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STUDIES ON BIOLOGICAL TREATMENT AND BIOMARKERS IN SYSTEMIC LUPUS ERYTHEMATOSUS-LONGTERM EFFECTS, RISK FACTORS AND PREDICTORS OF RESPONSE

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Studies on biological treatment and biomarkers in Systemic Lupus Erythematosus-longterm effects, risk factors and predictors of response
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Keep Ithaka always in your mind.
Arriving there is what you’re destined for.

(C. Kavafis)

«"O frati," dissi, "che per cento milia
perigli siete giunti a l'occidente,
a questa tanto picciola vigilia

d'i nostri sensi ch'è del rimanente
non vogliate negar l'esperïenza,
di retro al sol, del mondo sanza
gente.

Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza".»

Dante Alighieri (1265-1321)
Inferno XXVI, 112-120

To my Family

ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune disease in which several immune mechanisms are involved in a complex interplay. B-cells are considered to be a major player in the pathophysiology of the disease, and the advent of B-cell depletion therapy (BCDT), has brought much promise. Yet, after twenty years of use of the depleting agent Rituximab (RTX), BCDT still has to find its precise location and role in SLE therapy. Among SLE clinical manifestations, lupus nephritis (LN) carries a significant burden in terms of morbidity, and requires optimised approaches. Identifying clinical and biological biomarkers, may contribute to improving the clinical management of the disease and its major organ involvement.

The aims of this thesis were the following: 1) to contribute to a definition of RTX's role in SLE treatment, by studying its deep immunological effects, immunogenicity and side effects, aiming at identifying possible biomarkers of efficacy and safety which might be implemented in daily practice; 2) to identify possible non-invasive biomarkers of specific organ involvement, such as LN, which may also in the near future be implemented in daily practice for identifying LN, and to monitor disease activity and response to treatment.

In **Paper I**, deep immunological effects of RTX on recently defined B- and T- cell subsets were explored, through a multicolour flow cytometry approach. In particular, we investigated whether RTX affects the age-associated B-cells (ABCs) which are plasma cells precursors; and the T-follicular and T-peripheral helper T-cells (T_{FH}, T_{PH}). We showed that transient reduction of the frequencies of the B-cell phenotype age-associated B-cells (ABCs) is induced by the treatment during the early phases of B-cell depletion. This corresponded to a reduction within the DN2 compartment, which contains the ABCs cells. By contrast, no early significant changes of the T_{FH} and T_{PH} followed the administration of RTX. A reduction of a subset of CD4+ with high expression of the marker PD-1 was shown at later follow-up. Examining the behaviour of these cell subsets in patients who developed antibodies against the drug, we showed that immunogenicity was associated to lower frequencies of double negative (DN) B-cells and to a wider expansion of plasma blasts at early follow up.

In **Paper II**, we investigated the occurrence of anti-drug antibodies (ADAs) towards RTX, in a cohort of 66 SLE patients after their first course of treatment, in comparison with 22 first-ever treated ANCA-associated vasculitis (AAV) patients. After the first course of RTX almost 38% of SLE patients developed ADA, while in AAV no ADA were shown. The SLE patients developing ADA were younger, had a longer disease duration, and a more active disease at the time of treatment initiation. They were mostly treated for LN and had a serologically active profile. Despite an overall reduction of disease activity upon RTX treatment, the presence of ADA in SLE was associated with higher counts of B-cells in the peripheral blood at the 6 month follow-up, and with higher residual disease activity. At retreatment, ADA-positive SLE patients experienced more frequent infusion reactions, both of immediate and late-onset occurrence.

In **Paper III**, we investigated the occurrence of late onset neutropenia (LON) in 107 patients with SLE treated with RTX. We found a rate of occurrence as high as 30%. In most cases the laboratory finding was discovered in routine controls and did not cause complications. However, around 40% of the patients experienced clinical consequences with occurrence of neutropenic fever and infections, some requiring intensive care. The clinical characteristic associated with the risk of LON was high disease activity before treatment.

At a biological level, the appearance of LON was associated with the increase of BAFF which follows the disappearance of B-cells in the peripheral blood.

In **Paper IV**, we explored the use of the soluble protein Galectin-3 Binding Protein (Gal-3BP), as urinary biomarker in LN, comparing the urinary concentration of this protein in 86 patients with active LN, 63 patients with active SLE without LN, 73 inactive SLE patients, and 48 matched population based controls. Samples were tested separately for the concentrations of other biomarkers: Galectin-3 (Gal-3), neutrophil gelatinase associated lipocalin (NGAL), osteopontin (OPN) and kidney injury molecule-1 (KIM-1). Each of the biomarkers was assessed as absolute concentration and as concentration normalized for the urine-creatinine (adjusted concentration). We found that the levels of both non-adjusted and adjusted u-Gal-3BP were significantly higher in the urine of patients with active LN as compared to all the other groups. Higher levels of adjusted u-Gal-3BP were found in proliferative and membranous forms of LN as compared to mesangial forms. We also found a moderate correlation between the adjusted u-Gal-3BP and the histological activity index as evaluated in kidney biopsies. This correlation was stronger when we considered patients not receiving immunosuppressive treatments at the time of kidney biopsy. Current treatment with oral corticosteroids was associated with lower urinary levels of Gal-3BP in active LN patients. In patients with proliferative LN this association was found also regarding ongoing treatment with antimalarials. In a subset of ten patients with active LN, significant reduction of Gal-3BP levels was observed at repeated analysis after treatment.

In **Paper V**, we explored the presence in the urine of LN patients (n=13) of extracellular vesicles (EVs) carrying an array of molecules of interest, all with a putative role in inflammation. We found that all the tested EVs were detectable in the urine, although at lower concentrations as compared to blood. We preliminary adopted a cut-off of EVs concentration of 50x10^6/L and evaluated the concentration of EVs carrying the cargo molecules of interest above the above mentioned cut-off. We considered the expression of EVs with respect to the histological activity. No correlation was found, but the concentration of EVs carrying the split complement molecule C5a, was found to be significantly higher in patients classified as active at renal biopsy as compared to those classified as inactive. We then examined the expression of urinary EVs with respect to having a predominant proliferative histologic pattern with respect to non-proliferative pattern. In proliferative LN, urinary EVs expressing C3a, C4, C4d, C5a, Mitochondrial antigens, Lactadherin, NGAL and TWEAK were found in significantly higher concentrations as compared to patients with non-proliferative forms.

In conclusion, the thesis here presented adds some elements of knowledge on the immunological effects and consequences of the use of RTX in SLE, and on aspects of safety associated with immunogenicity. It also adds knowledge on the magnitude of LON as a safety aspect of RTX use as a therapy for SLE. Finally, it contributes to the definition of potential urinary biomarkers which might be implemented in clinical practice in the future.

LIST OF SCIENTIFIC PAPERS

I. **Rituximab in systemic lupus erythematosus: transient effects on autoimmunity associated lymphocyte phenotypes and implications for immunogenicity**

Francesca Faustini*, Natalie Sippl*, Ragnhild Stålesen, Karine Chemin, Nicky Dunn, Anna Fogdell-Hahn, Iva Gunnarsson and Vivianne Malmström
Submitted manuscript
**equal contribution*

II. **First exposure to rituximab is associated to high rate of anti-drug antibodies in systemic lupus erythematosus but not in ANCA-associated vasculitis**

Francesca Faustini, Nicky Dunn, Nastya Kharlamova, Malin Ryner, Annette Bruchfeld, Vivianne Malmström, Anna Fogdell-Hahn and Iva Gunnarsson
Arthritis Res Ther (2021),23:211

III. **Rituximab-mediated late-onset neutropenia in systemic lupus erythematosus – distinct roles of BAFF and APRIL**

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Lupus (2018), 27:1470-78

IV. **Urine Galectin-3 Binding Protein is a promising marker of nephritis activity in systemic lupus erythematosus**

Francesca Faustini, Helena Idborg, Anders Larsson, Wen-Rong Lie, Sven Pötzsch, Shinji L Okitsu, Elisabet Svenungsson, Iva Gunnarsson
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Manuscript

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LIST OF ABBREVIATIONS

PRRs	Pattern recognition receptors
PAMPs	Pathogen associated molecular patterns
SLE	Systemic lupus erythematosus
TCR	T-cell receptor
BCR	B-cell receptor
MHC	Major histocompatibility complex
IgM	Immunoglobulin M
PD-1	Programmed cell death-1
T-reg	T-regulatory cells
LN	Lupus nephritis
ESRD	End-stage renal disease
ANA	Anti-nuclear antibodies
Anti-dsDNA	Anti-double strand DNA antibodies
ENA	Extractable nuclear antigens
C3/C4/C1q/C2/	Complement fraction 3,4,1q,2
ACR	American College of Rheumatology
ARA	American Rheumatism Association
anti-Sm	Anti-Smith antigen antibodies
SLICC	Systemic Lupus Erythematosus International Collaborating Clinics
EULAR	European League Against Rheumatism
SLEDAI-2K	SLE disease activity index 2000
TGF-β	Transforming growth factore-beta
IL-	Interleukin-
NETs	Neutrophil extracellular traps
DNA	Deoxyribonucleic acid
LDG	Low density granulocytes
pDC	Plasmacytoid dendritic cell
CD	Cluster of differentiation
TLRs	Toll-like receptors
IFNs	Interferons
APCs	Antigen presenting cells
Bm	B-mature
DN	Double negative B-cells

SSA/SSB	Sjögren's syndrome related antigen A/B autoantibody
AMBc(s)	Atypical memory B-cells
HIV	Human immunodeficiency virus
ABCs	Age-associated B-cells
Syk	Spleen tyrosine-kinase
RA	Rheumatoid arthritis
mTORC1	Mammalian mechanistic target of rapamycin complex 1
BAFF	B-cell activating factor
APRIL	A proliferation inducing ligand
BAFFR	B-cell activating factor receptor
TACI	Transmembrane activator and calcium-modulator and cytophilin ligand interactor
BCMA	B-cell maturation antigen
T_H1	T-helper 1
T_H2	T-helper 2
T_H17	T-helper 17
RORγt	Retinoid-acid-receptor related orphan receptor gamma
TNF	Tumour necrosis factor
T_{FH}	T-follicular helper
T_{PH}	T-peripheral helper
IC	Immune complex(es)
GC-	Germinal centre-
CNS	Central nervous system
AMA	Anti-malarial agents
HCQ	Hydroxichloroquine
IS	Immunosuppressive (drugs)
AZA	Azathioprine
MMF	Mycophenolate mofetil
MTX	Methotrexate
CNI	Calcineurin inhibitors
NIH	National Institutes of Health
BSA	Body surface area
RTX	Rituximab
ANCA	Anti-neutrophil cytoplasmic antibodies

MS	Multiple sclerosis
RCT	Randomized clinical trial
Fab	Antigen-binding fragment
Fc	Fragment crystallizable
Fc-γR	Fc-gamma receptor
CDC	Complement-dependent cytotoxicity
ADCC	Antibody-dependent cytotoxicity
FcRn	Fc-receptor neonatal
BCD	B-cell depletion
DKO	Double knock-out
DEF-6	Differentially expressed in FDCP 6 homolog
SWAP-70	Switch-associated protein 70
IRF-5	Interferon regulatory factor 5
CXCR5	Chemokine CXC morif receptor 5
CXCL13	Chemokine CXC motif ligand 13
ICOS	Inducible T-cell co-stimulator
MAF	Transcription factor maf
ADA	Anti-drug antibodies
HACA	Human anti-chimeric antibodies
FDA	Food and Drugs Administration
SDF-1	Stromal cell-derived factor-1
RNA	Ribonucleic acid
MCP-1	monocyte chemoattractant protein-1
NGAL	Neutrophil gelatinase-associated lipocalin
OPN	Osteopontin
KIM-1	Kidney injury molecule-1
Gal-3	Galectin-3
Gal-3BP	Galectin-3 binding protein
CRD	carbohydrate recognition domains
Mac-2	Macrophage-2 antigen
EVs	Extracellular vesicles
PBMCs	Peripheral blood mononuclear cells
MPO	Myeloperoxidase
C3a/C5a	Complement split factors 3a/5a

HMBG-1	High-mobility group protein-1
Axl	AXL receptor tyrosine kinase
TWEAK	TNF-related weak inducer of apoptosis
EDTA	Ethylene-diamino-tetra-acetic acid
ECL	Electro-chemiluminescence (assay)
RECL	Relative-electro-chemiluminescence
ELISA	Enzyme linked immune-sorbent assay
IQR	Interquartile range
SD	Standard deviation
ROC	Receiver operating curve
COVID-19	Coronavirus disease-19
ICU	Intensive care unit
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor

INTRODUCTION

1 THE IMMUNE SYSTEM

The immune system undoubtedly is the component of human physiology that has received the most unprecedented attention in the scientific field, and in the media during the last two years. This attention, which almost marks today's *Zeitgeist*, has been driven by the global experiencing of the Coronavirus pandemic. However, the study of the immune system, or immunology, as a scientific discipline, has developed over more than a century since the last decade of the 19th century (1). Its evolution has witnessed enormous progresses since the pioneering discoveries of Elias Metchnikoff, Emil Behring and Paul Ehrlich, the latter being the first to introduce the concept of autoimmunity through the syntagma 'horror autotoxicus' (2).

The immune system can be defined as the set of mechanisms that our body utilizes to defend us from external attacks by pathogens, and more in general from potential injuries. In fact, the immune system can activate itself when internal danger arises, as for example when the heart muscle develops ischemia and infarction, and thus death cell debris must be eliminated: in this condition, the immune system sets up inflammatory-like mechanisms in the area to clean it of damaged tissue debris, and it promotes repair and scarring.

Our immune system has developed through evolution in two main branches, the innate immune system, and the adaptive immune system. The former is composed of cells and molecular mechanisms that can eliminate pathogens but cannot recall them, while the latter has the capacity of holding memory, which is the most relevant ability of our immune system. This dichotomous division mainly serves as scheme to simplify an enormous biological complexity, in which the innate and adaptive immune system tightly interact in a 'dance in the storm', as eloquently described in a recent book by the Italian immunologist Antonella Viola (*Danzare nella Tempesta*, Feltrinelli Editore, Milan, 2021).

The immune system, in order to deploy its defensive actions, must bear two main characteristics. It has to be sensitive, activating itself in the presence of even very tiny stimuli, and specific, which means that it has to have the ability to distinguish among signals of danger and become activated only in in the face of real dangers.

The signals that the immune system recognizes, and that drive its activation are of two main sorts. The antigens, generally represented by small segments of proteins (peptides), are recognized by the T-cells and B-cells through their specific receptors. The innate immune system can sense the presence of pathogens using highly conserved proteins that are globally defined as pattern recognition receptors (PRRs), and are able to sense repetitive motifs of molecules expressed by microbes, such as glycoproteins, lipopolysaccharide and nucleic acids. These molecular motifs are known as pathogen associated molecular patterns (PAMPs) (3).

The ability to distinguish between self and non-self is the heart of our immune system's functioning, together with its other main feature, immunological memory. In fact, an encounter with an antigen, will in most cases be followed by the activity of the immune system's remembering the antigen, which will make possible a response to it at a subsequent encounter.

As will become clearer below, the immune system can, in certain conditions, lose the ability of distinguishing between self and non-self and possibly give rise to a wide range of diseases, globally known as autoimmune diseases. These can be divided into two main categories: organ-specific diseases-in which the attack on the self causes inflammation, damage and functional impairment in a single organ-and systemic autoimmune diseases, in which pathological processes can involve several, if not virtually, all organs and tissues in the body.
A paradigmatic example of the latter type of autoimmune disease is systemic lupus erythematosus (SLE), which is the main focus of the present work.

2 AUTOIMMUNITY: FROM BREAK OF TOLERANCE TOWARDS THE SELF TO DISEASE

The immune system is designed to recognize antigens, generally represented by segments of proteins (peptides), and repetitive motifs of non-protein structures on microbes (PAMPs). As previously mentioned, in order to protect us, it has to be specific, being able to distinguish foreign antigens and ignore self-antigens. The ability to avoid reacting to an antigen, maintaining a status of unresponsiveness towards it, is generically defined as immunological tolerance. When addressed towards self-antigens, it is defined as tolerance towards the self. Tolerance is relevant to avoid attacking and damaging of the body, but also to mitigate damage to our own body when responding to foreign antigens (4). Since the number of possible antigens we can encounter is potentially infinite, the immune system has evolved in a manner that ensures its ability to generate a wide repertoire of cells and antibodies that can mount the immunological response towards potentially any unknown antigen. This ability resides in the process of gene rearrangement which allows the generation of the specific antigen recognition receptors of T- and B-lymphocytes (T-cell receptor, or TCR; and B-cell receptor, or BCR). Likewise, gene rearrangement is the key to the generation of the broad repertoire of circulating antibodies the immune system can provide towards pathogens. As a downside, such a complex genetic mechanism of diversity generation, can be affected by errors, which mistake self-antigens for external antigens and threats. Through evolution, the immune system has also developed mechanisms to counteract these errors, and suppress the emergence of autoreactive immune cells (4).

These mechanisms act at different stages in the natural evolution of immune cells, namely the T- and B-lymphocytes. During early phases of lymphocyte ontogeny, what is called ‘central tolerance’ acts. This occurs for B-lymphocytes in the bone marrow and for T-lymphocytes in the thymus, and basically consists of testing the ability of the antigen receptors to recognise self-antigens. For T-lymphocytes, thymic T-cell precursors are challenged in two ways. The TCR can recognize antigens only when presented as peptides in an immunological synapsis formed with the molecules of the major histocompatibility complex (MHC). These molecules are expressed on all nucleated cells and are of two types: type I, ubiquitous; and type II, expressed only by immune cells with the function of antigen-presenting cells. The first event of selection is called ‘positive selection’, which ensures the survival of T-cell precursors with high affinity for the MHC molecules. Once this step is performed, the pool of T-lymphocyte precursors will contain a part of autoreactive cells, which later will be tested again and eliminated by clonal deletion (negative selection). For B lymphocytes, in the bone marrow, autoreactive cells will undergo three types of event to be handled: clonal deletion, silencing through the induction of an unresponsive state (anergy), or receptor editing of their BCR (which

is membrane-bound IgM). This last type consists of changes in the sequence of its light chain that lead to changes of its specificity (5).

Despite the evolution of such control mechanisms, a relevant amount of autoreactive T- and B-cells egress the primary immune organs where they are generated, and reach the peripheral blood and secondary lymphoid organs. Here they can, upon favourable circumstances, initiate subsequent steps of autoimmunity. To prevent this, mechanisms of peripheral tolerance have been developed by our immune system. Among these mechanisms is the expression of inhibitory molecules, a relevant example of which is the molecule PD-1 (programmed cell death-1). While this molecule can also prevent the activation of self-reactive immune cells, its blockade results in promoting elimination of altered cells of the self, like tumour cells, a molecular event that has led to the development of what are called 'checkpoint inhibitors'. The most problematic side effects of these drugs, reside just in the onset of autoimmune phenomena (6). In addition, the induction of anergy (which can be elicited for example by engagement of the TCR in the absence of co-stimulation) is also an active mechanism in peripheral tolerance, and also involves B-lymphocytes. Moreover, a mechanism defined as clonal ignorance can allow autoreactive lymphocytes to avoid encountering antigens normally sequestrated in immunological niches. Finally, specialized lymphocytes are generated both centrally and in the periphery, which have the role of balancing and counteracting the activation of effector lymphocytes. These are within the T-cell compartment the T-regulatory cells or T-reg (7).

The surge of autoimmunity occurs when even mechanisms of peripheral tolerance are inefficient. First, autoantibodies can appear, which can be detected also in apparently healthy individuals, without a correlate of clinically overt pathology. Secondly, autoimmune disease can develop. Autoimmune diseases encompass a range of inflammatory disorders, with an estimated prevalence as high as 7-9% within the general population. Their aetiology is based on multiple genetic and environmental factors (6).

3 SYSTEMIC LUPUS ERYTHEMATOSUS

The word 'lupus' is the Latin word for 'wolf', the wild animal. The use of this word with reference to a medical condition can be traced to the Middle Ages, when it generally referred to ulcerous lesions, mostly of the lower limbs. Only in the later Middle Ages was the word used to refer to facial lesions. This use of the word probably encompassed not only the skin manifestations that we attribute to SLE today, but also other disease entities such as cutaneous tuberculosis. The nosological name of 'systemic lupus erythematosus' was conferred to the disease only in the Modern Era when, in the second half of the 19th century, the systematic descriptions of cutaneous lesions and their association with extra-cutaneous manifestations were progressively accumulated, and the systemic nature of the disease became known (8). Only in the 20th century was the association of SLE with autoimmunity established (9, 10).

Today SLE is considered to be the prototypical systemic autoimmune disease, characterized by the presence of several autoantibodies and the possible involvement of the majority of organs and tissues in the body. As a multiorgan disease, SLE bears a high degree of complexity, on both pathophysiological and clinical levels.

3.1 Epidemiology

Data on SLE incidence and prevalence vary widely in the reports available in the scientific literature. The most informative data on SLE epidemiological features come from studies conducted in the United States and Europe, while data from other continents are less solid, if not lacking, as in the case of African countries. In Europe, the estimates set the incidence of the disease at 1-4.9 cases/100,000 inhabitants per year, and the prevalence at 28-97/100,000 inhabitants, depending on the case definition used. In the US, the incidence ranges from 2 to 7.6 cases/100,000 per year and the prevalence is estimated 19-159/100,000). Epidemiological studies highlight other characteristics of the disease. First, data from the US and UK show how in certain ethnic groups incidence and prevalence are higher. This is the case of Afro-American and Afro-Caribbean individuals, who not only are more often affected by the disease compared to Caucasians, but are even more prone to develop certain disease manifestations such as renal involvement, and with a higher disease burden (11). Recent registry studies from USA show that individuals of Hispanic and Asian descent have a higher incidence and prevalence of SLE than Caucasians, but lower when compared to Afro-Americans (12). Moreover, data from the US also highlight the impact of socio-economic status on epidemiological features of SLE, especially on morbidity and mortality. Irrespective of which population is examined, SLE is a disease that predominantly affects women, especially of childbearing age. The female to male ratio in adult patients is as high as 9:1, with a lower ratio in childhood (3:1) (11).

Also data from the Nordic countries, which have predominantly Caucasian populations, show lower incidence rates for males (2.35/100,000/year) with respect to females (3.96/100,000/year) (13). In a recent analysis from southern Sweden, which took into account two sequential periods, global incidence of SLE decreased in the second examined period with a peak of incidence registered for females of 25-34 years of age, age-related incidence of 2.8 cases/100,000 inhabitants per year, and a punctual prevalence of 65/100,000. The authors noted no changes in the phenotypical presentation of the disease (14). Other trends, pointing towards a rise in the prevalence of SLE over time, have been highlighted in other studies; this increase might be explained by improved diagnostic capability, which enables identification of even milder forms of the disease (15).

3.2 Clinical manifestations

SLE, as a systemic disease, can cause a wide range of clinical manifestations, which involve several organs and tissues, either simultaneously or in different periods of the disease's course. Moreover, the severity of each manifestation can vary widely, ranging from mild to life threatening forms. Aside from specific organ manifestations, such as renal involvement, unspecific symptoms, such as fatigue or cognitive impairment, are a major problem in SLE patients' daily life (15, 16). Figure 1 illustrates the most common clinical manifestations of the disease.

3.2.1 Lupus nephritis

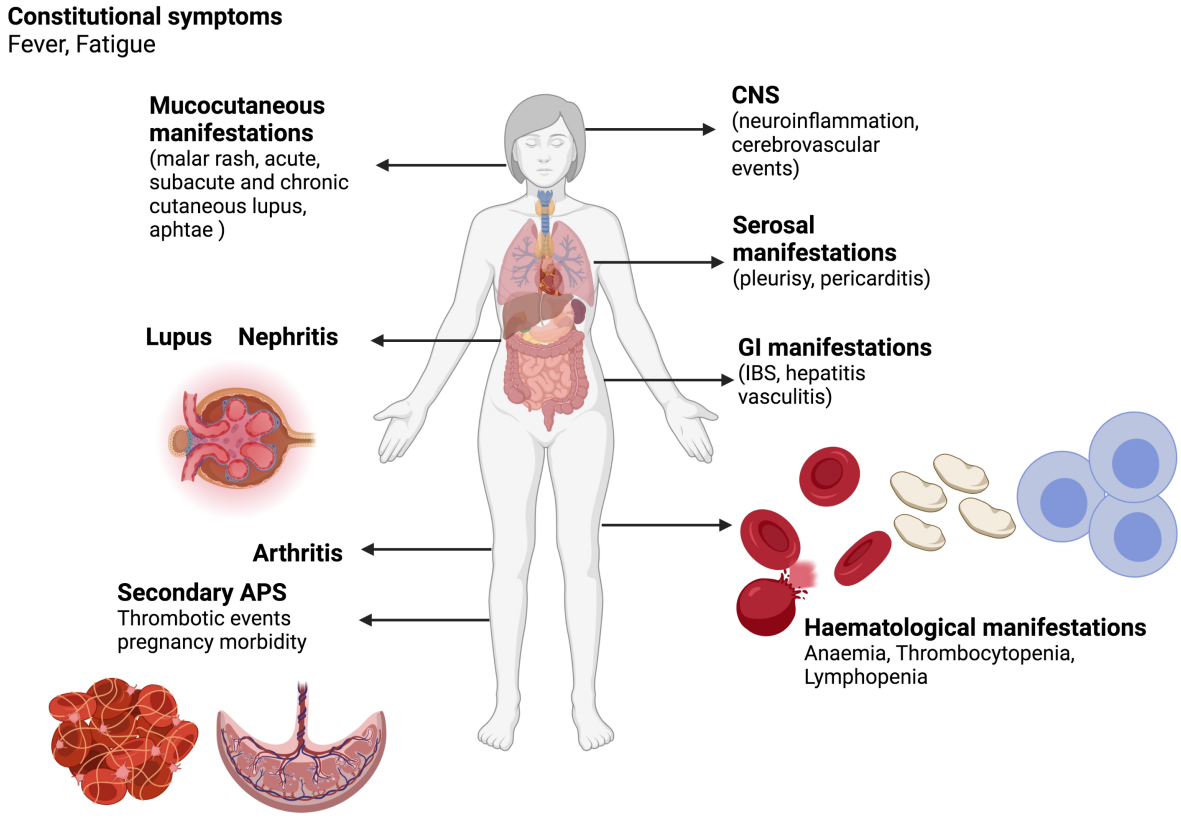
Lupus nephritis (LN) is among the major clinical manifestations of SLE, and accounts for a high burden of morbidity as well as it contributing to SLE-related mortality. Estimates of its prevalence among SLE patients vary, but reliably LN affects about half of SLE patients (17, 18). Despite an overall improvement of clinical management and early diagnosis, thanks to more systematic screening and increased awareness, LN is still a cause of end-stage renal disease (ESRD). It has been recently estimated that the global risk of ESRD at five years

improved in previous decades, reaching 11%; but after a period of plateau, it has again risen in the first decade of the 21st century. Moreover, aggressive forms of LN still carry a risk of ESRD which has been estimated at 44% at fifteen years from the diagnosis (19).

LN often occurs early in the course of the disease, although late presentation is not uncommon. The clinical picture can vary widely, with patients presenting only minor abnormalities in urine analysis to patients presenting with nephritic or nephrotic syndrome, or acute deterioration of renal function. The modality of presentation has over the years changed even in relation to the above-mentioned increased awareness, and owing to more frequent search for laboratory abnormalities. Overall, the features of LN at presentation have become less severe over time (20).

The most frequent laboratory finding leading to suspicion of LN and following diagnosis are proteinuria and haematuria, with associated casts in urine sediment. A repeated proteinuria above 500 mg/24h or a single determination above 1g/24h, or a deterioration of kidney function not otherwise explicable, are regarded as indications for a confirmatory kidney biopsy (21).

Figure 1: Spectrum of the clinical manifestations of SLE



The major clinical manifestations of SLE are reported in the figure. Their prevalence ranges between 20% (for serosal inflammation) and 70-80% of the patients (for joint and skin manifestations). Kidney involvement and haematological manifestations affect around half of the patients. (source of the data on frequencies: Kaul A, et al. Nat Rev Dis Primers 2016). *Created in BioRender.com*

3.3 Immunological hallmarks of SLE

3.3.1 Autoantibodies

A wide range of autoantibodies can be detected in SLE, with variable frequency and prevalence among patients (22). It is to date known that the appearance of autoantibodies predates the onset of clinically overt disease of years, a phenomenon in line with the concept of loss of tolerance preceding further events in SLE pathogenesis (23).

SLE-associated autoantibodies are directed towards a wide range of antigens (Figure 2.); however, the most characteristic ones recognize antigens originally located within the cells, in particular in the nucleus. The collection of such autoantibodies constitutes the anti-nuclear antibodies (ANA).

ANA are the hallmark of the disease: they are highly prevalent and sensitive, but not specific. In fact, a non-negligible prevalence in the general population is reported, and ANA can be detected in other autoimmune diseases. For this reason, ANA testing must be conducted with appropriateness in order to interpret results in the clinical context. Moreover, the methods used are of relevance in this respect. Today ANA is prevalently tested by indirect immunofluorescence assay, or by enzyme-linked immunosorbent assay (ELISA) or multiplex bead assay. ANA are today invariably required for the classification of SLE, with a cut-off of positivity of 1:80 (24, 25).

The second major immunological hallmark of SLE, and more specific than ANA, is anti-double strand DNA antibodies (anti-ds-DNA). These antibodies recognise and bind the DNA, either free or in macromolecular complexes formed with chromatin-associated proteins. The generation of anti-dsDNA, and the modalities by which the DNA becomes immunogenic, are at the core of SLE pathogenesis (26).

3.3.2 The complement system

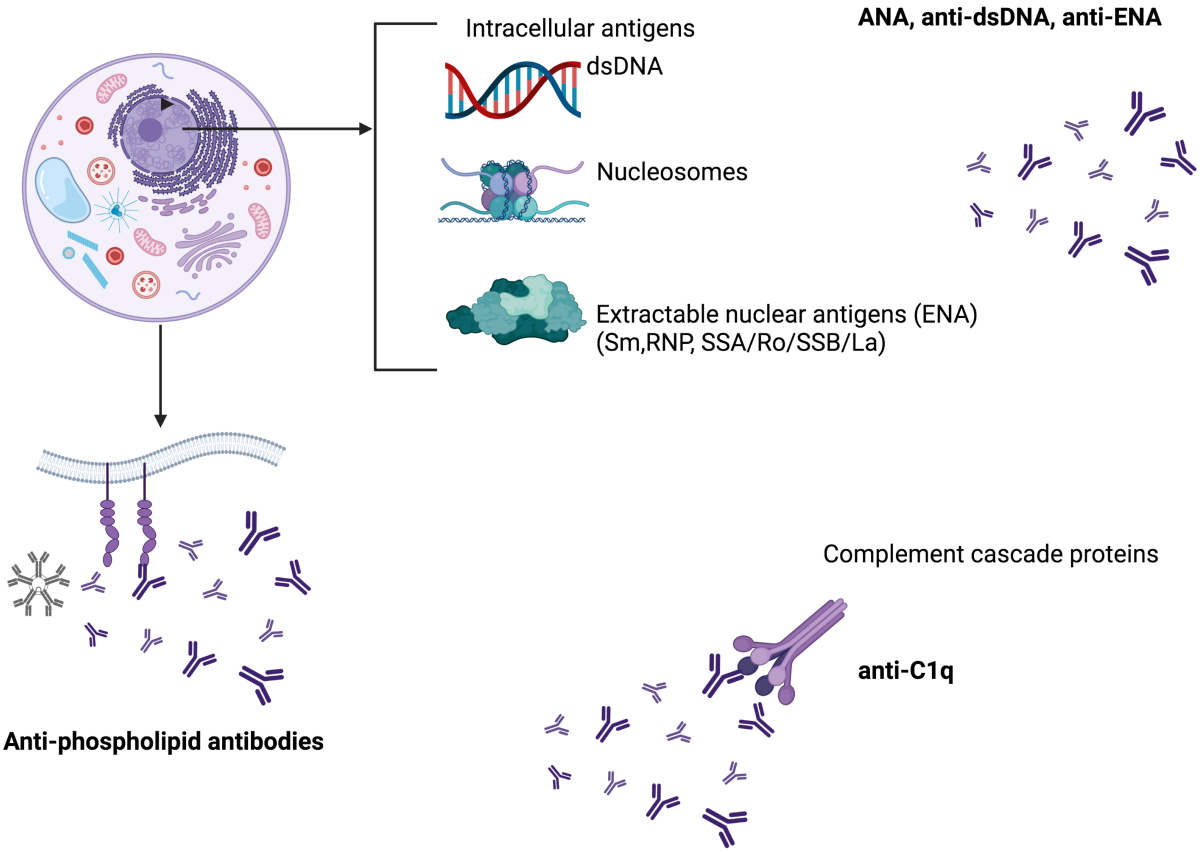
Among the clinical and laboratory features of SLE is the frequent finding of complement activation, with thus low serum levels of C3 and C4, and other complement components.

The role of complement in SLE is complex, and encompasses three main aspects. First, genetic defects and variations within components of the complement system play a role as predisposing factors to SLE. It is known that single gene defects involving genes encoding for proteins of the classical pathway of the complement system result in monogenic forms of SLE. These forms are usually described in children, as homozygous defects of C1q, which causes lupus-like manifestations with a third of the patients experiencing nephritis. In such cases, the dominant pathophysiological mechanism linking the genetic defect and SLE manifestations is the defective clearance of apoptotic bodies, with consequent overload of autoantigens (27). Defects involving the gene of the complement fraction C4 have been extensively studied. This gene reveals a high level of complexity, and variation of the copy number of it has been linked to genetic predisposition to SLE (28, 29). Defects of the complement component C2 have also been linked to the occurrence of SLE (30).

Secondly, complement activation in SLE is a main contributor to tissue inflammation and damage, especially in LN, where deposition of immune complexes activates the complement cascade. Third, complement activation can potentially serve as a biomarker of disease.

Conventional measurements of C3 and C4 are routinely performed during assessment of SLE. However, their efficiency and reliability as biomarkers of disease has been questioned. Indeed, several factors can influence the amount of complement factors present in serum. Aside from genetic variations, concomitant inflammation can, for example, trigger increased expression of C3, resulting in normal to high levels of it (31). This kind of factor contributes to making complement determination a poor diagnostic marker for SLE. During the early disease course only 54% of patients show hypocomplementemia despite active disease (32). Moreover, longitudinal follow-up studies evaluating complement factors as biomarkers of disease activity and their role in prediction of flares have proved disappointing (31). Current research efforts are focusing on exploring the value of measuring several other products of the complement cascade activation, which are generated from the cleavage of the main components of the cascade and could better reflect the status of activation of complement (33).

Figure 2: SLE-associated autoantibodies



The most prevalent autoantibodies are ANA (~95%), followed by anti-dsDNA and anti-nucleosome (~50-90%). ENA have a lower frequency (10-50%, depending on the sub-specificity), anti-C1q is present in up to 50%, and anti-phospholipid are positive in about 30-40% of SLE patients (source Dema B. et al. Antibodies (Basel) 2016). *Created in BioRender.com*

3.4 Diagnosis and classification criteria

The diagnosis of SLE is clinical, based on the recognition of typical disease manifestations in the presence of pathognomonic immunological features such as ANA and anti-dsDNA antibodies.

Classification criteria, although useful in the diagnostic process, are meant primarily for including patients in research cohorts, assuring that patients included truly have the disease and can be comparable with other patients.

To date, different sets of classification criteria have become available, which differ in terms of sensitivity and specificity.

3.4.1 American College of Rheumatology criteria

This set of criteria, provided by the American College of Rheumatology (ACR), was published in 1982 (34) and represented an update on broadly used criteria, which had been introduced in 1971 by the American Rheumatism Association (ARA). The 1982 ACR criteria set introduced the immunological item of the positivity of anti-nuclear, anti-Sm and anti-ds-DNA antibodies for the classification of SLE, while removing clinical features of low specificity and sensitivity (alopecia, Raynaud's phenomenon) from the previously proposed criteria. The sensitivity and specificity of these criteria were both as high as 96%. The patients included in the Karolinska SLE cohort have been primarily classified using this criteria set, which is reported below (Table 1).

In 1987, an update of the ACR criteria was published, in order to incorporate into the set recent advancements in the immunological knowledge of SLE (35). The old 'positive LE cell preparation' item in the section 'Immunological disorder' disappeared to leave place for the introduction of anti-phospholipid testing, which was set by the side of the false positive Syphilis serology item (Table. 2). The removal of the LE phenomenon did not impact in a relevant way on sensitivity and specificity, which were 96.1% and 93.4%, respectively.

3.4.2 Systemic Lupus International Collaborating Clinics (SLICC) criteria

These criteria were published in 2012, with the purpose of overcoming certain limitations of the ACR criteria. These limitations include, e.g.: unbalance of the weight given to the relevance of some clinical manifestations (such as skin rash) over others (nephritis); and omission of some possible neurological manifestations and of some immunological aberrations (e.g. complement consumption). The set comprises eleven clinical and six immunological criteria. For a patient to be classified as having SLE according to the SLICC criteria, four criteria, of which at least one clinical and one immunological, must be met cumulatively (i.e. the criteria must not be present at the same time). Alternatively, the criteria are satisfied if a patient has biopsy-proved lupus nephritis in the presence of ANA or anti-dsDNA antibodies. The SLICC criteria are highly sensitive (96.7%) but less specific (83.7%) (36).

3.4.3 2019 European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) criteria

This set of classification criteria is of more recent introduction and therefore not used as extensively as the above-described criteria for SLE. With respect to the older criteria, in these

criteria sets, ANAs constitute an entry criterion, which means a patient can be evaluated only if she or he has tested positive for the antibodies. The additional criteria present in this classification set are also divided into clinical and immunological, and include clinical manifestations such as fever, which before was not considered a criterion manifestation. Moreover, as a relevant novelty in comparison to the other criteria set, in the 2019 EULAR/ACR criteria set, each manifestation is given a numerical weight, so that different organ manifestations yield different scores. A patient will be classified as having SLE when a score of ten in the count of additional criteria is reached (24).

Table 1: The ACR 1982 classification criteria for SLE

	Criterion	***Definition***
1	Malar rash	Fixed erythema, flat or raised, over the malar eminences, sparing the nasolabial folds
2	Discoid rash	Erythematous raised patches with adherent keratotic scale and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4	Oral Ulcers	Oral or nasopharingeal ulceration, usually painless, observed by a physician
5	Arthritis	Nonerosive arthritis involving 2 or more joints, characterized by tenderness, swelling or effusion
6	Serositis	Pleuritis-convincing history of pleuritic chest pain or rub heard by a physician or evidence of pleural effusions *OR* Pericarditis-documented by electrocardiogram or rub or evidence of pericardial effusion
7	Renal disorder	Persistent proteinuria, either >0.5 g/day or>3+ if quantitation not performed *OR* Cellular casts-may be red blood cell, haemoglobin, granular tubular or mixed
8	Neurologic disorder	a. Seizures-in the absence of offending drugs or known metabolic derangements (e.g. uremia, acidosis or electrolyte imbalance) *OR* b. Psychosis-in the absence of offending drugs or known metabolic derangements (e.g. uremia, acidosis or electrolyte imbalance)
9	Hematologic disorder	a. Hemolytic anemia with reticulocytosis *OR* b. Leukopenia <4000/mm^3 in 2 or more occasions *OR* c. Lymphopenia <1500/mm^3 in 2 or more occasions *OR* d. Thrombocytopenia <100.000/mm^3 in the absence of offending drugs
10	Immunologic disorder	a. Positive LE cell preparation *OR* b. Anti-DNA: antibody to native DNA in abnormal titer *OR* c. Anti-Smith antibody-presence of antibody to Sm nuclear antigen *OR* d. False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponema antibody absorption test
11	Anti-nuclear antibodies	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with drug-induced lupus syndromes

The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have SLE if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation. (Reproduced with modifications from Tan EM, et al., Arthritis & Rheum, 1982)

Table 2: Extract of the 1987 revision of the 1982 ACR criteria for SLE

	Criterion	*Definition*
10	Immunologic disorder	a. Anti-DNA antibody-antibody to native DNA in abnormal titer *OR* b. Anti-Smith antibody-presence of antibody to Sm nuclear antigen *OR* c. Finding of antiphospholipid antibodies based on (1) abnormal serum concentration of immunoglobulin IgG or IgM anticardiolipin antibodies, (2) positive test result for lupus anticoagulant using a standard method, or (3) false-positive serologic test result for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test

(Reproduced with modifications from Hochberg MC, Arthritis Rheum, 1997)

3.4.4 The diagnosis of lupus nephritis

The mainstay of LN diagnosis is kidney biopsy. This is usually conducted under ultrasound guide and is a relatively safe procedure. The major complications of kidney biopsy are bleeding within the renal capsule, with consequent haemodynamic instability, and formation of infra-capsular haematoma (37). The standard requirements for an adequate and informative kidney biopsy are established. An adequate sample should contain at least ten glomeruli, and be examined by haematoxylin-eosin, special staining, and electron microscopy (38).

Biopsy samples from LN patients are currently evaluated according to a standard classification system, the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 (39). This classification system identifies six major histological classes of LN, based on the description of the pathological findings at glomerular level (Table 3). The histopathological evaluation also comprises assessment of the degree of activity and chronicity of the glomerular lesions (40).

The value of kidney biopsy, aside from confirming the diagnosis, is to provide information that can be useful for treatment decision-making. Not all the subclasses of LN need therapeutic intervention (class II); however some classes must be treated in a timely manner, such as the proliferative forms (class III and IV). Class V, which gives mostly proteinuria with or without nephrotic manifestations, necessitates of dedicated therapeutic interventions, and its treatment is today rather challenging (17, 41).

Relevant information can be collected from pathological evaluations which concern the presence of other pathological features caused by comorbidities, such as hypertension and diabetes, or the presence of anti-phospholipid associated pathology. Moreover, it has become evident over the years that LN is not exclusively a pathology of the glomerulus.

Tubulointerstitial lesions and inflammatory infiltrates in the renal interstitium have been found to be of high relevance also in terms of prognosis (42-44).These acquisitions led to a proposal for a revision of the classification system, which has reconsidered some pitfalls in the established description of pathological findings and suggested implementation of the assessment of tubule-interstitial and vascular pathology (45).

Another issue, which is the subject of research and discussion within the scientific community, is the informative role of repeated biopsy, in terms of prognostic evaluations and prediction of subsequent flares on one side, and planning of drug withdrawal on the other side (46). In a

study from our group, it was shown that, despite the clinical response, residual histological activity was not an uncommon finding (47). To date, the practice of repeating biopsy has not yet been implemented in clinical recommendations, but it is the subject of active research and debate (21, 48).

Table: 3 International Society of Nephrology and Renal Pathology Society Classification Criteria of Lupus Nephritis (2004)

Class II	**Mesangial proliferative LN** Purely mesangial hypercellularity of any degree or mesangial matrix expansion by LM with mesangial immune deposits Possibly a few isolated subepithelial or subendothelial deposits visible by IF or EM but not by LM
Class III	**Focal LN*** Active or inactive focal, segmental and/or global endo-or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations
Class III (A)	Active lesions: focal proliferative LN
Class III (A/C)	Active and chronic lesions: focal proliferative and sclerosing LN
Class III (C)	Chronic inactive lesions with glomerular scars: focal sclerosing LN
Class IV	**Diffuse LN**** Active or inactive diffuse, segmental or global, endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental (S), when ≥50% of the involved glomeruli have segmental lesions, and global (G) when ≥50% of the involved glomeruli have global lesions. Segmental is defined as a lesion that involves less than half of the glomerular tuft. Global is defined as a lesion that involves more than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation
Class IV-S (A) or IV-G (A)	Active lesions: diffuse segmental or global proliferative LN
Class IV-S (A/C) or IV-G (A/C)	Active and chronic lesions: diffuse segmental or global proliferative and sclerosing LN
Class IV-S (C) or IV-G (C)	Chronic inactive lesions with scars: diffuse segmental or global sclerosing LN
Class V	**Membranous LN** Global or segmental subepithelial immune deposits or their morphologic sequelae by LM and by IF or EM, with or without mesangial alterations. It may occur in combination with class III or IV in which case both will be diagnosed. It shows advanced sclerosis.
Class VI	**Advanced sclerosis LN** ≥90% of glomeruli globally sclerosed without residual activity

Legenda
LN=lupus nephritis
LM=light microscopy
IF=immunofluorescence
EM=electron microscopy
*indicate the proportion of glomeruli with active and with sclerotic lesions
**indicate the proportion of glomeruli with fibrinoid necrosis ad/or cellular crescents
Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions. Class I excluded because corresponding to normal histological finding on optic microscopy
(*Reproduced with modifications from Weening JJ, et al J Am Soc Nephrol. 2004*)

3.5 Disease activity measurements and outcome measures

Several indexes of disease activity have been proposed and implemented in clinical practice. Among them, an extensively used clinimetric index is SLEDAI-2K (SLE disease activity index 2000), which evaluates specific manifestations in nine organ systems, and assigns scores to descriptor manifestations if present in the ten days preceding the clinical evaluation. In this evaluation system, a score of 6 is deemed clinically significant (49). Evaluating clinical response in SLE is a matter of great complexity, and a review of the proposed and endorsed indexes is not within the scope of this thesis. For what concerns LN, several definitions have been proposed. Among those in use, within the Karolinska SLE cohort, the EULAR response criteria proposed in 2012 are used (50, 51).

3.6 The pathogenesis of SLE

The pathogenesis of SLE is a topic of extreme complexity, and therefore very difficult to summarize in a short and comprehensive manner. Virtually every component of the immune system can contribute to SLE pathogenesis. Multiple aberrations have been described, which involve both the innate and the adaptive arms of the immune system. Below are brief descriptions of some of these immunological aberrations. The description is mainly focused on cellular aspects of SLE pathogenesis, and leaves out environmental, genetic and epigenetic factors, for the sake of brevity, although these are also relevant aspects (52).

3.6.1 Innate immunity

Innate immunity is involved in SLE pathogenesis in four main aspects: 1) the abundance of autoantigens via defective clearance of apoptotic debris; 2) the processing and presentation of autoantigens to the adaptive immune system; 3) the modulation of adaptive immunity activation via the production of cytokines; and 4) the participation in effector mechanisms and contribution to tissue damage.

Defects in clearance of apoptotic debris have been identified as crucial in SLE pathogenesis. In physiological conditions, cells undergo repeated turnover, and cell remnants are removed within the tissues by near vital cells or by the intervention of professional phagocytes. This takes place in a non-inflammatory milieu, with phagocytes, as macrophages and neutrophils, engulfing remnants of apoptotic cells, and activation of inappropriate immune responses suppressed via downregulating cytokines (TGF-β, IL-10) (53).

If this process of removal is unbalanced, apoptotic cells removal is delayed, which may lead to the initiation of a secondary necrosis in apoptotic cells. This may lead to the creation of neoantigens, from exposed intracellular content, especially nucleic acids. These neoantigens may initiate a process of stimulation of the immune response, and be presented in the secondary lymphoid organs to T-cells, giving rise to a self-perpetuating process of inflammation (54-56). A typical situation in which defects of the pathways presiding removal of apoptosis remnants may lead to an overwhelming exposure to such neoantigens is sun exposure, which is recognised as a risk factor for SLE flares. Defective clearance of apoptotic debris can also be contributed to by genetic defects of the complement pathway, such as defects in C1q, known to be associated with monogenic forms of SLE (53). Cell death dysregulation seems to be relevant in the exposure of nucleic acids towards which in SLE the main autoimmune response

is generated. One particular form of cell death has received much focus in recent years: NETosis.

NETosis is per se one of the modalities by which neutrophils perform their killing actions against pathogens. It consists in the extrusion from the cells of DNA and chromatin which form nets, also defined as neutrophil extracellular traps (NETs), which contain enzymes with killing actions, usually located in the granules of these cells (such as myeloperoxidase). In such nets, the pathogens can be entrapped, and the action of the microbicide enzymes will favour killing. NETosis can take place in two main modalities, one leading to the death of the neutrophils and one leaving these cells vital (57). Involvement of NETosis in SLE has been sustained by several experimental studies. Being per se a protective process, once NETosis has acted and led to cell death, the debris needs to be removed. Defects of apoptotic cell clearance may make some individuals unable to remove such debris, an outcome which has been shown experimentally linked to impairment of enzymes degrading DNA, and to correlate with LN (58). An impaired removal of NETs would lead to persistent exposure to autoantigens, which seems to be the case for SLE, where NETosis potently induces interferon signalling (59). Recent advances in the biology of neutrophils have led to the description of a particular subset of these cells, called low-density granulocytes (LDG), which has a marked proinflammatory profile, is prone to form NETs and stimulates T-cells towards activation (60, 61).

Another cell type within innate immunity which is crucial to SLE is the plasmacytoid dendritic cell (pDC). This type of cell, originating in the bone marrow, is defined as plasmacytoid for its morphological resemblance to plasma cells. Differently from usual dendritic cells, this cell type lacks the expression of CD11c. Its relevance in SLE pathogenesis lies in the capacity of pDC to produce large amounts of type I interferons, along with other pro-inflammatory cytokines (62). To date, pDC are considered the main source of interferon in SLE; and it is well demonstrated that they can be activated via stimulation of toll-like receptors (TLRs), and can subsequently drive the differentiation of B-cells into antibody-producing cells in the context of extrafollicular responses. These events are key to the generation of anti-dsDNA antibodies (63).

Interferons (IFNs), mentioned here in several passages, are a family of cytokines which is involved in the host's defence against viruses. They are produced by several immune cells and are divided into subclasses (type I, II, III IFNs). IFNs have become increasingly important in the study of the immunobiology of SLE, because of growing understanding of their activity in the disease, which has led to developing new therapeutics targeting this pathway (64). It is widely recognised that IFNs are highly expressed in SLE, in particular those belonging to the type I subclass of these proteins. The hyperactive IFN-signalling typical of SLE leads to an over activation of genes (interferon regulated genes) which codify for a wide range of products, which in turn regulate the activity of the immune system. The status of enhanced expression of IFN regulated genes is known as 'interferon signature'. This is a feature of about 90% of children and of half to two-thirds of adult SLE patients (52, 65, 66).

3.6.2 Adaptive immunity

Adaptive immunity, the part of the immune system which is able to specifically recognize antigens and has memory, is centrally involved in the pathogenesis of SLE, as described below.

B-cells

B-cells (or B-lymphocytes) were for a long time been believed to play a secondary role in autoimmunity, and as mere producers of autoantibodies. This restricted view has widely changed over the last decades. It has become clear that B-cells do not just contribute to autoimmunity, by generating plasma cells and hence producing autoantibodies. Two other main functions of B-cells have been identified, one being their ability to act as antigen presenting cells (APCs), the other being their capacity to produce proinflammatory cytokines (67). In SLE, B-cells show a wide range of abnormalities, which involve aberrations of their ontogenesis and distribution into different subsets, and their being hyperactive, with dysregulated signalling (68).

The understanding of the role of B-cells in SLE pathogenesis has developed through contributions from both clinical and pre-clinical research. Animal models of the disease have also contributed to this understanding. A detailed review of what animal models have taught us is beyond the scope of this chapter, but it should be noted that through animal models several pathogenic events have been identified. Among these are the modalities of self-tolerance breakdown, and the definition of B-cell development and B-cell subsets, and the cross-talk of B-cells with other immune cells, including T-cells (69).

In human SLE, an immediate approach to understanding the contributions of immune cells to disease pathogenesis is to study the cells directly in the blood. This is made possible by methods such as flow cytometry, which has become fundamental for this kind of work (70). In the case of B-cells, this method allows the recognition of discrete subsets which correspond to various functional and developmental stages that the cells acquire during their maturation.

One approach to immune phenotyping that has become standard for B-cells, was first applied to interrogate the diversity of human B-cells in tonsils. This system (Bm, for B mature) subdivides the cells on the basis of the expression of two main markers, IgD and CD38. In this way, four main subsets can be defined: IgD+CD38-(naïve B-cells), IgD+CD38+ (pre-germinal centre), IgD-CD38+ (germinal centre B-cells), and IgD-CD38- (memory B-cells). By the addition of other markers, from this basic subdivision, several other subsets have been defined and deeper characterization attained. One of the markers added early to this approach is CD27, which for a long time was considered to be a universal marker of memory B-cells (71, 72). Memory B-cells, expressing CD27, can be further subdivided based on IgD expression into unswitched memory (IgD+CD27+) and switched memory (IgD-CD27+). This paradigmatic subdivision was valid until the discovery of the double negative memory B-cells, which lack CD27 expression (DN, IgD-CD27-) (73). The discovery and addition of an increasing number of surface markers allowed scientists to dissect the diversity of B-cells.

The introduction presented here is necessary for understanding the description of the main findings in the study of B-cells in human SLE that are mentioned throughout this thesis. An overview of B-cell development and the main phenotypes is provided in Figure 3 to help the reader in understanding the significance of the B-cell aberrations which is described below.

One main characteristic of human SLE is the presence of lymphopenia. However, in the context of a reduced count of lymphocytes, the balance of B-cell subsets in SLE differs with respect to healthy individuals. Adult SLE patients in fact show a pronounced reduction of naïve B-cells, with a relative expansion of memory B-cells. Moreover, in the active phases of the disease, cells with high expression of CD27 are predominant within the B-cell pool, and show features

of activation and differentiation towards subsequent stages of B-cell development, namely plasma blasts (74). This finding of plasma blasts expansion in relation to disease activity characterizes also paediatric SLE. However, children do not exhibit the same unbalanced distribution of naïve and memory cells as adult patients (75). They are still profoundly lymphopoenic, with a composition of mature B-cells skewed towards a relative dominance of pre-germinal centre cells, which actively recirculate between blood and secondary lymphoid organs (76).

Another concept that has emerged from studying peripheral B-cell subsets is that the disturbances of B-cell homeostasis are a function of the disease duration and severity. This means that, along the disease course and with repeated flares, an expansion of cells which are antigen-challenged occurs. This results in expansion of plasma blasts, which in number and frequency correlate with disease activity. These cells generate plasma cells of both short and long life spans. These two types of plasma cells correspond to two types of antibody. Short-lived plasma cells can give rise to anti-dsDNA, while long-lived ones are responsible for the production of antibodies such as anti-SSA, -SSB, and -Sm. It is common experience in clinical practice that anti-dsDNA titres fluctuate over time, while the other specificities are more stable. To account for this diverse behaviour, there are diverse biological features of the cells producing antibodies (77). Moreover, this diversity is reflected by the location of such events in different anatomical compartments. B-cells are instructed to undergo terminal differentiation either in germinal centres (GC) in secondary lymphoid organs (follicular response), or outside of them (extra-follicular response), e.g. in mucosal environments. This type of event does not occur once in the life span of a B-lymphocyte. Cells recirculate continuously between peripheral blood and tissues, and antigen-cell and cell-cell interactions are reiterated.

However, in a simplified way, biological events can be described by highlighting cardinal steps. When naïve B-cells are challenged by an antigen, their subsequent destiny is dichotomous. One part of their progeny will generate plasma blasts and plasma cells; the other will generate memory B-cells. These will be ready to respond to reiterated challenges. In SLE, memory B-cells are of particular interest. They are in fact a putative reservoir of plasma blast and plasma cell generation, so it is highly desirable to tackle them therapeutically. However, early observations have shown that these cells are resistant to treatment (74). To understand how to modulate or exclude memory cells involved in autoimmunity, it is necessary to understand their generation.

Memory B-cells are the subject of intense investigation, which has led to an understanding of a wide diversity within this subset, with the emergence of diverse phenotypes collectively known as ‘atypical memory B-cells’ (AMB-c) (78, 79).

The standard view of memory B-cells is that these cells have the ability to rapidly respond to antigenic stimulation and show superior proliferative capacity compared to naïve B-cells. They are capable of interacting with T-cells and rapidly generate new plasma blasts and plasma cells. All these features are essential in ensuring efficient secondary responses (80). On a phenotypical level, classical memory B cells have been defined by the expression of the marker CD27 (81). This paradigm has been in part superseded by new discoveries in the field.

AMBcs are phenotypically different from typical memory B-cells. They lack surface markers which are typical for the memory status, such as CD27. Moreover, they are characterized by the absence of CD21, a complement receptor, which normally acts as a positive regulator of BCR signalling (82, 83). Another main feature of these cells is their dependence on the

transcription factor t-bet, which implies modality of activation and further differentiation in response to cytokine stimulation and TLR engagement. Moreover t-bet, which was first discovered in T-cells, was later found instrumental for class switching in B-cells (84). Another marker investigated in these cells is the integrin receptor CD11c (85). Given the simultaneous investigations by several research groups with different specific focuses, there is a certain degree of heterogeneity in the field of AMBcs, which encompasses phenotypical characterization, functional analysis, and nomenclature. Collectively, AMBcs have been described in situations of chronic antigenic stimulation, such as infections (HIV, malaria) and in the context of autoimmunity (79, 86). Their attribution to the memory compartment, aside from the lack of expression of typical surface markers, is due to the evidence that these subsets of cells, although heterogeneous, share typical memory features, such as class switching and somatic hypermutation. These features indicate that these cells are antigen experienced (78, 84, 85). Moreover, further phenotypical and functional characterization has led to highlighting that AMBcs overexpress inhibitory receptors, such as CD95 (Fas), and globally have a profile of reduced BCR activation and signalling, which are features of anergy/exhaustion (79). This terminology might be misleading, since it sounds like of inactivity, which contrasts with the concept of an association with autoimmunity. This aspect might be regarded in the perspective of chronic antigenic stimulation. In this situation, the expression of markers usually associated with a pro-apoptotic profile should be interpreted not as a primary defect, but as a reaction to chronic stimulation itself.

In the field of SLE, three main phenotypes of AMBcs have emerged in the studies performed in recent years (79). The first phenotype of interest is denominated ‘double negative’ (DN). These are memory B-cells lacking both IgD and CD27(73). Their belonging to the memory compartment has been demonstrated by evidence of somatic hypermutation and class-switching features. They have been discovered in the context of SLE. Subsequent studies have enabled definition within DN B-cells of two main phenotypes, denominated DN1 and DN 2 (87). Deeper characterization of these cells has revealed that they correspond to a phenotype of B-cell which was first identified as age-associated B-cells (ABCs), whose features will be described in more detail in the background of Paper I in this thesis.

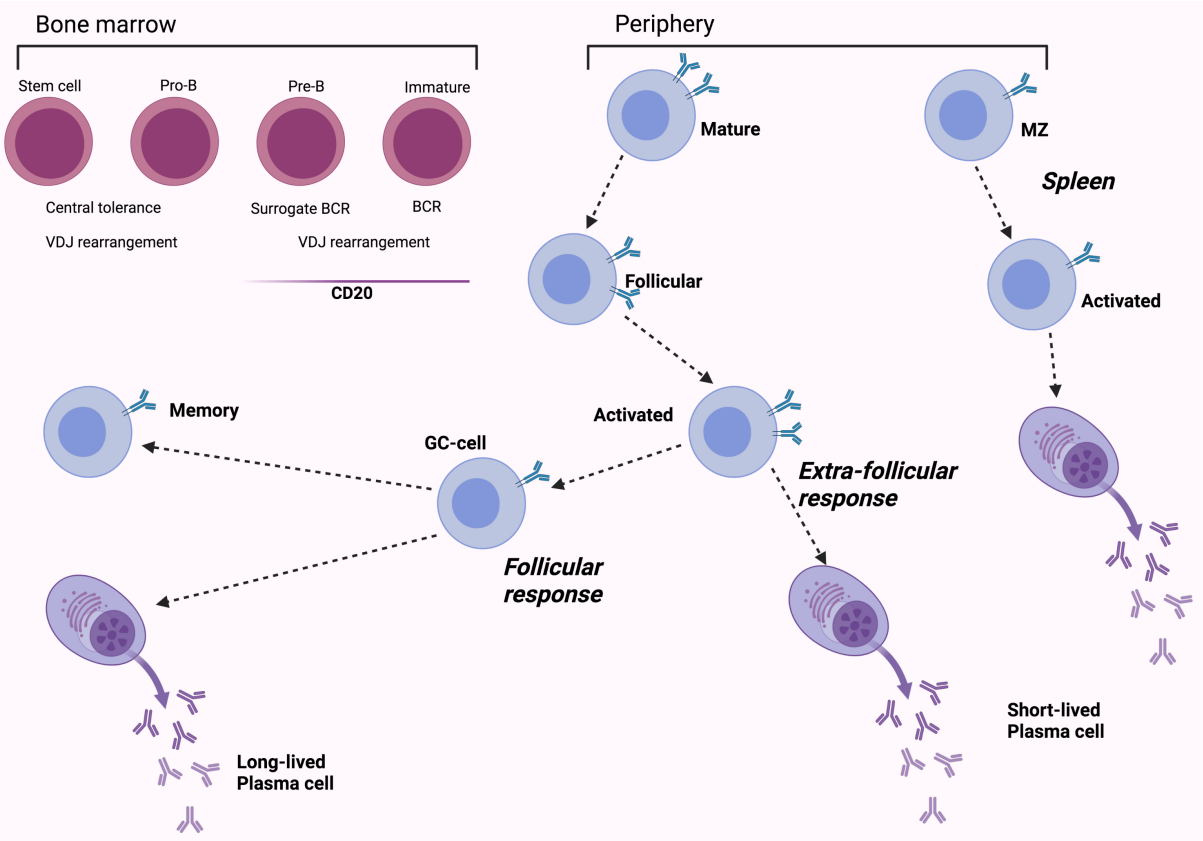
Another phenotype of AMBcs which has been described in the context of SLE is Syk high. Syk (spleen tyrosine-kinase) is an intracellular molecule which mediates the process of downstream signalling of the BCR, and becomes phosphorylated upon BCR engagement. The analysis of B-cells with respect to the intracellular expression of Syk, revealed a subset of CD27- memory B-cells characterized by increased intracellular expression of Syk. Moreover, this subset was shown to be significantly more represented in SLE patients than in controls and in comparator autoimmune patients (RA and Sjögren’s). These memory cells did not show significant associations with clinical parameters of disease activity in the study revealing them. However, stimulation experiments showed that they can evolve into antibody secreting cells (88).

An interesting recent study has attempted to gain further insights into the complexity of the AMBc subsets in SLE. In this study, conducted on newly diagnosed treatment naïve SLE patients, a population of AMBcs with variable expression of CD27 but absent CD21, and positive CD11c, was found expanded in the patients. The expansion was even more pronounced in patients with renal involvement, and a correlation with disease activity and antibody profiles was shown. These cells were characterized on a functional level, which led to the evidence of an altered BCR mediated activation. The analysis of the downstream signalling upon BCR engagement revealed hyperactivation of the mTORC1 (mammalian mechanistic target of

rapamycin complex 1) pathway, an observation which offers a translational perspective for targeting these cells (89).

Differentiation and survival of B-cells is regulated throughout their ontogeny by a wide range of stimuli, which comprise not only the interaction with other cells, but also the action of cytokines, which intervene in promoting the passage of B-cells through differentiation and activation stages. Among these cytokines, the B-cell activating factor (BAFF) and a proliferation-inducing ligand system (APRIL), are of critical importance. Briefly, BAFF is the main survival factor for B-cells. It acts through three types of receptor (BAFFR, TACI, BCMA), expressed on B-cells at diverse stages of their ontogeny. BAFFR only interacts with BAFF, while the other two can bind APRIL as well. BAFF is produced by various cells, including monocytes, and acts promoting the steps of B-cell maturation from immature to mature, and the subsequent evolution after antigen encounter. APRIL has critical roles in favouring plasma cell survival. A dysregulated signalling and excessive production of BAFF and APRIL in mouse models and human SLE has been investigated, and led to the definition of a crucial role for these two cytokines in SLE pathogenesis, and consequently to the pharmaceutical development of drugs targeting this biological system (90-92).

Figure 3: Ontogenesis of the B-lymphocytes



B-cell development is articulated in a bone marrow phase and post-bone marrow phase, taking place in secondary lymphoid organs and in tissues. Expression of CD20 begins in the bone marrow at the Pre-B stage, and will be lost at plasma cell stage. Mature cells until the activated stage express both IgD and IgM on their surface. IgD is lost at the germinal centre (CG) stage. T-cell independent development in the spleen. T-cell dependent development can progress either as follicular or extrafollicular response. *Created in BioRender.com*

Plasma cells

The other component of the cellular biology of the B-lineage that has relevance to SLE is the effector of the humoral arm of adaptive immunity: the plasma cell, which will be briefly addressed.

As touched on earlier, plasma cells can be generated after interactions between B- and T- cells, which takes place as intrafollicular or extrafollicular responses. Both these pathways can be involved in autoimmune responses in SLE and generate plasma cell precursors which evolve into cells producing antibodies with diverse degrees of affinity (93). A third possibility is the generation of plasma cells in a T-independent modality. In mice, the evolution of B-cells into plasma cells in the absence of T-cell help is a modality followed by B1-cells. It is not clear which human counterpart of these cells takes part in the generation of T-independently generated plasma cells in humans. This modality ensures a rapid production of antibodies, is associated with a low grade of maturation affinity and low grade of memory generation. In protective immune responses, this modality is often used by our organism to respond to polysaccharidic antigens (93-95).

On a phenotypical level, plasma cells are characterized by the loss of markers such as CD20 and CD22, and the acquisition of other surface molecules, such as CD138. They can express specific homing receptors and activate transcription factors. Plasma cells are tissue-based cells which do not replicate. Long-lived plasma cells usually reside in immunological niches, e.g. in the bone marrow, and from their location they steadily produce antibodies which they release into the blood. This means that plasma blasts, generated in the sites of inflammation, migrate to find the niches in which they will reside as plasma cells. The survival in the niches is a function of the milieu, and of the interaction with the stroma and other cell populations present in the bone marrow. At a cytokine level, plasma cell survival is dependent on APRIL, and IL-6. In the situation of inflammation, plasma cells not only can be generated in inflamed tissues, but also they can there find adequate survival conditions, as in the case of LN. This possibility suggests that the concept of niches refers rather to a functional than a physical location (95-97).

Targeting plasma cells in autoimmune diseases is an area of growing interest in which data are so far limited. In SLE, some preliminary studies have evaluated the use of the proteasome inhibitor bortezomib, including in cases of refractory LN, with some encouraging results, although there are some safety concerns. Larger studies are needed. Another molecule, daratumumab, currently approved for multiple myeloma, has been used in refractory patients (98-102).

T-cells

T-cells participate in SLE pathogenesis through a wide range of mechanisms, which reflect their enormous biological complexity, generated during life. As for B-cells, the study of peripheral blood T-cells has led to the identification of several subsets, which play diversified roles in homeostasis and disease. The integration with animal studies, and the study of tissues, has allowed understanding of the tissue distribution and how the different subsets localize in anatomic sites and interact with other cells (103).

The aberrations of T-cells contributing to SLE pathogenesis involve their number, phenotype, and actualization of their effector functions. In this respect, CD4+ T-cells have been more

extensively studied, while fewer studies have been performed on the role of CD8+ T-cells in SLE. One relevant aspect of such defects concerns the effector functions of CD8+ cells. In SLE, CD8+ subsets appear to be unbalanced, with a minor representation of CD8+ memory T-cells in the peripheral blood. Moreover, these cells are functionally impaired, being less able to effectively release granzyme and perforins, the main mediators of cytotoxicity (104).

CD4+ T-cells, the core of the adaptive immune system, are central to autoimmunity and SLE. One fundamental property of CD4+ T-cells is plasticity, which can be defined as the ability to start and revert different pathways of differentiation, in relation to the stimuli received by the near milieu. In these pathways, CD4+ naïve T-cells can give rise to different subsets, each one characterized by a set of cytokines and transcription factors preferentially expressed (105).

Differentiation in the presence of TGF-β generates the subset known as T-reg, or regulatory T-cells. This subset carries out a transcriptional programme led by the transcription factor FoxP-3, and is highly dependent on IL-2 stimulation. In SLE, T-regs are defective, which contributes to the failure of peripheral tolerance mechanisms. The primary driver of T-reg impairment in SLE seems to be a reduced availability of their main differentiation and survival factor: IL-2. This element has led to attempting therapeutic approaches aimed at rectifying this imbalance by providing IL-2 in low doses (106).

In recent years, novel subsets have emerged from the characterization of CD4+ cells, with the classical dichotomous division between T_H1 and T_H2 becoming at least in part superseded. Among the most intensively studied CD4+ subsets in SLE pathogenesis, and in general in autoimmune diseases, are the T_H17 cells, and the T-cell subsets interacting with B-cells in the context of secondary lymphoid organs and extra-follicular interactions.

T_H17 cells are a subset of pro-inflammatory CD4+ T-cells, naturally involved in immune defence against fungi, and intimately interconnected to a cytokine pathway known as IL-17/-23 axis, (105, 107). T_H17 differentiate in the context of naïve T-cell stimulation by IL-6, IL-21 and TGF-β, and through the activation of the transcription factor RORγt. Their survival depends on IL-23 stimulation, and their effector functions consist in the production of IL-17A, IL-21, TNF, and IL-22, which in turn stimulate various cell types and induce the expression of inflammatory mediators (105).

T_H17 have been a hot topic in autoimmunity for quite some years, and have been investigated in SLE, where they have been shown to have relevant biological roles. Consequently, therapeutic approaches targeting the IL-17/-23 axis have entered clinical evaluation in several trials. The involvement of T_H17 and the associated cytokines has emerged at different levels.

First, in peripheral blood of SLE patients, T_H17 cells have been found in higher levels, as have IL-17 and IL-23. Moreover, in patients with LN, pre-treatment serum levels of IL-17 were associated with poor clinical outcome. At tissue level, IL-17 colocalization with CD3+ infiltrates has also been demonstrated (108-110).

The growing evidence of the involvement of T_H17, and the cytokines which are critical in their biology, has encouraged the conduct of clinical trials to evaluate drugs directed at IL-17 and IL-23 in SLE, with some encouraging results for what concerns inhibiting IL-23 (111, 112). Clinical trials evaluating IL-17A blockade are currently ongoing.

Along with the accumulation of data in favour of a role of T_H17 and the related cytokine system in SLE pathogenesis, other subsets have been described. Among them, those involved in the interactions with B-cells have also been extensively investigated. These subsets, namely follicular (T_{FH}) and peripheral (T_{PH}) helper T-cells will later be described further, since they are relevant background to Paper I. Figure 4 concludes this chapter and gives an overview of the pathogenesis of SLE and of the cellular types described below.

3.6.3 Pathophysiological aspects relevant to LN pathogenesis

LN is an immune-complex (IC) mediated glomerulonephritis. This means that damage induced by inflammation in the renal parenchyma is initiated upon deposition of IC into the glomerular tuft. This event will elicit the activation of the complement cascade, and initiate the recruitment of inflammatory cells. This in turn will amplify inflammation and produce damage to the glomerular structure. This sequence of events, although correct, tells only one side of the story, the other protagonist of it being the tubulo-interstitial area of the renal parenchyma.

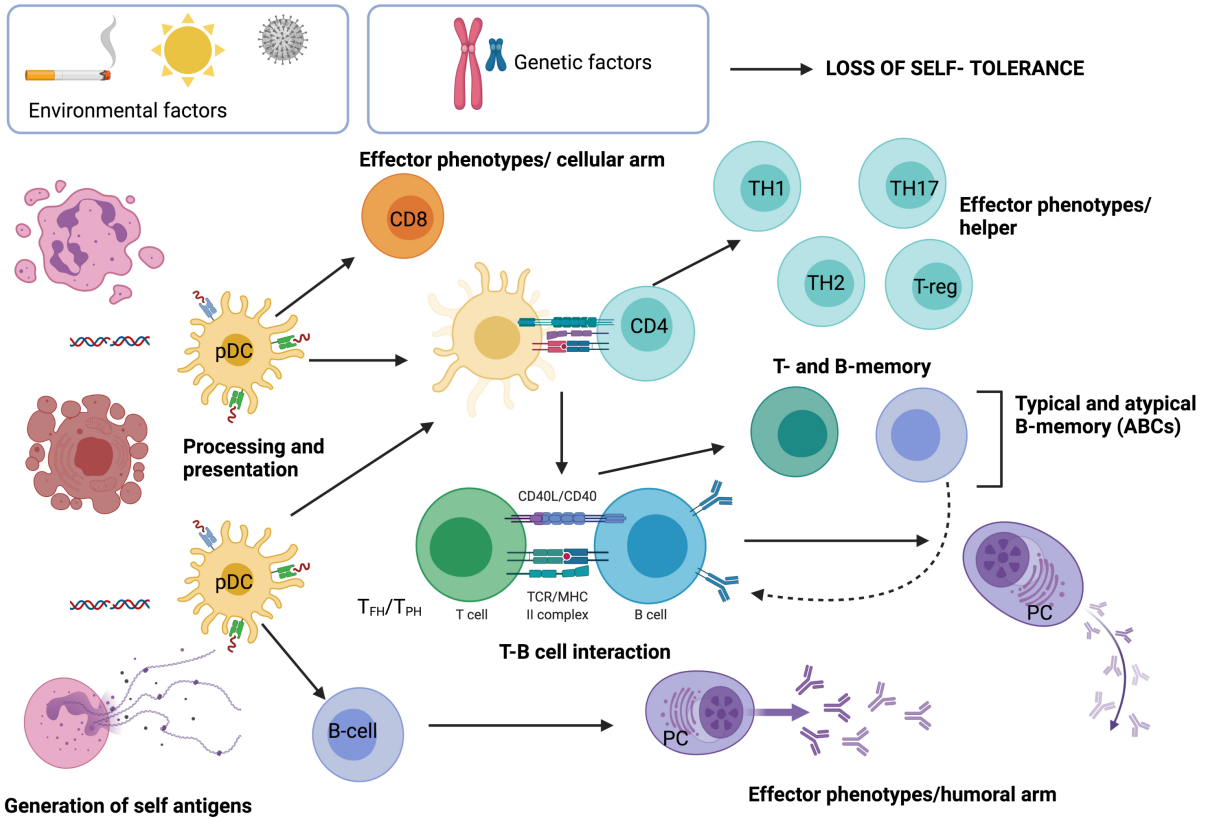
In LN several pivotal moments or events can be identified: first, the formation and deposition of IC containing nuclear antigens; second, the initiation and progression of inflammation; and third the structural damage to the nephrons which leads to their loss and the attempt to repair tissue which in turn ends in scarring and fibrosis (66).

Regarding the first aspect, there has long been investigation and debate regarding whether the deposition of IC is due to circulating IC which get caught in the glomeruli, or to the exposition of nucleic acids related antigens in the kidney itself, or to the binding of autoantibodies to cross reactive antigens in the renal tissue. The mechanisms of pathogenicity and tropism for the renal tissue of the anti-dsDNA and chromatin-directed autoantibodies have as well long been investigated (26, 113). Recent discoveries may help shed light on these mechanisms. One of these discoveries links the concept of excessive cellular death with the precipitation of circulating IC into the glomeruli. Current research is investigating whether autoantigens generated during cell death may circulate in form of IC with fragments of cells and apoptotic blebs. These structures, referred to as microparticles or extracellular vesicles, may contribute to LN pathogenesis (114). The location of the IC within the glomerular structure depends on the physical and chemical characteristics of the IC and their interaction with the glomerular basal membrane. The final location of the IC in a subendothelial or subepithelial position will produce different pathogenic consequences, with podocyte injury on one side, and inflammation with recruitment of inflammatory cells on the other side. Ultimately, damage will be mediated by cytokines, and other inflammatory mediators. The possibility exists that IC deposit into the mesangium and elicit proliferation of mesangial cells (115).

The damage to the glomeruli is intimately linked to the damage to the tubuli, which will be affected by the ischemia generated at glomerular level during the progression of inflammation. The presence of interstitial damage and tubular atrophy has relevant impact on long-term outcomes of LN (116). The participation of the kidney epithelial cells in the pathogenic events of LN is currently under investigation. In a recent transcriptomic analysis of the LN cellular actors, tubular cells showed an IFN-response signature, and in patients resistant to treatments, a transcriptomic profile poised towards the expression of profibrotic molecules was identified (117).

The other relevant aspect of LN pathophysiology is the presence of inflammatory infiltrates. Immune cells infiltrate the glomeruli, including neutrophils and macrophages. Transcriptomic analysis has recently revealed a composite presence of myeloid cells in kidney tissues from LN patients, with both infiltrating and resident cells (118). Moreover, immune cell infiltration is present in the tubule-interstitial area, where aggregates of B- and T-cells can architecturally dispose as germinal centre-like (GC-like) structures and give rise to extrafollicular responses (119). Also, recent transcriptomic analysis has revealed an abundance of cells deemed to be involved in B-T cell interactions (118).

Figure 4: Predisposing factors and cellular players involved in SLE pathogenesis



The action of environmental factors on genetic predisposition leads to loss of self-tolerance.
Generation of self-antigens is consequence of an excess of cell death/inability of removing cell remnants. NETosis is also a relevant contributor to the generation of autoantigens. Antigen-presenting cells (here are represented plasmacytoid dendritic cells, pDC), process and present autoantigens to the adaptive immune system, and different effector and memory cells are generated. T-B cell interactions are fundamental for the generation of long-lasting memory and plasma cells producing high affinity antibodies. *Created in BioRender.com*

3.7 Treatment of SLE: The present standard of care

The therapeutic arsenal of SLE has been object of intense research for the last six decades, with numerous attempts to introduce new drugs into clinical use. Many of these efforts have proved disappointing. Despite a considerable number of molecules which have been tested at different levels of experimentation, only one drug has reached full approval. This drug is the anti-BAFF

fully human monoclonal antibody belimumab, which reached authorization for its use in SLE in 2011(120, 121).
The current standard of care of SLE treatment is a multifaceted combination of pharmacological and non-pharmacological interventions, which primarily aim at inducing remission, preventing flares and impacting on damage accrual. Moreover, additional drugs such as ACE-inhibitors, anti-platelet drugs, anti-coagulants, or statins find room for application in correcting specific consequences and risk factors associated with the SLE morbidity and mortality burden. Life style interventions are also part of the current recommendations for SLE management (122). A brief review of the drugs currently being used in SLE is provided below.

3.7.1 Corticosteroids

The discovery of cortisone was a major breakthrough in the history of medicine, and the treatment of inflammatory conditions has largely benefited from it. This is generally true also for SLE, where the introduction of corticosteroids has had an enormous impact on SLE-related survival, which has risen from 17% to 55% at five years between the pre- and post-cortisone era (123). Soon after the start of corticosteroid use, the side effects of these drugs also became evident. Therefore, the optimisation of their use, together with the management of the large variety of short- and long-term side effects, has become a major need for the global SLE management. Indeed, corticosteroids strongly contribute to treatment-related mortality in SLE, increasing the risk of infections, accelerating the development of atherosclerosis and ultimately contributing to damage accrual (124-126).

Today, corticosteroid use still represents the first line of treatment for major flares and life threatening organ manifestations, typically LN and CNS involvement. In these situations, rapid improvement is achieved by intravenous boluses (250-1000 mg of 6-metylprednisolon over three consecutive days), and this approach constitutes a bridging therapy for major immune-suppressants as cyclophosphamide. Oral corticosteroids usually follow the administration of intravenous boluses, or can be given for managing minor flares, with the aim of tapering the dose and maintaining chronic administration below the threshold of 7.5 mg of prednisone equivalent daily, if not withdrawn completely (122).

3.7.2 Antimalarials

Antimalarial agents (AMA), have become a cornerstone of SLE treatment. The most used AMA molecule is hydroxychloroquine (HCQ), first introduced in medical use in the 1950s and recommended today for all SLE patients at a dose of 5 mg/Kg/daily (122, 127). Its use is supported by several pieces of evidence, showing that it modulates SLE disease activity by preventing flares, and has favourable impact on survival and reduction of damage accrual. Moreover, the use of HCQ in pregnancy is safe, and a positive impact on pregnancy outcomes has also been demonstrated (128).

The pharmacological properties of AMA have been extensively investigated. To date, the exact pharmacodynamic of these drugs is still to be defined in vivo. In vitro observations have discovered there is a direct interference of the drug with the mechanisms presiding in antigen-presentation by specialized cells within the immune system (APCs) (127).

In practice, AMA are drugs with a favourable safety profile. Chronic use requires the monitoring of few side effects, the one of major relevance being retinal toxicity, which is dependent on the cumulative dose and time of exposure to the drug (129).

Among the most recent developments in AMA research is the possibility to monitor blood concentrations of HCQ. This practice has not yet entered routine application, but appears to be a valuable tool for assessing drug adherence, a problem of high practical relevance in SLE management (122).

3.7.3 Immunosuppressive drugs

Current evidence encourages the use of different immunosuppressive drugs (IS) with the aim of treating specific organ manifestations, avoiding corticosteroid use, and maintaining disease control after disease flares. The choice of the drug can be guided by different principles. Aside from the level of evidence in tackling specific organ manifestations, considerations on teratogenicity and impact on fertility are a major issue when operating therapeutic decisions.

The drugs most used are azathioprine (AZA), mycophenolate mofetil (MMF), methotrexate (MTX), and calcineurin inhibitors (CNI) (15).

The latter are today an area of active investigation. Their use has been limited due to their safety profile, with side effects such as drug induced hypertension and possible renal toxicity of cyclosporine. More recently, tacrolimus has been more largely investigated, even as part of multitargeted approaches in which synergic effects were attained using lower doses of each used drug (130).Pharmacological progress in this class of drugs has led to the development of voclosporine, which has been recently tested in combination with mycophenolate mofetil for active LN (131, 132).

3.7.4 Cyclophosphamide

Cyclophosphamide (CYC) finds its main use in the two most challenging manifestations of SLE, LN and CNS lupus. Two alternative regimens are in use of CYC, the NIH protocol (monthly infusions of 0.5-0.75 g/m^2 of BSA for six months), and the Eurolupus protocol (500 mg intravenously every two weeks for six times) (133, 134).

3.7.5 Biologic drugs: Belimumab

Given the cellular and molecular complexity of SLE pathogenesis, the general principle of intercepting molecular events to control the disease has robust biological foundations. Unfortunately, the path of different candidate drugs from the preclinical to the clinical phase of drug development has often been a bumpy ride. Many drugs have witnessed a disappointing arrest of their development, and only one drug has so far been officially approved.

This drug is belimumab, a monoclonal antibody blocking the action of the soluble B-cell activating factor BAFF. Following the studies published in 2011, belimumab has been approved by the major regulatory authorities for managing moderately active SLE without LN or CNS manifestations, and in this indication has been used over the last decade (120, 121). Only recently, the indications for its use have been extended to LN based on the results of a LN dedicated clinical trial (135).

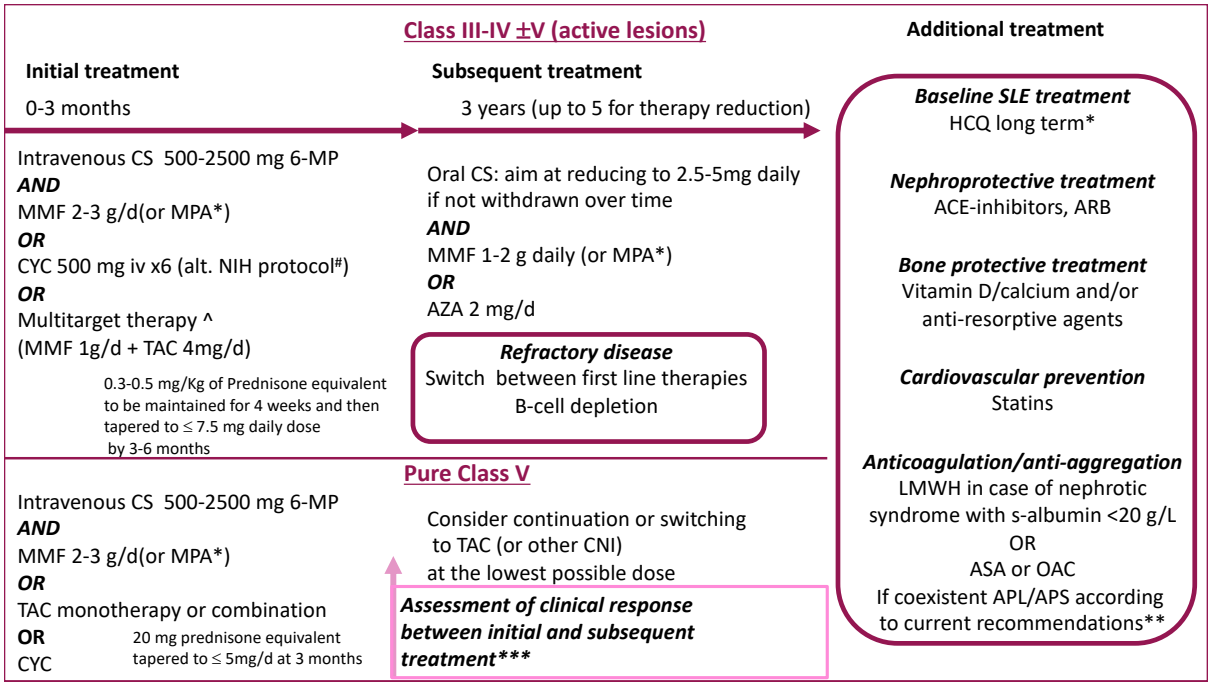
3.7.6 Special considerations on the treatment of LN

LN is a major therapeutic challenge in today's management of SLE. The primary goal of treating LN is to halter nephron loss and prevent end stage renal disease (ESRD), which can develop as a consequence of repeated flares and accumulation of organ damage. Among the histological classes of LN, proliferative forms (i.e. classes III and IV) are those mostly associated with nephron loss over time, and therefore require aggressive treatments. Class V, characterized by prevalent membranous changes, and associated with proteinuria and nephrotic syndrome, is also a therapeutic challenge (41).

As of today, the major rheumatological societies provide a set of recommendations, periodically updated, to guide clinicians in LN management. The general approach is articulated into an initial and subsequent treatment (Figure 5), in which immunosuppressive drugs, nephroprotective drugs and additional measures are integrated (21). A treat-to-target approach is strongly advised by some authors based on that achieving a proteinuria below 700 mg/daily at one year from treatment start is associated with a better long-term outcome in terms of preserving renal function (136).

In today's approach to LN (and SLE in general), refractoriness to standard treatments is the niche of utilization of RTX. In fact, despite global improvement in the diagnosis and management of LN, there is still a significant number of LN patients who do not achieve response. The frequency of this problematic situation varies across studies; estimates report a frequency of 14-33% (137). An exact definition of refractoriness is not generally provided by recommendations, and varies across response criteria. Moreover, a systematic approach in clinical research is lacking, which means that solid ground on how to treat refractory LN is missing. Another aspect to consider is that LN is a heterogeneous disease. Therefore, time and magnitude of clinical response may be different, based on the different histotypes and characteristics of the patients. Patients' related factors influencing the outcome of treatment can be of different nature, e.g. genetic, as in the case of certain polymorphisms associated with higher risk of progression in Afro-American patients (137-139).

Figure 5: Synopsis of the current joint European League Against Rheumatism and European Renal Association/European Dialysis and Transplant Association (EULAR-ERA/EDTA) recommendations for the management of LN



CS: corticosteroids; 6-MP: 6-metyl prednisolone; MMF: mycophenolate mofetil; MPA: mycophenolic acid; CYC: cyclophosphamide; NIH: National Institutes of Health; TAC: tacrolimus; AZA: azathioprine; CNI: calcineurin inhibitor; HCQ: hydroxychloroquine; ACE: angiotensin converting enzyme; ARB: angiotensin II receptor blockers LMWH: low-molecular weight heparin; ASA: acetyl-salicylic acid; OAC: oral anti-coagulants; APL: anti-phospholipid antibodies; APS: antiphospholipid syndrome

*at equivalent dose. Data suggest comparable efficacy ; # especially recommended for acute kidney injury, and cases of histological findings of cellular crescents and/or fibrinoid necrosis; 0.5-0.75 g/m^2 of BSA (body surface area); ^in cases of proteinuria within nephrotic range; *in case adjusted according to renal function (GFR); ** Tektonidou M, et al ARD 2020; *** Complete clinical response (CRR) proteinuria at 12 months 500-700 mg/24hours; partial clinical response (PCR) improvement of proteinuria (with normalization/stabilization of renal function) at 3 months and at least 50% reduction of proteinuria at 6 months; frames extended to 6-12 months if starting condition is nephrotic range proteinuria

(source: Fanouriakis A, *et al.*, ARD 2020)

3.8 Rituximab

Rituximab (RTX) is a chimeric mouse-human monoclonal antibody, structurally belonging to the IgG1 subclass of immunoglobulin G. Its molecular target is the surface antigen CD20, a pan-B-cell marker, expressed by committed B-cell precursors through subsequent maturation stages, and progressively lost at the terminal stages of differentiation (Figure 3). The molecule is expressed both by normal and by pathological B-cells within neoplasms (140). CD20 is a transmembrane molecule whose function is not entirely understood, but it appears to function as a calcium channel, and in participate to BCR activation, colocalizing with it in areas of the cell membrane rich in glycosphingolipids, denominated lipid rafts (141).

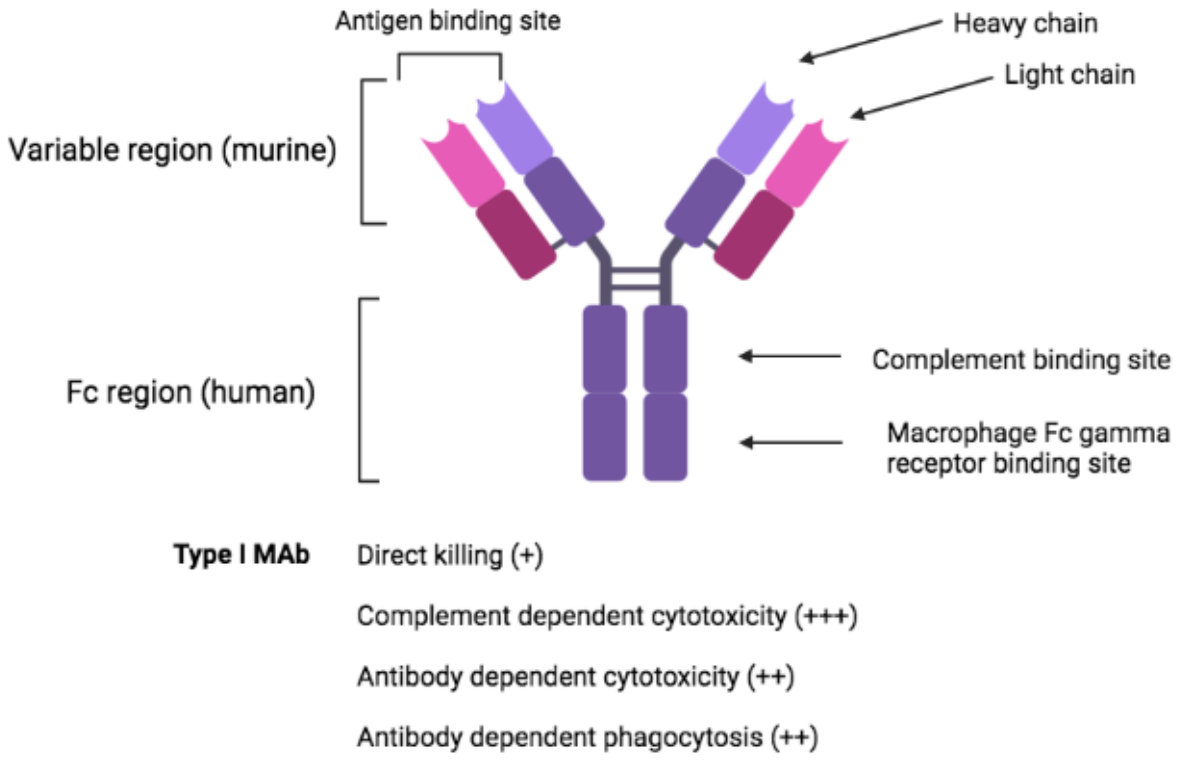
RTX today has a wide spectrum of clinical applications, which comprise approved use for haematological malignancies (non-Hodgkin's lymphoma and chronic lymphocytic leukaemia), RA, ANCA-associated vasculitis and pemphigus vulgaris (142-146). Aside from the canonically approved applications, RTX is used off label for a plethora of other immune-mediated diseases, including SLE and multiple sclerosis (MS) (147, 148).

RTX use in SLE has faced controversial moments, beginning with the two randomized clinical trials (RCT) meant to lead to its approval, but failing to meet their primary endpoints. The two trials, known as EXPLORER and LUNAR were designed to evaluate RTX use in non-renal and renal SLE respectively (149, 150). The reasons for the failure of the two trials have been widely discussed in the research community, highlighting the need for better trial design, and for reconsidering how to define endpoints for a biologically and clinically complex disease such SLE. Despite the disappointing results of the two trials, post-hoc analysis revealed a capacity of the drug in preventing major flares, and in the following years a large body of real-life data has accumulated, leading to the current recommendation of using RTX as a rescue therapy (122, 151).

3.8.1 Pharmacodynamics and pharmacokinetics

The structure of RTX is that of a chimeric IgG1k, carrying 75% of human and 25% of murine aminoacidic sequence. The murine part is located in the antigen binding fragment (Fab). The Fc fragment contains the site of interaction with the complement and the terminus of the molecule interacting with the Fc-γ-Receptor (Fc-γR), responsible for both the action and catabolism of the drug (see Figure 6). The mechanisms of action of RTX are deemed to be multiple, with complement dependent cytotoxicity (CDC) being the prevalent one, followed by antibody dependent cytotoxicity (ADCC), while direct killing is less relevant. Structural studies have enabled characterization of the dynamics of RTX interaction with its target, and with the complement, in comparison to other anti-CD20 monoclonal antibodies. To carry out CDC, RTX binds to CD20 which is present as homodimers in the cell membrane, and the steric interaction promotes a circular concatenation of six RTX molecules and six CD20 homodimers, with each CD20 bound by two RTX molecules (Fab). In this way, the Fc fragments of RTX are in an adequate conformational status which allows interaction with the complement fraction C1q, a hexa-heterodimeric structure, which upon this interaction initiates the activation of the complement classical pathway (152, 153).

Figure 6: Basic structure of Rituximab

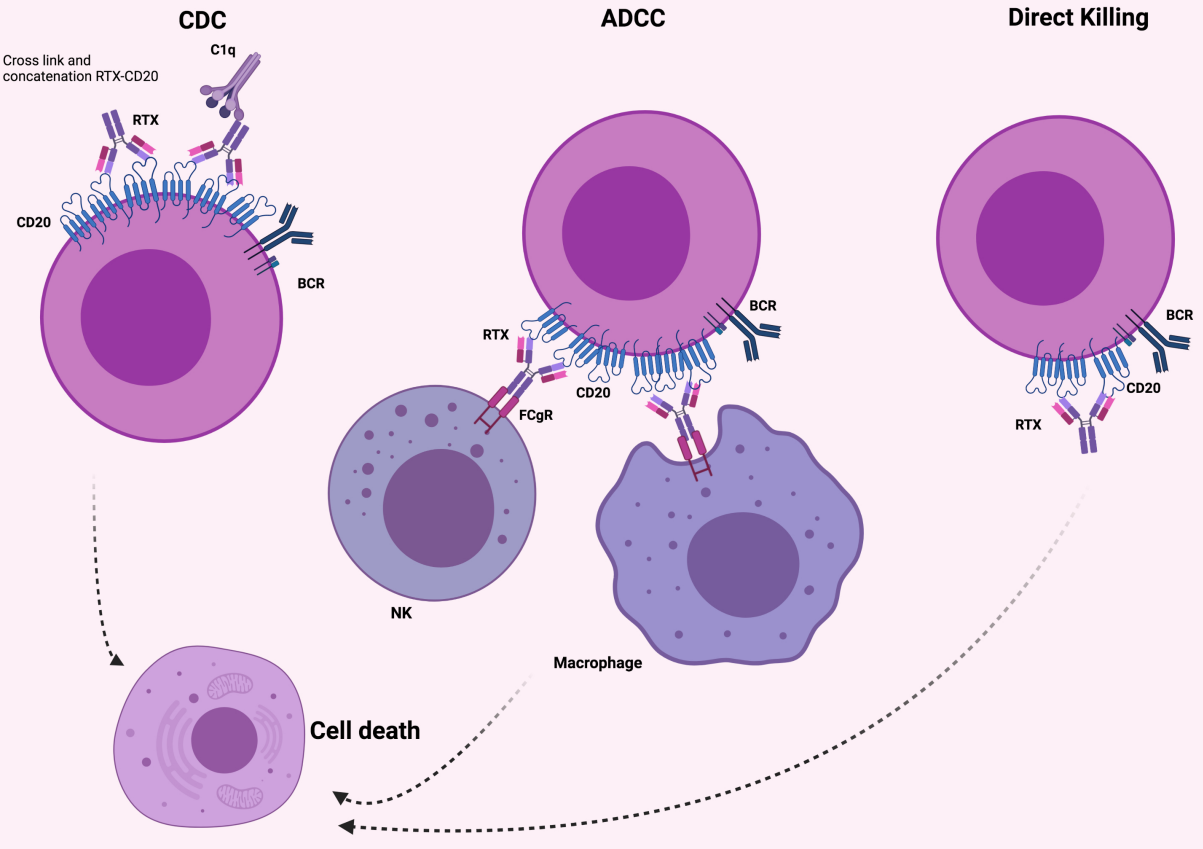


The basic molecular structure of RTX is of an IgG1 antibody. *Created in BioRender.com*

The diverse mechanisms of action of RTX are depicted in Figure 7. The efficiency by which RTX can carry out its cell lysis activity can be influenced by several factors. On the target cell side, there is the level of expression and density of the CD20. On the patient side, the availability of complement, fundamental to RTX action, may be a factor limiting its efficiency. It has been clearly demonstrated in leukaemia that RTX consumes large amounts of complement very soon after its administration (154). On the other hand, for the ADCC, polymorphisms in the Fc-γRIII (CD16) may influence the affinity of interaction of the drug with this receptor, influencing the magnitude of ADCC, an element that appears relevant in SLE (155, 156).

Data on the pharmacodynamics of RTX in SLE are scarce. Older studies in RA showed that the pharmacokinetics of RTX is not influenced by concomitant administration of anti-rheumatic drugs such as MTX or CYC. Moreover, the half-life of RTX was of about three weeks and the drug showed slow clearance and low distribution volume (157). In SLE, serum drug concentrations were found in general to be dose dependent, but also to vary largely among individuals (158). The drop in B-cells was demonstrated already after the first infusion, and B-cell levels remained low throughout the time of observation up to one year (150). The maintenance of RTX blood levels, its tissue distribution, recirculation and degradation are dependent on the interaction of its Fc fragment with one particular Fc-γR, the neonatal (FcRn) one or Brambell receptor. By this receptor, RTX is internalised by several types of cells by pinocytosis. Depending on the intracellular trafficking following internalization, the drug can be either again released in the blood or the intercellular space, or undergo degradation (159).

Figure 7: Mechanisms of action of Rituximab



RTX kills the B-cells expressing CD20. CDC: complement mediated cytotoxicity; cross-link and concatenation of RTX-CD20 creates the conformational structure which gives access to C1q; ; ADCC: antibody dependent cytotoxicity; through the FCγR, natural killer (NK) and macrophages bind the FC fragment of the drug and start their killing action; Direct killing: the interaction with the CD20 directly induces the cell into apoptosis; *Created in BioRender.com*

3.8.2 Efficacy and safety

A phase I/II dose escalation trial showed promising results in terms of clinical improvement and safety of RTX, and evaluated the immunological response induced by the drug. In this study patients achieving profound B-cell depletion showed significant reduction of the disease activity in the short term and this improvement was maintained at one year (158). In the two main RCT, although efficacy could not be proved, the administration of RTX was in general well tolerated; no higher frequency of adverse events, consisting in infusion reactions or infections, was registered in the treatment arm (149, 150).

Before and after the publication of these studies, several other studies investigated the efficacy and safety of RTX in SLE. These studies diverge in many aspects, from study design and number of patients enrolled, to modality of RTX use (alone or as add-on with other medications) and dosages of RTX. Moreover, definitions of clinical response adopted in the studies can be diverse. Taken together, these elements make it difficult to draw conclusions on the real effectiveness of RTX, especially in the setting of LN (160). Nevertheless, the use of RTX in real life in Europe represents a small but significant part of therapeutic arsenal currently

applied to control the disease, with differences often reflecting different regulatory approaches, rather than evidence (161).

Single-centre experiences, multicentre studies and data from registries provide the available real- life evidence of the utility of RTX in SLE, in terms of clinical response, surrogate measures of efficacy, immunological effects and safety.

In an early open study by Leandro et al. (162), a significant reduction of the disease activity score was achieved at six months in a small cohort of 24 SLE patients, refractory to standard treatments. This finding was subsequently confirmed in more recent reports. A recent retrospective analysis of 115 patients from the same academic centre confirmed efficacy in terms of significant improvement of the disease activity measures after the first and subsequent cycles of treatment (163). Moreover, data of the British registry on the use of biologics in SLE found as many as half of the patients responding at three and six months from treatment start. In addition, the authors observed significant improvement in the SLEDAI-2K in about 70% of the patients, a finding in line with previously reported figures in the French registry (164, 165). Other registries, such as the German GRAID (German Registry of Autoimmune Diseases), reported an efficacy rate of 46.8% as defined by physician's judgement, together with significant improvement of the SLEDAI score (166). High response (62.9% at six months) was also reported by a retrospective analysis of a multicentre study conducted in Spain (167). As surrogate measures of efficacy, the possibility to reduce over time the consumption of corticosteroids has emerged as a possibility from register studies (164).

In addition, several studies have considered as an element in favour of RTX therapeutic potential, the measurable effects on immunological parameters indicative of SLE activity. A reduction of the titres of anti-dsDNA antibodies and an increase of the levels of complement fractions have been observed in several studies (162, 168, 169).

Concerning the safety aspects of RTX use in SLE, the larger studies (multicentre and registries) indicate that RTX is highly tolerable, with a global rate of infusion reactions and low figures for what concerns the occurrence of severe infections (165-167).

Some particular safety aspects have received more attention in recent years, which concern late infusion reactions and their possible relation to the immunogenic potential of the drug. Moreover, late onset neutropenia (LON) has emerged as a possible side effect of RTX. These two types of safety concerns are of specific interest for this thesis and are examined in more detail in sections below.

3.8.3 Efficacy in LN

The question of whether RTX is effective in LN is of particular interest. The available literature offers a large amount of data, although the studies are considerably different in design, interventions, evaluation of RTX as monotherapy or in combination, and in modalities of assessment of RTX effects and renal response. Therefore, comparing results among studies and drawing conclusions about the real efficacy of RTX in LN is once again complex and difficult.

Nevertheless, interesting observations have been made by several authors, and encompass the evaluation of clinical efficacy as well as the correlation with immunologic and histopathologic effects. The analysis of the first seven patients with proliferative LN treated in the Karolinska SLE cohort showed improvement of the histological activity and a change in histopathological

class at repeated biopsy (170). Moreover, interstitial infiltrates examined at renal biopsy showed a reduction of B- and T-lymphocytes in the tissue, concomitant with peripheral B-cell depletion. A following report, in which the local cohort was pooled with the cohort of another academic institution, suggested even a benefit in class V LN (171). A later analysis of the first 25 refractory LN patients confirmed efficacy in terms of significant reduction of proteinuria, reduction of the activity index at repeated kidney biopsy, and change of the histological class from a proliferative to a mesangial form, in a considerable number of patients. Moreover, this study linked clinical response with the duration of B-cell depletion (172). The utility of RTX treatment in class V LN was more recently highlighted by the observation of a major degree of immune deposit resorption in RTX-treated patients, compared to patients undergoing conventional therapies (173).

Only a few randomized studies have been conducted in LN, aside from the LUNAR study, leading to conflicting results (160). Some studies have directly compared RTX as monotherapy against other standard treatments, namely CYC and MMF. In one of these studies, RTX produced complete remission in about 70% of the enrolled patients, a result comparable with the rate of response observed for the other two drugs under investigation. The authors also highlighted how baseline characteristics of the RTX-treated patients were less favourable with respect to the other groups of patients (174).

Given the relevance of corticosteroid side effects in the particular SLE subgroup with LN, a study named RITUXLUP was undertaken to explore the possibility of inducing remission with RTX and avoid oral corticosteroids. In the protocol, RTX 1 g two weeks apart was administered together with corticosteroid pulses, followed by oral MMF with no further oral corticosteroids. The report on the first 50 patients treated with this approach, showed a rate of complete remission of 72% at 36 weeks of follow up (175). Unfortunately, further patient recruitment proved challenging in this study, leading to early interruption and thus lack of confirmation of the preliminary results.

It has been argued that, considering the absence of reliable conclusions in several available studies and the pitfalls in study designs, new randomized clinical trials should be encouraged. Learning from the mistakes of the past and developing more appropriate trial designs could help better define how to use RTX in LN and for which categories of patients to administer it to, a question still unanswered after two decades of use of the drug (160).

Meanwhile one further matter has been investigated, which is those of the possible benefits of combining RTX with Belimumab. The biologic rationale for this is to be found in the rise of BAFF under B-cell depletion, which promotes the reappearance of transitional B-cells in the peripheral blood. Preliminary analysis of data from a phase II clinical trial did not show a clinical benefit adding Belimumab to RTX in association with standard of care. However, safety data showed the combination as feasible. The immunological changes showing a decline in the rate of autoreactive B-cells in the Belimumab arm so far found no clinical correlate (176).

3.8.4 Immunological consequences of RTX use in SLE

The administration of intravenous RTX is rapidly followed by a decline of the peripheral B-lymphocytes expressing on their surface the marker CD20. The consequences of B-cell depletion and the reconstitution of the peripheral B-cell pool (repopulation of the peripheral blood) have been studied in the different diseases for which RTX has been tested.

In general, B-cell depletion (BCD, defined as the reduction of B-cell counts below 5 cells/microliter or a percentage of CD19+ cells below 0.5) is maintained for periods ranging around 6-9 months, and repopulation (i.e. the return of B-cell counts to the pre-treatment levels or in any case above the threshold for BCD) occurs at around twelve months from RTX start (177).

Since the target molecule CD20 is expressed by B-cells at defined maturation stages, not all the cells composing the B-cell pool are susceptible to the killing action of RTX, which indeed spares the early stages (pro-B-cell) and the very late stages of B-cell differentiation (plasma cells). Moreover, the degree of CD20 expression decreases in pre-plasma cell stages, making cells at these points of the B-cell ontogeny less effectively depleted, if not resistant to depletion. For this reason, it is naïve B-cells that are the most affected by RTX action, and a drop in their counts is invariably observed in all the diseases for which the drug is used (177).

In patients treated for lymphoma, it has been demonstrated that the reappearance of B-lymphocytes in the peripheral blood is dominated by the inflow of immature cells with features of antigen unexperienced cells (transitional phenotype IgD-CD27-CD38^{high}CD24^{high}), with a less represented presence of memory B-cells in the periphery. This dynamic is similar to the normal ontogeny of the B-lymphocytes and in line with what has been observed during the immune reconstitution that follows bone marrow transplantation. However, while in normal ontogeny memory B-cells progressively accumulate in the peripheral blood, RTX-treated patients exhibited a prolonged reduction of memory cells with a delay in the replenishing of this compartment. This dynamic was particularly prolonged in a subset of patients with normalization of autoantibody titres and long-lasting clinical effect. It has been questioned whether such dynamic might have consequences in terms of safety of repeated cycles of RTX (178, 179).

In the field of autoimmune diseases, where RTX has found large use, the regeneration of the B-cell pool has also been investigated. In RA the reappearance of B-cells in the peripheral blood has been clearly shown to be dominated by immature cells, with naïve B-cells reaching pre-treatment levels after about one year from RTX infusion. By contrast, memory B-cell recovery was delayed for several months with this phenotype still as high as 50% of the baseline levels up to two years after treatment (180).

Studies concerning how RTX affects the peripheral B-cell homeostasis in SLE patients have been conducted over the last two decades. It should be kept in mind that the rationale of BCD is to promote a renewal of the B-cell pool both in the peripheral blood and in the lymphoid organs and bone marrow, that leads to the inflow of new clones less enriched in autoreactivity and leads to a rebalance of the disturbances of B-cell homeostasis which characterize the disease. Early observations in a phase II trial have highlighted how an efficient BCD is linked to the reappearance of a peripheral B-cell pool with restored abnormalities. In fact, while pre-treatment lymphopenia was mainly attributable to a reduced quote of naïve cells, reconstitution after efficient BCD was associated to increased counts of naïve cells close to healthy control levels (181). In this study, the authors detected for the first time in SLE patients a population of IgD-CD27- double negative (DN) B-cells, which later was better characterized in the disease as mainly belonging to the memory compartment (73). This latter study provided insights into the phenotypical and functional features of these cells, demonstrating that they are antigen-experienced, able to be activated upon BCR stimulation, and with a chemokine receptor profile which is indicative of a migration capacity directed towards sites of inflammation. Further

characterization of DN cells has been performed by more recent studies, highlighting how a particular sub-phenotype, denominated DN2, is prevalent in SLE (87).

The dynamics of the B-cell subsets after RTX have been investigated in depth by several studies. Comparing such dynamic changes in SLE and RA, similar patterns of reappearance of the B-cell pool in the peripheral blood have emerged, with comparable elevation of BAFF levels during the status of depletion. APRIL levels were affected by RTX treatment in SLE undergoing a significant reduction concomitantly to peripheral blood depletion, a change not observed in RA (182).

Different studies concur in the finding that plasma blasts are the phenotype dominating during B-cell depletion. However, persistence of naïve and memory B-cells is also described. Moreover, baseline levels of B-cells are indicative of the duration of depletion and early reappearance or persistence of memory B-cells and plasma blasts associated with the occurrence of a relapse. An association with clinical response achievement and baseline levels of plasma blasts has also been described (183-185). Also, in SLE convergent observations testify that repopulation is dominated by an immature phenotype of B-cells (181, 184).

4 SPECIFIC BACKGROUND OF THE THESIS PAPERS

In this section, the scientific literature background of the papers included in the thesis is presented.

4.1 Introduction to the concept of biomarker

A biomarker is a biological characteristic of a given pathological process that can be objectively measured and used to indicate the presence of the process itself, its evolution, and possibly the modification of such process upon treatment. An ideal biomarker is thus usable for diagnostic and prognostic purposes, and for evaluation of treatment response. Given the complexity of the majority of the pathological processes taking place in nature, a single biomarker which encompasses all the above-mentioned features is rather difficult to identify. Therefore, research should aim at identifying several forms of biomarker that could reflect different aspects of the pathologic events under examination, and could be used combined in panels to express diversified significance and inform clinical evaluation and decision-making.
In this thesis, the general core of the papers presented, is the search for biomarkers that could reflect efficacy and safety aspects of rituximab treatment. In addition, in lupus nephritis we searched for biomarkers that could be used in the near future for diagnostic and prognostic purposes, and for monitoring disease activity and treatments effects. The implications of Papers I and II go beyond the sole concept of biomarker, and also include pathogenic aspects of SLE and pharmacological features of RTX. In Paper III, safety aspects are the focus. In Papers IV and V, the explored biomarkers of LN have relevant pathogenic implications.

4.2 Specific background of Paper I: Newly described lymphocyte subsets

Advances in B- and T-cell biology have led to the definition of new subsets of lymphocytes which are relevant to the pathogenesis of SLE, and other immune-mediated diseases. Important acquisitions have also been provided by the study of immunological effects of RTX. In this context the subset of memory B-cells, denominated DN, has for the first time emerged (181).
Paper I relies on acquired knowledge, provided by previous studies on immunological changes of the B-cell subsets induced by RTX in SLE, and investigates whether RTX influences more recently defined subsets, in both the B- and the T-cell compartment.
The cellular subsets of interest for this paper are the age-associated B-cells (ABCs) within the B-cell compartment and the DN, and the T_{FH} and T_{PH}, within the T-cell compartment. Given the growing body of studies, the interest on these new phenotypes, and the lack of knowledge on how they are affected by B-cell directed therapies, we undertook the study described in Paper I.

ABCs

The ABCs have been extensively studied in mice, the species in which they were first described by two groups of researchers almost at the same time. The isolation of these cells in aging mice is the reason for the adoption of the moniker ABCs. A complete description of their characterization in animal models goes beyond the scope of the present literature review. However, some studies will be here mentioned in light of their translational relevance for human pathology.

The first description of the ABCs was provided by Hao et al. (186), who described a population of B-cells accumulating with age among mouse mature splenocytes, which they phenotypically characterized as CD21-. The cells were demonstrated to be weakly activated upon canonical stimulation of the BCR and CD40, but to show maximal activation upon synergistic engagement of both BCR and TLRs (namely TLR-7 and TLR-9). The authors defined some functional properties of the cells, including the ability to evolve into antibody-forming cells, and the capacity of producing cytokines, and of acting as APCs. In this work it was even pointed out how ABCs might derive from mature ancestors within the B-cell pool, and how they do not seem to represent an aberration of the fate of normal B-cells but accumulate over time during immune senescence. In parallel, Rubtsov et al. (187), working on the sexual dimorphism typical of autoimmunity, demonstrated the age-related accumulation of a population of CD11c+ B-cells in wild type female mice, and the major expansion of them in strains of lupus-prone mice. In the same study, they could translate their findings in humans, detecting their presence in female patients with autoimmune diseases. Moreover, these cells, expressing the integrin CD11c+, were demonstrated to be able to secrete autoantibodies, to be activated upon engagement of the intracellular sensor TLR-7, and to possess a transcriptional programme led by the transcription factor t-bet. The relation with autoimmunity was further explored in a subsequent study on the lupus mouse model Mer -/-. This mouse strain, lacking the expression of the tyrosine kinase Mer, develops spontaneous lupus-like disease with production of antibodies to nuclear antigens. The authors demonstrated the dependency of ABCs accumulation on the expression of TLR-7, showing different frequencies of ABCs depending on the presence or absence of the expression of the TLR-7 gene (188). A similar approach was adopted in a recent work in which ABC accumulation in relation to TLR-7 expression was studied in a double knock-out mouse model (DKO) lacking the expression of the proteins DEF-6 and SWAP-70 (SWEF), whose role is to counterbalance the activity of the transcription factor IRF5 (Interferon Regulatory Factor 5) (189). This transcription factor signalling was shown to be critical for the accumulation of ABCs in the SWEF DKO mouse, and thus to be a possible regulator of ABCs, alternative to t-bet, which has in any case been shown to be fundamental for ABC generation and autoimmunity related pathology in mice (190). Another interesting observation, which emerged from the study of murine models, is the ability of ABCs to interact with T-cells in the GC, which leads to aberrant T_{FH} generation and impaired selection of high-affinity B-cell clones, in conditions of excess of ABCs. In particular, in a mouse model known as $Ship^{\Delta B}$, ABCs are detectable in high proportions in younger mice, with no evidence of aberrant T-cell activation. Only later in life do the mice show aberrant T-cell activation and T_{FH} deregulation, which could be experimentally linked to excess of ABCs and their activity of APCs. Moreover, in human SLE, a positive correlation between ABCs and T_{FH} in peripheral blood was shown (191).

Moving our attention to human studies, things become complex. In general, accumulating evidence points towards a correspondent relevance of ABCs in human immune senescence, autoimmunity and chronic infections, all settings characterized by chronic antigenic stimulation (86). In this respect, the feature of the association with age loses its centrality and leaves room to the concept of reiterated antigenic stimulation.

Based on current evidence, ABCs are a cellular phenotype which can be included in a larger (and yet to be fully dissected) spectrum of atypical/non-canonical memory B-cells, as earlier described. One aspect of complexity in the field derives from the multiple phenotyping approaches used to characterize these cells.

In humans, ABCs are mostly characterized as CD11c+CD21- within the CD19+ B-cells. They show features of antigen experienced (isotype switched) cells, which suggests their

emergence from T-cell driven immune responses although formal demonstration of this interaction in vivo is missing. Nonetheless they appear to remain as memory B-cells, or at least to belong to a spectrum of phenotypes of non-canonical memory B-cells. Their exact progenitor has not yet been identified, although their evolution appears once again dependent on TLR-7 stimulation and on further signals such as IL-21 and INF-gamma stimulation (86).

In human SLE, the studies focusing on dissecting B-lymphocyte subsets converge towards the identification of ABCs within the subset of DN cells, described to be expanded and to correlate with disease activity (73). Further interrogation of the DN compartment has permitted identification of two major subsets within the DN compartment. A first subset, denominated DN1 is largely represented in healthy individuals, while a second, denominated DN2, is predominant in SLE patients, especially of Afro-American ancestry. This DN2 subset shares phenotypical and functional features of the ABCs. In fact, it is defined as DN CD11c+CD21-, shows activation upon TLR-7 stimulation, and is transcriptionally regulated by t-bet. Moreover, CXCR5, a chemokine receptor critical for homing into the GC is lacking on DN2 cells, suggesting they are part of an evolutionary pathway addressed towards extrafollicular immune responses, which appear relevant in SLE. In this context, DN2/ABCs appear as an intermediate of a differentiation pathway, in which ABCs are precursors of antibody forming cells and they derive from naïve activated cells, as suggested by transcriptomic analysis showing how a single evolutionary trajectory can be traced within these phenotypes. Their fate towards the generation of plasma-cells is regulated by activation via TLR-7 stimulation and the action of IL-21 (87). In the context of autoimmunity, ABCs are enriched in autoreactive clones and are poised towards a migratory phenotype, as suggested by their asset of chemokine receptor expression (CD11c, absence of CXCR5). They correlate with disease activity and specific organ manifestations. Moreover, they can be identified in affected tissues such as skin and kidney (192). In a recent analysis of kidney tissue, with single cell transcriptomic technique, active disease was found to be associated with the expression of 21 subsets of leukocytes, and among B-cells by a dominating ABC profile (118).

In a recent longitudinal analysis of B-cells conducted by our group with mass cytometry on Belimumab treated SLE patients, the decline of ABC-like cells (defined as CD11c+CD21-), together with switched memory IgA+ B-cells, was associated with response to treatment. By contrast, these cells remained unaltered in non-responders (193).

Using the same method of analysis, a recent study performed a detailed analysis of CD11c+ B-cells in human blood, comparing SLE patients, Sjögren's patients and healthy controls. A significant expansion of CD11c+ B-cells was found in SLE, which confirms flow cytometry observations. The study revealed, however, that CD11c+ cells, although abundant in the memory compartment, are not exclusively clustering with memory cells. Examination of the B-memory compartment, revealed that CD11c+ cells cluster among the DN cells. Moreover, when examining CD21 expression, CD11c+ cells were intimately connected with CD21- cells, and an enrichment could be found among CD21-CD27- in SLE. This study also evaluated the expression of check-point molecules, as PD-1 and PD-1ligand on CD11c+ B-cells, and could identify a unique profile of co-expression of this type of marker in SLE patients (194).

T_{FH} and T_{PH}

In the context of physiological immune responses, and by extension also in pathological immune activation, the interaction of T- and B-cells is crucial for the generation of a progeny of efficient antibody-producing cells. These interactions take place in secondary lymphoid

organs (such as the germinal centres (GC) of the lymph nodes) or outside of the lymphatic system, e.g. in the mucosae, where immune cells aggregate without a containing structure (such as the lymph node capsule), but form GC-like structures. This second case corresponds to what is called 'extrafollicular response'. The follicular and extrafollicular B-T cell interactions lead to the generation of two different variants of humoral response, which differ in terms of the cells and the antibodies which are generated. In fact, while classical GC interactions generate highly mutated and high affinity antibodies and long-lived plasma cells, extrafollicular responses lead to the generation of short-lived plasma cells which produce antibodies with lower affinity and limited degree of somatic mutations (94).

The concept of the need for the B-cells to receive the help of T-cells in order to undergo terminal differentiation is not new in immunology. However, the subsets of T-cells responsible for such interactions have been defined in the last two decades. It was in the first years of this century that T_{FH} were first identified, and they were progressively characterized as a distinct subset over the first decades (195-197). These cells localize in the lymph nodes following a chemokine gradient. The GC stroma is in fact rich in CXCL13, which attracts the cells expressing the CXCR5 homing receptor, as is the case of the T_{FH}. Once in the GC area of the lymph nodes, the T_{FH} interacting with the B-cells will induce their evolution into antibody forming cells. This means that the B-cell will activate the molecular mechanisms responsible for class switching, affinity maturation and somatic hypermutation which precede their evolution into memory cells or plasma blasts and then plasma cells. The communication between B- and T-cells takes place through cell-cell contact via ICOS and through the production of IL-21 (198).

Over the years, the studies performed have revealed that T_{FH} are extremely plastic cells, capable of adapting to the environment in which they evolve, activating multiple transcriptional factors and producing a wide range of cytokines. In virtue of this property, T_{FH} show common features with other subsets. This has led to sub-classification of T_{FH} based of the cytokine profile they share with other subsets (199).

The relevance of T_{FH} in SLE has emerged from several studies. These cells are phenotypically defined as CD4+PD1^{high}CXCR5+, and can be further characterized by the expression of the costimulatory molecule ICOS and the intracellular expression of the transcription factor bcl-6, which functions as master regulator of transcription in these cells (198).

Frequencies of T_{FH} cells have been investigated in patients with SLE and in relation to clinical features. Translating into human SLE the observation made in a mouse model which exhibits spontaneous formation of GCs and high frequencies of T_{FH} in blood, CD4+CXCR5+PD^{high} were identified in a subset of SLE patients. The presence of these cells was associated to the presence of clinical manifestations such as LN, thrombocytopenia and thromboembolic events. Moreover, higher T_{FH} frequencies correlated with titres of anti-dsDNA antibodies and with the presence of anti-phospholipid and anti-ENA (200). A correlation with titres of autoantibodies was confirmed in further studies (201). Other studies confirmed such observations and found correlation of T_{FH} and of expression of the marker PD-1 with disease activity. A correlation with disease activity and peripheral expression of DN B-cells was found for the phenotype denominated T_{FH2} (202). Moreover, dynamic changes in the peripheral blood frequencies of T_{FH} were observed upon corticosteroid pulse therapy (203).

In SLE, the events taking place in the inflamed tissues are deemed critical for the pathogenesis of the disease and the production of organ damage. Therefore, the characterization of extrafollicular T-B cell interactions is extremely interesting. Recent works have identified a new phenotype of cells which are relevant to such interactions. The first

characterization of these cells, for which the name T-peripheral helper (T_{PH}) has been created, has been provided by a study dissecting the cellular composition of synovial infiltrates in RA. T_{PH} are phenotypically defined as CD4+PD-1^{high}CXCR5- (204). These findings have been subsequently translated into SLE. In an excellent study, T_{PH} cells frequencies were found to be correlated with disease activity and presence of a CD11c+ peripheral blood B-cell population. On a functional level, T_{PH} cells were shown to be able to drive plasma cell differentiation in vitro via the secretion of IL-21 (this ability being dependent on the activation of the transcription factor MAF). The authors confirmed the presence of the T_{PH} cells in the cell infiltrates in kidney biopsies, where the abundance of these cells correlated with the abundance of B-cells (205). Support for the concept of extreme plasticity of this subset of cells came in a recent study describing a further phenotype of T_{PH} active in tubule-interstitial infiltrates in LN and able to provide B-cell help. These cells (T_H10) can provide B-cell help by modalities different from T_{FH} and T_{PH}, i.e. independently of IL-21, while releasing IL-10 and succinate, a product of mitochondrial metabolism (206).

4.3 Specific background of Paper II: immunogenicity of RTX, and potential clinical consequences

Immunogenicity can be defined as the property of a given drug of eliciting an unwanted immune response in the organism by which the same drug is received. The result of the activation of such an immune response will be the production of anti-drug antibodies (ADA). For RTX, as for other biotherapeutics, the basis for being immunogenic is that the drug is a protein (hence a structurally complex molecule), which is synthetized by a foreign hybrid cell line. Moreover, being a chimeric antibody, RTX contains sequences of murine origin in its variable regions, which can be recognized by the immune system as foreign epitopes. Also, during the production of the drug in bioreactors, post-translational modifications (taking place in the cell line) may contribute to structural changes with potential creation of new epitopes. In addition, impurities and formation of aggregates, which can elapse between production and clinical use, can be a factor in immunogenic phenomena (207).

The potential immunogenicity of biotherapeutic agents is a known problem that is taken into consideration during drug development, already at a preclinical level. Regulatory agencies require thorough assessment of the immunogenic potential of new-candidate biologic drugs. Furthermore, strategies for monitoring immunogenicity are to be implemented for marketing authorization. Regulatory agencies also instruct pharmacovigilance to aim to collect information on clinical consequences.

Despite several interventions that can be carried out during pre-clinical development, industrial proceedings, and once the drug is on the market, biologic drugs still have a residual immunogenic potential. This is intrinsic to their nature of proteins, as explained above, but can also depend on other factors. The development of humanized and fully human monoclonal antibodies represents one of the advancements made to reduce the immunogenic potential of biotherapeutics. Increased attention has been drawn by the introduction of biosimilars into clinical applications (208).

Several factors can contribute to the immunogenic potential of a drug, as depicted in Figure 8.

There are multiple possible consequences of the formation of ADA (Figure 9). In fact, ADA can directly bind to the idiotype, i.e. the part of the drug responsible for the interaction with its molecular target. Alternatively, they can bind in the vicinity of the active site of the drug, and hinder the interaction with the target. This will invariably interfere with the action of the

drug. Those ADA directly binding with the target (neutralizing ADA) will hence prevent the pharmacodynamic effect of the drug. On the other hand, while forming ICs with the drug, ADA will lead to a decrease of the serum concentration of free drug, reducing therapeutic exposure, and ultimately reducing the half-life and accelerating the catabolism of the drug. Hence, formation of ADA has potential pharmacodynamic and pharmacokinetic consequences (208, 209).

The impact on the pharmacological properties of the drug and the mechanisms leading to ADA formation, have potentially clinically meaningful consequences. In fact, hindrance to drug-target interaction and alterations of the pharmacokinetics of the drug can impact on drug efficacy, leading to loss of a detectable clinical effect. On the other hand, the very process of immune reaction towards the drug implies the possibility of immune-mediated reactions to drug re-exposure, with the occurrence of infusion reactions and other adverse reactions.

The principles illustrated above are valid for all biotherapies, and indeed in recent years much research effort has been devoted to investigating the clinical consequences of immunogenicity. In the fields of rheumatology and gastroenterology, the formation of ADA against TNF-inhibitors, has been to a great extent investigated, given the broad use of these drugs in inflammatory diseases.

Regarding the immunogenicity of RTX, the issue has been addressed in a limited manner. Some information has been provided by older clinical trials: here ADA are more often referred as HACA (i.e. human anti-chimeric antibodies). In lymphoma, the reported frequency of HACA was of one out of 37 tested patients in a clinical trial; and it has been argued that the profound immune-suppression due to concomitant chemotherapy may explain such low figures (210). In RA, occurrence of HACA was 2.7-4.3% in the active arms of two RTCs with no evident clinical correlates (142, 211). In ANCA-associated vasculitis, as reported by the FDA prescribing information, 23% of patients enrolled in the RAVE study were positive for HACA, however no clear clinical consequences emerged (143, 212).

In SLE, higher HACA occurrence has been reported. In the phase I/II dose escalation trial by Looney, et al., 64.7% out of 17 tested patients showed positive results, with high titre HACA (>100 ng/ml) found to be associated with Afro-American ancestry, higher baseline disease activity, less efficient B-cell depletion and accelerated clearance of the drug (158). In the EXPLORER trial, 26% of patients in the RTX arm tested positive for HACA. Three of the HACA positive patients developed serum sickness reaction, one of which registered as a serious adverse event (149). In the LUNAR trial's active arm, 15.1% of the patients developed HACA, at a median titre of 113 ng/ml, with one patient developing a severe infusion reaction upon re-exposure to the drug (150).

Real life studies have partly contributed to the understanding of the magnitude of the immunogenicity of RTX. In particular, systemic diseases appear to be more prone to ADA formation. In a recent analysis of data from a French study, 14.7% of systemic disease patients treated with RTX had at least one detection of positive ADA, which was significantly higher than in RA. In this study, systemic diseases were prevalently represented by Sjögren's syndrome, while SLE cases were few. The presence of ADA was associated with infusion reactions at retreatment and loss of clinical effect (213).

A recent analysis of 57 SLE patients initiating RTX, showed a rate of ADA occurrence as high as 37%. The authors detected an association of this finding with younger age at diagnosis and RTX start, with males, and lack of reduction of anti-dsDNA antibody titres. In addition, they detected a clear association between the presence of ADA and the occurrence of infusion

reactions at retreatment, most of them of moderate severity, but with also a few of high-grade severity (214). The detection of ADA to RTX was also found in association with lack of response to retreatment in a previous study, which suggested the switch to a humanized anti-CD20 as a possible rescue strategy for the patients who experienced loss of efficacy, a suggestion advanced also by other authors (185, 213).

Infusion reactions are a common event when RTX is administered. A recent retrospective study found them to occur in almost 6% of all infusions given for SLE treatment. Their severity is variable, ranging from mild to life-threatening, and can reflect diverse putative pathophysiologic mechanisms (215).

Some reactions can occur days after the administration of the drug (delayed adverse reactions). Among this type of reaction is serum sickness. The clinical definition of serum sickness is the appearance of a triad of fever, arthralgia and skin rash at about 5-10 days from an immunological trigger, in this particular case represented by the treatment. This reaction has been reported in several anecdotical cases in the literature, associated with diverse background diseases for which RTX is usually administered (216-222).

On an immunological level, serum sickness is a classic example of type III delayed immune reaction. The mechanistic key to serum sickness development is the formation of circulating ICs which, precipitating in tissues with a permeable capillary network, lead to the onset of an inflammatory response, further amplified by the ability of the ICs to activate the complement cascade and the innate immune cells via the engagement of the FcγR (209).

Figure 8: Factors potentially contributing to the risk of immunogenicity of biological drugs

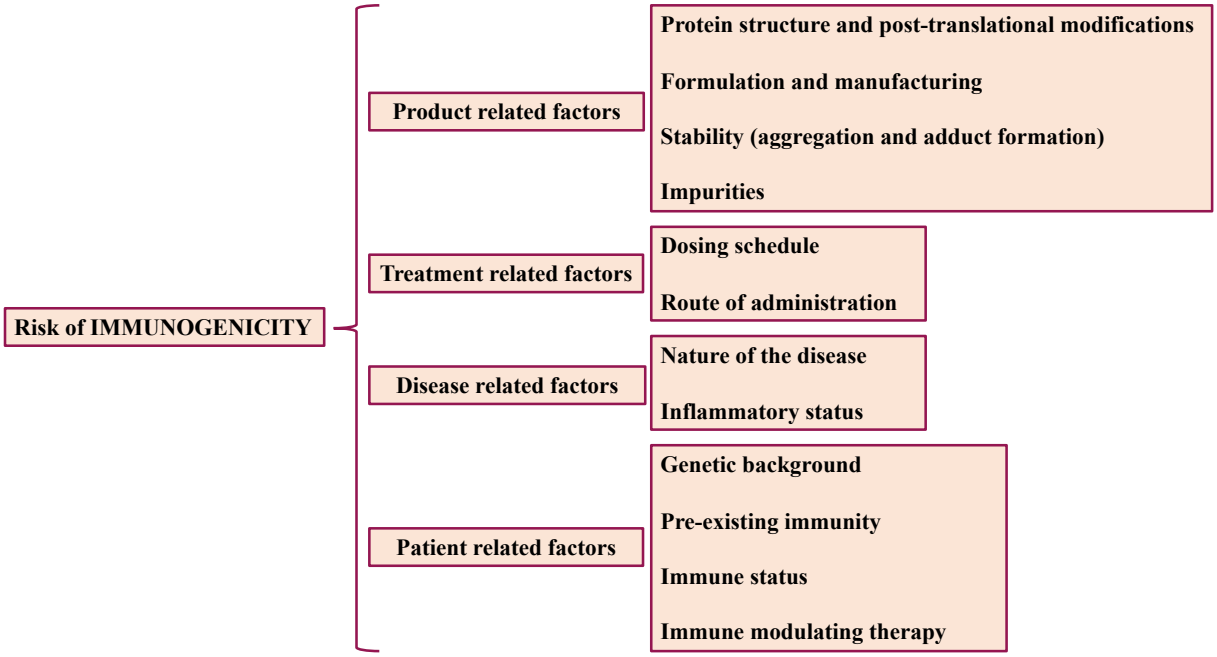
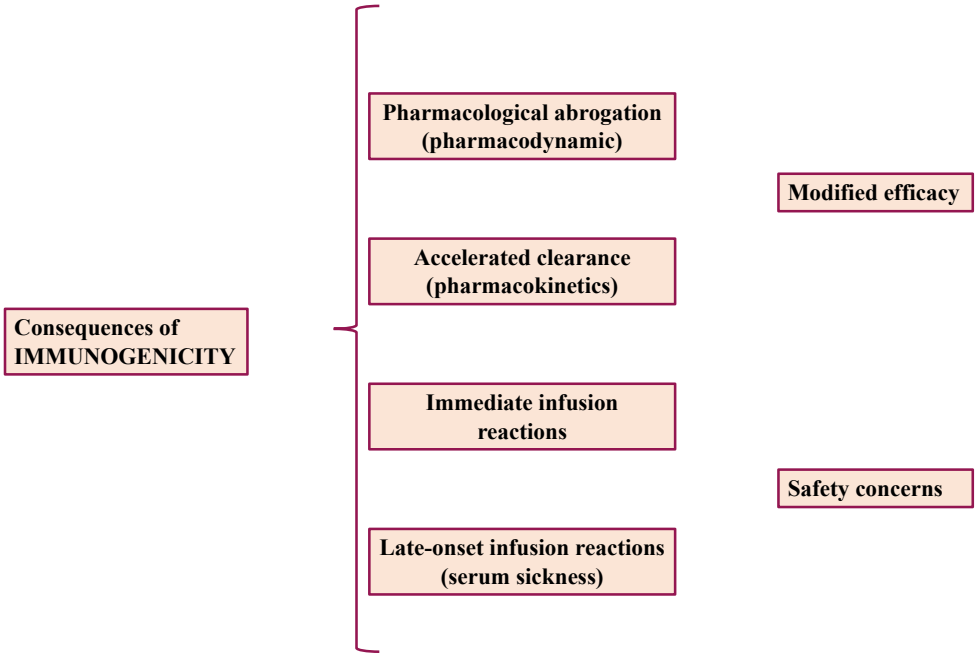


Figure 9: Consequences of Immunogenicity



4.4 Specific background of Paper III: RTX-induced late-onset neutropenia

Late-onset neutropenia (LON) is defined as the occurrence of low neutrophil counts which develop during the follow-up of RTX treatment. In our study it was defined as neutropenia (neutrophil count below 1500 cells/μL) occurring at least one month and up to two years after RTX administration, or even later provided that peripheral B-cell depletion was persisting at the time of detection. To be attributable as RTX-induced, the occurrence of neutropenia must not be otherwise explainable.

This event has been detected since early studies on haematological malignancies. Early clinical trials on lymphoma reported an incidence of 4% for episodes of neutropenia (223). This datum, however, might be underestimated, given the short duration of clinical trials, with respect to longer observation time in real life. Indeed, after the introduction of RTX in clinical use, this entity has been described in the literature in several reports; and at least for haematological malignancies, LON appears to occur in up to 27% of the patients, and at variable median time after exposure to the drug, depending on the stringency of neutropenia definitions used (224-227).

Later on, with the extension of RTX use to autoimmune diseases, LON has been described also in these settings. The event appears to be less frequent among RA patients, with reported incidences of 1.3-4.6%, while it is more represented in other autoimmune diseases, with peaks of around 20-23% in systemic diseases such as ANCA-associated vasculitis and SLE (228-230). In a recent retrospective survey from Sweden, the incidence of LON was as high as 11.9% of the cases of RTX-treated ANCA-associated vasculitis (231). In a newly published analysis of a single-centre retrospective cohort, the cumulative incidence of LON at one year in systemic and glomerular diseases was of 6.6.%. SLE, along with concomitant exposure to CYC emerged as an independent risk factor for the event (232). Due regard being the persistence of a status of B-cell depletion, LON seems to occur in the mentioned studies within a median time from RTX start of 3-5 months (228, 230, 231).

The clinical consequences of LON occurrence have also been investigated. In some studies, rates of hospitalization have been reported high, in association with superimposed sepsis. In other studies, the majority of LON cases are reported as incidentally discovered, with the remaining cases being symptomatic for febrile neutropenia or occurrence of infections requiring hospitalization (230, 232).

The mechanistic insights of LON are intriguing. Indeed, since the first observations of this phenomenon, it was evident that LON cannot be ascribed to a direct cytotoxic effect of RTX on neutrophils or their precursors. Indeed these cells do not express CD20 (224). An immune-mediated pathogenesis was suggested by early reports, but this hypothesis has not been supported by similar findings in subsequent investigations (224, 233). Therefore, much attention has been devoted to exploring how the depletion status and the biology of repopulation of the B-cells may interfere with granulopoiesis. An early investigation suggested a role for the stromal derived factor-1 (SDF-1), which acts at bone marrow level regulating early B-cell development and the egress of neutrophils from the bone marrow. Accelerated B-cell recovery appeared in this study inversely correlated with neutrophil counts, and blood levels of SDF-1 were shown to increase upon RTX treatment, and to correlate with dynamic changes in B-cell counts. These explored reciprocal changes suggested that, while implicated in the dynamics of B-cell recovery, SDF-1 may act on neutrophils by blocking their egress from bone marrow and ultimately producing LON (225). The thorough study of a single case of LON in a patient treated for Waldenström's macroglobulinaemia suggested instead a role for BAFF in LON. In this case, bone marrow histology revealed a depressed granulopoiesis and serial analysis of serum and whole blood cytokines revealed that LON occurrence could be dated approximately at the peak of BAFF levels after RTX. This observation suggested a dynamic of events in which lymphopoieses, competing with granulopoiesis would overcome the latter (234). A very recent study, again conducted in haematologic patients, confirmed an implication of BAFF, which was (together with granulocyte-colony stimulating factors levels, G-CSF) found in higher levels in patients with LON at onset of the complication (227). Genetic factors, namely polymorphisms of the FcγRIIIa, have been shown to correlate with the risk of post-RTX LON in haematological malignancies. The mechanistic correlate of this association would reside in the polymorphisms influencing the efficiency of ADCC killing mechanisms of RTX, due to the expression of FcγRIIIa with various degrees of affinity for the IgG1 antibodies, to which RTX structurally belongs. In the presence of a high affinity variant of FcγRIIIa, a more efficient ADDC may take place. This, in a context of high lysis as in haematological settings, may lead to high release of effector cytokines and granzymes, which in turn would explain neutrophils killing as a bystander effect (235). An influence of this receptor as a determining factor of RTX efficiency in SLE has been suggested for, among other diseases, SLE (156). In a study investigating the role of the polymorphisms of the gene encoding for FcγRIIIa in rheumatic diseases, carrying a certain allele of the gene encoding for this receptor, was associated with an increased risk of LON, which was dependent on the number of alleles inherited. This study included 23% of SLE in the study population. In the population examined (not restricted to SLE patients), LON and the 158V/V allele of FcγRIIIa were associated with longer flare-free survival, an observation which suggests that LON is a side-effect intimately linked to the very efficiency of RTX actions at a pharmacodynamic level (236).

4.5 Specific background of Papers IV-V: urine biomarkers in lupus nephritis

4.5.1 General concepts on biomarkers and urinary biomarkers in lupus nephritis

As explained earlier, the mainstay of LN diagnosis is represented by kidney biopsy. Its value is unquestionable for confirming the presence of renal involvement. Repeating kidney biopsies may help in verifying effects of treatment, and guide decision-making on when to stop treatment. However, the precise role of this practice is yet to be defined (48).

Using other clinical or laboratory parameters that can assist in the diagnosis and management of LN is one of the unmet needs of this condition. This role is demanded for routine analyses such as proteinuria, urine sediment, or renal function tests. None of these is specific to LN, nor are they sensitive enough to inform attentive and successful decision-making. Reaching a proteinuria target of 700 mg per day and below is known to be associated with a better outcome; however proteinuria has some limitations (136). One of these limitations is that it cannot discriminate completely between active inflammation and chronic damage. Other parameters, such as rise in anti-dsDNA and fall in complement levels, may be useful in some patients, but are not a valid tool for all patients (237). Therefore, alternative or complementary biomarkers that could be more informative are greatly needed in LN.

The idea of using urine-derived biomarkers for LN is in fact natural. Urinary biomarkers are likely to reflect pathogenic events taking place in the renal parenchyma. Collecting urine is a non-invasive procedure; and urine can contain several forms of biomarker, from single molecules (proteins, cellular antigens, cytokines) to cells or subcellular bodies (extracellular vesicles/microparticles), to nucleic acids (messenger and non-coding RNA) (238). So far, a growing body of studies has explored several forms of urinary biomarkers in the context of LN. However, their implementation in daily practice has not yet developed, and only a few have been validated in large cohorts of LN patients (239). From a pathophysiological perspective, urinary biomarkers can have two main meanings. First, they can reflect immune pathogenic events taking place in the kidney (markers of inflammation). Secondly, they can reflect the consequent reaction of the kidney tissue to inflammation (markers of damage).

Inflammatory markers can be produced by cells infiltrating the renal parenchyma, and pass into urine. Among this type some have been studied with promising results. An example is Monocyte chemoattractant protein-1 (MCP-1). This molecule is produced upon inflammation by epithelial cells at both the glomerular and tubular levels. In active LN, urinary levels of this marker are higher than in quiescent LN, SLE patients without LN and controls, according to the conclusions of a recent meta-analysis (240).

In study IV, we used some urinary biomarkers for which more extensive literature can be found in relation to renal diseases and LN, as comparators for a new putative biomarker.

These comparator biomarkers were neutrophil gelatinase associated lipocalin (NGAL), osteopontin (OPN), and kidney injury molecule-1 (KIM-1). We also used Galectin-3 (Gal-3), which will be described together with galectin-3 binding protein (Gal-3BP), which was the molecule of interest in our study. NGAL, OPN and KIM-1 have been evaluated in the context of SLE and LN either alone or in combination with other biomarkers. Some of the most relevant studies are described below.

NGAL, is considered to be a marker of tubular injury, since it can be expressed by tubular epithelial cells during damage (241). This molecule, which was initially evaluated in children with LN, has been tested in adult patients with LN in some studies. Its urinary excretion

fraction has shown predictive value for renal flare in a study evaluating NGAL in both serum and urine, using a double design, with a transversal and a longitudinal cohort (242). In a recent study conducted on 109 active LN patients, in comparison with 45 LN patients in remission and healthy controls, NGAL was evaluated in a panel with KIM-1 and MCP-1. Active LN was associated with high urinary levels of the three markers with respect to controls and inactive LN patients. The markers presented association with some clinical features: in particular, KIM-1 and MCP-1 were associated with acute renal failure, while NGAL was associated with proteinuria and presence of nephrotic syndrome. Both NGAL and MCP-1 were associated with leukocyturia. Interestingly, associations with histopathological parameters were also demonstrated. Aside from associations with glomerular pathology, this study found that all three markers were significantly higher in urine samples of patients exhibiting active tubulointerstitial cell infiltrates, with respect to patients with only chronic damage (tubular atrophy and/or interstitial fibrosis) (243).

KIM-1 was investigated in urine samples from LN patients in a study that also evaluated its tissue expression by immunohistochemical staining. In this study, the urinary levels of KIM-1 were higher in patients as compared to healthy controls. Moreover, when examining LN patients stratified as active and non-active at the time of sampling, some differences emerged. Inactive patients had significantly lower urinary levels of KIM-1 as compared to active patients. In addition, tissue expression of the molecule was absent in inactive LN specimens, while active LN patients expressed the molecule at tubular level. This finding is in line with KIM-1 being an inducible molecule, expressed in response to damage. A significant association with active interstitial infiltrates was also found in the study. Moreover, KIM-1 urinary levels at baseline correlated with renal function parameters after induction treatment (244).

OPN is an extracellular matrix protein which is involved in several physiological processes and largely studied in renal diseases (245). This molecule was evaluated as biomarker in LN in a recent study. Here both serum and urine levels of OPN were evaluated in a small cohort of patients, stratified as active LN, inactive LN and SLE patients without renal involvement. Serum OPN levels were able to discriminate LN patients from healthy controls and SLE patients with no renal involvement. However, no differences were evident between active LN and non-active LN. All groups examined presented urinary levels of OPN significantly higher than serum levels, but no differences emerged across groups. This study included very small numbers in each group examined (246). Notwithstanding these results, OPN is a molecule which has been extensively studied in SLE, and more in general in renal pathology. In a recent analysis of the SLICC inception cohort, in which 344 SLE patients were included, serum levels of OPN were shown to be significantly higher than in controls, and to be associated with the presence of LN. Moreover, OPN showed a correlation with disease activity (247).

4.5.2 Galectin-3 and Galectin-3 binding protein

Galectin-3 binding protein (Gal-3BP) was first identified in cancer cell lines by several research groups aiming at identifying ligands of Galectin-3 (Gal-3), which is a protein belonging to the galectins, in turn members of a larger family of proteins (lectins). The galectins are evolutionary-conserved proteins, with the peculiar property of binding beta-galactoside residues expressed by other proteins (248).

The members of this family (to date 15 proteins have been identified in mammals) are expressed by several tissues, and are likely multifunctional proteins. On a structural level, they are characterized by the presence, at the C-terminus, of one or more carbohydrate

recognition domains (CRD), and can be found in diverse conformational configurations based on the number and organization of the CRDs. They can be expressed both intracellularly (in nucleus and cytoplasm), and at extracellular level (this modality known as non-classical pathway) (249). Gal-3 is the most studied member of the galectin family, and was first identified as a surface antigen (denominated then Mac-2), in murine peritoneal macrophages (250).

Gal-3 and other members of the lectin family have been investigated largely in the fields of cancer and immunity, because they mediate several functions, from cell-cell interactions to cell-matrix interactions, that are fundamental for tumorigenesis, growth and progression and metastasis formation(250). Moreover, a large body of literature, has focused on the use of members of this protein family as possible disease activity and prognostic markers (IBD, cardiovascular diseases, thrombosis), and on their structural and biochemical characterization for further utilization as drug targets. A certain number of heparin derived (with no anticoagulant activity) Gal-3 inhibitors have been synthetized and are in various stages of pharmacological development (251).

Aside from cancer biology and broad immunological research, Gal-3 has been investigated in renal pathology and in lupus nephritis. In renal tissue, Gal-3 is expressed prevalently in tubular cells, and an increase of its expression has been demonstrated in vivo after ischemia-reperfusion (249). The protein has been investigated in the context of acute kidney injury, chronic renal failure, diabetic nephropathy, renal fibrosis and other pathological conditions, including inflammatory nephropathies. In this context, Gal-3 has been implicated in the expansion of the mesangial cells (249).

Renal expression of Gal-3 has been investigated in a study conducted on 88 SLE patients with biopsy proved lupus nephritis, in which pathological biopsies were compared to normal kidney tissue (252). The biopsy specimens were prevalently represented by class IV LN (61.4%). Immunohistochemical analysis was able to confirm the expression of the protein confined to tubular cells in normal kidneys, while in SLE specimens, glomerulonephritis was accompanied by the expression of Gal-3 at glomerular level, in the mesangium, in the capillary loops and in the parietal part of the Bowman capsule, with a pattern of staining suggesting intracellular expression rather than extracellular deposition. However, the definition of precisely which cells were expressing the protein was not possible. The levels of intraglomerular expression of Gal-3 correlated with the activity index but not with the chronicity index. Interestingly, Gal-3 directed autoantibodies had been described in patients with LN by the same research group (253).

The first observation of a relevant serum expression of Gal-3BP in SLE individuals in comparison to healthy subjects was made in a study investigating Gal-3 and Gal-3BP as biomarkers in Behçet's disease. In this study, twenty SLE patients were used as comparators (254). In recent years, this finding was confirmed in a larger study in which Gal-3BP was measured both in plasma and serum, in patients from three SLE cohorts (255). Moreover, this second study linked one relevant aspect of Gal-3BP biology to pathogenic aspects of SLE, exploring the role of Gal-3BP as a surrogate marker of type I interferon activity in SLE. In fact, it is widely known that SLE is characterized by what is called interferon signature, and that the interferon signalling is abnormally activated in the disease. Among the proteins whose related genes are interferon-inducible, is Gal-3BP. Another aspect of relevance, which makes Gal-3BP an interesting biomarker to quantify the degree of interferon activation, is that, while the majority of interferon inducible genes codify for intracellular proteins, Gal-3BP is a soluble protein, whose concentration is thus easily measurable in biological fluids (248).

Subsequent studies have focused on the expression of Gal-3BP on circulating extracellular vesicles (EVs). These studies are mentioned in detail in the next section, since they are relevant to the background of Paper V. In brief, Gal-3BP can be found on EVs and localized in immune complexes in LN (256). In a very recent work, serum Gal-3BP and Gal-3 levels were investigated in the context of cutaneous manifestations of SLE. In this study, SLE patients showed a higher serum concentration of the two markers, with respect to controls, while no major differences were evident among SLE patients stratified based on the presence of skin lesions. There was, however, a clear correlation between serum levels of Gal-3BP and disease activity (257).

4.5.3 Extracellular vesicles

One of the most interesting novel acquisitions in the field of cell biology has been the discovery of extracellular vesicles (EVs), membranous structures derived from cells, which can be found in biological fluids. After they were first described, the common interpretation of their biological meaning was that of inert material. EVs were considered to be the equivalent to waste products of cells, or remnants of dying cells generated during apoptosis. This interpretation is no longer valid (258).

EVs can be defined according to their size, cellular origin, modality of generation, content, and function into three main categories (259):

1. Exosomes (30-150 nm in diameter), derived from intracellular organelles
2. Microvesicles (100-1000 nm in diameter), derived from the outer cell membrane
3. Apoptotic bodies (50-5000 nm in diameter), derived from dying cells

It has been recognised that EVs are not simple fragments that cells dispose of, but active players in inter-cellular communications. By means of the release of EVs, cells can in fact exchange molecules, such as lipids, proteins (membrane-bound or cytosolic), and nucleic acids, with other cells. This modality of cell-cell interaction is part of the normal physiology of the organism, but is also present in pathological conditions (259).

In the field of autoimmunity, EVs have gained increasing attention in recent years, and the possible role of EVs as carriers of autoantigens has generated growing research. In SLE, this possibility is attractive, since the identification of the sources of autoantigens which trigger autoimmune responses has several implications. It is relevant for understanding pathogenic mechanisms, and, by virtue of this, for identifying potential targets for therapeutic interventions, or biomarkers for disease identification and monitoring (260).

Early studies have investigated the possible relation of EVs with apoptosis. The generation in vitro of EVs, from culture cell lines in which apoptosis is obtained by irradiation, has allowed to verify that EVs carry nucleic antigens and that these can be bound by specific antibodies. Moreover, adding plasma from SLE patients to the EVs generated in vitro, has shown the formation of complexes with IgG contained in SLE plasma, which has led to the confirmation that, in vivo EVs circulate in form of IC with IgG (261). EVs generated in vitro have also been shown to interfere with the removal of apoptotic bodies by phagocytes. This generates the hypothesis of a role of an excess of EVs in vivo in prolonging cell death and exposing nuclear antigens to secondary necrosis and generation of autoantigens (262). Moreover, exposing pDC and monocytes from healthy donors to apoptotic EVs isolated from plasma of SLE patients with active LN, induces the production of cytokines, and the upregulation of costimulatory molecules. Similarly, the combined stimulation of neutrophils

form healthy donors with such EVs and bacterial lipopolysaccharide potently stimulates the formation of NETs and respiratory burst in neutrophils (263, 264).

Subsequent studies have investigated the presence of EVs in blood of SLE patients, showing that the composition of EVs in SLE patients is different from that of healthy subjects. Aside from amounts of EVs, SLE patients show an abundance of EVs of cell derivation (defined by the ability to bind Annexin-V), with a significant higher load of IgG, IgM, and C1q, which confirms their presence in blood in circulating IC, and therefore their carrying putative autoantigens. Moreover, IgG-positive EVs have been shown to correlate with levels of autoantibodies and complement activation (265, 266). Further qualitative analysis of EVs in SLE has confirmed the diversity in protein content and types of proteins in comparison to healthy individuals and patients with other autoimmune diseases. In such studies, the presence of EVs carrying Gal-3BP has emerged, which has been the subject of deeper analysis. It has in fact emerged that SLE patients exhibit higher levels of EVs carrying Gal-3BP, and that these EVs can be localized in electron-dense depositions in the renal tissue of SLE patients with LN (256, 267). The interest on Gal-3BP is motivated by the fact that the protein is encoded by an interferon-regulated gene, as explained above. Further mechanistic insights have been provided by the same investigators, who have shown that EVs co-expressing Gal-3BP and dsDNA can be generated from peripheral blood mononuclear cells (PBMCs) of healthy donors, using agonists of TRL-9 (268). Stimulating PBMCs isolated from patients with active LN with agonists of TLR-9 together with recombinant IFN-α, produces an increased release of EVs, with a prevalence of EVs co-expressing Gal-3BP and dsDNA. Moreover, the load of single EVs in Gal-3BP is remarkably higher in EVs derived from LN patients, compared to both healthy individuals and SLE patients without nephritis. Blocking IFN-α in these experiments did not affect the process of generation, nor did the simultaneous engagement of TLR-7, which suggests a prominent role for TLR-9 in the generation of EVs in SLE, and particularly in LN (269).

Taken together, these and other evidence reported in the literature, suggest that autoantigens in autoimmune diseases, including SLE, are present not as single biomolecules, but in complexes with other biomolecules and cellular components (260). The cellular sources of these EVs are multiple, and the modality of generation comprise cell death and cell activation.

Given the possibility to detect higher levels and the altered quality profile of EVs in SLE, the question arises as to whether EVs can serve as biomarkers of disease, and show associations with clinical parameters. In a large study on 280 SLE patients and matched controls, blood samples of SLE patients showed a six-fold higher amount of circulating EVs compared to controls. Phosphatidylserine-negative EVs were predominant among SLE patients, with respect to phosphatidylserine-positive ones. EVs from patients were enriched in inflammation and thrombosis markers of derivation from leukocytes, platelets and endothelial cells. The prevalent phosphatidylserine-negative EVs showed significant associations with gender, current smoking, and proinflammatory cytokines (270).

In a recent study, the possibility of tracking larger EVs containing antigens of mitochondrial derivation, has been explored in a large SLE population of 327 patients. In this study, SLE patients were confirmed to express higher amounts of EVs with a cargo of nuclear components and IgG. Moreover, they displayed a population of larger EVs in which mitochondrial components were present. In this large-sized subpopulation, IgG were co-expressed, which suggests that EVs of mitochondrial derivation circulate in the blood of SLE patients in complex with IgG. Hence even mitochondrial antigens might act as autoantigens in SLE. These EVs showed significant associations with disease activity, and the presence of anti-dsDNA antibodies (271).

Another aspect of interest is the possibility of localizing on EVs fractions of the complement cascade, given the relevance of complement activation in SLE. In a recent study, this was explored in ANCA-associated vasculitis. In this setting, EVs expressing myeloperoxidase (MPO, autoantigen in the disease and marker of neutrophils) were studied, and it was found that they could co-express the complement split factors C3a and C5a in a significantly higher proportion than healthy controls. Moreover, while the co-expression of C3a in MPO-positive EVs was not able to distinguish between patients with active and non-active disease, it could identify patients with renal involvement within disease-active patients. C5a co-expressing MPO-positive EVs followed the same pattern, being higher in patients compared to the control group, and being associated with active renal involvement (272).

Moving from the above-described data generated by our group on blood expression of EVs, both in SLE and in small vessel vasculitis, from interesting data from other groups on EVs and, from previous studies on serum biomarkers of LN (273), we proceeded to explore the possibility of detecting EVs in urine of patients with LN, and to test the expression of a series of molecules of interest.

The study of cell-derived EVs in urine samples of LN patients is to date very limited. One approach to evaluating whether EVs in urine might help in identifying patients with LN has been to study EVs derived from non-classical monocytes, which are known to infiltrate kidneys (274). These cells express high levels of high motility group box-1 (HMGB-1), a non-histone protein involved in DNA binding and chromatin regulation, already known to be present in EVs (275). Patients with LN show higher frequencies of circulating EVs which are positive for HMGB-1, with frequencies of 15.7% of these EVs being able to discern patients with LN from patients without the clinical manifestation. High frequencies of EVs carrying HMGB-1 were also confirmed in the urine of LN patients, and could identify patients with active LN (274). Another study has instead focused on another player in LN: the podocyte. Urinary EVs were characterized as deriving from podocytes when positive for both Annexin-V and the podocyte specific protein podocalyxin. Comparing urine samples in LN patients with urine from healthy controls, higher levels of these EVs emerged in SLE patients, and were shown to correlate with clinical parameters of disease activity, immunological features (anti-dsDNA positively and complement levels in an inverse fashion), and with parameters of kidney involvement (proteinuria). In a sub-analysis of patients with histological data available, patients with proliferative forms of LN excreted higher amounts of podocyte-derived EVs in urine, compared to membranous forms, which might reflect different types of podocyte injury in the diverse LN histotypes (276).

We added to the panel of explored cargo molecules two other markers, Axl and TWEAK. Axl is a receptor tyrosine-kinase, whose addition was motivated by the findings of recent work by our group, in which serum Axl was evaluated as a biomarker in LN. In this study, 52 patients with proliferative LN, and 12 patients with membranous LN, were evaluated for serum Axl in the context of clinical and histological parameters. Biopsies were available both before and after induction treatment. Aside from being significantly higher in the whole cohort of LN patients compared to controls, serum Axl could clearly discriminate between patients with class IV LN from those with class III. Moreover, a significant decrease in post treatment levels of the marker could be shown, which was due mainly to class IV LN patients. Post treatment levels were evaluated in relation to long-term outcomes, and it was shown that lower levels were associated with better long-term outcome (273) .

TWEAK is a molecule belonging to the TNF superfamily of cytokines, which has been investigated in LN for several years, in studies with variable sample size and designs.

According to a recent meta-analysis, the marker may be promising for identifying patients with active LN, although validation in larger cohorts is needed (277).

5 RESEARCH AIMS

The overall aims of this thesis were:

1. To increase understanding of the role of RTX therapy in SLE through the study of its deep immunological effects, immunogenicity and safety, with the goal of identifying possible biomarkers of efficacy and safety which might be implemented in daily practice.
2. To identify possible non-invasive biomarkers of specific organ involvements, such as LN, which in the near future may also be implemented in daily practice for identifying LN, and also used to monitor disease activity and response to treatment.

Specific aims

Paper I: to investigate whether RTX exerts specific effects on lymphocyte phenotypes which have been recently identified, and which are deemed critical for autoantibody production. These phenotypes are represented by ABC cells (within the B-lymphocytes), and T_{FH} and T_{PH} (within the T-lymphocytes).

Paper II: to investigate the immunogenicity of RTX in SLE, its impact on safety and efficacy at a clinical level.

Paper III: to investigate the occurrence and frequency of late-onset neutropenia (LON) induced by RTX in the context of SLE.

Paper IV: to evaluate the use of urine Galectin-3 binding protein (u-Gal-3BP) as biomarker of renal involvement in SLE in patients with biopsy-proved LN.

Paper V: to screen for the presence, in urine samples, of EVs carrying molecules possibly implicated in SLE pathogenesis, and to identify candidate biomarkers of LN.

6 MATERIALS AND METHODS

6.1 Patients cohorts, data sources, clinical information, inclusion criteria, and study designs

All the patients included in the studies belong to the Karolinska SLE cohort, which has been recruiting SLE patients for more than two decades. The patients included fulfilled the 1982 ACR classification criteria and/or the SLICC criteria for SLE (34, 36). Sub-cohorts have been created over time with the aim of studying specific subgroups of patients, either in relation to organ involvement (LN) or particular treatments (RTX).

Paper I: in this work, frozen PBMCs stored in the institution's repositories over time, have been used. The biological material belongs to 15 patients who have received RTX treatment during the last two decades. These patients were also included in Paper II. PBMC samples included baseline and follow-up timepoints up to two years. Clinical information was mainly retrieved from the electronic clinical records. Patients were included at treatment baseline, when samples were obtained before the first course of RTX, and followed-up longitudinally every three months for the first year and then with a lower frequency according to clinical needs and physician's judgement. SLE disease activity was evaluated using the SLEDAI-2K index, and efficacy was established based on physicians' judgement and/or SLEDAI-2K improvement (49).

Paper II: in this work we used serum samples from patients belonging to the part of the SLE cohort treated with RTX. We selected 66 SLE with available serum samples obtained before treatment initiation and after at least 6 months from start, to ensure drug concentration at trough and therefore minimize the risk of drug interaction in ADA determination.

We analysed n=62 baseline samples and n=66 follow-up samples. As comparator group, 22 patients with ANCA-associated vasculitis (AAV), also treated with RTX, were included in the study. In addition, for establishing the disease-specific cut-point for ADA determination, sera from additional 19 AAV naïve to RTX were included (see also Figure 11). Clinical information was retrieved for both disease groups from the cohort databases (SLE cohort and VASKA cohort) and from the electronic clinical records for completeness.

Paper III: in this study 107 SLE patients were included. All patients had received RTX, during a period of fifteen years. The patients were included in the study if they had been followed longitudinally for at least six months. Patients had received RTX once or more times, which resulted in examining 225 cycles of treatment. Clinical information was retrieved from the cohort databases and the electronic clinical records.

Paper IV: the study was conducted on urine samples collected and stored frozen, from a total of 270 individuals in a cross-sectional manner. The 270 individuals were divided in the following way: the study cohort of 86 active LN patients, the disease comparators represented by 63 patients with active SLE without present or previous history of renal involvement, 73 patients with inactive SLE (and no history of renal involvement) and 48 population-based controls matched for age and sex to the study cohort. For the LN patients, urine samples were collected at the time of confirmatory kidney biopsy, while for the other SLE categories they were obtained at the visits which marked the inclusion in the SLE cohort. Data were extracted from the cohort databases and from the electronic clinical charts when verification was needed, or data were missing in the databases. The recruitment of the population-based

controls was conducted according to a standardized procedure. In such processes, population registries are consulted to single out individuals who match study patients for sex and date of birth, as well as for site of residence. Such individuals are contacted and, after giving consent, are called for recruitment in the control group. They are selected on the basis of the absence of the disease under study (in this case SLE) but are not necessarily in full health.

Paper V: this is a pilot study conducted on a small group of patients (n=13), belonging to the SLE nephritis cohort. As in Paper IV, the collection of urine samples had taken place at the time of confirmatory kidney biopsy, and urine samples had been preserved frozen for subsequent studies. Clinical data were extracted from electronic medical records.

6.2 Treatment protocols in Papers I, II and III

RTX was administered intravenously in each patient. However dosing regimens and concomitant medications have changed over the years.

During the first years of use of the drug, the protocol assumed the administration of RTX according to the haematological dosing regimen (i.e. 375 mg/m^2 of body surface area administered on days 2, 9, 16, and 23), and previous premedication with hydrocortisone 100 mg. On days 1 and 23 boluses of 6-Metylprednisolone (250 mg) and of CYC (0.5 g/m^2 of body surface area) were administered (170).

The haematological dosing schedule was changed after years, and the first cycle was then to follow the RA-like schedule with two infusions of 1g RTX given two weeks apart. In such cases, the drug would either be added to intravenous CYC given according to the Eurolupus protocol, or given alone, with or without corticosteroid pulse therapy, according to the physician's judgement.

6.3 In vitro methodology

6.3.1 Handling of the blood samples (Papers I, II, III and V)

Blood samples used in the studies were obtained concomitantly with clinical assessments. Once collected in appropriate vials, the samples were transported to the laboratory for processing.

For what concerns the PBMCs, after dilution with PBS (phosphate buffered saline), addition of a density gradient medium (Ficoll) and centrifugation, separation of the cellular and acellular fractions of the blood was obtained, with the density gradient medium interposed between erythrocytes and granulocytes and PBMCs, which were then carefully obtained from the buffy coat. The PBMC fraction was then frozen and stored following a standardized internal protocol. The freezing medium consisted of: 10% dimethyl sulfoxide, 40% media, 50% fetal calf serum. The storing was performed in freezing containers, and the freezing was achieved by a gradual temperature reduction to -80°C, then the vials were transferred in liquid nitrogen.

At the time of analysis, the protocol of thawing followed standardized steps. The cells were then stained together with one buffy coat from a healthy donor. As the procedure of freezing and thawing could result in a certain amount of dead cell debris, the cells were stained with LIVE DEAD Fixable NEAR IR Dead Cell Stain kit (Invitrogen), in order to identify non-viable cells. Viable cells were then further stained with fluorochrome-labelled monoclonal antibodies directed against surface markers of B- and T-cells.

For serum samples, the blood specimens, first collected in EDTA (therefore anticoagulated) were allowed clotting for 30 minutes and then centrifugated within one hour. After that, partitions were performed and the separated vials underwent freezing at -80°C.

In Paper III, the serum samples were collected with careful attention to cases of previous utilization, owing to the intention to analyse cytokines, whose stability in solution is known to be sensitive to repeated cycles of freezing and thawing.

In Paper V, blood vials preserved in citrate were used.

6.3.2 Multicolor flow cytometry

Flow cytometry is a technique that is fundamental for immunological studies and has accompanied the development of immunology over the last decades, finding both clinical routine and research applications. This technique allows for the recognition of different cell types (or fractions of cells such as extracellular vesicles).

The general principle by which flow cytometry works is the interaction of laser light with a material (in this case a cell) and the consequent scattering and emission of light by the material. In the flow cytometer, cells suspended in solution, and are directed into a flow cell, where they pass through an interrogation point with the aid of laminar flow and hydrodynamic focussing. Here they are hit by electromagnetic radiation in the form of laser light. The cells reflect the laser light in multiple directions, and this can be used for obtaining information on their size and intracellular content, while emitted light from fluorochromes gives information on functional properties.

Specifically, the reflected light will slightly deflect from the direction of the incident beam (forward scatter). The detection of this light will be processed by the software associated with the cytometer, and give information on the size of the cells and particles examined. Part of the incident beam will be reflected with an angle of 90 degrees (side-scatter), since the cells are translucent. The side-scatter light will be detected and will give information about intracellular complexity, including the presence of organelles and other intracellular properties.

For the purpose of immunological studies, aside from cell size, functional information is fundamental. Here we come to the concept of phenotyping. Flow cytometry can be used for interrogating functional and phenotypical features of cells, through the labelling of surface or intracellular molecules. Labelling is performed through the use of monoclonal antibodies directed against the molecules of interest, and conjugated with fluorochromes, i.e. substances able to absorb electromagnetic energy and subsequently-emitting electromagnetic waves of length and energy dependent on those of the incident beam and the material they are made of. In this way it is possible to separate different populations of blood cells which express a wide range of markers. The markers (usually the cluster of differentiation-CD-molecules) can characterize the cells based on their origin and developmental pathway (lineage markers), their activation status (activation markers), stage of differentiation (e.g. naïve vs memory for lymphocytes), or propensity to migrate into tissue (chemokine receptors and homing markers) or they can identify intracellular functional properties (e.g. master regulator of transcriptions) (70, 278).

Flow cytometry has the main advantage of being relatively easy and fast to use. However it requires training of the operator in addressing its possible limitations and pitfalls, avoiding artefacts.

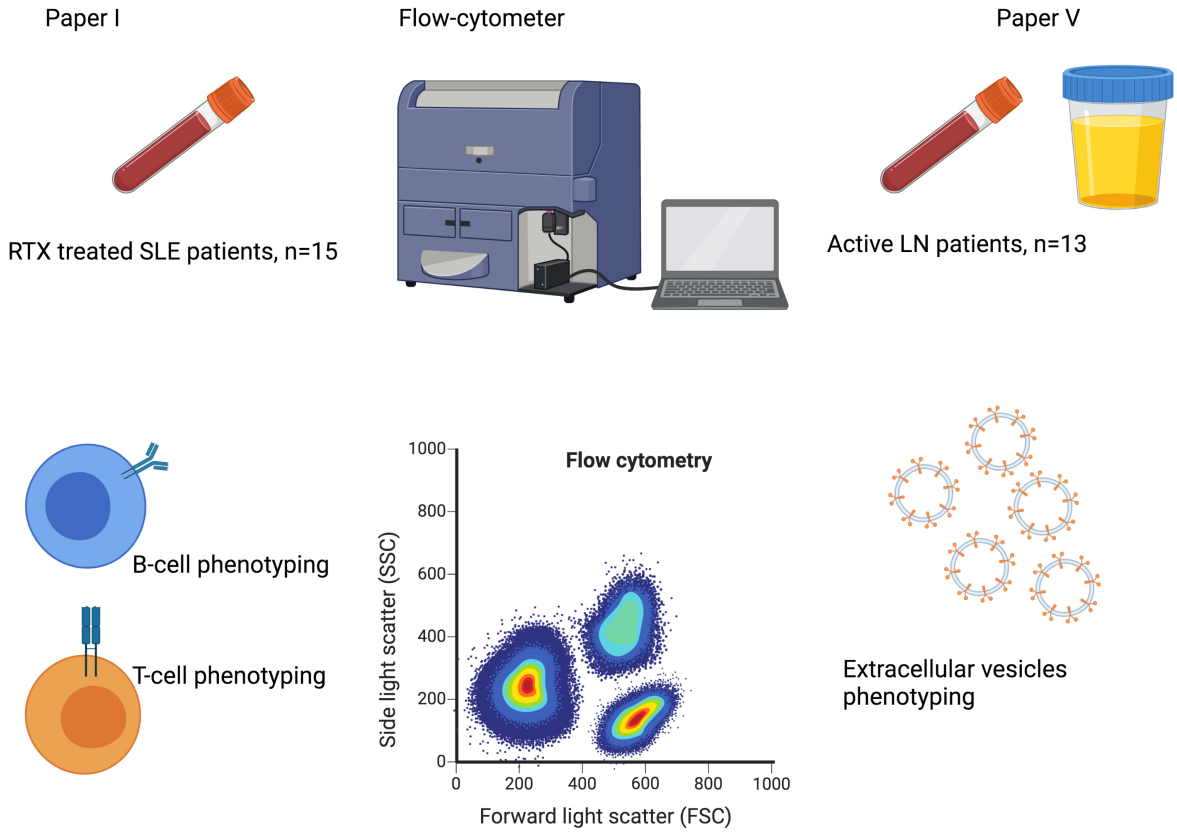
6.3.3 Use of flow-cytometry in Paper I

Here flow cytometry was used for the purposes of immunophenotyping and distinguishing different sub-populations of T- and B-lymphocytes (Figure 10). Lymphocytes were defined by size, using forward scatter-based definition. Monocytes were excluded by adding the labelling for CD16 and CD14. Then cells were further interrogated with sequential separation based on surface marker expression. For T lymphocytes CD3+ cells were subsequently separated by expression of CD4 and CD8. Each population was then subdivided according to the differential expression of CCR7 and CD45RA, PD-1 and CXCR5. For B-cells, expression of CD19 was first detected, and then reciprocal separation of subsets was performed by analysis of IgD, CD38 and CD27. The defined double negative, IgD-CD27- cells, were further analysed for expression of CD11c and CD21, and in a subset of 10 patients, for CXCR5.

6.3.4 Use of flow cytometry in paper V

In Paper V the application of flow cytometry was specifically meant to inform the detection of extracellular vesicles (Figure 10). The definition was based on dimensions, defined by forward scattered technique. EVs were defined as subcellular structures of 0.1-1 μm of diameter. Furthermore, immunophenotyping was obtained through monoclonal antibody-based labelling of markers which may indicate the cell type from which EVs were generated.

Figure 10: Use of flow-cytometry in Paper I and V



Flow-cytometry assays were performed on blood and urine samples for the phenotypical analysis of immune cells and EVs; *Created in BioRender.com*

6.4 Electro-chemiluminescent immunoassay for determination of ADA (Paper II)

ADA determination was carried out utilizing an electro-chemiluminescent assay (ECL). This method is based on a bridging principle, and uses streptavidin coated plates (see Figure 11). To ensure that ADA are picked by the assay to the maximum extent, the possibility that ADA bound in IC might be missed is reduced by the application of a passage of acidic dissociation. After this passage, a master-mix containing biotinylated capture drug and sulfo-tag conjugated drug is added and the mix is incubated for two hours. If ADA are present in the mix, a concatenation of streptavidin, biotinilated drug, ADA and sulfo-tag conjugated drug takes place, which triggers an electrochemical reaction in the solution, with emission of a signal which will be then detected.

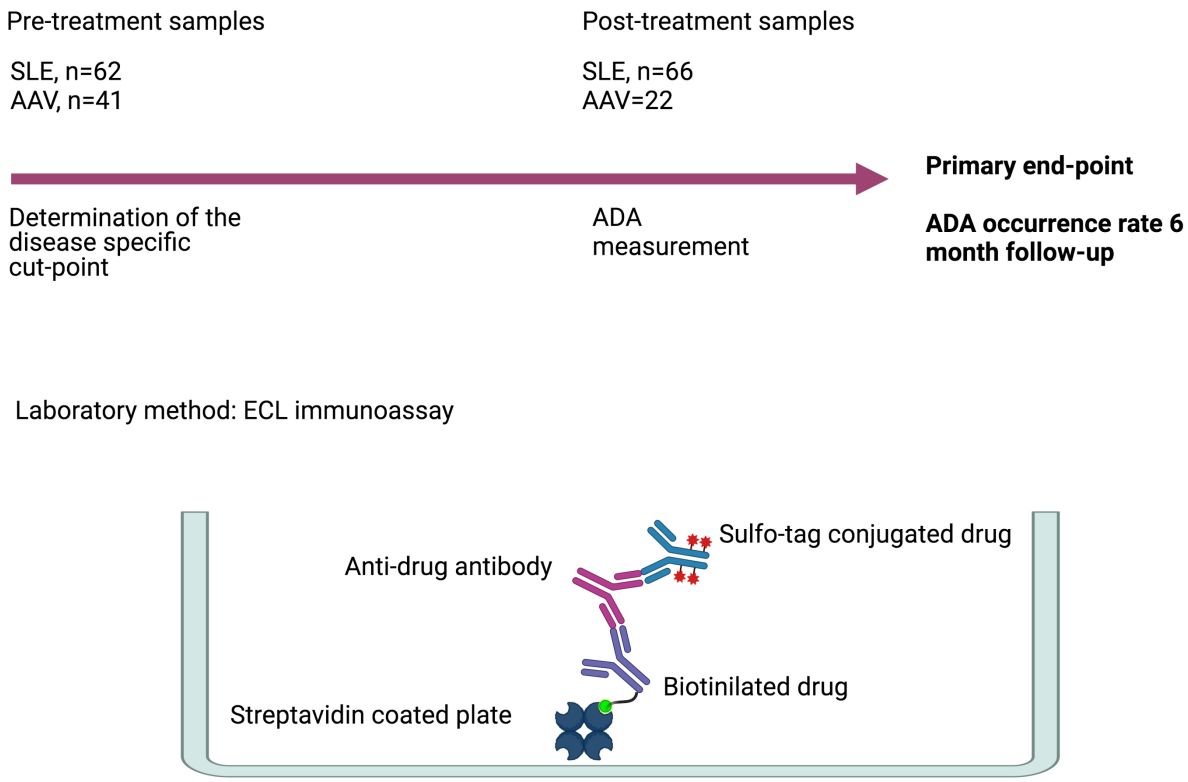
This method is among those recommended for ADA detection and has some advantages and disadvantages. Among the advantages are the high throughput and the suitability for examining solutions containing monoclonal antibodies. Among the disadvantages the fact that it might not detect low-affinity ADA. In addition, it is drug sensitive, hence affected by the presence of residual drug (208). To remedy this weakness we chose to select samples obtained at about six months follow-up, when it could be assumed the drug concentration had reached its trough.

The specific method used in this work had been previously used in a study investigating the occurrence of ADA in multiple sclerosis (279). The method has been validated in-house and is based on a three-tiered approach. This means that the analysis proceeds through three sequential steps: screening, confirmation and characterization.

The screening assay gives a first determination of ADA, which is further confirmed in a competitive assay, which makes it possible to ascertain that the detected antibodies are specific to the drug. In these first stages also samples from patients not exposed to RTX were tested. This allowed determination of a disease specific cut-point, which corresponded to a RECL (relative electro-chemiluminescence) of 1.44 for SLE and 1.18 for ANCA-associated vasculitis.

The third step of characterization consisted in ADA titration, the titre being expressed as arbitrary units per millilitre (AU/ml). For some samples, positivity was confirmed but their RECL was below the titration cut-point (relative ECL, RECL <3.72). These samples were assigned a titre of <2 AU/ml. We did not characterize ADA by defining the isotypes, their affinity, or their neutralizing capacity. To ascertain this, a specific cell based assay evaluating cell killing and complement consumption should be applied.

Figure 11: Use of the ECL immunoassay in Paper II



In Paper II, two sets of samples were analysed. The first consisted of naïve samples (i.e. unexposed to RTX) which were used for determining a disease specific cut-point for both the SLE and AAV cohort. The second set consisted in post-treatment samples and was meant to examine the rate of occurrence and titre of ADA after RTX (primary endpoint). The lower panel of the figure depicts a schematic view of how the ECL assay works. *Created in BioRender.com*

6.5 ELISA method for determination of serum cytokines and urinary soluble markers (Papers III and IV)

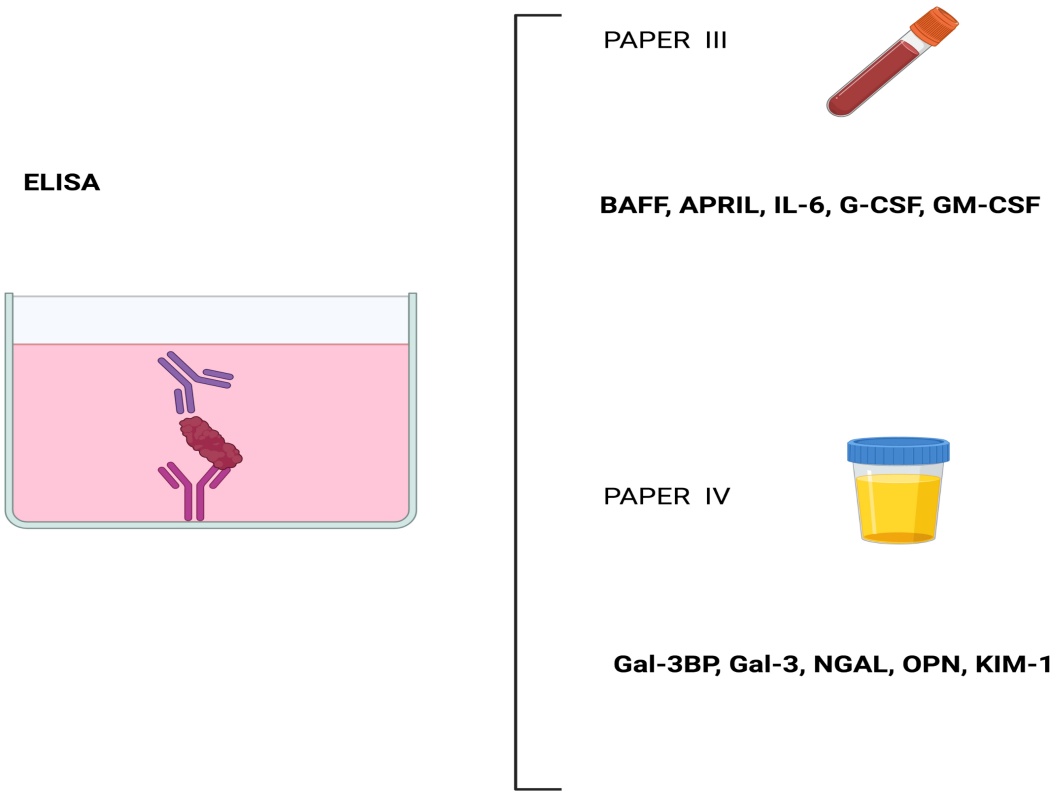
In Paper III, enzyme linked immunosorbent assay (ELISA) was used to determine serum levels of BAFF, APRIL, IL-6, GM-CSF and G-CSF. For each determination, commercially available kits were used according to manufacturer's instructions. Similarly, in Paper IV, ELISA was used for determining the urinary concentrations of Gal-3BP, Gal-3, NGAL, OPN and KIM-1. For Gal-3BP, an ELISA method developed by industry partners was applied.
ELISA (see Figure 12) is a commonly used method in both the research setting and in clinical routine, to detect a wide range of biologically active molecules (proteins, antibodies, hormones). The principle by which ELISA works is similar to that explained for ECL in Paper II. In fact, the possibility to detect a biomolecule is based on the interaction of it with a specific antibody.

There are different types of ELISA based on how the analyte of interest is immobilized into the wells of the plate in which the solution containing it is analysed, and on the modality of detection of such analyte. The immobilization of the analytes can occur either via absorption to the surface of the plates' wells or by antibodies fixated to the bottom of the wells. Once fixated, the presence of the analyte is revealed by the addition of an antibody labelled with a reporter substance, usually an enzyme, which will initiate a biochemical reaction once its substrate is added to the solution. The signal emitted and amplified via the reaction will reveal

the presence of the substance of interest and allow its quantification. The ELISA-based method offers a series of technical advantages and disadvantages, and potential pitfalls (280).

In our case, the main potential pitfall in using ELISA for determining the presence of protein analytes (cytokines, urinary biomarkers) was represented by the pre-analytical phase. We worked with frozen serum and urine samples, which had to be thawed. It is known that cycles of freezing and thawing may alter the integrity of this type of analyte and cause protein denaturation, hence modifying the real concentration of the analyte and influencing the results of the testing (281). To obviate this potential pitfall, we made sure to use samples that had been processed and frozen but not thawed before use in our studies.

Figure 12: Use of ELISA in Paper III and IV



Simplified view of the ELISA's principle of functioning (on the left). Use of the method in the two papers, relative matrix material, and evaluated analytes. *Created in BioRender.com*

7 STATISTICAL ANALYSIS

Descriptive statistics were performed with prior verification of the distribution of the continuous variables as gaussian or non-gaussian, using the Kolmogorov-Smirnov test. When not normally distributed, continuous variables are descripted as median and interquartile range (IQR). In figures representing the concentration of urinary biomarkers in Paper IV, we show median and 5^{th} to 95^{th} percentile. If they were normally distributed, mean and standard deviations (SD) were used as central measures of the value distribution.

Given the preponderance of not-normally distributed continuous variables in our studies, inferential statistics were conducted applying non-parametric tests. To compare central measures between unpaired groups, Mann-Whitney U test was applied as appropriate. In cases of comparison of more than two groups Kruskal-Wallis H test was applied. When analysing data concerning continuous variables in the same patient group, but collected at separate time points, the Wilcoxon signed rank test was applied. To measure the correlation between two variables the Spearman ρ (rho) test was applied. Categorical variables are generally presented as numbers and/or percentages. Comparisons of categorical variables were performed applying the either the Fisher's exact test or the Chi-square test as appropriate. In Paper III, univariate analysis was followed by a multivariate analysis based on a logistic regression model.

In Paper IV, calculations regarding urine concentrations of the analytes of interests were conducted before and after normalization of the measured values for the concentration of urine creatinine. Moreover, to assess the performance of u-Gal-3BP as biomarker, a receiver operating characteristic (ROC) curve analysis was applied.

In all studies the level of significance was set at 5%, i.e. p values of less than 0.05 were deemed significant.

8 ETHICAL APPROVAL

All the studies included in this thesis were conducted under approval of the projects by the Stockholm County Ethics Committee (Etikprövning mindigheten, Region Stockholm). Each patient provided informed consent prior to enrolment in the SLE cohort, and/or at the occasion of kidney biopsy (lupus nephritis sub-cohort) or the start of RTX treatment (rituximab sub-cohort). Patients with ANCA-associated vasculitis included in the VASKA study gave informed consent at the time of enrolment in the specific study. Each study was conducted in conformity to the principles of the Declaration of Helsinki guidelines on studies performed on human subjects.

9 RESULTS AND DISCUSSION

9.1 RTX transiently reduces DN/ABCs memory B-cells frequencies in the peripheral blood (Paper I)

DN/ABCs in the context of CD19+ B-cells and modifications of frequencies upon RTX

The general behaviour of CD19+ cells in our cohort, followed what is expected in SLE, with a significant reduction of B-cells at a group level and some patients not depleting according to the common definitions of depletion.

The subject of interest of our study was the newly defined subset of ABCs, since these cells are deemed to be precursors of antibody forming cells, and have been demonstrated to be present in tissues affected by SLE related pathologies, such as kidney and skin (118, 192). ABCs, as memory B-cells, express CD20, the molecule targeted by RTX; therefore, it is reasonable to ask at which extent they can be affected by RTX (5).

In our cohort, 20.4% of the DN cells at baseline corresponded to the ABCs phenotype (IgD-CD27-CD11c+CD21-, or DN2). Moreover, in the subset of patients in which we added the labelling of the chemokine receptor CXCR5, the DN2 cells (here defined as CD11c+CD21-CXCR5-) accounted for 19% of the DN pool.

From the baseline percentage of 20.4%, the frequency of DN2 decreased to 11.3% at the early follow-up time point of 2-4 months, to return to pre-treatment frequencies at the 9-10 month follow-up. At subsequent time points, we saw apparent reduction, which was not statistically significant when compared to the baseline, and could have been influenced by the number of available samples. Enlarging our consideration to the entire subset of CD11c+CD21- cells, irrespective of CD38, IgD and CD27 expression, we did not observe significant changes in the cell frequencies upon RTX. This could reflect the actual complexity of the cells expressing CD11c in human blood, which are expanded in SLE in comparison to healthy individuals, and whose characterization goes beyond their sole location in the DN compartment (194).

In our study, we only considered frequencies, therefore we cannot affirm with certainty whether the transient reduction we observed corresponds to an actual reduction of absolute numbers of the DN2 cells. This is therefore an observation which needs further confirmation. However, it also points towards a possible relevance of these cells for the outcomes of B-cell targeting treatments. In fact, if ABCs express CD20, one should expect a more stable reduction of these cells between treatment and full repopulation. An early return to baseline levels, as we observed, may suggest that B-cell depletion does not significantly impact on the molecular mechanisms and/or the precursors that generate these cells. This, considering the critical role they play in autoimmunity, is not of little relevance.

Moreover, since a relative increase of plasma blasts frequency is a typical effect of RTX, and ABCs are poised towards plasma blasts generation, their transient decrease may actually signify that these cells are relatively resistant to RTX, for reasons other than the sole CD20 expression, and be a reservoir of plasma blasts expansion during the early status of peripheral B-cell depletion.

Our exploratory analysis of the interrelation of the DN subsets and the presence of anti-drug antibodies to RTX (ADA) may offer some insights. These are discussed below.

Before that discussion, another observation which came from our data analysis of the DN compartment needs to be discussed.

In a recent paper, another subset of DN cells has been defined and named DN3. These cells are double-negative for CD11c and CD21, and were described while comparing SLE patients and patients with COVID-19 infection. They were found to be consistently expanded in patients with SLE and COVID-19 patients with severe disease and hospitalized in the ICU. Their characterization suggests that DN3 cells may be, together with activated naïve and DN2 B-cells, active players in extrafollicular response, which are relevant for SLE, and autoimmunity in general (87, 94, 282).

In our study, the CD11c-CD21- subset showed a significant increase in frequencies, even when characterized for the absence of CXCR5, at the early follow-up, suggesting a rebalancing of the different components of the DN compartment under B-cell depletion. Given the relevance of these cells for extra-follicular immune responses, our observation warrants further exploration of this cell population, not only in the sole peripheral blood but even at the target tissue level. The origin of DN3 deserves further studies, as underlined in the work which reported its first detection. In fact, DN3 in that study showed a certain overlay with DN1 cells and continuity with switched-memory B-cells (282).

9.2 ADA development is associated with different perturbations of the B-cells under B-cell depletion (Paper I)

Since there was an overlap in the study populations of Papers I and II, we used some of the data generated in Paper II (discussed more in detail below), to explore the possible influence of RTX immunogenic consequences on the homeostasis of B-cells during depletion.

The ability of RTX to generate an immune response primarily resides in its nature as a protein, and therefore in the possibility to carry several epitopes capable of eliciting immune response.

The part of the drug that mostly is immunogenic is the idiotype, which is located in the murine derived part of the Fab in the RTX molecule. However, other epitopes are possible, depending for example on the presence of post-translational modifications. These could modify the Fc region at an extent which is sufficient for generating new epitopes, at least in theory. In a recent study which used CD4+ T-cells extracted from healthy donors, nine possible epitopes of RTX were identified by stimulating naïve T-cells with APCs loaded with either RTX or IFX (infliximab) derived peptides. Some of the T-memory cells obtained in healthy donors by multiple runs of T-cell antigenic stimulation in vitro, could also stimulate T-cell response in samples obtained from patients with ADA (283). This type of experiment is an example of the relevance of identifying T-cell epitopes for strategies aimed at reducing immunogenicity, but also more simply is indicative of which events lead to ADA formation.

In a schematic way, RTX would induce an immune response as any other T-dependent antigen. It would be picked up by an antigen presenting cell, and presented in the form of peptide to a T-helper lymphocyte, which in turn would prime a naïve B-cell. This latter, evolving into plasma blasts and plasma cells, would then give rise to the effector step of this sequence of events, the production of antibodies, and to a specific B-cell memory clone. At a new encounter with the antigen, in this case through retreatment, memory cells would

expand, giving rise to a subsequent run of immune response, in a manner similar to a vaccination boost.

However, the generation of ADA during the condition of B-cell depletion is somehow in contrast with the elimination of naïve cells which could be poised to respond to RTX. In fact, it could be argued that, in the absence of naïve B-cells, ADA formation should not be elicited. A logical explanation would be that naïve B-cells are primed upon their return, in the presence of persistent antigenic stimulation, which in this case should correspond to the residual circulating drug. Another possibility is that factors intrinsic to individual patients can result in primary resistance to depletion, which leaves in the circulation and tissues naïve B-cells which can mount the response against RTX, already during the early phases after drug administration. This is an intriguing hypothesis, but without deep analysis of the residual cells in the first weeks after RTX administration, we cannot confirm whether this is the case.

In our cohort, at the time of depletion (2-4 month follow-up), the patients who showed positive ADA at the subsequent time point (6-8 months), had lower frequencies of DN B-cells as compared with those who were ADA-negative.

All the ADA-negative patients (n=7) showed an increase in the frequencies of DN B-cells at the first follow-up. Among the ADA-positive patients (n=8), the increase appeared to be more contained, although no longitudinal differences could be detected in the Wilcoxon test. Instead, when operating a punctual comparison of the two groups at the 2-4 month follow-up, the Mann-Whitney test detected a significant difference ($p=0.02$).

When focussing on the DN/ABCs (CD11c+CD21-) and DN2 (CD11c+CXCR5-), we did not observe significant changes between timepoints in each group, nor could we detect differences between groups at correspondent time points. However, the reduced number of the samples might account for a lack of power in detecting differences. The direction of the changes in DN/ABCs and DN2 was the one of a reduction in the frequencies of these subsets.

A significant expansion of plasma blasts between the baseline and the early follow-up time point corresponded, in the ADA-positive patients, with lower frequencies of DN at the 2-4 month follow-up. With the limits given by the reduced sample size, which may have contributed to not detecting important differences, this observation is in our perspective relevant. In fact, the cells undergoing activation and generating plasma blasts, plasma cells and memory cells to RTX, must be contained within the residual cells left when the drug has exerted its action. From pharmacological studies, we know that the CD19 positive cells, in particular the naïve cells, drop consistently after the first infusion of RTX (158). Our observation suggests another hypothesis with respect to the intrinsic resistance of naïve B-cells discussed above. It is possible that DN cells function as a biological reservoir for plasma blasts expansion during B-cell depletion, and possibly, they could house the B-cells which are primed by RTX.

Here however another question arises. DN are considered to be mainly memory cells, and they are enriched in autoreactivity in SLE patients. Autoreactive clones are the villains in autoimmunity, but their presence is not completely without significance from an evolutionary perspective. They could indeed be used to tackle unknown antigens via mechanisms of clonal redemption (284).

9.3 Effects of RTX on T-cell phenotypes associated with B-cell help is unclear (Paper I)

As illustrated in previous sections, great emphasis has been devoted to the characterization of T-helper cells involved in B-cell help as major players in SLE immune pathogenesis. Moreover, a population of CD4+PD-1^{hi}CXCR5-, consistent with the previously detected T_{PH} population found in synovial tissue of RA patients, had been found expanded in SLE patients at the start of our study (204, 205). Therefore, we aimed at exploring how these cell populations behaved in our cohort, and whether RTX could influence them, given the relevance they have for B-T cell interactions.

In our cohort, which was small, T_{FH} did not show significant association with disease activity nor disease manifestations: they were in fact not different at baseline between LN and non-LN patients. Moreover, no impact exerted by RTX was observed during the longitudinal follow-up in our cohort.

Similarly, when turning our attention to the T_{PH}, no differences at baseline based on disease activity or organ involvement could be detected, and again no longitudinal shifts were detected. It could be argued that, given the relevance of T_{FH} and T_{PH} in tissue-based interactions, this factor, coupled with our sample size, may influence the results. In fact, peripheral blood, although easily accessible, may not truly reflect the dynamics of these two lymphocyte subsets.

When we considered high expression of PD-1 within the CD4+ compartment, we observed a strong trend towards an association with nephritic organ involvement. Indeed, the frequencies of PD-1^{hi}CD4+ T-cells in LN were higher compared to non-LN, at the limit of significance ($p=0.055$). In this subset, we detected some statistically significant fluctuations, at later follow-up, which remain of uncertain significance.

These cells, when examined in the context of ADA development, showed a trend towards higher frequency in ADA-positive patients. In this case, as for other observations in the paper, the small sample size may have played a major role in not disclosing statistical differences in biologically meaningful comparisons.

9.4 ADA development is a frequent event after RTX, already after the first course of treatment (paper II)

In Paper II, we explored the occurrence of ADA to RTX in SLE, and we used a cohort of AAV as disease comparator. We found a percentage of ~38% of ADA positivity in our SLE cohort after first exposure to RTX, while in AAV, in a cohort of 22 patients, none was found positive for ADA at follow-up. The rate of ADA occurrence in our cohort was as high as that detected by other authors, who used the same laboratory method, and in line with the rate of ADA found in MS patients by our collaborators from the Neuroimmunology Laboratory at KI (214, 279).

We observed that ADA occurrence was not influenced by the concomitant medications received by the patients at the time of treatment, such as high-dose corticosteroids and cyclophosphamide. This finding is in contrast with what acquired in the field of RA, where administering MTX together with infliximab, another chimeric monoclonal antibody, can prevent ADA formation (285). The different biology of the two diseases might explain the different propensity to elicit antibodies. In fact, ADA to RTX in RA are a relatively rare occurrence, in general not associated with clinical consequences of relevance (208). This

lower incidence (also true for AAV) might reside in the less pronounced level of B-cell aberrations and hyperresponsiveness, and in a less broaden immunologic repertoire RA and AAV have with respect to SLE (286). The hyperactivity of B-cells typical of SLE might also explain the presence of pre-existing antibodies against the drug, as highlighted by our finding of pre-exposure positivity in some patients.

From a laboratory perspective, the finding of ADA-positive results in non-exposed samples must be considered in terms of specificity of the tests used. In the method that we used, the confirmatory step ascertained the specificity of ADA for the drug. Other methods, less specific but more sensitive, may instead give higher rate of false positive results. In terms of pre-analytical factors, the quality of SLE sera is also important. These sera are in fact rich in polyreactive antibodies, which create a higher level of 'noise' in the test. An example of this type of interference is the high rate of false positive COVID-19 serology tests obtained from samples donated by SLE patients years before the pandemic (287).

Moreover, our data shows that the patients with greater propensity for ADA development, are those more in need of receiving RTX, which is still used as rescue therapy in SLE. These patients were younger, had shorter disease duration, exhibited higher disease activity, and were mostly presenting with LN, which was the most represented indication for RTX in our cohort. Younger age at RTX initiation was also found associated with ADA by other authors (214). We also found an association of ADA development with positivity of anti-dsDNA at baseline. This may be a consequence of the skewness of our cohort towards a predominance of LN patients, but once more it highlights that the immunologic status of an individual, in this case due to the major immunologic disruption intrinsic to SLE, plays a major role in determining susceptibility to ADA.

Another consideration of relevance concerns the association with complement: we did not find that complement consumption at baseline was associated with a higher frequency of ADA occurrence at follow-up. Although more patients with complement consumption at baseline developed ADA at follow-up, this rate was not statistically different from those not consuming complement before treatment. This finding was rather unexpected, given the relevance of complement for the pharmacodynamic of RTX (154). It could be speculated that in individuals with low complement levels, RTX would consume the residual available complement early, which would lead on one side to lower efficiency of CDC, and on the other side to a prolonged persistence of circulating drug. This, from an immunological perspective, would correspond to a prolonged antigenic stimulation of naïve T-cells, and naïve B-cells not efficiently killed through CDC.

Of course, this speculation, given that B-cells in SLE are overall depleted by RTX, raises the question of which among the possible killing mechanisms exerted by the drug prevails in vivo. On the other hand, it also fits with the higher efficiency in depleting B-cells hold by the glycoengineered anti-CD20 obinutuzumab, whose B-cell killing action is less dependent on complement and consequently on CDC (288).

9.5 ADA are associated with higher B-cell proportions and counts at follow-up

As introduced above, the development of ADA in a condition of B-cell depletion is a counter-intuitive event, which takes place during a striking reduction of naïve B-cells which should be poised to respond to RTX. Therefore, the determination of which cells actually give rise to the response towards the drug is intriguing, as discussed above. On the other hand, from a pharmacodynamic perspective, we had not expected an impact of ADA on the B-cell counts

at follow-up. The reasoning behind this expectation was that, if any effect would arise, this might be detectable upon retreatment.

In fact, we assumed that ADA would appear upon B-cell return. Therefore retreatment would act as a new immunological challenge eliciting a secondary immune response, and an increase in ADA titres which would impact on RTX action.

We observed instead that ADA-positive patients, showed a significantly higher CD19+ count and percentage in peripheral blood at around the 6-month follow-up, with respect to ADA-negative patients. Since we studied patients after their first RTX treatment, it is difficult to interpret this observation precisely from a biological point of view.

We do not know if this reflects an accelerated B-cell repopulation or an inefficient B-cell depletion. We could examine B-cell counts and percentages only for a subgroup (n=37) of the 66 SLE patients with ADA status determined at follow-up. This reduced availability of data affected what we could conclude. Indeed, we could not demonstrate a linear correlation between higher ADA titres and lower B-cell counts and/or percentages at follow-up. Similarly, within the limits of detection possible in our laboratory, we could not establish a clear association between ADA status and whether or not peripheral BCD was achieved.

These limitations in our study make it not possible for now to clarify the real impact of ADA on B-cell counts as a surrogate of efficacy of RTX. It might be speculated that the impact of ADA on B-cell counts at follow-up does not follow a linear kinetic. In fact, while the action of RTX and the development of ADA may proceed in parallel, at a certain moment ADA would interact with the residual circulating drug in a stoichiometric fashion, forming IC and saturating the residual drug in excess, not impacting further on its pharmacodynamic. This aspect is closely related to an unanswered question, regarding what the correct dose of RTX should be for SLE and LN patients.

Further studies focusing on the early events after RTX administration might help unravel the sequence of events leading to this disparity. Such studies should not only aim at identifying which cells mount the response to the drug, but should also define the dynamic of ADA appearance and the magnitude of the immune response. Moreover, they should aim at defining which epitopes in the molecular structure of RTX are effectively responsible for the formation of neutralizing ADA, the ones which are deemed to have a major impact on the drug's pharmacodynamics.

9.6 ADA may have a role in the occurrence of adverse reactions to a subsequent course of RTX

In the SLE cohort studied, 31 out of 66 patients received a second course of RTX. Of these 31 retreated patients, 12 (38.7%) had tested positive for ADA at follow-up, while 19 (61.3%) had tested negative. The time to retreatment was highly variable, with a median of 17.5 months (IQR:10.2-66.7) for the ADA-positive patients, which was similar to the ADA-negative group median time to retreatment of 19.0 months (IQR: 9.7-67.0).

We were interested in exploring whether adverse reactions at retreatment could be associated with ADA development. We therefore revised the clinical records to trace immediate infusion reactions occurring during retreatment. This type of infusion reaction could be identified in about 25% of the retreated ADA-positive SLE patients. In contrast, no immediate infusion reactions were traced among the ADA-negative patients.

The second type of infusion reaction we considered in the study was late-onset reactions such as serum sickness. To define the occurrence of such reactions, we proceeded with a retrospective analysis of the clinical records. We found clinical descriptions compatible with typical serum sickness reaction after retreatment in two patients who were ADA-positive and one patient who was ADA-negative. Given the retrospective mode we used for answering the question, it is highly probable that our study underestimates the real proportion of the phenomenon. Nevertheless, as our observation is in line with others in the medical literature, it raises awareness of this clinical manifestation and its relation to RTX-induced immunogenicity (289). On a practical level, this should be translated into the implementation of active monitoring of such events together with implementation of ADA screening.

9.7 ADA may be implicated in loss of efficacy of RTX at retreatment in LN patients

In our study, 42 patients were included who had LN as the main indication for RTX treatment. Seventeen of them underwent retreatment, of which seven were within 18 months (early retreatment). Of these seven patients undergoing early retreatment, five were retreated owing to a new nephritic flare. Four of these patients were ADA-positive after the first course of RTX. We looked at renal response rates at 6 and 12 months from retreatment for these patients. Three of them were non-responders at the two time points, while one was a partial responder. All the patients had experienced a measurable clinical effect after the first course of RTX. However, the question of whether ADA played a role in the subsequent non response of the retreated patients remains. The numbers in our study were too small to draw conclusions, and this aspect should be further explored in larger cohorts of retreated patients.

9.8 Late-onset neutropenia (LON) is a frequent event after RTX in SLE patients and not always free from clinical consequences

Paper III explored the incidence and clinical consequences of RTX-induced LON in SLE. On a study population of 107 RTX-treated patients the cumulative incidence of LON was as high as ~30%. This figure corresponded to 32 patients, of whom 20 experienced the event after the first exposure to RTX, while 10 experienced it after the second cycle, and two after the third and fourth courses of treatment, respectively.

In the majority of the cases (59.4%), the surveyed patients did not experience any symptoms, and low neutrophils counts were detected serendipitously in concomitance with control blood tests. The remaining 40.6% of the patients with low neutrophil counts instead required hospitalization, either because of presence of fever, or because they presented with symptoms of infection. In some cases, hospitalizations were motivated by the very low neutrophils counts which warranted clinical observation. Major infections were observed in three patients (two with sepsis and one with necrotizing fasciitis requiring long stay in the ICU), caused by Staphylococci, Pseudomonas and Streptococci respectively. The degree of neutropenia was not found predictive of symptom appearance, nor of the duration of hospitalization.

The frequency of LON observed in our study, in comparison with the frequency of LON reported for RA, ANCA-associated vasculitis and lymphoma, sets SLE as a condition intrinsically at risk for LON, an observation which finds further confirmation in a study published more recently (232). This study also confirms our observation that the majority of LON events pass as asymptomatic. However, there is a consistent minority of patients with LON experiencing clinical manifestations (fever) and complications (major infections). This aspect warrants that neutrophil counts be monitored in clinical practice, especially when re-exposing patients with a history of post-RTX LON.

9.9 High disease activity at RTX start is the main predisposing factor for LON development

The clinical characteristics of the patients studied in Paper III were analysed in order to define possible predisposing factors for LON development. In patients developing LON after the first cycle, the main factors associated with LON occurrence at univariate analysis were a higher dose of oral corticosteroids at baseline, and a higher disease activity (as measured by the SLEDAI-2K score). Of these two factors, only the SLEDAI-2K score was confirmed as an independent factor associated with LON, and it conferred an odds ratio of 1.1 of developing the complication. Further analysis confirmed that a SLEDAI-2K score at treatment start over the value of 8 conferred an odds ratio of 4.1 for the development of LON.

This observation is unique to our study, since it is focused on SLE patients. In previous reports, SLE patients were present among other patient categories and did not represent the predominant subgroup investigated (230, 232). Moreover, in line with the findings in Paper II, SLE appears, among the conditions for which RTX is used, more prone to complications, possibly immunologically driven. Although the global safety profile of RTX is fairly good, its use in certain disease contexts characterized by major immunological disruptions should raise awareness of the possibility of certain complications.

9.10 LON with agranulocytosis is mainly associated with the cumulative dose of RTX and exposure to cyclophosphamide

Neutropenia before first exposure to RTX did not qualify as a risk factor for RTX-induced LON in the study. For patients developing LON upon repeated exposures to RTX, the cumulative dose in patients ever experiencing LON was significantly higher with respect to patients who, although retreated, never developed LON. Similarly, the cumulative dose of RTX was higher in patients manifesting agranulocytosis. Moreover, agranulocytosis was associated with higher exposure to CYC.

Exposure to CYC in concomitance to RTX was found to increase the risk of LON by a hazard ratio of 1.98 in multivariate analysis in a recently published work, which did not find an association with cumulative dose as we observed (232). However, the study did not include only SLE patients, so direct comparison of the results is hard to perform.

On a more general perspective, the lack of association with previous episodes of neutropenia might be explained by the fact that such anamnestic events may reflect a different pathogenesis. Neutropenia is a rare possible manifestation of SLE, where it can coexist with other cytopeanias of immune origin under the eponym of Evan's syndrome (290). Moreover, myelosuppression is encountered as a common side effect of immunosuppressants (for example, AZA), and in such cases it is mostly determined by pharmacogenomic and metabolic factors (291).

CYC by now has a long history in the treatment of systemic diseases, and at the doses used for rheumatological indications it has an acceptable safety profile. However, it is known that cumulative doses impact on long-term safety and increase the risk of malignant transformation (292). From the perspective of the action of the drug, since it is able to interfere with several cell types at high turnover, it is intuitive to consider that repeated cycles may impact on the bone marrow, reducing the availability of myeloid precursors.

9.11 B-cell related cytokines behave differently in patients developing LON

LON occurs in a context of higher levels of BAFF

Baseline serum levels of BAFF did not differ between patients later developing LON and those not experiencing the event. Therefore, BAFF at baseline could not serve as a predisposing or predicting factor to LON. As expected, BAFF levels rose in the whole study population following RTX administration. However, when examining this increase in a transversal fashion (LON at the time of the event vs non-LON at comparable median time of follow-up), the post-treatment levels were significantly higher in patients who developed LON.

In four patients who developed agranulocytosis, the granulopoiesis curve was reported as left-shifted, which means the myeloid line in the bone marrow was mostly represented by immature precursors. This finding supports the theory of a maturation arrest of the granulocytes as a major determinant of the peripheral blood neutropenia, as suggested by previous observations (234). The participation of the BAFF-APRIL system in SLE pathogenesis is now common knowledge, and it represents a solid rationale for targeting B-cells in the disease (92).

BAFF is fundamental for B-cell maturation and survival, and acts on B-cells once egressed from the bone marrow. The rise of BAFF levels following rituximab is a known phenomenon . BAFF can be produced by various cells, including neutrophils (293). These cells display a high degree of heterogeneity and capacity of interplay with B- and T-cells, which have been described (294). Their involvement in SLE pathogenesis has received much emphasis given the relevance of NETosis as a potential source of autoantigens and ability to drive INF-α production by pDC (62). This propensity towards an interplay with B-cells concerns not just the effector mechanisms of mature cells. At the bone marrow level, neutrophils in SLE produce IFN-α, BAFF and APRIL, in the context of an interferon signature. This upregulation of BAFF and APRIL results in alteration of B-cell ontogeny, with a suppression of early B-cell precursor and a maturation curve skewed towards an excess of transitional B-cells (295). Given these premises it is not unreasonable to hypothesize that the rapid and abundant rise of BAFF under B-cell depletion, in a context in which the interferon signature already causes disruption in the homeostasis of the bone marrow, may lead to an aberrant feed-back on the granulopoiesis. In other words, if neutrophils can be a source of BAFF and therefore influence the development of B-cells in different physiological and pathological conditions, BAFF can also exert autocrine and paracrine actions on neutrophils. In this perspective, rather than being a side effect, LON would be an intrinsic secondary effect of RTX, strictly linked to its mechanism of action. In this context, the altered bone marrow homeostasis favoured by the interferon signature, might explain the propensity of SLE towards LON in comparison to other diseases. With this in mind, it would be interesting to explore the occurrence of LON with other anti-CD20, since this might be a class effect of these drugs, as suggested by recently published data (296).

APRIL levels are elevated at baseline but not at LON occurrence

APRIL levels at baseline were higher in patients belonging to the LON group, and in contrast to BAFF levels did not show significant dynamic changes upon treatment (before vs post-treatment comparison). The discrepancy remained when considering post-treatment levels between the two groups of patients. However, within each group no longitudinal dynamic changes were detected.

In the BAFF-APRIL system, the principal role of APRIL seems to act on plasma cell survival. The finding of higher APRIL levels at baseline in patients later developing LON is a more complex interpretation. Regarding LON development, the higher baseline levels may have a predictive role, although we did not ascertain a clinically meaningful cut-off level associated with an increased risk. On the other hand, high levels of APRIL might be intrinsic to SLE and relate to disease activity, which in turn we were able to associate with LON development. Similar observations on APRIL levels were not known in the literature, and more recent data on LON in lymphoma did not reveal higher levels in patients developing LON after RTX in this clinical indication (227).

IL-6 and granulopoiesis-related cytokines' behaviour in LON share common grounds with BAFF excess

Serum levels of IL-6 were not different between LON and non-LON patients, either before or after treatment. However, while in patients developing LON the post-treatment levels remained comparable to the pre-treatment levels, in patients with no LON, a significant decrease was observed. A similar behaviour, with a significant decline from pre- to post-treatment phase was observed for GM-CSF levels but not for G-CFS levels.
The differential dynamic changes in these cytokines may suggest two main processes: they may reflect inflammatory activity and be associated with poor response; alternatively, an inflammatory response may be a bystander phenomenon driven by BAFF rising levels. Moreover, if GM-CSF as G-CSF are also inflammatory markers, they primarily are haematopoietic growth factors; therefore, their staying elevated during LON may also be, at least in part, explained as a reactive phenomenon induced by neutropenia itself.

9.12 Urinary Gal-3BP is a promising marker of LN (Paper IV)

Levels of u-Gal-3BP are higher in active LN patients compared to patients with active non-renal disease, inactive disease and population based controls

Urinary levels of the biomarkers investigated were analysed both as absolute concentration and normalized for urine creatinine concentration. In both analyses, the levels of u-Gal-3BP were higher in LN patients, compared to the other examined groups. The finding indicates that the presence of ongoing inflammation in the renal parenchyma is reflected by the possibility to detect Gal-3BP in the urine of patients with LN. In addition, the levels of u-Gal-3BP in LN were higher than in active SLE disease without renal manifestations and inactive disease. Hence u-Gal-3BP levels normalized for urine creatinine concentration can identify LN patients against other disease categories and controls.

This observation constitutes a first affirmative answer to the main question of the study, i.e. whether u-Gal-3BP can serve as biomarker of the presence of ongoing LN. Moreover, it indicates that the protein reflects pathogenic events of kidney inflammation.

As illustrated in previous sections, Gal-3BP is encoded by a gene whose expression is regulated by interferon. The interesting aspect of this gene, among the interferon-inducible genes, is that it encodes for a soluble protein, which can be measured in biological fluids (248). By contrast, the majority of interferon-inducible genes encodes for proteins which exert their action within the cells.

The fact that Gal-3BP can be detected in urine, tells us that inflammation is present in the kidney. However, it does not address the question of what the cellular source is of the protein

within the inflamed renal parenchyma. An interferon signature, being present in peripheral blood, can be 'transferred' into the renal parenchyma by infiltrating immune cells, either in the glomeruli or in the interstitium. The latter is not only site of immune cell infiltration, but is also theatre of activation of resident cells. In a recent study, an interferon signature has been detected also in the tubular cells as a consequence of damage (117).

Levels of u-Gal-3BP are different across histopathological phenotypes of LN

In our study, u-Gal-3BP concentrations were higher in patients presenting with proliferative and membranous LN with respect to those with mesangial forms. The difference was evident across the groups, but single comparisons revealed a significant difference between proliferative and mesangial forms, and no difference between proliferative and membranous forms. To account for the lack of differences, we would need to consider the reduction of group size caused by the stratification of patients according to histological subtypes that we operated. The absence of a detectable difference between proliferative and membranous forms might imply either a problem of power in the statistical calculations, or a true absence of difference. This would suggest an extraglomerular origin of the protein, and more precisely an origin from cells not infiltrating the glomerulus, given the paucity of cells in the glomeruli in class V LN. Rather this might suggest the production of Gal-3BP by immune cells infiltrating the renal interstitium, or the upregulation of the protein expression in the tubular cells responding to inflammation. The latter case would suggest the role of a marker of damage, rather than a marker solely of inflammation, and it would be in line with the recent finding of an interferon signature in tubular cells as mentioned above (117).

To understand which cellular source at kidney level is responsible for expression of the protein, further investigation would be necessary. One first step would be to perform immunohistochemistry studies on renal biopsies, to localize Gal-3BP expression within areas of the renal tissue. This, given the secretory nature of the protein, would probably not be sufficient. Therefore, deeper analysis of the transcriptional activities within infiltrating and resident cells might help clarify the issue.

In proliferative forms of LN, u-Gal-3BP correlates with the histological index of disease activity

We detected a moderate correlation between u-Gal-3BP levels and the degree of histological activity (i.e. the activity index) in kidney biopsies in patients with proliferative forms of LN. This correlation appeared more robust when patients not receiving immunosuppressants at the time of kidney biopsy were taken into consideration, despite the fact that we did not restrict for the use of corticosteroids.

This finding suggests that u-Gal-3BP reflects the degree of disease activity as defined at histological evaluation, which means that it might serve as biomarker not only of the presence of LN, but also of its severity.

Levels of u-Gal-3BP are influenced by ongoing treatments with corticosteroids and, for proliferative LN, antimalarials

The levels of u-Gal-3PB were analysed with respect to ongoing treatments. When considering the entire group of LN patients, the main difference which emerged was the influence of ongoing oral corticosteroid treatment on u-Gal-3BP. Being on such treatment was associated with significantly lower levels of the protein. No differences emerged when

comparing u-Gal-3BP levels in patients with LN with respect to being on immunosuppressants or antimalarial treatment at the time of kidney biopsy.

We repeated the calculations, further stratifying the LN patients according to their histopathological subtype. In patients with proliferative LN, the influence of corticosteroids was still evident, with untreated patients showing higher levels of u-Gal-3BP. This difference was instead lost in the patients with membranous and mesangial LN. The absence of a difference in u-Gal-3BP levels in relation to ongoing immunosuppressants was confirmed in all three major histopathological subtypes. Instead, while the lack of influence of antimalarials was still evident for membranous and mesangial forms, in proliferative LN a difference was detected. In fact, patients with proliferative LN on antimalarial drugs showed significantly lower levels of u-Gal-3BP compared to those not on antimalarial drugs.

These findings are interesting for two reasons. First, regarding use of the protein as a urinary biomarker, larger studies should address the question of a possible correlation with the ongoing dose of corticosteroids. In our study, we looked for such a correlation, but we did not find one. This might reflect the fact that not only is the dose at the time of determination relevant, but so is the cumulative exposure of being on a certain dose for a certain time in terms of suppression of the synthesis of the protein and by extension of the interferon pathway (297). Second, the observation of a reduction of u-Gal-3BP in relation to both corticosteroid and antimalarial treatment introduces some pathophysiological and biological considerations. In fact, it is compatible with a pharmacological effect of both these types of drugs on the interferon-mediated activity. Since Gal-3BP is an interferon-inducible protein, its reduced expression in the presence of these treatments implies an action of corticosteroids on the pathways responsible for the protein's expression.

Levels of u-Gal-3BP reduces over time upon treatment

In a subset of ten patients, repeated kidney biopsies were available. When the procedure was performed, new urine samples were obtained. It was therefore possible to compare the concentrations of the urine biomarkers under examination in our study pre- and post-treatment. This comparison showed a significant reduction of the levels of u-Gal-3BP upon treatment.

We did not observe similar behaviour for the other biomarkers, with the exception of KIM-1 which significantly decreased upon treatment.

This finding confirms that the urinary levels of u-Gal-3BP are sensitive to change, which is a feature that allows its use in monitoring treatment effects. Given the reduced sample size on which this observation was made, its confirmation in larger cohorts is warranted. Also, the reduction observed upon treatments needs to be evaluated with respect to achievement of clinical response or not.

9.13 Extracellular vesicles (EVs) may be used as urinary biomarkers of LN (Paper V)

EVs carrying inflammatory markers as cargo molecules can be found in urine of LN patients

Paper V is an exploratory study testing the possibility to detect in urine samples EVs previously demonstrated to be abundant in the blood of SLE patients. The study confirmed this possibility.

In the 13 SLE patients enrolled in the study, paired analysis of blood and urine samples confirmed the presence in the urine of all the EVs carrying the molecules of interest. The amount of EVs in the urine samples was generally lower than in blood, with the most notable difference regarding IgG carrying EVs, which were very low in urine samples.

Patients with active LN showed significantly higher levels of EVs carrying C5a, a complement fraction with potent pro-inflammatory activity.

In further analysis, stratifying patients as having a proliferative or a non-proliferative form of LN, all the EVs tested showed higher levels in the proliferative forms. From this analysis were excluded IgG and AxL-carrying EVs based on the choice of an arbitrary cut-off of urine levels, which was considered of interest.

Proliferative LN was associated with higher levels of EVs carrying all the complement products we considered (C3a,C4,C4d, C5a), which suggests the origin of EVs from cells which undergo complement attack. Moreover, high levels of EVs carrying mitochondrial antigens, lactadherin, NGAL and TWEAK were detected in higher proportion in proliferative LN as compared to non-proliferative LN.

These results, although preliminary, appear promising. In fact, they confirm that EVs present in urine carry molecules involved in immune pathogenetic events (as complement fractions and split products and other immune markers as NGAL and TWEAK) that could be associated with disease activity. Moreover, the presence of EVs carrying mitochondrial antigens also points towards the passage in urine of bigger EVs carrying potential autoantigens.

Moreover, the presence of cargo molecules such as mitochondrial antigens and lactadherin fits into the role of apoptosis in SLE pathogenesis and is in line with previous reports on the relevance of these molecules in EVs pathobiology (260, 271).

This study was meant only to be an exploratory one. The purpose was indeed to test whether molecules of interest would be found expressed on urinary EVs. Among the tested markers, the one that seem to be promising as indicative of active disease at histological examination is C5a. The other markers are found in higher levels in proliferative nephritis, which is somehow expected. The findings need further confirmation in larger cohorts, which could better reveal how specific EVs might relate to the histotype, the degree of activity and be evaluated in terms of clinical correlate. Much research is also needed to better define the pathogenetic role of EVs in the context of LN.

10 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis sought to contribute elements of knowledge in two main areas of research on the complex disease of SLE. The unifying concept behind the papers as presented here is utility of biomarkers in SLE care. The general aim of the studies can be summarized in the search for biologic and clinical elements which may be used for predicting efficacy, and anticipating safety concerns, when using specific treatment options or, more in general, when dealing with clinical manifestations of high impact for SLE patients, especially LN.

In **Paper I**, we explored how RTX treatment would impact on recently defined B- and T-cell subsets, which were not known when the drug was first introduced into clinical use.

We demonstrated that ABC cells are transiently reduced upon RTX treatment, which might correspond to an effective transient depletion, given that these cells, as memory cells, express CD20. This observation is of interest because ABCs have received great attention during the recent years, and they are thought to represent a precursor of antibody-producing cells. Very recent insights into the phenotype of B-cells characterized as CD11c+CD21- has shown that within this phenotype is present a wide complexity (194).

We were also able to provide insights into the relation of certain B-cell phenotypes with the development of immunogenicity, which we further explored in Paper II. In particular, we observed that in the presence of ADA, frequencies of DN B-cells are higher; and that the relative expansion of plasma blasts which is normally seen in the early phase of RTX-induced changes of B-cell peripheral subsets, is more pronounced.

These data are of interest, since one major question in the treatment of autoimmune conditions, is that of how effectively they target autoreactive clones, sparing the clones not directly involved in the autoimmune process. Moreover, they suggest that the cellular source of immunogenicity, aside from the recognition of drug related epitopes by the CD4+ T-cells, resides in the residual B-cells.

RTX has been used for about two decades in SLE, outside a formal indication. While several real-life studies point towards a general benefit of the drug for the treatment of the disease, formal demonstration of its efficacy is still absent. New trials would be of utmost importance for defining when, how and whom to treat with RTX, alone either in combination with BAFF blockade or other approaches. Also, a renewed analysis of the deep immunological consequences of RTX administration, in light of newly defined phenotypes of B-cells, may help to define the place of this drug in the therapeutic armamentarium of SLE, as it would to better classify patients in terms of immunological abnormalities within the B-cell lineage, defining e.g. patients with predominant hyperreactive B-cells and those with a predominant plasma cell related immunopathology (298).

In **Paper II**, we defined how immunogenicity is a frequent consequence of RTX administration in SLE, providing insights into this phenomenon and into its pharmacological and clinical implications. We were able to show that the presence of ADA was associated with higher B-cell counts at follow-up, which may imply a reduced efficacy on depleting B-cells or an accelerated repopulation, which in turn may signify a shorter duration of the pharmacodynamic action of the drug. We were also able to link the presence of ADA with the occurrence of serum sickness, a reaction often missed owing to its delayed appearance.

The safety aspects we were able to highlight in our study encourage a more active surveillance of infusion reactions, especially serum sickness. Patients should be educated about the recognition of such reactions, and to report them for being treated in appropriate ways and be screened for ADA. An active surveillance would of course require an active recording of these reactions, which could help in defining the real magnitude of the phenomenon.

We could most of all demonstrate which clinical characteristics of the SLE patients receiving RTX were mostly associated with ADA development, linking high clinical disease activity, a serologic-active profile, and nephritis to immunogenicity. Since we also confirmed no apparent role for comedications such as CYC in limiting ADA occurrence, the identification of the patients at risk for immunogenicity causing meaningful clinical consequences is, in our view, of great relevance. We firmly believe that our findings should encourage further research and also the implementation of systematic screening for ADA, especially in nephritis patients.

ADA are most probably of different isotypes and polyclonal, and can be developed at variable titres. These features may reflect diverse consequences in terms of safety and efficacy. Therefore, determining the isotypes and identifying and quantifying neutralizing ADA may be relevant for characterizing and stratifying the risks associated with immunogenicity for individual patients. Future developments should see the implementation of integrated programmes, in which ADA are tested and further evaluated in the laboratory to verify their activity (299). An evolution of immunogenicity evaluation in real life which integrates the bench and the bedside is not only motivated by research scopes. It has immediate clinical repercussions and not least pharmacoeconomic ones. From a clinical perspective, identifying patients who develop neutralizing ADA, may lead to avoiding retreatments which would have doubtful efficacy and promote the switch to alternative treatments or humanized anti-CD20 molecules. These drugs, such as obinutuzumab, are in principle less immunogenic than RTX. However, ADA to RTX may also be able to bind humanized monoclonal antibodies. Performing laboratory tests to ascertain this ability of ADA would help in selecting a more appropriate treatment strategy, which aside from the clinical benefit, would impact on treatment costs, a concern considering current costs of the new generation of anti-CD20 molecules. Developing and implementing algorithms for immunogenicity assessment to be used in clinical routine is a highly desirable goal for better utilization of biotherapeutics.

In **Paper III**, we found that post-RTX LON is also a frequent consequence of RTX use in SLE, and we provided insights into its clinical meaning. In line with other studies, SLE patients appear more prone to this complication as compared to other patient categories. Moreover, although asymptomatic in a majority of cases, LON is not always free from clinical consequences, causing fever and in some cases, major infections related to the profound neutropenia. In addition, we provided some mechanistic insights into LON as a biological phenomenon. The particular susceptibility to LON of SLE patients may have a biological basis in the same biology as that of the disease. The high production of BAFF which follows RTX may explain the interference with granulopoieis and act on an already defective bone marrow.

Our findings indicate a benefit in recommending active surveillance of SLE patients receiving RTX for the occurrence of LON, given the possibility of infectious complications. Also, linking the occurrence of LON to inevitable and desired biological modifications of the B-cell compartment and related cytokines, our study raises awareness for LON as a side-effect that could be shared by other anti-CD20. Among the molecules of new generation, obinutuzumab produces a more profound B-cell depletion compared to RTX (288). This

might be a key to a higher efficacy and the possibility to produce more profound depletion also at tissue levels. However, deeper depletion would at least in theory signify an even more pronounced burst in BAFF levels. The introduction of new generation anti-CD20 in clinical practice will probably reveal whether more efficient B-cell depletion will be accompanied by even higher incidence of LON. Data on obinutuzumab use in lymphoma seem to point towards this direction (296).

In **Paper IV** we moved from the field of RTX, but remained in that of biomarkers. We were able in this paper to demonstrate that Gal-3BP measured in urine is a valuable biomarker of LN. It can indicate the presence of organ involvement and its severity, and has a good sensitivity to change, which makes it also a good tool for monitoring treatment effects.

In **Paper V** we were able to confirm the presence of EVs carrying a range of molecules possibly involved in pathophysiological processes in SLE in urine samples from LN patients.

Both papers (IV and V) contribute to a wide and active field of research. Searching for urinary biomarkers of renal pathology, and in particular of LN, is important in many respects. Kidney biopsy has an essential role in the clinical management of LN, and it is currently not possible to envisage that it could be replaced by only urine analysis evaluating single or combined biomarkers.

In the scientific debate on the matter, often the terminology 'liquid biopsy' is used. It is possible that, in the future, advanced techniques able to generate deep and comprehensive analysis will enable the possibility of replacing kidney biopsy. This is the case with proteomic and transcriptomic approaches, which today mainly find room of application in the research setting, but could allow to dissect LN and classify the disease on a molecular level, which in the future may allow personalized approaches to the management of LN patients (300).

These modalities generate enormous amounts of data, and are truly fascinating. However, big amounts of data also raise the question of how to manage information. On the other hand, it is necessary to think about how to implement possibilities in daily life. Advanced methods, such as OMICs and flow cytometry, are not available everywhere, and require experienced operators. Also, they require clinicians who can handle and interpret the results, which requires a cultural step-forward.

Even if the care of LN patients is often remanded to tertiary centres, the availability of methods and personnel, along with the scalability of the techniques, may make their implementation challenging. In this respect, implementing tests of rapid and more flexible execution and interpretation may be an advantage.

Panels of biomarker, in which single molecules can reveal different components of renal pathology, are surely a good way forward. However, the validation of single and combination urine biomarkers in multiple cohorts is a need that cannot be overlooked.

Also, it will be necessary to explore each biomarker thoroughly to understand how the molecule truly reflects the subtype of renal inflammation and its histological activity.

The search for reliable biomarkers of LN is in conclusion a fascinating area of research, which offers a wide range of translational perspectives. There is however 'a long a winding road' to chase until the definition of the best type of biomarker, both in terms of its capacity to be fully informative about the pathology of LN, and of its being cost-effective and easy to perform.

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