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Comprehensive analysis of immune dysregulation of peripheral blood mononuclear cells involved in SLE pathogenesis

Humbel Morgane

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UNIL | Université de Lausanne
Faculté de biologie et de médecine

Department of Immunology and Allergy

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Doctoral Thesis in Life Sciences (PhD)

Presented to the Faculty of Biology and Medicine of the
University of Lausanne
by

Morgane HUMBEL

Master of Biological Sciences

PhD Committee

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Dr. Denis Comte, Thesis Director
Prof. George Tsokos, Expert Harvard University
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Expert·e·s	Madame	Prof.	Déla	**Golshayan**
	Monsieur	Dr	Alexandre	**Harari**
	Monsieur	Prof.	George	**Tsokos**

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Comprehensive analysis of immune dysregulation of peripheral blood mononuclear cells involved in SLE pathogenesis

Lausanne, le 11 février 2022

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Caroline Pot-Kreis

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

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Comprehensive analysis of immune dysregulation of peripheral blood mononuclear cells involved in SLE pathogenesis

Morgane Helena Humbel
PhD Thesis Examination
2018-2022

Laboratory: Division of Immunology and allergy
Chief, Giuseppe Pantaleo, M.D.
Professor of Medicine
CHUV
Rue du Bugnon 46

Supervisor: Division of Immunology and allergy
Denis Comte, M.D.
CHUV BH10-527
Rue du Bugnon 46

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Abstract English

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease. The disease etiology remains unclear. Alterations of the innate and the adaptive immune system contribute to a loss of tolerance, which promotes the production of autoantibodies leading to tissue damage. The symptoms presented by SLE patients are very heterogenous, making the diagnosis complicated. The treatment of SLE patients mainly relies on immunosuppressive drugs.

The aim of this project was to better understand the pathophysiology of SLE with the perspective of identifying novel diagnostic and therapeutic approaches. To do this, we first performed single cell mass cytometry to study the cellular phenotype of peripheral blood mononuclear cells (PBMC), focusing on signaling lymphocytic activation molecule family (SLAMF) receptors, in SLE patients, healthy and autoimmune diseases (sarcoidosis, Sjögren's syndrome and multiple sclerosis) controls. We identified that the frequency of SLAMF4+ monocytes and SLAMF4+ natural killer (NK) inversely correlated with SLE disease activity, whereas the frequency SLAMF1+ CD4+ terminally differentiated effector memory T (TDEM) cells directly correlated with disease activity. Accordingly, these cell subtypes could be measured to determine SLE disease activity. Furthermore, we found that SLAMF1+ B (SLEB1) cells, SLAMF1+SLAMF3+SLAMF5+SLAMF6+ switch memory B cells (SLESMB) and SLAMF1+SLAMF3+SLAMF5+SLAMF6+ circulating T follicular helper (SLEcTFH) cells were specifically increased in SLE compared to all controls. The combined measurement of SLEB1 cells or SLESMB cells with SLEcTFH cells allowed discriminated SLE patients from autoimmune controls in 90% of the cases. These results suggest that the measurement of these cell subsets in peripheral blood can be used to accurately diagnose SLE. Our data also highlight the importance of SLAMF receptors in the pathophysiology of SLE.

In the second part of this project, we investigated the role of NK cells in SLE pathogenesis. We examined the extracellular phenotype of NK cells and observed that SLE NK cells express a higher level of CD38 and do not properly upregulate SLAMF7 upon activation compared to their healthy counterparts. We further investigated the role of these two receptors, engaging them with monoclonal antibodies, daratumumab and elotuzumab respectively. We demonstrated that both antibodies promote cytokine production and cytotoxicity of SLE NK cells. Finally, we evaluated how ligation of CD38 and SLAMF7 influences the interaction between NK cell and plasma cells, two cell populations that express high levels of these two receptors. Using a B-NK cell co-culture system, we showed that daratumumab promotes the specific killing of SLE circulating plasma cells. Therefore, targeting CD38 and SLAMF7 may represent potential therapeutic targets for SLE.

In conclusion, in this PhD thesis project, we identified new possible approaches to diagnose and treat SLE.

Abstract French

Le lupus érythémateux systémique (LES) est une maladie auto-immune inflammatoire chronique. L'étiologie de la maladie reste peu claire. Les altérations du système immunitaire inné et adaptatif contribuent à une perte de tolérance, qui favorise la production d'auto-anticorps conduisant à des lésions tissulaires. Les symptômes présentés par les patients atteints de LES sont hétérogènes, ce qui rend le diagnostic compliqué. Le traitement des patients LES repose principalement sur des médicaments immunosuppresseurs. L'objectif de ce projet est de mieux comprendre la physiopathologie du LES dans la perspective d'identifier de nouvelles approches diagnostiques et thérapeutiques. Pour ce faire, nous avons exploité la cytométrie de masse pour étudier le phénotype de surface des cellules mononucléaires du sang périphérique (PBMC), en nous concentrant sur les récepteurs de la famille des molécules d'activation lymphocytaire de signalisation (SLAMF), chez des patients LES, des contrôles sains et contrôles atteints de maladies auto-immunes. Nous avons identifié que la fréquence des monocytes SLAMF4+ et des cellules natural killer (NK) SLAMF4+ est inversement corrélée à l'activité de la maladie, tandis que la fréquence des cellules T CD4+ mémoires effectrices différenciées terminales SLAMF1+ est positivement corrélée à l'activité de la maladie. En conséquence, ces sous-types cellulaires pourraient servir pour déterminer l'activité de la maladie. En outre, nous avons constaté que les cellules B SLAMF1+ (SLEB1), les cellules B switch memory SLAMF1+SLAMF3+SLAMF5+SLAMF6+ (SLESMB) et les cellules circulantes T follicular helper SLAMF1+SLAMF3+SLAMF5+SLAMF6+ (SLEcTFH) sont spécifiquement augmentées dans le LES par rapport aux contrôles. La mesure combinée des cellules SLEB1/SLESMB avec les cellules SLEcTFH permet de discriminer les patients LES par rapport aux contrôles auto-immuns dans 90% des cas. Ces résultats indiquent que la mesure de ces sous-types cellulaires dans le sang périphérique peut être utilisé pour diagnostiquer le LES et suggèrent une implication des récepteurs SLAMF dans la physiopathologie du LES. Dans la deuxième partie de ce projet, nous avons étudié le rôle des cellules NK dans la pathogenèse du LES. Nous avons examiné le phénotype extracellulaire des cellules NK du LES et observé qu'elles expriment CD38 de manière accrue de CD38 et que SLAMF7 n'est pas régulé adéquatement lors de leur activation. Nous avons étudié le rôle de ces deux récepteurs, en les engageant avec des anticorps monoclonaux, daratumumab et elotuzumab respectivement. La liaison de ces anticorps favorise la production de cytokines et la cytotoxicité des cellules NK du LES. Enfin, nous avons examiné comment la liaison de CD38 et SLAMF7 influence l'interaction entre les cellules NK et les plasmocytes, en utilisant un système de co-culture cellulaire B-NK. Nous avons montré que le daratumumab favorise la destruction spécifique des plasmocytes circulants du LES. Par conséquent, SLAMF7 et CD38 peuvent représenter des cibles thérapeutiques potentielles pour le LES. En conclusion, ce projet de thèse de doctorat a permis d'identifier de nouvelles approches possibles pour diagnostiquer et traiter le LES.

List of abbreviations

ACR	American College of Rheumatology
ADCC	Antibody dependent cellular cytotoxicity
ANA	Anti-nuclear antibodies
APC	Antigen presenting cell
BILAG	British Isles Lupus Assessment Group
BICLA	BILAG-based Combined Lupus Assessment
CAR-T	Chimeric antigen receptor T cell
CB-CAP	Cell-bound complement activation products
CD	Cluster of differentiation
cTfh	irculating T follicular helper cells
CVD	Cardiovascular diseases
C1q/C4	Complement component 1q/4
DNA	Deoxyribonucleic acid
EAT2	Ewing's sarcoma-associated transcript 2
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immuno absorbent spot
EULAR	European Alliance of Associations for Rheumatology
FCγR	FC γ Receptor
FDA	Food and Drug Administration
FNA	Fine needle aspiration
GC	Germinal center
HLA	Human leukocyte antigen
HLA-DR	HLA DR isotype
IFN	Interferon
IFNAR1	Type I IFN receptor subunit 1
IL	Interleukin
IRF5	Interferon regulatory factor 5
ITSM	Immunobase tyrosine-based switch motif
LN	Lymph node
mDC	Myeloid dendritic cell
MS	Multiple sclerosis
M1	Classically activated macrophage
M2	Alternatively activated macrophage
NET	Neutrophil extracellular trap
NK	Natural killer cells
NSAID	Nonsteroidal anti-inflammatory drugs
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
RA	Rheumatoid arthritis
SAP	SLAMF associated protein
SLAMF	Signaling lymphocytic activation molecule family
SLE	Systemic Lupus Erythematosus
SRI-4	SLE responder index-4
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Tfh	T follicular helper cell
Tfr	T follicular regulatory cells
TNF	Tumor necrosis factor
TNFSF4	TNF ligand superfamily member 4
TLR	Toll like receptor
Treg	T regulatory cells
TREX1	Three-prime repair exonuclease 1
T1D	Type 1 diabetes
UV	Ultra violet

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1. Introduction

1.1. SLE prevalence

Systemic lupus erythematosus (SLE) is a chronic multi-system autoimmune disease. The prevalence of SLE ranges from 20 to 150 cases per 100’000 individuals. The exact prevalence is difficult to determine, as diagnosis remains challenging and clinical presentation is highly variable. SLE mainly affects women of the childbearing age. Furthermore, African, Asian, Hispanic and native-American populations are more affected, and experience higher mortality, compared to other ethnicities ([1]–[4]).

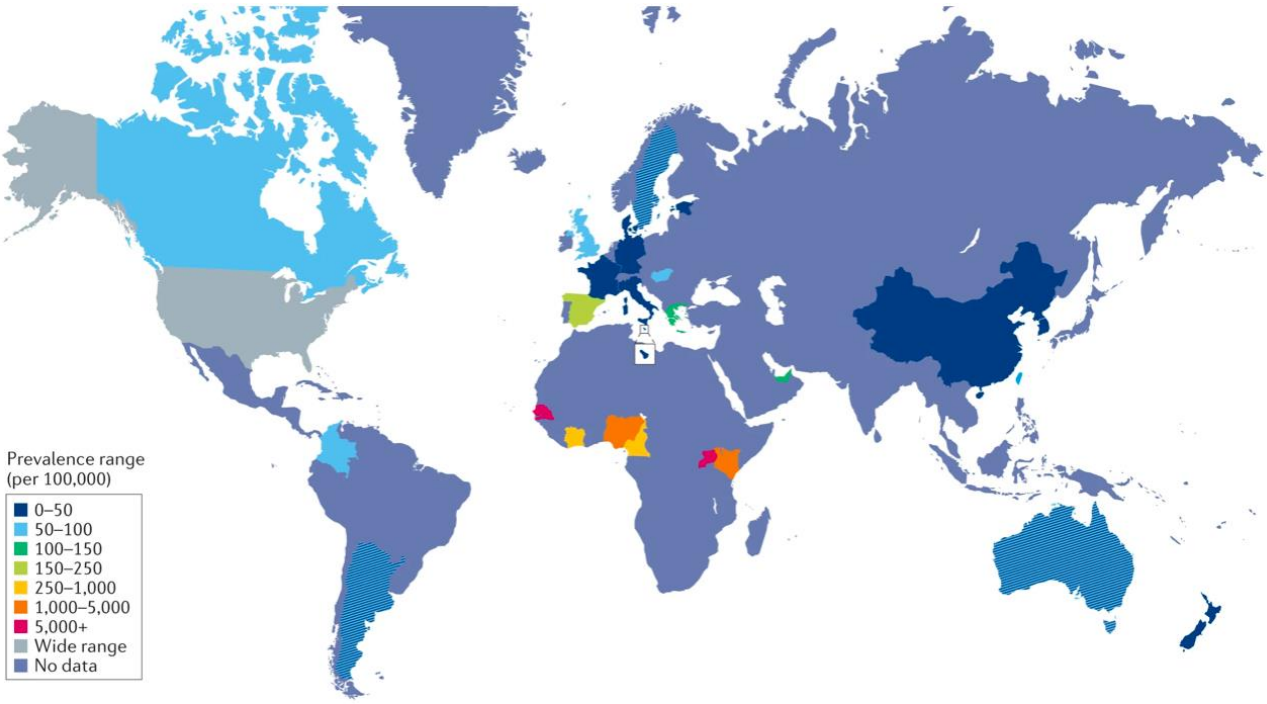

Figure 1. Prevalence range of SLE in the world. This figure (from Nature Reviews Rheumatology [4]) shows the prevalence of SLE in the world according to the reported global values.

1.2. SLE Susceptibility Factors

The etiology of SLE remains poorly understood, but involves environmental, hormonal, epigenetic and genetic factors which are associated with an increased susceptibility to developing the disease. A widely accepted model of SLE suggests that a genetic predisposition is essential for the disease to develop in the presence of the other factors mentioned above [5].

The **environmental** susceptibility factors are mainly exposure to UV light, smoking and infections.

Exposure to UV light can lead to the activation of several pathways, such as cellular apoptosis, immune complex formation, and inflammation, that can promote the symptoms of cutaneous lupus erythematosus. Furthermore, this could be the initial trigger that promotes the inflammatory and autoreactive environment at a systemic level ([6]–[8]).

Smoking promotes the generation of reactive oxygen species and subsequent genetic mutations. Altered DNA can be recognized as non-self by the immune system, promoting the generation of autoantibodies targeting double stranded DNA [9]. Furthermore, smoking promotes immune activation [10] and might contribute to the chronic inflammation seen in SLE patients.

Infections by viruses can induce an autoimmune response due to molecular mimicry. Some viral peptides are molecularly almost identical to self-peptides and can cause T and B cells cross-reactivity, leading to immune responses [11]. The best study example is chronic Epstein-Barr Virus (EBV) infection, which is associated with an increased susceptibility to develop autoimmune connective tissue diseases such as rheumatoid arthritis, primary Sjögren's syndrome and SLE [12].

Epigenetic changes, such as DNA acetylation and methylation, lead to changes in gene expression and activity. These modifications are influenced by environmental factors (including drugs, aging, toxins) and can contribute to normal development processes or neopathogenesis [13]. Inhibition of DNA methylation can lead to the generation of autoreactive T cells [14]. In SLE CD4+ T cells are hypomethylated [15], leading to the increased expression of several genes linked to the SLE pathophysiology (autoreactive T cells contribute to self-reactive B cell activation).

Gender and related hormones are among the most important susceptibility factors for SLE. Indeed, the disease is significantly more prevalent in women of childbearing age (men to women ratio 1:10) and pregnancy can worsen the disease [16]. This is thought to be partly due to the X chromosome, that carries many of the altered genes contributing to the susceptibility for SLE, and partly to the female hormones, such as estrogen, that have been associated with lupus severity [17]. It is of note, that although the disease is rarer in male individuals, they often present more severe symptoms, with a higher incidence of nephritic dysfunctions [17].

The role of **genetic factors** in lupus is undeniable. Studies have consistently observed variable degrees of heritability, and indicate that the disease may follow an additive model of polygenic inheritance [18]. SLE is not caused by a single dominant gene defect, although monogenic SLE has been described in rare cases, involving complement factors (C1q or C4 deficiency). In most cases, SLE is associated with a combination of variations in several genes. These variations have been observed in HLA and non-HLA genes. Genes variants associated to SLE mainly include complement genes but also HLA-DR2 and HLA-DR3 [19]. Other non-HLA variants include genes implicated in inflammatory pathways (i.e. IRF5, STAT4, C1q, TREX1) and T-B cell activation (i.e. FCγR,

TNFSF4, IL10) [19]. Based on current available data, it is likely that a genetic predisposition is required to develop SLE in presence of other susceptibility factors.

1.3. SLE Clinical Manifestations

The variability of the susceptibility factors and their possible combinations partly explain the heterogeneity of the disease symptomatology. SLE can virtually involve every organ of the body, and clinical manifestations vary widely from patient to patient, and during the disease evolution over time. Patients usually present constitutional symptoms, including fatigue, fever, and weight loss. In some instances, SLE clinical manifestations are mild, involving skin (rash, discoid skin manifestations) and joints (arthritis), whereas more severe forms, associated to high morbidity and mortality, present renal failure, hematological disorders and / or neuropsychiatric manifestations.

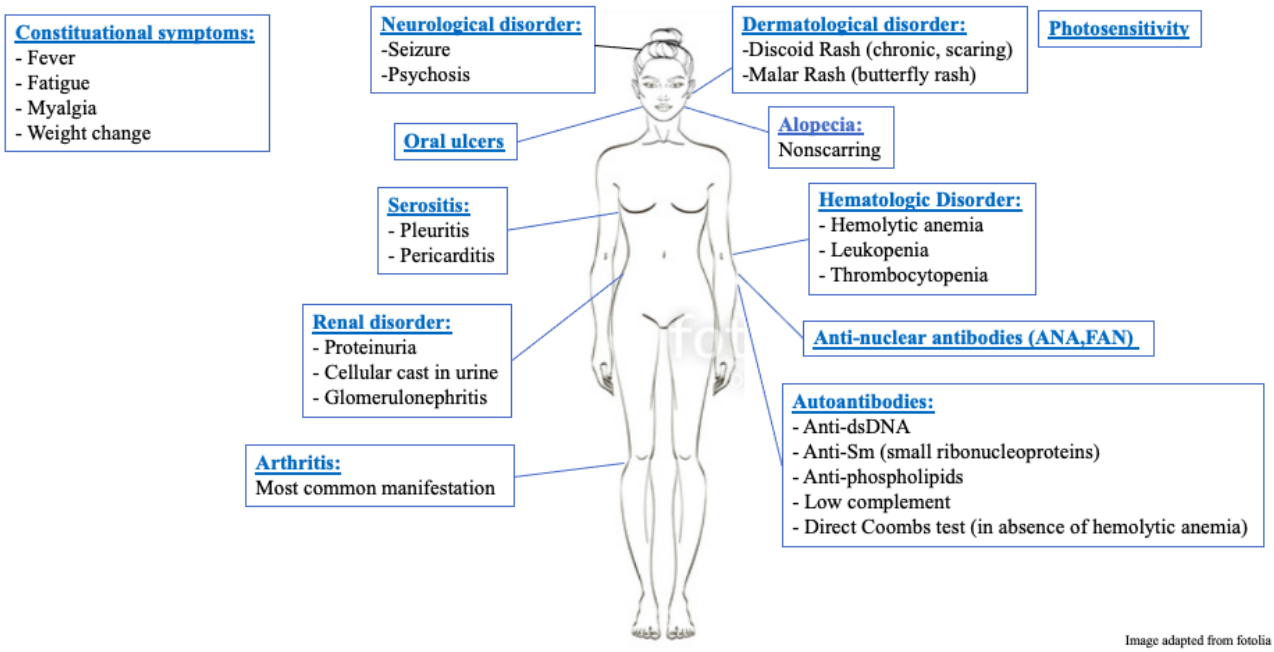

Figure 2. Clinical presentation of SLE. This figure shows details the different symptomatologic presentations of SLE.

In addition, SLE patients face serious complications related to the dysregulation of the immune system as well as long lasting immunosuppressive treatments. These complications include infections, increased rate of cardiovascular diseases and cancer.

Higher susceptibility to **infection** (bacterial, viral, and parasitic, in order of occurrence) is related mostly to the dysfunction of cytotoxic cells (both CD8+T cells and natural killer (NK) cells, further details on this subject can be found in Chapter 1.4). Patients are more susceptible to infections during disease flares and following long lasting treatment with immunosuppressive agents (see Chapter 1.6)

[20]. To prevent these complications, the main clinical approach is prevention, thus SLE patients are vaccinated against all main infectious agents [20].

The higher rate of **cardiovascular diseases (CVD)** in lupus patients has been associated (in addition to glucocorticoid use) with chronic inflammation and with a sedentary life style, which is due to fatigue and pain [21].

Patients with SLE are also more likely to develop several types of **cancers**, including lymphoma (Hodgkin's and non-Hodgkin's lymphoma), leukemia, multiple myeloma, lung and cervix cancer [22]. The precise underlying cause is not known. B-cell lymphomas may be linked to an overstimulation of B cells and defects in the immune surveillance [23]. From this point of view, defective SLE NK cells may be permissive to tumor cell proliferation and contribute to cancer development (see Chapter 1.4 for more information).

1.4. SLE pathophysiology

The pathophysiology of SLE remains incompletely understood. It involves complex dysregulations of both the innate and the adaptive immune system.

1.4.1. Innate immune cells in SLE pathophysiology

The **innate immune system** is the first line of defense of the human body. It is in charge of rapidly identifying and eliminating foreign pathogens or damaged cells, and of activating the adaptive immune system. Although the role of innate immune cells in SLE pathophysiology has been less studied compared to the adaptive immune system, studies show an impaired function of many innate immune cells.

Macrophages in SLE have reduced phagocytic activity, which contributes to the accumulation of cellular debris in the extracellular milieu. The balance between classically activated (M1) and alternatively activated (M2) is skewed in favor of classically activated cells. These cells are induced by IFNγ and are involved in inflammation and tissue damage [24].

Neutrophils in SLE have a reduced phagocytic capacity while the generation of NETs (neutrophil extracellular trap) is accelerated due to the presence of anti-ribonucleotide complexes and apoptotic particles in the extracellular milieu. In addition, the process responsible for the degradation of NETs is impaired, due to the presence of anti-NET antibodies and DNase inhibitors. Overall, the accumulation of NETs promotes the production of type I interferons and the activation of plasmacytoid dendritic cells [24].

Dendritic cells in SLE have aberrant capacities for maturation, antigen uptake and presentation. They play an important role in the processing and presentation of self-antigens to T cells, thus leading to the development of autoreactive T and B cells. Moreover, plasmacytoid dendritic cells (pDC), in response to NETs activation, internalize immune complexes. This in turn activates TLR7/9 leading to the production of IFNγ, which promotes the activation of the innate immune system and differentiation of cells of the adaptive immune system that contribute to the inflammatory state (i.e. generation of plasmablasts). Myeloid dendritic cells (mDC) secrete pro-inflammatory cytokines, such as IL6, which also promote B cell maturation into plasma cells [24].

Natural killer (NK) **cells** represent the third biggest lymphocyte population (after T and B cells). They were first identified as cells capable of killing infected or tumor cells, but today we know that they also have an important role in immunosurveillance [25].

Several studies have shown that NK cells play a role in autoimmune diseases, including type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), Crohn's Disease and SLE.

The exact role of NK cells in autoimmunity remains elusive, however, in all the above-mentioned diseases, quantitative and qualitative alterations have been observed in NK cells compared to healthy controls. More specifically, in SLE, NK cells are reduced in number, have reduced cytokine production and impaired cytotoxicity [25].

Although NK cells could contribute to organ/joint damage that occurs in autoimmune diseases, in many cases it is difficult to obtain organ biopsies to confirm this hypothesis. Conversely, NK cells may have a protective role and it would therefore be beneficial to increase their number and promote their function. This hypothesis is supported by a study in lpr murine model of SLE, which showed that adoptive transfer of NK cells delays the development of autoimmunity [26]. Furthermore, when low-dose IL2 was administrated to SLE patients, among the changes in the distribution of peripheral blood cell, an increase in NK cells number was observed. This increase was associated with a reduction of the disease severity [27]. This suggests that a higher number of NK cells may be linked to a favorable disease outcome. Furthermore, two studies on murine models of MS [28] and RA [29] have shown that, after activation, NK cells can attack autoreactive T cells, thereby reducing inflammation and diseases activity, suggesting that higher NK cells activity may be beneficial in autoimmune diseases. Studies on the importance of NK cells in SLE are still in their early stage and require further investigations.

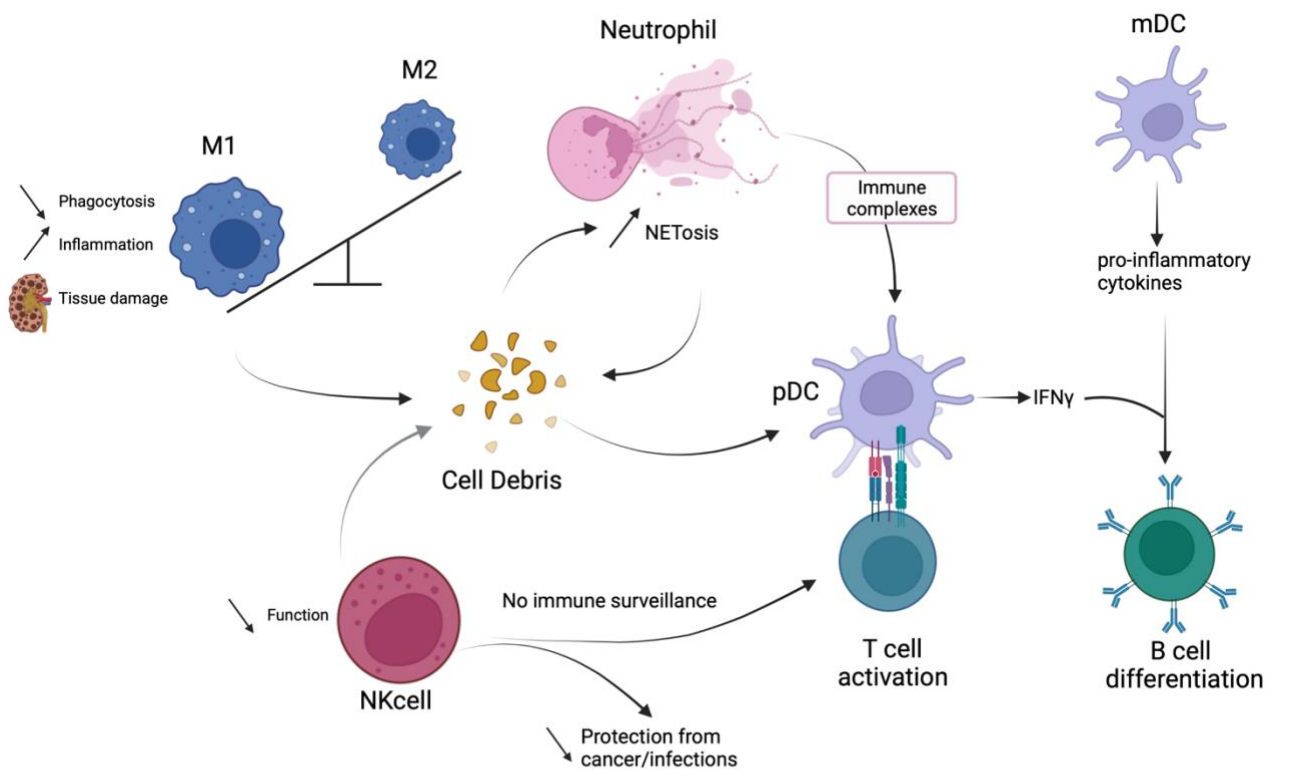

Figure 3. Innate immune cells in SLE pathophysiology. Summary of main alterations of the innate immune cells in SLE patients (created with BioRender.com).

1.4.2. Adaptive immune cells in SLE pathophysiology

The role of the **adaptive immune system** in the pathophysiology of SLE has been long established, however many questions remain unanswered.

T cells are classified into two main populations: CD8+ T cytotoxic cells and CD4+ T helper (Th) cells.

Cytotoxic T cells (CD8+ T cells) are responsible for the recognition and clearance of infected cells. Similarly to NK cells, their role in SLE is not completely understood. Several groups reported ([30], [31]) that SLE peripheral CD8+ T cells have reduced effector functions (cytolytic and cytokine production) contributing to the persistence of autoreactive B cells and increased risk of infection ([1], [32]). Studies on murine SLE models, showed that dysfunctional CD8+ T cells accelerate humoral autoimmunity [33]. This indicates that the cellular defect of SLE CD8+ T cells contributes to the pathophysiology. On the other hand, kidney biopsies from lupus nephritis present an important infiltration by tissue resident CD8+ T cells, which is linked to the disease activity ([34]–[36]). This suggests an active cytotoxic role of CD8+ T cells promoting tissue damage and accumulation of

cellular debris. Further research is necessary to completely understand the involvement of CD8+ T cells in SLE pathophysiology.

Interestingly, tissue resident CD8+ T cells that are exposed to elevated autoantigen stimulation can become **double negative T cells** (CD3+ CD4- CD8-). These cells produce IL17, interact with B cells to promote the production of autoantibodies and directly infiltrate tissues [37].

T helper cells (CD4+ T cells), which are responsible for helping B cell activation and maintenance, are divided into different populations, based on their cytokine production profile, extracellular receptor, and transcription factor expression. **SLE Th1** cells produce high levels of IFNγ, which is important for inflammation and T cell mediated activation of B cells, and reduced amounts of IL2, which is responsible for the survival of T regulatory cells (Treg) [38]. **SLE Th2** cells produce reduced levels of IL4, thus indirectly promoting the generation of autoantibodies. Furthermore, high levels of IL5 are observed in SLE skin inflammation [39]. Th17 cells mainly produce IL17A and IL23. **SLE Th17** are increased in number, leading to high levels of IL17A, which contributes to inflammation and autoantibody generation [40]. IL23 promotes the differentiation of Th cells into Th17 and limits the levels of IL2.

Regulatory T (Treg) **cells** are responsible for the maintenance of self-tolerance. Regulatory T cells do not function properly in SLE patients (which could be related to the reduced levels of IL2 observed in SLE patients), which may contribute to the persistence of autoreactive T cells [40]. Published data on the abundance of Tregs in the peripheral blood of patients with SLE diverge from study to study. This is due, at least in part, to inconsistent definitions of Treg, which rely either on the expression of phenotype markers (cell surface markers, transcription factors), analysis of the demethylation of the FoxP3 promoter or functional assays (*ex vivo* suppressive assays).

Because SLE is characterized by a high production of autoantibodies, examination of **T follicular helper cells** (Tfh) is of utmost importance. Tfh cells ($CXCR5^+ICOS^+PD1^+BCL6^+$) typically produce IL21, which is required for germinal center (GCs) development and generation of plasma cells.

In autoimmune diseases, GC arise spontaneously, contributing to the aberrant production of antibodies. Speculated mechanisms for the development of autoimmunity suggest that high levels of IL6, IL21 and IL23 upregulate BCL6 in CD4+ T cells, which promotes the differentiation of (self-reactive) Tfh cells, leading to spontaneous GC formation [41]. In addition, upregulation of Blimp1, responsible for inhibiting Tfh differentiation in response to IL2, is reduced in SLE Tfh cells due to a reduced availability of IL2 cytokine [41]. **T follicular regulatory cells** (Tfr) are cells derived from T regulatory cells and express Foxp3. Tfr cells represent the regulatory counterpart of Tfh that suppresses GC formation and are reduced in SLE secondary lymphoid organs [41].

Due to limited access to SLE lymph nodes and other secondary lymphoid organs, only few studies are available on SLE Tfh cells. Therefore, most human studies focused on SLE **circulating T follicular helper cells** (**cTfh**).

Circulating T follicular helper cells are classified in three main sub-populations that reflect the T helper subpopulation in their properties. Circulating Tfh Th1 like cells ($CXCR3^{+}CCR6^{-}$) that produce IFNγ and are considered non efficient helper cells. The other two subsets, Tfh Th2 like ($CXCR3^{-}CCR6^{-}$) that produce IL4, IL5, IL13 and cTfh Th17 like cells ($CXCR3^{-}CCR6^{+}$) that produce IL17A and IL22, are considered efficient helper cells.

Patients with active autoimmune diseases (like Sjögren's syndrome, RA and MS) display altered composition of cTfh sub-populations, showing a decrease in cTfh Th1 like cells and an increase in cTfh Th2 and cTfh Th17 like cells [42]. In SLE, cTfh cells ($CXCR5^{high}$ $ICOS^{high}$ $PD1^{high}$) are expanded [43] and their increase correlates with disease activity. This suggests an abnormal T-B cell interaction, which promotes activity of B cells and autoantibody production. Nevertheless, further studies are needed to clarify how this abnormality in the periphery is correlated to B cell maturation and autoantibody production at the level of secondary lymphoid organs.

B cells are probably the most important cell population involved in the pathogenesis of SLE, as they are responsible for the production of autoantibodies and contribute to tissue damage.

In SLE patients, B cells have exaggerated BCR signaling after activation and a lower activation threshold, resulting in highly activated and potentially autoreactive B cells [44]. After antigen recognition, some B cells become short-lived low affinity antibody producing cells that remain in periphery, whereas others migrate to the germinal center, where they interact with Tfh cells, undergo affinity maturation, and become memory B cells, high affinity plasma cells or long-lived plasma cells. The production of autoantibodies by both short- and long-lived plasma cells, is probably why drugs, such as rituximab, targeting short lived plasma cells (CD20+), only have limited therapeutic effect.

1.4.3. Antibodies and complement system in SLE pathophysiology

SLE patients are characterized by the presence of autoantibodies. Autoantibodies act in three ways to contribute to the pathophysiology of the disease. Firstly, they bind to self-cells leading to their destruction. Secondly, they bind to antigens to form immune complexes, which deposit in small blood vessels and trigger organ inflammation. Thirdly, they activate the complement system. The complement system is a part of the innate immune system that promotes the clearance of apoptotic bodies, infected and altered cells. There are three main pathways of the complement: classical, alternative and lectine pathway. All of these pathways rely on the presence of proteins produced by

the liver, which upon stimulation, are progressively cleaved leading to the end result of phagocytic cell activation, inflammation and the activation of the cell-membrane attack complex.

Patients with SLE present mainly with alterations of the classical pathway. Serum levels of some complement members, such as C1q and C4 are reduced in SLE serum (especially due to anti-C1q antibodies) and represent another reason for the accumulation of cellular debris.

Accumulated cellular debris contain self-nuclear antigens (i.e. DNA, RNA, histones, etc), which under physiological conditions are rapidly cleared. These self-antigens are accessible to the immune system thus leading to abnormal activation and the generation of autoreactive cells. Furthermore, autoantibodies promote activation of the complement system, thereby enhancing inflammation.

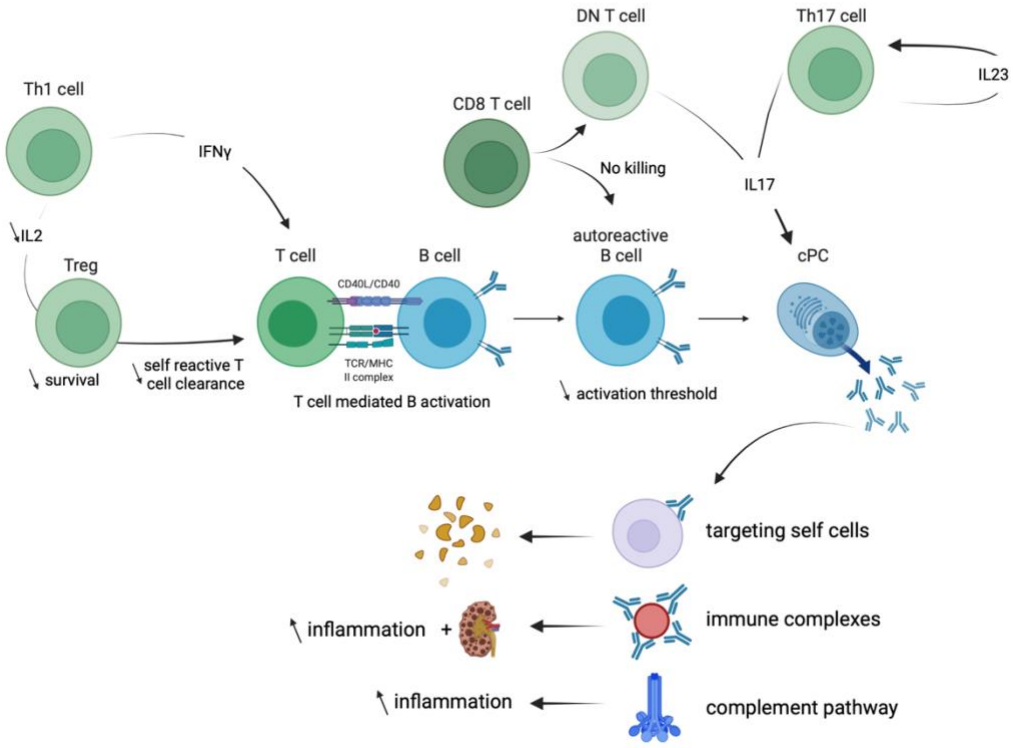

Figure 4. Adaptive immune cells in SLE pathophysiology. Summary of main alterations of the adaptive immune cells in SLE patients (created with BioRender.com).

1.4.4. Signaling lymphocytic activation molecule family receptors

A genome wide association study made at the turn of the century [45] identified genetic factors associated with a higher susceptibility to develop SLE. In this study, the authors describe a family of receptors encoded in the 1q21 region of chromosome 1 called signaling lymphocytic activation molecule family (SLAMF). SLAMF receptors are a family of nine members that belong to the CD2

superfamily of immunoglobulin containing molecules. This type I transmembrane glycoprotein receptors are expressed on all hematopoietic cells, are self-ligands (made exception for SLAMF2 and SLAMF4 which bind each other) and when bound provide a strong co-stimulatory or co-inhibitory signal [46]. Their function can be different depending on the cell type they are expressed on. Furthermore, alterations in the expression of these surface receptors have been reported in SLE patients.

SLAMF1 (CD150 or SLAM) is mainly expressed on memory CD4+, CD8+ T cells and dendritic cells [47]. Its expression is increased on total CD4+ T cells and B cells of SLE patients [48] and its ligation with a specific monoclonal antibody reduces T-B cell interaction and lowers IL6 production by B cells in both HC and SLE patients [49].

SLAMF2 (CD48) is structurally distinct from the other SLAMF members, since it lacks a cytoplasmic tail. This receptor is a component of lipid rafts and its engagement leads to an enhanced early TCR signaling. On the surface of SLE T cells, lipid rafts exist in a pre-aggregated form, which explains the rapid signaling and the high level of calcium flux that are observed in T cells [50]. SLAMF2 was observed to be increased in SLE CD4+ and CD8+ T cells independently of their differentiation status [48]. Furthermore, SLAMF2 overexpression probably allows the prolonged interaction between DCs and APCs observed in SLE [51].

SLAMF3 (CD229 or Ly9) is mainly expressed on T, B, and NK cells. In SLE patients SLAMF3 expression is decreased on NK cells and increased on naïve T cells (both CD4+ and CD8+) [48]. SLAMF3 has been shown to play a fundamental role in the maintenance of tolerance, since SLAMF3 deficient mice develop autoimmunity [52]. Furthermore, it was shown *in vitro* that the engagement of this receptor on CD4+ T cells via monoclonal antibodies promotes cell proliferation, inhibits the production of IFNγ and promotes the expression and signaling of IL2. This promotes the generation of functional regulatory T cells and therefore the generation of a tolerogenic environment [53]. In the context of SLE, CD4+ T cells have been shown to have a defective production and signaling of IL2, which could be normalized with anti-SLAMF3 antibodies, thus representing a potential therapeutic target [54] to restore the activity of regulatory T cells.

SLAMF4 (CD244 or 2B4) is mainly expressed on cytotoxic cells (CD8+ T and NK cells). Nonetheless it is also detected on double negative (CD4-CD8-) T cells, CD4+ T cells and monocytes [48]. SLAMF4+ cytotoxic cells are more active (production of IFNγ, granzymes, perforin) compared to SLAMF4- cells [50]. SLE patients have less CD8+ SLAMF4+ T cells compared to HC. The reduced number of these cells could explain the reduced protection against infections that characterizes lupus patients. Furthermore, CD8+ SLAMF4- cells are hypothesized to become double

negative T cells, a key contributor to SLE pathogenesis, which produce pro-inflammatory cytokines, for instance IL17, thus promoting organ damage [50].

Finally, it was shown that SLE patients present reduced frequencies of SLAMF4+ NK cells and monocytes [55].

SLAMF5 (CD84) is expressed on all major circulating cell subsets. The information on the role of this receptor in SLE are limited and contradictive. While one study on lupus nephritis patients showed decreased levels of SLAMF5 (together with SLAMF3 and SLAMF7) on CD8+ T cells and plasmacytoid dendritic cells, other studies did not find such divergence. Functionally, it was suggested that the engagement of SLAMF5 with monoclonal antibodies leads to the production of IFNγ, a cytokine that is highly present in SLE patients [50].

SLAMF6 (CD352, NTBA or SF2000 in human or Ly108 in mice) is one of the receptors of this family that has been less studied. Nonetheless, in recent years, studies suggested it could be an immune checkpoint inhibitor [56]. The group of Terhorst [57] demonstrated that the injection of a murine cancer model with anti-SLAMF6 antibody led to an activation of exhausted CD8+ T cells (as shown with an increased production of CD107a, IFNγ, granzyme B and IL2). In a study in 2017, Terhorst's group found that the injection of SLAMF6 negative CD4+ T cells in mice generated lupus-like autoimmunity, with the production of autoantibodies and increased frequencies of T follicular helper cells and germinal center B cells [58]. Conversely, a study on SLE patients with lupus nephritis, showed that CD4+ SLAMF6+ frequency was increased in active lupus patients compared to those in remission. In this study, it was also observed that patients with increased frequency of SLAMF6+ DN T cells did not respond to B cell depletion therapy. Studies on a larger scale will be necessary to confirm whether this information can be exploited for personalized therapy approaches [59]. A study [60] focusing on SLE patient's peripheral blood cells, found that SLAMF6 is a co-receptor important for the production of IFNγ and TNFα by T cells. Furthermore, they show that this ability is compromised in SLE T cells (although they found similar expression patterns of the receptor in HC and SLE patients PBMC).

SLAMF7 (CD319, CS1 or CRACC) is mostly expressed by cytotoxic cells and plasma cells. In SLE, its expression is decreased on CD8+ T cells and DN T cells, while it is increased on $CD56^{low}$ NK cells. The engagement of SLAMF7 with a monoclonal antibody on CD8+ T cells leads to an enhanced anti-viral response [30]. This receptor has also been greatly studied in oncology, where an anti-SLAMF7 specific monoclonal antibody is used for the treatment of multiple myeloma (MM). The humanized monoclonal antibody (elotuzumab) binds MM cells (derived from plasma cells) activating so antibody dependent cellular cytotoxicity (ADCC) and simultaneously binds and

activates NK cells. Elotuzumab was accepted by the FDA and SwissMedic for the treatment of MM but is normally used as a last resort and in combination with other drugs.

The non-conventional SLAMF family receptors, SLAMF8 and SLAMF9, have been identified more recently compared to conventional family members, which is why their role is less well studied. These two receptors both lack the cytoplasmic ITSM fragments that characterize the other SLAMF receptors.

SLAMF8 (CD353 or BLAME) expression has been reported on monocytes, dendritic cells and on mature B cells [61]. Although no information is available on its expression in lupus PBMC, it has been described in the context of oncology. SLAMF8 is overexpressed in glioma, ovarian and gastric cancer cells. It has been suggested that SLAMF8 suppresses the function of macrophages while promoting mast cell function (due to the lack of SAP and EAT-2 in the latter) [62]. In general, it probably promotes inflammation and immune cell activity, but further investigations are necessary to confirm this and to understand its implications for autoimmunity.

SLAMF9 (**CD84-H1 or SF2001)** is expressed by mononuclear phagocytes and plasmacytoid dendritic cells. Its expression increases with differentiation of monocytes into macrophages. It has been suggested that SLAMF9 is involved in the pDC differentiation and function [63] as well as in the initiation of inflammation [64]. Here too, further studies are warranted.

1.5. SLE diagnosis and biomarkers

Due to the highly heterogenous presentation of SLE and the absence of a specific test, diagnosis remains challenging.

The diagnostic criteria (ACR-EULAR 2019) of SLE are based on a combination of three immunological features (i.e. high ANA levels, antiphospholipid proteins and/or decreased complement proteins in periphery) and seven clinical symptoms (i.e. hematologic, neuropsychiatric, mucocutaneous, serosal, musculoskeletal, renal symptoms) that carry different weights in a scoring system. A minimum of ten points is required for the SLE classification. It is of note that several autoimmune diseases can present overlapping features and, according to the diagnostic criteria, it is up to the clinician to exclude any differential diagnosis.

In the absence of specific laboratory tests for SLE, a delay between initial symptoms and definitive diagnosis is often observed and associated with an unfavorable prognosis and permanent organ damages, especially in the case of brain or renal involvement [65].

Entry Criterion Anti-nuclear antibodies at a titer of >180 on Hep-2 or equivalent positive test			
Additive Criteria 1) Do not count a criterion if an explanation other than SLE is more likely 2) Occurrence of a criterion on at least one occasion is sufficient 3) At least one clinical criterion is required 4) Criteria need not occur simultaneously 5) Within each domain, only the highest weighted criterion is counted toward the total score			
Clinical domains and criteria	**Weight**	**Immunological domains and criteria**	**Weight**
Constitutional		**Anti-phospholipid antibodies**	2
Fever	2	Anti-cardiolipin antibodies or anti-β2GP1 antibodies or lupus anticoagulant	
Cutaneous		**Complement protein**	
Non-scarring alopecia	2	Low C3 or low C4	3
Oral ulcers	2	Low C3 and low C4	4
Subacute cutaneous or discoid lupus	4		
Acute cutaneous lupus	6		
Arthritis		**Highly specific antibodies**	
Either synovitis characterized by swelling or effusion in ≥two joints or tenderness in ≥two joints plus ≥30min of morning stiffness	6	Anti-dsDNA antibody Anti-Smith antibody	6 6
Neurological			
Delirium	2		
Psychosis	3		
Seizure	5		
Serositis			
Pleura or pericardial effusion	5		
Acute pericarditis	6		
Hematological			
Leucopenia	3		
Thrombocytopenia	4		
Autoimmune haemolysis	4		
Renal			
Proteinuria >0-5g/24h	4		
Renal biopsy class II or V lupus nephritis	8		
Renal biopsy class III or IV lupus nephritis	10		
Classify as systemic lupus erythematosus with score of 10 or more if entry criterion fulfilled			

Figure 5. EULAR-ACR diagnostic criteria. This table adapted from Dörner and Furie, 2019 [16] summarizes the 2019 EULAR-ACR diagnostic criteria, that are currently being exploited for SLE diagnosis.

In recent years, great effort has been devoted to the identification of new or optimization of current biomarkers, with the final goal of generating more specific and sensitive tests for the diagnosis of SLE.

For example, a decrease in the C4 fraction of complement represents a biological parameter that can help diagnose SLE. However, this test is not sensitive and the short protein half-life represent a major problem in the test interpretation. A new possibility proposed is the measure of cell-bound complement activation products (CB-CAP), that persist longer in the periphery and are associated with disease severity [66].

Other biomarkers currently evaluated aim at quantifying the characteristic type I IFN signature. In this perspective several aspects that can be altered by the IFN signature are considered: going from protein expression to gene expression and epigenetic alterations [67].

1.6. Treatment of SLE

1.6.1. General drugs

SLE is a chronic disease, for which no cure has yet been identified. Current medication aims to treat disease flares and maintain sustained remission [68]. The most common drugs used to treat SLE are glucocorticoids, nonsteroidal anti-inflammatory drugs (NSAID) and antimalarial drugs. Antimalarial drugs, such as hydroxychloroquine, reduce the inflammation by inhibiting the complex-activated TLR pathway [69]. Immunusuppressive agents, such as azatihoprine, cyclosporine, methotrexate cyclophosphamide and mycophenolate mofetil are used in severe cases. These drugs are accompanied by significant side effects, linked to their broad effect of suppressing the immune system (i.e. infections). Treatment regimen depends on organ involvement. Treatment goal is to achieve remission or low disease activity, prevent organ damage and improve the overall quality of life [16].

1.6.2. Targeted Drugs for SLE

In the past six decades only two new drugs have been approved by the FDA and SwissMedic for the treatment of SLE: belimumab (in 2011) and anifrolumab (in 2021 by FDA, evaluation ongoing by SwissMedic).

Belimumab is a fully humanized monoclonal antibody that targets BLyS, the B cell activating factor, leading to a reduced B cell survival and autoantibody production. The drug was shown to be safe and to reduce disease activity and limit the use of glucocorticoids [70]. Initally, approved as treatment for mucocutaneous and muscoskeletal SLE, it has recently also been accepted as adjunctive therapy for lupus nephritis [71].

Anifrolumab is a fully human monoclonal antibody targeting the IFN I pathway (directed against type I IFN receptor subunit 1, IFNAR1). The efficacy and safety of anifrolumab has been evaluated in the IIb clinical trial, MUSE and the phase III trial, TULIP-2, that met the primary endpoints. This is a good example of the importance of clinical trial design for SLE. Indeed the original phase III clinical trial, TULIP-1, failed. The evaluation of responsiveness was changed for TULIP-2 [69][72]. It should be kept in mind that TULIP-2 reported a considerable risk of infection in treated individuals compared to placebo controls.

Rituximab, another monoclonal antibody, directed aginst B cells (targeting CD20), showed benefice in case report and open-label studies but failed to reach the primary endpoint in phase III clinical

trials (EXPLORER and LUNAR), where it was tested in comparison to standard of care. Therefore, rituxmab is approved by the FDA to treat SLE [73] and the european league against rheumatism (EULAR) recommends the use of rituximab only when all other options failed for the management of severe lupus [74].

Voclosporin is a calcineurin inhibitor, aiming at reducing the T-B interaction (expression of CD40 ligand) and development of inflammatory cytokines (such as IFNγ, TNF and IL17). This drug has been accepted for the treament of lupus nephritis by the FDA in January 2021, following the success of the phase III clinical trial AURORA1. Indeed, the study showed a better renal response of voclosporin combined with mycophenolat mofetil and low dose steroids, compared to MMF and steroids alone ([75], [76]).

1.6.3. SLE novel therapeutic approaches

The recent advances in the understanding of SLE pathophysiology have contributed to the development of new therpeutic agents, which are currently at different stages of evaluation in clinical trials [69]. These agents aim at specifically targeting the mechanisms invovled in SLE pathogenesis by acting on intracellular signaling pathways, inflammatory cytokines, chemokines, cell surface co-stimulatory receptors and proteasomes.

Since belimumab, and rituximab to a certain extent, were successful, several new **treatments targetting B cells** aiming at limiting autoantibody production have been developed.

Several antibodies targeting CD20, including ocrelizumab, ofatumumab and obinutuzumab have been evaluated in SLE in recent years.

Ocrelizumab clinical trials were interruppted first because of high rates of infections and secondarily for low efficacy [77].

Ofatumumab showed good results in patients with active refractory lupus nephritis, and was suggested as a good alternative for individuals allergic to rituximab. Nevertheless, there are no ongoing clinical trials to evaluate its efficacy for systemic lupus [77].

Obinutuzumab showed good results in the treatment of lupus nephritis (NOBILITY study), with a general amelioration of symptoms and no reported important side effects [78].

Furthermore, a study (BLISS-BELIEVE) is currently investigating the possibility and efficacy of combining B specific therapies belimumab and rituximab [79]. The rationale behind it is that belimumab promotes the mobilisation of tissue resident B cells, which could then be targeted by rituximab, increasing the efficacy through a synergistic therapeutic action.

Initial findings (presented at ACRconvergence21 [80]) of the phase III clinical trial showed no additive efficacy when combining the two drugs. Furthermore, the side effects were similar to single treatment, but with a significant increased infection risk.

Researchers are also evaluating novel B cell targets, such as **CD22 (epratuzumab)** and **CD19 (XmAb5871),** however, no conclusive results are yet available. In the same perspective of reducing the production of autoantibodies, another approach was considered with the inhibition of the **proteasome (bortezomib)**, which helped alleviate several symptoms but had severe side effects [81]. Finally, drugs targeting **APRIL and BlyS**, aiming at reducing the differnetiation of activated and antibody producing B cells are being evalutated, such as **Telitacicept**. A phase IIb clinical trial showed promising results in this respect [82].

Due to the evident importance of type I IFNs for SLE pathophysiology other drugs, besides anifrolumab, **targeting the IFN pathway** are being investigated. **IFNα-kinoid** is a fusion of an inactivated IFNα to a carrier protein that allows the endogenous generation of anti-IFNα antibodies. Although it showed promising results, it did not meet the phase II clinical trial primary endpoint and novel investigations are on standby [83]. Another option that is being investigated is to block the pDC surface receptors, necessary for the production of type I IFNs, such as blood DC antigen 2 (BDCA2) with monoclonal antibodies (**BIIB059**). Preliminary results are promising and currently a phase III trial is underway in this respect (TOPAZ-1 study)[69].

Intracellular signaling can be targeted specifically to prevent perpetuation of pathogenic pathways. Targets considered include mTOR and JAK/STAT. The inhibition of **mTOR,** with **rapamycin (Sirolimus)** or **N-acetylcysteine,** blocks the cellular metabolic activity and has shown great promise. Studies report a suppression of IL17 production and increased Treg activity. Indirect inhibition of mTOR with **metformin** has furhtermore shown a reduced B cell differentiation into plasmacells. **JAK**/**STAT** inhibition (with **tofacitinib** or **baricitinib**) blocks cytokine production and reduces the activation of inflammatory cells, clinical trials are ongoing but no definitive results are available at this point.

Co-stimulation inhibition by targeting CD28 or CD40 has been evaluated to reduce the activation of autoreactive T and B cells. Nevertheless, clinical trials did not meet the primary endpoints and were terminated.

Cytokines have an important role in the pathophysiology of SLE and many different approaches have been investigated to specifically target cytokine that directly or indirectly contribute to tissue damage.

Two different approaches have been evaluated in SLE: either providing cytokines that are reduced or targeting cytokine pathways that are aberrantly increased.

Low-dose IL2 treatment was developed to promote the activity of T regualtory cells, thus improving the immune tolerance and reducing symptoms at a broad level. Initial clinical trials show good efficacy and relatively mild side effects [84].

Targeting the **IL12/23 pathway** with monoclonal antibody **ustekinumab**, was intended to reduce the development of Th17 cells and their production of IL17. Ustekinumab showed promising results in phase II, but investigations were discontinued following lack of efficacy compared to standard care in a phase III clinical trial [69].

Also state of the art therapeutic approaches, such as **CAR-T therapy** are being evaluated.

In August 2021, Schett and colleagues, published a case study on a patient with severe and refractory SLE who was treated with CD19 CAR-T cells and showed rapid resolution of symptoms without significant side effects related to the treatment [85]. Large-scale investigations are needed to evaluate the safety and efficacy of this type of approach, bearing in mind that it remains an expensive technique.

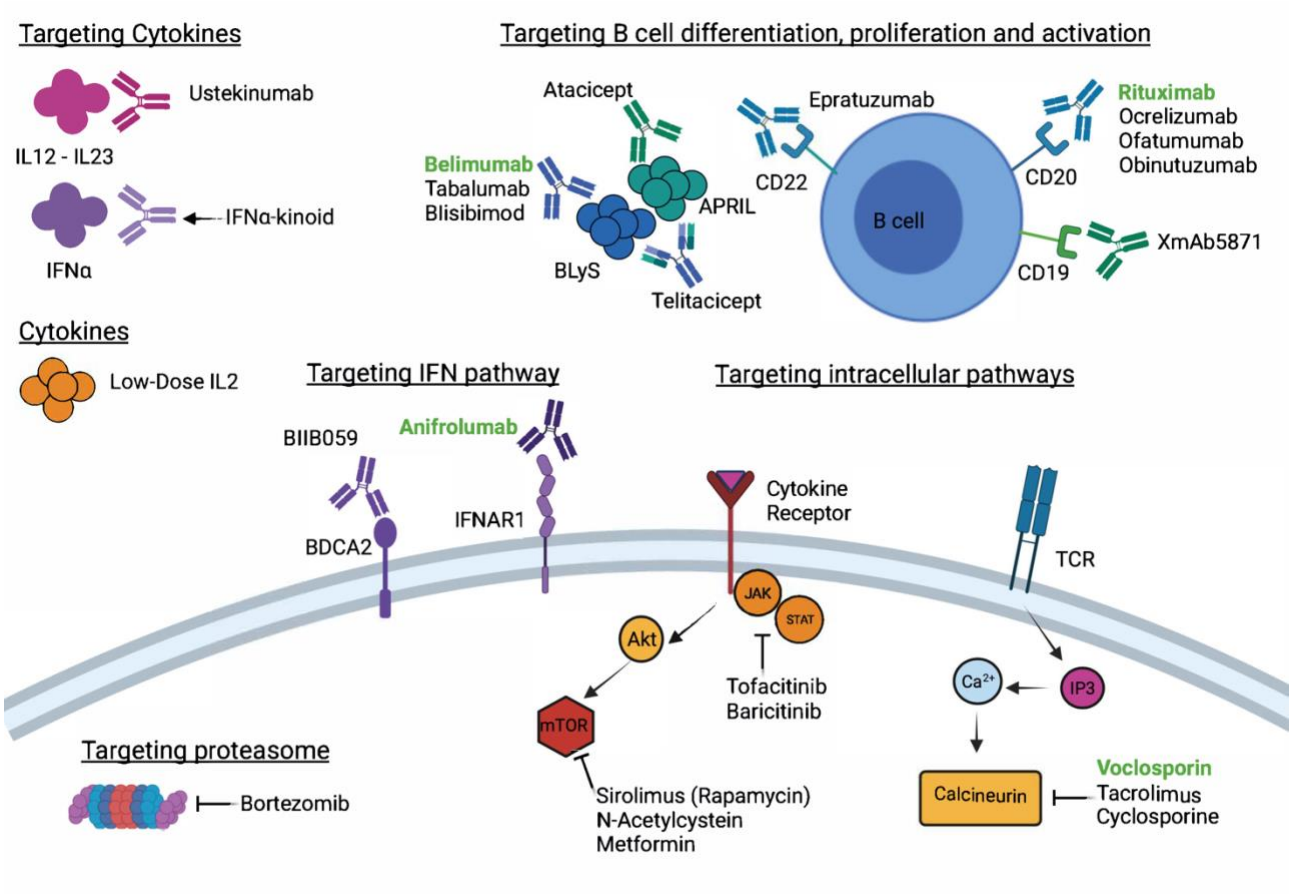

Figure 6. Novel therapeutic approaches for SLE. Graphical summary of targeted therapeutic approaches for SLE. In green the drugs that have been FDA approved or are currently used to treat SLE (created with BioRender.com).

2. Aim, Novelty and Importance of the project

The two main goals of this project are the identification of a novel biomarker and therapeutic target(s) for SLE. As elaborated in the introduction, there is an urgent need for better diagnostic tools, which enable early diagnosis and predict disease flares. Furthermore, although the identification of novel therapeutic targets for SLE is skyrocketing, only a few drugs reach the primary endpoint in clinical trials and long-lasting remission is difficult to achieve in some patients. New knowledge on the pathophysiology of SLE may provide innovative approaches to modulate and restore normal function of the immune system in patients with SLE.

This PhD project focuses on SLAMF receptors, a family of receptors, which has been shown to be important in the maintenance and proper function of the immune system and to contribute to cell function alterations seen in SLE. Although many studies have been conducted in the past to better understand the role of SLAMF receptors in the pathophysiology of SLE, a comprehensive analysis of SLAMF receptor expression on all major peripheral blood mononuclear cells has not yet been performed.

Our first hypothesis is that the altered expression of SLAMF receptors on SLE PBMC represents a specific immune signature. In this work, we exploit mass cytometry, which allows to detect more than 40 parameters at once. We compare the expression of these receptors at single cell level between SLE and controls (healthy and diseases controls). This approach aims at defining new biomarkers that are specific for SLE (and not shared with other autoimmune diseases, i.e. sarcoidosis).

Our second hypothesis is that the functional alterations of SLE NK cells are linked to the aberrant expression of cell surface receptors (including SLAMF receptors). Additionally, targeting certain of these NK cell surface receptors – with monoclonal antibodies – can restore the immune function of SLE NK cells.

Overall, this projepct will provide a comprehensive analysis of the phenotypic and functional alterations of SLE PBMC, in the context of SLAMF receptors, which will help to understand the pathogenesis of the disease at a deeper level. Furthermore, it will allow to detect the phenotypic and functional alterations of NK cells characterizing SLE patients. Taken together this project will allow to identify novel diagnostic and therapeutic approaches for SLE.

3. Results: Summary of publications

3.1. Humbel *et al*., under review

The aim of this study was to determine whether altered co-/expression of SLAMF receptors on PBMC identifies cell populations characteristic of SLE. To this end, single cell mass cytometry and bioinformatic analysis were exploited to compare SLE patients to healthy and autoimmune diseases controls (sarcoidosis, Sjögren's syndrome and multiple sclerosis).

First, the expression of single SLAMF receptors on PBMC populations were investigated. We observed that the frequency of SLAMF1+ B cells (referred to as SLEB1) was increased in SLE compared to controls (healthy and autoimmune controls). Furthermore, the frequency of SLAMF4+ monocytes and SLAMF4+ NK were inversely correlated with disease activity, whereas the frequency SLAMF1+ CD4+ TDEM cells were directly correlated with disease activity.

Consensus clustering analysis identified several PBMC co-expressing SLAMF receptors in healthy controls and SLE patients (confirmed by manual gating). Only two cell clusters were significantly altered in frequency in SLE patients compared to HC and autoimmune controls: switch memory B cells expressing SLAMF1, SLAMF3, SLAMF5, SLAMF6 (referred to as SLESMB) and circulating T follicular helper cells expressing the same SLAMF receptors (referred to as SLEcTFH). Finally, ROC curve analysis was implemented to determine the robustness of the identified cell populations as biomarkers for SLE. The combined measurement of SLEcTFH and SLEB1 cells or SLEcTFH and SLESMB cells allowed to discriminate SLE patients in 90% of cases.

In conclusion, this study identified 3 SLAMF based immune signatures for SLE, further highlighting the involvement of SLAMF receptors in the pathogenesis of SLE.

My contribution to this publication:

I performed most experiments, performed all gating, and bioinformatic analysis that allowed to identify the populations of interest. I wrote the manuscript and prepared the figures.

3.2. Humbel *et al.*, 2021

The aim of this article was to decipher the role of NK cells in the pathophysiology of SLE.

First, we confirmed previous data on SLE NK cells, showing that their number in the peripheral blood is reduced and that SLE NK cells are dysfunctional. The function of NK cells largely depends on the surface markers they express, therefore we analyzed SLE NK cells' extracellular phenotype with single cell mass cytometry. We observed that CD38 is significantly increased in SLE NK cells compared to their healthy controls. Next, we investigated the extracellular phenotype following cell activation. Of all the receptors studied, only two showed a significant difference between HC and SLE NK cells: SLAMF1 and SLAMF7. Indeed, these receptors were not properly upregulated following the activation with cytokines.

Second, we examined how the altered expression of these receptors is linked to their impaired function in SLE. In that interest, we engaged the receptors with monoclonal antibodies: anti-SLAMF1 (clone A12), anti-SLAMF7 (one used in research (clone 162.1) and the drug elotuzumab), anti-CD38 (daratumumab). Our results show that the engagement of SLAMF7 with elotuzumab and of CD38 with daratumumab, respectively, restores the function of SLE NK cells (degranulation and cytokine production).

Our data show that the expression of SLAMF1, SLAMF7 and CD38 are highly expressed on healthy circulating plasma cells (cPC) and increased on SLE cPC. Thus, we set up a NK-B cell co-culture system to observe how the ligation of elotuzumab or daratumumab influences the interaction between these two cellular populations. We identified that the engagement of NK cells with daratumumab or elotuzumab enhances the killing of cPC of healthy individuals. Nevertheless, when the same experiment was performed with cells isolated from SLE patients, only daratumumab (but not elotuzumab) significantly promoted the specific killing of circulating plasma cells.

In conclusion, our results indicate that the altered expression of SLAMF1, SLAMF7 and CD38 on SLE NK cells contributes to an altered interaction between NK cells and circulating plasma cells, thus potentially contributing to the production of autoantibodies. Accordingly, targeting SLAMF7 or CD38 with monoclonal antibodies could represent novel therapeutic approaches for SLE.

My contribution to this publication was: aid in experimental setup, performance of experiments and bioinformatic analysis, preparation of figures, significant contribution to writing of the manuscript.

4. Publications

4.1. Humbel *et al*., under review

SLAMF receptor expression identifies an immune signature that characterizes systemic lupus erythematosus

Morgane Humbel[1], Florence Bellanger[1], Alice Horisberger[1], Madeleine Suffiotti[1], Natalia Fluder[1], Mariko Makhumutova[3], Amandine Mathias[2], Renaud Du Pasquier[2], Craig Fenwick[1], Camillo Ribi[1], Denis Comte[1]

[1]Service of Immunology and Allergy, Lausanne University Hospital and University of Lausanne, Switzerland

[2]Service of Neuroimmunology, Department of clinical neurosciences, Lausanne University Hospital and University of Lausanne, Switzerland

[3]Machine Learning and Optimization Laboratory, EPFL, Lausanne, Switzerland

*** Correspondence:**
Dr. Denis Comte, Service of Immunology and Allergy, Department of Medicine, Rue du Bugnon 46, 1011 Lausanne, Switzerland, T +41 79 556 68 06, denis.comte@chuv.ch

Keywords: SLE, autoimmunity, SLAMF, immune-signature, biomarker

Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease of unknown etiology, linked to alterations in both the innate and the adaptive immune system. Due to the heterogeneity of the clinical presentation, the diagnosis of SLE remains complicated and is often made years after the first symptoms manifest, delaying treatment, and worsening the prognosis. Several studies have shown that signaling lymphocytic activation molecule family (SLAMF) receptors are aberrantly expressed and dysfunctional in SLE immune cells, contributing to the altered cellular function observed in these patients. The aim of this study was to determine whether altered co-/expression of SLAMF receptors on peripheral blood mononuclear cells (PBMC) identifies SLE characteristic cell populations. To this end, single cell mass cytometry and bioinformatic analysis were exploited to compare SLE patients to healthy and autoimmune diseases controls.

First, the expression of each SLAMF receptor on all PBMC populations was investigated. We observed that SLAMF1+ B cells (referred to as SLEB1) were increased in SLE compared to controls. Furthermore, the frequency of SLAMF4+ monocytes and SLAMF4+ NK were inversely correlated with disease activity, whereas the frequency SLAMF1+ CD4+ TDEM cells were directly correlated with disease activity. Consensus clustering analysis identified two cell clusters that presented significantly increased frequency in SLE compared to controls: switch memory B cells expressing SLAMF1, SLAMF3, SLAMF5, SLAMF6 (referred to as SLESMB) and circulating T follicular helper cells expressing the same SLAMF receptors (referred to as SLEcTFH). Finally, the robustness of the identified cell populations as biomarkers for SLE was evaluated through ROC curve analysis. The combined measurement of SLEcTFH and SLEB1 or SLESMB cells identified SLE patients in 90% of cases.

In conclusion, this study identified an immune signature for SLE based on the expression of SLAMF receptors on PBMC, further highlighting the involvement of SLAMF receptors in the pathogenesis of SLE.

1 Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory heterogenous autoimmune disease that mostly affects women of childbearing age [1]. Over the past decade, great strides have been made in understanding the pathogenesis of the disease. However, the etiology remains unidentified, making the development of new diagnostic tests and therapeutic approaches challenging. Currently, the diagnosis of SLE relies on a combination of clinical manifestations and diagnostic tests of low sensitivity and/or specificity. Thus, the diagnosis of SLE remains complicated and is often made years after the first symptoms manifest, delaying treatment, and worsening the prognosis. Therefore, the development of easily accessible and specific diagnostic tools is of paramount importance.

In recent years, research has focused on identifying novel biomarkers for SLE. Most of the suggested biomarkers are proteins involved in cellular communication, including cytokines, chemokines and growth factors, as well as cell surface receptors ([2], [3]). From this point of view, signaling lymphocytic activation molecule family (SLAMF) receptors are type I glycoprotein surface receptors expressed on all hematopoietic cells [4]. This receptor family includes nine members: SLAMF1 (CD150 or SLAM), SLAMF2 (CD48), SLAMF3 (CD229 or Ly9), SLAMF4 (CD244 or 2B4), SLAMF5 (CD84), SLAMF6 (CD352, NTBA or SF2000 in human or Ly108 in mice), SLAMF7 (CD319, CS1 or CRACC), SLAMF8 (CD353 or BLAME) and SLAMF9 (CD84-H1 or SF2001). SLAMF receptors represent a complex system implicated in cell-to-cell contact and cell activation. They have the unique property of being self-ligands (except for SLAMF2 and SLAMF4 that bind each other) and they can act as a ligand or a receptor depending on the cell by which they are expressed[5]. Each hematopoietic cell expresses three to five different SLAMF molecules and they signal via recruitment of adaptor proteins to provide a co-stimulatory or co-inhibitory message that influences cell activation [5]. Genome wide association studies have identified that SLAMF receptors are located in the 1q23 locus on chromosome 1, which was identified as a susceptibility locus for SLE [6]. Furthermore, various studies have evaluated the alteration of SLAMF expression and function in peripheral blood mononuclear cells (PBMC) from SLE patients ([7; 8; 9; 10; 11; 12; 13; 14; 15; 16; 17]). So far, most studies on SLAMF receptors in SLE focused on one receptor at a time and few data examined the co-expression of multiple SLAMF receptors at a single cell level [4].

In this research project, single cell mass cytometry was exploited to perform in-depth immunophenotyping of SLE PBMC to determine the expression of all SLAMF receptors at single cell level. The pattern of expression of SLAMF receptors was compared to healthy and autoimmune diseases controls. We hypothesize that the altered pattern of expression of SLAMF receptors on PBMC contribute to the impaired cell activation and cell-to-cell contact that lead to the development of autoimmunity. Accordingly, SLAMF receptor expression patterns define a SLE specific immune signature.

2 Materials and methods

Cohorts

SLE patients were diagnosed according to the American College of Rheumatology classification criteria and/or the Systemic Lupus International Collaborating Clinics (SLICC) criteria ([18; 19]), and were recruited from the Division of Immunology and Allergy at Centre Hospitalier Universitaire

Vaudois (CHUV). Current or past use of rituximab was an exclusion criterion. All patients and controls were included in the Swiss Systemic Lupus Erythematosus Cohort Study (SSCS)[20]. Disease activity score was measured using the SLE Disease Activity Index (SLEDAI) scoring system. We categorized patients into three groups of disease activity: inactive (SLEDAI 0-3), moderate (SLEDAI 4-10) and active (SLEDAI >10).

Two distinct cohorts were examined: cohort 1 included 28 SLE patients and age-, sex-, and ethnicity-matched healthy controls (Supplementary Table 1A). Cohort 2 included 10 patients with SLE, 10 age-, sex-, and ethnicity-matched healthy controls, 10 patients with biopsy-proven sarcoidosis (SAR), 10 patients with Sjögren's syndrome (SJS; based on the 2002 American-European Classification Criteria) and 10 patients with multiple sclerosis (MS; based on the 2017 McDonald Criteria) (Supplementary Table 1B). For MS patients, treatment with corticosteroids within three months before the blood draw was an exclusion criterion.

Cell isolation

Analysis of absolute cell count was performed on fresh blood by flow cytometry according to standard diagnostic measurements.

For mass cytometry analysis, peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation (FICOLL 400, Merck, Switzerland), from peripheral blood, and then cryopreserved in liquid nitrogen.

Single cell mass cytometry

Samples were stained according to a previously published approach [16]. Briefly, cryopreserved PBMC from SLE patients and controls (healthy and autoimmune) were thawed, resuspended in RPMI (completed with 20% heat-inactivated serum). Cells (1 Mio per individual on average) were stained for live/dead with cisplatin 50 µg (5min, room temperature (RT)), barcoded with CD45-metal conjugated antibodies (20min, RT, Supplementary Tables 2A-B) and then pooled. For cohort 1, two HC and two SLE were pooled, for cohort 2 one HC, SLE, SAR, SJS, MS sample were pooled in each experiment. Next, cells were incubated with metal conjugated antibody mix for the extracellular staining (20min, RT). The panel consisted of 39 markers, including markers for SLAMF receptors and for the main PBMC populations (CD4+ T cells, CD8+ T cells, double negative T cells (DN), B cells, natural killer (NK) cells, dendritic cells (DC) and monocytes) and differentiated subsets (Supplementary Table 2C). Cells were washed and fixed with 2.4% paraformaldehyde (10 min, RT). Labeled samples were acquired on a Helios Cytof System (Fluidigm). For each experiment at least 500'000 cells were acquired per patient. Flow cytometry standard (FCS) files were normalized to EQ Four Element calibration beads using CyTOF software.

Data analysis and statistics

Data were debarcoded on Cytobank software (Beckman Coulter) and fcs files were generated. The fcs files were then analysed using FlowJo™ software (version 10.2, Becton, Dickinson and Company). All major PBMC populations and subpopulations were gated according to the gating strategy presented in Supplementary Figure 1. The data were then processed using GraphPad prism (version 8), R software (version 3.5.1) or Python (version 3.8.5). Statistical analysis was performed with GraphPad prism. Specifications of test exploited and sample size are specified in the figure descriptions. In general, data (cell subset frequencies) were transformed into $\log_{10}(x+1)$ and normality was assessed with Shapiro-Wilk test. Two groups were compared using Welch's T test (or Mann-Whitney T test if

normality test failed). One-way ANOVA was used for multiple group comparison with normal distribution and p-values were adjusted for multiple testing using Tukey's test (comparison between all groups) or Dunnett's test (comparison to a control group). Correlations were assessed using Pearson's correlation. All data are presented as mean "±" standard error of the mean (SEM). A p-value ≤ 0.05 was statistically significant.

Manually gated cell sub-/populations were imported in R studio environment and processed as previously described [16]. Briefly, single cell expression was transformed using hyperbolic inverse sine (with cofactor 5) [21]. Dimensionality reduction and 2-dimensional visualization were done using the Barnes-Hut implementation of t-stochastic neighboring embedding algorithm (Rtsne package). Unsupervised clustering analysis was performed on previously gated PBMC using self-organizing map in combination with consensus clustering (FlowSOM package). The parameters used for clustering were SLAMF1, SLAMF3, SLAMF4, SLAMF5, SLAMF6 and SLAMF7. The analysis was repeated on subpopulations of cells to ensure consistency of findings. Manual gating was then performed on to confirm the existence of an identified clusters. A minimum of 100 cells was required for a cell subset to be considered for further analysis.

Python (Scikit-Learn library) was used to normalize cell frequencies (min-max normalization) of SLEB1, SLEcTFH and SLESMB population. The normalized frequencies were then summed and averaged to obtain combination of the different measurements. ROC curve analysis was used to determine the ability of these measures to distinguish a patient with SLE from a healthy or autoimmune controls. The area under the curve (AUC) represents the accuracy of a measurement in distinguishing SLE from controls, and was therefore used as an indicator of separation between groups. Youden index was used to determine the optimal cut-off to separate SLE patients from controls. This cut-off was then applied to cohort 2 to determine the specificity of the approach in identifying SLE patients among patients affected by other autoimmune diseases.

Study Approval

Informed written consent was obtained from all participants prior to inclusion and the study was approved by the Institutional Review Board (SwissEthics 2017-01434 and 2018-01622), in compliance with the Declaration of Helsinki.

3 Results

Distribution of PBMC populations is altered in SLE patients

The pathophysiology of systemic lupus erythematosus (SLE) is characterized by alterations of the innate and adaptive immune system. To identify an immune signature for SLE, we performed single cell mass cytometry analysis. We included markers for all major PBMC populations, markers of differentiation and markers of activation. First, we assessed the distribution of the main populations of PBMC in healthy controls (HC) and SLE patients: CD4+ T cells, CD8+ T cells, double negative (DN) T cells, B cells, natural killer (NK) cells, dendritic cells (DC) and monocytes (Figure 1A and Supplementary Figure 1). Consistent with previous studies [22; 23], we observed significant lymphopenia in SLE patients compared with HC (Figure 1B) and significant decrease in all lymphocyte subpopulations (including CD4+ T cells, CD8+ T cells, DN T cells, B cells, NK cells and DC) (Figure 1C and D), validating our technical approach. No difference was observed in abundance of monocytes in SLE patients compared to HC (Figure 1C). Interestingly, there was no association between lymphocyte count and the severity of the disease (data not shown). We proceeded by analyzing the subpopulations of CD4+ T, CD8+ T and B cells (Supplementary Figure 1). The following populations

were considered for CD4+ T cells: CD4+ T naïve, effector memory (EM), central memory (CM), terminally differentiated effector memory (TDEM), Th1, Th2, Th17, circulating T follicular helper cells (cTfh), regulatory T cells (Treg). T helper subsets were defined on the basis of cell surface chemokine receptor expression (Supplementary Figure 1). For CD8+ T cells, naïve, EM, CM and TDEM cells were included. Finally, for B cells, naïve, switch memory (SM), non-switch memory (NSM), double negative (DN) and circulating plasma cells (cPC) were included in the analysis (Supplementary Figure 1). We observed that the frequency of Treg cells (CD127-CD25+), cTfh cells (CD45RO+CXCR5+) and cPC (CD27+CD38+) were significantly increased in patients with SLE (Figure 1C). The frequency of NSM B cells (CD27+IgD+) was significantly reduced in SLE patients. No other significant alterations in subset frequency were observed.

Distribution of the single expression of SLAMF receptor on PBMC is altered in SLE patients

Several studies indicate that SLAMF receptors play a role in the pathophysiology of SLE, as mentioned above [4]. We hypothesize that SLAMF receptors expression defines an immune signature unique to SLE. To investigate this, we first examined the individual expression of each SLAMF receptor (SLAMF1, SLAMF3, SLAMF4, SLAMF5, SLAMF6, SLAMF7) on all main populations and subpopulation of PBMC from SLE patients included in cohort 1 (Figure 2A).

SLE patients showed a significant increase in the frequency of CD4+ T cells- and B cells-expressing SLAMF1, as well as CD4+ T cells-, B cells- and monocytes-expressing SLAMF7. Furthermore, there was a decrease in the frequency of DN T cells positive for SLAMF3 and SLAMF4, and of the percentage of DN T cells-, B cells- and monocytes-expressing SLAMF6 (Figure 2B). Next, we investigated SLAMF receptors expression on CD4+ T cell, CD8+ T cell and B cell subsets. We found that the percentage of SLAMF1-expressing cells was increased in all SLE CD4+ T cell subsets, including naïve T cells, CM, EM, TDEM, Th1, Th2, Th17, Treg and cTfh cells. Furthermore, the frequency of CD4+ TDEM-expressing SLAMF7 was significantly increased in SLE (Figure 2C). No significant alteration was observed in the expression of SLAMF receptors in SLE CD8+ T cell subsets (Supplementary Figure 2). Analysis of SLE B cell subsets indicated an increase in the frequency of naïve, NSM, SM and DN (CD27- IgD-) B cells-expressing SLAMF1. In addition, the frequencies of SM and DN B cells-expressing SLAMF7 were increased in SLE, whereas naïve B cells-expressing SLAMF6 were reduced in SLE patients compared to HC (Figure 2C).

Expression of a single SLAMF receptor may be linked to SLE disease activity

Given the suggested relationship between SLAMF receptor expression and the pathophysiology of SLE, we questioned whether single SLAMF expression could serve as marker for disease activity. To answer this question, we first evaluated the individual expression of SLAMF on each population and subpopulation of PBMC. This analysis showed that the frequency of NK cells expressing SLAMF4 NK cells (Figure 3A) and of monocytes expressing SLAMF4 (Figure 3B) inversely correlated with disease activity. Furthermore, the percentage of TDEM CD4+ T expressing SLAMF1 positively correlated with SLE disease activity (Figure 3C).

Identification of the co-expression of multiple SLAMF receptors at single-cell level in SLE patients

We evaluated the simultaneous expression of all SLAMF receptors at single-cell level (Figure 4A). To run this analysis, we performed unsupervised clustering analysis based on SLAMF expression on pre-gated major PBMC populations. This analysis was followed by unbiased clustering analysis of pre-gated subpopulations of CD4+ T cells, CD8+ T cells, NK cells, monocytes, and dendritic cells. Populations that were consistently discovered after applying sequential unbiased analysis were

manually gated. Based on their relative cell abundance they may be of biological significance (supplementary Figure 4A).

Accordingly, our analysis of CD4+ T identified the following cell subsets, which did not differ in their frequency between SLE and HC, as potentially relevant: naïve CD4+T cells and CM CD4+ T cells co-expressing SLAMF3 and SLAMF6, Th1 CD4+ T cells co-expressing SLAMF1, SLAMF3 and SLAMF6, Th2 CD4+ T cells co-expressing SLAMF5 and SLAMF6, Th17 CD4+ T cells co-expressing SLAMF3, SLAMF5 and SLAMF6 (Supplementary Figure 3). The presence of the following population was confirmed after manual gating and their frequency was increased in SLE patients compared to HC: EM CD4+ T cells co-expressing SLAMF1, SLAMF3 and SLAMF5, Treg CD4+ T cells co-expressing SLAMF1 and SLAMF5, and cTFH CD4+ T cells co-expressing SLAMF1, SLAMF3, SLAMF5 and SLAMF6 (Figure 4B). Finally, Th1 CD4+ T cells co-expressing SLAMF3 and SLAMF6 were significantly decreased in SLE patients (Figure 4B).

The analysis of CD8+ T cells and subsets identified that EM CD8+ T cell co-expressing SLAMF1, SLAMF3, SLAMF6 and SLAMF7, were significantly decreased in SLE patients (Figure 4B).

Analysis of B cells and B cell subsets, identified one cell subset as potentially relevant, whose frequency was not significantly different between HC and SLE patients: naïve B cells co-expressing SLAMF3 and SLAMF6 (Supplementary Figure 3). Furthermore, naïve B cells co-expressing SLAMF1 and SLAMF3 and SM B cells co-expressing SLAMF1, SLAMF3, SLAMF5 and SLAMF6 were significantly increased in SLE, while cPC co-expressing SLAMF4 and SLAMF6 were reduced (Figure 4B).

From our analysis, we did not identify any SLAMF-based clusters in NK cells, DC and monocytes that exhibit altered frequency in patients with SLE compared to HC. However, CD16+PD1+ monocytes co-expressing SLAMF1, SLAMF5 and SLAMF7+ showed a tendency to be increased in patient with SLE. Furthermore, CD16high NK cells co-expressing SLAMF4, SLAMF6 and SLAMF7 are consistently identified by unbiased clustering analysis and their presence was confirmed by manual gating (Supplementary Figure 3).

SLAMF expression and co-expression characterizes patients with SLE compared to other autoimmune diseases

In order to identify an immune signature specific to SLE, we considered a second cohort of patients (cohort 2), which included patients with SLE, HC and patients with the following autoimmune diseases: sarcoidosis (SAR), Sjögren's syndrome (SJS) and multiple sclerosis (MS) (Figure 5A).

In patients included in the cohort 2, we examined SLAMF-based cell populations that were identified in the cohort 1. We first focused our analysis on single SLAMF receptor expression. We observed that, among all the populations of interest identified in the cohort 1, only B cells-expressing SLAMF1 (identified as SLEB1) were significantly increased in SLE compared to healthy and autoimmune diseases controls (Figure 5B). Then, we examined the frequencies of population defined by the co-expression of multiples SLAMF receptors as characteristics of SLE in cohort 1. This analysis showed that two populations are significantly increased in SLE patients compared to healthy and autoimmune diseases controls (Figure 5C): SM B cells co-expressing SLAMF1, SLAMF3, SLAMF5 and SLAMF6 (identified as SLESMB) and cTfh CD4+ T cells co-expressing SLAMF1, SLAMF3, SLAMF5 and SLAMF6 receptors (identified as SLEcTFH).

Identification of an immune signature for SLE based on the expression of SLAMF receptors by PBMC

Overall, analysis of single-expression and co-expression of SLAMF receptors in PBMC identified three subsets of cells with altered frequencies in SLE compared to healthy and autoimmune controls. We investigated the potential of each of these populations, taken individually or in combination, to distinguish SLE patients from healthy individuals and patients with other autoimmune diseases. The populations of interest were present in healthy and autoimmune disease individuals. Therefore, we used their frequencies and evaluated them as continuous variables. To compare SLE and HC, the frequency of each cell subset was normalized (min-to-max normalization). Then, we determined which cell populations taken separately was the best marker to differentiate SLE patients from healthy controls. ROC curves showed that the measurement of the SLEcTFH population was the best individual marker (AUC = 0.92) to distinguish SLE from HC (Figure 6A). Then, we combined the normalized values of the different populations to determine which combination best discriminates SLE from HC. We observed that the measurements of SLESMB together with SLEcTFH increases the performance from SLESMB AUC = 0.83, SLEcTFH AUC = 0.92 to AUC = 0.94 (Figure 6A).

Secondly, we examined which cell population (normalized frequencies) best distinguishes SLE from other autoimmune diseases using subjects included in the cohort 2. We observed that the single measurement of SLEB1 and SLESMB better discriminates SLE from autoimmune controls compared to SLEcTFH (SLEB1/SLESMB AUC = 0.81 vs SLEcTFH AUC = 0.72, Figure 6B). Furthermore, the combined measurements of SLEB1 and SLEcTFH taken together was the best to differentiate SLE from autoimmune diseases (AUC = 0.847, Figure 6B). We then calculated the ideal cut-off to distinguish SLE from autoimmune diseases controls in cohort 2. Using the Youden index, we determined that individuals with a score greater than 0.282 (for both the SLEB1-SLEcTFH and SLESMB-SLEcTFH combinations) can be diagnosed as SLE. Overall, our data indicate that the combination of SLEB1 and SLEcTFH measurements or the combination of SLESMB and SLEcTFH, both correctly diagnosed 90% of SLE samples (Figure 6C). These results show that the expression of SLAMF receptors by PBMC can represent a powerful diagnostic tool for SLE.

4 Discussion

We exploited single-cell mass cytometry to perform an in-depth analysis of PBMC populations expressing or co-expressing SLAMF receptors with the aim of identifying a SLE immune signature.

Our data identified that the frequency of SLAMF1+ B cells (SLEB1) is significantly increased in SLE patients compared to all controls (healthy and autoimmune). Moreover, consensus clustering analysis identified alteration in the frequencies of several populations co-expressing SLAMF receptors in SLE patients compared to healthy controls. The frequencies of SMB cells and cTFH cells co-expressing SLAMF1, SLAMF3, SLAMF5 and SLAMF6 (identified as SLESMB and SLEcTFH, respectively) were significantly increased in SLE compared to all controls (healthy and autoimmune). We showed that the increased frequency of SLEB1, SLESMB and SLEcTFH is sufficient to discriminate SLE patients from sarcoidosis, Sjögren's syndrome and multiple sclerosis patients. Furthermore, the combined measurements of SLEB1-SLEcTFH or SLESMB-SLEcTFH increased the accuracy of discrimination. Indeed, 90% of the individuals identified with this approach were diagnosed with SLE. Interestingly, SLE patients identified by this approach have varying clinical characteristic and are treated heterogeneously. In the majority of the cases, these differences do not prevent their identification using the above-mentioned markers. Our data identified three cell subsets that correlated with disease activity: the frequency of SLAMF4+ NK cells and SLAMF4+ monocytes was inversely correlated with SLEDAI, while the frequency of SLAMF1+ TDEM CD4+ T cells was directly correlated with disease activity. Overall, our data show that the expression of SLAMF receptors defines an immune signature that is specific to SLE. Moreover, our data further suggest a role of SLAMF

receptors in the pathophysiology of SLE, as previously shown in human and murine models [8; 9; 10; 14; 24; 25; 26; 27]..

The expression of single-SLAMF receptors by PBMC population and subpopulations closely matched previously published results [17]. There are minor differences between the two studies, which mainly concern the expression of SLAMF6 and SLAMF7. These distinctions may be related to variations in the composition of the cohorts and to differences in the technique used (flow vs mass cytometry).

SLAMF1 has been shown to be increased in T and B cells of SLE patients upon activation [17; 24]. In addition, this receptor is implicated in B cell proliferation, differentiation and Ig production [28]. Targeting SLAMF1 has been proposed as a therapeutic target for SLE since anti-SLAMF1 monoclonal antibody can reduce the T-B interaction, B cell production of IL6 and B cell differentiation into plasma cells [8].

To our knowledge, no study to date has examined the co-expression of SLAMF receptors in SLE PBMC. Our analysis shows that two cell populations, defined on the basis of the co-expression of SLAMF receptors, are altered in frequency in SLE patients compared to healthy and autoimmune controls. These populations are SLESMB and SLEcTFH cells, which both co-express SLAMF1, SLAMF3, SLAMF5 and SLAMF6. Since SLAMF receptors act as self-ligands and are expressed on both populations, they likely play a role in the cellular interaction of SLESMB and SLEcTFH cells. Functional studies will be essential to deeper understand the role of these cell populations in SLE patients. From this point of view, a study on mice with disrupted SLAMF1, SLAMF5 and SLAMF6 genes showed that these receptors synergistically contribute to humoral immunity control[29]. Indeed, SLAMF1-SLAMF5-SLAMF6- mice exhibited an increased T-dependent and T-independent production of antibodies compared to the wild type. Moreover, SLAMF3 deficient mice develop autoimmune features, including the expansion of Tfh cells and germinal center B cells and the production of autoantibodies, suggesting a role in the regulation of humoral immunity[7]. Although no studies has evaluated the absence of all four receptors at the same time, these murine models suggest that SLAMF1, SLAMF3, SLAMF5 and SLAMF6 may be responsible for the fine-tuning of regulation of humoral immunity.

SLE is a very heterogeneous disease with great variability in susceptibility factors and symptoms. For this reason, there is often a significant delay between the first symptoms and the definitive SLE diagnosis. This can delay adequate medical management and lead to permanent organ damage. Accordingly, discovering biomarkers that are both specific and sensitive enough to identify all patients suffering from SLE is an important goal to achieve. The markers we propose here can identify the vast majority of SLE patients, despite significant clinical presentation heterogeneity. However, cytometers that allow simultaneous analysis of the large number of cell surface markers needed for this approach might not be readily available to most diagnostic laboratories.

The major limitations of this research are the relatively small size of the cohorts studied and that the patients included are almost exclusively Caucasian. Further studies are needed to confirm the validity of our findings in other ethnic populations. In addition, examining a larger cohort may better define the optimal cutoff for our biomarkers and increase the specificity of the test. Furthermore, a cohort including more patients with active disease is warranted to confirm the findings on the correlation of SLAMF4+ NK cells, SLAMF4+ monocytes and TDEM CD4+ SLAMF1+ with disease activity. Moreover, patients with active organ involvement should be included to evaluate if these cell subsets could be used to predict organ involvement.

In conclusion, this study identified an immune signature based on the expression of SLAMF receptors by PBMC, which is specific for SLE and may represent a biomarker to identify the disease and its severity.

5 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

6 Author contribution

DC: study design. MH, FB: conducting experiments. MH, MS, MM: data analysis. CR, AH: recruitment of HC and SLE, SAR, SJS patients. CF: responsible for CyTOF facility. DC, MH, NF: writing and editing of manuscript. All authors reviewed the manuscript.

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9 Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation

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11 Figure Legends

Figure 1: Distribution of PBMC in SLE patients. A) T-stochastic neighboring embedding analysis of main PBMC populations on 3 representative SLE patients (with active disease) and mean expression of lineage markers (blue: low expression, red: high expression). B) Lymphocyte abundance in SLE patients (n=22) compared to normal healthy range (represented as median with interquartile range of HC n=15, Student T-test, **p=0.007). C) Abundance of innate immune cells in peripheral blood of SLE patients compared to HC (Welch's T test, NK cells (HC n=15, SLE n=22, ***p<0.001), DC (HC n=15, SLE n=22, ***p<0.001), monocytes (HC=15, SLE n=28, ns p=0.46). D) Abundance of adaptive immune cells in peripheral blood of SLE patients compared to HC (Welch's T-test, CD4+ T cells (HC n=15, SLE n=22, ***p<0.001), CD8+ T cells (HC n=15, SLE n=22, **p=0.03), DN T cells (HC n=15, SLE n=22, **p=0.009), B cells (HC n=15, SLE n=21, ***p<0.001). Abreviations: Healthy controls (HC), Systemic lupus erythematosus patients (SLE), double negative (DN) T cells, natural killer (NK) cells, dendritic cells (DC).

Figure 2. Single SLAMF expression in SLE. A) Graphical abstract of technical approach B) Dotplot of SLAMF expression in main PBMC of SLE patients (frequency and mean intensity, left) and presentation of significant differences in frequency of single SLAMF expressing PBMC between HC and SLE patients (n=28, Welch's T tests on log10 transformed data, right). C) Dotplot of SLAMF expression in CD4+ T (top) and B (bottom) cell subpopulations of SLE patients (frequency and mean intensity, left) and presentation of significant differences in frequency of single SLAMF expressing subpopulations between HC and SLE patients (n=28 for CD4+ T cells and n=26 for B cells, Welch's T tests on log10 transformed data, right). Abbreviations: DN T: double negative T cells, CM: central memory cells, EM : effector memory cells, TDEM: terminally differentiated effector memory cells, Th1, 2, 17: T helper type 1, 2, 17 cells, cTFH: circulating T follicular helper cells, NSM: non-switch memory cells, SM: switch memory cells, DN: double negative B cells, cPC: circulating plasma cells, Min: minimum mean intensity of marker expression, Max: maximum mean intensity of marker expression.

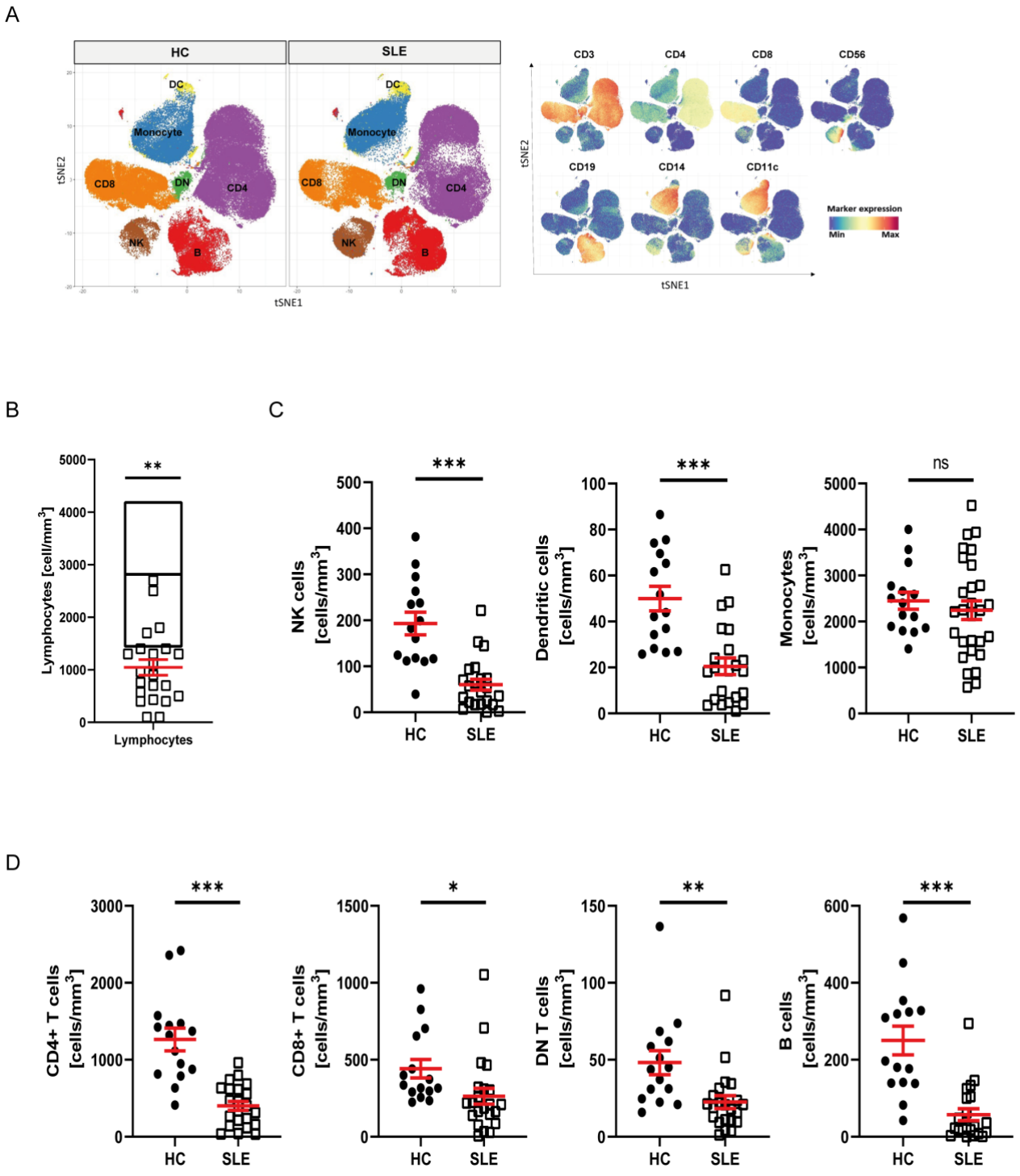
Figure 3. Single SLAMF link to disease activity. A) Frequency of SLAMF4+ natural killer (NK) cells according to disease activity categories (one way ANOVA with Tukey's multiple comparison test, left) and correlation between frequency of SLAMF4+ NK cells and SLEDAI (Pearson's

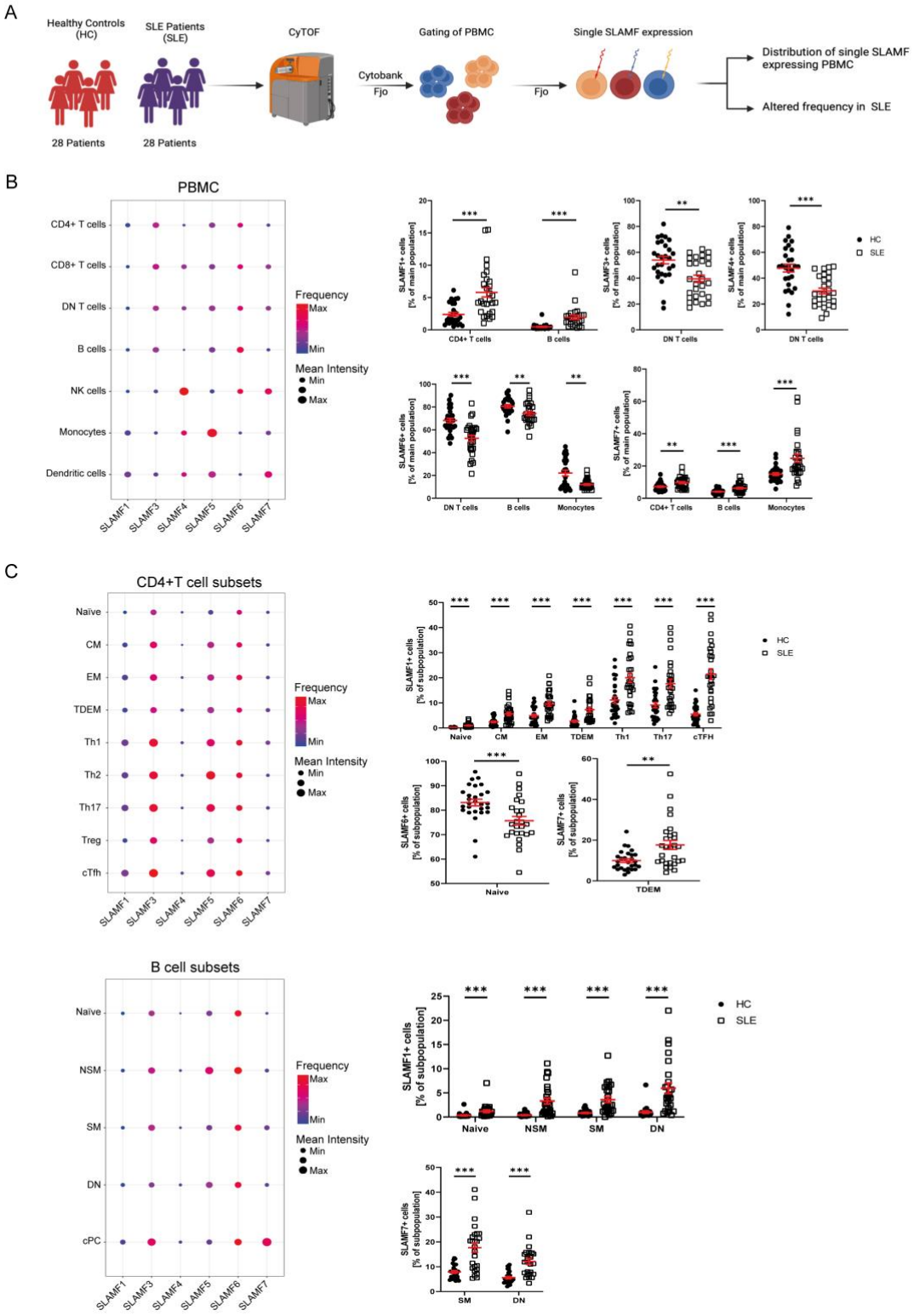
correlation, p=0.003, right). B) Frequency of SLAMF4+ monocytes according to disease activity categories (one way ANOVA with Tukey's multiple comparison test, left) and correlation between frequency of SLAMF4+ monocytes and SLEDAI (Pearson's correlation, p<0.001, right). C) Frequency of SLAMF1+ terminally differentiated effector memory CD4+ T (TDEM) cells according to disease activity categories (one way ANOVA, left) and correlation between frequency of SLAMF1+ TDEM and SLEDAI (Pearson's correlation, p=0.04, right). Data presented as log10 transformed values,*p=0.02, **p=0.002, ***p<0.001.

Figure 4. SLAMF co-expressing PBMC in HC and SLE patients. A) Graphical abstract of technical approach. B) Frequency of SLAMF co-expressing populations identified by consensus clustering (T test on log10 transformed data, Welch's T test if normal, Mann-Whitney if not normal distributed).

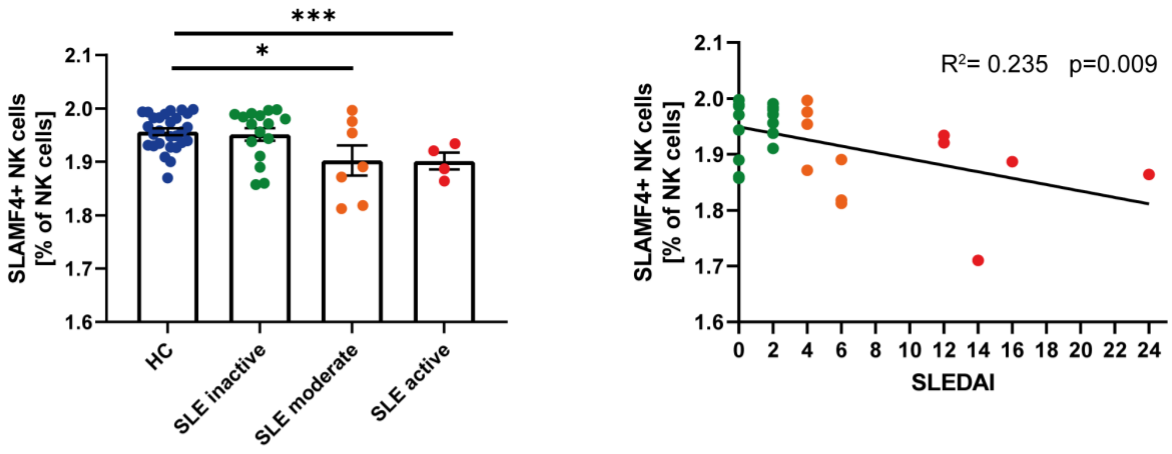
Figure 5. SLE specificity of SLAMF immune signature. A) Graphical abstract of technical approach. B) Frequency of SLAMF1+ B cells over B cells in cohort 2 (n=10 per group, one way ANOVA with Dunnett's multiple comparison test on log10 transformed data, *p=0.02, **p=0.002, ***p<0.001). C) Frequency of circulating T follicular helper cells expressing SLAMF1+3+5+6 over memory CD4+ T cells (left) and switch memory B cells expressing SLAMF1+3+5+6+ over SM B cells (right) in cohort 2 (n= 10 per group, one way ANOVA with Dunnett's multiple comparison test on log10 transformed data, *p=0.02, **p=0.002, ***p<0.001).

Figure 6. Predictive value of SLAMF expressing populations for SLE. A) ROC curves of SLESMB, SLEcTFH and SLEB1 and of their combinations in cohort1. B) ROC curves of SLESMB, SLEcTFH and SLEB1 and of their combinations in cohort 2. C) Samples of cohort 2 identified by combining SLESMB-SLEcTFH and SLEB1-cTFH.

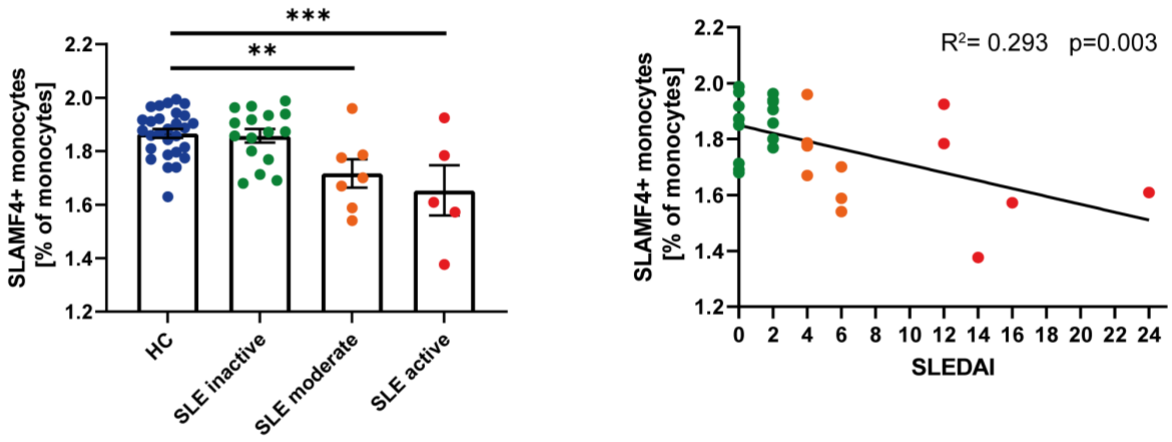

A
HC
SLE
DC
Monocyte
CD8
DN
CD4
NK
B
tSNE1
tSNE2
CD3
CD4
CD8
CD56
CD19
CD14
CD11c
Marker expression
Min
Max
B
Lymphocytes [cell/mm3]
Lymphocytes
C
NK cells [cells/mm3]
Dendritic cells [cells/mm3]
Monocytes [cells/mm3]
ns
D
CD4+ T cells [cells/mm3]
CD8+ T cells [cells/mm3]
DN T cells [cells/mm3]
B cells [cells/mm3]



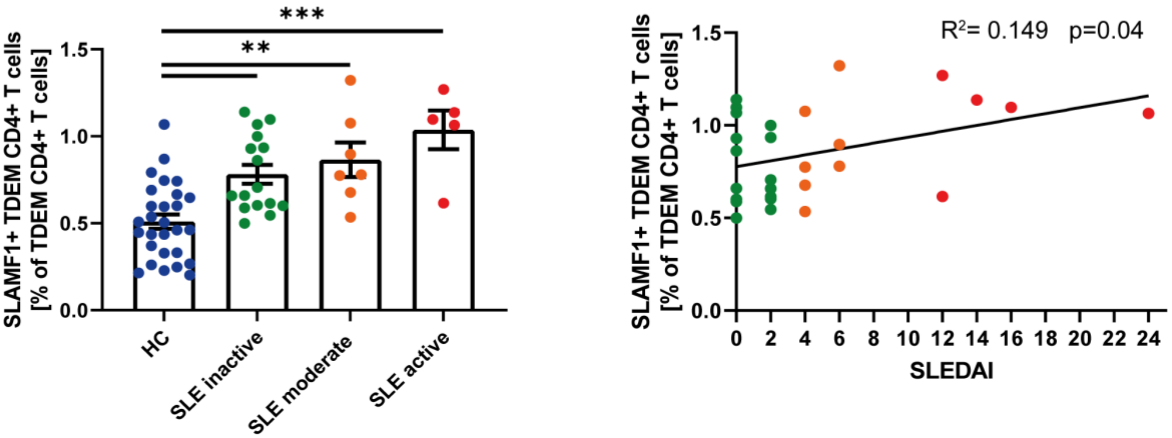
A


***
*
SLAMF4+ NK cells [% of NK cells]
2.1
2.0
1.9
1.8
1.7
1.6
HC
SLE inactive
SLE moderate
SLE active
R²= 0.235 p=0.009
SLEDAI
0 2 4 6 8 10 12 14 16 18 20 22 24

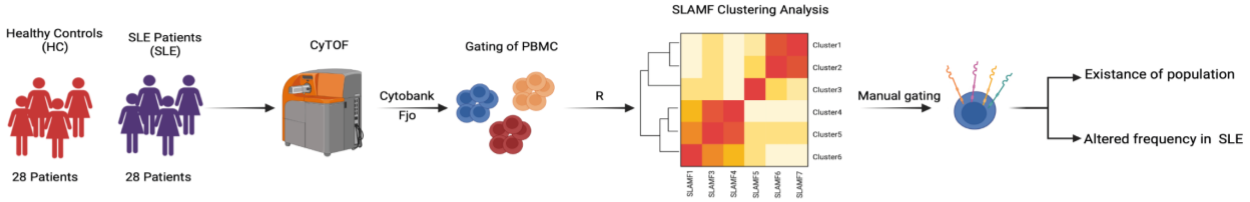
B


***
**
SLAMF4+ monocytes [% of monocytes]
2.2
2.0
1.8
1.6
1.4
1.2
HC
SLE inactive
SLE moderate
SLE active
R²= 0.293 p=0.003
SLEDAI
0 2 4 6 8 10 12 14 16 18 20 22 24

C


***
**
SLAMF1+ TDEM CD4+ T cells [% of TDEM CD4+ T cells]
1.5
1.0
0.5
0.0
HC
SLE inactive
SLE moderate
SLE active
R²= 0.149 p=0.04
SLEDAI
0 2 4 6 8 10 12 14 16 18 20 22 24

A



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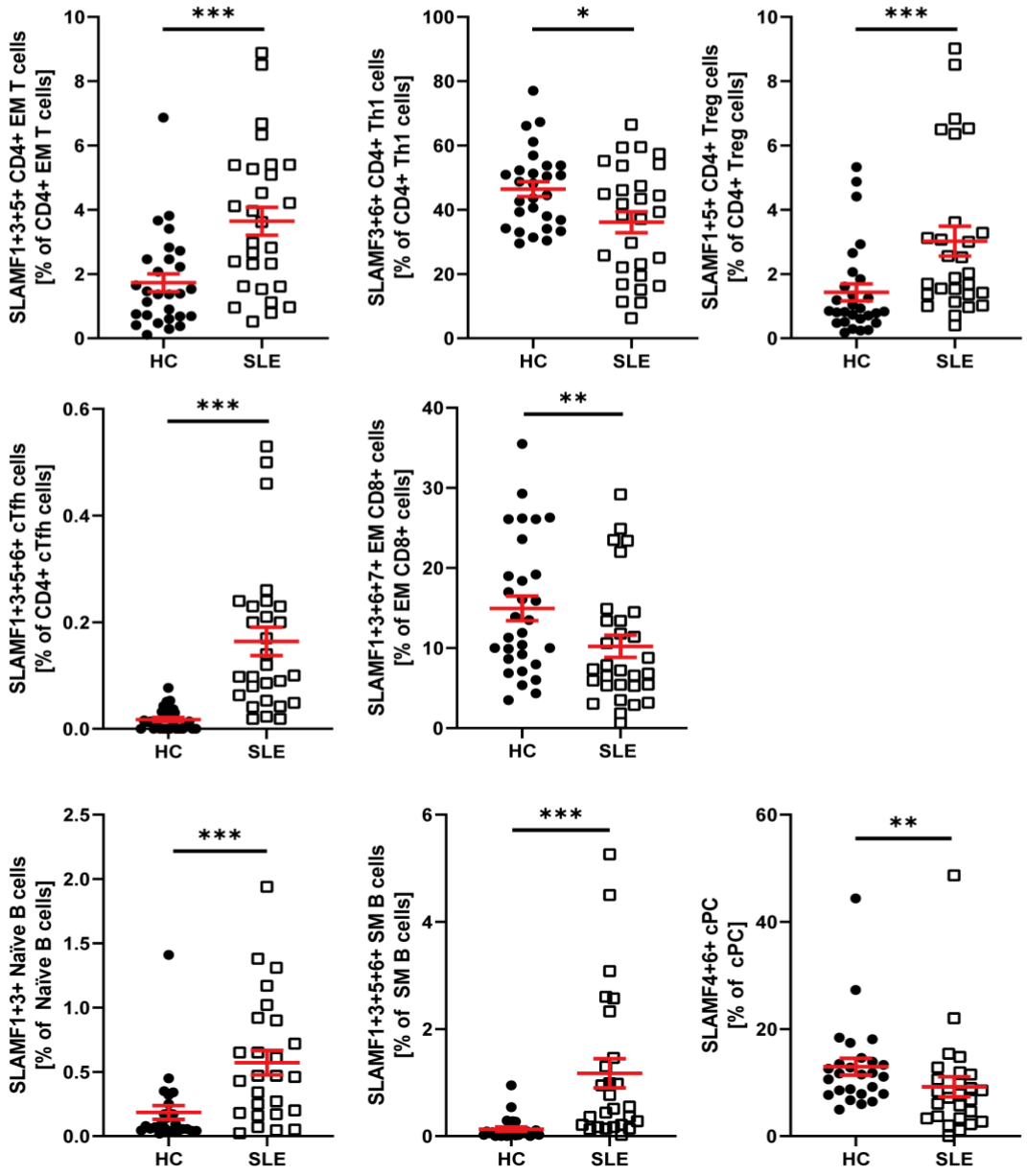
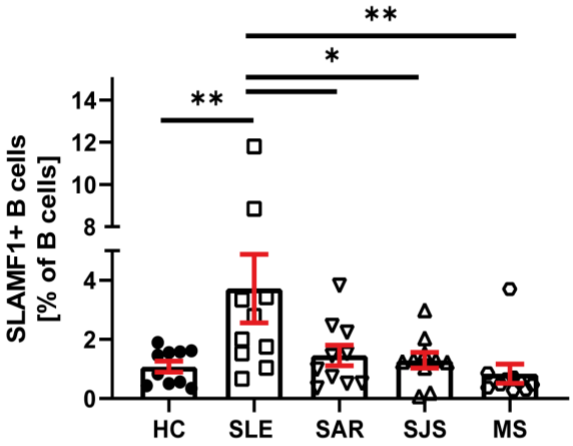


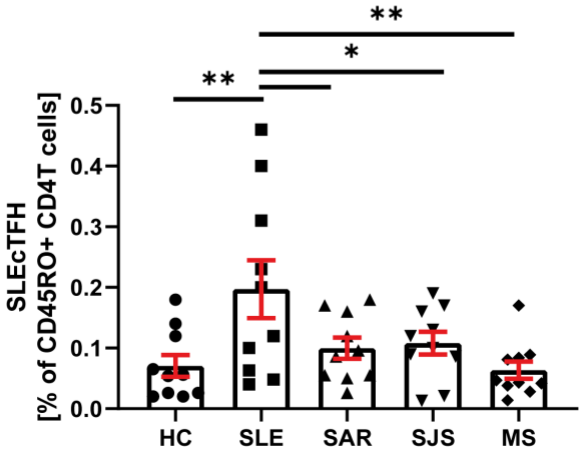
A

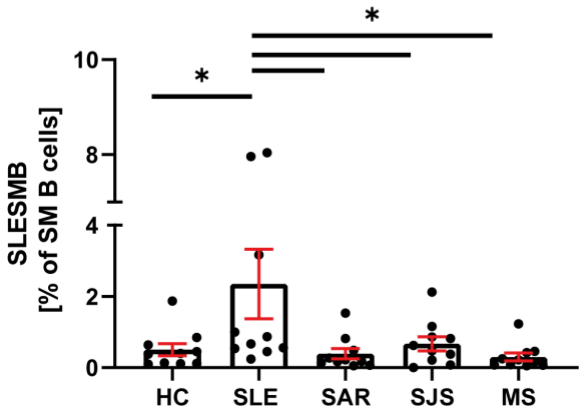

Healthy Controls (HC)
SLE Patients (SLE)
Sarcoidosis (SAR)
Sjögren's Syndrome (SJS)
Multiple sclerosis (MS)
10 Patients
10 Patients
10 Patients
10 Patients
10 Patients
CyTOF
Cytobank
Fjo
Gating of popuation of interest
Signautre of SLE

B

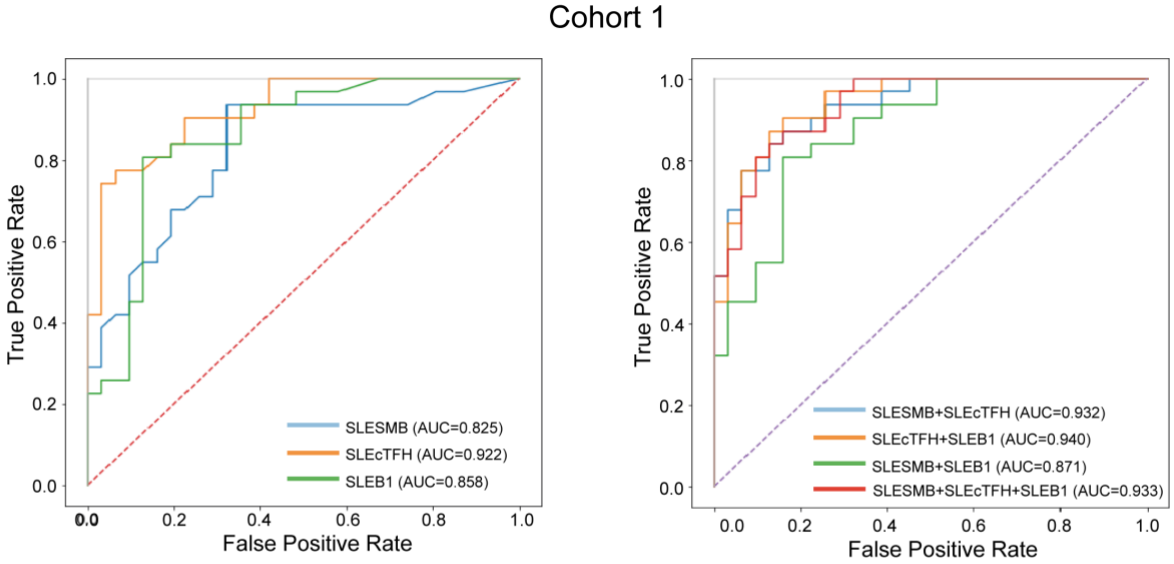

SLAMF1+ B cells
[% of B cells]
HC
SLE
SAR
SJS
MS

C

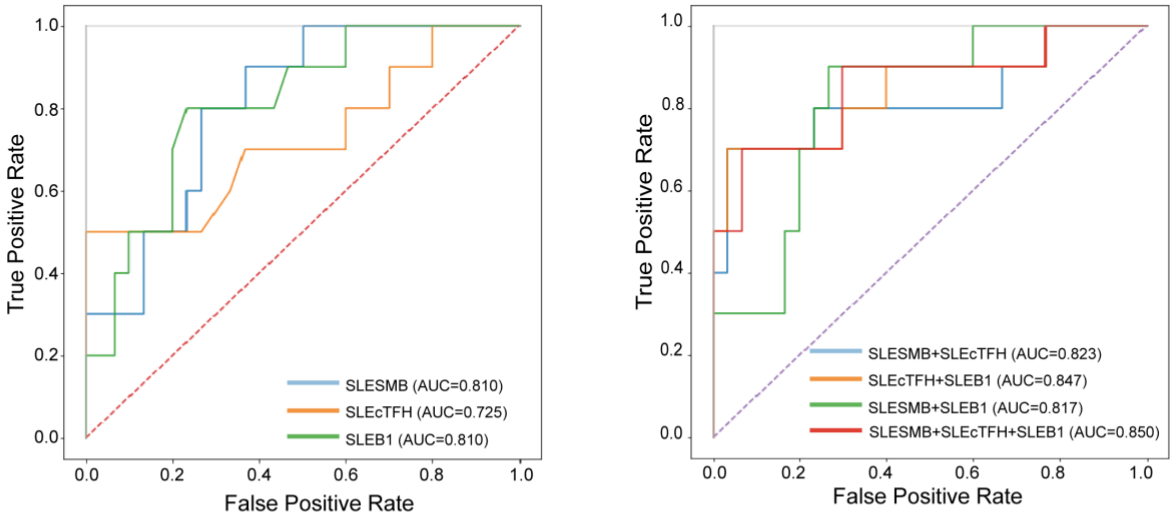

SLEcTFH
[% of CD45RO+ CD4T cells]
HC
SLE
SAR
SJS
MS


SLESMB
[% of SM B cells]
HC
SLE
SAR
SJS
MS

A



B



C

Patients identified with SLESMB + SLEcTFH

Patient	Disease	Disease Activity	Therapy	SEX	Ethnicity
1	SJS	PGA=0	All treatments	F	Caucasian
2	SLE	0	All treatments	F	Caucasian
3	SLE	0	All treatments	F	Caucasian
4	SLE	2	All treatments	F	Caucasian
5	SLE	4	All treatments	F	Caucasian
6	SLE	10	Naive	M	Hispanic
7	SLE	19	Naive	M	African
8	SLE	18	Anti-malarial	M	Caucasian

Patients identified with SLEB1 + SLEcTFH

Patient	Disease	Disease Activity	Therapy	SEX	Ethnicity
1	SAR	PGA=0	All treatments	F	Caucasian
2	SLE	0	All treatments	F	Caucasian
3	SLE	0	All treatments	F	Caucasian
4	SLE	2	All treatments	F	Caucasian
5	SLE	4	All treatments	F	Caucasian
6	SLE	10	Naive	M	Hispanic
7	SLE	19	Naive	M	African
8	SLE	18	Anti-malarial	M	Caucasian

Supplementary Material

Supplementary Table 1. Patient information

A. Cohort 1 Patient information

Information	HC (n=28)	SLE (n=28)
Age, mean (SD)	42 (14)	42 (14)
Gender (% female)	82%	82%
Ethnicity (% Caucasian)	89%	89%
Disease Activity (n)		
Active SLE	--	5
Moderate SLE		7
Inactive SLE		16
Therapy		
Naïve		6
Antimalarials only	--	8
All else*		14

HC: healthy controls, SLE: Systemic lupus erythematosus, Disease activity (Active SLE: SLEDAI>10, moderate SLE: SLEDAI 3-10, inactive SLE: SLEDAI<3), Therapy: naïve was considered patients with no rituximab ever and no immunomodulatory treatment during month prior to sampling; antimalarials only (Hydroxychloroquine, average daily dose (263pm, SD=106), no cytotoxic drug or biologic agent), *all else (corticosteroids (oral, n=11), hydroxychloroquine (n=10, average daily dose 274pm, SD=85), azathioprine (n=5, average daily dose 75, SD=35), mycophenolate mofetil (n=5, average daily dose 1000, SD=612).
SD: Standard Deviation.

B. Cohort 2 Patient information

Information	HC (n=10)	SLE (n=10)	SAR (n=10)	SJS (n=10)	MS (n=10)
Age, mean (SD)	36 (17)	37 (17)	55 (12)	50 (18)	38 (8)
Gender (% female)	80%	70%	40%	100%	60%
Ethnicity (% Caucasian)	100%	80%	100%	90%	--
Disease Activity (%)					
High	--	30%	30%	40%	--
Moderate		20%	40%	20%	40%
Low		50%	30%	40%	60%
Therapy (% naïve)	--	20%	50%	50%	100%

HC: healthy controls, SLE: Systemic lupus erythematosus, SAR: sarcoidosis, SJS: Sjögren's Syndrome, MS: multiple sclerosis, PGA: Physician Global Assessment, EDSS: Expanded Disability Status Scale, SD: standard deviation. Disease activity per disease is categorized as follows: SLE SLEDAI (high<10, medium 4-10, low<3), SAR and SJS PGA (high=2, medium=1, low=0), MS EDSS (severe disability>3, moderate disability 2.5-3, 0-2 low disability). For therapy naïve patients with no rituximab and no immunomodulatory drugs in the month proceeding sampling were considered.

Supplementary Table 2. Antibody list

A. Barcoding Cohort 1

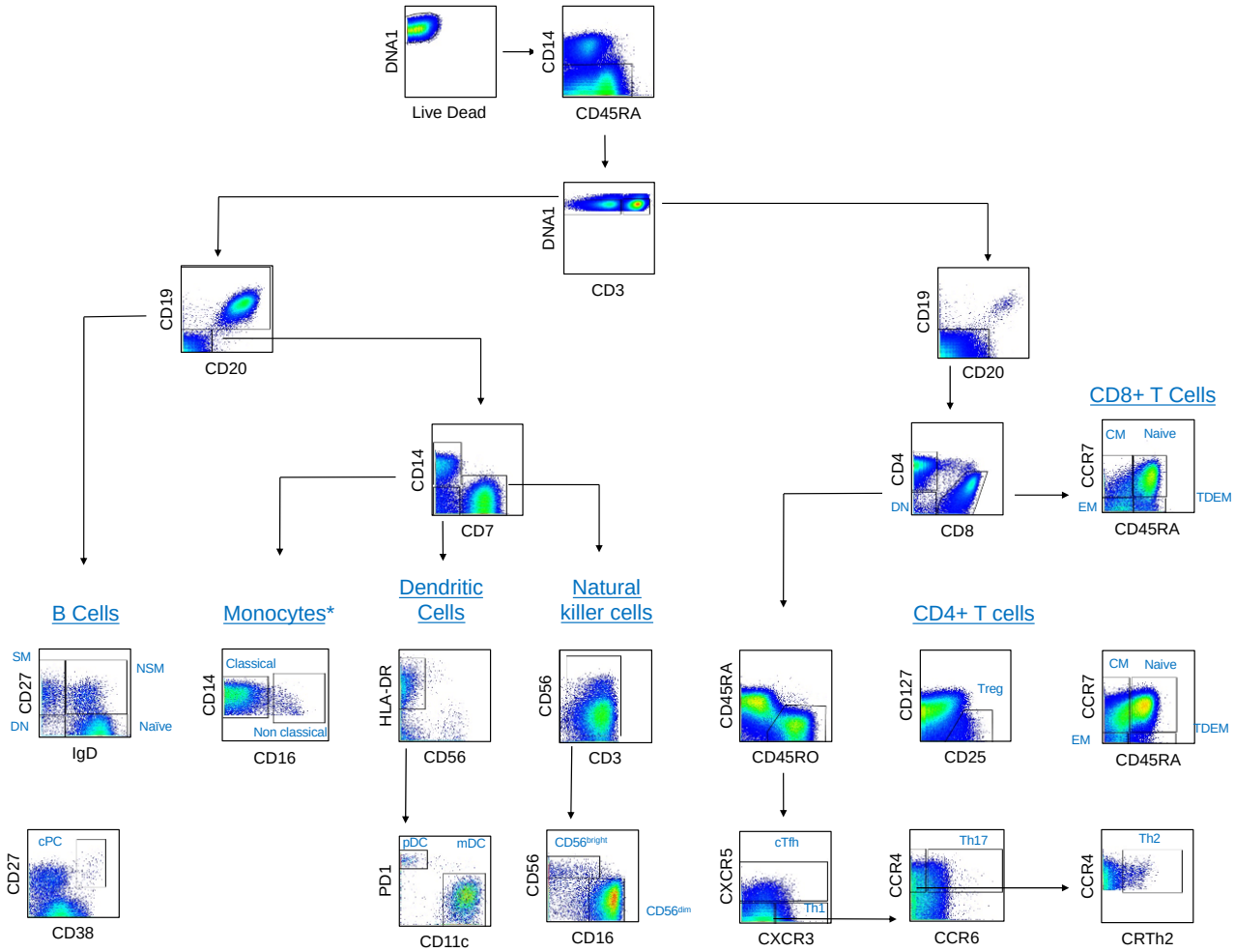
Mass Cytometry Antibody	Format	Clone	Company
CD45	89 Y	HI30	Fludigm
CD45	148 Nd	HI30	Conju-Biolegend
CD45	166 Er	HI30	Conju-Biolegend
CD45	198 Pt	HI30	Conju-Biolegend

B. Barcoding Cohort 2

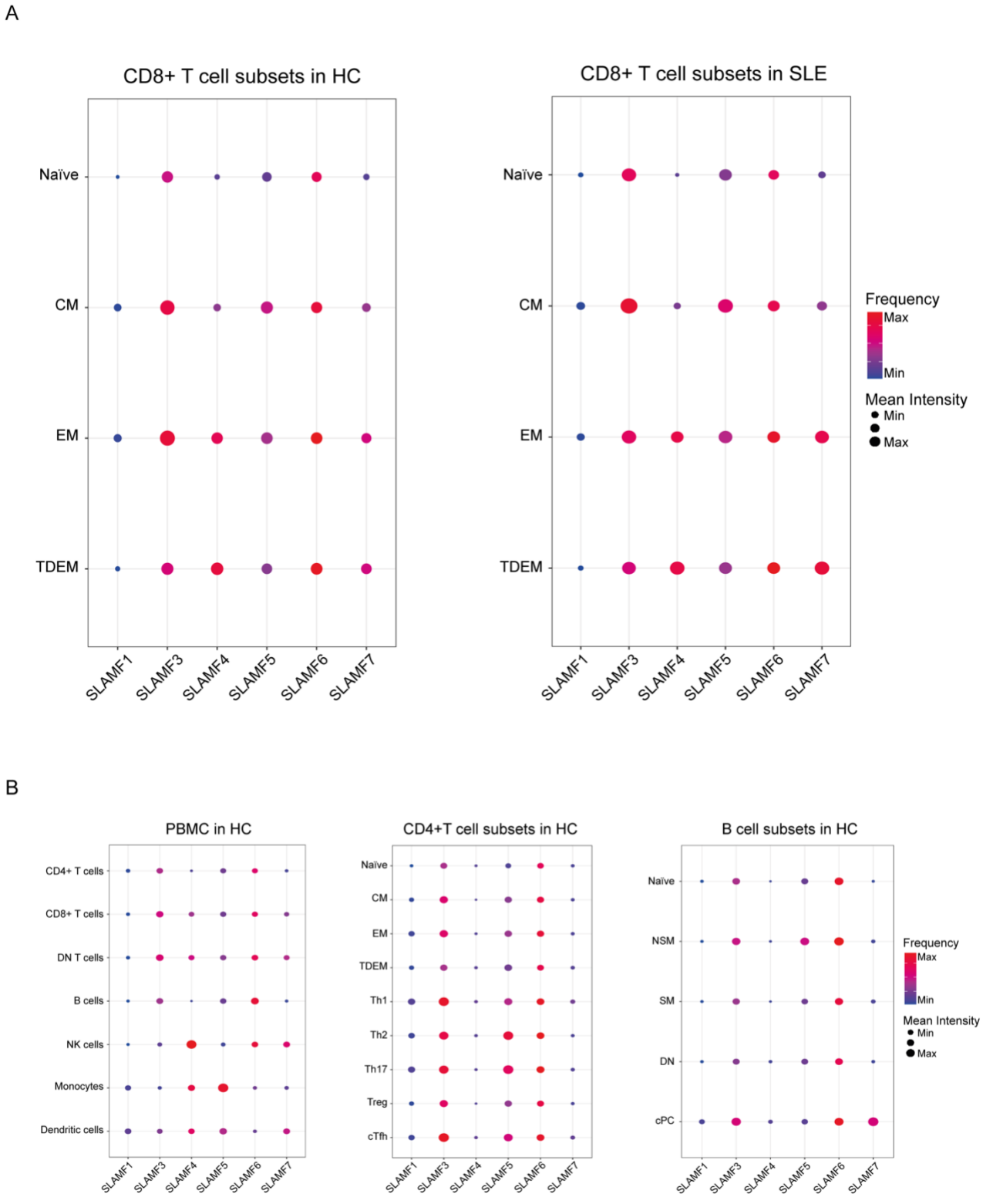
Mass Cytometry Antibody	Format	Clone	Company
CD45	89 Y	HI30	Fludigm
CD45	194 Pt	HI30	Conju-Biolegend
CD45	195 Pt	HI30	Conju-Biolegend
CD45	196 Pt	HI30	Conju-Biolegend
CD45	198 Pt	HI30	Conju-Biolegend

C. Extracellular phenotyping Panel

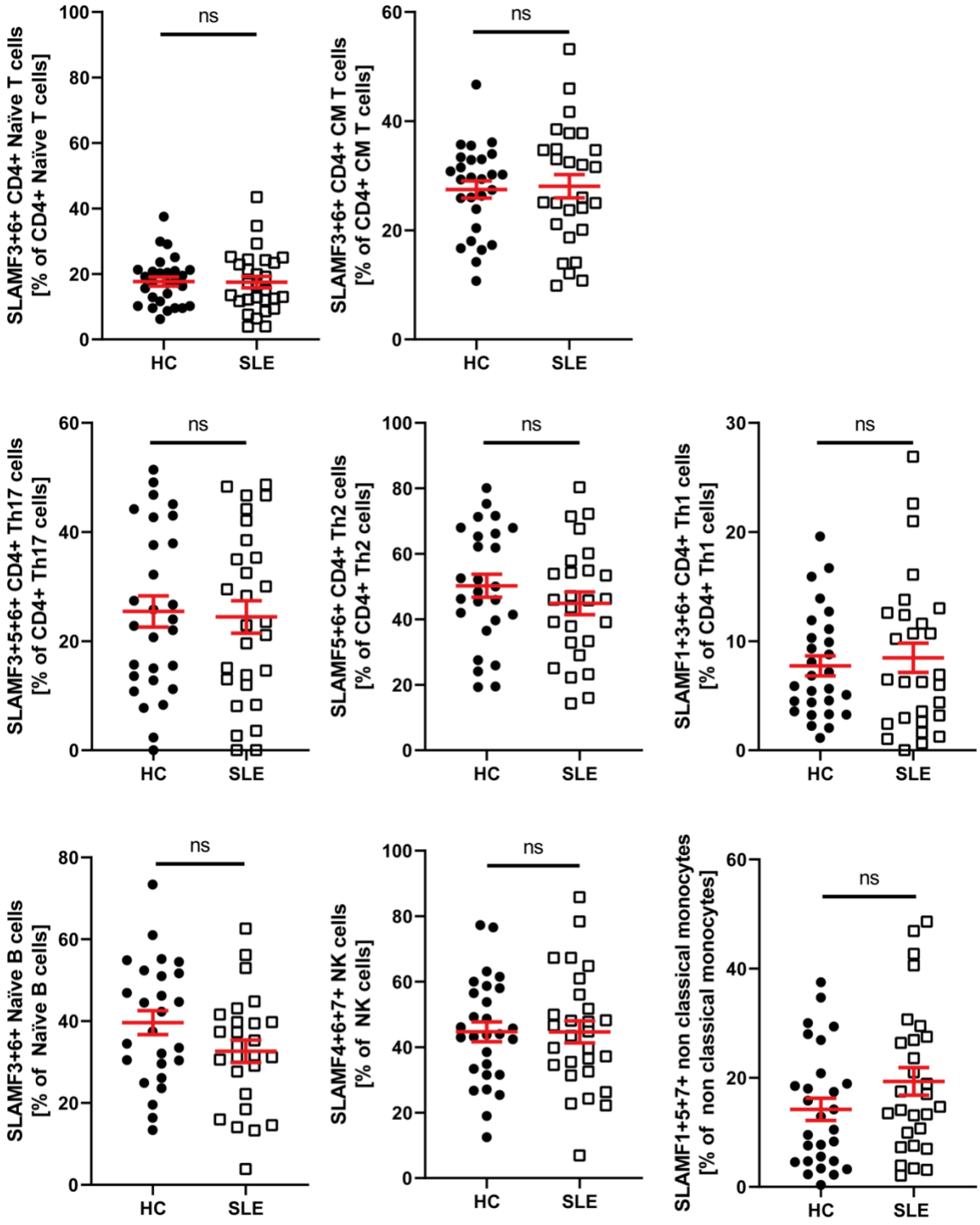
Mass Cytometry Antibody	Format	Clone	Company
Live/Dead	103Rh	-	Fludigm
CD8	113 In	RPA-T8	Biolegend
CD4	115 In	RPA-T4	Biolegend
CD196/CCR6	141 Pr	11A9	Fludigm
CD19	142 Nd	HIB19	Fludigm
CD352 / SLAM 6	143 Nd	NT-7	Fludigm
CD38	144 Nd	HIT2	Biolegend
CD127	145 Nd	A019D5	Biolegend
IgD	146 Nd	IA6-2	BD bioscience
CD7	147 Sm	CD7-6B7	Fludigm
CCR4	149 Sm	205410	Fludigm
CD3	150 Nd	UCH-T1	BD bioscience
CD123	151 Eu	6H6	Fludigm
PD-1	151 Eu	EH12.2H7	Biolegend
CD21	152 Sm	BL13	Fludigm
CD45RA	153 Eu	HI100	BD bioscience
CD84 / SLAM 5	154 Sm	CD84.1.21	Fludigm
CD27	155 Gd	L128	Fludigm
CD319/SLAMF 7	156 Gd	162.1	Biolegend
CXCR3	158 Gd	1C6/CXCR3	BD bioscience
CCR7	159 Tb	G043H7	Biolegend
CD14	160 Gd	M5E2	Fludigm
CD150 / SLAM 1	161 Dy	A12(7D4)	Biolegend
CD11c	162 Dy	clone 3.9	Fludigm
CRTh2 (Fluidigm)	163 Dy	BM16	Fludigm
CD48 / SLAM 2	164 Dy	BJ40	Biolegend
CD45RO	165 Ho	UCHL1	Fludigm
CXCR5	167 Er	RF8B2	Biolegend
ICOS	168 Er	C398.4A	Biolegend
CD25	169 Tm	2A3	Fludigm
TCR va24-Ja18 (6B11)	170 Er	Witek	Fludigm
CD20	171Yb	2H7	Fludigm
TCRαβ	172 Yb	IP26	Biolegend
CD353/ SLAM8	172 Yb	REA394	Conju-Miltenyi
HLA-DR	173 Yb	L243	Fludigm
CD229 / SLAM 3	174 Yb	HLy9.1.25	Fludigm
CD244 / SLAM 4	175Lu	C1.7	Biolegend
CD56	176Yb	R19-760	Fludigm
CD57 (CHUV)	194 Pt	NK1	Conju-BD bioscience
CD16	209Bi	3G8	Fludigm



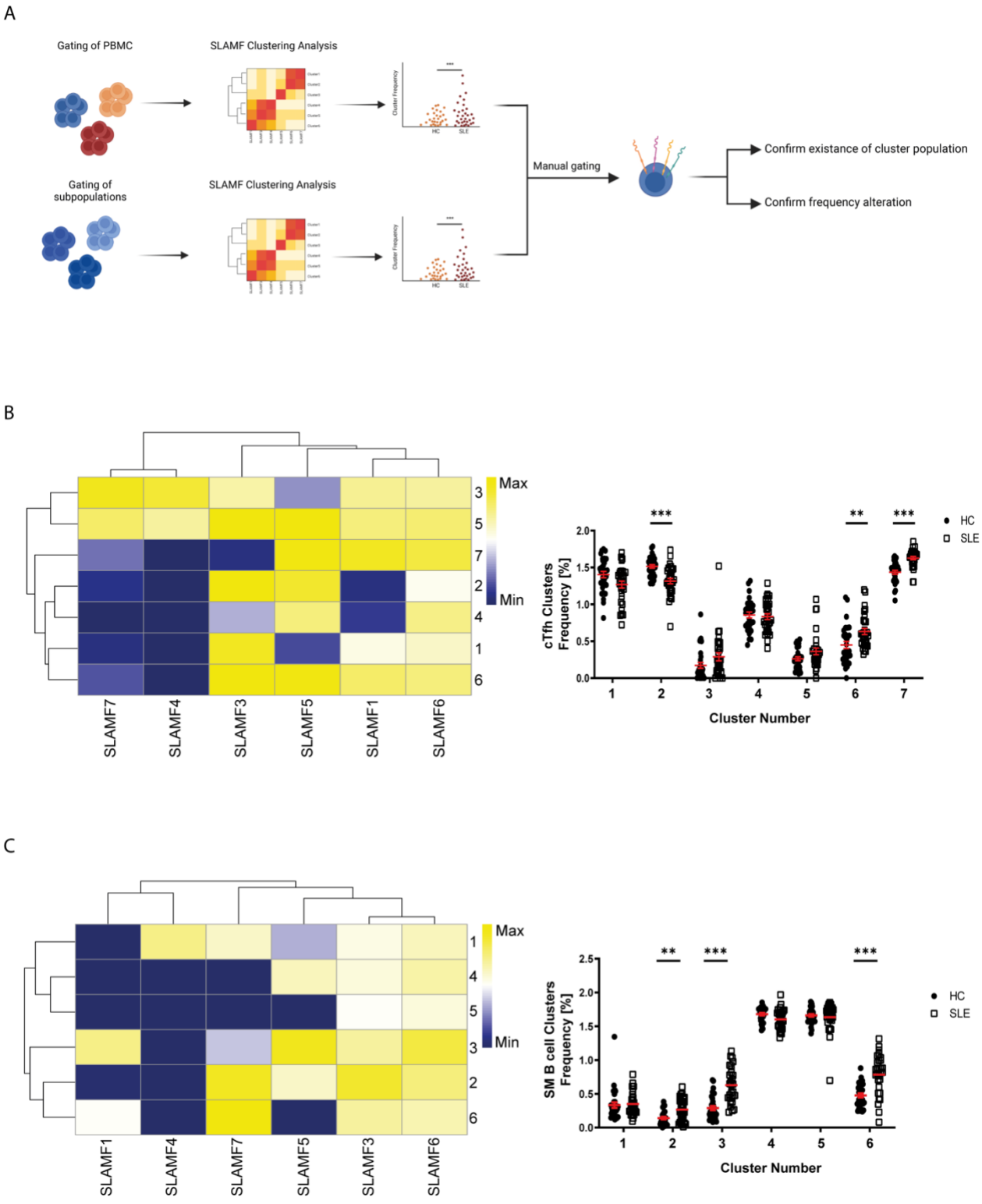
Supplementary Figure 1. Gating Strategy. Following debarcoding and removal of doublets, cells were gated as shown in this representative example. NSM: non-switch memory, SM: switch memory, DN: double negative, cPC: circulating plasma cells, pDC: plasmacytoid dendritic cells, mDC: myeloid dendritic cells, cTfh: circulating T follicular helper, Th1/2/17: T helper type 1/2/17, Treg: regulatory T, CM: central memory, EM: effector memory, TDEM: terminally differentiated effector memory.



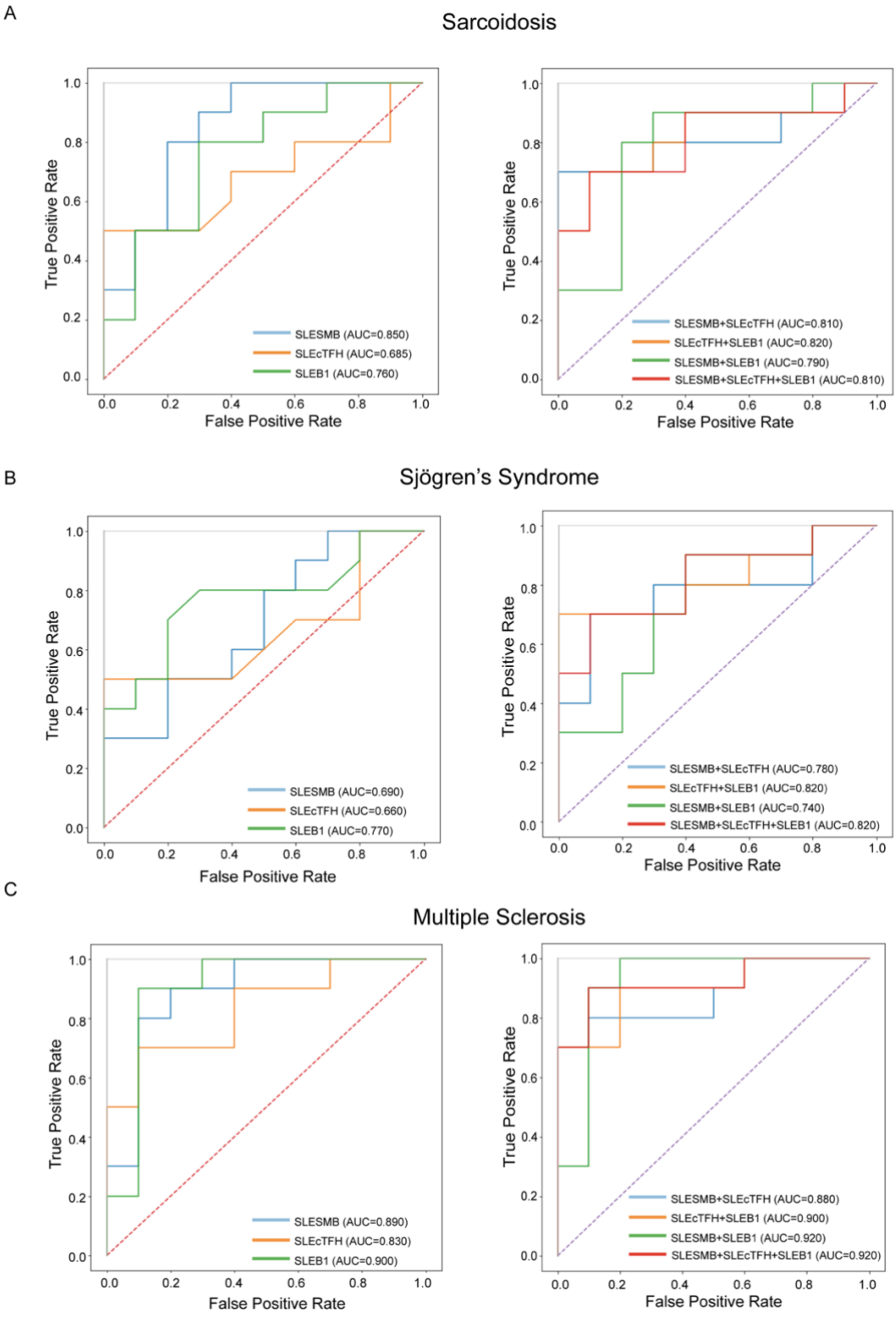
Supplementary Figure 2. SLAMF expression in SLE and in HC. (A) Dotplot of SLAMF expression in CD8+ T cell subpopulations in HC (n=28, left) and SLE patients (n=28, right) showing frequency and mean intensity. (B) Dotplots of SLAMF expression in PBMC (left), CD4+ T cell subsets (center) and B cell subsets (right) of healthy controls, showing frequency and mean intensity (n=28). Min: minimum mean intensity of marker expression, Max: maximum mean intensity of marker expression.



Supplementary Figure 3. Newly identified SLAMF co-expressing cell subsets. Frequency of SLAMF co-expressing populations identified by consensus clustering of cohort 1 and confirmed by manual gating, but not presenting significant alterations in SLE patients compared to HC (n=28, Welch T test on log10 transformed data).



Supplementary Figure 4. Consensus clustering analysis identifies populations of interest. (A) Graphical abstract of technical approach. (B) Heatmap of clustering analysis of cTfh cells (left) and frequency of clusters in HC and SLE patients (n=28, Welch T test on log10 transformed data, right). (C) Heatmap of clustering analysis of switch memory B cells (left) and frequency of clusters in HC and SLE patients (n=26, Welch T test on log10 transformed data, right).



Supplementary Figure 5. ROC curve analysis for SLE against autoimmune diseases. (A) ROC curve analysis for SLESMB, SLEcTFH, SLEB1 in SLE versus sarcoidosis (left) and for the combined measurements of SLESMB, SLEcTFH and SLEB1 (right). (B) ROC curve analysis for SLESMB, SLEcTFH, SLEB1 in SLE versus Sjögren's syndrome (left) and for the combined measurements of SLESMB, SLEcTFH and SLEB1 (right). (C) ROC curve analysis for SLESMB, SLEcTFH, SLEB1 in SLE versus multiple sclerosis (left) and for the combined measurements of SLESMB, SLEcTFH and SLEB1 (right).

4.2. Humbel *et al.*, 2021

frontiers in Immunology

ORIGINAL RESEARCH
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Restoration of NK Cell Cytotoxic Function With Elotuzumab and Daratumumab Promotes Elimination of Circulating Plasma Cells in Patients With SLE

*Morgane Humbel, Florence Bellanger, Natalia Fluder, Alice Horisberger, Madeleine Suffiotti, Craig Fenwick, Camillo Ribi and Denis Comte**

Service of Immunology and Allergy, Lausanne University Hospital, University of Lausanne, Lausanne, Switzerland

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****Correspondence:***
Denis Comte
denis.comte@chuv.ch

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Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by multiple cellular and molecular dysfunctions of the innate and adaptive immunity. Cytotoxic function of NK cells is compromised in patients with SLE. Herein, we characterized the phenotypic alterations of SLE NK cells in a comprehensive manner to further delineate the mechanisms underlying the cytotoxic dysfunction of SLE NK cells and identify novel potential therapeutic targets. Therefore, we examined PBMC from SLE patients and matched healthy controls by single-cell mass cytometry to assess the phenotype of NK cells. In addition, we evaluated the cell function of NK cells (degranulation and cytokine production) and the killing of B cell subpopulations in a B cell-NK cell *in vitro* co-culture model. We found that SLE NK cells expressed higher levels of CD38 and were not able to adequately upregulate SLAMF1 and SLAMF7 following activation. In addition, ligation of SLAMF7 with elotuzumab or of CD38 with daratumumab on SLE NK cells enhanced degranulation of both healthy and SLE NK cells and primed them to kill circulating plasma cells in an *in vitro* co-culture system. Overall, our data indicated that dysregulated expression of CD38, SLAMF1 and SLAMF7 on SLE NK cells is associated with an altered interplay between SLE NK cells and plasma cells, thus suggesting their contribution to the accumulation of (auto)antibody producing cells. Accordingly, targeting SLAMF7 and CD38 may represent novel therapeutic approaches in SLE by enhancing NK cell function and promoting elimination of circulating plasma cell.

Keywords: systemic lupus erythematosus (SLE), SLAMF, CD38, elotuzumab, daratumumab, NK cells, CD150/SLAMF1 receptor, CD319/SLAMF7/CS1

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disease that mainly affects women of childbearing age (1, 2). The pathogenesis remains elusive but includes alterations of the immune system leading to the production of autoreactive cells, autoantibodies and the formation of immune complexes that ultimately damage organs (1, 3). Although important progress was made over the last decades toward the development of new treatments, management of SLE still relies on the use of corticosteroids and immunosuppressive agents that non-specifically target immune cells. Despite the well-established importance of autoreactive B cells and autoantibody production in the pathogenesis of the disease (1), treatments based on B cell depletion have only been moderately successful so far (4). In this context, understanding the role of other immune cells involved in the pathogenesis of SLE and their link with antibody-producing cells is taking a center stage in the development of new therapies. Among the various cellular abnormalities that characterize SLE, Natural Killer (NK) cells' dysfunction has been supported by various studies (5–8). NK cells are innate lymphocytes that play a pivotal role in the immune surveillance (9), through the recognition of healthy cells and the elimination of damaged or infected cells. NK cells from SLE patients are reduced in number in the peripheral blood, show impaired cytokine production upon stimulation, reduced cytotoxicity, and defective antibody-dependent cellular cytotoxicity (5). However, their exact role in the pathogenesis of lupus remains elusive.

In the present study, we used single-cell mass cytometry to perform a comprehensive phenotypic analysis of healthy and SLE NK cells. We sought to identify how these alterations are linked to the altered function of SLE NK cells and might represent therapeutic options to treat SLE.

MATERIALS AND METHODS

SLE Patients and Controls

SLE patients (N=44) were diagnosed according to the American College of Rheumatology classification criteria and/or the Systemic Lupus International Collaborating Clinics (SLICC) criteria (10, 11), and were recruited from the Division of Immunology and Allergy at Centre Hospitalier Universitaire Vaudois (CHUV). All patients and controls were included in the Swiss Systemic Lupus Erythematosus Cohort Study (SSCS) (12). Characteristics of the SLE patients included in this study are provided in **Table 1**.

Age-, sex-, and ethnicity-matched healthy individuals were chosen as controls. Disease activity score was measured using the SLE Disease Activity Index (SLEDAI) scoring system. We categorized patients into three groups of disease activity: inactive (SLEDAI 0-3), moderate (SLEDAI 4-10) and active (SLEDAI >10).

Cell Isolation

Peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation (FICOLL 400, Merck, Switzerland). PBMC were cryopreserved in liquid nitrogen.

TABLE 1 | Demographic characteristics of SLE patients (N=44) included in the study.

Characteristic	Value
Age, years	
Median	46
Range	24-73
Sex	
Female	37
Male	7
Ethnicity	
Caucasian	40
Asian	3
Hispanic	1
SLE disease activity	
Inactive (0-3)	21
Moderate (4-10)	15
Active (>10)	8
Treatments	
Naïve	7
Hydroxychloroquine only	11
Other immunomodulatory drugs	25

Cell Culture

Cells were cultured in RPMI (Gibco; Life Technologies) containing 10% heat-inactivated FBS (Institut de Biotechnologies Jacques Boy), 100 IU/ml penicillin and 100 µg/ml streptomycin (Bio Concept), hereafter referred to as complete RPMI (cRPMI).

Antibodies

A complete list of mass cytometry, flow cytometry and purified antibodies is provided in the **Supplementary Table 1**.

Some antibodies for the mass cytometry assay were conjugated in our facility (MaxPar® X8 multimetal labeling kit, Fludigm). Briefly, the MaxPar® polymer is loaded with the metal, and then the antibody is partially denatured to allow its conjugation to the polymer. Finally, the metal bound polymer is conjugated to the antibody.

Mass Cytometry

Cryopreserved PBMC from SLE patients and matched healthy controls were thawed, resuspended in cRPMI, stimulated with cytokines or left unstimulated as mentioned in the figures. Cells were stained for live/dead with cisplatin 50 µg (5min, room temperature (RT)), barcoded with CD45-metal conjugated antibodies (20min, RT) and then pooled. Next, cells were incubated with metal conjugated antibody mix (20min, RT). Cells were washed and fixed with 2.4% paraformaldehyde (10 min; RT). Labeled samples were acquired on a Helios Cytof System (Fluidigm). Flow cytometry standard (FCS) files were normalized to EQ Four Element calibration beads using CyTOF software. FCS files were debarcoded using Cytobank (Beckman Coulter).

Mass Cytometry Data Analysis

Manual gating of FCS files was performed using FlowJo™ Software version 10.2 (Becton, Dickinson and Company; 2019). Data analysis was performed using R software (version 3.5.1.). Manually gated cell populations were imported into R

environment and single cell expression data were transformed using hyperbolic inverse sine (with cofactor 5) (13). Dimensionality reduction and 2-dimensional visualization were done using the Barnes-Hut implementation of t-stochastic neighboring embedding algorithm (Rtsne package). Unsupervised clustering analysis on cell populations were performed using self-organizing map in combination with consensus clustering (FlowSOM package) in order to define 4 different clusters.

For the analysis of NK cells, we merged two experiments designed with two different panels using CytofMerge (14) with default settings. The CytofMerge methodology is based on the k-nearest neighbor algorithm and a set of common markers in order to impute the value of missing markers by taking the median values of from the k most similar cells.

NK Cells Cytokine Production and Degranulation

PBMCs were thawed and resuspended in cRPMI. For evaluation of degranulation, NK cells were stimulated with IL-15 (50ng/ml), IL-18 (50ng/ml) or a combination of IL-2 (50ng/ml) and IL-12 (20ng/ml). For evaluation of NK cell activation with monoclonal antibodies cells were resuspended in cRPMI with IL-15 (1ng/ml). Cells were then stimulated with or without cytokines (IL-2 and IL-12, 50ng/ml and 20ng/ml respectively) with the following antibodies: SLAMF1 A12 (5µg/ml), SLAMF7 162.1 (5µg/ml), elotuzumab (0.1µg/ml), daratumumab (1µg/ml), elotuzumab and daratumumab (0.1µg/ml and 1µg/ml respectively) and incubated for either 6 or 18 hours at 37°C. BD GolgiPlug™, BD GolgiStop™ and CD107a-PE were added 6h before readout.

After incubation, cells were stained with Live/Dead Aqua and cell surface antibodies:CD3-BUV737, CD4-PB, CD8-BV605, CD19-FITC, CD56-BUV395. After permeabilization with BD Cytofix/Cytoperm™ kit, cells were stained with IFNγ-AF700, TNFα-APC. Finally, cells were fixed in BD CellFIX™ and stored at 4°C until data acquisition on LSR Fortessa™ (BD Bioscience).

NK and B Cells Co-Culture

PBMC cells from HC were thawed and sequential positive selection of CD19+ and CD56+ cells was performed (human microbeads, Miltenyi positive selection kits) using the AutoMACS® ProSeparator (Miltenyi Biotec). B cells were stained with CFSE (LifeTech). All cells were resuspended in cRPMI with IL-15 (1ng/ml).

NK cells were incubated for 30minutes at 37°C with the following stimulation conditions: unstimulated, SLAMF1 A12 (5µg/ml), SLAMF7 162.1 (5µg/ml), elotuzumab (0.1µg/ml), daratumumab (1µg/ml), elotuzumab and daratumumab (0.1µg/ml and 1µg/ml respectively). After incubation NK cells were washed and B cells were added (in cRPMI with IL-15) at a 2:1 ratio (NK min 500'000 cells, max 1Mio; B cells min 250'000, max 500'000 cells) and incubated for 5.30hours. After incubation cells were washed and stained with Live/Dead Aqua, CD56-BUV395, CD20-PB, CD21-AF700, CD27-PeCy7, CD38-ECD, SLAMF7-PE. Finally, cells were fixed in CellFIX™ and stored at 4°C until data acquisition on a LSR Fortessa™.

Depletion Assay

PBMCs were thawed and CD3 negative cells were isolated (EasySep™ Human CD3 Positive Selection Kit II, StemCell Technologies). Cells were resuspended in cRPMI with IL-15 (1ng/ml), and the following stimulation conditions were added: not stimulated, SLAMF1 (5µg/ml) with cytokines (IL-2 (50ng/ml) and IL-12 (20ng/ml)), elotuzumab (0.1µg/ml) with cytokines, daratumumab (1µg/ml) with cytokines and HLA-DR (0.005µg/ml) with cytokines. CD3 negative cells were then incubated for either 6 or 18 hours at 37°C. After incubation, cells were stained extracellularly with Live/Dead Aqua, CD3-BUV737, CD19-FITC, CD20-PB, CD27-AF700, CD38-ECD, CD56-BUV395, SLAMF7-PE. Cells were then washed in annexin buffer (10X Annexin V Buffer, BD Pharmingen) and stained with Annexin V-APC. Cells were stored at 4°C until data acquisition on LSR Fortessa™, for maximum 2h.

Statistics

Statistical analysis were performed using GraphPad Prism (version 8). Specifications of tests exploited and sample size for each experiment are mentioned in the figure descriptions. In a general manner, Mann-Whitney test was used for comparison between two groups with non-normal distribution (normality was assessed with Shapiro-Wilk test). Kruskal-Wallis test was used for the comparison of multiple groups with non-normal distribution and p-values were adjusted for multiple tests using Dunn's method. One-way ANOVA was used for the comparison of multiple groups with normal distribution and p-values were adjusted for multiple tests using Sidak's method. Two-way ANOVA was exploited for the comparison of multiple groups and p-values were adjusted for multiple tests using Sidak's method. Two-way ANOVA was exploited for multiple comparisons within a group and p-values were adjusted for multiple tests using Tukey's method. A p-value lower than 0.05 was considered significant.

Study Approval

Informed written consent was obtained from all participants prior to inclusion and the study was approved by the Institutional Review Board (SwissEthics 2017-01434), in compliance with the Declaration of Helsinki.

RESULTS

NK Cells Are Reduced in Numbers and Their Function Is Impaired in SLE Patients

As previously reported, SLE patients display a significant decrease in the absolute numbers of NK cells compared to sex-, age- and ethnicity- matched healthy controls (HC; **Figures 1A, D**). Percentage and/or absolute numbers of CD56+CD16+ and CD56hiCD16- NK cell subsets are reduced in SLE patients (**Figures 1B, D**). Decreased NK cell numbers correlate with disease activity, as patients with higher disease activity display a more profound reduction in NK cell numbers compared to patients with inactive disease and HC (**Figure 1C**).

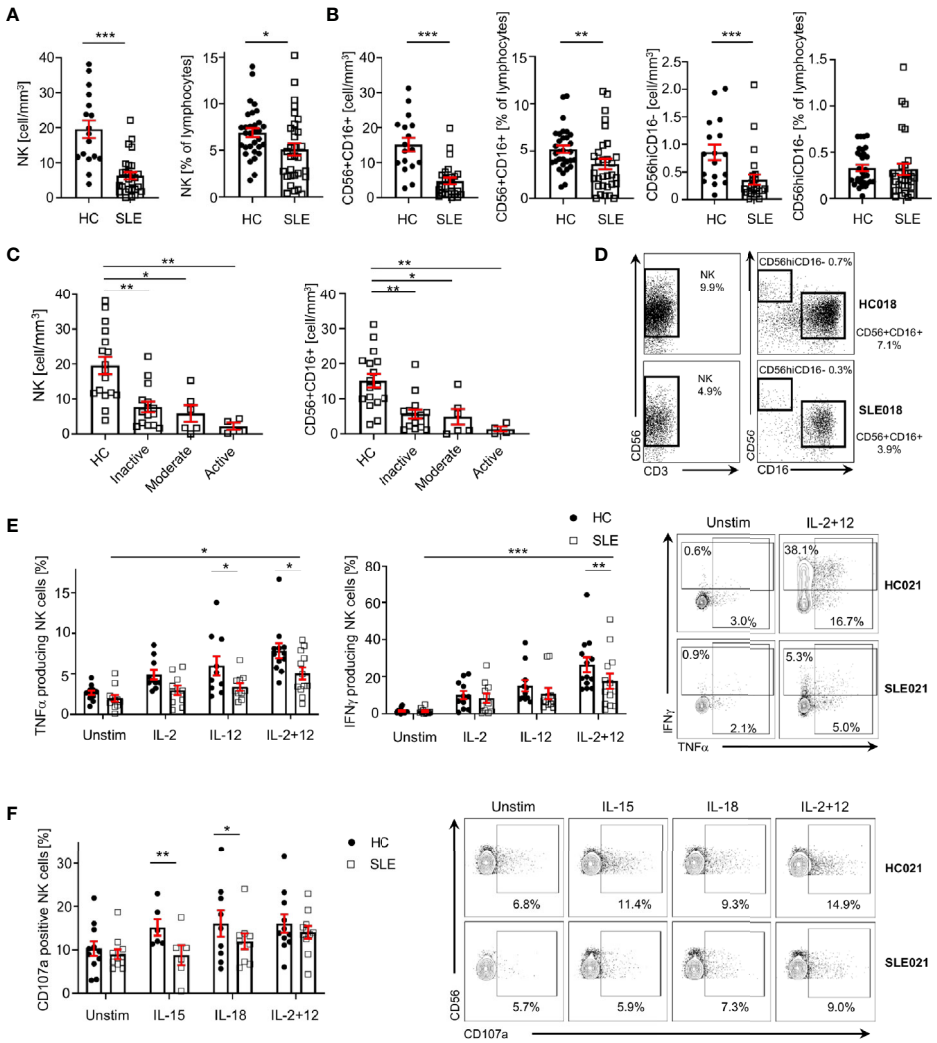

FIGURE 1 | NK cells are decreased and dysfunctional in patients with SLE. **(A)** Total NK cells and **(B)** CD56+CD16+ and CD56hiCD16- NK subpopulations in SLE patients and HC are shown as absolute number (HC=17, SLE=27; Mann-Whitney Test) and percentage of total lymphocytes (HC=31, SLE=31; Mann-Whitney Test). **(C)** Total NK cells and CD56+CD16+ absolute number according to SLE disease activity (HC=17, Inactive=15, Moderate=6, Active=4; Kruskal-Wallis Test with Dunn's multiple comparison test). **(D)** Representative dot-plot of NK cells (left) and subpopulations (right) staining gated on live CD45+CD14-CD7+CD20-CD19- cells. The percentages of NK, CD56+CD16+ and CD56hiCD16- refer to % of total lymphocyte count. **(E)** Cumulative results and representative dot-plot showing NK cell cytokines production in SLE and HC after overnight stimulation (IFNγ HC=12, SLE=12; TNFα HC=13, SLE=13; mixed-effects analysis and two-way ANOVA with Sidak's multiple comparison test). **(F)** Cumulative results and dot-plot showing NK cell degranulation (CD107a+ cells) after overnight stimulation in SLE and HC (HC=11, SLE=11; two-way ANOVA and Sidak's multiple comparison test). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

To examine the function of NK cells in SLE, we stimulated NK cells with a combination of IL-2 and IL-12, which promoted the production of IFNγ and TNFα by SLE NK cells (**Figure 1E**), although significantly less compared to HC (**Figure 1E**). In response to IL-15 and IL-18, the degranulation of SLE NK cells is impaired compared to HC, as illustrated by the reduced frequency of CD107a+ NK cells in SLE patients (**Figure 1F**). Stimulation with IL-2 and IL-12 provided a strong enough stimulation to activate SLE NK cells degranulation as effectively as in HC (**Figure 1F**). Collectively, our data indicates that SLE NK cells display impaired cytokine production and reduced degranulation in response to activation with different cytokines.

Phenotypic Alterations of SLE NK Cells

We exploited single-cell mass cytometry to decipher the extracellular phenotype of SLE NK cells. Our panels include lineage markers for T cells, B cells, NK cells, monocytes and dendritic cells. The gating strategy is presented in **Supplementary Figure 1**. Various markers that characterize NK cells subpopulations, as well as markers that have been shown to be aberrantly expressed on other cell subsets in SLE were examined.

These include CD25, CD38, PD-1, activation receptors (NKp46, NKG2D, DNAM-1), inhibitory receptor (NKG2A. KIR2DL, KIR3DL) and receptors belonging to the SLAMF family, including SLAMF1 (CD150), SLAMF2 (CD48), SLAMF3 (CD229), SLAMF4 (CD244, 2B4), SLAMF5 (CD84), SLAMF6 (CD353, NTB-A) and SLAMF7 (CD319, CRACC, CS-1).

Our data indicates that CD38 is expressed at a higher level in total SLE NK cells (CD3-CD14-CD7+CD19-CD56+) (**Figure 2A**; **Supplementary Figure 2A**). This difference is independent of disease activity (**Figure 2B**) and is also observed in CD56+CD16+ and CD56hiCD16- NK subsets (**Figures 2C, D**). Similar results were found for treatment-naïve SLE patients (**Supplementary Figure 2B**), suggesting that this alteration is not drug-related. We applied clustering analysis on pre-gated NK cells and identified four cell clusters (**Figure 2E**) that do not differ in frequency between HC and SLE, indicating that there is no NK subpopulation that is characteristic of SLE patients and could be used as a biomarker. Interestingly, we observed that cluster 2 has a CXCR5 expressing subpopulation and cluster 4 one expressing KIR3DL1, which are only present in SLE patients (**Figure 2E**). Further research is warranted to understand the pathophysiological importance of these subpopulations.

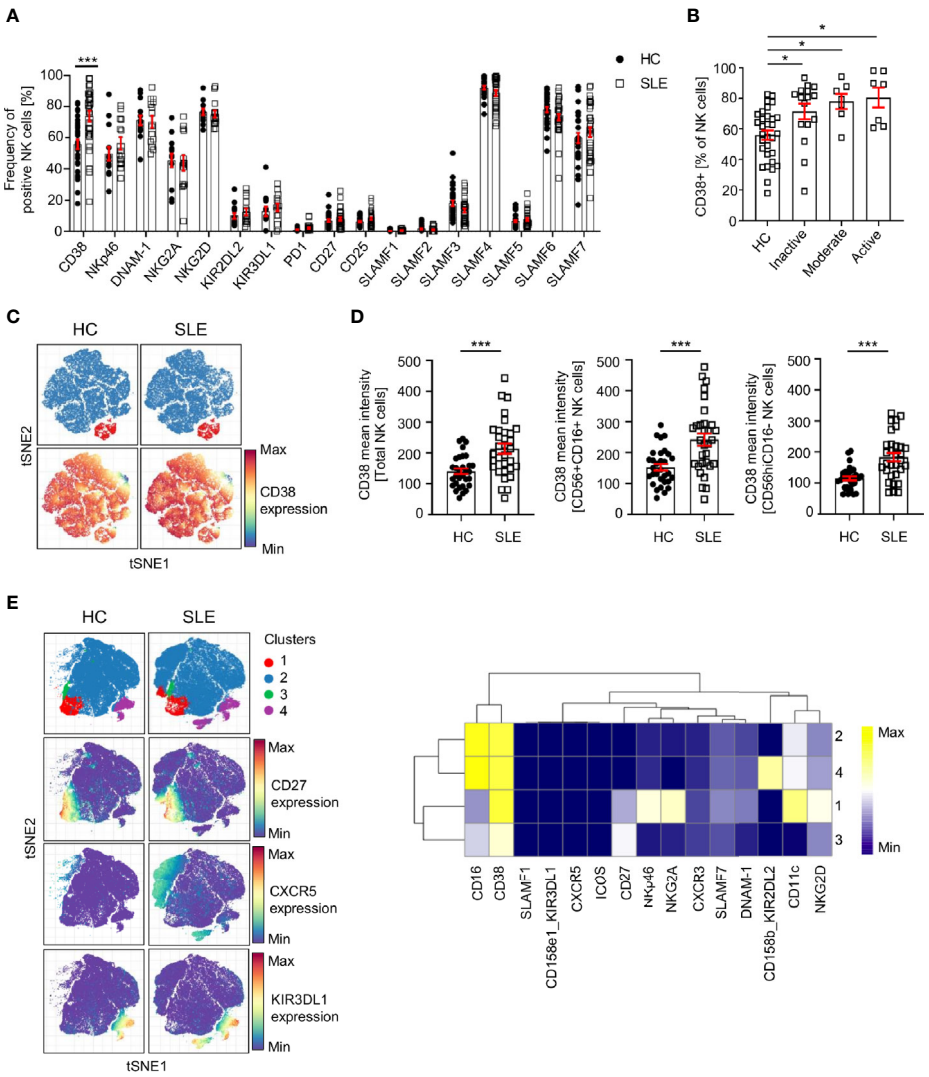

FIGURE 2 | Analysis of NK cell surface markers in SLE patients and controls by single-cell mass cytometry. **(A)** Percentage of NK cells expressing the indicated cell surface markers in HC and SLE (HC=33, SLE=33; two-way ANOVA and Sidak's multiple comparison test). **(B)** Frequency of CD38+ NK cells in SLE patients according to the SLE disease activity (HC=31, Inactive=16, Moderate=7, Active=7; Kruskal-Wallis Test with Dunn's multiple comparison test). **(C)** Representative t-SNE analysis showing the expression of CD38 on SLE and HC NK cells (down-sample HC=30'000 cells, SLE=30'000 cells; blue: CD56+CD16+ NK cells; red: CD56CD16hi NK cells). **(D)** Cumulative results showing the expression (mean intensity) of CD38 on total NK, CD56+CD16+ and CD56hiCD16- NK cells (HC=31, SLE=31; Mann-Whitney test). **(E)** t-SNE analysis (down-sample HC=10'000 cells, SLE=10'000 cells) and heatmap showing NK cell clusters in HC and SLE patients. Data represent mean ± SEM (*P < 0.05, ***P < 0.001). HC, healthy controls.

SLE NK Cells Fail to Upregulate SLAMF1 and SLAMF7 in Response to Cytokine Stimulation

Since the response of SLE NK cells to cytokine stimulation is impaired and considering that the function of NK cells relies on their extracellular phenotype (9), we examined the expression of NK cells surface receptors following stimulation with IL-2 and IL-12 for up to 48h in SLE patients and matched HC. We observed a marked upregulation of SLAMF1 and SLAMF7 on NK cells from HC, 11-fold and 9-fold respectively at 48h of cytokines stimulation, compared to unstimulated cells (**Figure 3A**). Interestingly, PD-1 also shows a 5.6-fold increase at 48h of stimulation (**Figure 3A**). Of note, CD38 is not significantly upregulated after NK cells are activated with cytokines (**Figure 3A**). However, NK cells from SLE patients failed to upregulate certain cell surface receptors to the same extent as HC (**Figures 3B, C**; **Supplementary Figure 3**). More specifically, although SLAMF1 expression is also upregulated on SLE NK cells upon cytokine stimulation, the upregulation is less prominent than that observed in HC (**Figures 3B, C**). Similarly, NK cells from SLE patients fail to upregulate SLAMF7 and PD-1 to the same extent as HC (**Figures 3B, C**; **Supplementary Figure 3**). Overall, our data shows that NK cells from SLE patients fail to adequately upregulate SLAMF1 and SLAMF7 in response to cytokine stimulation.

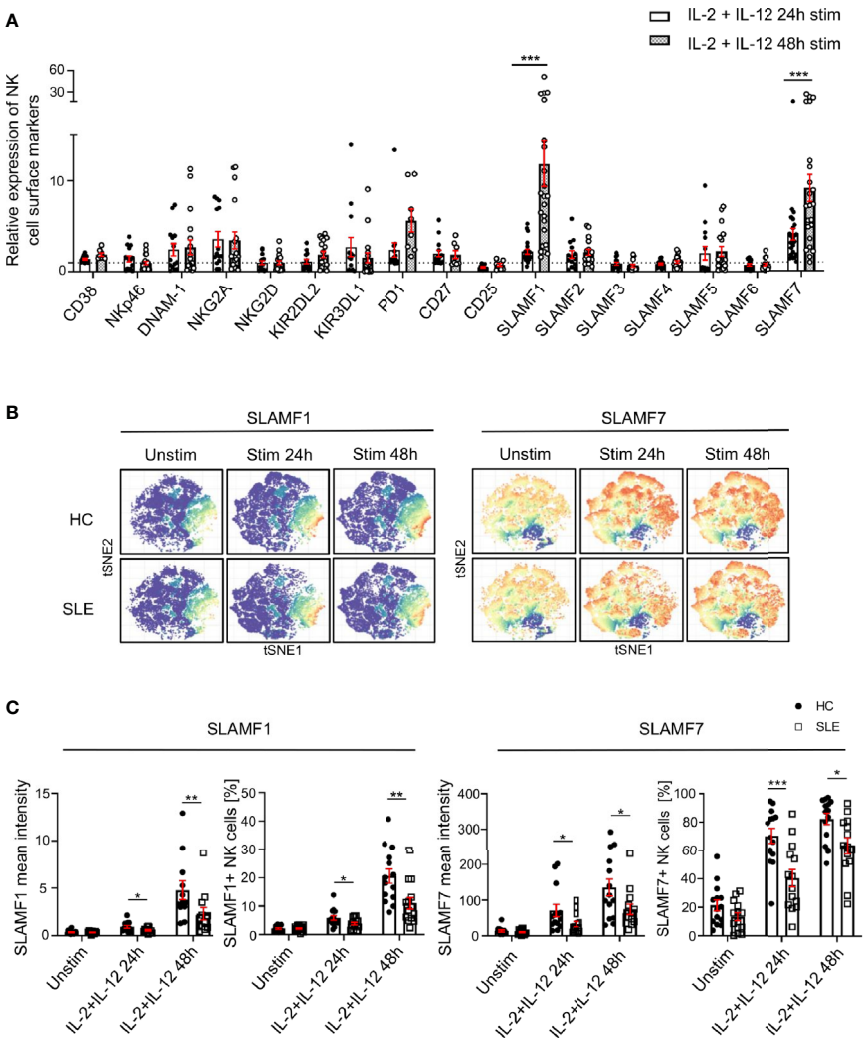

FIGURE 3 | SLAMF1 and SLAMF7 fail to be properly upregulated on the surface of SLE NK cells after activation with cytokines. **(A)** Expression of surface markers after 24h and 48h of stimulation with cytokines on healthy NK cells, standardized to their level of expression on unstimulated cells (HC=23, SLE=23; Mixed-effects analysis with Sidak's multiple comparison test). **(B)** t-SNE presentation of the expression level of SLAMF1 and SLAMF7 in HC and SLE patients before and after stimulation with cytokines (down-sample HC=12'000 cells, SLE=12'000 cells). **(C)** Comparison of expression of NK cell surface markers after 24h and 48h of stimulation with cytokines between HC and SLE patients as mean intensity (above) and frequency (below) (HC=14, SLE=14; two-way ANOVA and Sidak's multiple comparison). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

Engagement of CD38 and SLAMF7 With Specific Monoclonal Antibodies (mAb) Enhances the Function of Healthy and SLE NK Cells

We investigated how the engagement of SLAMF1, SLAMF7 and CD38 with mAb influences NK cell function, by examining the production of cytokines, degranulation and cell viability after 6h and 18h. Ligation with elotuzumab, a humanized anti-SLAMF7 mAb approved to treat relapsing multiple myeloma (15), promotes NK cells degranulation and IFNγ production after 18h, whereas no significant NK cells activation was observed at 6h of stimulation in HC (**Figure 4A**). Another clone of anti-SLAMF7 mAb (clone 162.1), which has been shown to enhance the cytotoxic response of SLE CD8+ T cells in response to viral antigen (16), did not produce any significant effect on NK cells degranulation (**Figure 4A**). NK cells stimulation with daratumumab, a mAb that agonizes CD38, strongly enhanced NK cells degranulation, IFNγ and TNFα production (**Figure 4A**). Interestingly, in healthy controls daratumumab effectively promotes NK cell degranulation and production of IFNγ and TNFα after 6h of stimulation, whereas elotuzumab takes longer to activate NK cells (18h) and only promotes degranulation and production of IFNγ, but not TNFα. Stimulation of NK cells with anti-SLAMF1 mAb (clone A12) did not result in any effect on degranulation or cytokine production (**Figure 4A**).

Next, we examined the effect of SLAMF7 ligation with elotuzumab and of CD38 with daratumumab on NK cells from SLE patients. Based on our results from healthy controls, we used anti-SLAMF1 (clone A12) as negative control. We observed that in SLE NK cells both daratumumab and elotuzumab promote degranulation, after 6h and 18h respectively, to the same extent as in HC (**Figures 4B, C**). However, compared to results obtained in healthy controls, daratumumab and elotuzumab do not promote cytokine production by SLE NK cells (**Supplementary Figure 4A**). Furthermore, the magnitude of degranulation at 18h is, for both HC and SLE NK cells, more prominent following ligation with daratumumab (6-fold) compared to elotuzumab (4-fold) (**Figure 4C**). In addition, we examined NK cells viability after stimulation with elotuzumab and daratumumab. Both antibodies lead to a slight increase in mortality of NK cells compared to the control condition (**Supplementary Figure 4B**). Eventually, we examined the effect of elotuzumab and daratumumab on other lymphocyte subsets and observed no effect on the viability or activation of

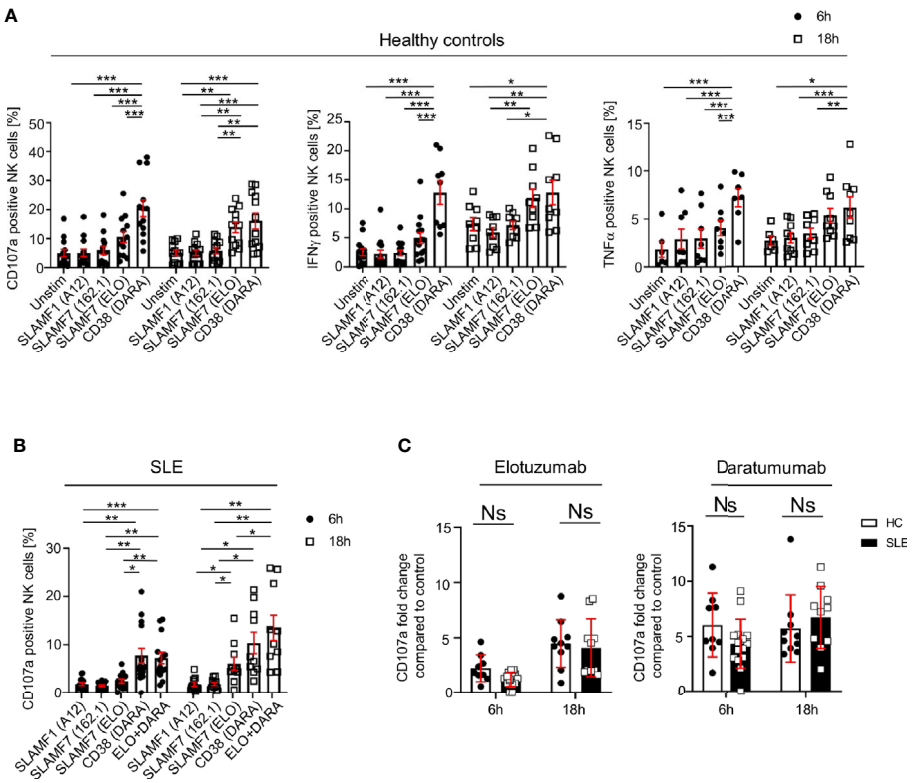

FIGURE 4 | Engagement of SLAMF7 and CD38 with specific mAb enhances the function of healthy and SLE NK cells. **(A)** Degranulation (CD107a) and production of cytokines (IFNγ and TNFα) in NK cells of healthy controls after stimulation with daratumumab (N=14) and elotuzumab (N=12, Mixed-effect analysis with Tukey's multiple comparison test). **(B)** Degranulation (CD107a+) in NK cells of SLE patients after stimulation with daratumumab (6h N=15, 18h N=10) and elotuzumab (6h N=14, 18h N=10; two-way ANOVA analysis and Sidak's multiple comparison test). **(C)** Fold change of CD107a compared to control after 6h or 18h stimulation with elotuzumab or daratumumab in HC and SLE patients (HC 6h=9, HC 18h=10, SLE 6h=15, SLE 18h=10; mixed-effect analysis with Sidak's multiple comparison). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

CD4+, CD8+ T cells and B cells (**Supplementary Figures 5** and **6**).

Altogether, our data shows that elotuzumab and daratumumab specifically activate SLE NK cells by promoting their cytotoxic activity.

Expression of CD38, SLAMF1, and SLAMF7 Characterizes SLE Circulating Plasma Cells

The above-mentioned cell surface receptors are important in cell-to-cell contact. Therefore, to understand their relevance for the pathophysiology of SLE, we investigated their expression on other major lymphocyte populations. We manually gated on CD4+, CD8+, DN T cells (CD4- CD8- double negative T cells), B cells and NK cell. We then exploited t-SNE analysis to visualize the expression of CD38, SLAMF1 and SLAMF7 on these cell populations (**Figure 5A**) and quantified their relative expression levels in SLE patients and HC (**Figure 5B**).

Other than NK cells, our data indicates that all three receptors are expressed at higher levels on SLE B cells compared to HC. CD38 expression did not show any difference in its expression between SLE and HC in any other lymphocyte population included in this study (**Figures 5A, B**). The expression of SLAMF1 is significantly higher on B cells and on CD4+ T cells from SLE patients as previously described (17). In addition, our data shows that SLAMF7 is increased on total B cells from SLE patients, despite a low expression level compared to other lymphocytes such as NK cells, CD8+ and DN T cells. We further examined the expression of these receptors on B cell subpopulations. A t-SNE analysis of naïve B cells (CD19+ CD27- IgD+), non-naïve B cells (CD19+ which are not CD27- IgD+) and circulating plasma cells (CD19+ CD20- CD27+ CD38+ IgD-), showed that all three molecules are expressed at a higher level on circulating plasma cells compared to other B cell subpopulations (**Figures 5C, D**). Moreover, the level of expression of all the three receptors is increased in SLE circulating plasma cells compared to HC, suggesting that these molecules could contribute to the dysfunction of SLE B cells.

Activation of SLE NK Cells With mAb Directed Against CD38 and SLAMF7 Promotes the Killing of Peripheral Blood Plasma Cells

We evaluated whether the activation of NK cells can promote the killing of SLE peripheral blood plasma cells. We generated a NK-B cell *in vitro* co-culture system, in which we pre-stimulated NK cells of HC with elotuzumab or daratumumab, then co-cultured them with autologous B cells and measured the mortality of B cell subsets.

First, our data shows that elotuzumab (18h) and daratumumab (6h) can efficiently kill circulating plasma cells, leading to 2.1 and 2.7 fold more dead cells compared to negative control (SLAMF1 stimulation) respectively (**Figure 6A**). Furthermore, when NK cells are activated with either mAb they kill circulating plasma cells specifically, sparing other B cell subpopulations, such as naïve, activated, resting and tissue like memory cells (**Figure 6A**). Second, we observed that the presence of NK cells is necessary to achieve significant killing of circulating plasma cells, although both mAb alone have a minor impact on circulating plasma cell mortality (**Figure 6B**).

Due to the restrictions in the SLE sample size that we can obtain, we could not repeat this assay in SLE patients. Accordingly, we isolated CD3- cells and stimulated them with mAb. We observed that at 6h, treatment with daratumumab significantly killed circulating plasma cells of SLE patients to the same extent as in the matched HC (**Figure 6C**). In conclusion, our results strongly suggest that these mAb act through the activation of SLE NK cells and effectively kill SLE circulating plasma cells.

DISCUSSION

We exploited single-cell mass cytometry to decipher the phenotypic alterations that characterize SLE NK cells. Our data identified CD38 as being highly expressed on SLE NK cells compared to HC. Moreover, we observed that SLE NK cells fail to properly upregulate SLAMF1 and SLAMF7 when activated with cytokines; two receptors that play an important role in cell-to-cell interaction. We showed that these three receptors are also highly expressed on SLE peripheral blood plasma cells, a cell population that contributes to the production of autoantibodies in SLE. In addition, we demonstrated that mAb directed against CD38 and SLAMF7 receptors enhance the degranulation of SLE NK cells and selectively promote the killing of peripheral blood plasma cells. Overall, our data suggests that the dysregulation of SLAMF1 and SLAMF7 on the surface of SLE NK cells contribute to their dysfunction and might impair their interaction with plasma cells, resulting in an accumulation of autoantibody producing cells. Additionally, targeting NK cells with activating mAb may represent an attractive direction to eliminate autoantibody-producing cells in SLE.

SLAMF1 and SLAMF7 belong to the signaling lymphocytic activation molecule family receptors. A unique feature of these two SLAMF members is that they act as self-ligand (18). The involvement of SLAMF molecules in SLE pathogenesis has been repeatedly reported (16–24) as well as their importance in NK cells activation and interaction with other cell types (25, 26). SLAMF1 has been reported to be expressed at a higher level on SLE B cells and CD4+ T cells compared to their healthy counterparts and its importance for SLE B cell function was previously reported (26). However, its potential role on SLE NK cells was not previously described. SLAMF7 has been shown to be highly expressed by cytotoxic cells and plasma cells. SLAMF7 displays an altered expression, function and/or regulation on SLE NK cells and CD8+ T cells (16, 19, 22), supporting a role of this molecule in SLE pathogenesis. The importance of SLAMF7 was described in multiple myeloma, where elotuzumab was approved to treat disease relapse (15). The binding of elotuzumab contributes to the elimination of myeloma cells, through various mechanisms including the activation of NK cells cytotoxic response and antibody dependent cell-mediated cytotoxicity (27). A previous

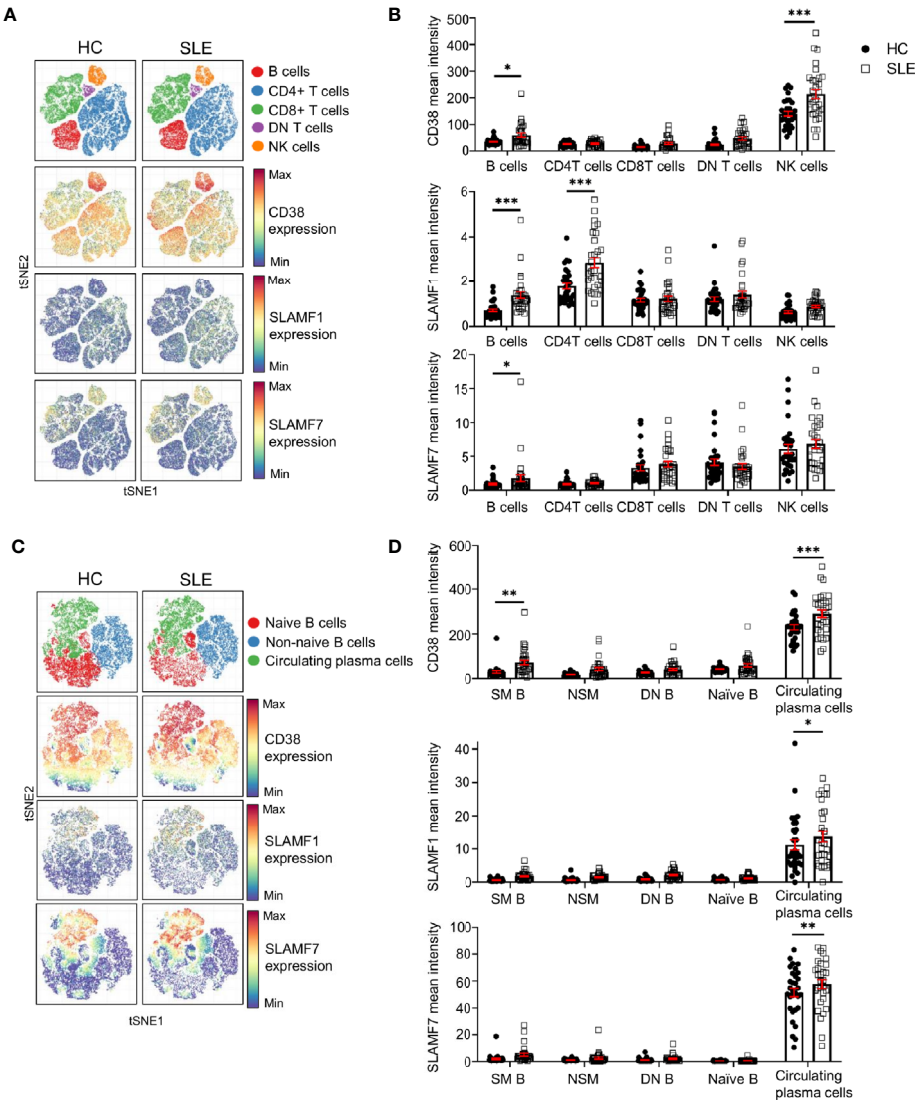

FIGURE 5 | Expression of CD38, SLAMF1 and SLAMF7 characterizes SLE circulating plasma cells. ***(A)*** t-SNE presentation of the expression level of CD38, SLAMF1 and SLAMF7 in main lymphocyte populations for HC and SLE patients (down-sample HC=100'000 cells and SLE=100'000 cells). **(B)** Comparison of mean expression level of CD38, SLAMF1 and SLAMF7 between HC and SLE patients in main lymphocyte populations (HC and SLE=31; two-way ANOVA with Sidak's multiple comparison test). **(C)** t-SNE presentation of the expression level of CD38, SLAMF1 and SLAMF7 in B cell subpopulations for HC and SLE patients (down-sample N=10'000 cells per subpopulation HC and SLE=26). **(D)** Comparison of the mean expression level of CD38, SLAMF1 and SLAMF7 between HC and SLE patients in B cell sub-populations (HC=31, SLE =31; two-way ANOVA with Sidak's multiple comparison test, SM, switch memory; NSM, non switch memory; DN, double negative). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC, healthy controls.

study has shown that the ligation of SLAMF7 in SLE promotes the degranulation of CD8+ T cells in response to viral antigens, therefore empowering the antiviral response that is compromised in patients with SLE (16), highlighting the potential therapeutic benefit of targeting SLAMF7.

CD38 is a surface glycoprotein with ectoenzymatic functions and is expressed at high levels on plasma cells. Like SLAMF7, CD38 has been identified as a target for mAb to eliminate myeloma cells in patients with relapsing multiple myeloma with the use of anti-CD38 daratumumab (28). A recent report has shown that daratumumab represents a potential therapeutic approach to eliminate antibody-producing plasma cells in SLE patients (29). Furthermore, it has been shown to ameliorate clinical manifestations and to eliminate antibody producing plasma cells in two patients with refractory SLE (30). A subset of SLE patients who are highly susceptible to infections, exhibit an altered CD8+ T cells cytotoxic response and express a high level of CD38 on their surface (31), thus further underlining the potential benefits of targeting CD38. Our data stresses a preponderant role of NK cells in the process leading to plasma

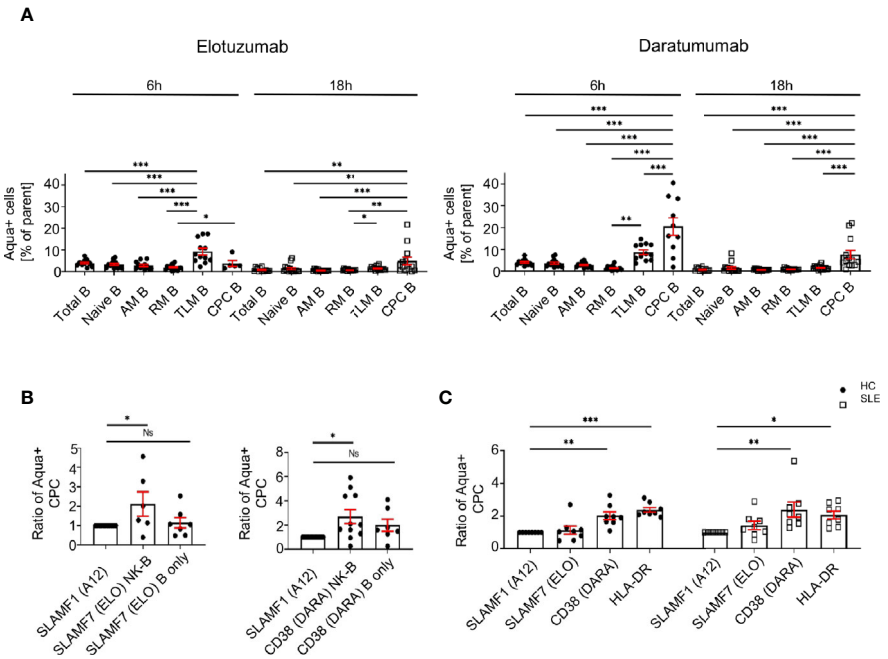

FIGURE 6 | Activation of SLE NK cells with mAb directed against CD38 and SLAMF7 promotes the killing of peripheral blood plasma cells. **(A)** Frequency of dead cells in a NK-B cell co-culture system after 6h (N=14) and 18h (N=16) following stimulation with SLAMF1, elotuzumab or daratumumab (one-way ANOVA with Tukey's multiple comparison test). **(B)** Fold increase of dead circulating plasma cells following stimulation with daratumumab 6h (N=12) or elotuzumab 18h (N=7) in either B cells alone or B cells co-cultured with pre-stimulated NK cells (one-way ANOVA with Sidak's multiple comparison test) **(C)** Ratio of dead cells over control condition after 6h stimulation with elotuzumab or daratumumab in HC and SLE patients (HC=8, SLE=8; two-way ANOVA, Sidak's multiple comparison test). Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001). HC=healthy controls.

cell depletion by daratumumab, as stimulation of isolated NK cells with daratumumab is sufficient to promote the killing of circulating plasma cells in culture, whilst the sole exposure of B cells to the drug isn't.

As previously reported, PD-1 is also upregulated on NK cells in response to inflammatory cytokines (32). This increase is significantly altered in SLE NK cells and likely reflects SLE NK cells compromised activation status. Further investigation is warranted on this aspect.

Our study reveals interesting differences between daratumumab and elotuzumab. Elotuzumab promotes degranulation of NK cells and IFNγ production, but not the production of TNFα by NK cells. Since elevated TNFα levels have been described in SLE patients and may contribute to the pathogenesis of organ damage (33), this property could be of interest if elotuzumab was to be considered as a therapeutic option in SLE. On the other hand, SLE NK cell degranulation and elimination of antibody-producing cells *in vitro* is more robust when NK cells are activated with daratumumab compared to elotuzumab.

Our study has several limitations. First, the use of single-cell mass cytometry limits the identification of cell surface receptors to the antibodies included in our panels. Compared to RNA seq, this method monitors fewer targets but directly identifies cell surface proteins that can be targeted by therapeutic mAb. Second, further experiments are warranted to identify the individual implications of the three cell surface markers evaluated in this study in the interaction between NK cells and circulating plasma cells. So far, this aspect remains unexplored due to the limited number of circulating plasma cells available from the peripheral blood of patients and controls. We are working on plasma cell line culture system that will allow to individually silence each receptor. Finally, examination of secondary lymphoid organ and bone marrow aspirations would allow examination of B cells during their maturation process and long-lived plasma cells. However, these tissues are difficult to obtain.

In conclusion, the failure of SLAMF1 and SLAMF7 regulation on SLE NK cells might contribute to an impaired interaction between NK cells and plasma cells. This might lead to the accumulation of antibody producing plasma cells that characterizes SLE. From this point of view, restoration of NK cell cytotoxicity may contribute to the elimination of SLE plasma cells. Targeting SLAMF7 with elotuzumab and CD38 with daratumumab contributes to the elimination of antibody producing cells *in vitro* and this elimination occurs, at least in part, through the restoration of SLE NK cells degranulation. Because both elotuzumab and daratumumab are safe when used to treat multiple myeloma and appear to be well-tolerated when administrated to SLE patients, their utilization should be evaluated in controlled studies to assess their efficacy to treat SLE.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Commission cantonale d'Ethique de la Recherche sur l'être humain CER-VD (SwissEthics 2017-01434). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DC: study design and data analysis. MH, FB, and NF: conducting experiments, data acquisition and analysis. CR and AH: recruitment of SLE patients and healthy controls. CF: responsible of CyTOF facility. MH and MS: bioinformatics analysis. DC and MH: writing and editing of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.645478/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5. Conclusion and Future perspectives

Systemic lupus erythematosus is a rare chronic inflammatory autoimmune disease whose etiology and pathophysiology remain elusive to this date. Patients with SLE present alterations in the innate and adaptive immune system, which elicit a loss of tolerance and the generation of autoantibodies. These immune alterations lead to a plethora of symptoms in SLE patients, making the diagnosis complicated. Due to this, there is often a considerable delay between the initial symptom and the final diagnosis, which may be associated to a belated treatment and cause permanent organ damage. There is currently no cure for the disease and only few targeted treatments. A deeper understanding of SLE pathophysiology is fundamental to obtain better diagnostic and therapeutic approaches. Furthermore, it may allow to classify SLE into subcategories and individualize medical care.

5.1. SLE Diagnosis: SLAMF receptors define a SLE immune signature

The first aim of this PhD project was to gain a better understanding of the phenotypic alterations characteristic of SLE PBMC, with the perspective of identifying a SLE immune signature. Previous studies on SLE PBMC showed that SLAMF receptors expression and the frequencies of the respective subpopulations are altered in patients afflicted by the disease [50] . Furthermore, it was shown that SLAMF receptors have an important role in the function of healthy lymphocytes and could be exploited to restore the function of SLE PBMC. For example, CD8+ T cells in SLE have reduced cytotoxic abilities, leading to a reduced immune surveillance and viral protection. The engagement of SLAMF7 with monoclonal antibodies allows to recover the normal CD8+ T cell anti-viral function [30].

We hypothesized that, since the pathologic function of SLE cells is linked to an altered SLAMF expression, these receptors could define a SLE specific immune signature. In this perspective, we investigated the extracellular phenotype of the main PBMC populations (CD4+ T cells, CD8+ T cells, DN, B cells, monocytes, dendritic cells, and natural killer cells) and their subpopulations in SLE patients, by focusing especially on SLAMF receptors.

Our investigation identified three cell subsets that are significantly increased (frequency over parent population) in SLE compared to healthy controls and autoimmune diseases controls (sarcoidosis, Sjögren's syndrome and multiple sclerosis): SLAMF1+ B cells (SLEB1), SLESMB cells and SLEcTFH cells.

Increased levels of SLAMF1 in T cells and B cells, similar to the ones observed here, were reported by Karampetsou *et al.* [49]. In the same study the group suggested that monoclonal anti-SLAMF1 antibodies could be used to treat SLE, since they reduce T-B interactions, the production of IL6 by B cells and B cell differentiation into plasmablasts.

It is possible that the IL6 production by SLAMF1+ B cells promotes Tfh cell differentiation and spontaneous generation of germinal centers (GC). This hypothesis and the potential impact of this cell population on SLE circulating Tfh cells should be investigated in the future.

Our analysis, further identified that switch memory B cells and circulating T follicular helper cells co-expressing SLAMF1, SLAMF3, SLAMF5 and SLAMF6 are increased in SLE (referred to as SLESMB and SLEcTFH respectively). Considering that these two cell subsets express the same SLAMF receptors, it is likely that they interact via SLAMF1, SLAMF3, SLAMF5 and SLAMF6. Studies on how co-expression of SLAMF receptors impacts the function of single SLAMF receptors are limited. A murine model with SLAMF1, SLAMF5, SLAMF6 knockout genes showed that these receptors synergistically contribute to humoral immunity control [86]. Murine models with SLAMF3 knockout also indicated its role in the negative regulation of humoral immunity [52]. Although no studies have been made on the expression of all four receptors, these murine models indicate that SLAMF1, SLAMF3, SLAMF5 and SLAMF6 are responsible for the fine tuning and regulation of the humoral immunity. It is possible that in SLE this control is lost, thus promoting B cell differentiation into antibody-producing cells. Contrarily, these cell subsets are expanded in SLE, in an attempt of the body to moderate the self-reactive humoral response observed in patients. Further studies are necessary to assess the role and function of these two cell subsets in the SLE pathophysiology.
The functional role of SLESMB cells should be investigated. More precisely it would be of interest to determine whether the SLESMB cells have a lower BCR activation threshold compared to SM B cells and if this leads to a higher titer of antibody production. Furthermore, co-culture assays should be performed to determine if and how SLESMB interact with SLEcTFH cells.

Our research identified a total of three cell subsets that have an increased frequency (over parent) in SLE patients. In view of identifying a SLE immune signature we calculated the predictive factor of these three populations for SLE. ROC curve analysis showed that the measurement SLEcTFH allows to distinguish SLE patients from healthy controls with an accuracy of 92%. Furthermore, the combined measurements two or three of these cell subsets further increased the predictive value. We found that the combination of SLEcTFH and SLESMB measurements promoted the accuracy to 94%. The same analysis was performed to determine the ability of this cell subsets to distinguish SLE from other autoimmune diseases. We found that the measurement of SLEB1/SLESMB allow to distinguish SLE from autoimmune controls with an accuracy of 81%. When we combined the mesaurements, we found that the predictive value could be increased to 84%. We calculated the minimal cell frequency that a patient must have to be considered as SLE and applied to the autoimmune disease cohort. The

combination of either SLESMB with SLEcTFH or SLEB1 with SLEcTFH allowed to discriminate SLE patients from autoimmune controls in 90% of the cases. Interestingly the SLE patients identified using this approach had different: gender, age, ethnicity, disease activity, organ involvement and treatment regiments. This indicates that the SLAMF signature we observed is a general feature of all SLE patients, not of a specific sub-category, and specific to this autoimmune disease. This aspect is a very important characteristic for a biomarker and should be taken as an incentive for future researchers to validate the SLAMF signature on a larger scale. These results show the usefulness of SLAMF receptors in the diagnosis of SLE and underline their involvement in the SLE pathophysiology.

5.2. Future perspectives: Understanding the lymph node of SLE

Recent evidence suggests that alterations in Tfh cells are essential in the development of autoimmunity, including SLE ([87], [88]). Tfh represent a population of memory CD4+ T helper cells that play a pivotal role in GC formation, antibody production and humoral memory establishment through their ability to interact with B cells within follicles [89]. However, no study to date has systematically examined the secondary lymphoid organs of patients with SLE, and the data on genuine Tfh cells and their interaction with B cells is only anecdotic. A major limitation to our understanding of the role of Tfh cells and on B cell maturation is that most data come from the peripheral blood [90] and few information is currently available on cells present in the secondary lymphoid organs of SLE patients. Due to the important contribution of Tfh in the maturation of B cells, understanding their alteration is an important aspect that warrants deeper research. Accordingly, we recently established a safe, easy and minimally invasive procedure to access lymph cervical lymph nodes from patients. This procedure consists of performing fine-needle aspiration (FNA) under ultrasound monitoring, which allows to obtain enough cells to perform mass cytometry analysis and identify the major lymphocytes populations of LNs. Access to the lymph nodes through FNA will provide insight into the quantitative and qualitative alterations of follicular T helper cells, follicular T regulatory (Tfr) cells and B cells.

The first thing that could be investigated, by exploiting mass cytometry, are phenotypic alterations of cells composing the germinal centre in SLE. In parallel mass cytometry analysis of peripheral blood should be performed, to examine the potential correlation between the secondary lymphoid organ cells and peripheral blood cells. Furthermore, this information could in the future be exploited to draw preliminary conclusion of the LN cellular composition following initial blood drawing and indicate if a FNA is warranted. In addition, to understand the contribution of LN resident cells to the SLE

pathophysiology, serum inflammatory cytokines (Luminex multiassay) and autoantibodies (anti-dsDNA, anti-nucleosome) should be evaluated concomitantly.

According to our data, markers allowing for a careful examination of SLESMB and SLEcTFH will be included. Previously SLAMF1, SLAMF5 and SLAMF6 co-expressing Tfh cell and GC B cells have been described in healthy individuals [91]. Furthermore, it was reported in mice that these Tfh and GC B cells can suppress GC formation but promote the development of antigen-specific B cells as well as antibody production [92]. These studies indicate that an increased frequency of SLESMB and SLEcTFH may directly promote spontaneous GC formation via the production of pro-survival effectors (such as BCR and BCL2).

Furthermore, we expect to identify an increased frequency of Tfh cells and a decrease in the Tfr/Tfh ratio in SLE patients compared to healthy controls. This increase in Tfh cells may be correlated with an increased frequency of memory B cells (in particular SM B cells) and serum autoantibodies.

If the frequency of Tfh cells were to be increased, it would be interesting to study why. One hypothesis is that the increase in the number of Tfh cells in the SLE GC is due to a deficiency in IL2 in the LN microenvironment. From this point of view, numerous studies have shown that the production of IL2 by effector T cells is impaired in SLE and that the bioavailability of secreted IL2 is reduced [93]. Other studies have shown that IL2 strongly inhibits Tfh cell differentiation, by activating Tfr cells [41]. Because IL2 acts in paracrine and autocrine ways to influence nearby cells, the IL2 production by T cells that compose the LN should be investigated. To perform this analysis, LN lymphocytes could be stimulated *in vitro* and IL2 production could be examined by intracellular staining. We expect to observe a decrease in the production of IL2 by effector T cells present in the LN from SLE patients compared to healthy controls. The examination of SLE LN will provide important new insights into the pathophysiology and possibly provide novel therapeutic targets for SLE.

5.3. Therapeutic approaches for SLE: considerations

The second aim of this project was to identify novel possible therapeutic targets for SLE. For a long time, only immunosuppressive drugs were available to treat SLE patients. Although they help maintain disease remission, they have severe side effects. Considerable efforts went and still are going into the development of targeted drugs for SLE.

The blatant role of autoantibodies in the pathophysiology of SLE led to intensive studies on treatments that reduce B cell activation and differentiation. This brought the first SLE targeted drug to the market: belimumuab. Targeting the activation and differentiation of B cells to reduce the autoreactive B cell activity, is nevertheless, going to affect almost all B cells, since BAFFR is

expressed by all B cells except for plasma cells [94]. Similarly, rituximab, which is still an off-label drug for SLE, affects all B cells expressing CD20.

Future research should focus on the development of drugs that specifically target B cell subsets that are involved in the production of autoantibodies, such as long and short lived antibody producing cells. A good candidate in this regard might be daratumumab, targeting CD38, which is expressed by all antibody-producing cells.

SLE patients present a characteristic type I IFN signature. Genetic alterations, abnormal cell activation and accumulation of cellular debris contribute to the production of type I IFN. In turn, these IFN promote the inflammatory environment and accumulation of cellular debris. Thus, it remains to be determined whether the IFN signature is a cause or a consequence of the pathophysiological alterations observed in this disease. In either scenario, blocking IFN signaling reduces inflammation and inflammation related pathologic pathways (such as cell differentiation, antigen presentation, antibody production, for instance), as shown by studies on anifrolumab. Nevertheless, this approach significantly increases the risk of infection, which can be very dangerous for these individuals, as mirrored by the fact that infections are the leading cause of mortality in SLE patients.

The generation of better treatments for SLE is a challenging task. The most ambitious task is to identify a potent therapeutic target that, when engaged, elicits a response in all SLE patients, independently of their heterogeneity. The identification of novel therapeutic targets relies on intense scientific effort to decorticate the pathophysiology of the disease. The heterogeneity of the disease makes it complicated to find a common factor between all patients that could be targeted and to define primary endpoints of clinical trials.

It stands to reason that the best way of treating SLE patients is not to target one cell type or one pathway, but to intervene on several pathological cells/pathways. For this purpose, it may be interesting to target receptors, which are expressed on multiples cells with different functions, such as SLAMF receptors, for example. Furthermore, combination therapies should be considered to normalize several pathogenic mechanisms at the same time. For example, combining rituximab (reduce autoantibody production) with anifrolumab (reduce chronic inflammation). Another option could be to combine anifrolumab with elotuzumab (monoclonal antibodies targeting SLAMF7). The main side effect of anifrolumab was shown to be an increased risk to infection. Elotuzuamb allows to restore the function of cytotoxic cells in SLE patients and could therefore also reduce the risk of infection. Combination therapy in the context of targeted therapies for SLE nevertheless needs to be cautiously tested. Considering heterogeneity of patients and the complex pathophysiology it may have unforeseen side effects or only be efficient in specific SLE subcategories.

5.4. Therapeutic approaches for SLE: NK cells

In the second part of our project, we focused on the importance of natural killer cells in SLE. We observed that NK cells are altered in number (frequency over lymphocytes and absolute numbers) and function (degranulation and cytokine production). This could partly explain why SLE patients present higher incidence of infections and cancer, compared to the healthy population ([20], [22]).

The analysis of the extracellular phenotype of NK cells, that included NK cell specific markers, differentiation markers and SLAMF receptors, showed alterations of two surface receptors. Indeed, SLE NK cells present an increased expression of CD38 and a dysfunctional upregulation of SLAMF7 upon cytokine activation, compared to healthy NK cells. Functional investigations indicated that the engagement of both receptors with specific monoclonal antibodies, elotuzumab (anti-SLAMF7) or daratumumab (anti-CD38) restores the function of SLE NK cells (cytokine production and degranulation). In addition, An NK-B co-culture system allowed to observe that daratumumab and elotuzumab ligation on SLE NK cells promotes the specific killing of circulating plasma cells of HC in a NK-B cells *in vitro* co-culture system. Daratumumab had the same effect on SLE NK cells. Our study showed interesting difference in the kinetic and magnitude in NK cells activation by daratumumab and elotuzumab. Indeed, daratumumab activates NK cells after 6 hours of incubation, whereas elotuzumab enhances NK cells degranulation and cytokine production after 18 hours of stimulation. Furthermore, daratumumab elicits a stronger response compared to elotuzumab in all aspects investigated (cytokine production, cytotoxic activity, cPC killing). Interestingly, *in vitro*, the two drugs did not show a synergic effect. In the future, it will be interesting to understand why these two antibodies act differently.

The low NK cell activation induced by elotuzumab, suggests that it could be evaluated in clinical trials for patients with mild to moderate SLE disease, as an adjunctive therapy to hydroxychloroquine for example.

Elotuzumab and daratumumab present a different mechanism of action compared to current drugs. Indeed, SLAMF7 and CD38 are highly expressed on circulating plasma cells but also on NK cells. Both antibodies bind and activate NK cell cytotoxicity. Furthermore, they bind to cPC, promote ADCC and stabilize NK-cPC contact, . Furthermore, activation of NK cells could help prevent infections in SLE patients, which would be a unique feature among SLE treatments. For these reasons SLAMF7 and CD38 could be powerful therapeutic targets.

In a general manner, this study identified two novel potential therapeutic targets for SLE. Interestingly, concomitant to our study, a German group [95] treated two remittent SLE patients with daratumumab, observing a resolution of most symptoms. In addition, elotuzumab and daratumumab have been accepted by the FDA and SwissMedic for the treatment of multiple myeloma. In this

context, clinical trials showed that both drugs present minimal secondary effects (mostly reactions to injection).

5.5. Future perspectives: Understanding SLE NK cell dysfunction

To deeper understand the alterations that characterize SLE NK cells, we plan to examine their cellular metabolism. Metabolic alterations are closely related to cell phenotype and function ([96], [97]). The metabolism of NK cells relies mainly on glycolysis and oxidative phosphorylation (OXPHOS). No data is available on SLE NK cells.

We already performed several preliminary experiments in this respect. Previous research focused on SLE CD4+ T cells and showed increased glycolysis and OXPHOS at basal level and after TCR activation compared to their healthy counterparts ([98], [99]). Our preliminary data indicate that SLE NK cell have a normal glycolytic activity, but an increased OXPHOS compared to healthy controls. The potential relationship between the alteration in OXPHOS and altered NK cell function needs to be investigated in the future.

Furthermore, it would be interesting to determine if the alteration in SLE NK cellular metabolism is a primary defect or linked to the chronic inflammatory environment characterizing SLE patients (which includes increase in TNFα, IFNγ, IL6, IL17) ([100], [38]).

To answer this question, two assays can be performed. First, incubate healthy NK cells with serum from SLE patients to examine if the altered metabolic phenotype of SLE NK cells can be reproduced. Second, healthy NK cells could be incubated with various cytokines mixtures and soluble factors (especially IFNγ) to examine how it affects their metabolism. Additionally, recently we identified that SLE patients display an increase in certain serum adipokines (mainly leptin and adiponectin), that have the capacity to alter SLE NK cells metabolism and cytotoxicity [101].

A further explanation of the alteration in SLE NK cellular metabolism could be the alteration of expression of extracellular metabolite transporters. Among the numerous surface receptors that characterize NK cells and contribute to their function, some are implicated in the transport of extracellular metabolites [102]. Three receptors have been shown to be induced on activated cytotoxic NK cell to fuel the cell and meet the nutritional needs: GLUT1, CD98 and CD71 [103]. They, respectively, contribute to glucose transport, amino-acid transport, iron transport (via transferrin binding). Their importance in fueling mitochondrial ATP production has been previously shown [103]. The expression of these receptors in SLE NK cells has not been evaluated to date. The potential alteration of SLE NK cells to transport extracellular metabolites is an important issue to be addressed in the context of cellular metabolism studies.

Our preliminary data indicate that OXPHOS is increased in SLE NK cells. Accordingly, it would be intriguing to evaluate the fitness of mitochondria in SLE NK cells by evaluating the mitochondrial mass, mitochondrial membrane potential (MMP) and mitochondrial structure. To investigate these aspects, two different approaches could be used: assessment of the mitochondrial mass and MMP by MitoTracker^{TM} staining and analysis by flow cytometry and confocal microscopy. We hypothesize that SLE NK cells present a normal mitochondrial mass, but alterations in the mitochondrial membrane potential, which would explain the increment in OXPHOS we found in our preliminary SLE NK analysis. Additionally, it would be interesting to examine potential alterations in mitochondrial ultrastructure (shape, cristae fragmentation, disarrangement of interior structure) by using transmission electron microscopy (TEM).

A further option, to decipher the metabolic alterations of SLE NK cells, would be to perform proteomic analysis. This could identify which metabolic related pathways are altered in SLE NK cells.

In our previous work [104] we showed that the engagement of SLAMF7 and CD38 with elotuzumab and daratumumab respectively, enhances NK cell degranulation and cytotoxic activity, although at different magnitude. To understand the how these antibodies activate NK cells, we plan to examine how they interfere with NK cellular metabolism. Interestingly, our preliminary data indicate that daratumumab primarily promotes glycolysis, whereas elotuzumab mainly enhances OXPHOS, suggesting that the two antibodies act upon distinct signaling pathways to activate NK cells. To study this further, one could also observe the effect of the two drugs on NK surface receptors. Preliminary data suggest that daratumumab and elotuzumab promote the expression of GLUT-1, CD98 and CD71. In addition, both monoclonal antibodies strongly enhance intracytoplasmic calcium mobilization to similar levels. These data warrant confirmation but suggest that there are important differences in how these monoclonal antibodies activate NK cells.

Another way to understand how daratumumab and elotuzumab affect NK cell metabolism is to culture NK cells under conditions of limited fuel availability and observe if the drugs can stabilize the response. To perform these experiments, healthy NK cells can be cultured in the presence of various media complemented with pyruvate, glucose, glutamine, or all of the above together.

From our preliminary data, fueling for NK cells OXPHOS depend mainly on the presence of glucose and pyruvate in the extracellular medium. Stimulation with elotuzumab appears to enhance the maximal respiration of NK cells to levels that are similar to mitostress medium, in the presence of glucose or pyruvate only, suggesting that elotuzumab also acts after glucose is metabolized into pyruvate, and probably at level of TCA cycle.

As previously mentioned, activation of SLE NK cells with elotuzumab is less effective than activation by daratumumab, and is not sufficient to promote the killing of cPC in SLE. Interestingly, our preliminary experiment indicate that daratumumab is able to enhance glycolysis in SLE NK cells, but elotuzumab has no significant effect on OXPHOS, and especially on maximal respiration, which is already aberrantly increased in SLE NK cells. These data provide a support to explain why elotuzumab is less effective to activate SLE NK cells than healthy cells.

All the experiments on the effect of elotuzumab and daratumumab proposed here should also be performed on SLE NK cells (examination of cell surface receptor for metabolites, calcium influx, limitation of fuel availability, and global proteomic analysis).

Furthermore, it would also be interesting to examine SLE NK cell transcriptomic by single cell sequencing analysis, compared to their healthy counterparts. This will help to identify gene expression changes associated with disease condition and open the field for additional hypothesis on the alteration of NK cells in SLE.

5.6. Concluding remarks

In conclusion, this PhD project identified novel possible diagnostic markers and therapeutic approaches. The relevance of SLAMF receptors in SLE pathophysiology is further underlined by this work. In this perspective, it would be interesting to systematically investigate the function of SLAMF8 and 9 in healthy immune cells and their alterations in autoimmunity and SLE especially.

The development of new technical approaches such as genomics, RNAomics, proteomic and metabolomic analysis are likely to be take a central stage in the deeper understanding of SLE pathophysiology. Furthermore, they could become part of routine diagnostic testing and could be used to take personalized therapy to the next level.

Decorticating complex pathological systems is an interesting task and could provide insights into healthy pathways. This could then be implemented for innovative therapeutic approaches, such as immunotherapy.

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