as ideal candidates for drug discovery in order to suppress the inflammatory response. However, the main concern of the TLR-focused effective treatment strategy should be to maintain the generic function of TLRs as they are important receptors for indicating and responding to a viral infection and other cell abnormalities of various pathology. It is of great interest to explore what happens when we suppress the response of TLRs to treat several autoimmune diseases while a viral infection occurs, as a balancing act is pivotal in order to restore homeostasis. In addition, other PRRs, such as MDA5 and STING, also play a significant role in the response to viral infections as well as autoimmunity, but their therapeutic targeting seems to be more challenging than anticipated. Attenuating STING and other PRR activities, which are important regulators of the immune response to ameliorate inflammation in the context of autoimmunity, may give rise to new challenges in order to combat viral infection.

Several molecules have a significant role in the signal transduction downstream of PRRs, including MyD88, IRAK4, TRAF6, TAK1, TRIF, TBK1, and NF-κB, as mentioned previously. The development of inhibitors targeting these molecules is of great importance in order to treat autoimmune diseases. Three IRAK4 inhibitors have entered clinical trials with promising results for the treatment of RA [206,207]. Many studies use mouse models

in order to explore the treatment efficiency of the inhibitors. For example, polyphyllin I (PPI) is an NF-κB inhibitor, as it diminished the phosphorylation of the NF-κB subunit p65 and the subsequent p65 accumulation in the nucleus. It was revealed that PPI seem to ameliorate synovial inflammation by suppressing the NF-κB-induced inflammatory signaling observed in macrophages in an RA mouse model [208]. Taraxasterol (TAR) diminished IL-1β-induced synovial inflammation in human fibroblast-like synoviocytes RA (HFLS-RA) in vitro and the progression of the disease in a RA mouse model in vivo. TAR seems to be a considerable therapeutic compound for RA by suppressing the NF-κB and NLRP3 inflammasome-induced synovial inflammation [209]. In addition, the role of IRFs in the regulation of the immune response makes these transcriptional regulators important therapeutic candidates for drug discovery [210]. Recently, it was reported by Li et al. [211] that dysregulated IRF5 activity is a driver of SLE disease onset and severity. Preclinical treatment of NZBWF1 mice with an IRF5 inhibitor led to reduced antinuclear autoantibodies, dsDNA titers, and circulating plasma cells and attenuated SLE pathology to improve survival. In ex vivo human studies, the inhibitor blocked SLE serum-induced IRF5 activation and reversed basal IRF5 hyperactivation in SLE immune cells [212].

To conclude, chemical agents targeting several TLRs and downstream effector molecules and cytokines offer novel opportunities for the prevention of or intervention against virus-induced infectious diseases and autoimmunity. Targeting PRRs with drugs may also induce harmful immune activation or unwanted immunosuppression dampening antiviral responses. From this perspective, targeting selective innate immune cell types, such as monocytes, macrophages, DCs, or neutrophils, that play critical roles in host defense against viral compounds could result in improved therapeutic outcomes in the treatment of viral infection and autoimmunity.

4. Synthesis, Concluding Remarks, and Open Questions

In this review, we discussed the effects of an excess or a deficiency of PRR signaling in autoimmune diseases and its relation with viral infection, focusing on the cells of the innate immune arm, such as monocytes, macrophages, DCs, and neutrophils, and the cells that bridge the innate with adaptive immune responses, such as NK cells. In autoimmune diseases, aberrant cytokine production, tissue damage, and hyperinflammation inflammation along with the genetic predisposition and environmental factors influence the function of PRRs and the expression of downstream molecules. The aberrant innate immune response influences the adaptive immune cells leading to a disrupted antiviral response and increasing the risk for autoimmunity or disease flares when an autoimmune disease is already present. Further research is needed to expand our knowledge regarding the influence of abnormal PRR signaling in the pathogenesis of autoimmune diseases.

Over the last years, the emerging role of antiviral responses in innate immune cells during autoimmune manifestations has posed new challenges and questions about the host defense mechanisms. To provide insight into the PRR cascade in innate immune cell inflammatory response in autoimmunity, it is crucial to determine whether PRR alterations drive or exaggerate autoreactive phenotype (e.g., inflammatory cytokine production, interplay with adaptive immune cells to promote NET formation, and autoantibody production) and if these signals imprint on the inflammatory cascade. Moreover, it is of interest to determine whether the currently used therapeutic agents or drug candidates for the treatment of autoimmune disorders may deregulate the antiviral response in innate immune cells. Regarding the targeting of TLRs for the treatment of autoimmunity, it is essential to preserve their physiological function in the context of viral immunity while ameliorating their effects on autoimmune responses. In this context, it is of great importance to delineate whether the antiviral and/or inflammatory response pathways that are activated differ during systemic autoimmunity as compared to organ-specific autoimmunity. Modulating specific PRR activity in innate immune cells in patients suffering from such PRR-dependent autoimmune diseases, such as SLE, RA, SSc, and Sjogren's syndrome, may be of therapeutic value. To accomplish that, it is essential to focus on the discovery of inhibitors or

other molecules that could efficiently modulate the immune response and with reduced off-target effects. Addressing such questions may revolutionize therapeutic approaches for autoimmune diseases.

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Transcriptomic and proteomic profiling reveals distinct pathogenic features of peripheral non-classical monocytes in systemic lupus erythematosus

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Transcriptomic and proteomic profiling reveals distinct pathogenic features of peripheral non-classical monocytes in systemic lupus erythematosus

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ABSTRACT

Peripheral blood monocytes propagate inflammation in systemic lupus erythematosus (SLE). Three major populations of monocytes have been recognized namely classical (CM), intermediate (IM) and non-classical monocytes (NCM). Herein, we performed a comprehensive transcriptomic, proteomic and functional characterization of the three peripheral monocytic subsets from active SLE patients and healthy individuals. Our data demonstrate extensive molecular disruptions in circulating SLE NCM, characterized by enhanced inflammatory features such as deregulated DNA repair, cell cycle and heightened IFN signaling combined with differentiation and developmental cues. Enhanced DNA damage, elevated expression of p53, G_0 arrest of cell cycle and increased autophagy stress the differentiation potential of NCM in SLE. This immunogenic profile is associated with an activated macrophage phenotype of NCM exhibiting M1 characteristics in the circulation, fueling the inflammatory response. Together, these findings identify circulating SLE NCM as a pathogenic cell type in the disease that could represent an additional therapeutic target.

1. Introduction

Monocytes represent a myeloid lineage product and a major component of the innate immune system, and exert diverse immunological functions in orchestrating inflammation. Three major populations of monocytes are identified both in mice and humans; the classical monocytes ($CD14^+CD16^-$) that make up to ~85% of the total circulating monocyte pool, whereas the remaining ~15% consists of the intermediate monocytes ($CD14^+CD16^+$) and the non-classical monocytes ($CD14^{dim}CD16^+$) [1]. In monocyte differentiation, there is a linear trajectory starting with the classical monocytes (CM) that exit the bone marrow and give rise to intermediate monocytes (IM) and sequentially to non-classical monocytes (NCM) in peripheral blood circulation [2–4] with NCM representing the most mature monocytic subpopulation [5]. Circulating monocytes replenish tissue macrophages through M1/M2 polarization process and dendritic cells (DCs) both in steady-state and inflammation [6–8]. Monocytes possess migratory properties and contribute to the ongoing inflammation by infiltrating tissues. It is believed that the local environment, especially the cytokines milieu, has a pivotal role in influencing their differentiation process [9].

Systemic lupus erythematosus (SLE) is a chronic, multisystem autoimmune disease that manifests diverse clinical and molecular abnormalities attributed to the loss of self-tolerance to nucleic acids and endogenous antigens and sustained autoantibody production [10,11]. Excessive and irreversible tissue damage caused by autoantibodies or immune-complex deposition affects multiple organs leading to significant morbidity and increased mortality [12,13]. Recent findings underscore the pivotal role of innate immune cell subsets in the initiation

Abbreviations: CM, Classical Monocytes; IM, Intermediate Monocytes; NCM, Non-classical Monocytes; IFNα, Interferon α; IFNγ, Interferon γ; JAK/STAT, Janus kinase/Signal transducer and activator of transcription; IL2, interleukin 2; IL6, interleukin 6; TLR, Toll like receptor; IRF1, Interferon regulatory factor 1; STAT1, Signal transducer and activator of transcription 1.

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and propagation of the systemic autoimmune response and the development of end-organ damage in SLE [14]. Aberrations of monocytes underlie the SLE pathology and lead to perpetuation of inflammation and tissue injury [15–20]. Recent studies emphasize the involvement of terminally differentiated immune cell subsets on tissue damage and severity of the disease [21,22]. For instance, it has been reported that $CD16^+$ DCs arise from NCM in lupus skin contributing to the development of cutaneous lupus erythematosus (CLE) [23]. In addition, circulating $Ly6C^{low}$ non-classical monocytes undergo differentiation into M1 macrophages and are recruited to the joint, playing a pivotal role in coordinating the progression of autoimmune joint inflammation [24]. Furthermore, TLR-activated NCM infiltrate the glomerulus, representing important regulators in glomerular inflammation and kidney injury in SLE [25]. Despite the compelling evidence that NCM substantially contribute to disease progression in the target tissues, they remain the least explored monocytic cells.

Understanding the molecular pathways involved in NCM activation and function in the periphery of patients with active SLE, before migrating into the tissues can guide the development of targeted therapies to modulate their effects. To this end, various cell subset-specific molecules recently introduced in the therapeutic field, such as belimumab or anifrolumab affect monocytic function or their cross-talk with other immune cells. A better understanding of the core immune responses of NCM, could provide further insights for the design of more targeted therapeutic schemes.

In this study, we used transcriptomic and proteomic analyses to investigate disease-specific signatures of the three monocytic subsets in SLE. Herein, we report inflammatory features and extensive perturbation of NCM of patients with active SLE, including deregulated DNA repair, cell cycle, and metabolism combined with enhanced IFN signaling, differentiation and developmental cues. *Ex vivo* functional analysis suggest that enhanced DNA damage, elevated expression of p53, G_0 arrest of cell cycle and increased autophagy support the differentiation potential of SLE NCM. Immune profiling and cytokine production analysis indicate that NCM of patients with active SLE exhibit an activated M1 macrophage-like phenotype. Our findings highlight that NCM acquire a pro-inflammatory profile reminiscent of M1 macrophages in the periphery of patients, fueling the inflammatory response in SLE.

2. Material and methods

2.1. Experimental model and study participant details

2.1.1. Human subjects

Peripheral blood samples were collected from patients with SLE [$n = 37$, classified by the 1997 American College of Rheumatology criteria] and healthy individuals ($n = 42$). At the time of sampling, all patients had moderate to high disease activity (SLEDAI $\geq$8 and/or PGA $\geq$ 1.5) and, in the vast majority, had not received cytotoxic drugs 12 months before donation. All patients and healthy individuals were recruited through the Rheumatology and Clinical Immunology Unit, fourth Department of Internal Medicine, "Attikon" University Hospital and the Department of Rheumatology in Athens, Greece. Our study is in agreement with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all individuals before sample collection (Athens, Greece, protocol 10/22-6-2017). All patients omitted any treatment dose for at least 24 h before blood drawing. Clinical and demographic characteristics are summarized in table S1.

2.1.2. Animal studies

All procedures in mice were in accordance with institutional guidelines and were reviewed and approved by the Greek Federal Veterinary Office (1044/1319; Athens, Greece). New Zealand black (NZB) ♀ × New Zealand white (NZW) ♂ F1 mice (i.e., NZB/W-F1) spontaneously develop an autoimmune syndrome resembling human SLE. NZB/OlaHsd and NZW/OlaHsd mice were purchased from Envigo. NZB/W-F1 mice were considered diseased when exhibiting $\geq$100 ng/dl of urine protein (after 6 months of life) and prediseased at 10 weeks old. All animals were maintained in the BRFAA animal facility. All NZB/W-F1 mice used in the experiments were female. All animal procedures were in concert with the ARRIVE guidelines and were conducted following the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2.2. Human cell isolation from peripheral blood

Peripheral blood (12 ml) was obtained from patients with SLE and gender matched healthy individuals. Human peripheral blood mononuclear cells (PBMCs) were enriched by density gradient centrifugation of peripheral blood through a Lymphosep (Biowest, Cat. No. L0560–500) gradient. Shortly, blood was diluted 1:1 with phosphate-buffered saline (PBS) and layered over Lymphosep solution. Falcon tubes were centrifuged at 500*g* for 30 min with no brake at room temperature. PBMCs were collected, and cells were washed with PBS. Monocytes were isolated from PBMCs via flow cytometry. For the cell sorting of the three monocytic subsets, single-cell suspensions from fresh human PBMCs were stained with conjugated antibodies against: HLA-DR (1:100, 307,618, Biolegend), CD14 (1:100, 21,620,144, Biolegend), and CD16 (1:100, 302,012, Biolegend). Classical monocytes are defined as $HLA\text{-}DR^+CD14^+CD16^-$, intermediate monocytes as $HLA\text{-}DR^+CD14^+CD16^+$, and non-classical monocytes as $HLA\text{-}DR^+CD14^{dim}CD16^+$ cells.

2.3. Flow cytometry and cell sorting

For analysis and isolation of immune cell subsets, single-cell suspensions from fresh human PBMCs were stained with conjugated antibodies against: HLA-DR (1:100, 307,616, 307,608, Biolegend), CD14 (1:100, 21,620,144, 301,840, Biolegend), CD16 (1:100, 302,037, 302,048, Biolegend), CD36 (1:100, 336,232, Biolegend), CD163 (1:100, 333,626, Biolegend), CD123 (1:100, 306,017, Biolegend), CD11c (1:100, 301,608, Biolegend), CD80 (1:100, 305,227, Biolegend), CD64 (1:100, 305,042, Biolegend), and CD206 (1:100, 321,106, Biolegend). All cells were Zombie negative (1/1000; 423,110, BioLegend). For the intracellular staining including Foxp3, γH2AX (Ser^{139}) (1:50, 613,420, Biolegend), Ki67 (1:50, 350,504, Biolegend), and cleaved caspase-3 (Asp175) (1:50, 9669S, Cell Signaling Technology), cells were fixed and stained using the Foxp3 Staining Set (Cat. No. 00–5523-00, eBioscience) according to the manufacturer's instructions. For the respective mouse experiments: CD11b (1:200, 101,212, Biolegend), CD11c (1:200, 117,318, Biolegend), Ly6C (1:200, 108,430, Biolegend). Cells were sorted on a FACSAria III (BD Biosciences) using the BD FACSDiva v8.0.1 software (BD Biosciences). Analysis was performed with FlowJo software. Key resources table includes details on the antibodies (fluorochrome, clone, source, and identifier).

2.4. RNA-sequencing

2.4.1. Sample preparation

Peripheral blood mononuclear cells (PBMCs) of patients with SLE ($n = 7$) and HI ($n = 8$) were stained with the following conjugated antibodies: HLA-DR (1:100, 307,618, Biolegend), CD14 (1:100, 21,620,144, Biolegend), and CD16 (1:100, 302,012, Biolegend). The 3 monocyte subsets (classical, intermediate, non-classical) were sorted using fluorescence-activated cell sorting (FACS), according to their relative expression of HLA-DR, CD14 and CD16. Sorted subsets were stored in lysis buffer at -80 °C before RNA isolation. RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Cat. No. KIT0202) as per the manufacturer's protocol. The quality of the RNA samples was performed with the Agilent Bioanalyzer RNA nano kit. RNAseq experiments were

carried out in the Greek Genome Center (GGC) of the Biomedical Research Foundation of the Academy of Athens (BRFAA). RNAseq libraries were prepared with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, Quality Control was performed with the Agilent bioanalyzer DNA1000 kit, and Quantitation with the qubit HS spectrophotometric method. Approximately 25 Million 100 bp Single-End reads were generated for each sample in the Illumina Novaseq 6000 system.

2.4.2. Differential expression analysis

FastQC software was used to check the quality of sequencing data (version:0.11.9, RRID:SCR_014583) [26]. Sequences of adapters and low quality bases (Q < 30) of the 3′ end were removed using Cutadapt (v:1.18, RRID:SCR_011841) [27]. Reads were aligned to the human reference genome (v:hg38) using STAR (v:2.6.1b, RRID:SCR_004463) [28] and annotated using GENCODE (v:39, RRID:SCR_014966) [29]. Samtools (v:1.9, RRID:SCR_014966) was used to sort bam files [30]. Gene expression was quantified using HTSeq (v:0.11.0, RRID:SCR_005514) [31]. Differential expression (DE) analysis was performed using the edgeR [32] software (v:3.38.1, RRID:SCR_012802, qlmQLFtest function) in R (v:4.2.0, RRID:SCR_001905) [33]. Genes with fold change $|FC| \geq 1.5$ and P value ≤ 0.05 were considered as significantly differentially expressed genes (DEGs). Heatmaps, volcano plots and Venn diagrams were generated using the Complex Heatmap, ggplot2 and ggVennDiagram packages (v3.4.1, RRID:SCR_014601) [34].

2.4.3. Enrichment analysis

We performed pathway and gene ontology (GO) enrichment analysis using the g:Profiler web-server to explore the function of DEGs. Pathways were considered enriched when Benjamini-Hochberg corrected P value ≤ 0.05. Gene Set Enrichment Analysis (GSEA) was also performed against the Hallmark set of MSigDB (v:2022.1.Hs, RRID:SCR_016863) [35] using GSEA software (v:4.2.2, RRID:SCR_003199) [36] to reveal enriched signatures in our dataset. Genes were ranked in decreasing order of the product of -log10(P-value) and FC. GSEA pre-ranked analysis was performed using the default settings and setting 149 as the analysis seed. Terms were considered significantly enriched when False Discovery Rate (FDR) < 25%. The full lists of differentially expressed genes and enriched terms are provided in table S2.

2.5. Proteomics

2.5.1. Sample preparation

Peripheral blood mononuclear cells (PBMCs) of patients with SLE and HI ($n = 9$ individuals per group) were stained with the following conjugated antibodies: HLA-DR (1:100, 307,618, Biolegend), CD14 (1:100, 21,620,144, Biolegend), and CD16 (1:100, 302,012, Biolegend). The 3 monocyte subsets (classical, intermediate, non-classical) were sorted using fluorescence-activated cell sorting (FACS), according to their relative expression of HLA-DR, CD14 and CD16. The three different FACS sorted cell populations were lysed in a buffer consisting of 4% SDS and 0.1 M DTT in 0.1 M TEAB and incubated for 5 min in a 99 °C. Subsequently, the samples were subjected to sonication in a water bath for 15 min. The protein extracts were processed by tryptic digestion using the Sp3 protocol, including an alkylation step in 100 mM iodoacetamide (Acros Organics). 20 μg of beads (1:1 mixture of hydrophilic and hydrophobic SeraMag carboxylate-modified beads, GE Life Sciences) were added to each sample in 50% ethanol. Protein clean-up was performed on a magnetic rack. The beads were washed two times with 80% ethanol and once with 100% acetonitrile (Fisher Chemical). The captured-on beads proteins were digested overnight at 37 °C under vigorous shaking (1200 rpm, Eppendorf Thermomixer) with 0.5 μg Trypsin/LysC (MS grade, Promega) prepared in 25 mM Ammonium bicarbonate. Next day, the peptides were purified using a modified Sp3 clean up protocol and finally solubilized in the mobile phase A (0.1% Formic acid in water), sonicated and the peptide concentration was determined through absorbance at 280 nm measurement using a nanodrop instrument.

2.5.2. LC-MS/MS

Samples were analyzed on a liquid chromatography tandem mass spectrometry (LC-MS/MS) setup consisting of a Dionex Ultimate 3000 nanoRSLC coupled in line with a Thermo Q Exactive HF-X Orbitrap mass spectrometer. Peptidic samples were directly injected and separated on an 25 cm-long analytical C18 column (PepSep, 1.9 μm3 beads, 75 μm ID) using a one-hour long run, starting with a gradient of 7% Buffer B (0.1% Formic acid in 80% Acetonitrile) to 35% for 40 min and followed by an increase to 45% in 5 min and a second increase to 99% in 0.5 min and then kept constant for equilibration for 14.5 min. A full MS was acquired in profile mode using a Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer, operating in the scan range of 375–1400 m/z using 120 K resolving power with an AGC of 3 × 106 and maximum IT of 60 ms followed by data independent acquisition method using 8 Th windows (a total of 39 loop counts) each with 15 K resolving power with an AGC of 3 × 105 and max IT of 22 ms and normalized collision energy (NCE) of 26. Each sample was analyzed in two technical replicas.

2.5.3. Data analysis

Orbitrap raw data were analyzed in DIA-NN v:1.8.1 (Data-Independent Acquisition by Neural Networks) through searching against the Human_Reference Proteome (downloaded from Uniprot, 20,583 proteins entries, downloaded 8/11/2022) using the library free mode of the software, allowing up to two tryptic missed cleavages and a maximum of three variable modifications/peptide. A spectral library was created from the DIA runs and used to reanalyse them (double search mode). DIA-NN search was used with oxidation of methionine residues, N-terminal methionine excision and acetylation of the protein N-termini set as variable modifications and carbamidomethylation of cysteine residues as fixed modification. The match between runs feature was used for all analyses and the output (precursor) was filtered at 0.01 FDR and finally the protein inference was performed on the level of genes using only proteotypic peptides. The identified peptides (e.g. proteotypic peptides) used in the analysis described were only those being assigned to only one particular protein (e.g. unique protein). Peptides (e.g. razor peptides) being assigned to more than one protein (e.g. protein groups) were omitted from the analysis.

2.5.4. Enrichment analysis of proteomic data

The proteomics data were processed in Perseus v:1.6.15.0. Values were log (2) transformed, a threshold of 70% of valid values in at least one group was applied and the missing values were replaced from normal distribution. For statistical analysis, Student's t-test was performed and permutation-based FDR was calculated and thus, the differential expressed proteins were assessed for enrichment analysis. FDR < 0.05 was used to determine significance for enriched Gene Ontology biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. The full lists of differentially expressed proteins and enriched terms are provided in table S3.

2.5.5. Binding analysis for regulation of transcription (BART)

We conducted Binding Analysis for Regulation of Transcription (BART) to discover the transcription factors (TFs) responsible for binding to the regulatory regions of differentially expressed genes (DEGs) exclusively present in SLE NCM. These TFs play a pivotal role in governing the corresponding proteomic signatures. BART identifies a genome-wide cis-regulatory profile for the query genes using the MARGE method [37] and subsequently correlates the profile with a transcription factor profile derived from 6000 publicly available transcription factor ChIP-Seq datasets.

2.6. Cell cycle assessment

For the cell cycle assessment via flow cytometry, single-cell suspensions from fresh human PBMCs were first stained extracellularly with conjugated antibodies against: HLA-DR, CD14 and CD16 in 200 μl of 5% FBS/PBS buffer for 10 min at room temperature and then, following washing with PBS, cells were fixed and stained for Ki67 (1:50, 350,504, Biolegend) using the Foxp3 Staining Set according to the manufacturer's instructions. At the end, cells were resuspended in 200 μl 5% FBS/PBS buffer and stained with 7-AAD cellular DNA content marker (5 μl per sample, 420,404, Biolegend). Cells were analyzed using BD FACS Celesta using the BD FACSDiva v8.0.1 software. Linear scale was used for 7-AAD. Analysis was performed with FlowJo software. Key resources table includes details on the antibodies (fluorochrome, clone, source, and identifier).

2.7. Immunofluorescence and confocal microscopy

For the cell immunofluorescence experiments, sorted cells were seeded in coverslips pretreated with poly-L-lysine (Sigma-Aldrich), fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature, and washed twice with PBS. Cells were fixed with ice-cold methanol for 10 min at room temperature. Then, cells were permeabilized by using 0.1% saponin (Sigma-Aldrich), and 2% BSA (blocking buffer). Next, cell-seeded slides were incubated with primary antibodies in blocking buffer for 1 h at room temperature, followed by three washes with blocking buffer and then by secondary antibodies for 40 min at room temperature in the dark. For visualization of the nuclei, 4′,6-diamidino-2-phenylindole (DAPI) (1/100, 10,236,276,001, Sigma-Aldrich) was used. Last, cells were mounted with mowiol mounting media (Thermo Fisher Scientific, Cat. No. P36961) and visualized using a x63 oil lens in inverted or upright confocal imaging system Leica SP5. Puncta/cell was calculated using a macro developed in Fiji software. The primary antibodies in the immunofluorescence were against LC3 (1:20, 0231–100/LC3-5F10, nanoTools), LAMP-1 (1:400, sc-19,992, Santa Cruz), and p62 (1:400, PM045, MBL) and the secondary antibodies were Alexa Fluor 555 anti-mouse IgG (1:500, A-21425, Invitrogen), Alexa Fluor 488 anti-rat IgG (1:250, A-11006, Invitrogen), and Alexa Fluor 647 anti-rabbit IgG (1:200, A-21246, Invitrogen). For the analyses, 50 to 100 cells per human individual (with at least four different areas on the coverslip) were examined for each marker using confocal microscopy. Key resources table includes details on the antibodies (fluorochrome, clone, source, and identifier).

2.8. Cytokines profiling

Released cytokines were measured with the LEGENDplex Human Macrophage/Microglia Panel (13-plex) (BioLegend, Cat. No. 740503) following the collection of supernatants at day 1 from cultured $CD14^{dim}CD16^{+}$ cells. Data acquisition was performed on FACSAria III (BD Biosciences) and the BD FACSDiva v8.0.1 software (BD Biosciences). Analysis was conducted utilizing the LEGENDplex Data Analysis Software.

2.9. Phagocytosis assay

Time-lapse imaging of phagocytic events was performed after introducing particles to classical and non-classical sorted monocytes (10.000 cells/well) seeded in μ-slide 8 well ibiTreat chambers (Cat. No. 80826) in technical duplicates, which have a channel volume of 300 μl. Stock solutions of pH-rodo™ Red *E. coli* Bioparticles (Thermo Fisher Scientific, Cat. No. p35361) were prepared at 1 mg/ml in Dulbecco's PBS. The suspension of particles was sonicated to disperse the particles and was added to the cell culture microplate in a 1:10 dilution before starting time-lapse imaging via Aurox microscopy. The pH-rodo™ Red *E. coli* Bioparticles have absorbance and emission maxima of 560 and 585 nm, respectively. Time lapse live cell images were acquired every 5–15 min for 1 h, five frames of z-stacking with 2-μm optic slice by Aurox confocal microscopy. Signal maximum was achieved at 30 min. On average 10–50 monocytes were analyzed using the Image J software, and the data were expressed as % of monocytes that phagocytose *E. coli* BioParticles.

2.10. Statistical analysis

Statistical analysis was conducted taking into account the experimental setup using either paired or unpaired Student's *t*-test and one-way or two-way analysis of variance (ANOVA) in GraphPad Prism v8.0.1 software, as specified in the Figure legends. Data are presented as means ± SEM, and a significance level of $P < 0.05$ was considered as statistically significant. In the figure legends, all P values and n are reported. All statistical analyses were performed using GraphPad Prism software (v9.0.0). The investigators were not blinded to the sample identities and the compared samples were collected and analyzed under the same conditions. Each experiment was repeated at least three times. For LEGENDplex experiments, it was one run per assay.

3. Results

3.1. NCM display widespread gene expression changes in SLE compared to other monocytic subsets

First, we assessed the transcriptome profile of the three monocytic subsets isolated from the peripheral blood of patients with active SLE and age/sex-matched healthy individuals (HI) (Figure 1A; Supplementary Table S1). Subsets were defined by the surface markers CD14 and CD16 into CM, IM and NCM (Supplementary Fig. S1A). Principal component analysis (PCA) demonstrates that monocytes are not a homogeneous population but can be clustered in smaller, transcriptionally distinct subsets. Cluster segregation both in HI and SLE patients reveals distinct gene expression of NCM compared to the other two subsets (CM and IM) (Fig. 1B), pointing that these transcriptomic changes demarcate distinct responses of the three related monocytic cell subsets in health and SLE. We also performed PCA on each monocytic subset in HI and patients with SLE (Supplementary Fig. S1B). In tandem, CM and IM do not exhibit distinct transcriptomic profiles in HI vs SLE patients. In contrast, the NCM display more widespread gene expression changes in patients with SLE compared to HI. Both in health and disease, IM clearly represent a transition state between CM and NCM (Supplementary Fig. S1C). Thus, the transcriptomic distance is longer between NCM and any other monocytic subset, being more pronounced in SLE. Moreover, gene expression analysis identified 859 differentially expressed genes (DEGs) in CM, of which 515 were up- and 344 down-regulated in SLE compared to HI. In IM, 724 DEGs were identified, of which 470 were up- and 254 down-regulated in SLE compared to HI and in NCM, 1122 DEGs were identified, of which 489 were up- and 633 down-regulated in SLE compared to HI, suggesting an extensive transcriptomic rewiring of NCM in SLE compared to the other two monocytic counterparts (Fig. 1C). Of interest, 103 genes were found to be common in all three SLE monocytic subsets, representing the core monocytic SLE signature, consistent with deregulated pathways such as inflammatory response, cytokine signaling and IFNα/β response (Fig. 1D, E; Supplementary Table S2). In reference to SLE NCM, the largest group of DEGs (766 genes) was found to be exclusively differentially expressed in SLE implicating cell communication, regulation of metabolic processes and signal transduction (Fig. 1D, E; Supplementary Table S2). Together, these data suggest that NCM display widespread gene expression changes in SLE compared to other monocytic subsets rendering them molecularly distinct in terms of cellular communication and signal transduction.

3.2. SLE NCM display enriched type-I IFN signaling, inflammatory cytokine production, and altered metabolism

To further delineate the transcriptomic disparities of monocytes in SLE, we identified four clusters of genes that are uniquely or commonly deregulated in each monocytic subset in SLE using K-means clustering algorithm (Fig. 2A). Gene ontology (GO) analysis of CM clustering revealed several cascades to be deregulated in SLE CM such as IL-10 signaling pathway and inflammatory response (Fig. 2B; Supplementary Table S2). IM depicted altered TLR2 and TNF signaling in SLE (Fig. 2B; Supplementary Table S2). NCM revealed several cellular processes to be deregulated in SLE such as IL-1 processing, and IFN signaling (Fig. 2B; Supplementary Table S2). Next, in order to investigate the biological differences of each monocytic subset in SLE, Gene Set Enrichment Analysis (GSEA) was performed. Several key biological cascades were found to be positively enriched in SLE monocytes related to inflammatory response and the immune system, such as IFNγ response, TNFα via NF-kB and JAK-STAT3 signaling, and complement activation. Metabolic pathways were differentially deregulated in the transcriptomic signature of each monocytic subset both in steady state and disease. For instance, oxidative phosphorylation was negatively enriched in SLE CM while hypoxia was negatively enriched in SLE IM and SLE NCM, indicative of differential metabolic needs of each monocytic subset (Fig. 2C). Finally, heatmaps of select genes of interest involved in deregulated mTORC1, p53, and hypoxia pathways reveal that SLE NCM pathogenicity is attributed to these processes in the inflammatory environment of the disease (Figs. 2D, E). Both hypoxia and mTORC1 signaling are involved in regulating cellular metabolism, albeit through different mechanisms [39,40]. Collectively, these findings suggest that NCM in patients with SLE possess a distinct transcriptomic signature characterized by type-I IFN signaling, diverse cytokine production and alterations in cellular metabolic processes.

3.3. Proteomic profiling unravels a phenotype transition of NCM in SLE

To decipher how the transcriptomic characteristics of monocytes can drive their functional features in SLE, we next assessed the proteome of the three sorted monocytic subsets from the peripheral blood of patients with active SLE and age/sex-matched HI (Fig. 3A; Supplementary Table S1). Proteome analysis identified 6154 unique proteins for CM, 5274 unique proteins for IM and 5389 unique proteins for NCM. PCA exhibited a fine subset-specific separation of monocytes together with a distinct distribution between healthy and SLE (Fig. 3B; Supplementary Figs. S2A and S2B). Cluster segregation reveals that monocytes can be distinguished based on their proteomic profile in the three subsets with the IM being more similar to NCM irrespective of health or disease, with a distinct protein expression profile of all monocytic subsets (Fig. 3B; Supplementary Figs. S2A and S2B). In addition, protein expression analysis identified 830 differentially expressed proteins (DEPs) in CM, of which 279 were up- and 551 down-regulated in SLE compared to HI. In IM, 146 DEPs were identified, of which 80 were up- and 66 were down-regulated and in NCM, 1714 DEPs were identified, of which 250 were up- and 1464 were down-regulated in SLE as compared to HI. Notably, NCM have a significant number of DEPs in SLE compared to the other two monocytic counterparts (Fig. 3C). Furthermore, NCM possess the largest group of proteins (1341 proteins) to be exclusively differentially expressed in SLE NCM. GO analysis in the unique up- and down-regulated proteins in NCM exhibited decreased metabolism and cell cycle and increased inflammatory responses, complement activation and cell differentiation (Fig. 3D; Supplementary Table S3). To identify transcription factors that bind at the regulatory regions of the DEGs found only in SLE NCM and govern these proteomic signatures, we performed Binding Analysis for Regulation of Transcription (BART) [41]. These TFs were further filtered retaining the ones linked with developmental and differentiation processes. Among the top enriched factors in SLE NCM were STAT1, IRF1 and E2F1 (Fig. 3E). Of note, STAT1 and IRF1 are associated with polarization of monocytes to classically activated macrophages [42,43]. Taken together, the proteomic analysis denotes that SLE NCM undergo an altered differentiation process. The disrupted expression patterns of those TFs known to regulate the polarization of monocytes into M1 macrophages likely reflects the pro-inflammatory profile that NCM have acquired in SLE.

3.4. Inflammatory features, DNA repair, cell cycle and differentiation underscore the pathogenic signature of SLE NCM

To further investigate the proteomic disparities of monocytes in SLE, we identified two main clusters of proteins that were uniquely or commonly deregulated in each monocytic subset in the disease (Fig. 4A). GO analysis of CM clustering revealed several cascades to be deregulated in SLE CM such as cellular metabolic processes, and type I IFN production and signaling (Fig. 4B; Supplementary Table S3). IM depicted altered response to virus and to type I IFNs (Fig. 4B; Supplementary Table S3). NCM revealed several cellular processes to be deregulated in SLE such as cell cycle, developmental processes, IFN and cytokine signaling and metabolism (Fig. 4B; Supplementary Table S3). To further examine the biological differences of each monocytic subset in SLE, GSEA was performed revealing key features of SLE pathogenicity to be significantly deregulated in all three monocytic subsets such as the inflammatory response, and IFNα/γ response (Fig. 4C). In this setting, distinctive features of SLE NCM pathogenicity were the positively enriched complement activation and IFN signaling and negatively enriched DNA repair, fatty acid metabolism (FAM) and oxidative phosphorylation (OXPHOS) (Fig. 4D). Though the PI3K/Akt/mTOR signaling did not reach statistical significance, many proteins involved in this pathway were found to be significantly decreased in SLE NCM such as RPTOR, RPS6KA1, RPS6KA3, STAT2, PTEN, and TRAF2. Of note, heatmaps of select genes of interest involved in complement activation, DNA repair and OXPHOS highlight that SLE NCM are biased towards these processes in the inflammatory environment of the disease (Fig. 4E). In summary, the proteomic signature of SLE NCM stands apart from the other monocytic counterparts, underscoring the importance of cell cycle, differentiation process, and DNA repair as pivotal characteristics that drive their pathogenic potential.

3.5. Immune profiling of peripheral blood reveals reduction of NCM during active disease and initiation of a differentiation program

Since DNA repair is perturbed in SLE NCM, we next sought to investigate the DNA Damage Response (DDR) in SLE monocytic subsets by flow cytometry using γH2AX, as a main indicator of DDR activation [44,45]. We observed increased levels of γH2AX only in NCM of patients with SLE as compared to HI, supporting exacerbated DDR in SLE NCM (Fig. 5A; Supplementary Fig. S4A). The activation of the p53 pathway in the transcriptomic signature of SLE NCM was validated in SLE NCM by confocal microscopy (Fig. 5B). Subsequently, we investigated whether the observed reduction in DNA repair, and heightened p53 pathway and DNA damage in SLE NCM, could impact cellular growth and cell cycle [46]. We found decreased frequency specifically of NCM in patients with SLE compared to HI (Figs. 5C, D). This profile was further validated in murine (NZB/W-F1) SLE peripheral monocytic subsets (Supplementary Figs. S3A and S3B). Since DDR can potentially trigger apoptosis and/or proliferation, coupled with the findings of reduced SLE NCM in the peripheral blood of patients with active disease, we investigated cell cycle dynamics within each monocytic subset in the context of the disease. Neither proliferation (Ki67) nor apoptosis (cleaved caspase-3) differed in SLE NCM when compared to HI as assessed by flow cytometry assays (Fig. 5E; Supplementary Fig. S4B). However, we found that SLE NCM accumulate in the G_0 phase of the cell cycle, implying differentiation potential (Fig. 5F; Supplementary Fig. S4C) [47–49]. Collectively, these data suggest that in SLE NCM, the increased DNA damage, p53 activation and the halting of the cell cycle support an inflammatory phenotype of NCM and a differentiation potential.

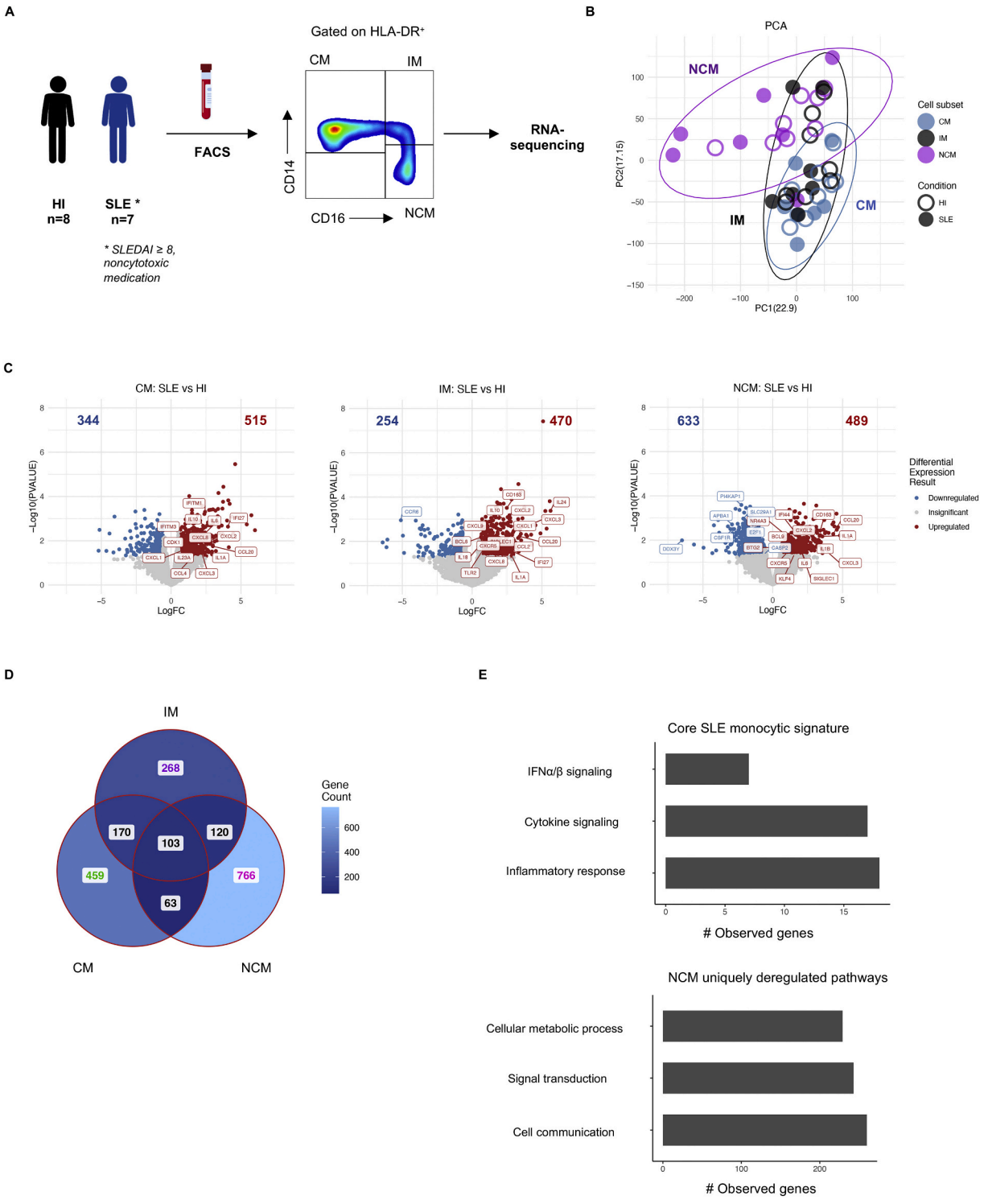

Fig. 1. NCM exhibit extensive perturbation in gene expression in SLE.
A. Graphical overview of our experimental setup. The 3 monocytic subsets were isolated using flow cytometry from the peripheral blood of patients with SLE ($n = 7$) and HI ($n = 8$) for transcriptomic analysis. **B.** PCA of gene expression profiles of the three monocytic subsets from SLE patients and HI. Point color represents cell subset (blue for CM, black for IM and purple for NCM) and point shape represents condition (hollow circle for healthy individuals, and filled circle for patients with SLE). **C.** Volcano plots of the three differential expression analyses (DEA) performed comparing (left to right), SLE CM vs HI CM, SLE IM vs HI IM, and SLE NCM vs HI NCM. Upregulated genes are denoted by red and downregulated genes are denoted by blue. Genes not reaching our significance threshold (P value <0.05) are shown in gray. **D.** Venn diagram comparing the DEGs of "SLE CM vs HI CM", "SLE IM vs HI IM", and "SLE NCM vs HI NCM". Color gradient correlates with gene count in each compartment. **E.** Enrichment analysis of Gene Ontology (GO) classification for core SLE monocytic genes (up) and expressed genes found only in SLE NCM (down).
See also Fig. S1 and Tables S1, and S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

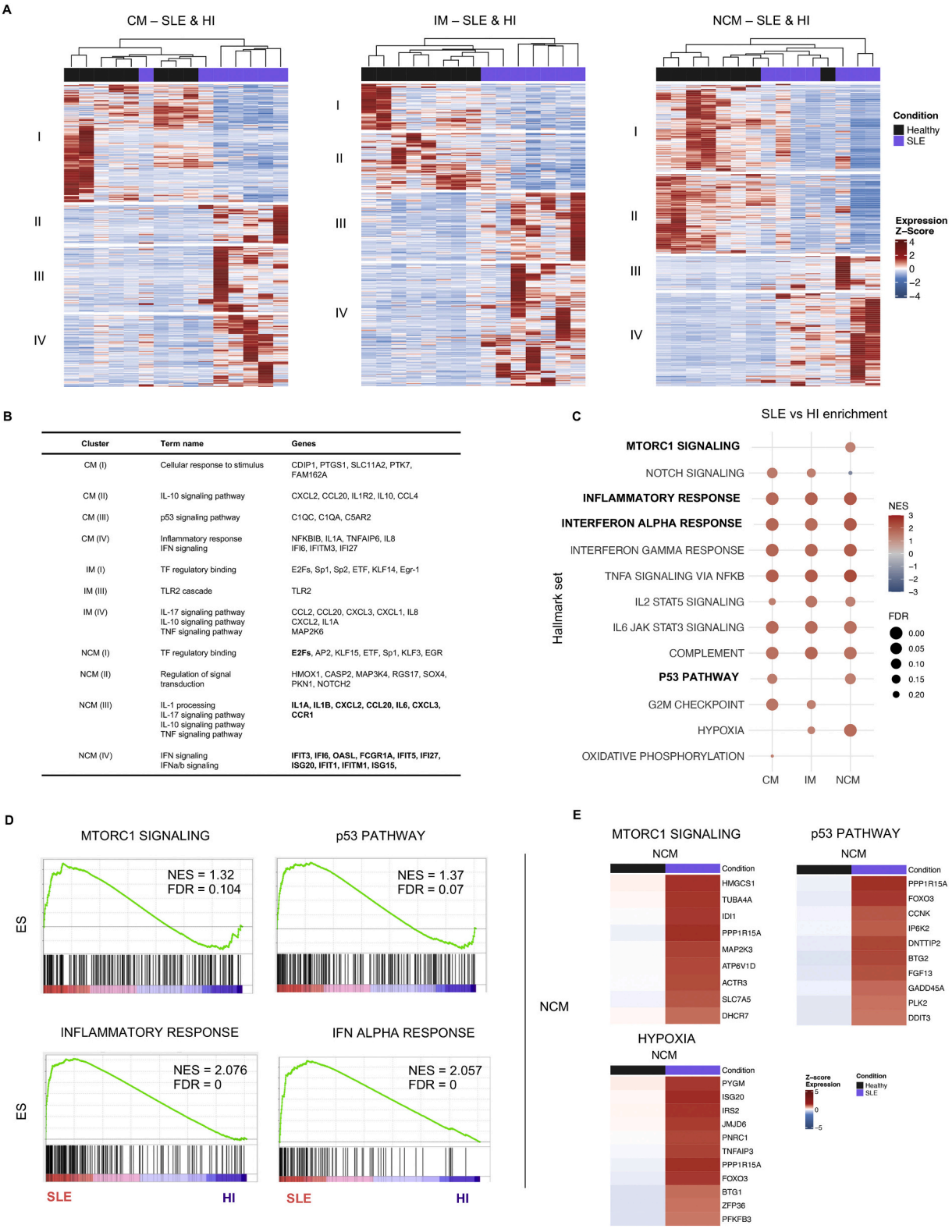

Fig. 2. Transcriptomic analysis identifies inflammatory response and IFN signaling as features of SLE NCM pathogenicity.
A. Heatmap showing the DEGs (from left to right) between "SLE CM and HI CM", "SLE IM and HI IM", and "SLE NCM and HI NCM". Expression values were z-score normalized. Top annotation row shows the condition of each sample colored black for HI and purple for SLE patients. **B.** Summary table showing gProfiler analysis networks of genes contained in each cluster and examples of signature genes in each cluster. **C.** Bubble plot of GSEA analysis representing enriched pathways associated with the Hallmark database in each monocytic subset in SLE patients. The size of the bubbles represents the statistical significance. **D.** GSEA plots for key pathways involved in the pathogenic features of NCM in patients with SLE (n = 7) compared to HI (n = 8).
See also Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

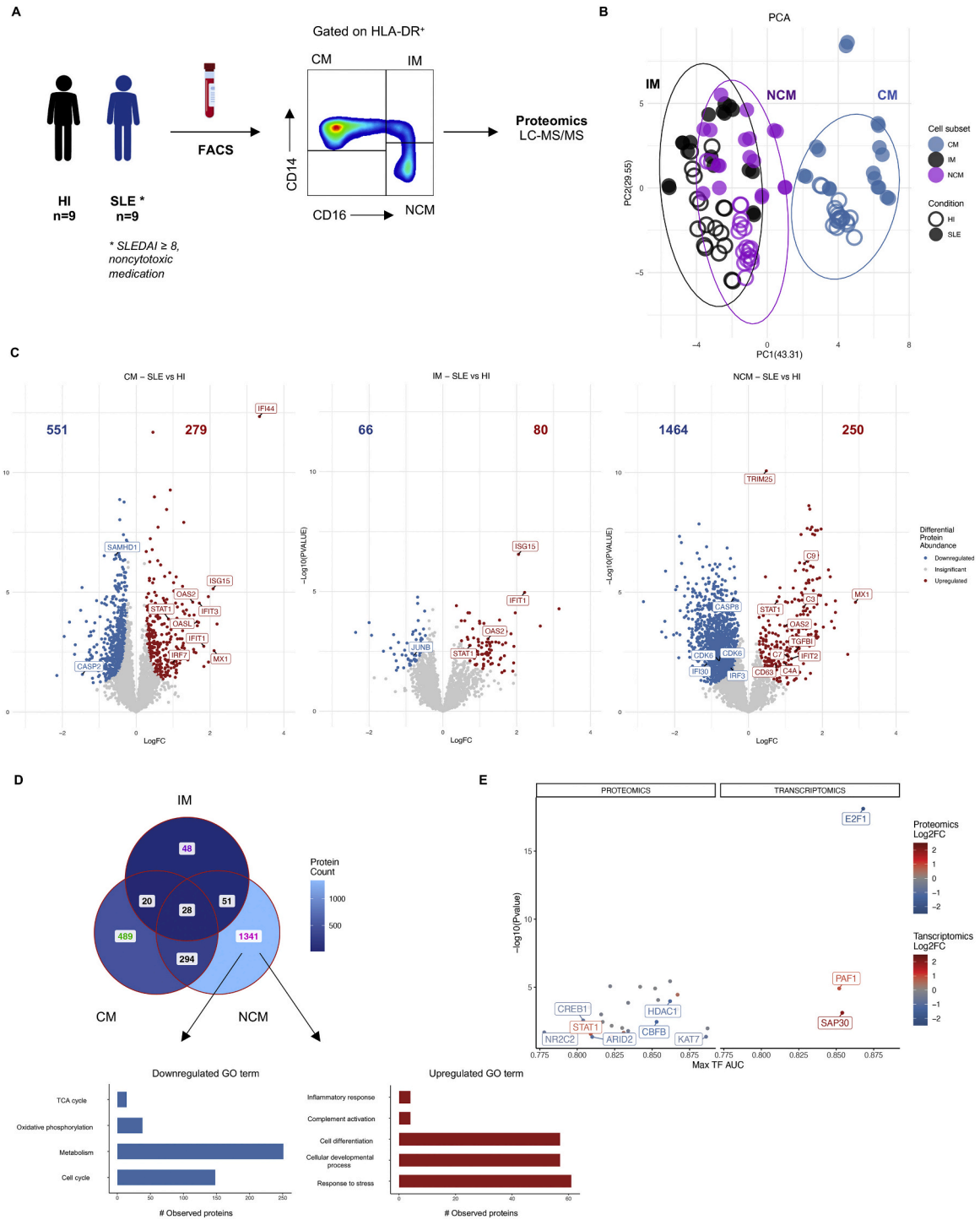

Fig. 3. NCM display the most widespread protein expression changes in SLE characterized by altered metabolism and enhanced differentiation.
A. Graphical overview of our experimental setup. The 3 monocytic subsets were isolated using flow cytometry from the peripheral blood of patients with SLE and HI ($n = 9$ individuals per group) for proteomic analysis. **B.** PCA of protein expression profiles of all monocytic subsets from SLE patients and HI in technical duplicates. Point color represents cell subset (blue for CM, black for IM and purple for NCM) and point shape represents condition (hollow circle for healthy individuals, and filled circle for patients with SLE). **C.** Volcano plots of the three differential expression analyses (DEA) performed comparing (left to right), SLE CM vs HI CM, SLE IM vs HI IM, and SLE NCM vs HI NCM. Upregulated proteins are denoted by red and downregulated proteins are denoted by blue. Proteins not reaching our significance threshold (P value <0.05) are shown in gray. **D.** Venn diagram comparing the DEPs of "SLE CM vs HI CM", "SLE IM vs HI IM", and "SLE NCM vs HI NCM". Color gradient correlates with the protein count in each compartment. Enrichment analysis of Gene Ontology (GO) classification for common downregulated (left) and upregulated (right) proteins found only in NCM. **E.** Transcription factors (TFs) related to cell differentiation and predicted to regulate the genes found as DE only in NCM SLE vs HI transcriptomic comparison ($n = 54$). The max TF AUC and P value were calculated by Binding Analysis of Regulation of Transcription (BART). On the left panel (proteomics) only TFs found to be differentially expressed using SLE vs HI NCM proteomic data are shown and on the right panel (transcriptomics) only TFs found to be differentially expressed using SLE vs HI NCM transcriptomic data are shown. Color scale represents the log2FC calculated during each comparison.
See also Fig. S2 and Tables S1, and S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

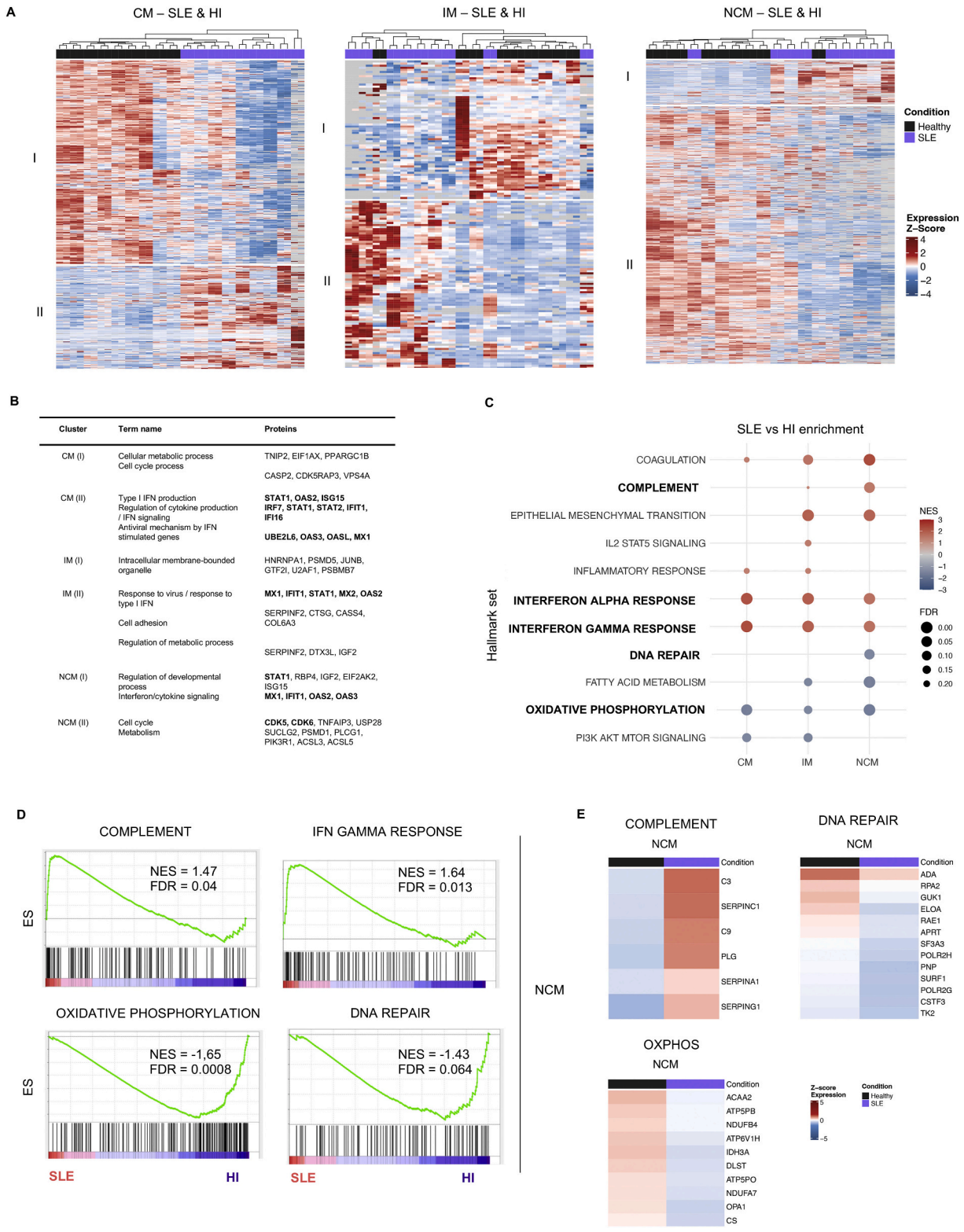

Fig. 4. The inflammatory profile of monocytes in SLE is characterized by common pathways derived from proteome and transcriptome profiling.
A. Heatmap showing the DEPs between (from left to right) "SLE CM and HI CM", "SLE IM and HI IM", and "SLE NCM and HI NCM". Expression values were z-score normalized. Top annotation row shows the condition of each sample colored black for HI and purple for SLE patients. **B.** Summary table showing gProfiler analysis networks of proteins contained in each cluster and examples of signature proteins in each cluster. **C.** Bubble plot of GSEA analysis representing enriched pathways associated with the Hallmark database in each monocytic subset in SLE patients. The size of the bubbles represents the statistical significance. **D.** GSEA plots for key pathways involved in the pathogenic features of NCM in patients with SLE compared to HI (n = 9 individuals per group).
See also Table S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

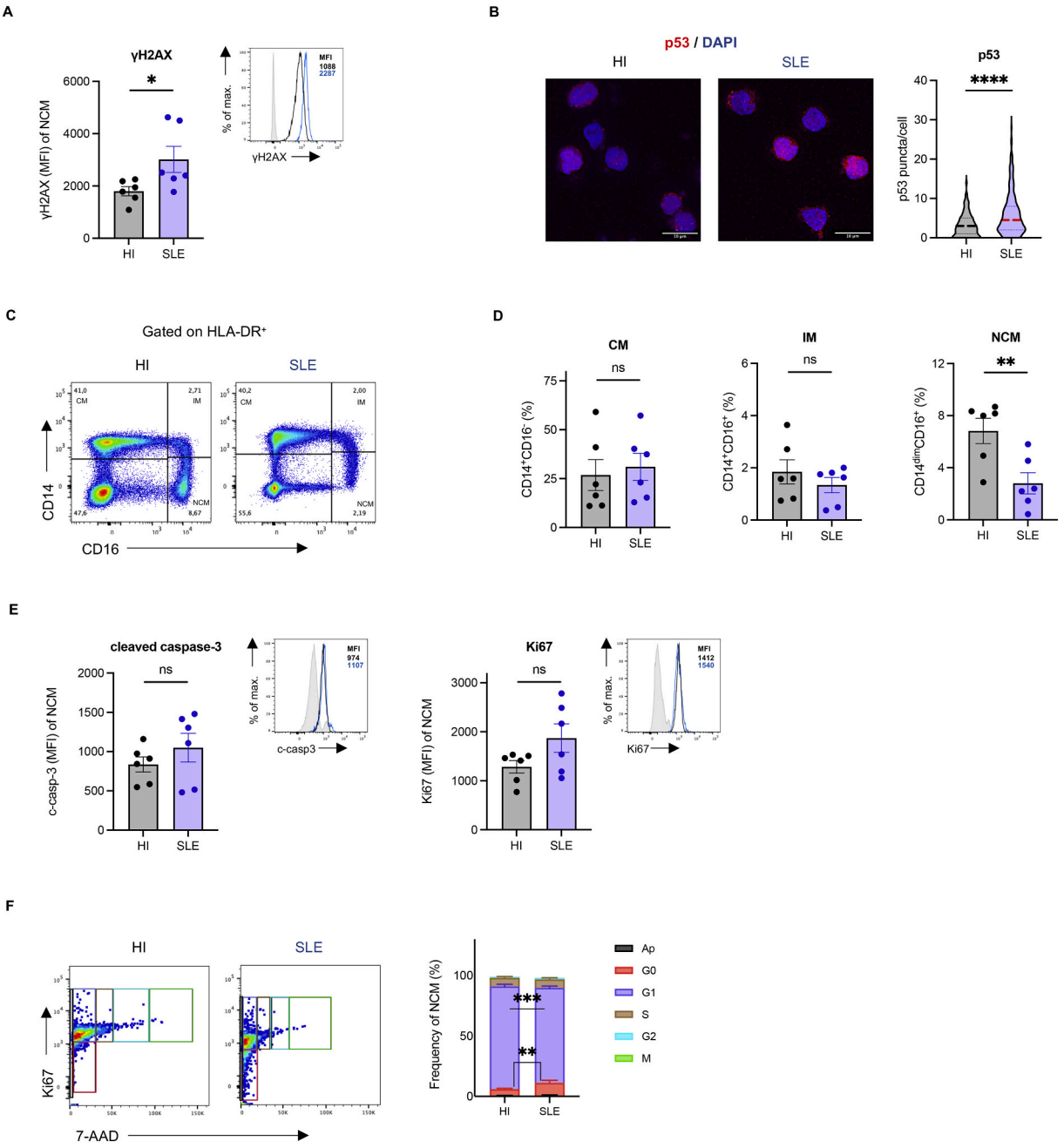

Fig. 5. Peripheral SLE NCM display hyper-inflammatory features and are prone to differentiate.
A. Flow cytometry assay of peripheral blood mononuclear cells (PBMCs) gated on $CD14^{dim}CD16^{+}$ cells for assessing DDR activation using γH2AX in NCM of SLE patients and HI ($n = 6$ individuals per group). Analyzed results are shown as mean of fluorescence intensity (MFI) per individual. Representative histogram showing overlay of unstained cells (gray), stained SLE (blue), and HI (black) cells is depicted. Results are expressed as mean ± SEM. Statistical significance was obtained by Mann-Whitney Student's *t*-test, * $P < 0.05$. **B.** Immunofluorescence confocal microscopy for p53 (red) and DAPI (blue) in sorted NCM from HI and SLE patients ($n = 3$ individuals per group). One representative experiment of 3 is shown. Analyzed results for p53 are depicted as mean puncta/cell. Scale bar, 10 μm. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired Student's *t*-test, **** $P < 0.0001$. **C.** Representative plots of gating strategies of the three monocytic subsets in SLE patients and HI, following PBMCs isolation from the peripheral blood, using flow cytometry. **D.** Monocytic subsets frequencies of HI and SLE patients (n = 6 individuals per group). Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired Student's *t*-test, ** $P < 0.01$. **E.** Assessment of apoptosis (cleaved caspase-3) and proliferation (Ki67) in NCM of SLE patients and HI (n = 6 individuals per group). Representative histogram showing overlay of unstained cells (gray), stained SLE (blue), and HI (black) cells are depicted. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired Student's t-test, $P \geq 0.05$ [not significant (ns)]. **F.** Flow cytometric analysis for assessing cell cycle phases in NCM from SLE patients and HI (n = 7 individuals per group). The cells were stained with 7-AAD and Ki-67 for cell cycle analysis. Representative plots showing the phases of cell cycle in HI NCM and SLE NCM. Results are expressed as mean ± SEM. Statistical significance was obtained by two-way ANOVA Student's t-test, ** $P < 0.01$, *** $P < 0.001$.
See also Figs. S3 and S4 and Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Enhanced autophagy may prevent SLE NCM from apoptosis and induce their differentiation towards an M1-macrophage profile

Results thus far imply that SLE NCM undergo a transition towards a terminally differentiated pro-inflammatory immune cell subset, while the proteomic profile further supports an enriched cell differentiation and developmental process. To this end, we mapped the differentiation process of NCM in patients with active SLE, by performing a broad immunophenotyping assessing the myeloid differentiation cues downstream of monocytes. SLE NCM demonstrated the most prominent increase in the expression levels of CD80 activation and M1 specific-surface marker and the most prominent decrease in the expression levels of CD123 and CD11c markers for the DC populations, implying enhanced activation and polarization towards an M1 phenotype (Fig. 6A; Supplementary Fig. S5A). An increased expression of CD163 scavenger receptor in all three monocytic subsets was also apparent, suggesting promotion of phagocytosis in SLE monocytes (Supplementary Fig. S5) [50,51]. Hence, we assessed the phagocytic activity of CM and NCM in SLE using *E. coli* BioParticles conjugates. Although non-significant differences were observed in the phagocytic abilities of CM and NCM in patients with SLE compared to HI, CM displayed a higher capacity for phagocytosis compared to NCM both in health and disease (Supplementary Fig. S6). In addition, M1-macrophages demonstrated increased frequency while $CD11c^+$ DCs revealed decreased frequency in the periphery of patients with active SLE (Fig. 6B; Supplementary Figs. S7 and S8A). We also observed increased frequency of M1-macrophage like NCM in SLE, indicating an amplified pro-inflammatory response (Fig. 6C; Supplementary Fig. S8B). Based on these findings, it can be inferred that NCM in the peripheral regions of individuals with SLE may adopt a macrophage phenotype resembling M1 characteristics, further adding to the inflammatory response and potential harm to vascular endothelium and tissues. In order to delineate the secretory phenotype of NCM in SLE, we sought to profile cytokine production in sorted NCM of patients with SLE and HI by assessing the release of various cytokines (IL-12p70, TNF-α, IL-6, IL-4, IL-10, IL-1β, Arginase, TARC, IL-1RA, IL-12p40, IL-23, IFN-γ, IP-10) which have been involved in SLE pathogenesis and monocyte function. IL-1β, IL-23, Arginase, and IL-10 levels were significantly upregulated in SLE NCM compared to HI, whereas IL-4 levels were significantly diminished (Fig. 6D; Supplementary Fig. S9). These data reiterate that NCM exhibit an activated macrophage-like and enriched M1 pro-inflammatory phenotype in the periphery of patients with active SLE. Since autophagy is essential for the differentiation of monocytes into macrophages [52] and thus enhance the differentiation potential of NCM in SLE, we next analyzed autophagy levels in sorted NCM from both HI and patients with active SLE. Indeed, we found upregulation of LC3 protein and a downregulation of p62 protein in patients with SLE compared to HI denoting enhanced autophagy in SLE NCM (Fig. 6E). Overall, these results suggest that enhanced autophagy may prevent SLE NCM from apoptosis and promote their skewing into a marked inflammatory phenotype.

4. Discussion

In this study, using proteomic and transcriptomic analyses of the three monocytic subsets from active SLE patients and HI, we provide evidence for an extensive perturbation of SLE NCM. Herein we report inflammatory features with type I IFN signature, complement and p53 pathway activation and metabolic deregulation with diminished oxidative phosphorylation and altered DNA repair of NCM of patients with active SLE. Our ex vivo data demonstrate that in SLE the NCM pathogenicity is characterized by increased DDR and p53 inflammatory responses, followed by G_0 cell cycle arrest and initiation of a differentiation program leading to enhanced autophagy and M1 macrophage polarization. We also provide evidence that enhanced autophagy may drive this differentiation process by preventing SLE NCM from apoptosis and support skewing of polarization. Collectively, these findings establish a connection between the pro-inflammatory responses of NCM in SLE with an M1-macrophage like profile, further fueling the inflammatory environment in the periphery and possibly promoting vascular injury prior to their infiltration into tissues.

Monocytes have a central role in several autoimmune diseases, yet disease-specific signatures of monocytes in the context of SLE and the role of NCM in the periphery of patients with active SLE remain elusive. Recent evidence underscores a pathogenic role of NCM in SLE, by identifying TLR-activated NCM as primary regulators in the development of glomerular dysfunction and kidney injury, but delineation of the molecular events that dictate pathogenicity of NCM in both the periphery and target tissues are poorly understood [25]. Herein, we extend these observations by depicting inflammatory features, IFN response, metabolic alterations and extensive perturbation of NCM in the periphery of patients with active SLE both in the transcriptome and in the proteome level.

Monocytes are not a homogeneous cell population being divided in three distinct subsets with the IM transcriptome being close both to CM and NCM. We observed that NCM display more widespread gene expression changes in SLE compared to any other monocytic counterpart, with a large cluster of unique genes being differentially expressed only in this subset. These findings highlight the diversity of each monocytic subset and the bidirectional impact of the disease on the NCM. Enrichment analysis highlighted that all monocytic subsets display pathogenic features with enhanced inflammatory response, IFNα and IFNγ response and cytokine signaling with activated IL2 STAT5 and IL6 JAK STAT3 cascades [53–56]. Single-cell RNA-seq data have revealed that SLE patients exhibit distinct cellular composition, with reduction of $CD4^+$ T cells, monocytes, pDCs and increase of $CD8^+$ T cells and B cells compared to healthy individuals. Moreover, monocytes represent the population with the largest fraction of high interferon stimulated gene expression signature in PBMCs. Enrichment of ISG-expressing monocytes was detected in patients of higher disease activity [57]. However, SLE NCM have a unique transcriptomic signature characterized - not only by increased inflammatory response and IFNα/γ signaling - but also by the elevated p53 pathway, mTORC1 signaling, and hypoxia. Disease-specific chromatin accessibility signatures characterize $CD4^+$ T cells of lupus patients, correlating with severity. Specifically, transcriptional aberrations of regulatory T (Treg) cells, featuring type I interferon-related functional exhaustion has been described through single cell transcriptomics [58]. By applying this technology to a different target tissue (renal cells), various leukocyte subsets -active in disease- were mapped, such as myeloid cells, T cells, natural killer cells and B cells, exerting pro-inflammatory and inflammation-resolving responses. Highly important evidence of monocyte differentiation within the kidney add up to their pathogenetic role in lupus nephritis [59]. Importantly, single-cell data strengthen the hypothesis that on the transcriptional level, CM and NCM produced the most prominent type I IFN signature among other immune cell types, placing these cells as key modulators in disease pathogenesis [60]. Of note, p53 has been implicated in autoantibody production and progression of autoimmunity in lupus mice [61]. In addition, the activation of the mTORC1 signaling cascade and the abnormalities of autophagy are closely related with the proliferation and differentiation of immune cell subsets and inflammatory cytokine signaling contributing to the pathogenesis of SLE [62–64]. Inhibition of mTOR in SLE monocytes reduced IFNα production and STING expression leading to the amelioration of the disease [65]. In addition, renal hypoxia leads to increased infiltration of T cells in the kidney of lupus-prone mice resulting in tissue damage [66]. All these enriched pathways in the transcriptome signature of NCM underscore a proinflammatory phenotype and position this cell subset as key player in the progression of SLE.

In support of our transcriptomic data, analysis at the proteome level further highlighted a significant separation of monocytes in health and SLE. Protein expression analysis revealed a more pronounced

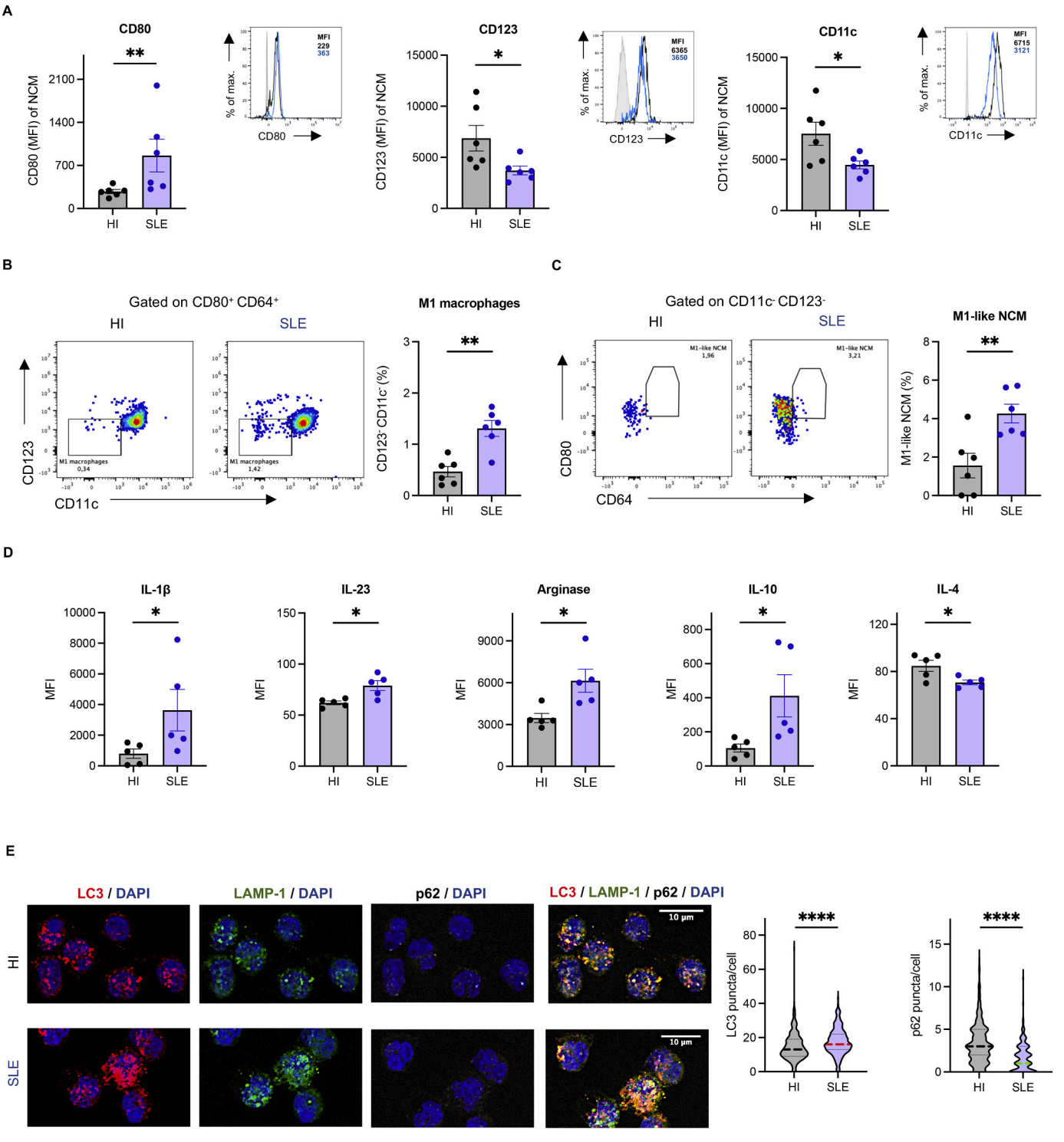

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Fig. 6. Enhanced autophagy may prevent SLE NCM from apoptosis inducing their differentiation towards an M1-macrophage profile.
A. Flow cytometry assay of peripheral blood mononuclear cells (PBMCs) gated on $CD14^{dim}CD16^{+}$ cells for assessing activation, M1 polarization (CD80) and DC polarization (CD123, CD11c) in NCM of SLE patients and HI (n = 6 individuals per group). Analyzed results are shown as mean of fluorescence intensity (MFI) per individual. Representative histogram showing overlay of unstained cells (gray), stained SLE (blue), and HI (black) cells is depicted. Results are expressed as mean ± SEM. Statistical significance was obtained by Mann-Whitney Student's t-test, * $P < 0.05$, ** $P < 0.01$. **B.** M1 macrophages frequencies of HI and SLE patients (n = 6 individuals per group). Representative plots of frequencies of M1 macrophages in SLE patients and HI, following PBMCs isolation from the peripheral blood, using flow cytometry. Results are expressed as mean ± SEM. Statistical significance was obtained by Unpaired Student's t-test, ** $P < 0.01$. **C.** M1-like macrophages frequencies of HI and SLE patients (n = 6 individuals per group). Representative plots of frequencies of M1-like macrophages in SLE patients and HI, following PBMCs isolation from the peripheral blood, using flow cytometry. Results are expressed as mean ± SEM. Statistical significance was obtained by Unpaired Student's t-test, ** $P < 0.01$. **D.** Detection of released cytokines (IL-1β, IL-23, Arginase, IL-10, and IL-4) using LEGENDplex technology through flow cytometry at day 1 of culture (n = 5 individuals per group). Analyzed results are shown as mean of fluorescence intensity (MFI) per individual. Results are expressed as mean ± SEM. Statistical significance was obtained by Mann-Whitney Student's t-test, * $P < 0.05$. **E.** Immunofluorescence confocal microscopy for LC3 (red), LAMP-1(green), p62 (white), and DAPI (blue) in sorted NCM from HI and SLE patients ($n = 4$ individuals per group). One representative experiment of 4 is shown. Analyzed results for LC3 and p62 are depicted as mean puncta/cell. Scale bar, 10 μm. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired Student's *t*-test, **** $P < 0.0001$.
See also Figs. S5, S6, S7, S8, and S9 and Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

perturbation of NCM in SLE compared to the other monocytic subsets. In particular, metabolism and cell cycle processes are downregulated, while inflammatory responses, cell development and differentiation are upregulated in SLE NCM. Binding analysis for the regulation of transcription revealed several TFs related to cell differentiation to bind and regulate the DEGs in SLE NCM. These TFs demonstrate altered expression levels as evidenced by SLE vs HI NCM proteomic and transcriptomic data. For instance, STAT1 being involved in M1-macrophage polarization was among the top enriched TFs that regulate DEGs in SLE NCM [42,67–69]. Moreover, RBM4 is downregulated in SLE NCM in our proteomic data; RBM4 expression suppresses polarization of macrophages towards an M1 phenotype, further supporting that this may coordinate cell identity during differentiation and cell cycle exit in SLE NCM [70]. Several other TFs that were found to regulate DEGs in SLE NCM such as IRF1, RAF1, and E2F1 are important regulators coordinating differentiation and cell cycle exit, a finding that underscores the interest of these pathways in SLE NCM [71–75]. In addition, the proteomic signature of the three monocytic subsets is also characterized by inflammatory features further validating the enriched transcriptomic pathways. Interestingly, SLE NCM displayed enriched complement, epithelial-mesenchymal transition (EMT) and IFN responses and diminished oxidative phosphorylation (OXPHOS), fatty acid metabolism (FAM) and DNA repair. EMT contributes to the development of fibrosis and it has been a feature of kidney damage in SLE [76–78]. NCM have an EMT signature in SLE suggesting that these cells may have proteins that interact with epithelial cells in the kidney mediating organ fibrosis and tissue damage. Mitochondrial OXPHOS and FAM is reduced in pro-inflammatory M1 macrophages which rely for their metabolic needs on glycolysis, blocking their conversion into an alternative/anti-inflammatory M2 phenotype [79,80]. Defective DNA repair and DNA damage is associated with an autoimmune phenotype and the development of SLE [81–85]. NCM seem to have a defective DNA repair, making them more susceptible to DNA damage and genomic instability. These signatures in SLE NCM highlight their inflammatory phenotype and imply their distinct metabolic needs within the autoimmune environment.

In our study, SLE NCM exhibit exacerbated DDR and increased levels of p53, supporting the enriched inflammatory responses, activation of p53 pathway and altered DNA repair observed in the proteomic and transcriptomic signatures. In addition, SLE NCM demonstrated decreased frequency in the periphery of patients with active SLE which was further validated in murine lupus peripheral monocytic subsets. The lower numbers of circulating NCM in the blood of patients may be associated with their harmful recruitment in several tissue targets including the liver as observed in patients with alcoholic hepatitis (AH) [86]. The interplay of immune cells in tissues is pivotal for the development of SLE, where the liver is a top causal tissue while NCM have been shown to play great role in SLE nephritic damage [25,87]. Of note, we have found that NCM are increased in the periphery of active PsA patients, providing additional evidence that the decreased frequency of NCM is specific to SLE and not secondary to the inflammatory milieu [88]. Since DNA repair is deregulated and DDR is enriched in SLE NCM, and these cascades are linked with cell cycle, we examined the cell cycle which is also altered in the proteomic signature of SLE NCM. SLE NCM accumulate in the G_0 phase of the cell cycle, suggesting initiation of a differentiation program. This has been demonstrated in Hematopoietic Stem Cells (HSCs) which are capable of differentiation into restricted progenitors, such as common myeloid progenitors, without entering the S phase of the cell cycle and undergoing cell division [48]. During terminal differentiation, most cells exit the cell cycle and enter a prolonged or permanent G_0 cell cycle arrest [47]. A link between monocyte to macrophage differentiation and induction of cell cycle arrest has been established [49]. The enhanced DNA damage and cell cycle arrest underlie a differentiation and developmental status of NCM in the inflammatory environment of SLE.

Since the proteomic signature of SLE NCM denotes a differentiation and developmental process – a finding further validated from our ex vivo studies, we next mapped the differentiation potential of these cells. SLE NCM display enhanced polarization towards an M1 phenotype, as evidenced by elevated expression levels of CD80, while exhibiting a reduced polarization towards the dendritic cell phenotype, as indicated by decreased expression levels of CD11c and CD123. This is a specific feature for SLE NCM, as the other two monocytic subsets did not display altered levels of these surface markers. Among the various immune cell populations examined, M1 macrophages demonstrated increased frequency whereas $CD11c^{+}$ DCs displayed decreased frequency in the periphery of individuals with active SLE. Our findings suggest that SLE NCM adopt a phenotype resembling M1 macrophages, further supporting the heightened pro-inflammatory responses. NCM secrete pro-inflammatory M1 cytokines such as IL-1β (also increased in the transcriptomic analysis only in SLE NCM), IL-23, and Arginase, supporting the inflammatory potential of NCM in the periphery of patients with active SLE. Monocytes act as a bridge, linking mononuclear phagocyte precursors in the bone marrow (BM) with terminally differentiated mononuclear cells [89,90]. Emerging literature implicates autophagy as a key regulatory pathway in preventing monocytes from apoptosis and inducing their differentiation towards macrophages [52]. Enhanced autophagy in SLE NCM may prevent these cells from apoptosis and induce their differentiation towards an M1-macrophage like profile in the disease. The differentiation journey of the monocyte/macrophage lineage begins from molecular switching that unfolds in the periphery, but in the context of the disease their relation to resident or infiltrating cells is yet to be thoroughly depicted [91–93].

There remain some limitations in the present study that leave questions to be tackled in future endeavors. Although we used flow cytometry to purify monocytic subsets, we employed bulk RNA-

sequencing technology- thus we cannot exclude the possibility that there is even greater heterogeneity inside each subset, yet to be described. Single studies on the already defined subsets of monocytes will add greater detail in the future. Because of the limited number of cells, we were unable to perform both transcriptome and proteome analysis in the same patients and healthy individuals. Patients had received a variety of therapies in the past although recent cytotoxic or glucocorticosteroid administration was avoided. Finally, we studied Caucasians in this study, so we cannot generalize our results to other ethnic groups. Notwithstanding these limitations, this is the first comprehensive transcriptomic and proteomic analysis of the three known subsets of peripheral monocytes in SLE combined with functional data and studies in animal models.

In conclusion, our study provides unique insights into the molecular signatures of SLE monocytes. We report here a distinct proteomic and transcriptomic profile of NCM of patients with active SLE with enhanced inflammatory features and extensive perturbation. The pathogenic profile of SLE NCM is characterized by deregulated DNA repair, cell cycle and enhanced IFN signaling, together with cell differentiation and developmental cues. Experimental screening underlined activation of p53 pathway and increased DDR in parallel with G_0 cell cycle arrest of SLE NCM corroborating initiation of a differentiation program. This defective profile of NCM of patients with SLE is linked with an activated macrophage-like and enriched M1 pro-inflammatory response. Our results suggest that enhanced autophagy may prevent SLE NCM from apoptosis and may support their skewing towards differentiation into a macrophage phenotype with M1 characteristics contributing to the inflammatory environment in the periphery where they may be damaging the endothelium prior to their migration to the tissue. Endothelial injury is a prominent feature of SLE linked vasculopathy in various tissues including blood vessels, brain, skin and kidneys [93,94]. Our findings highlight the importance of elucidating the molecular pathways that dominate each cell type in SLE in order to better map their functions in the disease and effectively target autoimmunity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2023.109765.

Author contributions

Conceptualization: E.M.S., T.M., D.T.B., and A.B. Performed experiments: E.M.S., and M.S. Data analysis: E.M.S., G.S., and M.S. Data interpretation: E.M.S., T.M., D.T.B., and A.B. Human sample collection: N.K., A.F, and D.T.B. Project administration: D.T.B. Supervision: D.T.B. and A.B. Writing - original draft: E.M.S. Writing - review & editing: E.M.S., T.M., D.T.B., and A.B.

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Declaration of Competing Interest

Funders were not involved in the study design, data collection, and analysis, interpretation, or preparation of this manuscript. The authors declare no competing interests.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The RNA-seq datasets for this study have been deposited in the European Genome-Phenome Archive under study number EGAS00001007304. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD042539 [38].

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Supplementary Information

Transcriptomic and proteomic profiling reveals distinct pathogenic features of peripheral non-classical monocytes in Systemic Lupus Erythematosus

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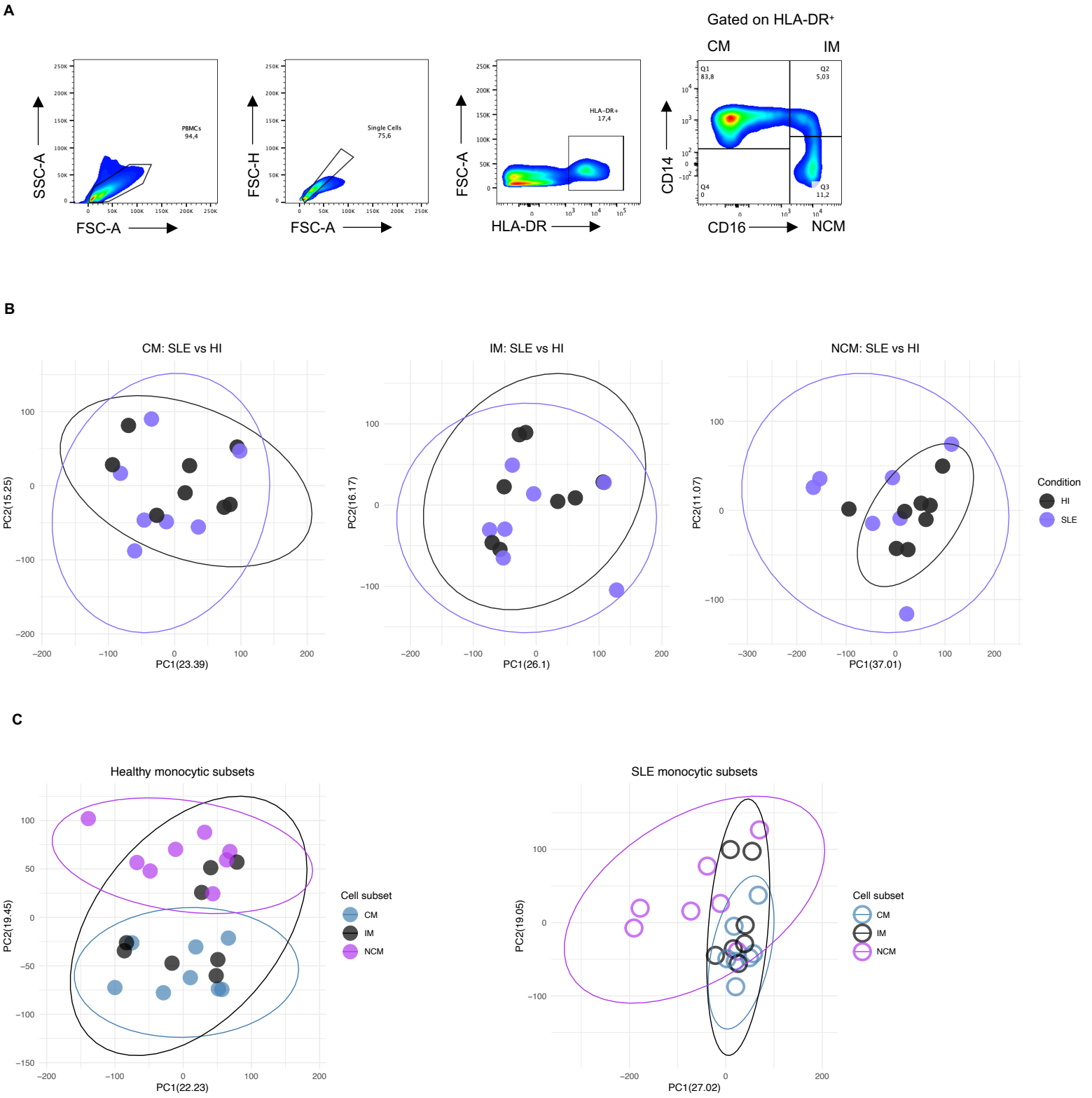
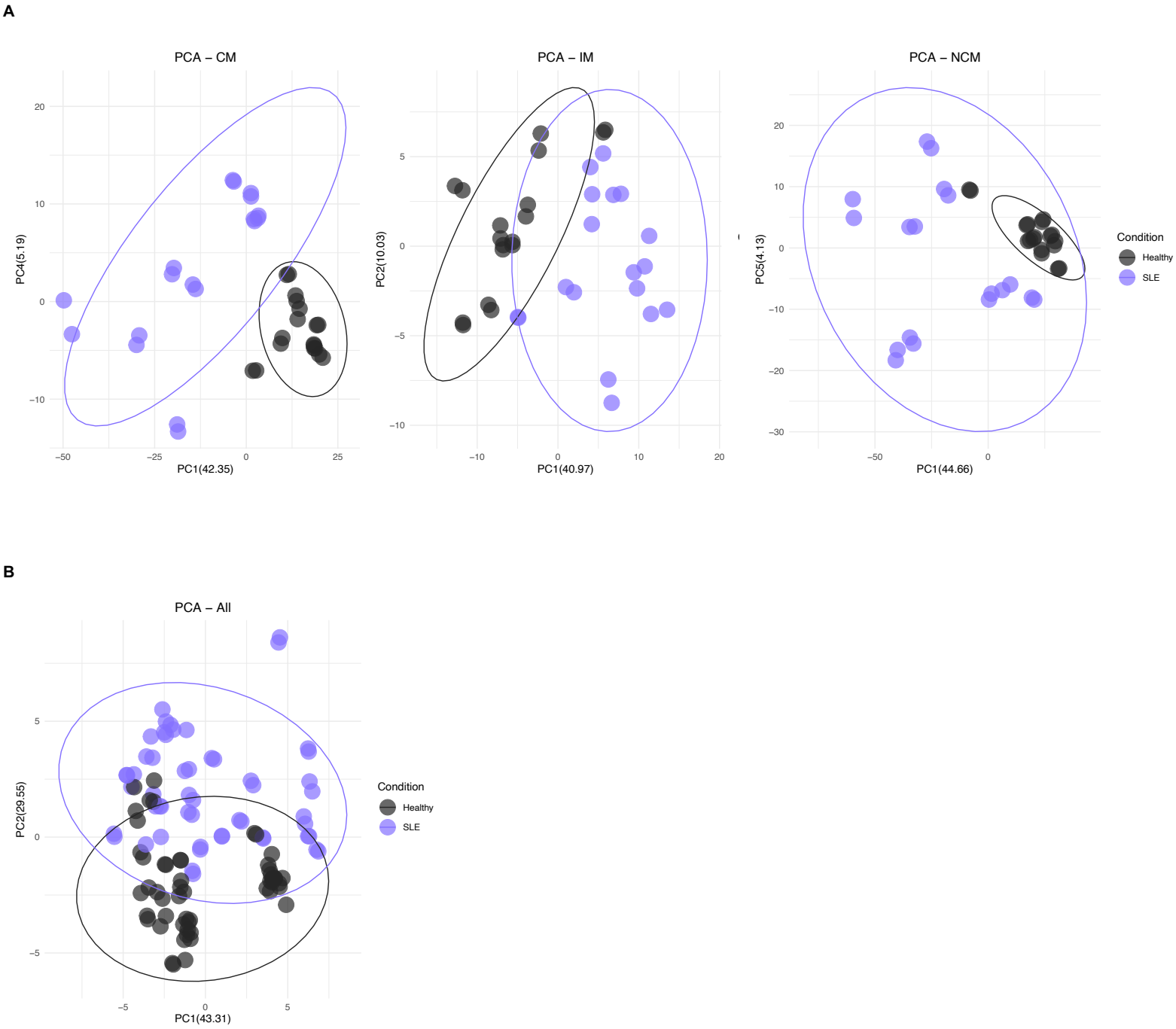
Contents **Page**

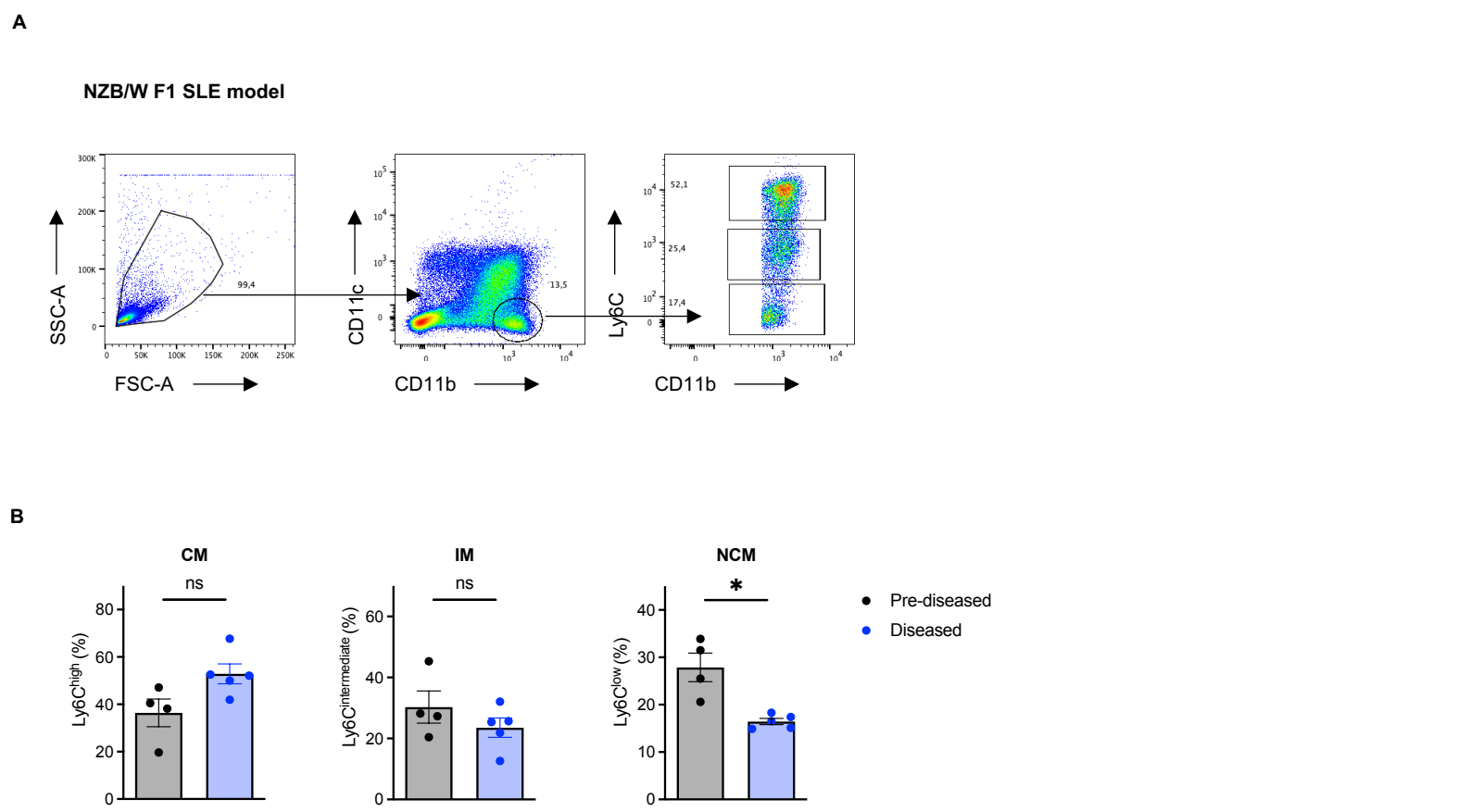


Supplementary Figure S1. Distinct transcriptomic profile of NCM compared to any other monocytic subset in health and SLE. Related to Figure 1.

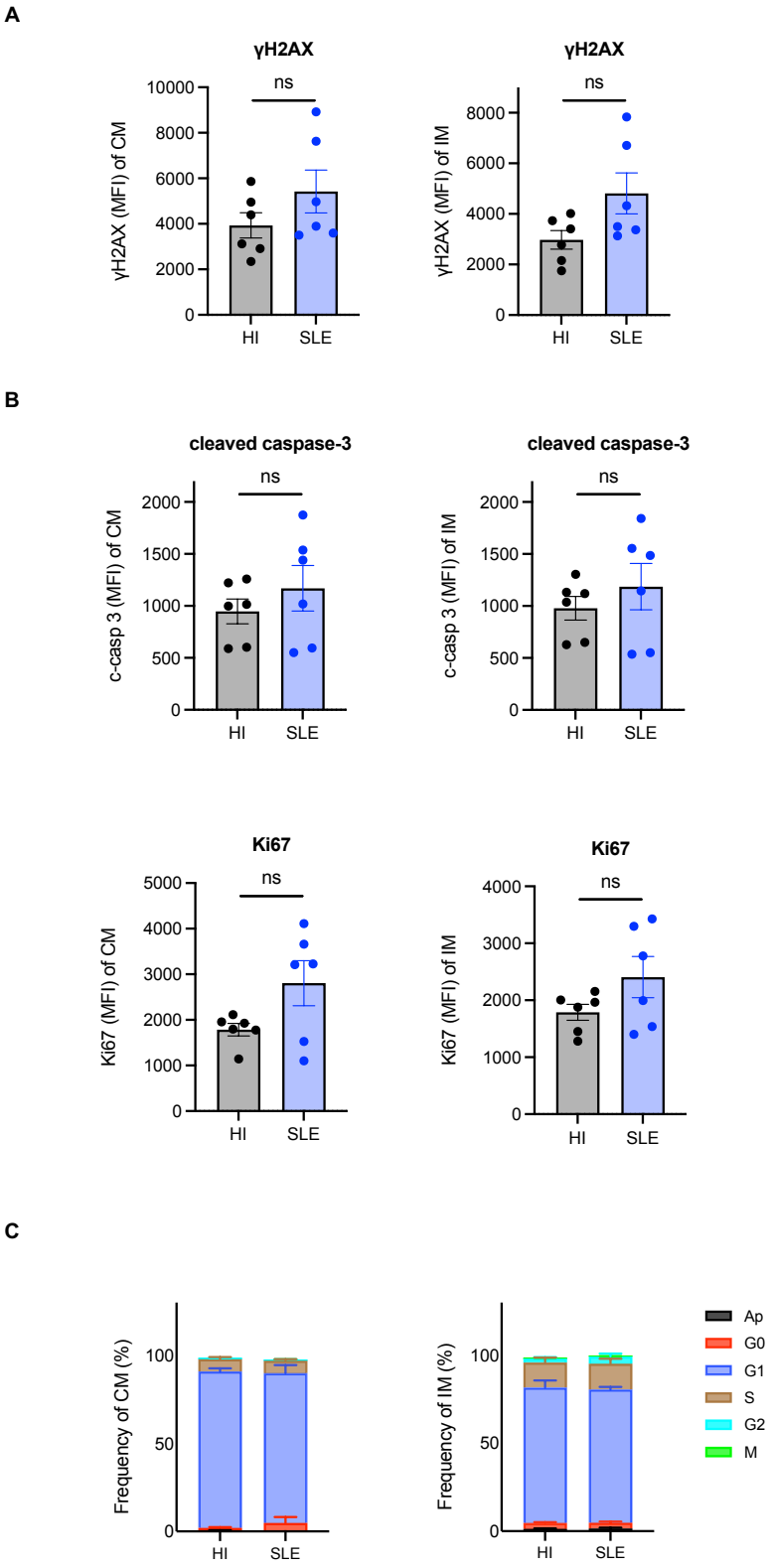
A. Gating strategy for the flow cytometry-sorted monocytic subsets, following PBMCs isolation from the peripheral blood and extracellular staining. **B.** PCA of gene expression profiles of (left to right) "SLE CM and HI CM", "SLE IM and HI IM", "SLE NCM and HI NCM". Point color represents condition (black for healthy individuals and purple for patients with SLE). **C.** PCA of gene expression profiles of the three healthy (left) and SLE monocytic subsets (right). Point color represents cell subset (blue for CM, black for IM and purple for NCM) and point shape represents condition (filled circle for healthy individuals, and hollow circle for patients with SLE).



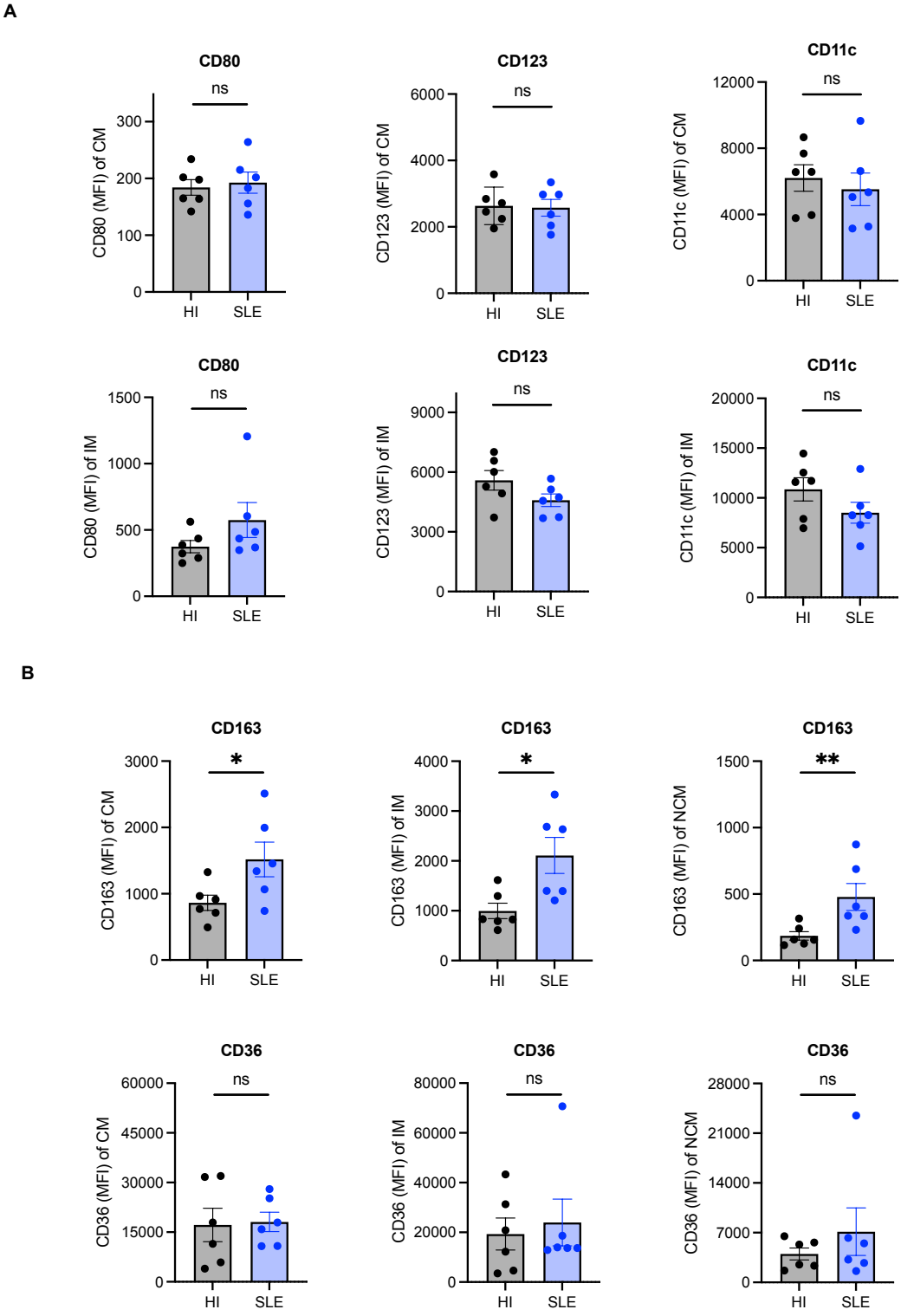
Supplementary Figure S2. Distinct proteomic profile of all monocytic subsets in health and SLE. Related to Figure 3.
A. PCA of protein expression profiles of (left to right) "SLE CM and HI CM", "SLE IM and HI IM", "SLE NCM and HI NCM" in technical duplicates. Point color represents condition (black for healthy individuals and purple for patients with SLE). **B.** PCA of protein expression profiles of all monocytic subsets from SLE patients and HI in technical duplicates. Point color represents condition (black for healthy individuals and purple for patients with SLE).



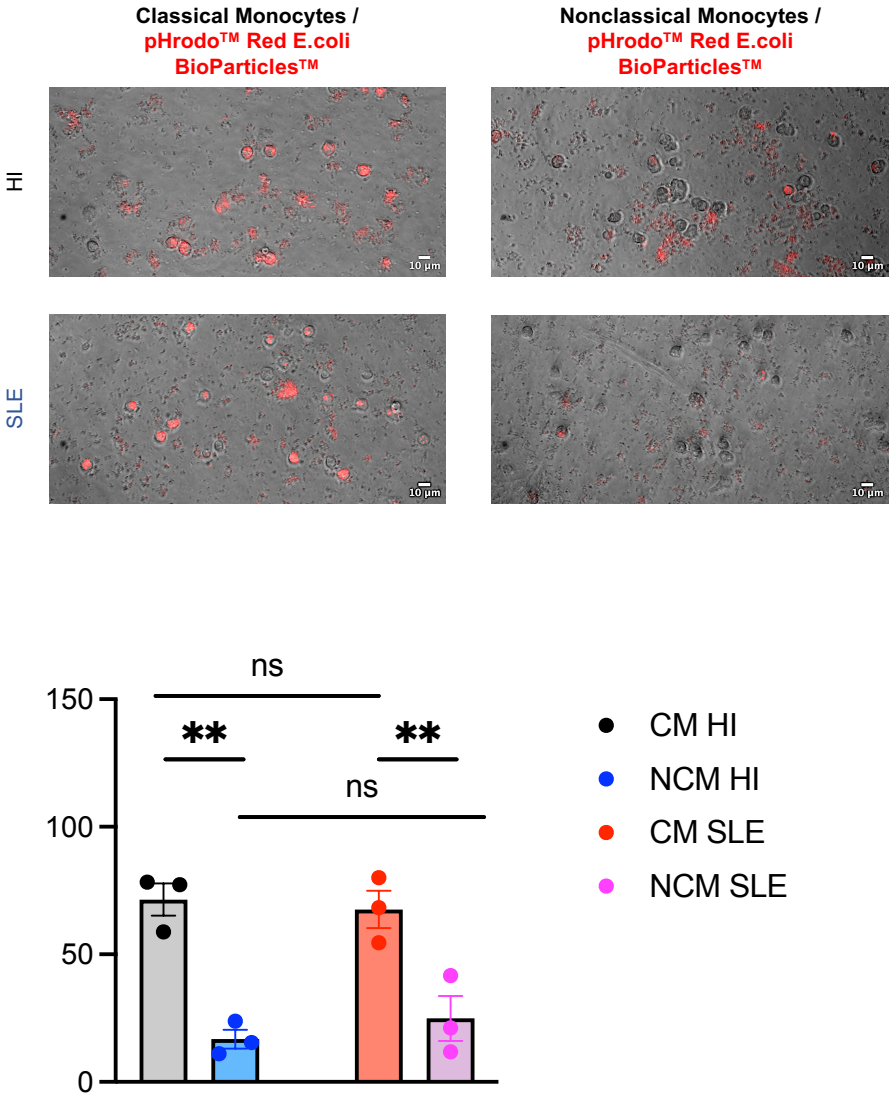
Supplementary Figure S3. NCM of lupus-diseased mice exhibit decreased frequency in the peripheral blood. Related to Figure 5. **A.** Representative plots of gating strategies of the three monocytic subsets in the peripheral blood of NZB/W F1 SLE diseased and pre-diseased mice, following PBMCs isolation, using flow cytometry. **B.** Monocytic subsets frequencies of SLE diseased (n=5) and pre-diseased mice (n=4). Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired and Mann-Whitney Student's *t* tests, * $P < 0.05$.



Supplementary Figure S4. DNA damage and cell cycle arrest are distinct features of SLE NCM pathogenicity. Related to Figure 5. **A.** Flow cytometry assay of peripheral blood mononuclear cells (PBMCs) gated on $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ cells for assessing DDR activation using γH2AX in CM and IM of SLE patients and HI (n=6 individuals per group). Analyzed results are shown as mean of fluorescence intensity (MFI) per individual. Statistical significance was obtained by unpaired Student's t test, $P \geq 0.05$ [not significant (ns)]. **B.** Flow cytometry assay of peripheral blood mononuclear cells (PBMCs) gated on $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ cells for assessing apoptosis (cleaved caspase-3) and proliferation (Ki67) in CM and IM of SLE patients and HI (n=6 individuals per group). Analyzed results are shown as mean of fluorescence intensity (MFI) per individual. Statistical significance was obtained by unpaired Student's t test, $P \geq 0.05$ [not significant (ns)]. **C.** Flow cytometric analysis for assessing cell cycle phases in CM and IM from SLE patients and HI (n=7 individuals per group). The cells were stained with 7-AAD and Ki-67 for cell cycle analysis. Representative plots showing the phases of cell cycle (left to right) in HI CM and SLE CM and HI IM and SLE IM. Statistical significance was obtained by two-way ANOVA Student's t test, $P \geq 0.05$ [not significant (ns)].

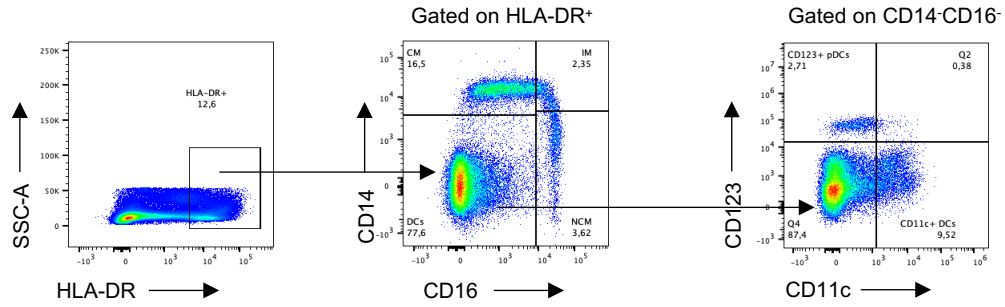


Supplementary Figure S5. M1 polarization and activation is a specific feature of SLE NCM. Related to Figure 6.
A. Flow cytometry assay of peripheral blood mononuclear cells (PBMCs) gated on $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ cells for assessing activation, M1 polarization (CD80) and DC polarization (CD123, CD11c) in CM and IM of SLE patients and HI (n=6 individuals per group). Analyzed results are shown as mean of fluorescence intensity (MFI) per individual. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired Student's t test, $P \geq 0.05$ [not significant (ns)]. **B.** Flow cytometry assay of peripheral blood mononuclear cells (PBMCs) gated on $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ cells for assessing phagocytosis (CD163 & CD36) in CM and IM of SLE patients and HI (n=6 individuals per group). Analyzed results are shown as mean of fluorescence intensity (MFI) per individual. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired Student's t test, $P \geq 0.05$ [not significant (ns)], * $P < 0.05$, ** $P < 0.0.1$.

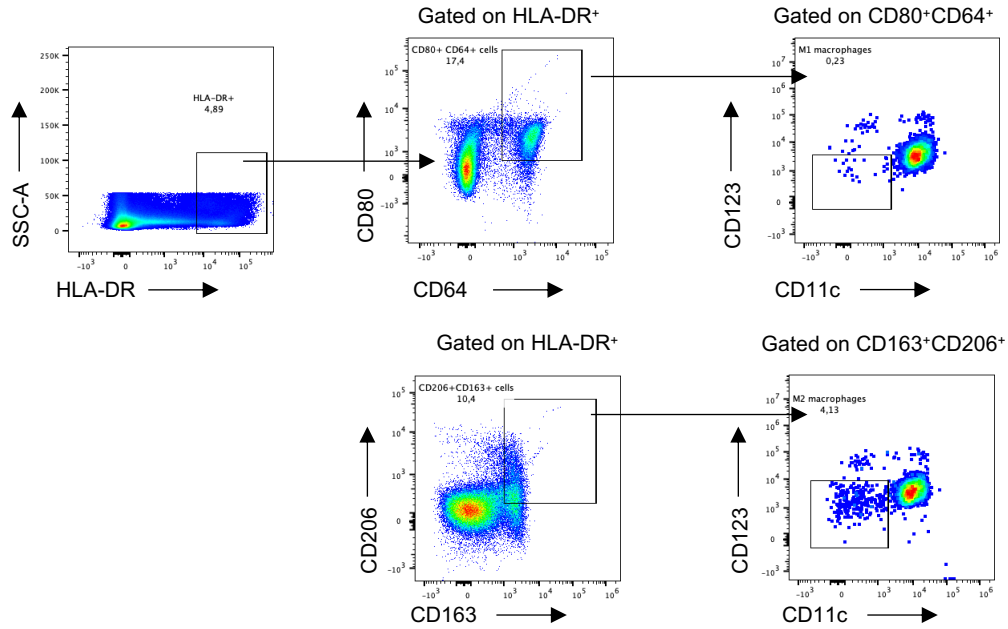


Supplementary Figure S6. CM are highly phagocytic compared to NCM both in health and SLE. Related to Figure 6.
Sorted CM and NCM from HI and SLE patients (n=3 individuals per group) were incubated with pHrodo™ *E. coli* BioParticles ® for 30min. One representative experiment of 3 is shown. Scale bar, 10µm. Results are expressed as mean ± SEM. Statistical significance was obtained by one-way ANOVA Student's *t* test, $P \geq 0.05$ [not significant (ns)], ** $P < 0.01$.

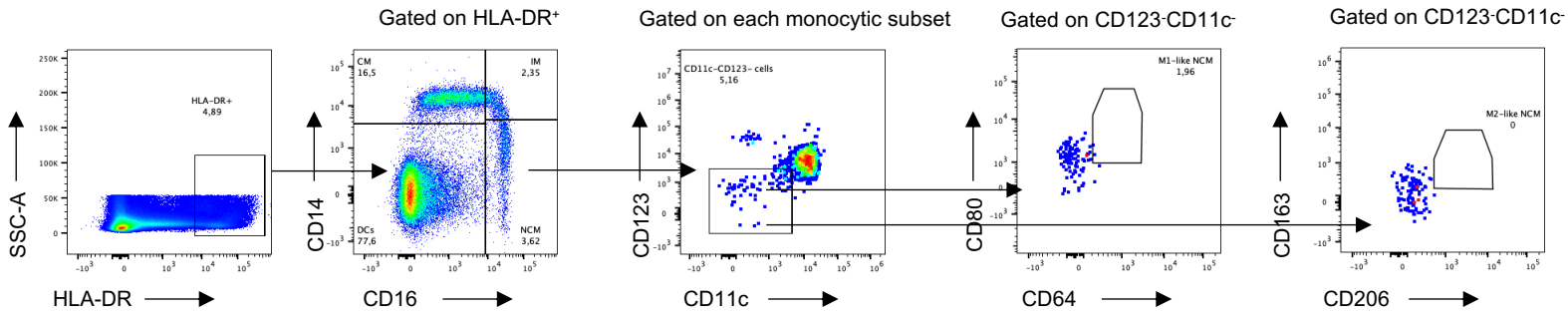
- *Monocytes, CD123+ pDCs, & CD11c+ DCs*



- *M1 & M2 macrophages*

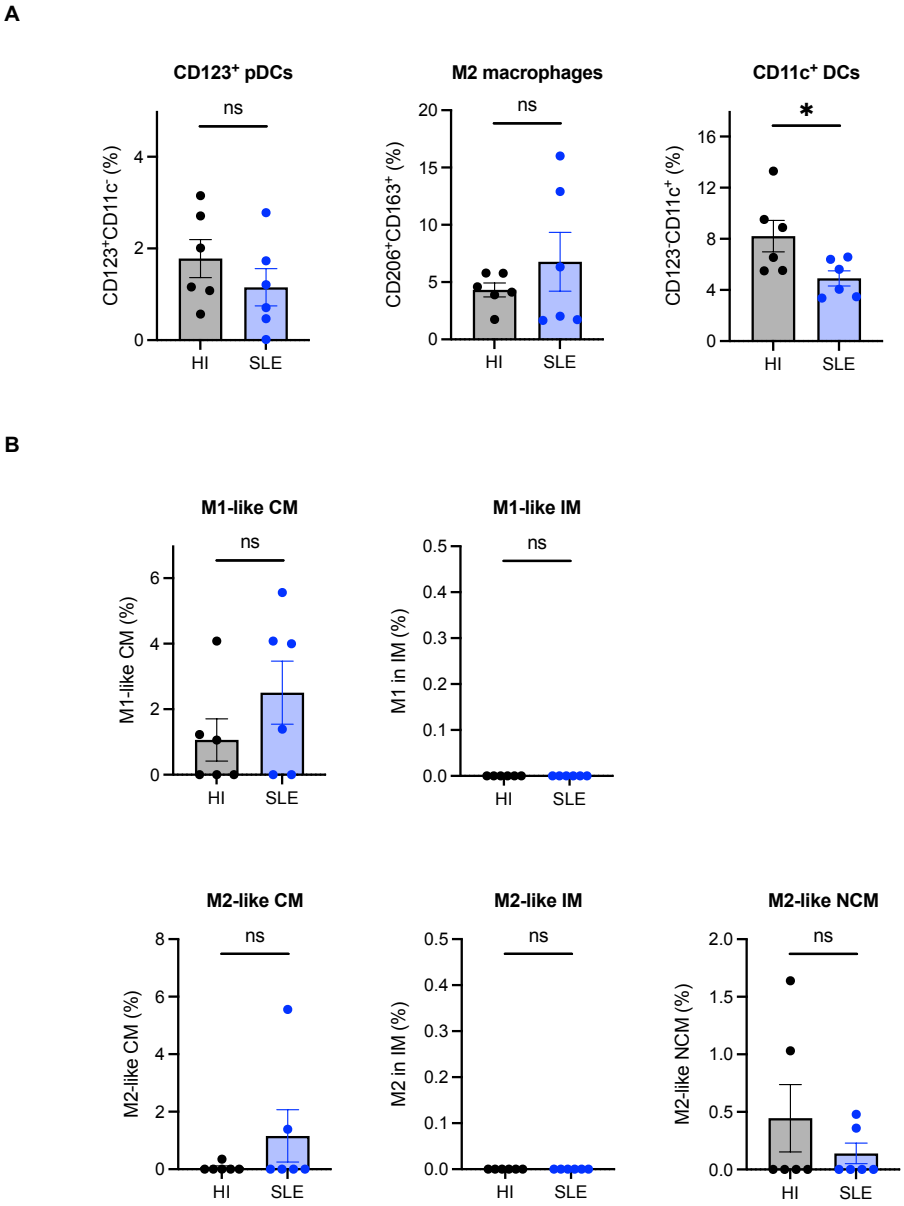


- *M1-like monocytes & M2-like monocytes*

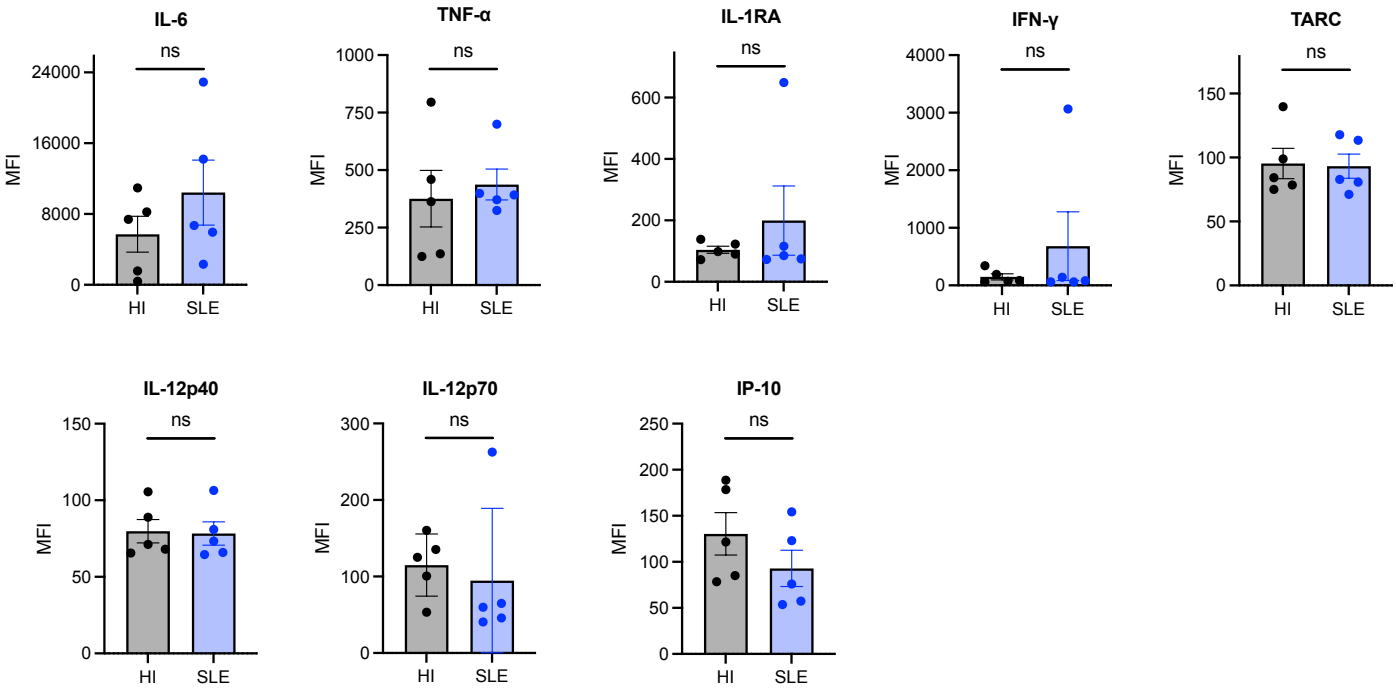


Supplementary Figure S7. Representative flow cytometry gating strategy for identification of immune cell subsets. Related to Figure 6.

Gating strategies for the flow cytometry-analyzed immune cells following PBMCs isolation from the peripheral blood of HI and SLE patients (n=6 individuals per group).



Supplementary Figure S8. Frequencies of the investigated immune cell types. Related to Figure 6.
A. Immune cell frequencies (SLE, HI; n=6 individuals per group). Results are expressed as mean ± SEM. Unpaired and Mann-Whitney Student's t tests, $p \geq 0.05$ (ns) & * $P < 0.05$. **B.** Immune cell frequencies (SLE, HI; n=6 individuals per group). Results are expressed as mean ± SEM. Unpaired and Mann-Whitney Student's t tests, $p \geq 0.05$ (ns).



Supplementary Figure S9. SLE NCM do not secret anti-inflammatory cytokines. Related to Figure 6.
Detection of released cytokines utilizing LEGENDplex^{TM} technology through flow cytometry at day 1 of culture (n=5 individuals per group). Results are expressed as mean ± SEM. Paired Student's t test, $p \geq 0.05$ (ns). MFI: mean fluorescent intensity

Legends for Tables S1-S3 (separate files)

Supplementary Table S1. Demographic and clinical characteristics of SLE patients and healthy individuals included in the study. Age and sex are depicted for patients with SLE and HI. For the patients with SLE, information on SLEDAI, PGA, dominant organ, disease status, and treatment at the time of sampling is also provided. SLE: Systemic lupus erythematosus; HI: healthy individual; SLEDAI: Systemic lupus erythematosus disease activity index; PGA: physician global assessment; F: female; M: male; LN: lupus nephritis; NPSLE: Neuropsychiatric lupus; MTX: methotrexate; HCQ: hydroxychloroquine; AZA: Azathioprine; GCs: Glucocorticoids, MMF: mycophenolate mofetil; CYC: cyclophosphamide, RTX: rituximab, LEF: leflunomide; * : for samples used for the RNA-sequencing experiment; ** : for samples used for the proteomic experiment

Supplementary Table S2. List of gene ontology (GO) analyses in the transcriptomic data in all monocytic subsets in SLE compared to HI

Supplementary Table S3. List of gene ontology (GO) analyses in the proteomic data in all monocytic subsets in SLE compared to HI

Discussion

In this study, we have employed transcriptomic and proteomic analyses to examine the three monocytic subsets from active SLE patients and HI. Our findings demonstrate an extensive perturbation of SLE NCM. Specifically, we report inflammatory characteristics marked by a type I IFN signature, activation of the complement system, the p53 pathway, as well as metabolic deregulation, which includes reduced oxidative phosphorylation and altered DNA repair mechanisms of NCM of active SLE patients. Our *ex vivo* experiments further elucidate the pathogenicity of NCM in SLE, revealing an augmented DDR and heightened p53-mediated inflammatory responses. These events are followed by G_0 cell cycle arrest and the initiation of a differentiation program, leading to increased autophagy and polarization toward an M1 macrophage phenotype. Moreover, our data provide evidence that enhanced autophagy may be instrumental in driving this differentiation process by preventing SLE NCM from apoptosis and support skewing of polarization. Collectively, these findings establish a link between the pro-inflammatory responses of NCM in SLE with an M1-macrophage like profile. This connection further contributes to the overall inflammatory milieu in the peripheral circulation and potentially promotes vascular injury prior to the infiltration of these cells into tissues.

Monocytes play a pivotal role in the pathogenesis of several autoimmune disorders. However, the specific molecular signatures of monocytes in the context of SLE and the function of NCM in the peripheral blood of patients with active SLE remain elusive. Recent evidence underscores a pathogenic role of NCM in SLE, by identifying TLR-activated NCM as primary regulators in the development of glomerular dysfunction and kidney injury, but delineation of the molecular events that dictate pathogenicity of NCM in both the peripheral blood and target tissues are ill defined [147]. Herein, we aim to expand upon these observations by depicting inflammatory features, IFN response, metabolic alterations and extensive perturbation of NCM in

the periphery of patients with active SLE both in the transcriptome and in the proteome level.

Monocytes constitute a heterogeneous cell population, which can be categorized into three distinct cell subsets. Remarkably, the transcriptome profile of IM closely resembles that of CM and NCM. Our findings reveal that, among the three monocytic subsets, NCM display more widespread gene expression changes in SLE. Notably, a substantial cluster of genes exhibits differential expression exclusively within the NCM subset. These findings underscore the inherent diversity within each monocytic subset and emphasize the bidirectional impact of the disease on the NCM. Enrichment analysis revealed that all monocytic subsets display pathogenic features with enhanced inflammatory response, IFNα and IFNγ response and cytokine signaling with activated IL2 STAT5 and IL6 JAK STAT3 cascades [161–164]. Analysis of single-cell RNA-sequencing data has depicted notable differences in cellular composition of SLE patients. Specifically, patients with SLE compared to HI demonstrate a decrease in $CD4^+$ T cells, monocytes, and pDCs, alongside an increase in $CD8^+$ T cells and B cells. Furthermore, it is noteworthy that monocytes constitute the population with the most prominent proportion of high ISG expression signature in PBMCs. Enrichment of ISG-expressing monocytes has been identified in patients with a more pronounced disease activity [165]. Nevertheless, it is important to emphasize that SLE NCM possess a distinctive transcriptomic signature. This signature is characterized not only by an augmented inflammatory response and enhanced IFNα/γ signaling but also by elevated activation of the p53 pathway, mTORC1 signaling, and hypoxia. Disease-specific chromatin accessibility patterns characterize $CD4^+$ T cells of lupus patients, correlating with severity. Notably, single-cell transcriptomic analyses have revealed aberrations of regulatory T (Treg) cells, marked by functional exhaustion related to type I interferon activity [166]. The application of this cutting-edge technology to a different target tissue, specifically renal cells, has enabled the mapping of various leukocyte subsets that play active roles in the disease process. These subsets include myeloid cells, T cells, natural killer (NK) cells and B cells, all of which engage in both pro-inflammatory and anti-inflammatory responses. Additionally, compelling evidence of the differentiation of

monocytes within the kidney further underscore their pathogenetic contribution to lupus nephritis [167]. Significantly, single-cell data strengthen the hypothesis that on the transcriptional level, both CM and NCM exhibit the most pronounced type I IFN signature among various immune cell types, placing these cells as key modulators in disease pathogenesis [168]. Of note, p53 has been implicated in the production of autoantibodies and the development of autoimmunity in murine models of lupus [169]. Furthermore, the activation of the mTORC1 signaling pathway and the abnormalities in autophagy are closely related with the proliferation and differentiation of immune cell subsets, as well as the signaling of inflammatory cytokines, all of which contribute to the SLE pathogenesis [170–172]. Inhibition of mTOR in SLE monocytes has been to shown to reduce IFNα production and STING expression, leading to the amelioration of the disease [173]. Additionally, renal hypoxia has been implicated with the increased infiltration of T cells in the kidney of lupus-prone mice, resulting in tissue damage [174]. All these enriched pathways in the transcriptome signature of NCM underscore their proinflammatory phenotype and position this particular cell subset as key player in the progression of SLE.

In support of our transcriptomic data, analysis at the proteome level underscores a significant separation of monocytes in HI and patients with SLE. Protein expression analysis revealed a more pronounced perturbation of NCM in SLE compared to the other monocytic subsets. Specifically, in SLE NCM, metabolic and cell cycle processes are downregulated, while inflammatory responses, cell development and differentiation are upregulated. Further analysis of the regulatory mechanisms governing transcription reveals several TFs associated with cell differentiation that bind and regulate the DEGs in SLE NCM. Notably, the expression levels of these TFs were altered, as evidenced by SLE vs HI NCM proteomic and transcriptomic data. For instance, STAT1, known to be involved in M1-macrophage polarization, ranks among the top enriched TFs that regulate DEGs in SLE NCM [175–178]. Furthermore, RBM4 is decreased in SLE NCM in our proteomic data. It is noteworthy that RBM4 expression suppresses the polarization of macrophages towards an M1 phenotype, further supporting that this may coordinate cell identity during differentiation and cell cycle arrest in SLE NCM [179]. Several other TFs that were found to regulate

DEGs in SLE NCM, including IRF1, RAF1, and E2F1, are important regulators that coordinate differentiation and cell cycle exit, a finding that underscores the significance of these pathways in SLE NCM [180–184]. In addition, the proteomic profile of the three monocytic subsets exhibits inflammatory features further that further substantiate the enriched transcriptomic pathways. Interestingly, SLE NCM demonstrate enriched complement, epithelial-mesenchymal transition (EMT) and IFN responses and diminished oxidative phosphorylation (OXPHOS), fatty acid metabolism (FAM) and DNA repair. The presence of an EMT signature in SLE NCM is of particular interest, as EMT has been implicated in the development of fibrosis, a recognized feature of kidney damage in SLE [185–187]. This suggests that NCM may have proteins that interact with epithelial cells in the kidney, potentially mediating organ fibrosis and tissue damage. Moreover, the observed reduction in mitochondrial OXPHOS and FAM in SLE NCM aligns with the metabolic profile of pro-inflammatory M1 macrophages, which rely on glycolysis for their metabolic needs, blocking their transition into an alternative/anti-inflammatory M2 phenotype [188,189]. Furthermore, deficiencies in DNA repair and DNA damage have been associated with an autoimmune phenotype and the development of SLE [190–194]. The apparent defect in DNA repair within NCM suggests that these cells may be more susceptible to DNA damage and genomic instability in SLE. Taken together, these signatures in SLE NCM underscore their inflammatory phenotype and suggest their distinctive metabolic needs within the autoimmune environment.

In our study, we observed that SLE NCM exhibit heightened DDR and elevated levels of p53, supporting the enriched inflammatory responses, activation of p53 pathway and altered DNA repair observed in the proteomic and transcriptomic signatures. Additionally, SLE NCM demonstrated reduced frequency in the peripheral blood of patients with active SLE which was further confirmed in a murine lupus peripheral monocytic subsets. The diminished presence of circulating NCM in the blood of SLE patients may be linked to their recruitment into various tissue targets, including the liver, as observed in patients with alcoholic hepatitis (AH) [195]. The interplay of immune cells within tissues holds pivotal significance in the pathogenesis of SLE, where the liver is identified as one of the primary causal tissues. Meanwhile, NCM

have been shown to play great role in the renal damage observed in SLE [147,196]. Of note, it has been revealed that NCM are increased in the periphery of active PsA patients, providing additional evidence that the decreased frequency of NCM is specific to SLE and not secondary to the inflammatory milieu [197]. Given the observed dysregulation in DNA repair and the heightened DDR in SLE NCM, and since these cascades are linked with cell cycle, we examined the status of the cell cycle, which also exhibits alterations within the proteomic signature of SLE NCM. We observed that SLE NCM accumulate in the G_0 phase of the cell cycle, suggesting the initiation of a differentiation program. A similar phenomenon has been observed in Hematopoietic Stem Cells (HSCs), which are capable of differentiating into restricted progenitors, such as common myeloid progenitors, without entering into the S phase of the cell cycle and undergoing cell division [198]. During terminal differentiation, most cells exit the cell cycle and enter a prolonged or permanent G_0 cell cycle arrest [199]. A link between monocyte to macrophage differentiation and induction of cell cycle arrest has been established [200]. The enhanced DNA damage and cell cycle arrest collectively reflect a state of differentiation and developmental alteration of NCM in the inflammatory environment of SLE.

Since the proteomic signature of SLE NCM denotes a differentiation and developmental process – a finding further validated from our *ex vivo* studies, we subsequently sought to delineate the differentiation potential of these cells. We revealed that SLE NCM display enhanced polarization towards an M1 macrophage phenotype, as indicated by elevated expression levels of CD80. Conversely, they displayed reduced polarization towards the dendritic cell phenotype, as evidenced by decreased expression levels of CD11c and CD123. Importantly, this particular feature is specific to SLE NCM, as the other two monocytic subsets did not exhibit altered levels of these surface markers. Furthermore, among the various immune cell populations examined, we noted an increased frequency of M1 macrophages and a decreased frequency of $CD11c^+$ DCs within the peripheral circulation of patients with active SLE. Our findings suggest that SLE NCM exhibit a phenotype that closely resembles that of M1 macrophages, thereby providing additional evidence for the heightened pro-inflammatory responses observed in these cells. Of note, we

demonstrated that NCM release pro-inflammatory M1 cytokines such as IL-1β (which was also found to be increased in the transcriptomic analysis exclusively in SLE NCM), IL-23, and Arginase, further underscoring the inflammatory potential of NCM in the periphery of patients with active SLE. It is important to note that monocytes serve as a critical link between mononuclear phagocyte precursors in the bone marrow (BM) with terminally differentiated mononuclear cells [121,201]. Emerging literature implicates autophagy as a key regulatory pathway in preventing monocytes from apoptosis and inducing their differentiation towards macrophages [125]. In the context of SLE, the enhanced autophagy observed in SLE NCM may prevent these cells from apoptosis and induce their differentiation towards an M1-macrophage like profile in the disease. The differentiation journey of the monocyte/macrophage lineage begins from molecular switching that unfolds in the periphery, but in the context of the disease their relation to resident or infiltrating cells is yet to be thoroughly depicted [120,202,203].

In summary, our study provides unique insights into the molecular signatures of monocytes in SLE. We report a unique proteomic and transcriptomic profile, specifically in the NCM, among patients with active SLE. This profile is characterized by enhanced inflammatory features and extensive perturbation. The pathogenic identity of SLE NCM is characterized by deregulated DNA repair, cell cycle and enhanced IFN signaling, together with cell differentiation and developmental cues. Experimental screening further emphasized the activation of p53 pathway and increased DDR, concomitant with G_0 cell cycle arrest in SLE NCM, reinforcing the notion of initiation of a differentiation program. This defective profile of NCM of patients with SLE is associated with an activated macrophage-like and enriched M1 pro-inflammatory response. Our findings suggest that enhanced autophagy may serve as a mechanism to prevent SLE NCM from apoptosis and support their skewing towards differentiation into a macrophage phenotype with M1 characteristics contributing to the inflammatory environment in the periphery where they may be damaging the endothelium prior to their migration to the tissue. It is noteworthy that endothelial injury is a prominent hallmark of SLE-associated vasculopathy, affecting various tissues, including blood vessels, brain, skin and kidneys [5,204].

Collectively, our findings underscore the significance of elucidating the molecular pathways that dominate each cell type in SLE. Such understating is imperative in order to better map their respective roles and functions in the disease and effectively target autoimmunity.

Limitations of the study

There remain some limitations in the present study that leave questions to be tackled in future endeavors. Firstly, despite using flow cytometry to isolate the monocytic subsets, we employed bulk RNA-sequencing technology, and as a result we cannot exclude the possibility that there is even greater heterogeneity inside each subset, yet to be described. Future studies focusing on these well-defined monocyte subsets as the single-cell level may provide further insights. Secondly, due to the limited number of cells, we were unable to concurrently perform both transcriptome and proteome analysis on the same cohort of patients and HI. Moreover, patients had received a variety of therapies in the past although recent cytotoxic or glucocorticosteroid administration was avoided. Finally, our study exclusively examined Caucasians, and therefore we cannot generalize our results to other ethnic groups. Notwithstanding these limitations, this is the first comprehensive transcriptomic and proteomic analysis of the three known subsets of peripheral monocytes in SLE combined with functional data and studies in animal models. Future research endeavors are poised to provide further clarity on these complex matters.

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