NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

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BSc in Biochemistry

T CELL-NEUTROPHIL CROSSTALK IN SYSTEMIC LUPUS ERYTHEMATOSUS

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

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To my family and friends for all the support!

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"Um bem-haja a todos!"

“Do not fear failure but rather fear not trying.” (Roy T. Bennett).

ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects mainly women of childbearing age. Both innate and adaptive immune systems are involved, however, a widely accepted major issue is the imbalance between cell death and the clearance of the biological debris created. Contributing to it are neutrophils that undergo a specific death program called NETosis and generate neutrophil extracellular traps (NETs), chromatin web-like structures, that have been reported as pathogenic in the disease. Neutrophils are a heterogenous population in which low-density neutrophils (LDNs) take a role in the pathogenesis of SLE contrasting with their counterparts the high-density neutrophils (HDNs).

Toll-Like Receptors (TLRs) 7 and 9 are pattern recognition receptors that recognize ssRNA and dsDNA, respectively, and are majorly expressed in innate immune cells. Nevertheless, late descriptions have been reported expression of TLRs in adaptive immune cells, among them T lymphocytes.

We hypothesized that TLR7 and TLR9 in $CD4^+$ T cells could play a role in SLE through the recognition of nucleic acids contained in NETs. To do so T cells were isolated from the blood of SLE donors and were stimulated with NETs from high- and low-density neutrophils.

Results of this study showed that both NETs from LDNs or HDNs upregulated the expression of TLR7 on $CD4^+$ T cells and induce a proinflammatory response through the secretion of TNFα, and especially of IL-21. In contrast, TLR9 is not engaged by NETs.

With this work a crosstalk between T cells and neutrophils was unraveled, where NETs play a central role in SLE and are the bridge between innate and adaptive immunity.

Keywords: SLE, T cells, LDNs, HDNs, NETs, TLRs

RESUMO

O Lupus eritematoso sistémico (LES) é uma doença autoimune crónica que afeta maioritariamente mulheres em idade fértil. Ambos os sistemas imunes inato e adaptativo estão envolvidos na doença. A disfunção mais aceite no LES relaciona-se com o desequilíbrio entre a morte celular e a remoção dos restos celulares por ela deixados. Contribuintes para isso são os neutrófilos, que sofrem um tipo específico de morte celular denominado NETose, gerando as armadilhas extracelulares de neutrófilos (NETs), estruturas em forma de rede composta por ácidos nucleicos, descritas como patogénicas no LES. Os neutrófilos são heterogéneos sendo constituídos pelos neutrófilos de baixa densidade (LDNs), que são patogénicos e pelos neutrófilos de alta densidade (HDNs), que não o são.

Os Recetores do tipo Toll (TLRs) 7 e 9 são recetores de reconhecimento de padrões, como o ARN de cadeia única e o ADN de cadeia dupla, que são maioritariamente expressos em células do sistema imune inato. No entanto, recentemente foi reportado que os TLRs podem ser expressos em células do sistema imune adaptativo, de entre elas nos linfócitos T.

Colocou-se a hipótese que os linfócitos T ao expressarem TLR7 e TLR9 poderiam ter uma função no LES através do reconhecimento dos ácidos nucleicos contidos nas NETs. Desta forma, células T do sangue de doentes de LES foram isoladas e estimuladas com NETs tanto dos neutrófilos de alta como dos de baixa densidade.

Este estudo mostrou que tanto as NETs dos LDNs como dos HDNs foram capazes de induzir a expressão do TLR7 nas células T, induzindo uma resposta proinflamatória pela secreção das citoquinas TNFα e IL-21. Em contraste, as NETs não reconheceram o TLR9.

Assim, foi descoberta uma interação entre as células T e os neutrófilos, onde as NETs desempenham um papel principal e são a ponte entre o sistema imune inato e o adaptativo.

Palavras chave: LES, Células T, LDNs, HDNs, NETs, TLRs

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ACRONYMS

ANAs	Anti-nuclear antibodies
ANCAs	Anti-neutrophil cytoplasmic antibodies
AP1	Activator protein 1
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
AZA	Azathioprine
BAFF	B cell activating factor of the tumor necrosis factor family
BCR	B cell receptor
BEL	Belimumab
BFA	Brefeldin A
C3	Complement protein 3
C4	Complement protein 4
CD	Cluster of Differentiation
CLRs	C-type lectin receptors
CpG	Cytosine triphosphate deoxynucleotide
CREB	Cyclic AMP responsive binding element
CXCR5	C-X-C chemokine receptor type 5

CYC	Cyclophosphamide
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DP	Doble-positive
dsNA	Doble-stranded nucleic acid
EBV	Epstein-Barr Virus
EDTA	Etinelediaminetetraacetic acid
ENAs	Extractable nuclear antigens
ER	Endoplasmic reticulum
Fas	FS-7 associated surface antigen
FasL	FS-7 associated surface antigen ligand
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable
FcRγ	Common γ chain of Fc receptor
FOXP3	Forehead box P3
GC	Glucocorticoids
HCQ	Hydroxychloroquine
HDNs	High-density neutrophils
HIV	Human immunodeficiency virus
IFN	Interferon
IL-21	Interleukin-21
Iono	Ionomycin
IQR	Interquartile median range
IRF	Interferon regulatory factor
LDNs	Low-density neutrophils

LL-37	Cationic antibacterial protein
LN	Lupus nephritis
MHC	Major histocompatibility complex
miRNA/miR	Micro RNA
MMF	Mycophenolate mofetil
MMP9	Matrix metalloproteinase 9
MPO	Myeloperoxidase
mRNA	Messenger RNA
MTX	Methotrexate
MyD88	Myeloid differentiation factor 88
NAFT	Nuclear factor of activated T cells
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
ng/mL	Nanogram per milliliter
NK	Natural killer
NLRs	Nucleotide-binding oligomerization domain Receptors
nRNP	Small nuclear ribonucleoprotein
PAD4	Protein arginine deaminase 4
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cells
PD-L1	Programmed death ligand 1
PDN	Prednisolone
Pen	Penicillin

PFA	Paraformaldehyde
PLL	Poly-L-lysine hydrobromide
PM	Polymyositis syndrome
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
R-848	Resiquimod
RBC	Red blood cells
RIX	Rituximab
RLRs	Retinoic-acid-inducible gene 1 receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Scl	Scleroderma
SD	Standard deviation
SLE	Systemic lupus erythematosus
SLEDAI	Systemic lupus erythematosus disease index
Sm	Smith
SSA	Sjogren's syndrome A antibody
SSB	Sjogren's syndrome B Antibody
ssRNA	Single-stranded ribonucleic acid
Strep	Streptomycin
Syk	Spleen tyrosine kinase
T-bet	T-box transcription factor TBX21
TBMs	Tingible body macrophages
T_C	T cytotoxic cell
TCR	T cell Receptor
T_{FH}	T follicular cell

T_H	T helper cell
TLRs	Toll-like receptors
TNFR	Tumor necrosis factor family receptor
TNFα	Tumor necrosis factor α
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
T_{reg}	T regulatory cell
TRIF	TIR-containing adaptor-inducing interferon β
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
UNC93B1	Unc-93 homolog B1 protein
ZAP70	ζ-chain associated protein 70
β_2GPI	β_2-glycoprotein

1

INTRODUCTION

1.1 Immune system

The immune system is what protects humans from pathogenic microorganisms, their toxins, and the damage they cause. It uses a complex array of protective mechanisms to control and eliminate these organisms and toxins.[1] Recognition of pathogens is the key to the host's health without damaging its tissues.[1]

The immune system can be divided into two arms:

i. Innate immune system or nonspecific response
ii. Adaptive immune system or specific response

1.1.1 Innate immunity

The innate immune system is the first line of defense of the host, and if effectively can eliminate the pathogenic agents, before a more specific response is recruited.[2] The innate immunity covers all host's immune defense systems encoded in their mature functional forms by the germ-line genes of the host. [1]

The first line of defense are the body surfaces such as skin and mucous secreted by epithelial cells of different organs and physiologic barriers such as enzymes, antimicrobial peptides, and cytokines.[1,2]

The next line of defense of innate immunity are the cellular players whose function is to destroy the invader.[3] These include neutrophils, eosinophils, basophils, mast cells, macrophages, monocytes, natural killer cells (NK), and dendritic cells (DCs).[3] The first four types are polymorphonuclear leukocytes or granulocytes. Neutrophils, eosinophils, monocytes, and DCs are phagocytes.[3] All phagocytes besides being able to destroy

microorganisms are critical to the specific immune response because of their action as antigen-presenting cells (APCs).

The above-stated cellular players, although not antigen-specific, sense microorganisms.[3] They express pattern recognition receptors (PRRs) that recognize and respond to evolutionary conserved microbial structures termed pathogen-associated molecular patterns (PAMPs).[3] PRRs comprise multiple families of receptors based on their molecular structures (Fig. 1.1). PRRs can be present on the cell surface, or within the cytoplasm. When activated they all lead to the activation of intracellular signaling pathways that end up altering gene expression to promote the elimination of the pathogen.[2] Toll-like receptors (TLRs) are the most studied and are involved in the recognition of viral, bacterial, fungal, and parasitic pathogens.[2]

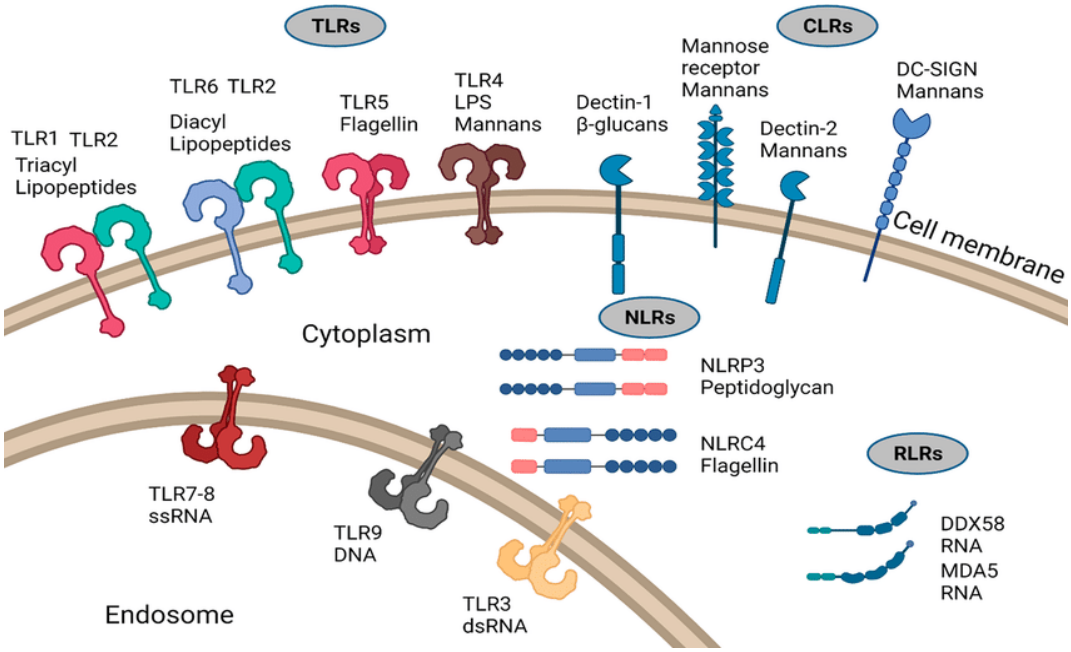

Figure 1.1 - Schematic representation of pattern recognition receptors (PRRs) and the pathogen-associated molecular patterns (PAMPs) each of them recognize: C-type lectin receptors (CLRs), Nucleotide-binding oligomerization domain receptors (NLRs), Retinoic-acid-inducible gene 1 receptors (RLRs), Toll-like receptors (TLRs). TLRs 1, 2, 5 and 6, and CLRs are membrane-bound receptors. TLRs 3, 7, 8 and 9 are endosomal receptors. NLRs and RLRs are cytoplasmic receptors. TLR4 can be membrane-bound or endosomal. Adapted from[4].

1.1.2 Adaptive immunity

Adaptive immunity enters into action when innate immunity fails to eliminate the pathogen of the organism.[3] Adaptive immunity is a more specialized and exquisite form of immunity, albeit slower.[1] The specificity of this response is the product of translational products of the host's genes that somatically rearrange and give rise to highly specific

cell receptors that recognize specific antigens of the pathogenic agent.[3] Adaptive immune cells include the B lymphocytes that mature in the bone marrow and the T lymphocytes that mature in the thymus.[5]

T lymphocytes express T cell receptor (TCR) that specifically binds an antigen.[3] The diversity of TCR is due to the genetic rearrangement.[3] However, TCR only recognizes an antigen when the latter is bound to major histocompatibility complex (MHC) molecules.[6] These are divided into two classes: MHC class I is expressed by all nucleated cells and presents endogenously generated proteins. MHC class II are majorly expressed by APCs and mainly present phagocytosed proteins processed in the endosomal pathway.[6] T cells can be further classified by their cell surface molecules expression - cluster of differentiation (CD). $CD8^+$ T cells are called cytotoxic T lymphocytes (T_C) and recognize antigens presented by MHC I, and $CD4^+$ T cells are the T helper lymphocytes (T_H) that only recognize an antigen presented by MHC II.[2,6]

Recognition by TCR of the antigen in the MHC context is required but not sufficient for a naïve lymphocyte to be activated.[2] A pair of costimulatory molecules need to be present such as the CD28-CD80/CD86 essential for interleukin (IL) -2 production.[7] In addition, lymphocytes need a third signal to become activated, in the form of cytokines (Fig 1.2).[2]

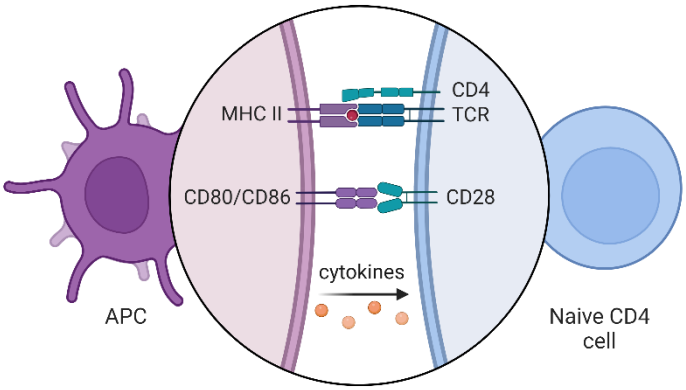

Figure 1.2 - Scheme of an antigen presenting cell activating a naive $CD4^+$ T cell (T_H cell). APCs expresses on its membrane MHC II, CD80/CD86 (B7 family ligands) and produces cytokines. Naïve T cell expresses in its cell surface TCR that recognizes the antigen- MHC II complexes, and the costimulatory molecule CD28. Adapted from BioRender.

Activated T_H cells do not have cytotoxic or phagocytic activity, they mediate the immune response by directing other cells to perform certain tasks and regulate the type of immune response that is developed.[6] According to the cytokines cells secrete they can

be classified into different subsets T_H1, T_H2, T_H17, and T_{FH}, which are the effector subsets or regulatory T cells (T_{reg}). [5]

T_H1 cells secrete tumor necrosis factor-α (TNFα), IL-2, and interferon (IFN) - γ , all involved in the activation of macrophages and $CD8^+$ T cells[8]; T_H2 cells secrete IL-4, IL-6, and IL-10, which can promote activation of B cells and induce isotype switching to IgG1[8]; T_H17 cells secrete IL-17, IL-22, TNF-α and IL-21.[8] IL-17 acts on fibroblasts, immune cells and epithelial cells, inducing the production of antimicrobial molecules, cytokines, chemokines, and matrix metalloproteinases, promoting the recruitment of neutrophils and other immune cells to sites of inflammation.[9] It also promotes B cell differentiation and survival[9]; T_{reg} modulates the function of effector T cells maintaining homeostasis; T follicular cells (T_{FH}) a $CD4^+$ T cell subset, localizes in germinal centers and stimulate B cells to differentiate into effector cells, through IL-21 and CD40L.[10]

B cells express membrane-bound antigenic receptor or B cell receptor (BCR), whose antigen specificity is also determined by gene rearrangement.[5] Upon antigen encounter, B cells proliferate, differentiate, and undergo isotype switching originating plasma cells or memory cells. Plasma cells produce and release soluble antibodies. Memory cells have a long lifespan and are easily activated.[5] B cells express MHC II on their surface meaning they also function as APCs.

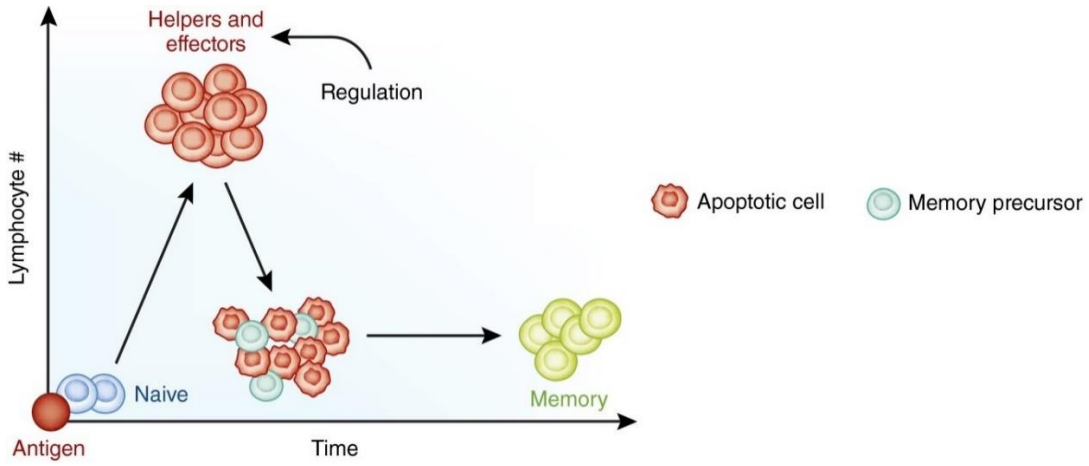

Figure 1.3 - Schematic view of the adaptive immune response. Non-self antigen (either directly in the case of B cells, or in the context of MHC molecules) binds to its specific naïve lymphocyte. This latter becomes activated and induces exponential proliferation. After clonal expansion lymphocytes differentiate into helper and effector cells. Regulation mechanisms enter in action and induce apoptosis of the cells, the ones that survive become memory cells. Adapted from reference [11].

The first encounter of the antigen with its specific lymphocyte primes the lymphocyte.[5] It then undergoes clonal expansion, originating many cells with the same specificity also called clones, that subsequently, differentiate into effector cells that will fight

the infection, or into memory cells.[5] These latter survive the resolution phase of the infection in which previously expanded cells dye through apoptosis (Fig 1.3).[11] Memory cells are less reliant on costimulatory stimuli, providing a quicker and heightened response to a secondary infection.[11]

1.1.3 Discrimination of self from non-self autoimmunity

Lymphocytes undergo an education termed maturation during their development in the primary lymphoid organs, where self-reactive lymphocytes are discarded.[3]

Immature B cells are tested for self reactivity through negative selection.[12] If a B cell expresses high reactivity to a self antigen it will be deleted through apoptosis.[12]

Immature T cells are doble-positive (DP) cells, expressing αβTCR, CD4, and CD8 on the cell surface.[12] DP cells interact with thymic epithelial cells that present thymic self-peptides through both MHC I or II, this is termed positive selection, and ensures cells express MHC molecules. Then they become committed either to CD4 or CD8.[12] Negative selection is followed where dendritic cells present self antigens to T cells, and cells whose TCR exhibits high affinity are eliminated.[12]

Occasionally autoreactive B and T cells may escape negative selection and traffic the periphery.[3] This can happen if a self antigen is not expressed at a high enough level or because the lymphocyte had a weak affinity to the self antigen during maturation.[3] In normal conditions, these cells are not a threat once they are clonally ignored, nevertheless, there are mechanisms in the periphery to overcome this loophole. [3]

One such mechanism is anergy. B cells become anergic by recognizing its specific antigen in the absence of costimulatory signals, failing to mount a response to the antigen. Anergic B cells do not enter lymph nodes because they have higher needs for B-cell activating factor of the tumor necrosis factor family (BAFF) and this is not provided by the organ, whereby undergoes apoptosis. [13] Regarding T cells, whenever one of the three signals required for its activation is not provided T cell becomes anergic.[13] Another compensatory mechanism is T_{reg} mediated suppression.[13] $CD4^+CD25^+$Forkhead box $P3^+$ (FOXP3) T_{reg} cells are considered the most relevant T_{reg} population.[13] They constitutively express CTLA-4 responsible for the inhibition of the immune response of $CD4^+$ T cells, $CD8^+$ T cells, B cells, dendritic cells, and NK cells.[3]

Despite the existence of these mechanisms, it is known that autoreactive lymphocytes arise and escape regulatory immune checkpoints and if activated can lead to autoimmunity.[3] Autoimmunity is caused by a wide array of combinations of genetic and environmental factors.[3]

An example of the failure of tolerance and responsible for autoimmunity are the anti-chromatin B cells that express TLR9. B cells can internalize the dsDNA for which its BCR is specific but in normal conditions, these cells are ignored by the immune system once the proper costimulatory signal is not provided.[14] However, since they express endosomal TLR9 and in an environment of increased cell death, the internalized dsDNA can have sequences that resemble the naturally PAMP recognized by TLR9 that is unmethylated dsDNA. [5,14] Therefore, TLR9 can serve as a costimulatory signal that was initially not provided and activate the ignored B cell (Fig 1.4).[14] This mechanism is described in Systemic Lupus Erythematosus (SLE) disease that will be addressed in the next chapters.

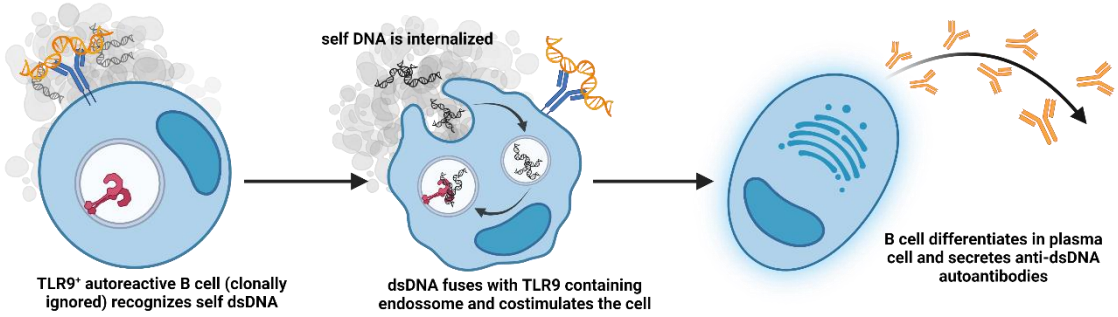

Figure 1.4 - Recognition of self DNA as pathogenic. Clonally ignored autoreactive B cell recognizes self DNA through BCR, promoting the internalization of the apoptotic unmethylated DNA. TLR9 present in the endosomes with the internalized DNA binds to the unmethylated regions of the dsDNA, and generates a costimulatory signal strong enough to activate the B cell and to differentiate into a anti-dsDNA antibody plasma cell. Adapted from reference [3].

1.2 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus is a chronic autoimmune disorder with a clinical heterogeneous phenotype. The disease is characterized by a break of tolerance of self-antigens that leads to the production of autoantibodies directed against nuclear and cytoplasmic antigens, driving an attack on healthy cells and tissues throughout the body affecting multiple organs.[15] SLE affects mainly women of childbearing age in a ratio of approximately six women to every one man.[16,17] Recent data reported the highest incidence - 23.2/100 000 persons - and prevalence - 241/100 000 persons in North America.[16] European countries have a lower incidence of SLE whilst Asia, Australasia and the Americas have a higher incidence.[16] The risk of mortality is higher for African Americans and native North Americans.[15] In Portugal, SLE affects 0.07% of the population, and 75% of the patient's ages are comprised between 16 and 49.[18]

1.2.1 Etiology of the disease

The etiology of the disease is multifactorial, combining ethnicity, genetic susceptibility, and environment. Nevertheless, the exact cause or the pathogenic mechanism of the disease is still substantially unknown.

Over the years approximately 90 genes have been implicated in SLE.[19] Monogenic SLE is due to single gene deficiencies, that encode proteins that do the clearance of biological debris.[20–22] However, most cases of SLE result from a combination or multiple gene variants[21] such as MHC 8.1 haplotype[17,23], interferon regulatory factors (IRFs), and even endosomal TLRs that sense nucleic acids[24].

Environmental factors have also been described to might trigger SLE. Infection with Epstein-Barr Virus (EBV) might induce the production of autoantibodies against homologous sequences between EBV-proteins and self-proteins.[17] Moreover, antibodies against Epstein-Barr nuclear antigen 1 can crossreact with host dsDNA, triggering autoimmunity.[17] Ultraviolet light can promote DNA breakage that can result in nucleic acid fragmentation, leading to cell death or altered gene expression through DNA reparation.[17] Tobacco smoking has also been associated with the development of SLE has already been studied.[25]

1.2.2 Clinical manifestations and diagnosis

The hallmark of the disease is the presence of autoantibodies due to immune system dysregulation[26], which are responsible for tissue injury[15]. The most implicated autoantibodies are the antinuclear autoantibodies (ANAs) that comprise various types of autoantibodies.[15] However, not all the patients that exhibit ANAs suffer from SLE.[17]

SLE can be present in a variety of ways (Fig 1.5).[27] The initial symptoms are weight loss, fatigue, fever, and arthralgias or arthritis.[15] Nevertheless, other symptoms like cytopenias, lymphopenias, cutaneous lupus[15], and renal involvement[27] are common. Lupus Nephritis (LN), a type of glomerulonephritis, is the most severe, and the leading cause of end-stage kidney disease and death in SLE. [28,29]

SLE diagnosis is complex because of the clinical heterogeneity and to facilitate it, a classification criterion was established for SLE[30] (Table 1.1) Each criterion is given weight and, in the end, is given a score, patients whose score ≥ 10 are classified as having SLE.[30]

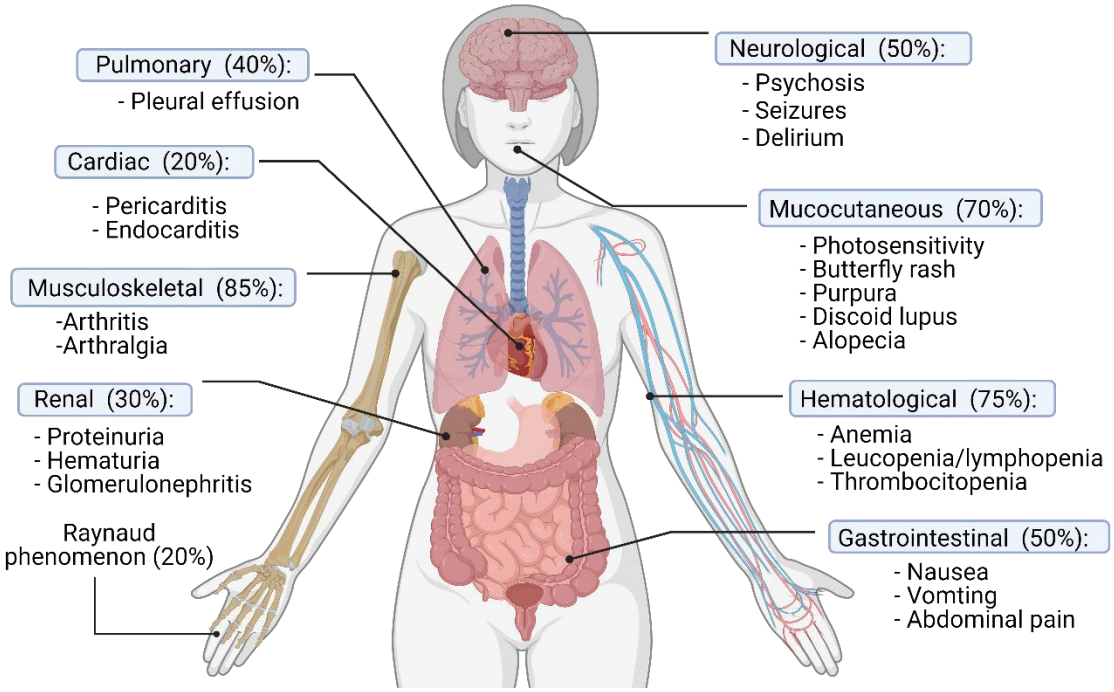

Figure 1.5 - Overview of clinical manifestations of Systemic Lupus Erythematosus. The systemic component of the disease allows the manifestations to be scattered around the body as shown in the figure. Adapted from references [15,17].

Table 1.1 - 2019 European League Against Rheumatology/American College of Rheumatology classification table for SLE. * = in an assay with 90% specificity against relevant disease controls. Anti-: β_2GPI = anti–β_2-glycoprotein; C3 = Complement protein 3; C4 = complement protein 4; anti-dsDNA= anti-double-stranded DNA. Adapted from reference [30].

Entry Criterion: ANA titer of ≥ 1:80 on Hep cells or an equivalent test: - If positive proceed to SLE diagnosis - If absent do not classify as SLE	
Immunological domains and criteria	**W**
Antiphospholipid antibodies	
Anti-cardiolipin antibodies OR	
Anti-β_2GP1 antibodies OR	
Lupus anticoagulant	2
Complement proteins	
Low C3 OR low C4	3
Low C3 AND low C4	4
SLE-specific antibodies	
Anti-dsDNA antibody * OR	
Anti-Smith antibody	6

Clinical domain and criteria	**W**
Constitutional	
Fever	2
Hematologic	
Leukopenia	3
Thrombocytopenia	4
Autoimmune hemolysis	4
Neuropsychiatric	
Delirium	2
Psychosis	3
Seizure	5
Mucocutaneous	
Non-scarring alopecia	2
Oral ulcers	2
Subacute cutaneous OR discoid lupus	4
Acute cutaneous lupus	6
Serosal	
Pleural or pericardial effusion	5
Acute pericarditis	6
Musculoskeletal	
Joint involvement	6
Renal	
Proteinuria > 0.5g/24h	4
Renal biopsy Class II or V lupus nephritis	8
Renal biopsy Class III or IV lupus nephritis	10

1.2.3 Treatment

The need for SLE treatment, the indication for glucocorticoids and immunosuppressive drugs, and the initial therapeutic dose are determined by the assessment of disease activity (SLE disease activity index - SLEDAI), major organ disorders, and complications such as infection and cardiac diseases.[31] The drugs used for SLE therapy are described in Table 1.2.

A high dose of glucocorticoids (GC) and immunosuppressive agents is recommended for patients with severe organ lesions and high disease activity.[31]

In asymptomatic patients with stable test results, no treatment is recommended. For patients with some symptoms but no major organ damage, hydroxychloroquine (HCQ) or low-dose glucocorticoids are recommended.[32]

Biological treatments such as Belimumab and Rituximab are recommended when patients fail to respond to standard therapy with HCQ, GC, and immunosuppressive agents, frequent relapses, or organ damage. [32]

1.3 Pathophysiology

The pathophysiology of SLE is complex and involves both arms of the immune system, innate and adaptive.

1.3.1 Innate immunity in SLE

Over the latest years apoptosis has been considered the major source of autoantigens in SLE. Apoptosis is a cellular death program characterized by being highly organized and immunologically silent.[33] It can be induced by the ligation of death receptors such as Fs-7 associated surface antigen (Fas) or tumor necrosis factor receptor (TNFR) or through a lack of essential survival signals that activate proteolytic caspases.[33] Apoptotic cells undergo various morphological alterations such as cytoskeletal disruption, cell shrinkage, DNA fragmentation, and plasma membrane blebbing.[33] Inside these blebs are concentrated many of the nuclear autoantigens that serve as targets in SLE.[33–35]

Lymphocytes, neutrophils, macrophages, and monocytes from SLE patients exhibit increased degree of apoptosis when compared to healthy controls, which directly correlates with disease activity measures through SLEDAI. [34,36–38] Also, serum from SLE patients induces a strong apoptosis response in macrophages, monocytes, and lymphocytes.[39] Furthermore, it has been shown that autoreactive T cells have an increased expression of

Table 1.2 - Therapeutic options reported in the literature for SLE patients. For each drug is described the recommended dosage and the mechanism of action. Adapted from references [15,31,32,40,41]. PDN -Prednisolone; HCQ - Hydroxychloroquine; AZA - Azathioprine; MMF - Mycophenolate Mofetil; MTX - Methotrexate; CYC - Cyclophosphamide; RIX - Rituximab; BEM - Belimumab.

Category	Drug	Adult Dosage	Mechanism of action
Corticosteroids	PDN	1-2 mg/kg/d	Anti-inflammatory steroid that blocks proinflammatory genes; Decreases expression of adhesion molecules in endothelial cells and leukocytes, reducing the accumulation of phagocytic cells in sites of inflammation; Leads to a rapid T cell depletion due to a combination of effects: enhanced circulatory emigration, induction of apoptosis, inhibition of T cell growth factors such as IL-2, and impaired release of cells from lymphoid tissues. T_H1 subset is the most affected and B cells also are reduced due to decreased T cell help.
Antimalarials	HCQ	400-800 mg/d	Pleiotropically modulates the immune response by inhibition of B cell and TLR signaling as well as intracellular TLR3 and 7 activations; Increases the lysosomal pH, interfering with antigen binding and secretion of cytokines; Exerts an anti-type I IFN response through the STING pathway.
Immuno-suppressive Agents	AZA	2 mg/kg/d	Purine analogue: it is converted in vivo in 6-thioguanine, that is incorporated in DNA and RNA inhibiting their synthesis. It may have a tolerogenic effect by inhibiting CD28-mediated signal in T cells
	MMF	1000-3000 mg/d	Preferentially depletes guanoside nucleotides in T and B cells inhibiting proliferation. It suppresses lymphocyte and monocyte recruitment to inflamed tissue and inhibits inducible nitric oxide synthase that can reduce tissue damage mediated by macrophages.
	MTX	7.5-15 mg/week	Antimetabolite that interferes with DNA synthesis, repair, and replication by irreversibly binding to dihydrofolate reductase, reducing purine synthesis. Low dose has pleiotropic effects: increased anti-inflammatory adenosine signaling, apoptosis of activated lymphocytes, reduction of circulating proinflammatory T cells, reduction of adhesion molecules on endothelial and synovial cells, reactive oxygen species, and others.
	CYC	1-5 mg/kg/d	Highly toxic alkylating agent that depletes T and B cell and suppresses antibody production;
Biologic Agents	RIX	1000 mg	Anti-CD20 monoclonal antibody that leads to peripheral B cell depletion. Also leads to the reduction of autoantibodies titers.
	BEM	10 mg/kg	Monoclonal antibody that binds and neutralizes soluble B-lymphocyte stimulators biologic activity (BAFF, IL-6, and others).

TNF-related apoptosis-inducing ligand (TRAIL), tumor necrosis factor-like weak inducer of apoptosis (TWEAK), and Fas Ligand (FasL), all three apoptotic ligands that directly mediate the apoptosis of monocytes.[42] This leads to the conclusion that an excess of apoptosis occurs in SLE patients. However, apoptosis is a physiological form of cell death, therefore the biological waste generated by apoptotic cells must be cleared. [33] This is done by phagocytes that induce an anti-inflammatory response.[43] An increased cell death *per se* does not explain the loss of tolerance against autoantigens.

To properly remove apoptotic debris well-functioning phagocytes are required. Curiously, SLE also demonstrates alterations in the clearance of this apoptotic debris. In SLE patients it has been reported impaired phagocytic activity of monocytes, macrophages, and granulocytes.[33] The phagocytic capacity of monocyte-derived macrophages in SLE is altered in about 50% of the patients.[38,44] Besides, the number of tingible body macrophages (TBMs) are strongly reduced in SLE patients.[45] TBMs make the uptake of apoptotic material of B cells that undergo apoptosis in the germinal centers.[45] If TBMs are reduced, apoptotic material can be presented to the follicular DCs and provide survival signals to autoreactive B cells.[37,38]

Serum proteins are also important in the removal of apoptotic debris. Opsonizing proteins such as IgM when at low levels correlate with higher SLE disease activity.[46] DNase I is responsible for degrading nucleic acids from apoptotic blebs.[47] When mutated or inhibited by anti-nucleic acid antibodies as in SLE, triggers autoantibodies production, type I IFN production, and can give rise to an anti-dsDNA immune complex.[47]

1.3.2 Adaptive immunity in SLE

T cells abnormalities in signaling, production of cytokines, proliferation, regulatory functions, and imbalance between cell populations have been reported in SLE.

CD3 that is aggregated to TCR is composed of many subunits, one of them the ζ subunit, which recruits the ζ-chain associated protein 70 (ZAP70) upon TCR engagement.[48] In SLE patients ζ chain expression is decreased[49] and replaced by the common γ chain of Fc receptor (FcRγ)[50]. FcRγ recruits' spleen tyrosine kinases (Syk) instead of ZAP70.[51] This leads to the migration of nuclear factor of activated T cells (NAFT) to the nucleus and consequently to a higher expression of the CD40L gene, therefore T cells become easily activated. [8] In fact, T cells from SLE patients maintain the expression of CD40L after activation for longer compared to healthy individuals.[52] An increased help is then provided by T cells for the activation and proliferation of B cells.[52] Moreover, engagement of TCR leads to transient hyperpolarization of the

mitochondria and depletion of adenosine triphosphate (ATP).[53] Repetitive T cell activation can be the cause of hyperpolarization and ATP depletion in SLE.[53] This hyperpolarization has been identified as a sensitizer for induced necrosis since ATP is required for the cell to undergo apoptosis.[53] This depletion of ATP can induce necrosis of T cells in the lymph nodes that occurs in SLE patients.[53] Necrosis is a cell death program in which cellular swelling results in lysis and releases oxidizing molecules, proinflammatory, chemotactic factors, increased availability of autoantigens, leading to the involvement of an immune response.

Under normal conditions, T_H1 and T_H2 subsets regulate each other and maintain balance.[8] In SLE this balance is altered and is believed that T_H1 is decreased in function and T_H2 presents hyperfunction, however, this is controversial.[8] Nevertheless, a study showed that T_H1 starts to dominate the imbalance as long as the disease turns chronic, more evident in patients with lupus nephritis IV.[54]

Regarding T_{reg} studies have reported reduced number of T_{reg} in SLE patients and their function is absent.[55,56] In normal circumstances, T_H17 and T_{regs} are in equilibrium, which is perturbed by SLE, where T_H17 expansion leads to the reduction of the number of T_{regs}. [55,56] In addition, IL-17, and BAFF synergically upregulate the differentiation and survival of B cells, resulting in upregulation of the production of autoantibodies.[8]

SLE patients have also shown an increased number of circulating $CD4^+$ expressing C-X-C chemokine receptor type 5 (CXCR5) T cells, presenting a phenotype similar to T_{FH} cells that correlate with anti-dsDNA antibodies and with disease activity.[57] Moreover the levels of IL-21 have also been reported as increased [58,59] and it plays a role in the pathogenesis of the disease since it is involved in the differentiation of B cells to plasma blasts.[60]

B cells are also players in SLE pathogenesis. In addition to increased help by T cells for B cell differentiation, improved B cell survival and proliferation (through BAFF, IL-6, and IL-21), and upregulated TLR signaling[17], enhanced BCR-mediated signaling can lower the activation threshold of peripheral B cells, promoting lupus cellular phenotypes.[8]

1.4 Neutrophils and NETosis

Neutrophils are the most abundant cell type in human peripheral blood and the first effectors to be recruited to sites of inflammation.[61] They are short-lived and target pathogenic microorganisms through reactive oxygen species from the respiratory burst, the release of bactericidal enzymes through degranulation, and phagocytosis, a process that engulfs the microorganism.[61]

More recently, it was discovered that neutrophils undergo NETosis and generate neutrophil extracellular traps (NETs) (Fig. 1.6). The neutrophil chromatin is decondensed by enzymes stored in the azurophilic granules, neutrophil elastase (NE), myeloperoxidase (MPO), and protein arginine deaminase 4 (PAD4).[62] Subsequently, the nuclear membrane is damaged, and chromatin expands inside the cell and is mixed with antimicrobial factors.[63] Finally, the cytoplasmic membrane breaks and the cell releases NETs.[63] They are web-like structures made of expelled intracellular DNA and proteins such as histones, calprotectin, and cathepsin G that provide antimicrobial properties to eliminate invaders.[64] NETs serve to trap, immobilize, inactivate and kill potential pathogens favoring the host's defense, however, when its formation is dysregulated it can lead to a cascade of inflammatory reactions, resulting in organ damage, cancer, tissue loss, and thrombosis.[64]

Although NETosis is a physiological cell death it has been widely accepted that it plays a role in autoimmune diseases where SLE is no exception.[33,65] In fact, NETs are a source of autoantigens that can even be more immunogenic than apoptotic material since the released DNA can be oxidized due to the proximity of reactive oxygen species (ROS) also released from neutrophils.[66] Several studies have identified increased levels of circulating DNA in the blood of SLE patients[67,68], and amongst the variety of autoantibodies produced in SLE, there are antineutrophil cytoplasmic antibodies (ANCA) directed against lysosomal proteins, proteinase 3, MPO, NE, and cathepsin G that have been associated with active disease[69] and with the presence of LN[70–72]. Moreover, some studies even documented impairment of NETs degradation by suboptimal concentrations of DNase1 in patients' serum that can be caused by NETs binding to C1q causing DNase1 inhibition.[73,74] Furthermore, the ability of patients' serum to degrade NETs is directly related to the presence of LN, low complement levels, and high titers of anti-dsDNA and anti-histone antibodies.[75] All these studies prove the role of NETs as a source of autoantigens contributing to the pathogenesis of SLE.

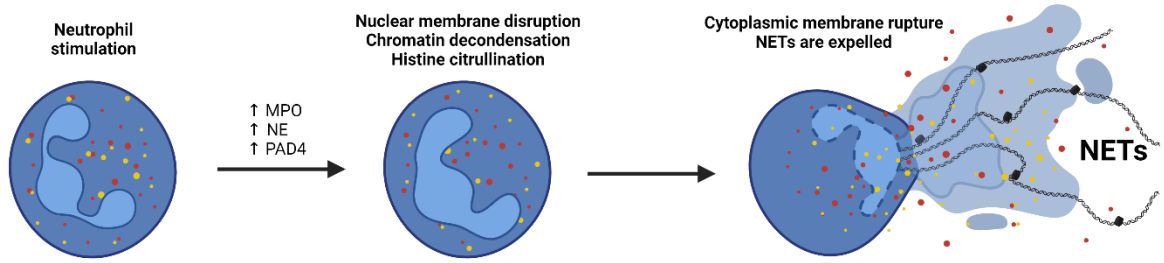

Figure 1.6 - Schematic representation of NETosis pathway. After stimulation, neutrophils adhere to the surface and the content of the granules (MPO, NE, PAD4) helps decondensing nucleic acids. Nuclear membrane is compromised and after cytoplasmic membrane suffers rupture and the intracellular content is released forming NETs. Adapted from [63,76,77].

1.4.1 The low-density neutrophils, NETs and SLE

Interestingly, heterogeneity in neutrophils has been discussed over the latest years and it has been shown that there are different populations in the neutrophils family.[61] One of those populations and quite relevant for SLE is the low-density neutrophils (LDNs) which have a smaller density than the rest of the neutrophils[61], and because of that are mixed with peripheral blood mononuclear cells after density gradient centrifugation[78]. Studies showed that the frequency of these LDNs in the peripheral blood mononuclear cells (PBMCs) layer is higher than in healthy individuals.[79–81] Supporting this is the fact that LDNs have been widely studied and a role in several diseases has been reported, whether their role is immunosuppressive or not depends on their phenotype and the disease in question.[82]

In SLE, LDNs are a pathogenic subset of neutrophils that synthesize more proinflammatory cytokines and type I interferons (TNF-α, IL-17, IFN-γ, and IFN-α) than their regular-density counterparts or also termed high-density neutrophils (HDNs)[82], and are toxic to endothelial cells.[81,83] More important is the fact that these LDNs have a higher capacity for generating NETs and are different from the ones generated by HDNs since they contain higher levels of autoantigens and immunostimulatory molecules such as a cationic antibacterial protein (LL-37), matrix mtalloproteinase 9 (MMP9), and dsDNA[74,83–85]. Additionally, LDNs NETs are more immunostimulatory and have more cytotoxic properties than those NETs originated from healthy neutrophils.[83]

1.5 Toll-like receptors

TLRs belong to the PRR family of the innate immune system and can be present in various immune cells such as monocytes, macrophages, dendritic cells, neutrophils, B lymphocytes and even in non-immune cells such as epithelial cells, endothelial cells, and fibroblasts.[86,87] Humans express TLR 1-10, however, the exact function of TLR10 is still unclear.[88] They can be further classified by their location and by their ligands (Fig 1.7), while TLR1, 2, and 6 are mainly found on the cellular surface and recognize accessible molecules of the pathogen membrane and induce inflammatory responses, TLR3 and TLR7-9 are primarily found in endosomes and recognize pathogens nucleic acids from bacteria or viruses and induce type I IFN and inflammatory responses.[89] TLR4 is a unique case since it is primarily expressed on the cell surface and upon recognition of its ligand is internalized and expressed in endosomes, inducing inflammatory and type I IFN responses. [90]

When TLRs are engaged by PAMPs, they can transduce the signaling to initiate either innate or adaptive immune responses (Fig. 1.7).[91,92] The cytoplasmic Toll/IL-1 receptor domain is responsible for recruiting one of two possible adaptor proteins: myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adaptor-inducing interferon-β (TRIF).[87] MyD88 leads to the activation of the transcription factors nuclear factor kappa B (NF-κB), cyclic AMP responsive binding element (CREB), and activator protein 1 (AP1) resulting in the production of proinflammatory cytokines. TRIF leads to the activation of IRF3 and IRF7, resulting in the production of type I IFNs.[93] Interestingly, TLR3 only recruits TRIF while TLR4 can recruit both TRIF and MyD88 in a time-dependent manner.[87]

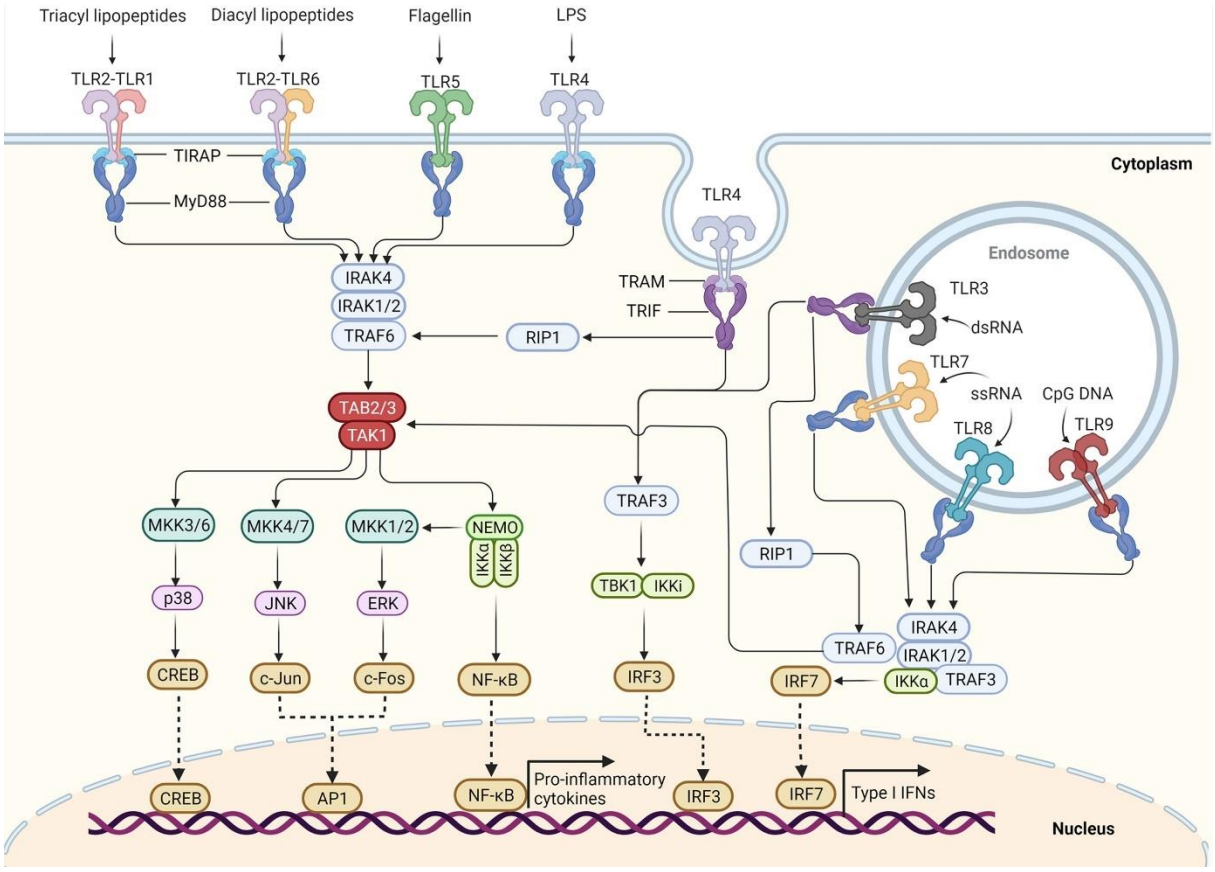

Figure 1.7 - TLR signaling in immune cells. TLR2-TLR1, TLR2-TLR6 together with TLR4 and TLR5 localize at the cellular membrane. TLR4 after recognizing its ligand suffers endocytosis. TLR3 and TLR7-9 are endosomal. All TLRs except for TLR3 and TLR4 signal through MyD88 that leads to activation of transcription factors NF-κB, CREB and AP1, resulting in transcription of proinflammatory cytokines. TLR3 and TLR4 signal through TRIF that results in activation of transcription factors IRF3 and IRF7 that leads to the transcription of type I IFNs. The endosomal TLRs can also promote the transcription of type I IFNs. Adapted from [93].

1.5.1 Toll-like receptors 7 and 9 in SLE

TLR7 and TLR9 are both endosomal TLRs that signal through MyD88 adaptor protein, resulting in the transcription of proinflammatory cytokines and type I IFNs. TLR7 senses single-stranded RNA (ssRNA), while TLR9 senses unmethylated cytosine triphosphate deoxynucleotide (CpG) -rich double-stranded DNA (dsDNA). Recent evidence has been proposing a relationship between endosomal TLRs and SLE pathogenesis.[94] In fact, it has been discovered that in B cells and plasmacytoid DCs (pDCs), the endosomal TLRs play an important role in the production of ANAs and type I IFNs.[95,96] In B cells TLR engagement increases antibody production, meanwhile, in pDCs, TLRs lead to IFN-α production, which causes myeloid DCs to release BAFF activating even more autoreactive B cells.[97] Furthermore, increased levels of TLR7 and TLR9 messenger RNA (mRNA) have been detected in PBMCs of SLE patients and their levels correlate with the expression of IFN-α.[98,99]

TLR7 has been proposed as a positive feedback mechanism to increase IFN-α, necessary for anti-microbial response.[100] In SLE this mechanism seems to be deregulated since patients' present features of a chronic viral infection in absence of a pathogen.[101] Indeed, upregulation of TLR7 was seen in healthy neutrophils when cultured with sera from active SLE patients. Also, pre-treatment with purified IFN-α resulted in a similar response.[102] Moreover, TLR7 may also be induced by serum-derived immune complexes containing TLR7 ligands.[103] TLR7 is preferentially increased in SLE patients with antibodies against RNA-associated antigens, while TLR9 induction correlated with anti-dsDNA antibody titers. [104]

While TLR7 has been accepted as a pathogenic mechanism of the disease, the role of TLR9 is still controversial. B cells and monocytes from patients with active disease express higher TLR9 levels compared to patients with inactive disease.[105,106] Moreover, studies have documented an increase in the frequency of TLR9-expressing B cells and correlated with the production of anti-dsDNA, anti-chromatin, and anti-nucleosome autoantibodies.[105,107–109] However, TLR9 deletion in lupus-prone models does not lead to amelioration of the disease but rather to its exacerbation, conferring a protective role in mice.[107,109,110] Curiously, although TLR7 acts parallelly with TLR9 on different types of autoantibodies, TLR9 suppresses TLR7-dependent RNA-associated autoantibodies.[107,111] A paradox is created when TLR9 expression is augmented in B cells and DCs from patients with severe disease but they present a lower response to TLR9 ligands.[86,110]

As stated above TLRs are present in innate immune cells and in B cells and are involved in SLE. However, TLRs expression in T cells is still unclear, and if TLR7 and TLR9 expressed on T

cells play a role in SLE pathogenesis is unknown. In fact, TLRs expressed in APCs play essential roles in innate response, but also in T cell-mediated adaptive responses through upregulation of MHC, costimulatory molecules, and by producing cytokines that modulate T cell differentiation into effector cells. [112] New evidence showed that TLRs can directly modulate T cell functions.[113] Contrarily to innate immune cells, TLRs *per se* did not induce activation of T cells.[114,115] Naïve $CD4^+$ T cells require TCR stimulation and TLR engagement simultaneously for them to be activated, acting as costimulators of $CD4^+$ T cells.[113–118] However, TLR engagement on effector T cells (T_H1, T_H17) results in strong activation by TLR ligands alone, without TCR stimulation.[114,119–121]

1.6 Aim of the study

This study pretends to address some gaps regarding TLRs expression in T cells and their potential role in SLE, and to explore NETs role as ligands of these TLRs. The first objective is to explore neutrophils phenotype in SLE patients. The second is to confirm the expression of TLRs in $CD4^+$ T cells, which has remained controversial along the years. The third objective is to assess TLR7 and TLR9 engagement in $CD4^+$ T cells by NETs generated from autologous low- and high-density neutrophils that provide ssRNA and dsDNA, the ligands of both TLRs in question. The fourth, objective is to evaluate the functional inflammatory profile caused by NET stimulation.

2

MATERIALS AND METHODS

2.1 Materials

All reagents and antibodies used are listed on the Tables 2.1 and 2.2

2.1.1 Reagents

Table 2.1 - List of reagents used with the respective description and supplier.

Reagent	Supplier
Brefeldin A, *Penicilliium brefedianum* (BFA)	Thermo Scientific
Bovine Serum Albumin HyClone™ (BSA)	Thermo Scientific
Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)	Sigma-Aldrich
Dextran from *Leuconostoc spp.*, Mr 450,000-650,000	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Biowest
Fixable Viability Dye eFluor 506™	Thermo Fischer
Interleukin-2 (IL-2)	NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Maurice Gately, Hoffman, LA Roche Inc
Ionomycin (Iono), Calcium Salt, *Streptomyces conglobatus*	Merck Millipore
L-glutamine	Gibco, Thermo Fischer
Lymphosep - Lymphocyte Separation Media	Biowest
MojoSort™ Human CD4 Nanobeads	Biolegend

MojoSort™ Streptavidin Nanobeads	Biolegend
Paraformaldehyde (PFA)	Sigma-Aldrich
Penicillin/Streptomycin (Pen/Strep)	Gibco, Thermo Fischer
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Phosphate Buffered Saline (PBS) 10x	VWR
Poly-L-Lysine Hydrobromide (PLL)	Sigma-Aldrich
RPMI Medium 1640 1x	Gibco, Thermo Fischer
Saponin	Carl Roth
RBC Lysis Buffer (Multi-Species) 10x	eBioscience
UltraComp eBeads™, Compensation Beads	Invitrogen, Thermo Fischer

2.1.2 Antibodies

Table 2.2 - Antibodies and respective clone, fluorochrome, and supplier list used for the experiments.

Antibody	Clone	Fluorochrome	Supplier
Anti-CD3, mouse IgG1	UHCT1	---------	Biolegend
Anti-CD3, mouse IgG1	UHCT1	PerCP	Biolegend
Anti-CD4, mouse IgG1	RPA-T4	FITC	Biolegend
Anti-CD10, mouse IgM	HI10a	PE/Cy7	Biolegend
Anti-CD15-Biotin, mouse IgM	HI98	---------	Biolegend
Anti-CD15, mouse IgG1	W6D3	FITC	Biolegend
Anti-CD16b	REAffinity	APC	Miltenyil Biotec
Anti-CD28, mouse IgG1	CD28.2	---------	Biolegend
Anti-CD38, mouse IgG1	HIT2	APC/Cy7	Biolegend
Anti-CD62L, mouse IgG1	DREG-56	PE	Biolegend
Anti-CD66b, mouse IgGM	G10I5	APC/Cy7	Biolegend
Anti-IL-2, mouse IgG1	3A3-N2	A647	Biolegend
Streptavidin	------	PE/Cy5	Biolegend
Anti-TLR7, mouse IgG1	4G6	PE	Novus Biologicals
Anti-TLR9, rat IgG2a	S16013D	BV 421	Biolegend
Anti-TNF-α, mouse IgG1	S16013D	PE/Cy7	Biolegend

A representative illustration of the materials and methods used in the experiences is represented in Fig. 2.1.

2.2 Isolation of human peripheral blood mononuclear cells

Peripheral blood was collected from SLE patients of Hospital Egas Moniz in Lisbon, in accordance the stipulated by the ethics committee of NOVA Medical School (84/2019/CEFCM) and Hospital Egas Moniz (20170700050). These donors provided consent for their blood cells to be used in research studies at NMS Research.

Human PBMCs were isolated from the blood by density gradient within hours after collection of peripheral blood. The blood in the collection tubes was transferred to a falcon and centrifuged in order to obtain a plasma aliquot. Blood was then diluted in a 1:1 proportion with PBS 1x (10% PBS 10x diluted in water) and homogenized. The diluted blood was carefully put on top of a Lymphosep separation media layer in a 2:1 proportion. After centrifugation, PBMCs were collected and washed with PBS 1x.

2.3 Magnetic separation of cell populations

2.3.1 Positive selection for $CD4^+$ T cells

The collected PBMCs were centrifuged, resuspended in MojoSort buffer 1x, prepared through dilution of MojoSort buffer 5x (50% PBS, 2.5% BSA, 10% EDTA) and incubated with CD4 human nanobeads for 15 minutes in a 1:20 proportion on ice. After cells were left in the magnet for 5 minutes and the unlabeled fraction was poured off, this step was repeated one more time.

After separation, $CD4^+$ T cells were cultured in complete RPMI 1640 medium (10% FBS, 1% Pen/Strep) at $2x10^6$ cells/mL medium with IL-2 (20 IU/mL).

2.3.2 Positive selection for low-density neutrophils

The unlabeled fraction from the previous separation was resuspended in MojoSort buffer 1x and incubated with biotin-CD15 anti-human antibody (0.5 mg/mL) for 15 minutes in a 1:20 proportion on ice, followed by a 15 minute incubation with streptavidin nanobeads also in 1:20

proportion on ice. Cells were then transferred to the magnet and left for 5 minutes, the unlabeled fraction was poured off, and this step was repeated one more time. $CD15^+$ fraction was then resuspended in 2 mL of MojoSort buffer 1x.

After separation, $CD15^+$ cells were resuspended in RPMI 1640 (1% Pen/Strep) supplemented with 1% of donor plasma at $5x10^6$ cells/mL.

2.4 Isolation of high-density neutrophils

From the reminiscent of the PBMCs density gradient centrifugation, granulocytes layer was collected and left to sediment with 10 mL 6% Dextran + 10 mL of PBS 1x for 1 hour. The supernatant was collected, centrifuged, and red blood cells were lysed with RBC lysis 1x for 10 minutes. Cells were centrifuged and washed.

Neutrophils were cultured in RPMI 1640 (1% Pen/Strep) supplemented with 1% of donor plasma.

2.5 NETs generation of LDNs and HDNs

NETs were generated according to a published protocol with some alterations.[122] LDNs and HDNs were stimulated with 500 nM of PMA overnight at 37ºC, 5% CO_2 to generate NETs. After stimulation supernatant and adhered material were collected and centrifuged to remove cells and obtain a cell-free supernatant. The supernatant was divided into Eppendorf's and ultracentrifuged. Pelleted NETs were then resuspended in a proportion of $20x10^6$/100 µL PBS 1x to obtain a stock solution of NETs. The concentration of nucleic acids in each stock solution was measured in Thermo Fischer's NanoDrop 2000/2000c.

2.6 Culture and stimulation assays of $CD4^+$ T cells

After isolation, $CD4^+$ T cells were cultured at $2x10^6$ cells/mL for 3 days (day 0 to day 3) in a 96-well round U-bottom plate previously coated with PLL (2 µg/mL), αβTCR (5 µg/mL) and CD28 (2 µg/mL).

On day 2 $CD4^+$ T cells were stimulated with 100, 250, and 500 ng/mL of NETs from LDNs or HDNs of the same donor.

On day 3 the previous NETs-stimulated $CD4^+$ T cells were again stimulated with 50 ng/mL of PMA and 500 ng/mL of ionomycin.

2.7 Phenotyping of CD4$^+$ T cells and cytokine production

After 2 hours of the last stimulation, cells were treated with a protein transport inhibitor (BFA, 2 μg/mL) for ~15 hours. Therefore NETs/PMA stimulated CD4$^+$ T cells were stained for phenotyping and cytokine production on day 4.

Cells were washed with PBS 1x and incubated with Fixable Viability Dye eFluor 506 20 min 4º C. Cells were washed twice with FACS buffer (PBS 1x + 2% FBS). Then, they were surface labeled with fluorescently conjugated primary antibodies for CD3 (2 μg/mL), CD4 (4 μg/mL), and CD38 (4 μg/mL) for 20 min at 4 º C. After that, cells were washed twice with FACS buffer, fixed with 1% PFA for 20 min at room temperature (RT), and washed once with FACS buffer. Cells were then permeabilized with 0.1% saponin for 30 min at RT so endosomal TLRs and cytokines can be stained. Then, they were intracellularly labeled with primary fluorescent antibodies for TLR7 (4,13 μg/mL), TLR9 (1 μg/mL), TNFα (2 μg/mL), and IL-21 (1 μg/mL) for 20 min at RT. Cells were washed and resuspended in FACS buffer.

After being stained, the CD4$^+$ T cells were analyzed by flow cytometry on BD FACS Canto II. Sample acquisition was then analyzed on FlowJo v10.8.1.

2.8 Phenotyping of low- and high-density neutrophils

Neutrophils from the PBMCs layer, LDNs after magnetic cell separation and HDNs were collected and stained for phenotyping on day 0.

Cells washed with PBS 1x and incubated with Fixable Viability Dye eFluor 506 20 min 4º C. Cells were again washed twice with FACS buffer (PBS 1x + 2% FBS). Then, they were surface labeled with fluorescently conjugated primary antibodies for CD15 (4 μg/mL), CD66b (4 μg/mL), CD10 (4 μg/mL), CD62L (4 μg/mL) and CD16b (concentration was not specified 1:50 is the brand recommend dilution) for 20 min at 4 º C. After that, cells were washed twice with FACS buffer, fixed with 1% PFA for 20 min at room temperature (RT), and washed once and resuspended in FACS buffer.

After being stained, neutrophils were analyzed by flow cytometry on BD FACS Canto II. Sample acquisition was then analyzed on FlowJo v10.8.1.

2.9 Statistical analysis

All statistical analysis and graphic preparation were performed using GraphPad Prism v.9.0.0 and IBM SPSS Statistics version 21 software's. First, normality of the data was tested by D'Agostino & Pearson normality test (n>6). If the samples, followed a normal distribution, the appropriate parametric test was chosen; If not, a non-parametric test was used. All statistical tests performed were two-tailed.

Overall, a p value ≤ 0.05 (α) was considered statistically significant. All the analysis considered a 95% confidence interval. The p values were calculated using the true distribution (exact p values). Results were considered significant at $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. For multiple comparisons adjusted p values were used.

For unpaired data, comparison between two groups: unpaired t-test (t) or Mann-Whitney test (U).

For paired data, comparison between two groups: Paired t-test (t) or Wilcoxon matched-pairs signed rank test (W) was used.

For paired data multiple groups comparison: Repeated measures one-way ANOVA with posttest Dunnett´s multiple comparisons or Friedman test with posttest Dunn's multiple comparisons.

For correlations, Pearson (r) or Spearman (r) was used.

The choice of test was dependent on the underlying distribution and is indicated in the legend of the figures. Data are presented as mean ± standard deviation (SD), for parametric statistical tests and median ± interquartile range (IQR) for non-parametric statistical tests. The number of biological replicates (n) is specified in the legend of the figure.

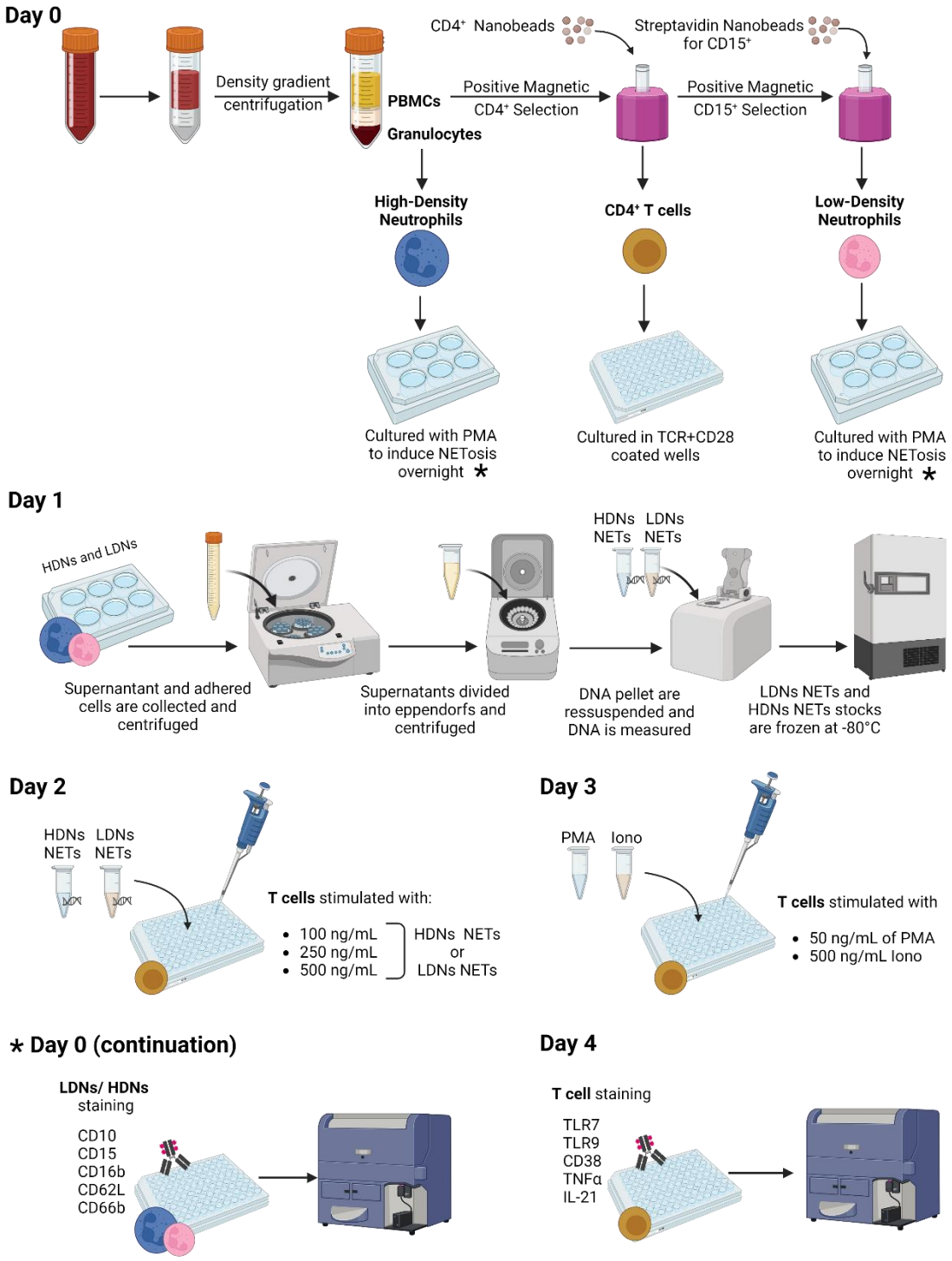

Figure 2.1 - **Representative scheme of the methods used in T cells and neutrophils experiences**. On day 0, all used populations of interest were isolated from fresh peripheral blood of SLE patients -PBMCs, LDNs, and HDNs - and properly cultured (*n*=14). A fraction of LDNs and HDNs was collected to stain with CD10, CD15, CD16b, CD62L, and CD66b, and acquired on cytometer for phenotyping. On day 1, the HDNs and LDNs NETs were isolated through two centrifugations to obtain two different stock solutions of NETs and measured on NanoDrop and frozen at -80°C. On day 2, T cells left in culture were stimulated with three different concentrations (100, 250, and 500 ng/mL) of LDNs or HDNs NETs. On day 3, all T cells were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL). On day 4, T cells were stained for TLR7, TLR9, CD38, TNFα, and IL-21, and acquired on cytometer for phenotyping.

3

RESULTS

3.1 Characterization of the SLE cohort

Demographic and clinical data for the SLE patients enrolled in this study are described in Table 3.1. The samples of peripheral blood (*n*=15) were mainly obtained from female patients (86.7%, *n*=13) in accordance with the ratio of the incidence of the disease (6:1, Female: Male). The mean age of the cohort was 57.0 ± 12.0 years and 86.7% (*n*=15) were of Caucasian ethnicity.

Only one patient was considered to have active disease since it had a SLEDAI of 5 while all the other participants had a SLEDAI of ≤ 2, making the mean of cohort 1.2 ± 1.4. As expected, and the hallmark of the disease, every patient exhibited ANA's and the most common ANA's titer was 1:2560 (45.5%). In addition, also every patient exhibited anti-dsDNA antibodies. Moreover, 57.1% of the cohort exhibited extractable nuclear antigens (ENA's: anti-Sjogren's syndrome A (SSA) antibodies, anti-Sjogren's syndrome B (SSB) antibodies, anti-Smith (Sm), anti-small nuclear ribonucleoproteins (nRNP) antibodies, or anti- polymyositis syndrome (PM) and scleroderma antibodies (Scl) antibodies).

Regarding treatment, only one patient was not subjected to any type of treatment whilst the rest of the patients were mostly having prednisolone (80.0%, *n*=12) and hydroxychloroquine (86.7%, *n*=13).

In the case of symptoms, the most prevalent is arthritis (86.7%), followed by mucocutaneous manifestations (alopecia 40.0%, *n*=6; malar rash 53.3%, *n*=8; purpura 36.7%; *n*=4, discoid lupus 6.7%, *n*=1) and hematological manifestations (leukopenia 60.0%, *n*=9; thrombocytopenia 33.3%, *n*=5; hemolytic anemia 33.3%, *n*=5).

Table 3.1 - Demographic and clinical characteristics of the patients of systemic lupus erythematosus from whom peripheral blood samples were collected (n=15).

	Features	Mean ± SD	Percentage (%)	(*n*)	Population range
Demographic Data (*n*=15)	*Sex*				
	Male		13.3	2	
	Female		86.7	13	
	Age (years)	57.0 ± 12.0		15	30-70
	Ethnicity				
	Caucasian		86.7	13	
	Melanodermic		13.3	2	
	Country				
	Portugal		66.7	10	
	Bulgaria		6.7	1	
	Brazil		13.3	2	
	Angola		6.7	1	
	Cape Verde		6.7	1	
Clinical Data (*n*=15)	*Age at diagnosis (years)*	43.7 ± 14.6		15	23-67
	Disease duration (years)	15.3 ± 15.8		15	2-52
	Smoking		20.0	3	
	SLEDAI	1.2 ± 1.4		15	0-5
	ANA's (positive test)		100.0	15	
	ANA titer (IU/mL) 1:320		27.3	3	
	ANA titer (IU/mL) 1:640		27.3	3	
	ANA titer (IU/mL) 1:2560		45.5	5	
	Anti-dsDNA (IU/mL)	44.3 ± 86.3		15	0.1-350.2
	ENA's (positive test)		57.1	8	
	ENA type (positive test) Anti-SSA		50.0	7	
	ENA type (positive test) Anti-SSB		21.4	3	
	ENA type (positive test) Anti-Sm		21.4	3	
	ENA type (positive test) Anti-nRNP		21.4	3	
	ENA type (positive test) Anti-PM/Scl		14.3	2	
	Treatment			14	
	Prednisolone		80.0	12	
	Hydroxychloroquine		86.7	13	
	Azathioprine		26.7	4	
	Methotrexate		6.7	1	
	Cyclophosphamide		6.7	1	
	Mycophenolate Mofetil		20.0	3	
	Belimumab		6.7	1	
	Symptoms				
	Arthritis		86.7	13	
	Malar Rash		53.3	8	
	Purpura		26.7	4	
	Discoid Lupus		6.7	1	
	Alopecia		40.0	6	
	Pericarditis		6.7	1	
	Class IV lupus nephritis		6.7	1	
	Hemolytic anemia		33.3	5	
	Leukopenia		60.0	9	
	Thrombocytopenia		33.3	5	
	Raynaud phenomenon		40.0	6	

3.2 Low-density neutrophils are present in the PBMCs layer of SLE patients

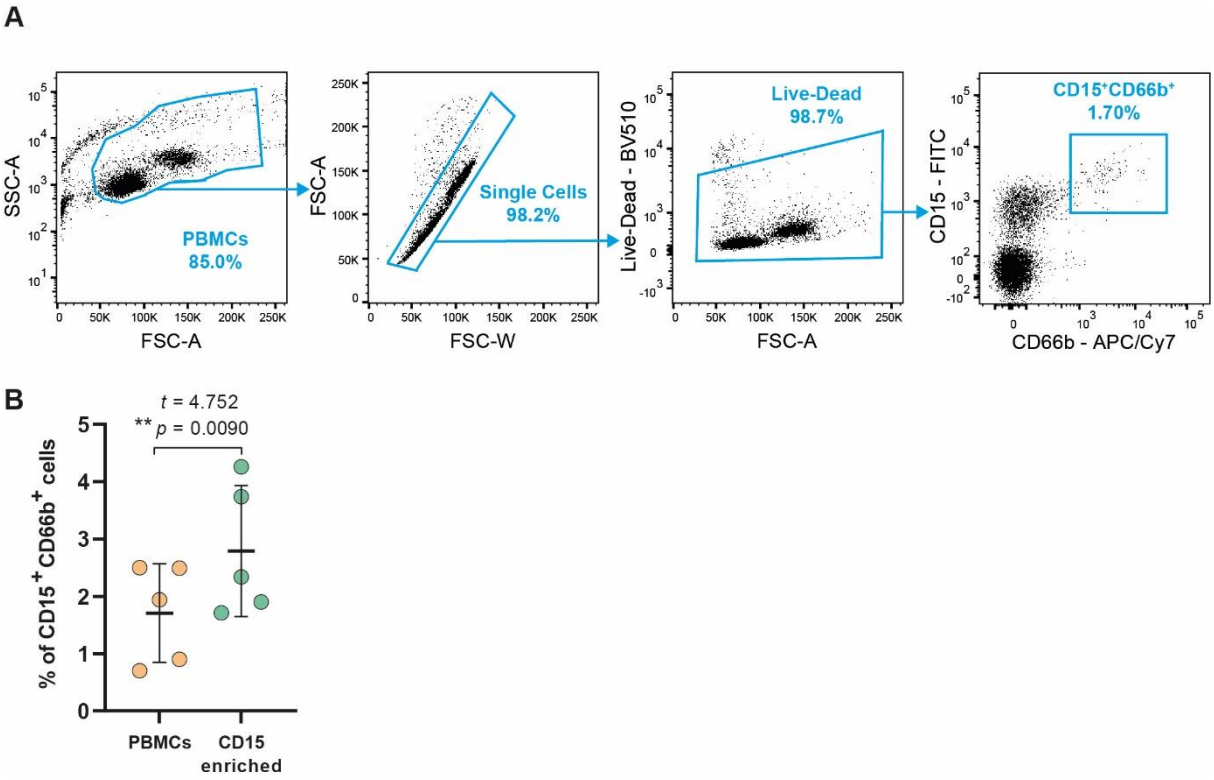

Figure 3.1 - **Low-density neutrophils in the PBMCs layer of peripheral blood. A** - Gating strategy of $CD15^{+}CD66b^{+}$ cells in peripheral blood mononuclear cells from freshly obtained peripheral blood; **B** - Cumulative frequency of $CD15^{+}CD66b^{+}$ cells in PBMCs (orange dots) and after CD15 enrichment by magnetic cell separation (green dots) (n=5). Data are presented as mean ± SD; Sample normality distribution was tested by Shapiro-Wilk normality test; P value $^{**}p \leq 0.01$ was determined by paired t-test (t).

In order to assess, the presence of SLE LDNs in the peripheral blood mononuclear cells as it is widely described in the literature, PBMCs of five samples of peripheral blood samples (n=5) were stained for CD15 and CD66b. CD15 allows the distinction of LDNs from monocytes when it is highly expressed[123], moreover, CD66b is a generic cell membrane granulocyte marker.[124,125] Both markers allow the identification of neutrophils ($CD15^{+}CD66b^{+}$ cells). Using a more wide-ranging gating strategy (Fig 3.1-A) it was possible to infer that LDNs constitute 1.7 ± 0.9 % of the PBMCs (PBMCs, 1.7 ± 0.9%) (Fig. 3.1B).

PBMCs were then CD15 enriched by magnetic cell separation to remove other cells that are also present in PBMCs layer such as monocytes, T cells, B cells NK cells, and dendritic cells by $CD15^+$ magnetic positive selection. $CD15^+CD66b^+$ cells are more abundant in the enriched fraction (CD15 enriched, 2.8 ± 1.1 %) than in the non-isolated counterpart (PBMCs, 1.7 ± 0.9%) (Fig. 3.1B). CD15 enriched fraction was called LDNs.

3.2.1 Positive selected low-density neutrophils purity

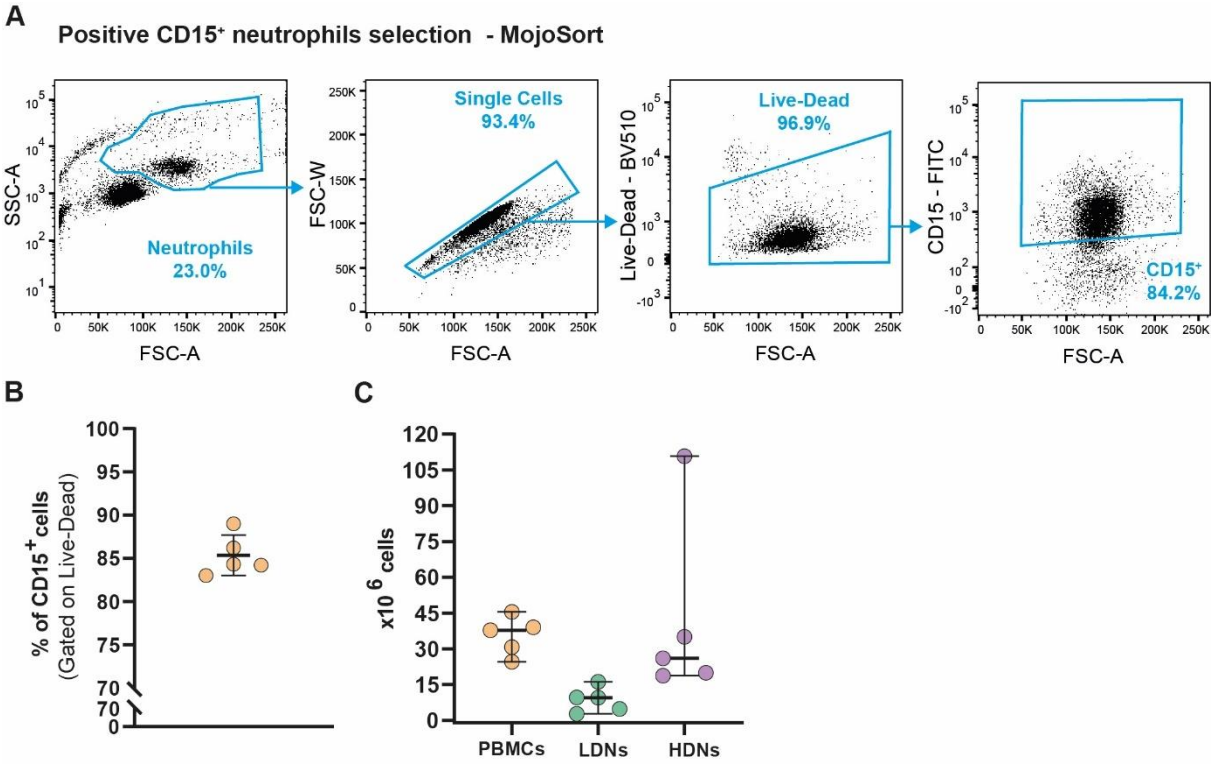

Figure 3.2 - MojoSort positive selected $CD15^+$ neutrophils purity. **A** - Gating strategy of $CD15^+$ magnetic positive selected cells from peripheral blood mononuclear cells of freshly peripheral blood; **B** - Cumulative frequency of purity of the $CD15^+$ magnetic positive selected cells from peripheral blood mononuclear cells of freshly peripheral blood (*n*=5); **C** - Cell counts of each isolated fractions - PBMCs, LDNs, and HDNs - from peripheral blood (*n*=5). Data are presented as mean ± SD.

To further work with LDNs the purity of the magnetic enriched cells was evaluated. Four fractions of LDNs (*n*=4) were stained for CD15 expression (Fig. 3.2A). A mean purity of 85.3 ± 2.3% (Fig. 3.2B) for $CD15^+$ expression was obtained that allowed to later proceed to NETs generation of NETs. From the same donor sample of peripheral blood besides LDNs isolated from

the PBMCs layer, the HDNs present on the granulocytes later after density gradient were isolated. Counts of all three cell populations (PBMCs, LDNs, and HDNs) are represented in Fig. 3.2C.

3.3 Low- and high-density neutrophils present a similar phenotype

According to literature neutrophils are a heterogenous family[61], especially LDNs which phenotype is different from disease to disease.[82] To understand which subsets of neutrophils were present on SLE, LDNs and HDNs were stained for CD10, CD16b, and CD66b (*n*=5) (Fig. 3.3A). CD10 is an enzyme called neutral endopeptidase[125] expressed in mature neutrophils and allows the differentiation between mature and immature neutrophils[80]. CD16b is a glycosyl phosphatidyl inositol-anchored protein that acts as a receptor for the Fc region of immunoglobulin gamma receptor exclusively expressed in neutrophils[125]. CD62L is a cell adhesion molecule also widely expressed in neutrophils and its absence allows to distinguish activated neutrophils.[125,126]

CD10 was found to be largely expressed either by LDNs (95.2% IQR:[95.6-97.0]) and HDNs (97.0% IQR [95.5-99.2]), suggesting that both types of neutrophils are mature. CD16b is similarly expressed in HDNs (97.8 ± 1.5%) and in LDNs (91.4 ± 2.2%). CD62L is definitely more expressed in HDNs (88.5 ± 4.0%) than in LDNs (68.0± 8.2%), proposing that part of the cells may be activated (Fig. 3.3C).

Although the percentages of expression of each marker differ between LDNs and HDNs, when evaluating mean fluorescence intensity (MFI) there is no statistical difference between them (HDNs: CD10 - 5646 ± 2624; CD16b - 7815 ± 3620; CD62L - 1920 ± 1078) (LDNs: CD10 - 4485 ± 2906; CD16b - 10070 ± 9753; CD62L - 990 ± 1033) (Fig. 3.3B; Fig.3.3D). This data suggests that HDNs and LDNs present a similar phenotype once all three markers are highly expressed in both cell populations. The bigger changes are related to CD62L which can suggest that part of the neutrophils are activated since it is common for this marker to be downregulated upon activation. The lower expression of CD62L indicates that the HDNs migrate more to the tissue than the LDNs, that are a more circulatory population.

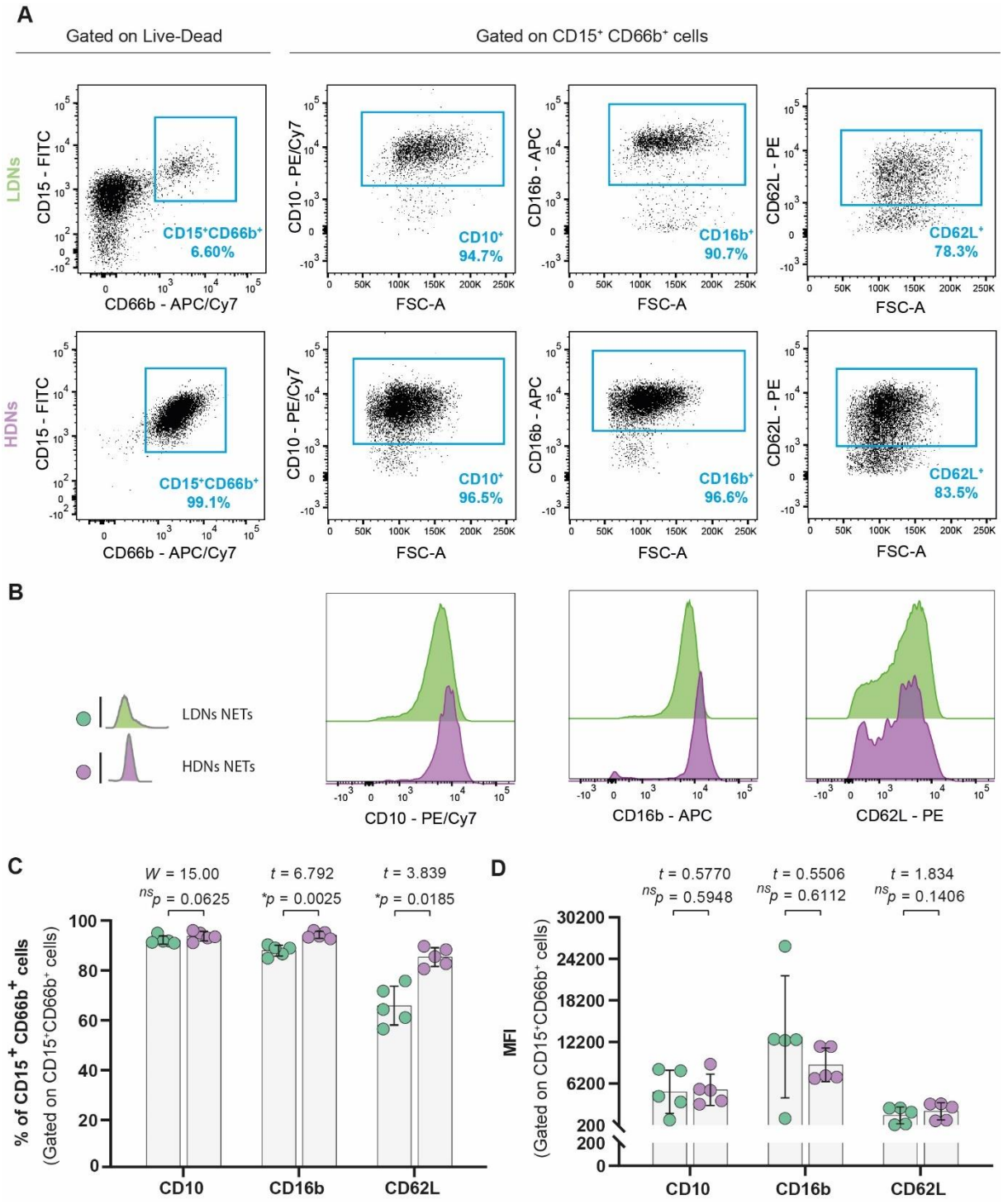

Figure 3.3 - Neutrophils phenotyping of LDNs and HDNs. A - Representative dot plots for each neutrophil maturation marker (CD10, CD16b, CD62L) gated on $CD15^{+}CD66b^{+}$ cells from LDNs (dot plot on top) and HDNs (dot plots underneath). **B** - Representative histograms of LDNs (green) and HDNs NETs (purple) for each neutrophil marker (CD10, CD16b, CD62L). **C-D** - Cumulative frequency and MFI of LDNs (green dots) and HDNs NETs (purple dots) for each maturation marker (CD10, CD16b, CD62L) gated $CD15^{+}CD66b^{+}$ cells (n=5). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using Shapiro-Wilk normality test. P value $*p \leq 0.05$ and *ns* (not significant) were determined by (**C**) Wilcoxon matched pairs signed (W); (**C-D**) paired t-test (t).

3.4 Low- and high-density neutrophils generate NETs

It has been reported that LDNs have a higher propensity to undergo NETosis and generate spontaneous NETs than HDNs [74,83–85]. To study NETs and their role when in contact with T cells both LDNs and HDNs were stimulated with 500 nM of PMA, a potent NETosis inducer[127], overnight. Since NETs are responsible for the release of the nuclear content to the extracellular medium, nucleic acids are exposed. This allows after centrifugations to generate a nucleic acid stock solution of either LDNs and HDNs (*n*=14) (Table 3.3).

LDNs NETs stock solutions show less DNA concentration (74.4 IQR [29.3-90.6] ng/µL) than HDNs NETs stock solutions (233.0 IQR [163.5-283.7] ng/µL) (Fig. 3.4).

Table 3.3 - Quantification of nucleic acids of NETs stocks generated from LDNs and HDNs isolated from peripheral blood (*n*=14).

Patient	LDNs NETs (ng/µL)	HDNs NETs (ng/µL)
056	15.2	157.2
057	21.3	239.1
059	78.9	298.2
060	25.2	81.6
061	30.6	165.6
062	69.8	167.8
063	253.1	454.1
064	111.2	362.6
065	44.9	216.6
066	86.9	193.2
067	61.6	278.8
068	83.1	255.6
069	101.7	107.2
070	82.1	229.3

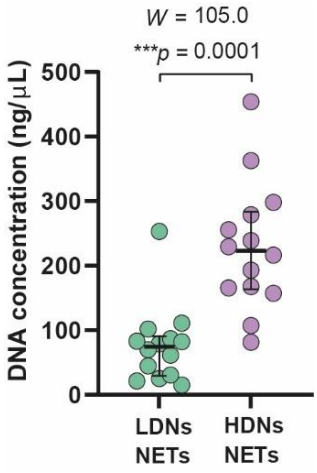

Figure 3.4 - DNA concentration of LDNs and HDNs stock solutions of NETs (*n*=14)**.** Data are presented as median ± IQR. Sample normality distribution was tested by using D'Agostino & Pearson normality test; *P* value ***$p \leq 0.001$ was determined by Wilcoxon matched-pairs signed rank test (*W*).

3.5 TLRs expression in resting CD4⁺ T cells

3.5.1 Positive selected CD4⁺ T cells purity

To address the question if CD4⁺ T cells would express innate immunity receptors such as TLRs, CD4⁺ T cells had to be isolated to remove contaminant cells as monocytes that are present in PBMCs layer. CD4⁺ T cells were then isolated through magnetic cell separation. Four fractions (*n*=4) of the isolated T cells were stained for CD3⁺ and CD4⁺. A mean purity of 88.36% ± 2.66% was obtained (Fig. 3.5A). High purity of T cells was necessary since cells were cultured with TCR+CD28 and treated with PMA and ionomycin after stimulation with NETs. PMA is typically known for its downregulation of CD4[128], and also the engagement of CD3 through antibodies can also cause its downregulation[129]. Once downregulation of CD3⁺ and CD4⁺ is possible, a high purity allows for further flow cytometry analysis to be gated directly in Live-Dead gate.

Cell counts of the CD4⁺ positive selected cells are also represented in Fig. 3.5A.

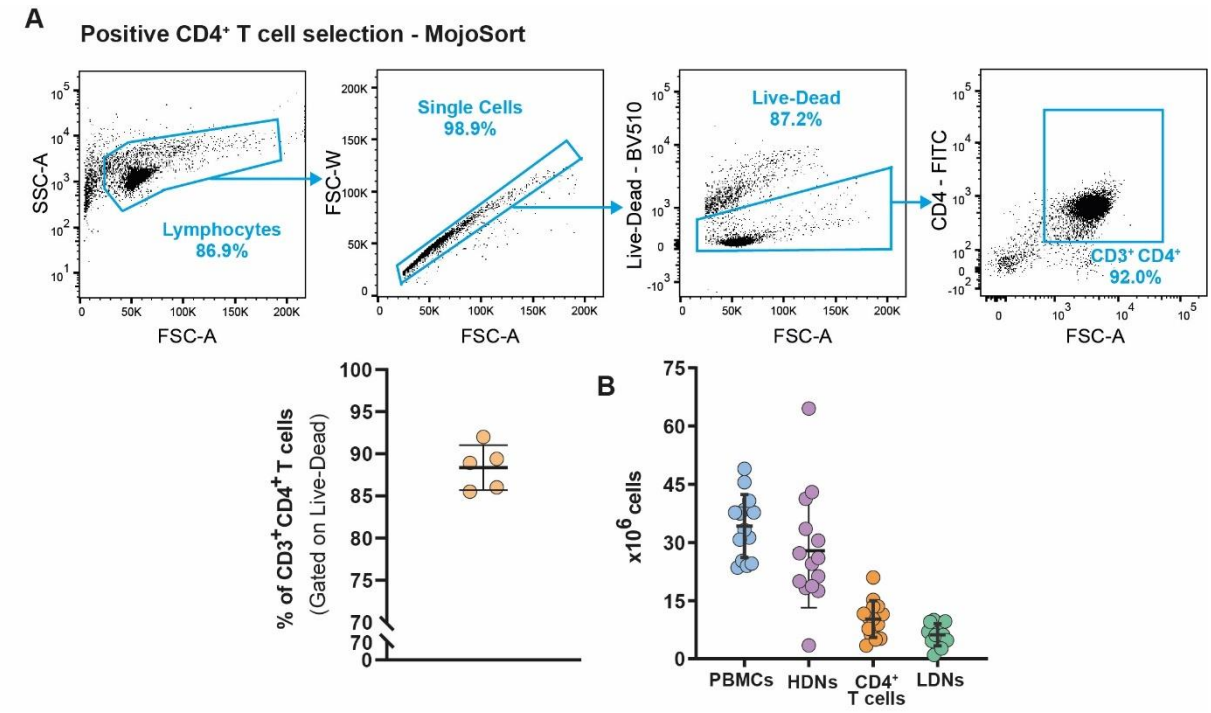

Figure 3.5 - MojoSort positive selected CD4⁺ T cells. A - Gating strategy and cumulative frequency of CD3⁺CD4⁺ magnetic positive selected T cells from peripheral blood mononuclear cells of fresh peripheral blood (*n*=5); **B** - Cell counts of each isolated fractions - PBMCs, HDNs, CD4⁺ T cells and LDNs - from peripheral blood (*n*=5). Data are presented as mean ± SD.

3.5.2 CD4⁺ T cells of SLE patients naturally express TLR7 and TLR9

The expression of TLRs and if they can directly act on T cells has been a controversial subject, however, it is now starting to be clear that these receptors are indeed expressed and that they can have a direct role. In fact, in this study, it is confirmed that T cells express TLR7 and TLR9 (*n*=14). TLR7 is quite expressed on resting T cells (88.7 ± 3.3%). TLR9 is also expressed but in less quantity (6.75 ± 2.95%) (Fig.3.6).

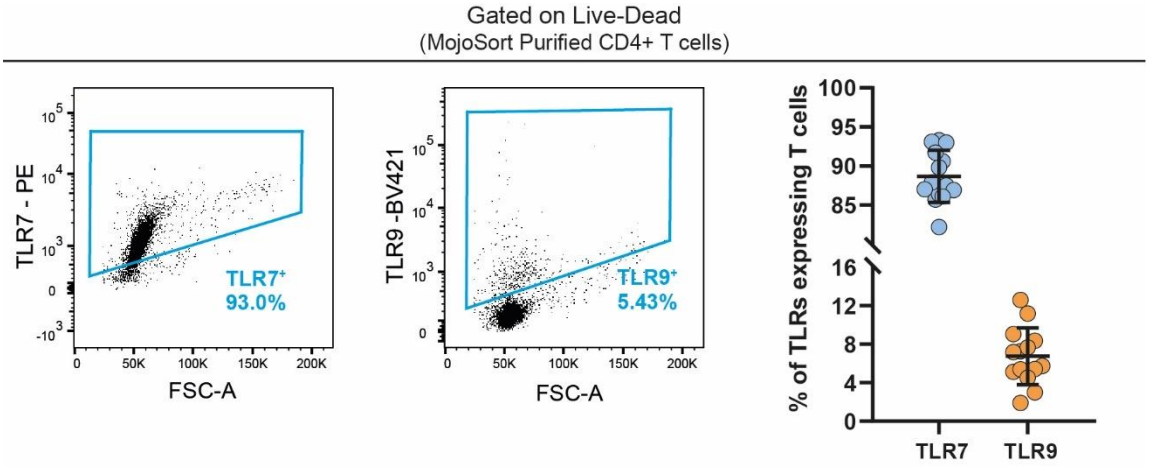

Figure 3.6 - TLRs expression on unstimulated conditions. Representative dot plots and cumulative frequencies of TLR7⁺ and TLR9⁺ on resting T cells (*n*=14). Data are presented as mean ± SD.

3.6 TLRs expression increases upon stimulation with LDNs and HDNs

To check if NETs could lead to an alteration of TLRs expression, T cells were firstly activated through TCR and CD28 and then stimulated with NETs from either LDNs or HDNs on day 2 of culture. On day 3 all NETs conditions were stimulated with PMA and ionomycin to maintain cells activated. NETs have been implicated in the pathogenesis of SLE [69–72], and patients have increased circulating NETs[67]. To demystify the effect of NETs quantity, T cells were stimulated with three doses of NETs, 100 ng/mL, 250 ng/mL, and 500 ng/mL (*n*=14).

3.6.1 TLR7 expression

As expected, on day 4 TLR7 expression was increased on all NETs stimulated conditions (Fig. 3.7A). Although not statistically significant LDNs NETs conditions (100 ng/mL - 94.1 ± 2.4%; 250 ng/mL - 94.0 ± 1.4%; 500 ng/mL - 93.6 ± 3.0%) had a higher TRL7 expression than HDNs NETs (100 ng/mL - 93.7 ± 2.0%; 250 ng/mL - 92.9 ± 2.4%; 500 ng/mL 91.7 ± 2.9%) (Fig. 3.7A). Nonetheless, all stimulated conditions were statistically significant when compared to unstimulated (88.7 ± 3.3%) (Fig. 3.7B). Also, not statistically significant, control condition PMA appears to promote expression of TLR7 (91.8 ± 3.7%) (Fig. 3.7A).

MFI TLR7 analysis supports the increased expression of TLR7 in T cells. Both T cell conditions stimulated with 250 ng/mL of either LDNs (1427 IQR [1024-2322]) or HDNs (1304 IQR [892-1304]) NETs showed a higher MFI than control conditions (unstimulated - 822 IQR:[910-1200]; PMA+Iono – 988 IQR [839-1206]) (Fig. 3.7B). In accordance with the data the TLR7 percentages data, PMA+Iono condition remains not significantly different compared unstimulated condition (Fig. 3.7B).

To make sure that the TLR7 increase was translated into activated cells, cells were checked for $CD38^{+}$, a T cell activation marker. Indeed, in all NETs stimulated conditions increased TRL7 expression was accompanied by a statistically significant increase in CD38 when compared to unstimulated condition (55.8 IQR [44.2-63.5]%) (Fig. 3.9A). As in TLR7 expression, $CD38^{+}$ appears to be more expressed in LDNs NETs (100 ng/mL - 85.2 IQR [72.3-90.2]%; 250 ng/mL- 85.3 IQR [71.5.2-93.1]%; 500 ng/mL - 81.8 IQR [75.0-91.1]%) stimulated conditions than in HDNs NETs (100 ng/mL - 80.2 IQR [73.6-86.0]%; 250 ng/mL - 81.1.8 IQR [73.5-86.6]%; 500 ng/mL - 79.6 IQR [74.1.2-92.7]%) (Fig- 3.8A). Curiously, PMA+Iono seems to downregulate CD38 expression (49.4 IQR [39.6-60.1]%) (Fig. 3.8A).

CD38 MFI shows congruity with the anterior results, where LDNs and HDNs NETs induced a highly activated status in T cells (unstimulated - 239 IQR [160-443]; PMA+Iono - 221 IQR [1401-406]; 250 ng/mL LDNs NETs - 2164 IQR [769-3804]; 250 ng/mL HDNs NETs - 1308 IQR [835-2163] (Fig. 3.8B).

The high TLR7 and CD38 expression in T cells upon stimulation with NETs suggests a role for TRL7 in the pathogenesis of SLE. In particular, LDNs NETs show a more homogenous expression of TLR7 and CD38 in comparison to HDNs NETs. Actually, the condition stimulated with 500 ng/mL HDNs NETs suggests a saturation of cells, proposing that HDNs NETs may not participate in SLE pathogenesis.

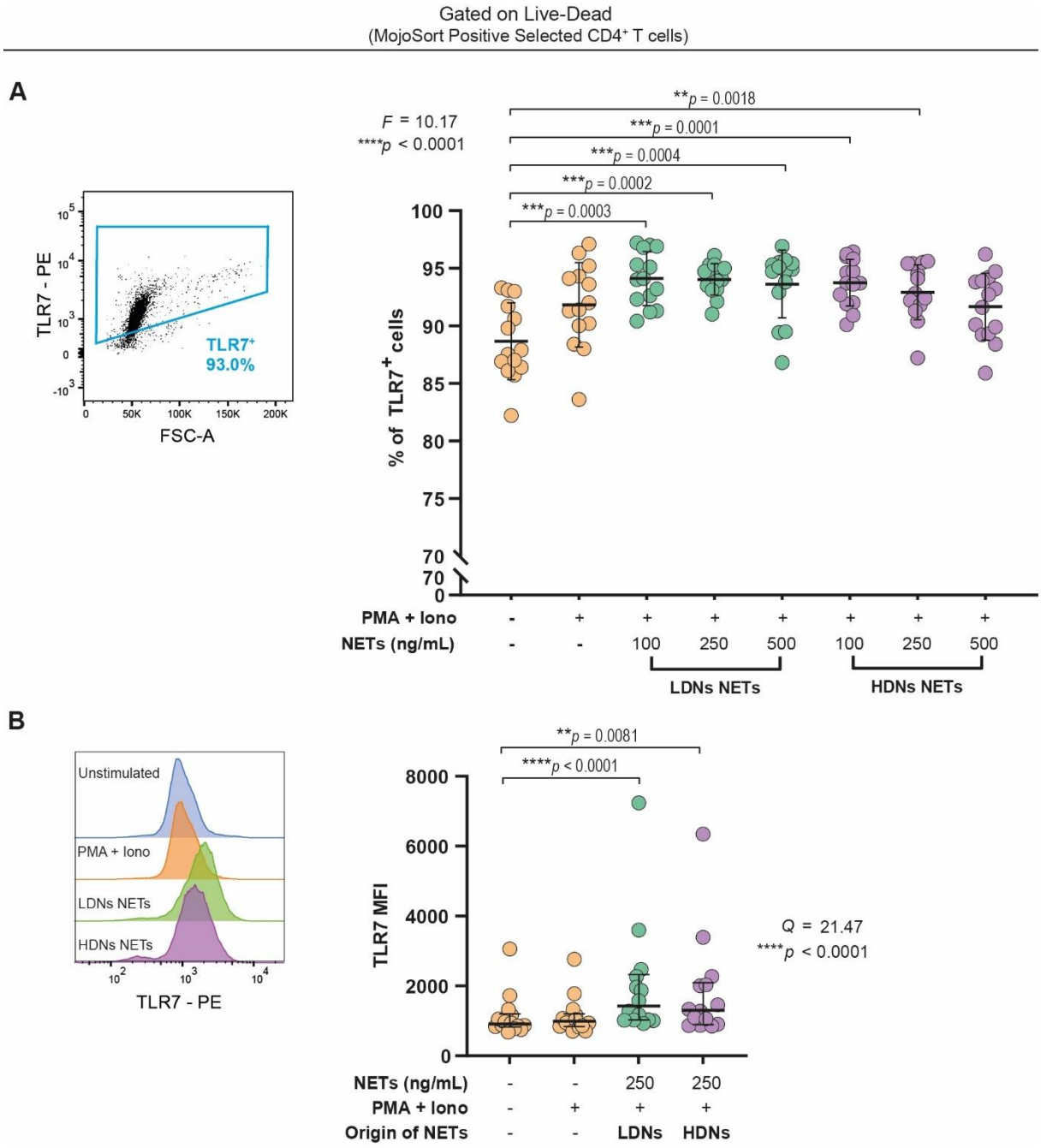

Figure 3.7 - TLR7 expression on T cells upon stimulation with NETs. A - Representative dot plot and cumulative frequencies of TLR7⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated condItions (LDNs (green) and HDNs (purple) NETs) (n=14); **B** - Representative histogram and cumulative frequencies of TLR7 MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (n=14). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. P values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ were determined by (**A**) Repeatead Measures one-way ANOVA (F) with posttest Dunnett's multiple comparisons; (**B**) Friedman (Q) test with posttest Dunn's multiple comparisons.

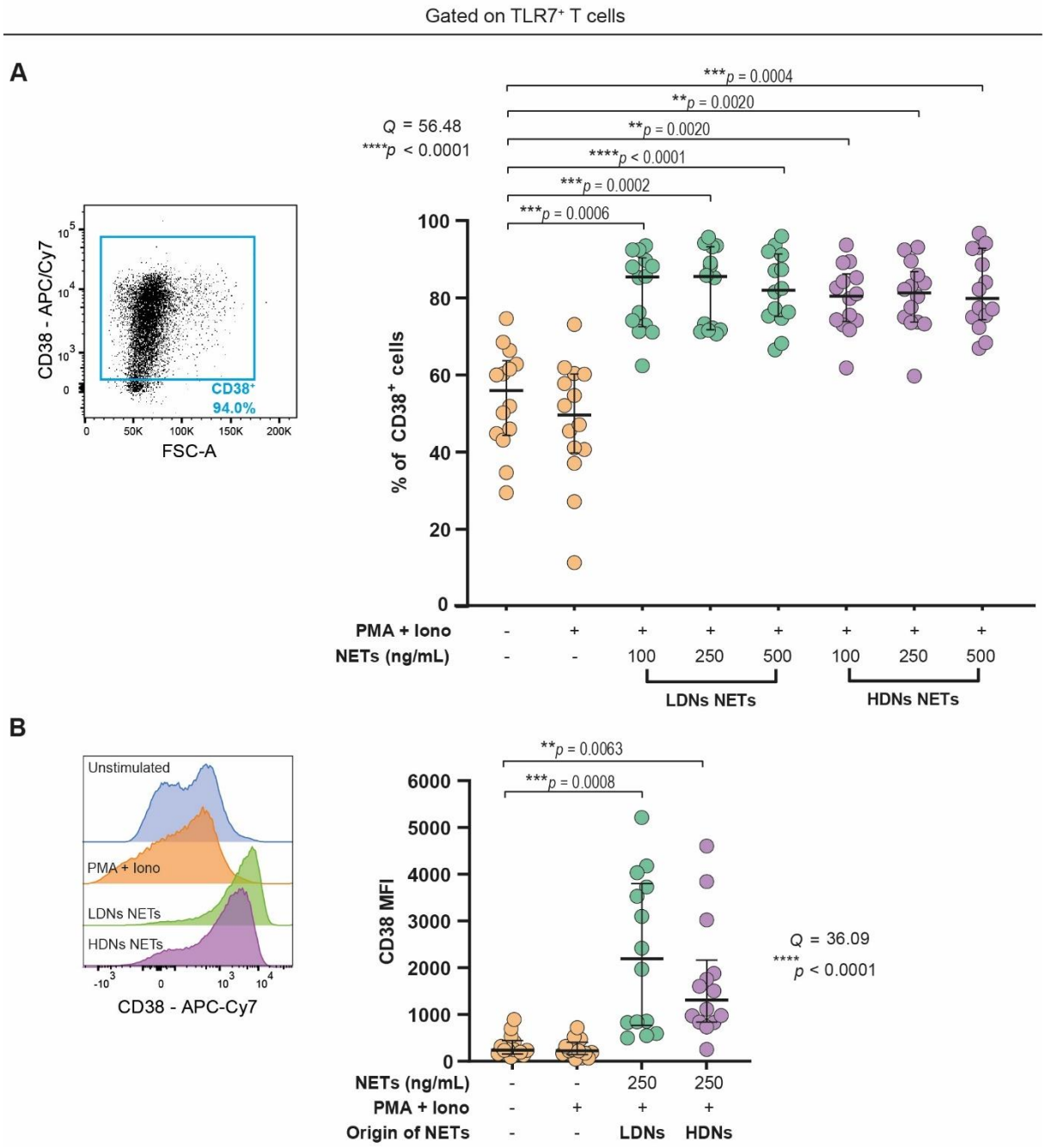

Figure 3.8 - Activation status of TLR7 T cells upon stimulation with NETs. **A** - Representative dot plot and cumulative frequencies of CD38 on TLR7⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated condItions (LDNs (green) and HDNs (purple) NETs) (n=14); **B** - Representative histogram and cumulative frequencies of CD38 MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (n=14). Data are presented as median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. P values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ were determined by Friedman (Q) test with posttest Dunn's multiple comparison.

3.7 TLR9 expression

Regarding TLR9 expression, the scenario is different. Contrarily, to TRL7 expression there are no statistically significant differences between unstimulated (6.5 IQR [5.0-8.5]%) and NETs-stimulated conditions (LDNs NETs: 100 ng/mL - 3.8 IQR [3.0-10.9]%; 250 ng/mL - 4.9 IQR [2.4.0-9.6]%; 500 ng/mL - 4.8 IQR [2.7-10.3]%) (HDNs NETs: 100 ng/mL - 4.6 IQR [3.0-11.4]%; 250 ng/mL - 6.1 IQR [3.3-10.6]%; 500 ng/mL - 4.2 IQR [3.0-11.4]%) neither between unstimulated and PMA+Iono condition (6.1 IQR [3.4-8.9]%) (Fig. 3.9A). However, it appears that NETs-stimulated conditions except for 250 ng/mL from HDNs NETs present less TLR9 expression than unstimulated and control conditions (Fig. 3.9A).

When looking at TLR9 MFI although there is a significant difference between unstimulated (135 ± 38) and 250 ng/mL of LDNs NETs (193 ± 77), but there is not a big shift in the fluorescence intensity in any of the conditions (PMA+Iono - 118 ± 38; 250 ng/mL HDNs NETs -182 ± 64) (Fig. 3.9B). This points to a weak induction of TLR9 by NETs.

The same approach of TLR7 was done in TLR9 T cells and CD38 activation marker was also checked. Despite only existing minor differences in $TLR9^{+}$ T cells, all cells showed a significant state of activation. NETs-stimulated (LDNs NETs: 100 ng/mL - 78.9 ± 9.9%; 250 ng/mL - 79.0 ± 8.1%; 500 ng/mL - 80.5 ± 8.9%) (HDNs NETs: 100 ng/mL - 75.0 ± 8.0%; 250 ng/mL - 78.3 ± 6.5%; 500 ng/mL - 79.72± 7.26%) cells showed higher activation status than unstimulated (57.5 ± 13.9%) and PMA control condition (51.0 ± 13.8%) (Fig. 3.10B). Interestingly, LDNs NETs-stimulated conditions appear to be more activated than their counterparts.

In consonance with the previous results CD38 MFI shows a big shift in fluorescence regards NETs-stimulated conditions (250ng/mL LDNs NETs - 1474 IQR [706-2660]; 250 ng/mL HDNs NETs – 1215 IQR [930-2032] compared to unstimulated (447 IQR: [234-564]) (Fig. 3.10B). Although not significant, fluorescence in PMA+Iono appears to be lower than unstimulated, which can prove that NETs promote a higher expression of CD38 (Fig. 3.10B).

These data showed that the expression of TLR9 in T cells is not altered by NETs stimulation, even when cells are highly activated. TLR9 expression remains more or less constant between stimulated and non-stimulated conditions. This proposes that TLR9 has no role in SLE or that its expression can be somehow affected or controlled by TLR7.

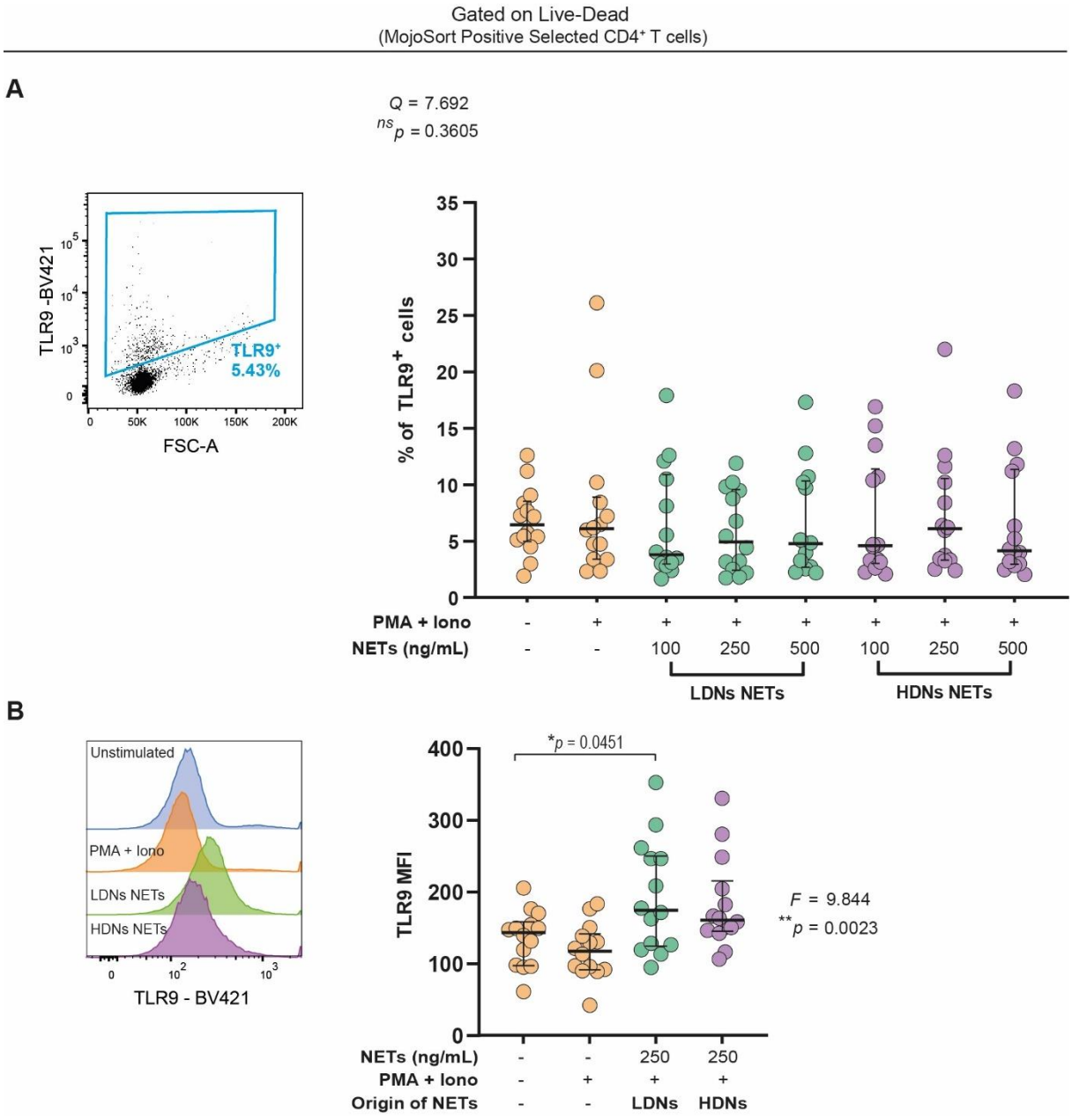

Figure 3.9 - TLR9 expression on T cells upon stimulation with NETs. A - Representative dot plot and cumulative frequencies of TLR9⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14); **B -** Representative histogram and cumulative frequencies of TLR9 MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* values $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ and *ns* (not sginificant) were determined by (**A**) Friedman (*Q*) test with posttest Dunn's multiple comparisons. (**B**) Repeated Measures one-way ANOVA (*F*) with posttest Dunnett's multiple comparisons.

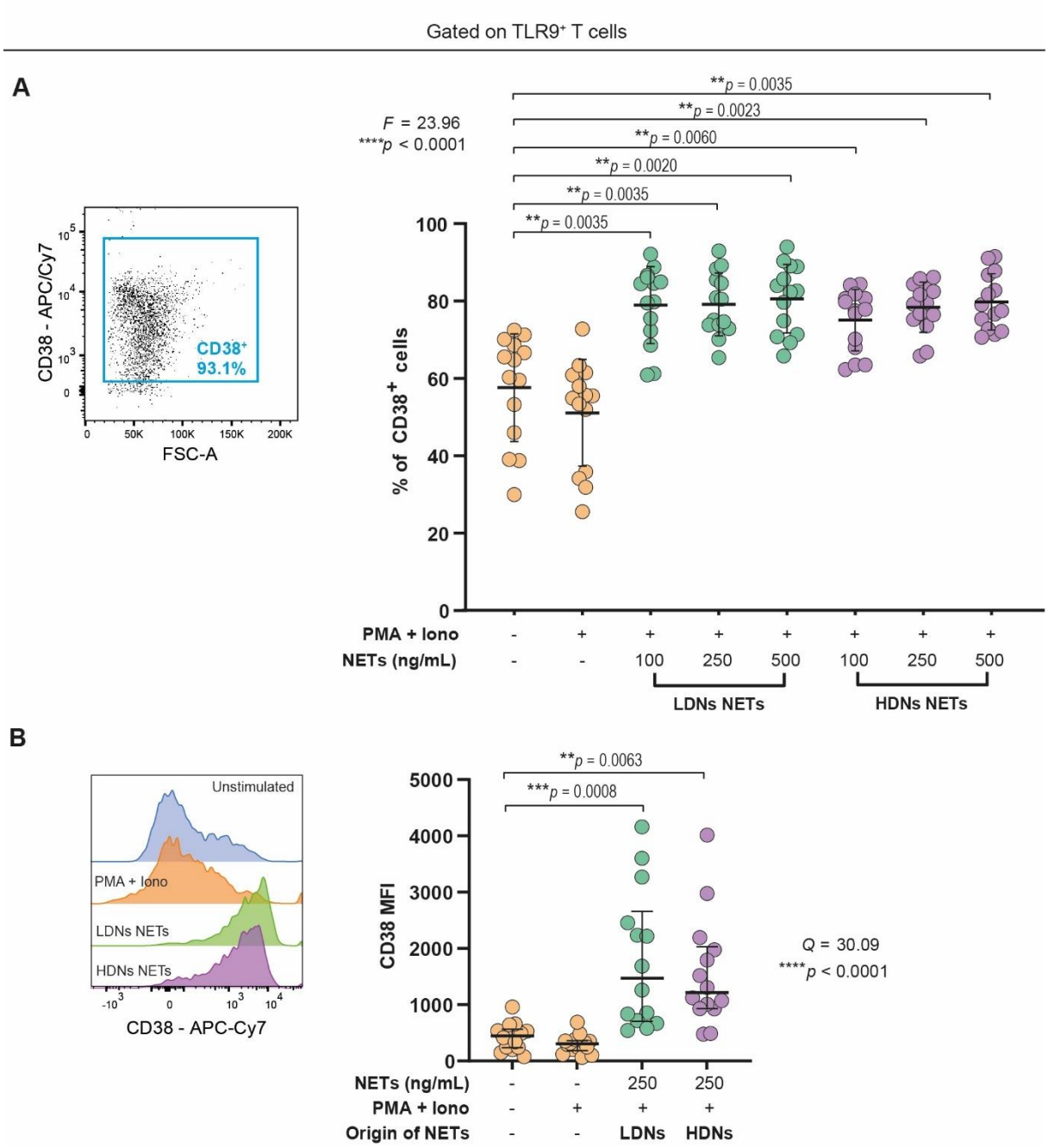

Figure 3.10 - Activation status of TLR9 T cells upon stimulation with NETs. A - Representative dot plot and cumulative frequencies of CD38 on TLR9⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated condItions (LDNs (green) and HDNs (purple) NETs) (n=14); **B** - Representative histogram and cumulative frequencies of CD38 MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (n=14). Data are presented as median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. P values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ were determined by (**A**) Repeated measures one-way ANOVA (F)with posttest Dunnett's multiple comparisons; (**B**) Friedman (Q) test with posttest Dunn's multiple comparisons.

3.7.1 TLR7 Vs TLR9 expression

The differences in TLR7 and TLR9 expression in NETs-stimulated conditions led to the question if there is a statistical difference between LDNs NETs- and HDNs NETs-stimulated T cells. In fact, there were no significant differences between the same concentration of each type of NETs nor between the different concentrations of the same type of NETs. Nonetheless, in TLR7^{+} T cells, there were significant differences between different types of NETs and concentrations of LDNs and HDNs (Fig. 3.11A). Even not being significant there seems to be a tendency for T cells to be saturated when stimulated when HDNs NETs (100 ng/mL - 93.7 ± 2.0 %; 250 ng/mL - 92.9 ± 2.4 %; 500 ng/mL - 91.7 ± 2.9 %) that does not happen with LDNs NETs (LDNs: 100 ng/mL - 94.1 ± 2.4 %; 250 ng/mL - 94.0 ± 1.4 %; 500 ng/mL - 93.6 ± 2.9 %) (Fig. 3.11A).

When it comes to TLR9, its expression does not vary between different doses of the same types of NETs, neither between the same dose of different types of NETs (Fig. 3.11B).

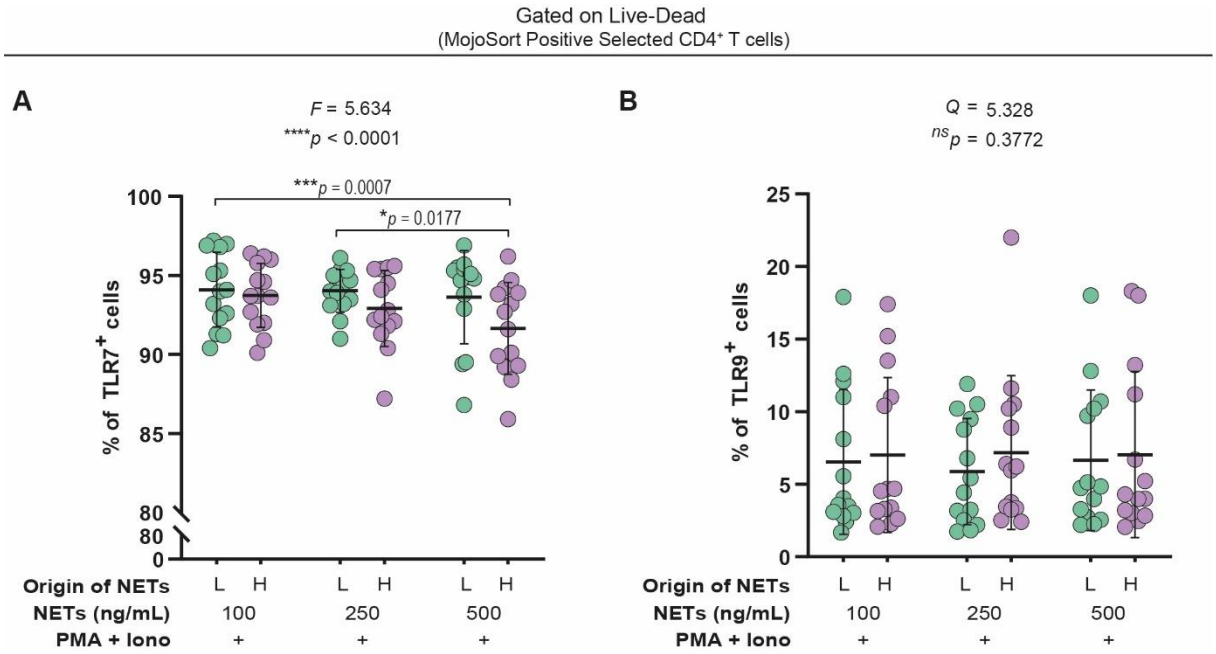

Figure 3.11 - TLRs expression in T cells upon stimulation with NETs. A - Comparison of cumulative frequencies of TLR7^{+} T cells between LDNs and HDNs NETs-stimulated condItions (*n*=14); **B** - Comparison of the cumulative frequencies of TLR9^{+} T cells between LDNs and HDNs NETs-stimulated condItions (*n*=14); Data are presented as mean ± SD, for parametrical statiscal tests. Sample normality distribution was tested by using Shapiro-Wilk normality test. *P* values ****$p \leq 0.0001$, ***$p \leq 0.001$, *$p \leq 0.05$ and *ns* (not significant) were determined by (**A**) Repeated measure one-way ANOVA (*F*) with posttest Sidaks's multiple comparisons; (**B**) Friedman (*Q*)with posttest Dunn's multiple comparisons.

3.8 NETs induce cytokine production by CD4$^+$ T cells

IL-21 is responsible for the differentiation of plasma blasts[60] and it has been described in SLE patients as increased in a T_{FH}-like subset [54,58]. TNFα is a pleiotropic cytokine, acting as a proinflammatory in SLE and it has been reported as increased.[130,131] Thus, cytokine profiling TLR-expressing T cells is important. For that matter, T cells were analyzed for IL-21 and TNFα production on day 4.

3.8.1 IL-21 and TNFα in TLR7$^+$ T cells

As a matter of fact, TLR7$^+$ T cells produce IL-21 (Fig. 3.13A). NETs-stimulated conditions produced IL-21 (LDNs NETs: 100 ng/mL - 5.9 IQR [4.4-8.6]%; 250 ng/mL - 5.6 IQR [3.5-11.7]%; 500 ng/mL - 5.30 IQR [4.2-8.5]%) (HDNs NETs: 100 ng/mL - 4.8 IQR [3.3-8.0]%; 250 ng/mL - 5.0 IQR [4.0-8.8]%; 500 ng/mL - 5.1 IQR [2.9-7.2]%) when compared to unstimulated (1.2 IQR [0.7-2.1]%) (Fig. 3.12A). Although not statistically significant, the lowest concentrations of LDNs NETs (100 and 250 ng/mL) appear to produce more IL-21 than their counterparts (Fig. 3.12A). This is sustained by the bigger statistical power of the *p* values of LDNs NETs. PMA+Iono condition also produces IL-21 but in smaller amounts (3.9 IQR [1.7-7.4]%) (Fig. 3.12A).

IL-21 MFI of TLR7$^+$ T cells indicates a higher shift in fluorescence for NETs-stimulated conditions when compared to unstimulated (149 ± 44), and LDNs NETs condition (250 ± 105) appears to have a higher shift than its counterpart (215 ± 79) (Fig. 3.12B). PMA+Iono condition fluorescence remains almost the same as unstimulated, supporting the production of IL-21 by NETs stimulated TLR7$^+$ T cells (Fig. 3.12B).

Regarding TNFα production, all conditions produce it. Even in unstimulated cells there is production of TNFα but not in a uniform way (61.3 IQR [41.2-77.4] %), some donors had already high production of TNFα while others had lower production (Fig. 3.13A). Nevertheless, PMA and ionomycin induce TNFα production (81.6 IQR [76.8-85.2] %) and, even though, there is no significant difference between PMA+Iono and NETs-stimulated conditions it seems that NETs potentiate even further TNFα production (LDNs: 100 ng/mL - 86.1 IQR [78.2-95.3]%; 250 ng/mL- 87.7 IQR [78.6-94.2]%; 500 ng/mL 85.9 IQR [79.2-93.2]%)(HDNs: 250 ng/mL - 84.5 IQR [80.0-91.5]%; 500 ng/mL - 85.20 IQR [80.5-92.4]%) with the exception of 100 ng/mL HDNs NETs condition (80.4 IQR [76.0-91.1]%) (Fig.3.13A).

TNFα MFI sustains the previous results, where TNFα production is majorly caused by PMA+Iono (2197 IQR [933-4212]) when compared to unstimulated cells (765 IQR [526-984]. This proves that reminiscent of TNFα production detected in NETs-stimulated condition is

caused by NETs (250 ng/mL LDNs NETs – 1477 IQR [1164-2623]; 250 ng/mL HDNs NETs - 1369 IQR [980-2965] (Fig. 3.13B).

In the case of doble positive production for IL-21 and TNFα, only LDNs NETs-stimulated conditions seem capable of inducing both cytokines in a statistically significant way (100 ng/mL - 5.20 IQR [2.92-6.75]%; 250 ng/mL - 5.64 IQR [3.34-8.02]%; 500 ng/mL - 4.31 IQR [3.73-7.11]%) in comparison to unstimulated (1.77 IQR [0.88-2.92]%, especially the lower concentrations (Fig. 3.14). Nonetheless, HDNs NETs (100 ng/Ml - 4.33 IQR [3.13-5.33]%; 250 ng/mL - 4.01 IQR [2.77-7.16]%; 500 ng/mL - 4.77 IQR [2.72-5.98]%) and PMA+Iono (3.25 IQR [2.30-5.26]%) also appear to induce both cytokines production but to a lesser extent (Fig. 3.14).

Taken together, NETs stimulation of $TLR7^{+}CD4^{+}$ T cells leads to secretion of the cytokine IL-21 and to some secretion of TNFα. This cytokine profile points to a pathogenic role of $CD4^{+}$ T cells when in contact with NETs, in particular, LDNs NETs.

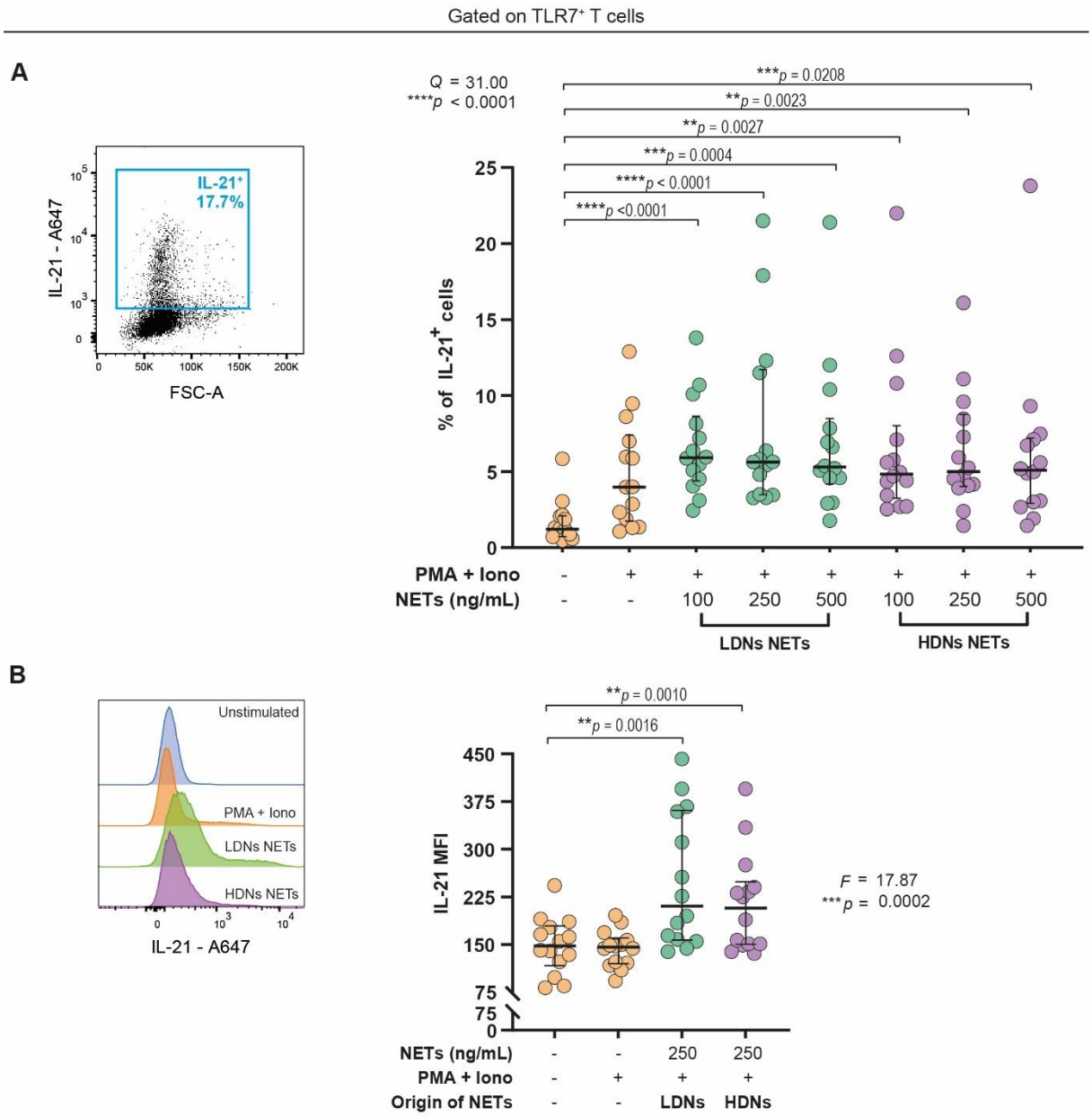

Figure 3.12 - TLR7⁺ T cells IL-21 production upon stimulation with NETs. Representative dot plot and cumulative frequencies of IL-21⁺TLR7⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14). **B** - Representative histogram and cumulative frequencies of IL-21 MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as mean ± SD, for statistical parametrical tests and median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality. *P* values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ were determined by (**A**) Friedman (*Q*) test with posttest Dunn's multiple comparisons; (**B**) Repeated measures one-way ANOVA (*F*)with posttest Dunnett's multiple comparisons.

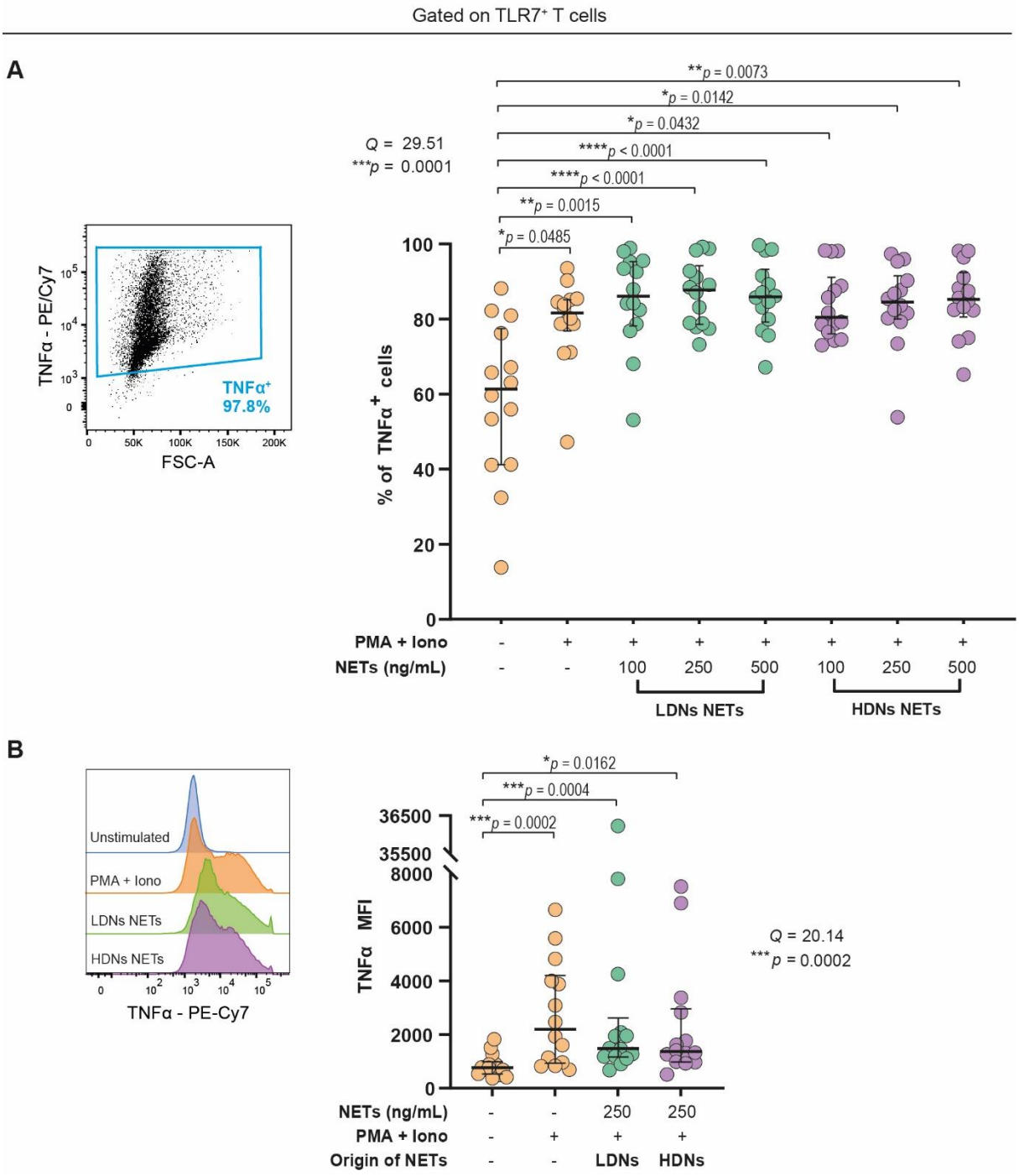

Figure 3.13 - TLR7⁺ T cells TNFα production upon stimulation with NETs. Representative dot plot and cumulative frequencies of TNFα⁺ TLR7⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14). **B** - Representative histogram and cumulative frequencies of TNFα MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality. *P* values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ were determined by (**A**) Friedman (*Q*) test with posttest Dunn's multiple comparisons.

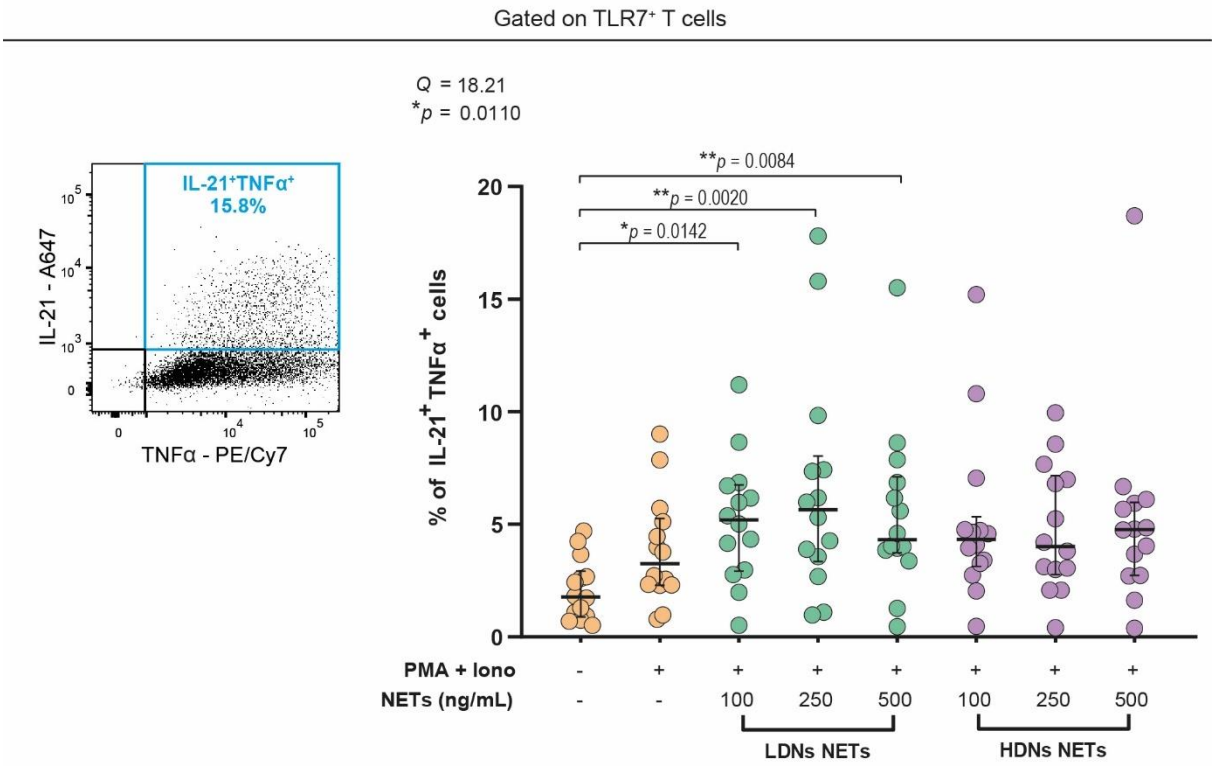

Figure 3.14 - TLR7⁺ T cells IL-21 and TNFα production upon stimulation with NETs. Representative dot plot and cumulative frequencies of IL-21⁺TNFα⁺ TLR7⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (n=14). Data are presented as median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality. P values $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ were determined by Friedman (Q) test with posttest Dunn's multiple comparisons.

3.8.2 IL-21 and TNFα in TLR9⁺ T cells

Considering TLR9 expressing T cells, LDNs NETs-stimulated conditions and 250 ng/mL of HDNs NETs produced IL-21 (LDNs NETs: 100 ng/mL - 8.9 ± 3.9%; 250 ng/mL - 10.5 ± 4.6% ; 500 ng/mL - 10.1 ± 5.4%) (250 ng/mL HDNs NETs - 8.7 ± 3.9%) compared to unstimulated (5.8 ± 3.0%) (Fig. 3.15A). The remaining HDNs NETs stimulated conditions were not statistically significant but they show some IL-21 expression (100 ng/mL - 9.1 ± 4.2%; 500 ng/mL - 8.8 ± 5.5%). In agreement with IL-21 production in TLR7⁺ T cells, IL-21 is also produced by TLR9 expressing T cells (Fig. 3.15A). PMA+Iono (7.1 ± 3.1%) does not seem to induce IL-21 besides the already produced in unstimulated (Fig. 3.15A). Interestingly, TLR9⁺ T cells present a higher basal expression of IL-21 on unstimulated cells (5.1 IQR [3.4-7.9]%) (Fig. 3.15A) compared to TLR7⁺ T cells (1.23 IQR [0.7-2.0]%) (Fig. 3.12A).

Taking a look at IL-21 MFI, both 250 ng/mL HDNs (185 IQR [145-267]) and LDNs (194 IQR [143-223]) NETs-stimulated conditions have a shift in fluorescence, albeit not big (Fig. 3.15B). PMA+Iono (136 IQR [114-181]) remains more or less equal to unstimulated (144 IQR [131-174) (Fig. 3.15B).

In regard to TNFα, it is produced in all NETs-stimulated conditions, (LDNs NETs: 100 ng/mL - 81.9 ± 9.4 %; 250 ng/mL - 82.8 ± 6.0 %; 500 ng/mL - 83.0 ± 8.1 %) (HDNs NETs: 100 ng/mL - 80.0 ± 7.6 %; 250 ng/mL - 821.1 ± 7.6 %; 500 ng/mL - 82.2 ± 9.1 %) (Fig. 3.16A). PMA+Iono condition (76.0 ± 9.8 %) looks like it stimulates TNFα production, even though, it is not statistically significant when compared to unstimulated (66.4 ± 13.6 %) (Fig. 3.16A).

Curiously, when checking TNFα MFI, PMA+Iono condition (1370 IQR [968-2342]) shows almost the same behavior as LDNs (1359 IQR [1093-2428]) and HDNs (1489 IQR [1105-3526]) NETs-stimulated cells in comparison to unstimulated (742 IQR [480-1164] (Fig. 3.16B). This suggests that PMA and ionomycin is the one promoting TNFα production as it was showed in in $TLR7^+$ T cells (Fig. 3.13A).

In the case of the doble positive T cells for IL-21 and TNFα, there are no statistically significant differences among the conditions, but the cumulative frequency shows that some cells produce both cytokines and others do not. Data is scattered and remains more or less constant across the conditions (Fig. 3.17).

Taken together, this data shows that $TLR9^+$ T cells when stimulated show IL-21 and TNFα production as it happens on $TLR7^+$ T cells. However, since TLR9 T cells are low expressed in SLE patients and are not increased upon stimulation with NETs, it questions if the IL-21 and TNFα detected is really produced by TLR9 engagement.

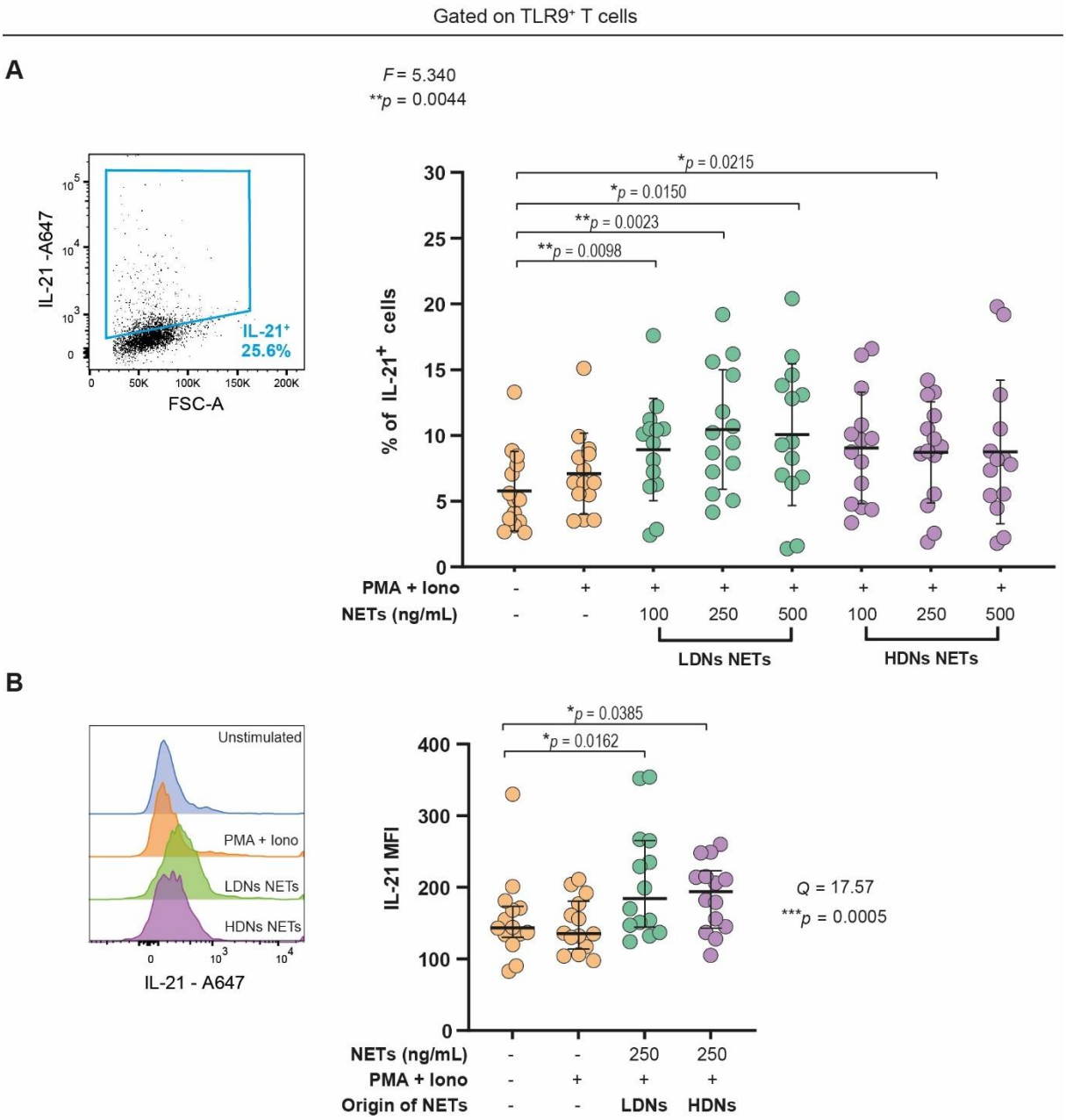

Figure 3.15 - TLR9⁺ T cells IL-21 production upon stimulation with NETs. A - Representative dot plot and cumulative frequencies of IL-21⁺TLR9⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (n=14). **B** - Representative histogram and cumulative frequencies of IL-21 MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* values ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ were determined by (**A**) Repeated Measures one-way ANOVA (*F*) with posttest Dunnett's multiple comparisons (**B**) Friedman (*Q*) test with posttest Dunn's multiple comparisons

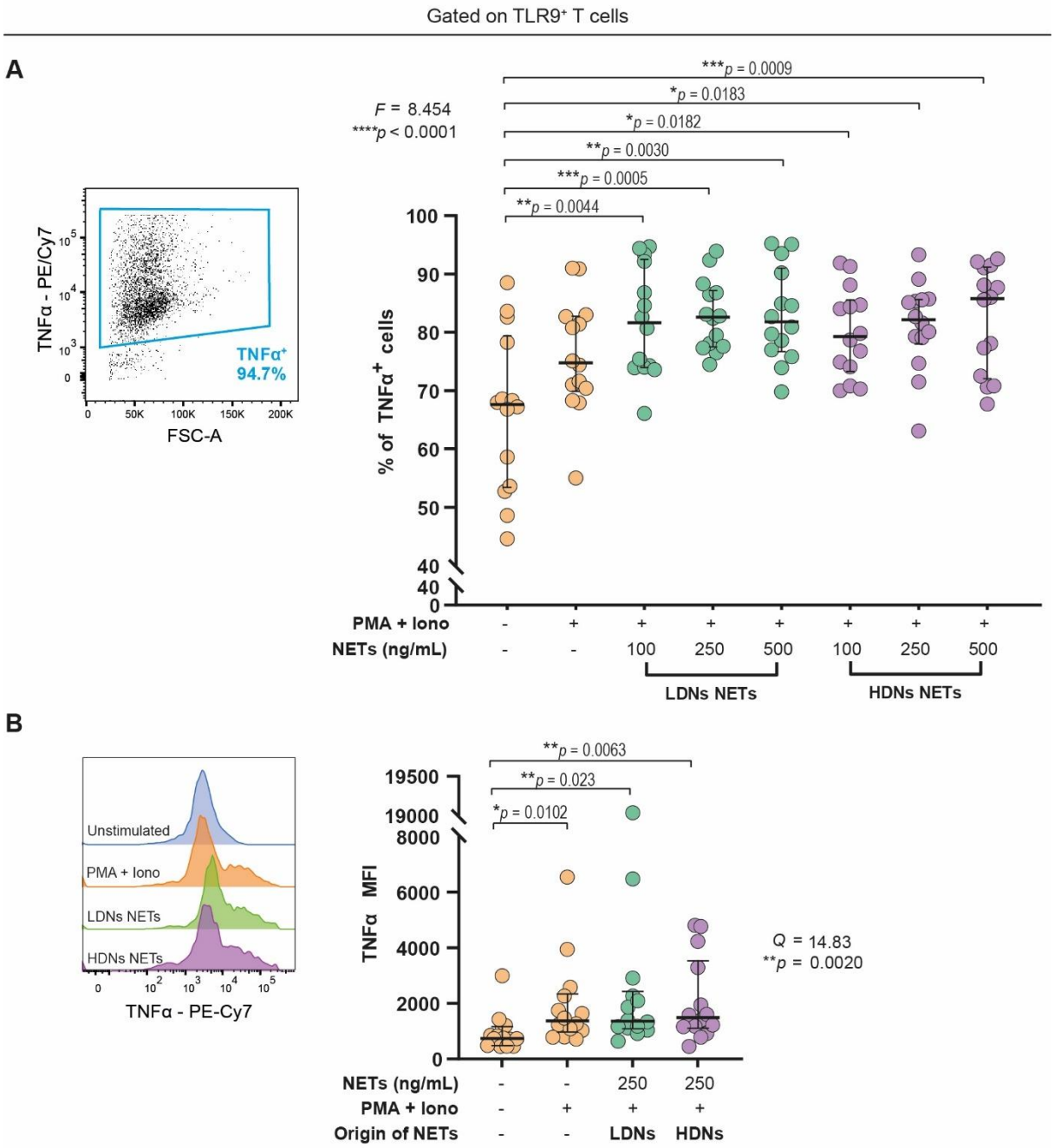

Figure 3.16 - TLR9⁺ T cells TNFα production upon stimulation with NETs. A - Representative dot plot and cumulative frequencies of TNFα⁺ TLR9⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (n=14). **B** - Representative histogram and cumulative frequencies of TNFα MFI for control conditions (unstimulated (blue/orange) and PMA+Iono (orange)) and NETs-stimulated conditions (250 ng/mL of LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ were determined by (**A**) Repeated Measures one-way ANOVA (*F*) with posttest Dunnett's multiple comparisons (**B**) Friedman (*Q*) test with posttest Dunn's multiple comparisons.

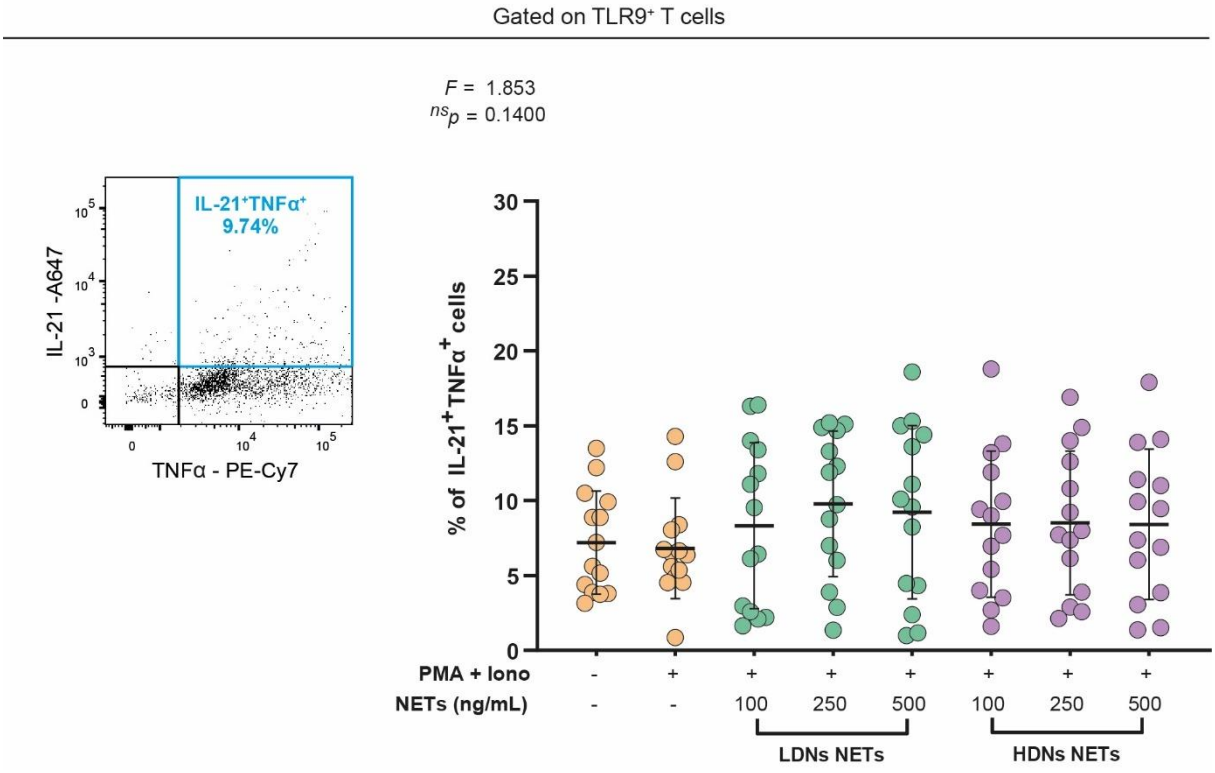

Figure 3.17 - TLR9⁺ T cells IL-21 and TNFα production upon stimulation with NETs. Representative dot plot and cumulative frequencies of Il-21⁺TNFα⁺ TLR9⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as mean ± SD, for parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality. *P* value *ns* (not significant) was determined by Repeated measures one-way ANOVA (*F*) with posttest Dunnett's multiple comparisons.

3.8.3 IL-21 in TLR- and non-TLR-expressing T cells

With the objective of understanding if IL-21 was produced by generic $CD4^+$ T cells, in other words, T cells that do or do not express TLRs, IL-21 was checked by directly gating in live-dead cells. IL-21 is produced by all NETs-stimulated (LDNs NETs: 100 ng/mL - 4.6 IQR [4.0-5.7]%; 250 ng/mL - 5.1 IQR [3.0-8.4]%; 500 ng/mL - 5.3 IQR [2.8-8.4]%) (HDNs NETs: 100 ng/mL - 4.4 IQR [2.7-5.6]%; 250 ng/mL - 3.9 IQR [3.2-7.9]%; 500 ng/mL: 4.1 IQR [2.1-5.4]%) conditions when compared to unstimulated (0.9 IQR [0.6-1.3]%) (Fig. 3.18A). Although not statistically significant LDNs NETs-stimulated conditions unveiled higher expression of IL-21 than HDNs NETs, in accordance with the data from IL-21 producing $TLR7^+$ T cells (Fig. 3.12A). PMA+Iono also exhibited production of IL-21 (3.6 IQR [2.0-7.0]% but not robust enough to be statistically significant.

TLR^+ and TLR^- T cells were also looked upon to dissect which fraction produced more IL-21, but this was done in 250 ng/mL LDN NETs-stimulated condition. This is justified by 250 ng/mL being the closest concentration of DNA detected in the plasma of SLE patients.[67] Consistently with all previous results, both $TLR7^+$ and $TLR9^+$ fractions produced more IL-21 than $TLR7^-$ and $TLR9^-$ fractions respectively ($TLR7^+$: 5.6 IQR [3.5-11.7]%; $TLR7^-$: 3.5 IQR [2.9-5.5]%) ($TLR9^+$: 11.3 IQR [6.1-18.3]%; $TLR9^-$: 3.6 IQR [1.6-6.4]) (Fig. 3.18B-C).

In sum, these results support a pathogenic role of TLR7 in T cells when the ligands of the receptor are provided by NETs, specially LDNs NETs, unveiling a crosstalk between neutrophils and T cells, where the major players are the NETs.

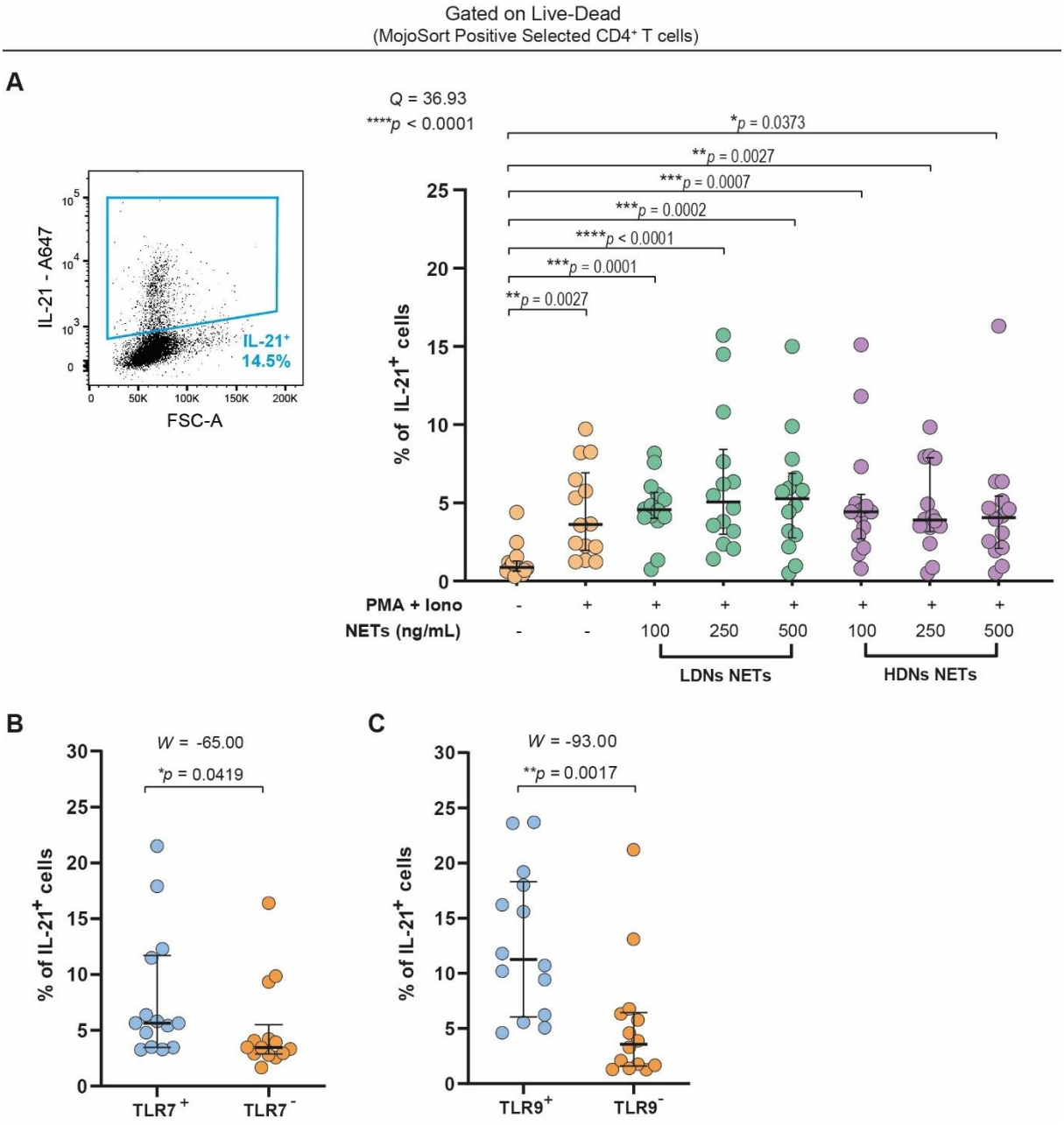

Figure 3.18 - IL-21 production in generic T cells. A - Representative dot plots and cumulative frequencies of IL-21$^+$ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (n=14). Cumulative frequencies of IL-21$^+$ producing CD4$^+$ T cells in TLR7$^+$/TLR7$^-$ (**B**) and TLR9$^+$/TLR9$^-$ (**C**) in 250 ng/mL LDN NETs-stimulated condition (n=14). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. P values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ were determined by (**A**) Friedman (Q) test with posttest Dunn's multiple comparisons. (**B,C**) Wilcoxon matched-pairs signed rank test (W).

3.8.4 TNFα in TLR- and non TLR-expressing cells

The same approach was done to TNFα. TNFα was also produced by generic $CD4^+$ T cells. TNFα was checked by directly gating in live-dead cells and it is produced by all stimulated conditions (PMA+Iono: 78.5 IQR [74.3-84.0]%) (LDNs NETs: 100 ng/mL - 81.7 IQR [76.3-94.3]%; 250 ng/mL - 82.2 IQR [77.0-90.7]%; 500 ng/mL - 83.9 IQR [76.4-90.6]%) (HDNs NETs: 100 ng/mL - 81.7 IQR [74.5-88.5]%; 250 ng/mL - 81.4 IQR [72.9-89.6]%; 500 ng/mL - 82.45 IQR [77.0-88.5]%) conditions when compared to unstimulated (60.4 IQR [39.7-81.6]%) (Fig. 3.19A).

Nonetheless, TLR^+ and TLR^- T cells were also looked upon to unveil which fraction produced more TNFα also in 250 ng/mL LDNs NETs stimulated condition. $TLR7^+$ T cells (86.7 ± 8.7%) produced more TNFα than $TLR7^-$ fraction (47.3 ± 14.9%) as expected. However, there was no significant difference for TNFα between $TLR9^+$ (82.84 ± 6.04%) and $TLR9^-$ (79.07 ± 12.13%) (Fig. 3.19B-C).

In sum, these results support that the augmentation in TNFα production seen in $TLR7^+$ T cells is majorly because of PMA and ionomycin, however NETs can stimulate some TNFα production. Since this cytokine is not highly induced by NETs it suggests that it may not play a crucial pathogenic role in SLE. Moreover, the lack of TNFα significant differences between $TLR9^+$ and $TLR9^-$ fractions also suggests once again, that this receptor may not be engaged by NETs, and the IL-21 that it is observed in $TLR9^+$ T cells may be due to simultaneous TLR7 and TLR9 expression.

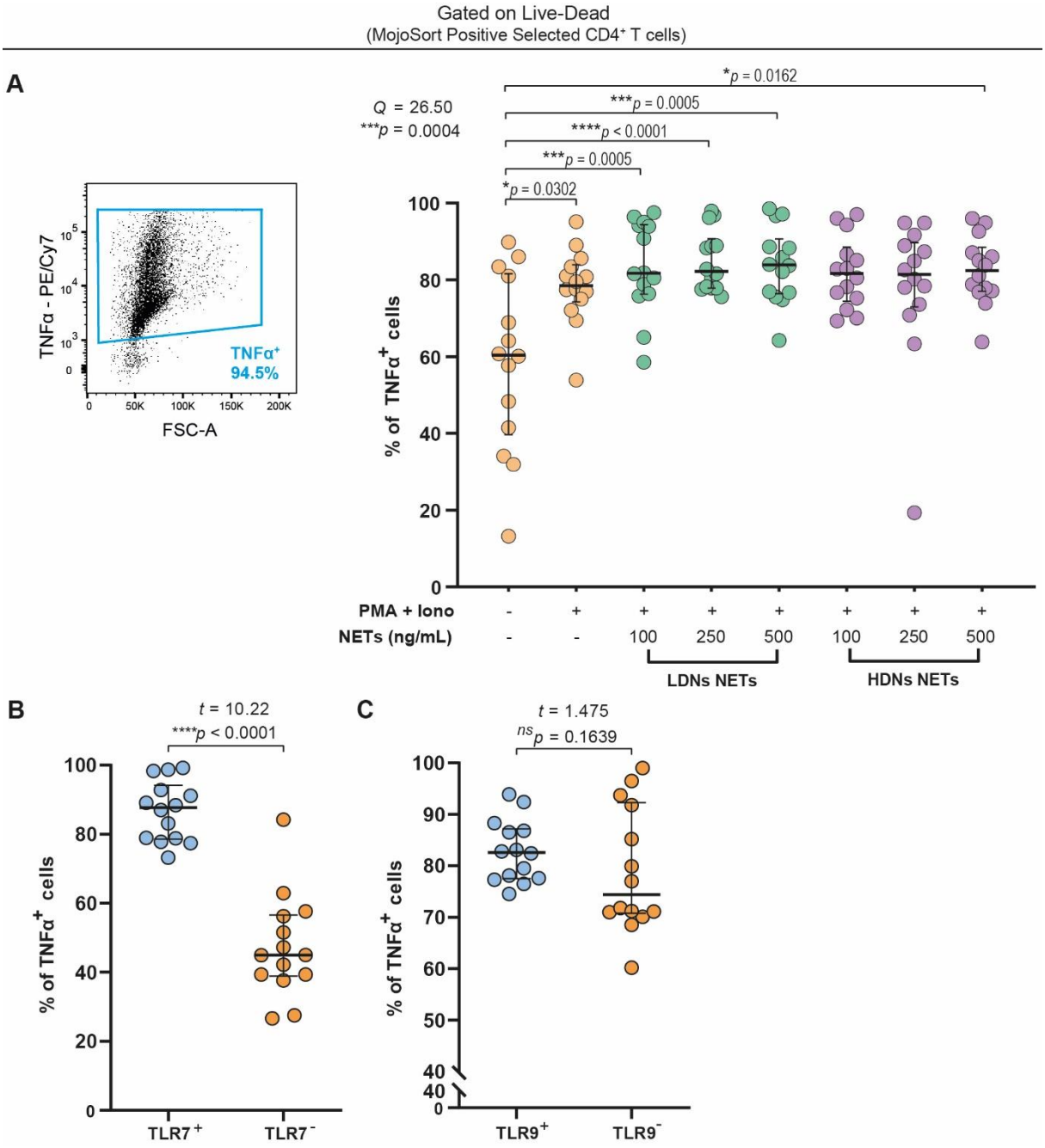

Figure 3.19 - TNFα production in generic T cells. A - Representative dot plots and cumulative frequencies of TNFα⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14). Cumulative frequencies of TNFα⁺ producing CD4⁺ T cells in TLR7⁺/TLR7⁻ (**B**) and TLR9⁺/TLR9⁻ (**C**) in 250 ng/mL LDN NETs-stimulated condition (*n*=14). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* values ****$p \leq 0.0001$,***$p \leq 0.001$, *$p \leq 0.05$ and *ns* (not significant) were determined by (**A**) Friedman (*Q*) test with posttest Dunn's multiple comparisons. (**B,C**) paired *t*-test (*t*).

3.9 Simultaneous expression for TLR7 and TLR9 in T cells

For further comprehension of the TLRs expression in T cells, doble positive TLR7 and TLR9 analysis were directly gated on live-dead gate. No significant differences were found amid the conditions (Fig. 3.20A). The percentages of $TLR7^{+}TLR9^{+}$ T cells are mostly constant in stimulated or non-stimulated cells (unstimulated - 4.9 ± 2.0 %; PMA+Iono – 4.2 ± 2.1 %)(LDNs: 100 ng/mL – 4.5 ± 2.5 %; 250 ng/mL – 4.0 ± 2.0 %; 500 ng/mL – 4.6 ± 2.5 %;) (HDNs: 100 ng/mL – 4.7 ± 2.5 %; 250 ng/mL – 4.7 ± 2.4 %; 500 ng/mL – 4.8 ± 2.9 %). Notwithstanding, $TLR7^{+}$ $TLR9^{+}$ T cells do not represent a lot of cells and to confirm this data, further analysis were done by gating directly in $TLR7^{+}$ T cells or $TLR9^{+}$ T cells.

TLR9 expression inside the $TLR7^{+}$ T cells (Fig. 3.20B) was concordant with the previous data of TLR9 expression directly gated on live-dead (Fig. 3.9A). Once again. there was no increase of TLR9 expression upon stimulation with NETs (Fig- 3.20B), suggesting once more that TLR9 might not be involved in the pathogenesis of the disease.

TLR7 expression inside $TLR9^{+}$ T cells was also checked (Fig. 3.21). Surprisingly, the majority of TLR9 T cells express TLR7 and there is indeed an upregulation of TLR7 in all NETs-stimulated conditions (LDNs: 100 ng/mL - 69.5 ± 12.0 % ; 250 ng/mL - 73.1 ± 12.8 %; 500 ng/mL - 71.5 ± 15.8 ;) (HDNs: 100 ng/mL - 68.7 ± 16.4 % ; 250 ng/mL - 68.4 ± 16.3 %; 500 ng/mL - 69.0 ± 16.6 % ;) (unstimulated - 51.5 ± 16.9 %; PMA+Iono – 57.6 ± 20.9 %) (Fig. 3.21).

Overall, this data concludes that TLR9 expression is not altered by NETs stimulation and that the IL-21 and TNFα detected in $TLR9^{+}$ T cells is produced due to the engagement of TLR7, inasmuch majority of $TLR9^{+}$ T cells also express TLR7.

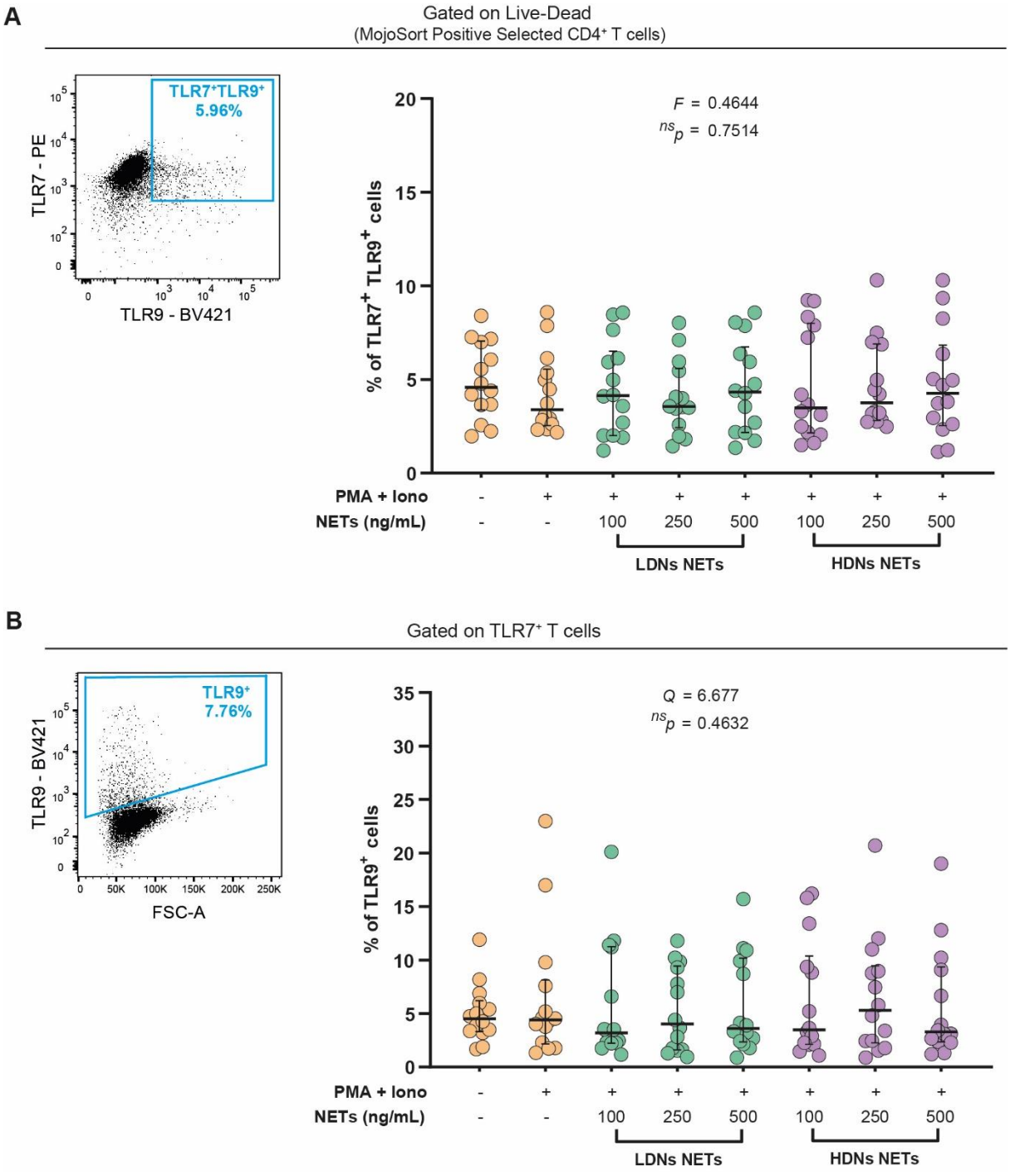

Figure 3.20 - Simultaneous expression of TLRs in T cells. A - Representative dot plots and cumulative frequencies of TLR7⁺TLR9⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14). **B** - Representative dot plots and cumulative frequencies of TLR9 expressing cells gated in TLR7+ T cell in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* value *ns* (not significant) was determined by (**A**) Repeated Measures one-way ANOVA (*F*) with posttest Dunnett's multiple comparisons (**B**) Friedman (*Q*) test with posttest Dunn's multiple comparisons.

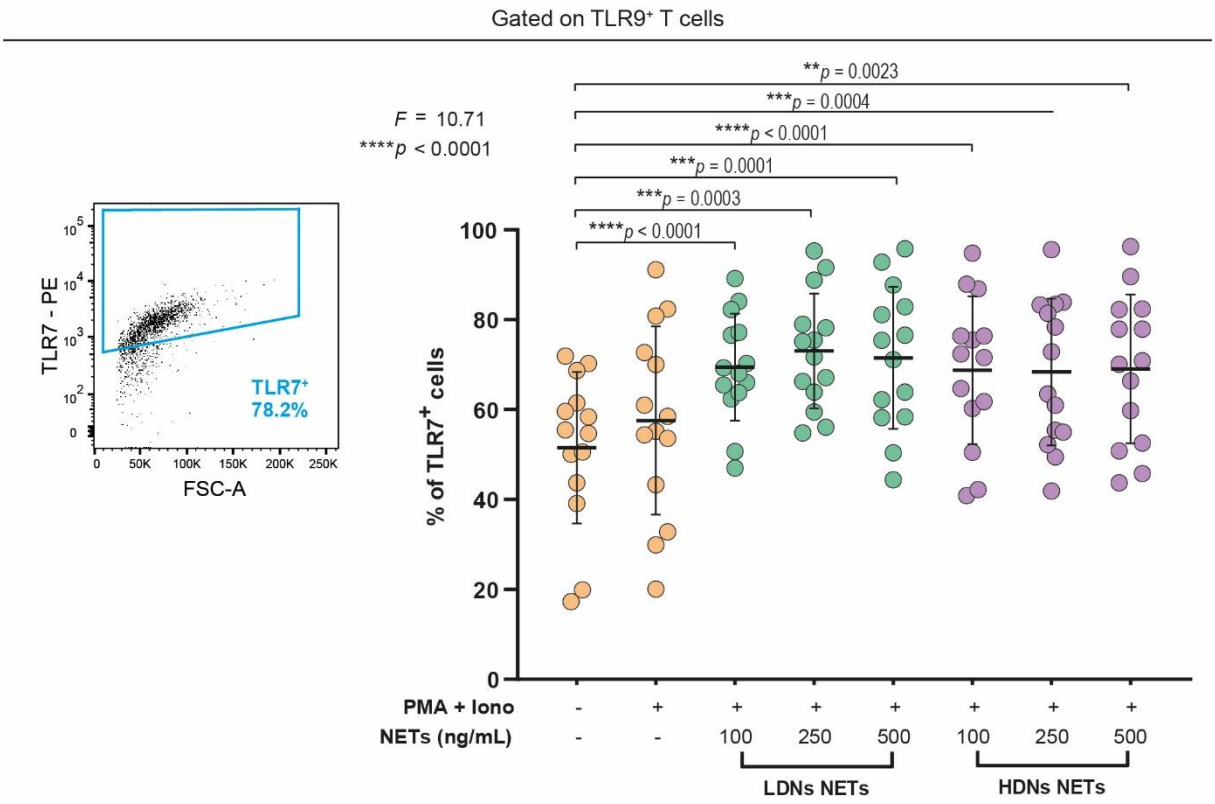

Figure 3.21 - TLR7 expression on TLR9⁺ T cells. Representative dot plots and cumulative frequencies of TLR9⁺ expressing cells gated in TLR7⁺ T cells in control conditions (unstimulated (orange) and PMA+Iono (orange)) and NETs-stimulated conditions (LDNs (green) and HDNs (purple) NETs) (*n*=14). Data are presented as mean ± SD, for parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* values ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$ were determined by (**A**) Repeated Measures one-way ANOVA (*F*) with posttest Dunnett's multiple comparisons.

3.10 Clinical and demographic influences in TLR-expressing T cells and respective cytokine secretion

Finally, a relation between TLR7 and TLR9 expression on naturally resting T cells and SLE demographics and disease presentation were pursued. Differences between age (bellow and above the mean age of the cohort) (Fig. 3.21A-B), the ANAs titer (below and above the median titer of the cohort) (Fig. 3.21C-D), the presence of extractable nuclear antigens (ENAs) (Fig. 3.21E-F) and the most common ENA[132], the anti-SSA (Fig. 3.21G-H) were analyzed. Also, differences between SLEDAI (bellow and above the mean SLEDAI of the cohort) (Fig. 3.21I-J) were analyzed. From the above-mentioned relations, only the percentage of TLR7 led statistically significant difference with SLEDAI (0 - 90.4 ± 2.6 %; ≥ 1 - 86.9 ± 3.2 %) (Fig. 3.22I). Patients with a more active disease express less TLR7. Regarding treatment, the cohort exhibited a heterogeneous combination of drugs that did not allow to perform any kind of relation with cumulative frequencies of TLR7 nor TLR9.

To further understanding if other clinical features could affect TLR7 and TLR9 expression correlations were performed with anti-dsDNA (Fig. 3.22A-B), the most common ANA, the complement protein 3 (C3) [133] (Fig. 3.22C-D) and complement protein 4 (C4)[133] (Fig. 3.22E-F) largely reported in literature as important in SLE. Surprisingly, the percentage of TLR9 correlated with the concentration of complement protein C4 (Fig. 3.22F).

Correlations between the percentage of IL-21 (Fig. 3.22G-H) and TNFα (Fig. 3.22I-J) production by either $TLR7^+$ or $TLR9^+$ T cells and anti-dsDNA were checked. Again, none of these correlations were statistically significant.

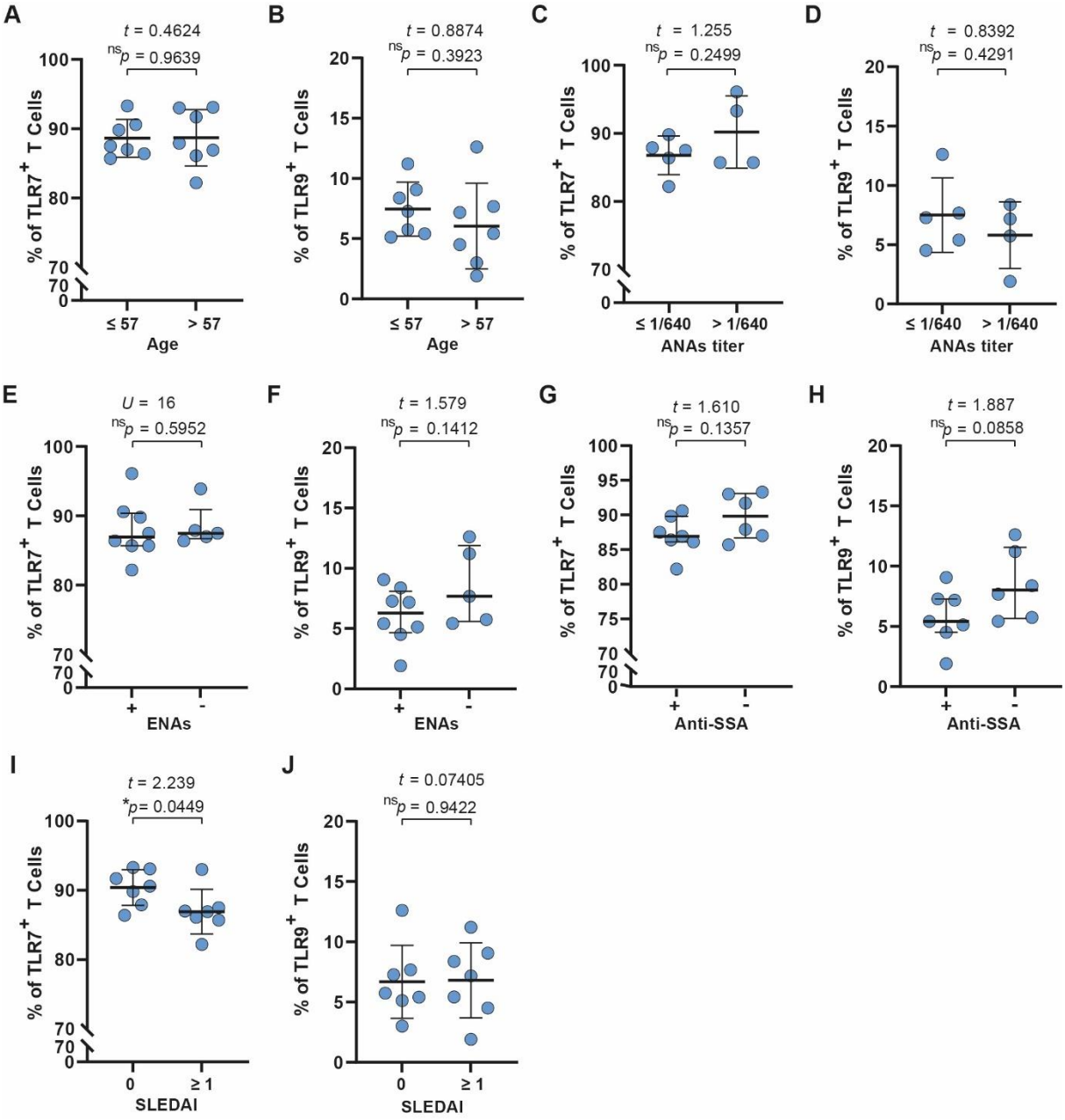

Figure 3.22 - Frequency of TLRs expressing T cells sorted by demographic and clinical features. Frequency of TLR7^{+} and TLR9^{+} T cells disaggregated by age (**A** - TLR7; **B** - TLR9; *n*=14; ≤ 57 years *n* =6; > 57 years *n*= 7), ANA's titer (**C** - TLR7; **D** - TLR9; *n*=9; ≤ 1/640 *n* =5; > 1/640 *n* =4), ENA's (**E** - TLR7; **F** - TLR9; n=13; ENAs^{+} n =8; ENAs^{-} *n*= 5), Anti-SSA (**G** - TLR7; **H** - TLR9; n=13; Anti-SSA^{+} n =7; Anti-SSA^{-} *n*= 6), SLEDAI (**I** - TLR7; **J** - TLR9; n=14; SLEDAI 0 n =7; SLEDAI ≥ 1 *n*= 7). Data are presented as mean ± SD, for parametrical statistical tests, or median ± IQR, for non-parametrical statistical tests. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* values *$p \leq 0.05$ and *ns* (not signficant) were determined by (**A,B,C,D,F,G,H,I,J**) unpaired *t*-test (*t*); (**E**) Mann-Witney test (*U*).

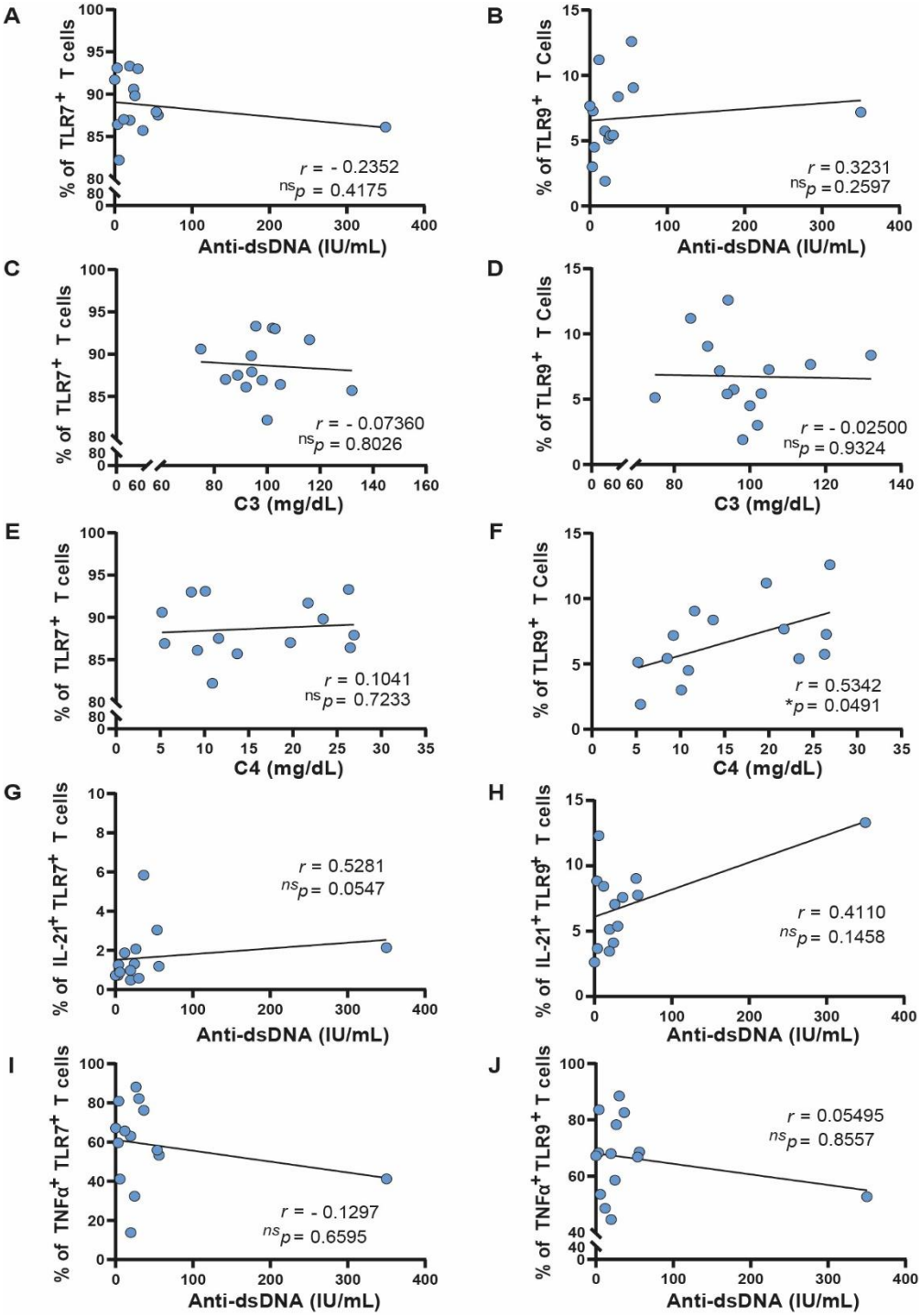

Figure 3.23 - Correlations between the frequency of TLRs expressing T cells and clinical features. A-D - Correlation between frequency of TLR7$^+$ and TLR9$^+$ T cells and anti-dsDNA antibodies (**A, B**), complement protein 3 (**C, D**) and complement protein 4 (**E, F**). **G-H -** Correlation between IL-21 production by TLR7$^+$ (**G**) or TLR9$^+$ (**H**) T cells and anti-dsDNA antibodies. **I-J** - Correlation between TNFα production by TLR7$^+$ (**I**) or TLR9$^+$ (**J**) T cells and anti-dsDNA antibodies. Sample normality distribution was tested by using D'Agostino & Pearson normality test. *P* values *$p \leq$ 0.05 and *ns* (not significant) were determined by (**A,B,G,H,I,J**) Spearmen correlation (*r*) and (**C-F**) Person correlation (*r*).

4

Discussion

Systemic lupus erythematosus is a chronic autoimmune disease characterized by dysregulation of T and B cells. B cells are responsible for the production of autoantibodies against nuclear and cytoplasmic antigens, the hallmark of the disease. High-affinity antibody production can only occur when T cells provide help to B cells. An increased help of T cells to B cells has been documented in SLE [52] as well as T cell populations imbalance [8,55–57] and aberrant signaling pathways. Nonetheless, innate immunity also plays a role. The most widely accepted issue in the disease is indeed the imbalance between increased cell death and the impaired clearance of biological waste (debris)[134]. More recently, neutrophils have been described as heterogenous [61] and key players in contributing to the increased cell death [33,65], especially the low-density neutrophils[82].

In this study it is demonstrated that SLE patients exhibit LDNs and that they can be found within peripheral blood mononuclear cells layer after density centrifugation. In fact, LDNs were firstly identified in SLE through the same process of centrifugation[135] and have been researched since then and identified as increased in the disease[49,50,79,81,136]. Indeed, LDNs have been reported as having a range of 1.2-54% of the total PBMCs, however the higher percentages observed belonged to patients that had skin involvement and synovitis.[81] We obtained a mean of 1.7 ± 0.9 % however, this fraction is bond to vary according to disease presentation.

Since neutrophils are heterogeneous the phenotype between LDNs and HDNs was compared. There is still not one single marker to identify neutrophils, to do it a panel is often used. CD15 and CD66b were used to mark neutrophil lineage since they are expressed in almost every neutrophil stage of maturation, while the other markers CD10 and CD16b are expressed in later stages of neutrophil maturation.[126,137] CD62L is also expressed in almost every stage of neutrophil maturation however its expression is rapidly decreased upon activation.[126] Here it is

shown that SLE LDNs exhibit a similar phenotype of HDNs being this phenotype $CD15^+$ $CD66b^+$ $CD10^+$ $CD16b^+$ $CD62L^+$, with some variations in percentage of CD62L but that can be due to the fact that neutrophils are fragile and easily activated cells that leads to variations in CD62L expression. The difference in the percentages of CD62L also shows that the LDNs are a more circulatory population than the HDNs. Our data is in accordance with previous studies where similar panels were used and either LDNs and HDNs expressed a similar phenotype.[61,138] Also similar high percentages of CD10, CD16 and CD15 between the two types of neutrophils were reported.[81] Curiously, a study revealed that SLE LDNs could be divided in two populations a $CD66b^+CD10^+$, the mature LDNs and the $CD66b^+$ $CD10^-$, the immature LDNs[80], albeit in this cohort none of the patients presented such phenotype. Although, we show that LDNs and HDNs possess a similar phenotype and are matured cells exhibiting the classical neutrophil markers, there may be other LDNs in the PBMCs of SLE that may express other markers like CD11, CD14, CD31, CD33, and CD45 used in other studies for neutrophil identification.[81,136–138]

Neutrophils undergo NETosis and generate NETs, nucleic acid web-like structures that are expelled providing greater exposure of adaptive immune cells to intracellular antigens contributing to the increased cell death observed in SLE.[84] In particular, LDNs have shown higher capacity to undergo NETosis.[83] Levels of nucleic acids due to increased cell death in SLE, particularly DNA, are augmented in SLE patients.[67,68] We evaluated the concentration of DNA after LDNs and HDNs were stimulated to generated NETs with PMA. After a first centrifugation to remove cells and a micro-centrifugation to pellet DNA we were able to obtain NETs stocks. LDNs and HDNs NETs stocks both presented nucleic acids. LDNs NETs presented a lower content of DNA than HDNs NETs. A possible reason for this difference may be the lower number of LDNs compared to HDNs or the first centrifugation may lead to some part of the nucleic acid to pellet together with cells and cell debris. Moreover, HDNs NETs were shown to display a bigger area than LDNs NETs[139] which can be related with a greater expelling of intracellular content and therefore a higher DNA concentration. This does not invalidate the results because, LDNs NETs were described as more immunogenic than HDNs NETs, due to higher content of autoantigens, immunostimulatory molecules, and its nucleic acid content being more immunogenic due to its proximity of ROS that oxidize the DNA, that in normal conditions does not occur. [74,83–85] In fact, recent studies are focused on the importance of the content of LDNs NETs and not in the higher capacity of generating NETs. MMP9 is found decorating LDNs NETs and induces endothelial death and vascular dysfunction.[84]

Toll-like receptors are innate immune receptors that when activated lead to a proinflammatory response and type I IFN response. TLR7 and TLR9 that sense ssRNA and dsDNA, have

already been widely studied in pDCs that are responsible for maintaining the IFN I signature leading to a cycle of inflammation. TLRs expressed in innate immune cells are able to directly activate them. Only recently, TLRs started to be studied and they were reported as strong inducers of effector T cells.[114,119–121] Albeit, little is known if TLR7 and TLR9 can be expressed in $CD4^+$ T cells. In this study we show that circulating resting $CD4^+$ T cells express TLR7 and TLR9. Our results are in line with previous reports showing that T cells express other TLRs like TLR1, TLR2, and TLR4.[119,121,140,141]

If NETs could contribute to SLE pathogenesis through activation of TLR7/9 T cells was not known. Our results showed that TLR7 is increased upon NETs T cell stimulation whether these latter are from LDNs or HDNs. Furthermore, TLR expressing T cells presented high levels of CD38, meaning a high activation status after NETs stimulations. $TLR7^+$ $CD4^+$ T cells show a tendency to express more TLR7 and CD38 than the ones stimulated with HDN NETs. The downstream effects of TLR7 were also checked through assessment of IL-21 and TNFα production. IL-21 was increased after stimulation of $TLR7^+$ $CD4^+$T cells with NETs by both types of NETs, particularly LDNs NETs showed a tendency to induce tenuous higher IL-21 production. This was even further sustained when IL-21 production was majorly detected in $TLR7^+$ $CD4^+$ T cells rather than $TLR7^-$ $CD4^+$ T cells. IL-21 is important in driving activation and differentiation of B cells.[60] Specially a population of $CD11c^{hi}$ T-box transcription factor $TBX21^+$ (T-bet) B cells that is significantly expanded in SLE.[60] The production of IL-21 through TLR7 engagement in T cells could be another mechanism contributing to the pathogenesis of the disease and responsible for its chronicity, since it can stimulate the production of autoantibodies from autoreactive B cells. TNFα was also produced but its production was vastly due to PMA and ionomycin stimulation of $TLR7^+CD4^+$ T cells. PMA and ionomycin have been used as an activating stimulus to induce T cell activation and cytokine secretion.[142] TNFα and IFNγ producing T cells are the ones consistently detected in substantial amounts after stimulation with PMA and ionomycin.[142,143] However, NETs stimulated conditions show a tendency for higher TNFα production meaning that TLR7 engaged by NETs also enhances TNFα production. In addition, $TLR7^+$ $CD4^+$ T cells produced more TNFα than the non-TLR7-expressing cells. Corroborating the production of this cytokine due to TLR7 engagement, is the fact that agonists of TLR7 lead to a higher expression of TNFα in macrophages[144] and PBMCs[145]. In addition, TNFα has also been described and used as an inducer of NETs in inflammatory disease condition[146], in healthy donors[147] and even in SLE human samples[148]. TNFα secretion by NETs can also be a mechanism by which the chronicity of SLE is promoted, the release of this cytokine can induce more NETosis and generate even more NETs, contributing to the imbalance between cell death and its clearance. All these

data are in line with already published results of TLR7 that has been predominantly studied in APCs, where its engagement induces the production of proinflammatory cytokines and upregulation of activation molecules.[149–151] Moreover, the few published reports addressing TLRs in $CD4^+$ T cells all revealed a positive costimulatory role for TLR signaling.[152,153] Specifically, one of those proved that direct stimulation of TLR7/8 through resiquimod (R-848) ligand upregulated proliferation and IFNγ production in memory $CD4^+$ T cells.[152] Furthermore, a TLR7 gain of function genetic variation is sufficient to induce B-cell driven autoimmunity lupus phenotype in a mouse model, supporting a pathogenic role of TLR7 in the disease.[154] Curiously, a recent report of TLR7 stimulation through synthetic ligands in human immunodeficiency virus (HIV) - 1 infected $CD4^+$ T cells induced a proliferation state similar to clonal anergy, unveiling an unknown role for this receptor.[155] This can possibly be explained by HIV $CD4^+$ T cells being more naïve than the ones in SLE being this latter a chronic disease and HIV causes immunodeficiency. Supporting even further our results and the role of TLR7 in autoimmunity is the fact that mice born with defects on Unc-93 homolog B1 protein (UNC93B1), a trafficking chaperone required for TLRs to exit endoplasmic reticulum (ER), exhibited hallmarks of systemic inflammation, activated T cells and development of anti-nuclear antibodies early in life. Also, a mutation in the N terminus of UNC93B1 enhances TRL7 signaling by augmenting its exportation from ER in detriment of TLR9.[156] UNC93B1 has a weaker interaction with TLR7 than with TLR9 that requires previous interaction with this chaperone to be released within endosomes and after activated by their ligands.[157] Besides, the different trafficking between TLR7 and TLR9, the more simple nature and the broader spectrum of TLR7 ligands can explain its major part in autoimmunity.[144,158]

TLR9 role in SLE is still unclear and remains controversial. TLR9 has been mainly studied in B cells. In mice models B-cell TLR9 intrinsic deletion decreases the levels of autoantibodies against DNA, increases the levels of class-switched antibodies targeting RNA-associated antigens, and broadens the autoantibody repertoire.[159] In human B cells inhibition of TLR9 through ST2825 ligand inhibits the plasma cells differentiation and antibody secretion in SLE.[160] Contrastingly, another mice model with TLR9-deficiency led to increased TLR7-dependent activation of B cells, and DCs showed increased response to TLR7 ligand, suggesting a protective phenotype for TLR9.[161] Also, B cell-specific *Tlr9* deficiency was identified as being a disease accelerator, and *Tlr9* overexpression resulted in ameliorated renal disease in mice.[110] In T cells TLR9 role is even more mysterious. The fact of existing very few studies of TLR9 expression in T cells adds a layer of difficulty in understanding the role of this receptor in these cells. TLR9 mRNA was showed to be expressed in T lymphocytes of healthy individuals.[152] Low levels of

TLR9 mRNA in T cells in comparison with other immune cells also were reported.[110] Although TLR9 mRNA can be expressed does not mean that the receptor will necessarily be expressed in the cell endosomes. Another study which assessed the expression of TLR9 in SLE PBMCs detected significantly higher expression of TLR9 T cells when compared to controls.[162] Our results for TLR9 expression after NETs exposure did not demonstrate significant alterations, contrastingly to what succeeded in TLR7$^+$ CD4$^+$ T cells. Although TLR9 expression was maintained, T cells exhibited a high expression of CD38, implicating an activated state. Surprisingly, IL-21 was detected in all three doses of LDNs NETs-stimulated T cells as well as in 250 ng/mL dose of HDNs NETs-stimulated T cells. TNFα, similarly to TLR7$^+$ T cells results, was highly produced in TLR9$^+$ T cells, however the PMA and ionomycin stimulated T cells did not exhibited such high production as TLR7$^+$ T cells did. To further understand the lack of responsiveness of TLR9 to simultaneous expression of both TLRs was pursued. TLR7 expression in TLR9$^+$ T cells showed that more than 40% of the cells stimulated with NETs were doble positive for both receptors. This led to the conclusion that the activation state and the secreted cytokines detected on TLR9$^+$ T cells were majorly due to the engagement of TLR7 and not TLR9. In line of thought with our results is Meås et al. work, where HIV-1 infected CD4$^+$ T cells were stimulated with TLR9 ligands and prevented TCR-mediated upregulation of activation markers and inhibited cytokine secretion.[163] Furthermore, the above-mentioned paper where TLR7 induced T cell anergy, also promulgated a decreased T cell proliferation upon TLR9 stimulation and a trend of decreased cytokine production.[155] This might be associated with the emerging protective role that it is being attributed to TLR9. In fact, B cells of SLE patients showed a defective TLR9 response to agonists.[164] Moreover, they also were less activated and secreted less IL-6, IL-10, TNFα after TLR9 triggering when compared to healthy donors B cells.[164] Interestingly, another study revealed a downregulation of TLR9 in SLE B cells which caused CpG (TLR9 agonist) unresponsiveness.[165] This downregulation was even more noticeable in patients with a SLEDAI ≥ 6 (active disease).[165] Although they are different types of cells than the ones presented in this work, the unresponsiveness and downregulation of TLR9 described in B cells could also be what explains TLR9$^+$ T cell lack of responsiveness when stimulated with NETs.

Interaction between neutrophils and T cells has already been studied. It was found that LDNs represented a pathogenic subset and activated CD4$^+$ T cells in a non-suppressive way, accompanied by T_H1 proinflammatory cytokines while HDNs did not drive CD4$^+$ T cell activation nor cytokine secretion in lupus donors.[136] In chronic graft-versus-host disease akin SLE, LDNs were mainly immature evaluated by CD10$^-$ also increased T cell activity and proliferation.[117] At the same time LDNs can induce the opposite effect. In cancer, LDNs express

programed death ligand 1 (PD-L1), an inhibitory immune checkpoint and exert an immunosuppressive effect on T cells.[139] This contrasting effect can be explained by the different outcomes of the contact between neutrophils with early-stage $CD4^+$ T cells that were shown to be suppressed or late-stage $CD4^+$ T cells were that activated.[166] Due to its chronicity SLE is more likely to possess more late-stage T cells than early-stage T cells. Taking this into account NETs are quite likely to induce a response by T cells. As expected, the results from this investigation both LDNs and HDNs NETs induced $CD4^+$ T cell activation, with special emphasis on LDNs NETs that showed a trend of higher TLR7 expression, activation through CD38 expression and cytokine secretion than their counterparts. Endorsing this are published data where resting $CD4^+$ T cells were cocultured with NETs and these latter led to lowered activation threshold for T cells.[167] The threshold was lowered in naïve and memory T cells, and they could be activated with suboptimal stimuli of CD3.[167] In SLE T cells are much likely to be already primed due to its chronicity and engagement of NETs with TRL7 will be enough to fully activate them as reported in our experiments. Supporting even more our data is Blanco et al. research where LDNs NETs externalize a micro-RNA (miRNA/miR), miR-let7b, a natural ligand of TLR7, that promoted inflammatory responses on endothelial cells of lupus patients.[168]

The cohort employed 15 SLE patients predominantly composed of female individuals, which in fact characterizes the disease. TLR7 gene is located at the X-chromosome, and it is known to escape inactivation doubling the dose of TLR7 expression in female cells, predisposing women to autoimmunity and offering a molecular explanation for the sex-bias of the disease.[169,170] A recent publication, described life-threatening coronavirus disease 2019 (COVID19) in a cohort of men under 60 years old and with recessive TLR7 deficiency.[171] This sustains not only the sex-bias but also the role of TLR7 in producing type I IFN in response to the viral infection to overcome it.[171] Thus it would be interesting to study TLR7 deficient individuals, specially men, to further investigate the pathogenic TLR7 role on SLE. Unfortunately, due to the small representation of men no relations or correlations could be performed. It would also be interesting to observe if men have an ameliorated phenotype of SLE due to the smaller dosage of TLR7 compared to women. Among the other clinical data, the percentage of $TLR7^+$ T cells was smaller in patients with a SLEDAI ≥ 1, in other words, with a more active disease and not in a state of total remission of disease. Hydroxychloroquine is a first line treatment for lupus, and it is known for interfering with the ligation of antigens to TLRs and with endosomal processing, leading to an inhibition of TLR mediated immune response.[172] This relation was performed on $CD4^+$ T cells without any type of stimulation, therefore hydroxychloroquine might be what is causing a decrease in patients with a SLEDAI ≥ 1. The frequency of $TLR9^+$ T

cells positively correlated with C4. C4 is a key player in the classical and lectin pathways of the complement system, and its absence can trigger inapt clearance of apoptotic debris and stimulate chronic activation of myeloid cells.[173] Decreased C4 has been linked to predisposing to autoimmune diseases, namely SLE being one of the strongest associations.[173] Although, C4 has been shown to negatively correlated with TLR9 and its downstream players, MyD88 and NF-κb p65[174] , higher expression of $TLR9^+$ T cells correlating with a higher expression of TLR9 goes in line of thought of its protective role.

5

Conclusions and Future Perspectives

In this work, LDNs were identified in SLE patients and exhibited a similar phenotype as the HDNs. These latter migrate more to the tissue while LDNs are more circulatory. Furthermore, both types of neutrophils can generate NETs. Regarding, $CD4^+$ T cells, TLR7 and TLR9 expression was confirmed, and considerable percentage of them even expresses both TLRs. These cells when stimulated by NETs led to engagement of TLR7, augmenting its expression and activating T cells, which does not occur with TLR9. Moreover, $TLR7^+$ T cells when engaged are able to induce an immune response through secretion of cytokines as TNFα and IL-21, that may take chronicity of the disease even further. Additionally, there were no statistical differences in both types HDNs and LDNs NETs in the activation of $CD4^+$ T cells, however, higher concentrations of NETs, specially HDNs NETs appear in induce saturation of the system. All things considered, a crosstalk between neutrophils and T cells through NETs was unveiled, sustaining the not so rigid division of immunity in the two composing arms, the innate and adaptive (Fig. 5.1).

This study has some limitations, regarding purity of the cell populations used. Sorted T cells and LDNs would guarantee a less possible contamination of monocytes, macrophages, NK cells, and eosinophils. On the other hand, in view of the high fragility of neutrophils flow cytometric cell sorting could compromise their cellular integrity. Additionally, NETs generation in a coverslip, followed by digestion of nucleic acids with DNAse and coculture of $CD4^+$ T cells might be more representative of the physiological conditions than using a NETs stock solution. Research of the content of NETs from LDNs and HDNs would also provide a better understanding of their pathogenic role in SLE. To confirm and to give robustness to the results obtained in this study stimulation of $CD4^+$ T cells with agonists and antagonists for each TLR, as well as assessment of the downstream signaling players like MyD88 or the transcription factors would

be an interesting path to follow. To further undercover the cytokine profile induced by TLRs engagement a broader spectrum of cytokines should be considered. Not also more cytokines should be evaluated, but their role in the pathogenesis of SLE, in particular, in understanding IL-21 and TNFα in the chronicity of the disease. Another limitation, and maybe the biggest one, is the size of the cohort, more patients would be more representative of the SLE population, and further relations and correlations with clinical and demographic data would be possible and more conclusive.

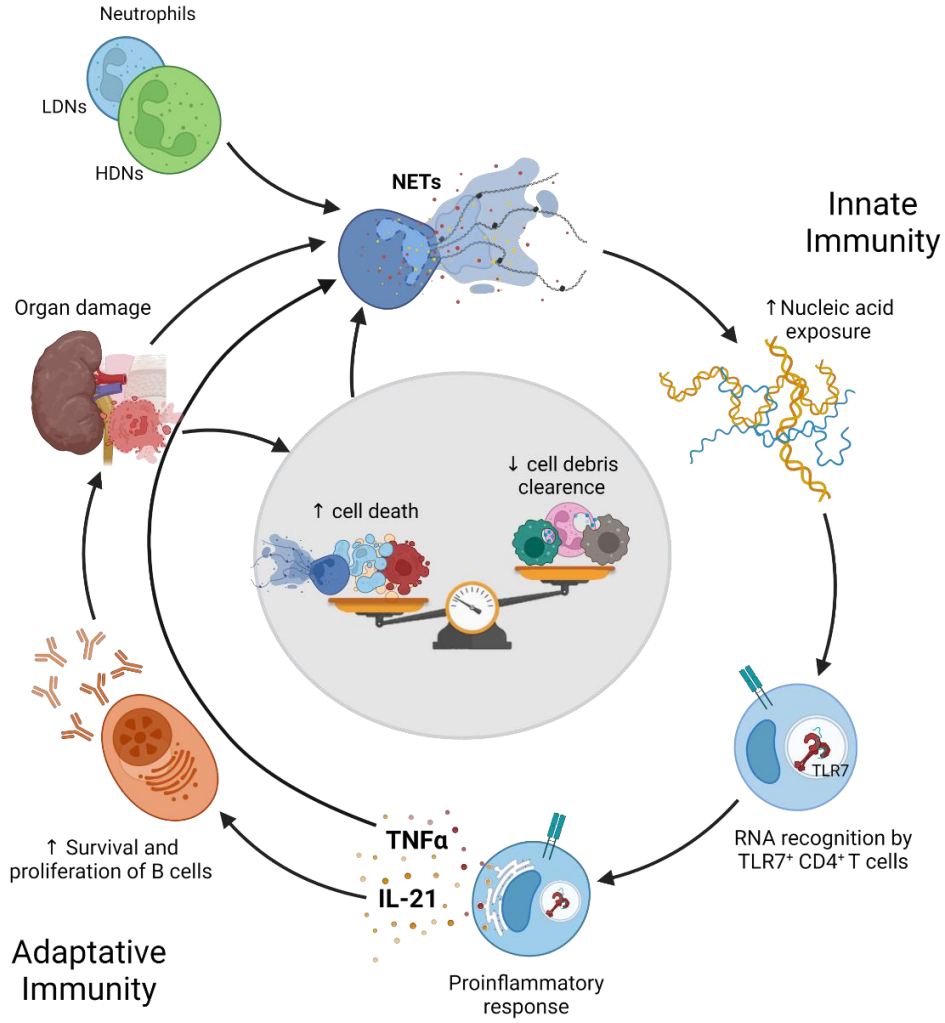

Figure 5.1 - Graphical abstract. Schematic resume of the crosstalk between T cells and neutrophils through NETs, indicating the interaction between innate and adaptive immunity. NETosis is increased in SLE, leading to increased NETs, providing a higher nucleic acid exposure to adaptative immune cells. T cells expressing TLR7 can recognize self RNA and induce a proinflammatory response through the secretion of IL-21 and TNFα, giving conditions for autoreactive B cells to enhance their survival, proliferation and to differentiate into plasma cells leading to the secretion of autoantibodies that cause even more cell death and organ damage.

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A

APPENDIX

A.1 Abstract accepted for poster presentation at the XLVII Annual Meeting of the Portuguese Society of Immunology

T cell-neutrophil crosstalk in Systemic Lupus Erythematosus

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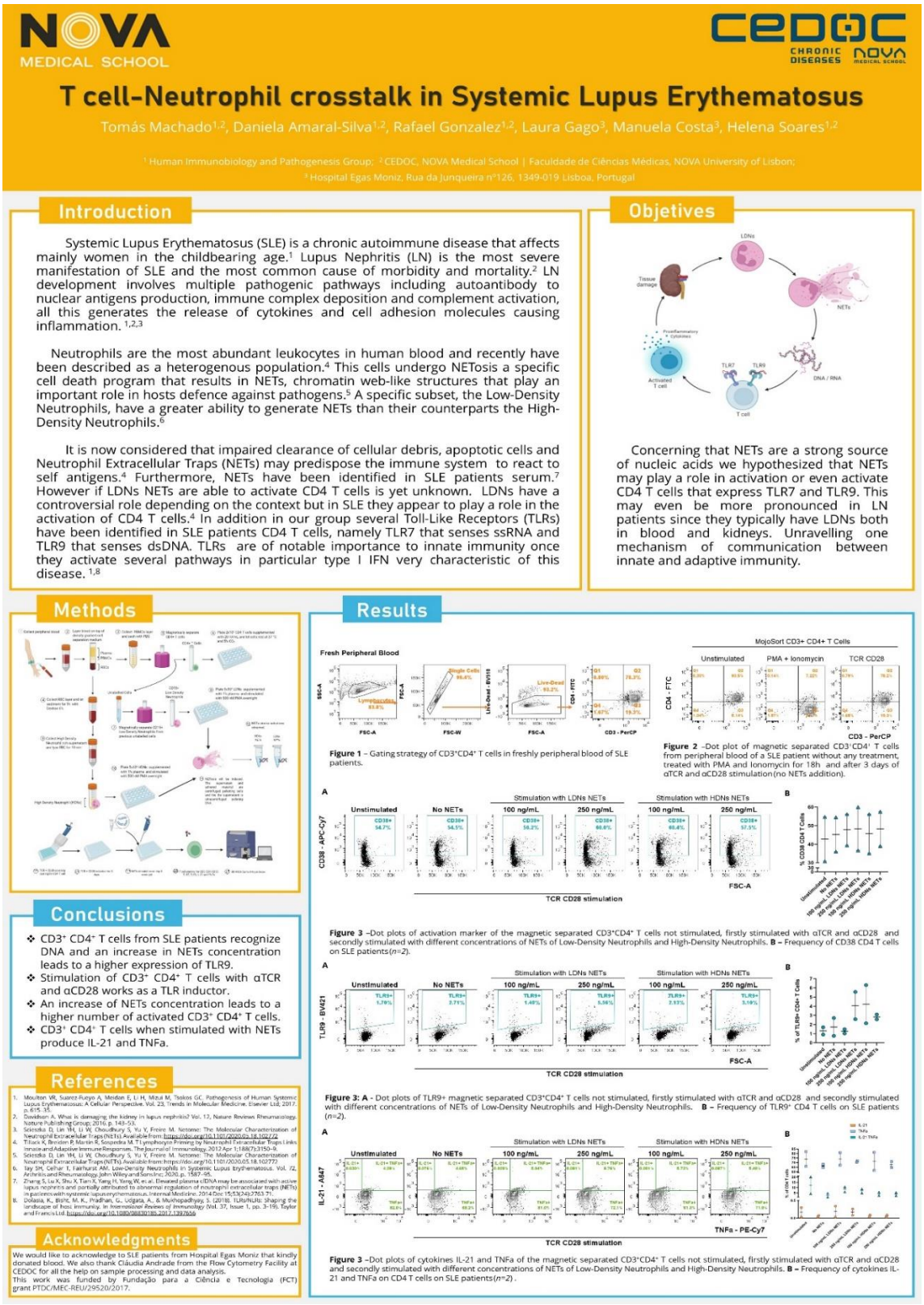
Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that affects mainly women in the childbearing age. Both innate and adaptive immune system have been described in disease pathogenesis, and it is known that impaired clearance of cellular debris, apoptotic cells, and Neutrophil Extracellular Traps (NETs) may predispose the immune system to react to self-antigens. This can lead to the deposition of immune complexes in the kidneys, resulting in the development of Lupus Nephritis (LN) the most severe manifestation of SLE. Immune complexes promote the release of proinflammatory cytokines and the recruitment of granulocytes like neutrophils to sites of inflammation. Neutrophils undergo NETosis, a specific

cell death program that results in NETs, chromatin web-like structures that are important in hosts defence against pathogens.

Neutrophils are the most abundant leukocytes in human blood and different phenotypes have been reported in inflammation, autoimmunity, and cancer. Low-Density Neutrophils (LDNs), a subset of neutrophils is typically found in kidneys and blood of LN patients and have a greater ability to generate NETs than their counterparts the High-Density Neutrophils. In LN, NETs from LDNs appear to have a role in promoting the activation of T cell through a mechanism yet unknown, being one of the bridges of interaction between innate and adaptive immunity. Preliminary data from our group showed that CD4 T cells from SLE patients express TLR7 and TLR9 which are able to sense ssRNA and dsDNA, respectively. Currently we are investigating the crosstalk between neutrophils and T cells. Our hypothesis is that T cells are able to sense nucleic acid released through NETs leading to their activation and possibly unravelling one of the pathogenic mechanisms of SLE.

A.2 Poster presented at the XVLII Annual Meeting of the Portuguese Society of Immunology



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T cell-Neutrophil crosstalk in Systemic Lupus Erythematosus

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Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that affects mainly women in the childbearing age.[1] Lupus Nephritis (LN) is the most severe manifestation of SLE and the most common cause of morbidity and mortality.[2] LN development involves multiple pathogenic pathways including autoantibody to nuclear antigens production, immune complex deposition and complement activation, all this generates the release of cytokines and cell adhesion molecules causing inflammation. [1,2,3]

Neutrophils are the most abundant leukocytes in human blood and recently have been described as a heterogenous population.[4] This cells undergo NETosis a specific cell death program that results in NETs, chromatin web-like structures that play an important role in hosts defence against pathogens.[5] A specific subset, the Low-Density Neutrophils, have a greater ability to generate NETs than their counterparts the High-Density Neutrophils.[6]

It is now considered that impaired clearance of cellular debris, apoptotic cells and Neutrophil Extracellular Traps (NETs) may predispose the immune system to react to self antigens.[4] Furthermore, NETs have been identified in SLE patients serum.[7] However if LDNs NETs are able to activate CD4 T cells is yet unknown. LDNs have a controversial role depending on the context but in SLE they appear to play a role in the activation of CD4 T cells.[4] In addition in our group several Toll-Like Receptors (TLRs) have been identified in SLE patients CD4 T cells, namely TLR7 that senses ssRNA and TLR9 that senses dsDNA. TLRs are of notable importance to innate immunity once they activate several pathways in particular type I IFN very characteristic of this disease. [1,8]

Objetives

Concerning that NETs are a strong source of nucleic acids we hypothesized that NETs may play a role in activation or even activate CD4 T cells that express TLR7 and TLR9. This may even be more pronounced in LN patients since they typically have LDNs both in blood and kidneys. Unravelling one mechanism of communication between innate and adaptive immunity.

Methods

Results

Figure 1 – Gating strategy of CD3⁺CD4⁺ T cells in freshly peripheral blood of SLE patients.

Figure 2 –Dot plot of magnetic separated CD3⁺CD4⁺ T cells from peripheral blood of a SLE patient without any treatment, treated with PMA and Ionomycin for 18h and after 3 days of αTCR and αCD28 stimulation (no NETs addition).

Figure 3 –Dot plots of activation marker of the magnetic separated CD3⁺CD4⁺ T cells not stimulated, firstly stimulated with αTCR and αCD28 and secondly stimulated with different concentrations of NETs of Low-Density Neutrophils and High-Density Neutrophils. **B –** Frequency of CD38 CD4 T cells on SLE patients (n=2).

Figure 3: A - Dot plots of TLR9+ magnetic separated CD3⁺CD4⁺ T cells not stimulated, firstly stimulated with αTCR and αCD28 and secondly stimulated with different concentrations of NETs of Low-Density Neutrophils and High-Density Neutrophils. **B –** Frequency of TLR9⁺ CD4 T cells on SLE patients (n=2).

Figure 3 –Dot plots of cytokines IL-21 and TNFa of the magnetic separated CD3⁺CD4⁺ T cells not stimulated, firstly stimulated with αTCR and αCD28 and secondly stimulated with different concentrations of NETs of Low-Density Neutrophils and High-Density Neutrophils. **B –** Frequency of cytokines IL-21 and TNFa on CD4 T cells on SLE patients (n=2) .

Conclusions

- CD3⁺ CD4⁺ T cells from SLE patients recognize DNA and an increase in NETs concentration leads to a higher expression of TLR9.
- Stimulation of CD3⁺ CD4⁺ T cells with αTCR and αCD28 works as a TLR inductor.
- An increase of NETs concentration leads to a higher number of activated CD3⁺ CD4⁺ T cells.
- CD3⁺ CD4⁺ T cells when stimulated with NETs produce IL-21 and TNFa.

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