Role of autoantigen-specific T cell subsets in disease pathogenesis and as therapeutic tool in systemic lupus erythematosus

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Die Naturwissenschaften braucht der Mensch zum Erkennen, den Glauben zum Handeln. Religion und Naturwissenschaft schließen sich nicht aus, wie heutzutage manche glauben und fürchten, sondern sie ergänzen und bedingen einander. ***Für den gläubigen Menschen steht Gott am Anfang, für den Wissenschaftler am Ende aller Überlegungen.*** *Wohl den unmittelbarsten Beweis für die Verträglichkeit von Religion und Naturwissenschaft auch bei gründlich-kritischer Betrachtung bildet die historische Tatsache, daß gerade die größten Naturforscher aller Zeiten, Männer wie Kepler, Newton, Leibniz von tiefer Religiosität durchdrungen waren.*

Max Planck (1858 – 1947)

My beloved father, Tito Kusnanto Hasan
1955 – 2012

Papah, salam rindu lestari ~

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass die von mir vorgelegte Dissertation mit dem Titel ***„Role of autoantigen-specific T cell subsets in disease pathogenesis and as therapeutic tool in systemic lupus erythematosus“*** zur Erlangung des akademischen Grades Doktor der Ingenieurwissenschaften (abgek. Dr.-Ing.) in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind.

Dimas Abdirama

Statement of contribution to jointly-authored works contained in the thesis

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis. I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, experimental design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis.

I used the phrase "we" instead of "I" throughout this thesis to appreciate any support and contribution of others to my thesis. The thesis represents a true collection of data generated in the laboratory of Prof. Gabriela Riemekasten at the medical clinic for rheumatology and clinical immunology, Charité Universitätsmedizin Berlin and at the Deutsches Rheuma-Forschungszentrum Berlin. Anyone in the group has contributed directly or indirectly to this thesis by any means, including discussion, brainstorming, or technical assistance. Nevertheless, I emphasize that the thesis is my original work.

The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award or any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Posters:

1. Poster presented at 4th European Congress for Immunology, September 6 – 9, 2015, Vienna, Austria.
   Title: *Loss of CD25 expression in genuine Treg from SLE patients is associated with reduced Treg activity, which can be restored by IL-2 therapy*
   Caroline von Spee-Mayer, Jens Y. Humrich, Dimas Abdirama, Anika Klaus, Angelika Rose, Andreas Radbruch, Gerd-Rüdiger Burmester, Gabriela Riemekasten
2. Poster presented at 36th European Workshop for Rheumatology Research, February 25 – 27, 2016, York, United Kingdom.
   Title: *Detection on autoantigen-specific CD4+ T cells associated with systemic lupus erythematosus*
   Dimas Abdirama, Philipp Enghard, Sebastian Tesch, Andreas Radbruch, Gerd-Rüdiger Burmester, Gabriela Riemekasten

3. Poster presented at 10th European Lupus Meeting, October 5 – 8, 2016, Venice, Italy.
   Title: *Frequency of SLE-Associated Autoantigen-Specific CD4+ T cells Correlates with SLE Disease Activity*
   Dimas Abdirama, Philipp Enghard, Sebastian Tesch, Jens Y. Humrich, Andreas Radbruch, Gerd-Rüdiger Burmester, Gabriela Riemekasten
4. Poster to present at 47th Annual Meeting of the German Society for Immunology, September 12 – 15, 2017, Erlangen, Germany.
   Title: *Nuclear autoantigen-reactive CD4+ T cells are expanded in SLE, correlate with disease activity, invade target organs such as the kidney, and outnumber their regulatory counterparts*
   Dimas Abdirama, Sebastian Tesch, Andreas Radbruch, Gerd-Rüdiger Burmester, Gabriela Riemekasten, Philipp Enghard
5. Poster to present at Cell Symposia – Human Immunity, October 22 – 24, 2017, Banff, Canada.
   Title: *Human CD4+ T Cells Reactive to Nuclear Antigens Are Expanded in Patients with Active SLE, Produce Effector Cytokines, Invade The Kidneys, and Outnumber Tregs*
   Dimas Abdirama, Sebastian Tesch, Andreas Radbruch, Gerd-Rüdiger Burmester, Gabriela Riemekasten, Philipp Enghard

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   Caroline von Spee-Mayer, Elise Siegert, Dimas Abdirama, Angelika Rose, Anika Klaus, Tobias Alexander, Philipp Enghard, Birgit Sawitzki, Falk Hiepe, Andreas Radbruch, Gerd-Rüdiger Burmester, Gabriela Riemekasten, Jens Y Humrich

Draft manuscript in preparation:

7. Title: *Nuclear antigen-reactive CD4+ T cells expand in active SLE, invade the kidneys and outnumber Treg*
   Dimas Abdirama, Sebastian Tesch, Anna-Sophie Grießbach, Caroline von Spee-Mayer, Jens Y Humrich, Ulrik Stervbo, Nina Babel, Alexander Scheffold, Falk Hiepe, Andreas Radbruch, Gerd-Rüdiger Burmester, Gabriela Riemekasten and Philipp Enghard

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This work is a heartfelt tribute to Papa. In the last days before I submit this thesis you came to me in my dream so many times.

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List of abbreviations

µCi	micro-curie
µL	microliter
ANA	anti-nuclear antibody
APC	Allophycocyanin
APRIL	A proliferation-inducing ligand
ARTE	antigen-reactive T cell-enrichment
Asp	Aspergillus fumigatus
BAFF	B cell activating factor
Bcl-2	B-cell lymphoma 2
BCR	B cell receptor
BILAG	british isles lupus assessment group scale
BLyS	B lymphocyte stimulator
BSA	bovine serum albumin
BV	Brilliant Violet™
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
c-Maf	proto-oncogene c-Maf
CMV	cytomegalo virus
CO_2	carbon dioxide
cpm	count per minute
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCR	chemokine (C-X-C motif) receptor
DNA	deoxyribonucleic acid
DMEM	Dulbecco Eagle's minimal essential medium
DRFZ	German Rheumatism Research Centre
dsDNA	double-strand DNA
EA	ethics authorities
EBV	Eppstein-Barr virus
EC_{50}	Half maximal effective concentration
ECLAM	european consesus lupus activity measure
EDTA	Ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FCS	forward scatter
FDA	food and drug administration
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead Box protein P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome wide association studies
Gy	gray

H_2O	water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	human leukocyte antigen
IC_{50}	half maximal inhibitory concentration
ICOS	Inducible T-cell costimulator
ICOS-L	Inducible T-cell costimulator ligand
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMEM	Iscove's Modified Dulbecco's Mediaúm
IRF-4	Interferon regulatory factor 4
IU	international units
KLH	Keyhole limpet hemocyanin
LAI	lupus activity index
LS	large size
MACS	magnetic-activated cell sorting
MHC	major histocompatibility complex
mL	mililiter
MMF	mycophenolate mofetil
MP65	mannoprotein 65
MS	medium size
MS	multiple sclerosis
NET	neutrophil extracellular traps
NK	natural killer
nM	nanomolar
NZB	New Zealand black mouse
NZW	New Zealand white mouse
O_2	oxygen
PB	Pacific Blue™
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	Phycoerythin
PerCP	peridinin-chlorophyll-protein complex
PHA	Phaseolus vulgaris
PLP	Proteolipid protein
PMA	phorbol 12-myristate 13-acetate
RNP70	ribonucleoprotein 70kDa
RORγt	RAR-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
SEB	Staphylococcus enterotoxin B
SIS	the national institute of health SLE index score
SLAM	systemic lupus activity measure

SLE	systemic lupus erythematosus
SLEDAI	SLE disease activity index
Sm	Small nuclear ribonucleoprotein
SmB	Small nuclear ribonucleoprotein B
SmD1	Small nuclear ribonucleoprotein D1
SSA	Sjögren's-syndrome-related antigen A
SSB	Sjögren's-syndrome-related antigen B
SSC	sideward scatter
STAT	signal transducer and activator of transcription
T1D	Type-1 diabetes
T2D	Type-2 diabetes
T-bet	Th1-specific T box transcription factor
Tcon	CD4+ conventional T cell(s)
TCR	T cell receptor
Tfh	T follicular helper cell
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
Tph	T peripheral helper
Tr1	Type 1 regulatory T cells
Treg	CD4+FoxP3+(CD127low) regulatory T cell(s)
Trm	tissue-resident memory cell
TSDR	Treg-specific demethylation region
U	unit
VLE	very low endotoxin

Zusammenfassung

Hintergrund/Ziele:
Systemischer Lupus Erythematodes (SLE) ist eine chronische und systemische Autoimmunkrankheit charakterisiert durch einen Verlust der Immuntoleranz gegen Kernantigenen wie SmD1, RNP70, Histone, Ro, und La. Neben der Erzeugung der Autoantikörper gegen Kernantigene könnten CD4+ T-Zellen mit Kernantigen-spezifischen T-Zell-Rezeptor Autoimmunreaktion auslösen und zur Organschädigung führen. Bis dato ist die Rolle der autoreaktiven CD4+ T-Zellen in SLE aufgrund der technischen Limitation wenig bekannt. Autoreaktive CD4+ T-Zellen stellen eine sehr seltene Population dar, so dass der Nachweis von solchen Zellen als sehr schwierig gilt. In der vorliegenden Doktorarbeit waren verschiedene Aspekte der autoantigen-spezifischen T-Zellsubpopulationen in SLE wie ihren Nachweis im peripheren Blut, ihre Antigenspezifität, ihre Frequenzen in Gesundheit und in Krankheit, ihre Effektorfunktionen, ihren Beitrag zu der Erzeugung von Autoantikörpern, ihre Infiltration in entzündete Gewebe, und ihre Regulationsmechanismen zu untersuchen, um ihre Rolle in der Krankheitspathogenese des SLE zu verstehen.

Methoden/Ergebnisse:
CD4+ T-Zellen mit Reaktivität gegen SmD1, RNP70, Histone, Ro, und La wurden mit polyklonalen T-Zellbibliotheken und Anreicherung der CD154-exprimierenden T-Zellen nach der *in vitro* Stimulation mit Antigenen nachgewiesen. Die Frequenzen dieser Zellen waren erhöht in den Patienten mit aktivem SLE und korrelierten mit der Krankheitsaktivität. Die Antigenspezifität solcher T-Zellen wurde durch die Generierung der T-Zell-Klone und T-Zell-Linien evaluiert. Durchflusszytometrische Untersuchung zeigte, dass diese Zellen Interferon (IFN)-γ, Interleukin (IL)-17, und IL-10 in den Patienten mit aktivem SLE produzierten, wobei IL-4 in der gleichen Kohorte weniger produziert wurde. Die Produktion von IFN-γ korrelierte darüber hinaus mit der Krankheitsaktivität. Durch die Generierung der Urin-CD4+ T-Zellbibliotheken konnte eine Akkumulation dieser Zellen im Urin nachgewiesen werden, die sich auf eine Infiltration dieser Zellen in entzündetes Nierengewebe zurückführen lässt. Eine Anreicherung der CD137-exprimierenden FoxP3+ CD4+ T-Zellen war nützlich für die Bestimmung der Frequenzen von SLE-assoziierten Kernantigen-spezifischen regulatorischen T-Zellen. Unsere Analyse zeigte, dass in den Patienten mit aktivem SLE die Frequenzen der Kernantigen-spezifischen CD4+ T-Zellen viel mehr waren als die Frequenzen der regulatorischen T-Zellen mit der gleichen Antigenspezifität. Diese Umstände korrelierten mit der Krankheitsaktivität.

Schlussfolgerungen/Relevanz:
Die vorliegende Doktorarbeit diskutiert die Basis von der Rolle der SLE-assoziierten autoantigen-spezisfichen T-Zellsubpopulationen in der SLE-Pathogenese und als therapeutisches Instrument für SLE, unter anderem mit Hinsicht auf ihre Antigenspezifität, ihre Frequenzen, ihre Infiltration in entzündete Organe, und ihre Regulationsmechanismen. Zum ersten Mal zeigten wir, dass diese Zellen im aktiven Lupus expandierten, Effektorfunktionen durch Zytokinproduktion ausübten, in entzündete Organe infiltrieren, und die Frequenzen der regulatorischen T-Zellen übertrafen. Diese Eigenschaften betonen, dass sich Kernantigen-spezifische CD4+ T-Zellen an der Krankheitspathogenese des SLE beteiligen, und dass sie ein interessantes Instrument für optimierte Behandlungsstrategien des SLE darstellen.

Abstract

Background/Aims:
Systemic lupus erythematosus (SLE) is a chronic and systemic autoimmune disorder characterized by loss of immune tolerance towards nuclear antigens such as SmD1, RNP70, Histone, Ro, and La. Besides the production of autoantibodies that directed to nuclear antigens, CD4+ T cells with T cell receptor specific to nuclear antigens may also trigger autoimmune responses leading to severe organ inflammation. To date, little is known about the role of autoreactive CD4+ T cells in SLE mainly because of technical limitations with respect to the detection of such cells which is extremely challenging. The thesis aimed to explore different aspects of autoantigen-specific T cell subsets in SLE such as their detection, their antigen-specificity, their frequencies in health and disease, their effector functions, their contribution to the autoantibody production, their infiltration to the inflamed tissues, and their regulatory mechanisms, in order to understand their role in disease pathogenesis of SLE.

Methods/Results:
CD4+ T cells reactive to SmD1, RNP70, Histone, Ro, and La were detected using libraries of polyclonal T cells and by enrichment of CD154-expressing T cells after *in vitro* antigenic stimulation. The frequencies of these cells were increased in patients with active SLE and correlated with disease activity. The antigen-specificity was evaluated by generating T cell clones and T cell lines. Flow cytometric analysis revealed that these cells produced interferon (IFN)-γ, interleukin (IL)-17, IL-10, but less IL-4 in patients with active SLE, in which the production of IFN-γ correlated with disease activity. Libraries consisting polyclonal urinary CD4+ T cells demonstrated an accumulation of these cells in the urine of active SLE patients with lupus nephritis, indicating their infiltration to the inflamed kidneys. An enrichment of CD137-expressing FoxP3+ CD4+ T cells after *in vitro* antigenic stimulation was useful to determine the frequencies of SLE-associated autoantigen-specific regulatory T cells. Our analysis revealed that SLE-associated autoantigen-specific CD4+ T cells outnumbered their regulatory counterparts in patients with active SLE and it correlated with disease activity.

Conclusions:
The thesis discusses the conceptual foundation about the role of SLE-associated autoantigen-specific T cell subsets in disease pathogenesis and as therapeutic tool in SLE, including but not limited to their antigen-specificity, their frequencies, their effector functions, their infiltration to the inflamed organs, and their regulatory mechanisms. We reported for the first time that in active SLE, CD4+ T cells reactive to nuclear antigens are expanded, acquire effector functions through cytokine production, infiltrate the inflamed organs, and outnumber the regulatory T cells. These features emphasize pathogenic characteristics of autoreactive CD4+ T cells that may contribute to the disease pathogenesis of SLE. Autoreactive CD4+ T cells represent an interesting target for optimised treatment strategies in SLE.

General introduction

Systemic lupus erythematosus (SLE) is a chronic and systemic autoimmune disorder characterized by loss of immune tolerance towards nuclear autoantigens. Nuclear components such as SmD1, RNP70, Histone, Ro, and La are the major autoantigens targeted in SLE. Patients with SLE develop high-affinity, somatically-mutated autoantibodies directed to nuclear antigens indicating they have arisen in the germinal centres as a result of antigen-specific B and T cell interaction. Investigations into cells from the adaptive immune system related to their implication in SLE have mainly focused on B cells. The role of CD4+ T cells specific to nuclear antigens in the disease pathogenesis of SLE is yet not fully known. Our current understanding of the factors that drive the pathology of SLE is limited, although great progress has been made over the past 50 years and mortality is currently only around 10% within the last ten years (compared with 50% within three years in the 1960s) [1].

Current state of science and technology on the immunological aspects of SLE:

1. It asserts that loss of tolerance and sustained autoantibody production towards nuclear antigens are the major determinants of SLE pathogenesis.
2. One of the key concepts in pathogenesis is an imbalance between apoptotic cell production and disposal of apoptotic material, including nuclear antigens.
3. Increased production of neutrophil extracellular traps and type I interferon is triggered by persistent apoptotic debris containing nuclear antigens and it is strongly associated with the disease progression.
4. Autoreactive B cells recognize nuclear antigens, and then they mature, expand, and begin to secrete more autoantibody, which enhances the adaptive immune responses.
5. HLA-genes confer the strongest genetic susceptibility for SLE sharpening indication of nuclear antigen-driven pathogenesis.
6. T cell-receptor (TCR) signalling abnormalities have been described in SLE and thought to be central to the disease process. TCR recognize antigen presented in HLA-molecule complexes, probably specific to nuclear antigens.
7. Deficient or defective regulatory T cells have been identified in SLE that leads to defective peripheral tolerance against autoreactive conventional T cells.
8. Efforts to elicit immunodominant T cell epitopes on nuclear antigens have been made, although the clinical consequences have not yet been determined.

Current knowledge underlines the importance of nuclear antigens and T cells in the pathogenesis. Due to limited available technologies, one of the last major frontiers in understanding systemic autoimmunity is to explain the specificity and functions of pathogenic T cells, bringing them on a par with present understanding of SLE pathogenesis, including but not limited to autoantibody production, because T cells provide more functions than just signals for B cells. Extensive and comprehensive research on nuclear antigen-specific CD4+ T cells in SLE is therefore necessary to elucidate their role in order to develop therapeutic tool against SLE. There is still a number of open questions remaining about autoantigen-specific CD4+ T cell subsets as enlisted in the main objectives of the thesis.

Main objectives and aim of the thesis:

1. Can SLE-associated autoantigen-specific human CD4+ T cells be detected?
2. How is the antigen-specificity of these cells?
3. How is the frequency of these cells in health and disease?
4. What kind of effector function do these cells exert?
5. Do these cells promote the production of autoantibodies?
6. Can these cells be detected in the inflamed tissue?
7. Can SLE-associated autoantigen-specific regulatory T cells be detected?

Answering the questions may help explain the role of SLE-associated autoantigen-specific CD4+ T cells in the disease pathogenesis of SLE, and by knowing their pathogenic role, therapeutic strategies by targeting these cells can be developed. Aim of this thesis is to answer the open questions enlisted above.

Working hypothesis:

CD4+ T cells specific to SLE-associated autoantigens (nuclear antigens such as SmD1, RNP70, Histone, Ro, and La) are pathogenic and contribute to the disease pathogenesis of SLE, thus targeting these cells may provide a therapeutic tool in SLE.

Outline of the thesis:

The thesis is subdivided into ten chapters. Because the immunological aspects of SLE are the scope and the focus in the thesis, an introduction to the immune system as well as to the cells in the immune system and general information about SLE are presented (**chapter 1**). The work within the thesis started with the detection of CD4+ T cells specific to an epitope on SmD1 protein, $SmD1_{(83-119)}$, which displays a target for anti-SmD1 autoantibodies (**chapter 2**). The detection of $SmD1_{(83-119)}$-specific CD4+ T cells revealed several issues. Analyses of TCR-specificity and MHC-binding to the autoantigen provide the explanation to the issues (**chapter 3**). However, CD4+ T cells reactive to a variety of SLE-associated autoantigens such as to full-length SmD1, RNP70, Histone, Ro, and La could be detected and their frequencies in the periphery of patients and healthy individuals could be determined (**chapter 4**). Following the detection of SLE-associated autoantigen-specific CD4+ T cells, this thesis explores their antigen-specificity (**chapter 5**) and cytokine production (**chapter 6**). Possible correlation between the frequencies of SLE-associated autoantigen-specific CD4+ T cells with the production of the autoantibodies is described (**chapter 7**). Furthermore, the presence of SLE-associated autoantigen-specific CD4+ T cells in the inflamed tissue could be predicted (**chapter 8**). Moreover, the frequencies of SLE-associated autoantigen-specific regulatory CD4+ T cells could be determined (**chapter 9**). Finally, by assembling the data and observation, the thesis discusses the conceptual foundation about the role of SLE-associated autoantigen-specific CD4+ T cell subsets in disease pathogenesis and as therapeutic tool in SLE, including but not limited to their antigen-specificity, their frequency, their effector function, their contribution to the antibody production, their infiltration to the inflamed organ, and their regulatory mechanism (**chapter 10**). The data in the thesis also increase the awareness that more knowledge on the immunopathogenesis of SLE may still be needed.

Chapter 1

Role of autoantigen-specific T cell subsets in disease pathogenesis and as therapeutic tool in SLE: an introduction

Abstract

The immune system is a remarkable biological feature possessed by virtually all metazoans, ranging from invertebrates to higher vertebrates. Besides the classical understanding about the function of the immune system which is defending the host by identifying and combating the infectious agents, the immune system is also about how to maintain homeostasis i.e. to keep balance between protections against harmful and tolerance to harmless foreignness. Moreover, it is also to keep its competence for elimination of harmful and ignorance of harmless self-body. Disturbance in the immune homeostasis results in various disorders including autoimmunity, in which the immune system fails to maintain immune homeostasis to self-particles. SLE is a prototypic systemic autoimmune disorder due to loss of immune tolerance against self-proteins from the cell nucleus. Almost all immune cell populations appear to be dysregulated in SLE, including CD4+ T cells, a cell type that is highly specialized in recognizing protein in form of an antigen via its antigen-specific receptor. Disadvantaged function of CD4+ T cells because of their receptor specificity to self-protein is believed to be one of the major causations that lead to the pathogenesis of SLE – through mechanisms that are still poorly understood until now. Hence, in this doctoral thesis, we aim to present our data and analyses about how CD4+ T cells mediate immunopathogenesis and immunotolerance through their specific interaction with self-proteins from the cell nucleus.

Keywords: *Immune system, SLE, CD4+ T cell, antigen-specific*

Contents

1. Mammalian immune system

Mammals, including humans, have developed a complex biological system to survive in any conditions in their environment. This system is sophisticated and involves a number of different cellular and molecular components with unique characteristics and functions. The identification of the immune system began in the late 18th century, when Edward Jenner observed that mild disease of cowpox could provide protection to mortality which is caused by this disease. A century later, Robert Koch discovered pathogenic microorganisms that led to infectious diseases, including viruses, bacteria, fungi, and parasites. Their findings were further explored for development of vaccination strategies by Louis Pasteur in the 1880s. Since then, discoveries in the immune system have been increasingly reported.

The immune system has been generally understood in the society as the ability to defend self from pathogenic foreignness. However, the immune system is actually more than that paradigm; it is about how to maintain homeostasis and to ensure that all components in the system work properly. In terms of infection, it is important for the immune system to perform rapid and efficient defence mechanisms to eliminate the pathogens. In terms of allergy, the immune system has to recognize the foreign materials as harmless agents to avoid unnecessary immune responses. The same is true in the case of tumour development, where the immune system needs to recognize this self-tissue as an enemy. And finally in the case of autoimmunity, the immune system has to establish tolerance mechanisms against harmless self-particles, since otherwise it would result in pathogenic, unwanted autoimmune manifestations.

In general, our immune system can be subdivided into two major groups, innate and adaptive immunity. The innate immune system is characterized by its rapid detection and early response against target particles. The cells from innate immunity include dendritic cells, macrophages, monocytes, neutrophils, basophiles, eosinophils, granulocytes, and natural killer (NK) cells that can recognize target particles through unspecific interaction. On the other hand, the adaptive immune system is highly specialized in recognizing the target particles through antigen-specific receptors. Hence, cells from adaptive immunity such as B and T lymphocytes (B and T cells) can perform specific and efficient responses against the target particles. Once the same target particles appear again, cells from adaptive immunity can memorize their structure and rapidly respond against it. The adaptive immunity works according to the receptor specificity. Due to somatic recombination processes and mutations of the gene segments encoding the receptors, cells of the adaptive immune systems have a great diversity of receptors that can recognize a high number of particles, also known as antigens.

1.1. Innate immunity

The early phase of an immune response is initiated by the cells from the innate immune system. These cells recirculate and locate mainly in the tissue to perform immune surveillance when foreign particles invade the host body. The defence mechanism depends mainly on unspecific, invariant pattern recognition receptors that can sense and detect common features of foreign particles such as their structures, composition, or shape of the molecules. An innate immune response is characterized for example by the development of complement, which are protein molecules that can

bind at target particles and recruit other innate immune cells. These cells then perform further actions such as killing, establishing local inflammation, and clearance. This early mechanism of protection is important but it does not lead to a perpetuated control because it cannot develop immunological memory that may be crucial for quick and enhanced response in a secondary infection.

One of critical molecular mechanisms performed by the cells from innate immunity is the process of antigen uptake and presentation. When encountering target particles, cells from the innate immune system such as monocytes, dendritic cells, or macrophages, can internalize the particles into their inner cell compartment (phagocytosis), either through invariant pattern recognition receptors such as Toll-like receptor (TLR), or directly by engulfing the particles into cytoplasm [2]. Cells with the ability to take the target particle, process it, and present its fragments are called antigen-presenting cells. The particles are then processed into small fragments in the endoplasmic reticulum. Then, the fragment binds to the major histocompatibility complex molecule (MHC; in human: human leukocyte antigen complex, HLA), and it is transported into the cell surface by the Golgi apparatus for presentation to the cells of adaptive immune system (T cells) [3]. There are two MHC subtypes known as the MHC class I and MHC class II molecule. In human, both structures are generated by HLA gene that is located in the short arm of chromosome 6 [4]. This gene segment has been associated with many autoimmune disorders, including SLE [5, 6]. The HLA gene region consists of three HLA class I genes (HLA-A, -B, and –C) and three HLA class II genes (HLA-DR, -DP, and –DQ). HLA genes have different allele groups and constitute a unique combination of HLA haplotypes for each individual known as HLA polymorphism. Different HLA haplotypes can bind differently to antigen fragments and its binding affinity itself depends on the structure of the fragments. MHC class I molecules can collect peptide fragments in the cytoplasm, thus they can display antigen fragments e.g. derived from viral proteins. MHC class II molecules can bind fragments derived largely from particles being processed in intracellular vesicles [7]. Although cells from the adaptive immune system such as B cell can also perform mechanism of antigen presentation, the process of antigen presentation by the innate immune cells serves as an important bridge between the innate and the adaptive immune system. In addition, the connection between innate and adaptive immunity is supported by the cytokines that are secreted by the cells from the innate immune system while they are encountering the target particles. These cytokines include pro-inflammatory type I interferon (IFN) such as IFN-α and IFN-β, interleukins (IL) such as IL-1, IL-6, IL-8, and IL-12, and tumour necrosis factor (TNF) [8].

Besides monocytes, macrophages and dendritic cells, another cell population from the innate immunity are granulocytes. These include basophiles, eosinophils, and neutrophils. Neutrophils are well recognized as one of the major players during inflammation with their role to promote protective or pathological immune responses in the tissue. Human neutrophils are short-lived cells with a half-life in the circulation of approximately eight hours. Neutrophils are able to eliminate target particles by multiple ways, both intracellularly by phagocytosis and extracellularly by degranulation and by releasing neutrophil extracellular traps (NET). NET are composed of DNA elements and nuclear proteins such as histones to immobilize target particles, thus preventing them from spreading [9]. Finally, NK cells are a crucial innate cell population. NK cells can produce a number of cytokines upon stimulation including IFN-γ, TNF-β, granulocyte-macrophage colony-stimulating

factor (GM-CSF), and IL-10, which are involved in the modulation of immune responses mediated by the cells from the adaptive immune system (T and B cells) [10].

1.2. Adaptive immunity

Adaptive immunity is initiated when innate immunity fails to encounter the target particles thereby the infection can become chronic. There are two major cell types in the adaptive immune system which are B and T cells. Both cell types originate from hematopoietic stem cells in the bone marrow. B cells undergo their maturation process in the bone marrow prior to their circulation in the periphery, whereas the maturation process of T cells occurs in the thymus. The crucial process in the adaptive immune system includes the selection and expansion of B and T cells bearing receptors that can recognize specific fragments of antigen. Through T cell receptors (TCR), T cells can recognize cognate epitopes of an antigen presented by the antigen-presenting cells within MHC-complexes. T cells are characterized by the expression of CD3 molecules on their surface and depending on the type of co-stimulatory molecule T cells are subdivided into two major subsets, CD4+ T cells and CD8+ T cells. Both cell populations have unique features and functions in immunity. CD4 and CD8 molecules can recognize parts of the MHC class II and MHC class I molecule, respectively. CD8+ T cells can identify the peptide presented by the MHC class I molecule which is mainly long-fragmented antigen, whereas CD4+ T cells can identify the peptide presented by MHC class II molecules which are by length shorter than the fragment bound on MHC class I molecule [11]. Upon activation, CD8+ T cells are able to eliminate infected cells by secretion of cytolytic molecules, such as granzymes and perforin, or by induction of apoptosis [12]. CD4+ T cells exert their function by modulating the function of other immune cells primarily through the secretion of effector cytokines, thus they are also called helper T (Th) cells. The Th cell population consists of different cell subsets with unique features and functions when they are activated by their cognate antigen [13]. Understanding the antigen-recognition in the adaptive immune system by T cells is therefore crucial for the control of allergies and autoimmune diseases, as well as for killing of tumour cells and for avoiding the rejection of transplanted organs.

A TCR is attached to the surface of T cells and it can only recognize antigens presented in a complex with MHC molecule. Unlike TCR, B cell receptors (BCR) can recognize soluble antigens. Upon antigen recognition by BCR and appropriate activation in the germinal centres of secondary lymphoid organs B cells can differentiate either to memory B cells or to antibody-producing plasmablasts or plasma cells [14]. In addition, B cells can endocytose the antigen either through their BCR or Fc-receptor and TLR, and process the antigen for presentation on MHC class II molecule to CD4+ T cells. Antigen-presentation by B cells is critical for B and T cell interaction in the germinal centres, where specialized subset of Th cells, follicular helper T (Tfh) cells secrete cytokines such as IL-21 upon antigen-specific activation to provide T cell-help for B cell differentiation into antibody-secreting plasmablasts and plasma cells [15]. Antibodies secreted by plasma cells are soluble forms of BCR that specifically recognize the same antigen. Antibodies are important to mediate humoral immunity by binding to the antigen and thereby triggering several different mechanisms aimed at antigen elimination.

1.3. CD4+ T cell subsets

After maturation in the thymus, CD4+ T cells circulate in the periphery and search for their cognate antigen. Cells that have never encountered any antigen are called naive cells. In the course of a primary immune response, naive CD4+ T cells are activated through antigenic stimulation by antigen-presenting cells, normally dendritic cells, in the secondary lymphoid organs. The quality of T cell activation is determined by antigenic, costimulatory, and cytokine signals by dendritic cells or other innate immune cells [16]. Activated CD4+ T cells are expanded and produce effector cytokines to modulate immune responses against the antigen. Only a small fraction of the expanded cells survives and persists for a lifetime as memory cells with preferential residence in the bone marrow [17]. These cells can remember the antigen thus they are able to proliferate efficiently and respond quickly in a secondary infection. Dissection of naive and different types of memory T cells has been possible using the expression of chemokine receptor molecules [18, 19].

Human CD4+ T cells have multiple subsets which can be dissected by their capacity to produce effector cytokines, their master transcription factor expression, and their homing receptor expression on the cell surface [20]. According to the phenotypical and functional heterogeneity of CD4+ T cells, several Th cell subsets have been identified now, such as Th1, Th2, Th17, Th9, Th22, Tfh, regulatory T (Treg) cells, and type 1 regulatory T (Tr1) cells [21]. Th1, Th2, and Th17 are appreciated as major types of differentiating CD4+ T cells with specific production of cytokines. Th1 cells produce IFN-γ and express T-bet for their role to combat intracellular pathogens, whereas Th2 cells produce IL-4, IL-5, and IL-13 and express GATA-3 for their role to attack extracellular parasites [22]. Th2 cells can produce IL-10 that can promote B cell proliferation [23, 24], but they are not the only IL-10 producer because almost all types of CD4+ T cells are actually able to secrete IL-10 as anti-inflammatory, self-limiting mechanism against unnecessary immune responses [25]. Th17 cells are primarily identified and characterized by their production of IL-17, IL-22, GM-CSF, and expression of RORγt [26] required for elimination of fungi and extracellular bacteria [27, 28].

Besides Th1, Th2, and Th17, CD4+ T cell subsets also include Th9 and Th22 cells, as well as CD4+ T cells with specialized function such as Tfh, Tr1, and Treg cells. Th9 cells are characterized by IL-9 production, driven by the master transcription factor Pu.1 and specialized in combating helminth infection [29]. A recent study reported IL-9 expression by Tfh cells that is crucial for memory B cell development in the germinal centre [30]. Th22 cells are characterized by their production of IL-22 and their ability to recognize lipid antigens presented by Langerhans cells in the skin [31]. A new subset of CD4+ T cells, Tr1 cells, has been recently identified with signs of strong immunosuppresive activity and ability to restore tolerance in several autoimmune diseases through secretion of IL-10 and granzyme B [32, 33]. These cells can be dissected by the expression of CD49b and LAG-3 [34]. Furthermore, Tfh cells mark another important CD4+ T cell subset. After cognate T-B cell interaction, antigen-specific B cells are expanded and differentiate into short-lived plasma cells which produce native antibodies usually of the immunoglobulin-M (IgM) isotype. This event is then followed by the generation of the germinal centre reaction, where Tfh cells promote proliferation, immunoglobulin isotype-switch, and affinity

maturation of antigen-specific B cells [35]. All CD4+ T cell subsets bear TCR with unique antigen-specificity hence their function is associated with antigen recognition.

1.4. Regulatory T cells

Regulatory T cells (Treg) are a subset of CD4+ T cells with the capacity to suppress an immune response. Emerging evidence shows that every adaptive immune response involves recruitment and activation of Treg besides B cells and conventional T cells. Autoimmune-preventive CD4+ T cells were firstly characterized through the expression of CD25 on the cell surface [36, 37], and furthermore these cells were found to express transcription factor FoxP3 which is important for their development and function [38, 39]. The critical function of FoxP3 in maintaining immune homeostasis in humans has been shown by a study involving individuals with FoxP3 mutations. These individuals suffer from the development of severe autoimmune disease, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [40, 41]. Recently, Treg are more characterized by epigenetic demethylation of CpG islands in the FoxP3 conserved non-coding region two (Treg-specific demethylation region; TSDR) [42]. Demethylation of the FoxP3 gene is described to reflect stable FoxP3 expression. Treg are generally produced in the thymus but they can also be induced from naive T cells in the periphery [43, 44]. It is important to distinguish between thymus-derived and peripheral-derived Treg thus nomenclature for Treg has been recommended [45]. Because of their beneficial properties in suppressing unwanted immune response and chronic inflammation, Treg have become an interesting focus for development of Treg-based immunotherapy in order to help patients with autoimmune disorders [46].

1.5. Immunologic tolerance and autoimmunity

A tight regulation is necessary to ensure that the immune system stays in a homeostatic balance. Because of the great heterogeneity of TCR as well as BCR it is possible for immune system to respond to a high variability of antigens, including self-antigens (also called autoantigens). Impairment in this balance leads to pathogenic immune activation against self-components marked by the activation of self-reactive (or autoreactive) T and B cells and production of autoantibodies. Aberrant activation of autoreactive cells and production of autoantibodies lead to severe tissue damage and organ failure known as autoimmune disorder. However, the immune system is attributed with immunologic self-tolerance mechanisms to avoid such reactions.

The main self-tolerance mechanism known as central tolerance occurs early in the thymus and bone marrow for T and B cells, respectively, before autoreactive T and B cells circulate in the periphery. In this mechanism, T or B cells with a high affinity receptor to an autoantigen are deleted by apoptosis or they become anergic in the process called negative selection [47]. Anergic autoreactive T and B cells escape the negative selection and enter the peripheral circulation. Although they are anergic, in some circumstances when the immune homeostasis is disturbed, they can become potential pathogenic cells, because they are capable of inducing autoimmunity. To support the central tolerance mechanism, the immune system developed secondary mechanisms known as peripheral tolerance. This tolerance is attained through a number of events involving cells with capacity to suppress excessive immune

response such as Treg or Tr1 cells. The mechanisms underlying peripheral tolerance includes depletion, self-ignorance, and suppression of activation [48].

Taken together, the immune system has pathogenic potential because of the existence of autoreactive cells that escaped the central tolerance in the thymus or bone marrow. In normal conditions, the immune system performs mechanisms to protect against the risk of autoimmunity through peripheral tolerance. Impairment in this system can result in autoimmune diseases. Autoimmunity can be induced locally in the specific tissue such as in type I diabetes or multiple sclerosis, or systemically such as in systemic lupus erythematosus (SLE) since the autoantigens are expressed broadly throughout the body.

2. Systemic lupus erythematosus

SLE is a chronic and systemic autoimmune disease with a broad spectrum of clinical manifestations affecting almost all organs and tissues. Some scientists and physicians propose a theory stating that SLE actually represents a syndrome rather than a single disease due to its extreme heterogeneity. Many patients with SLE have a milder disease, whereas others have a fatal and life-threatening presentation. Despite of the multiple faces of SLE, the disease is consistently characterized by loss of tolerance against autoantigens, predominantly towards nuclear antigens. This prototypic autoimmune disease is a decent model to investigate how the immune system works and in which circumstances the immune system fails to work properly.

2.1. Epidemiology, aetiology, pathogenesis, and pathophysiology

Prevalence rates in SLE are estimated ranging from 20 to 150 cases per 100,000 people depending on demography. Young women represent the most affected population, nine times more frequented than men [49]. Men with SLE appear to have more evidences of serositis but less photosensitivity [50]. The aetiology of the disease is not yet fully understood, but advancement in human genetics using genetic variant identification, lupus mouse models, gene expression studies, and epigenetic analyses suggest triggers from the environment, stochastic factors, and genetic susceptibility as possible aetiologies of SLE [49]. SLE may represent an inheritable disease observed in the evidence that monozygotic twins have a higher concordance rate of SLE than dizygotic twins supported by the fact that sibling recurrence risk ratio in SLE is high [51]. Recently, genome-wide association studies (GWAS) have successfully identified susceptibility genes for SLE using hundreds of thousands of single nucleotide polymorphism markers. It has revealed that alleles within the MHC locus are the strongest genetic susceptibility for SLE in the general population [52]. GWAS could identify three main molecular pathways that are responsible for SLE such as lymphocytes signalling (either within T or B cells), IFN signalling pathways, and clearance of immune complexes and other waste materials [53]. GWAS has successfully delivered information and key concepts around the pathogenesis of SLE although it has been criticized for its failure to identify target therapy or any other causative variants. Furthermore, changes in gene expression such as impaired process of DNA methylation has been identified in patients with SLE. this happens usually through the use of drugs such as procainamide that inhibits DNA methylation [54]. Notably, T cells from mice receiving this kind of drugs are capable of inducing lupus

in recipient mice [55]. These results not only emphasize the implication of epigenetics in SLE but also underline the pivotal role of T cells in the pathogenesis. Environmental factors such as ultraviolet light and viral infections are one of the most obvious triggers in SLE [49]. Epstein-Barr virus (EBV) has been identified as a possible factor that promotes SLE through its interaction with B cells or through possible molecular mimicry with SLE associated-autoantigen such as SmD1 [56]. Last but not least, hormonal factors may contribute to the disease progression of SLE. In murine models, addition of oestrogen or prolactin is able to induce an autoimmune phenotype with an increased frequency of mature high-affinity autoreactive B cells, suggesting its implication to the disturbance of immune homeostasis [57].

The pathophysiology of SLE is described by the loss of tolerance and perpetuated production of autoantibodies. As previously mentioned, recent advancements in the SLE research through GWAS have revealed the concept that (1) defective clearance of immune complexes and biological waste, (2) formation of NET, (3) nucleic acid sensing, (4) lymphocyte signalling, and (5) interferon production are critical to the loss of tolerance towards nuclear antigens [58]. Nuclear antigens are usually not accessible to the immune cells because they are normally cleared rapidly, but because of disturbed disposal of apoptotic materials, waste of nuclear antigens is accumulated and becomes causation of loss of tolerance towards nuclear antigens. Prolonged accumulation of apoptotic material containing nucleic acids and other nuclear antigens can induce an inflammatory response through the activation of TLR which can sense nucleic acid structures [59]. Nuclear components can promote B cell differentiation since B cells can respond to nucleic acids through direct antigen recognition as well as through structure of IgM receptors for proteins complexed with nucleic acids [60].

2.2. Diagnosis, clinical features, morbidity, and mortality

Criteria for SLE classification were developed for the classification and stratification of SLE patients. The criteria are helpful for diagnosis and for selecting suitable therapy for the patients. However, current criteria still have limitations as they were developed and validated for classification of patients with longstanding established disease thus may exclude patients with onset disease or disease affected to limited organs. Because SLE is a multifactorial disease with extreme heterogeneity of clinical symptoms, personalized therapy is of great interest [61]. The diagnosis of SLE usually relies on the serologic tests to measure the concentration of autoantibodies such as anti-ANA antibody, anti-dsDNA antibody and antibodies directed to nuclear antigens. However, most of the autoantibodies are not specific for SLE as they are also found in healthy individuals as well as in patients with other autoimmune diseases such as rheumatoid arthritis and Sjögren's syndrome. More precise diagnosis for reliable stratification of SLE patients is currently one of research focuses for SLE. Nevertheless, methods to assess disease activity in SLE are still helpful for the physicians as they form the basis for treatment decisions. Several validated global and organ-specific activity indices are now widely used, including European Consensus Lupus Activity Measure (ECLAM), the British Isles Lupus Assessment Group Scale (BILAG), the Lupus Activity Index (LAI), the National Institutes of Health SLE Index Score (SIS), the Systemic Lupus Activity Measure (SLAM), and the SLE Disease Activity Index (SLEDAI), with SLEDAI being the most convenient for use in daily practice [62].

Clinical features of SLE include mucocutaneous, muskuloskeletal, renal, cardiovascular, and haematological manifestations, as well as involvement the nervous system, pleura and lungs. Lymphadenopathy and splenomegaly can also be observed. The disease presentation includes malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorders, neurological disorders, haematological disorders and immunological disorders [63]. Renal involvement in SLE usually leads to severe lupus nephritis. Between 30-60% of patients with SLE develop renal involvement, in which 18% of the patients show five years of mortality and up to 15% of them suffer from kidney failure despite of treatment with therapies [64-66]. The morbidity in SLE is predominantly triggered by the disease itself, but notably also by the treatment. Treatment-related morbidity however is complex because it is not easily separable from disease-related morbidity. Most cases indicate that the side-effect of the drugs as the major determinant for morbidity and mortality in SLE. The use of immunosuppresive agents for example cyclophosphamide can increase the risk of infection in the SLE patients [63].

2.3. Current therapeutic strategies in SLE

Current and novel therapeutic strategies in SLE include the application of systemic inflammation-directed treatment, immune cell-targeted therapies, and treatment targeting costimulatory signalling pathways (reviewed in [67]). Several drugs have been developed to reduce inflammatory responses in SLE. Antimalarial drug-the hydroxychloroquine is a nonsteroidal anti-inflammatory drug effective in the treatment of mild SLE manifestations. The drug inhibits phagosome function, thereby inhibiting TLR activation, leading to the reduction of type I IFN production. Corticosteroids are standard drugs of treatment in patients with SLE. The drugs have strong anti-inflammatory effects that inhibit T and B cell responses and alter effector functions of monocytes and neutrophils. Cyclophosphamide is applied to patients with SLE to suppress excessive immune responses. These drugs however exert many dangerous side effects such as infertility, malignancy, haemorrhagic cystitis, and infection. Mycophenolate mofetil (MMF) is used to control DNA synthesis and block the proliferation of T and B cells. These drugs are a T cell-targeted therapy in SLE. Azathioprine drugs are administered to the patients as a sparing agent for corticosteroids that inhibit DNA synthesis and therefore prevent cell proliferation in the immune system.

Because the pathogenesis of SLE is induced by abnormal function of immune cells, therapies are also developed to target different immune cell populations such as B cells. Current therapies targeting B cells include the use of antibodies to B cell-surface antigens, tolerogens, blocking of costimulatory molecules, and inhibition of B cell-relevant cytokines. B cells express CD20 on their surface and by targeting this molecule e.g. by using anti-CD20 monoclonal antibody rituximab, B cells can be deleted. Rituximab has been approved by the US Food and Drug Administration (FDA) for the treatment of relapsed or refractory B cell-lymphoma. Besides CD20 expression on B cells, CD22 expression is also currently used as a therapeutic target. CD22 is involved in the regulation of B cell signalling, thus treatment with anti-CD22 monoclonal antibody epratuzumab may inhibit B cell function in SLE. Abetimus, a B cell tolerogen, becomes an alternative treatment since it can induce functional inactivation and deletion of B cells expressing autoreactive BCR. Cytokines with the

potential to induce B cell function such as BLyS (or also known as BAFF) has become therapeutic target. Belimumab binds to BLyS and thereby inhibit its action. Atacicept can also bind to BLyS and other soluble factor critical for B cell activity, APRIL.

Costimulatory signalling pathways also become therapeutic targets in SLE as reported by the use of blocking agents to CD40 and CTLA-4. The application of anti-CD40 agent can prevent the interaction between T cells (expressing CD154) with B cells (expressing CD40, the ligand for CD154). The blocking is useful to avoid autoantibody production in the germinal centres. Furthermore, blocking of CD28 on T cells that usually binds to CTLA-4 on B cells by abatacept has also been demonstrated to inhibit costimulatory signalling necessary for activation of T cells. Anti-cytokine therapies provide an alternative way to target immune cells by preventing their activation. However, the complexity of cytokines makes it difficult to evaluate the efficacy of the therapies. Application of anti-TNF agents in the rheumatoid arthritis patients or Crohn disease results in development of anti-ANA and anti-dsDNA autoantibody, as well as drug-induced lupus-like syndromes, all of which disappear after therapy is terminated. Anti-IL-10 therapy is introduced because of high serum level of IL-10 in SLE patients that indicates its role in B cell activation. However, it should be noted that IL-10 has also potent suppressive effects on antigen-presenting cells and can directly limit the degree of autoreactivity. Other anti-cytokines agents for therapeutic strategies in SLE include anti-IL-18, anti-IL-6, anti-IL-15, and anti-IFN-α agents, all of them focus on the reduction of cytokine-driven inflammatory response.

3. Autoreactive CD4+ T cells in the pathogenesis of SLE

As previously mentioned, GWAS data have identified defects in T cell signalling, more precisely aberrant signalling through the T cell receptor. This evidence is not intrinsic and can be induced from the environment as shown in an experiment where normal T cells were cultured with serum IgG from patients with SLE that resulted in aberrant T cell signalling [68]. T cells of patients with SLE show hyperactivity of the T cell receptor-signalling pathway [69]. Furthermore, CD4+ T cells are thought to play a central role in SLE disease pathogenesis mainly because of their association with MHC molecules. GWAS data also revealed that MHC genes are the most susceptible genes for SLE. Additionally, high prevalence of somatic-hypermutated isotype-switched autoantibody production in patients with SLE constitutes an indispensable role of CD4+ T cells at antigen-specific level. Nuclear autoantigens such as Sm/SmD1, RNP70, Histone, Ro and La have been described as targets for autoantibodies that progressively develop preceding onset of the clinical manifestations of SLE [70]. We have reported the detection of CD4+ T cells reactive to SmD1, whereas other groups have reported the detection of SmB/D-reactive, RNP70-reactive, Histone-reactive, and La-reactive CD4+ T cells isolated from patients with SLE [71-74]. Ro-specific T cell hybridomas have been successfully generated from HLA-DR3 transgenic mice [75], but the presence of Ro-specific CD4+ T cells in patients with SLE is not known. Despite various reports about the detection of SLE-associated autoantigen-specific CD4+ T cells, the results still provide lack of information about their specificity, detection quality, their frequency in health and disease, and their contribution to disease pathology.

4. Conclusion

The immune system is essential to protect the host from harmful foreign pathogens but its function can be disastrous when it identifies harmless self-particles as targets, the phenomenon known as autoimmunity. SLE is a prototypic autoimmune disorder marked by loss of tolerance towards nuclear antigens that leads to tissue inflammation and organ failure. CD4+ T cells play an important role in modulating the immune system thus they are thought to be central in autoimmune diseases such as SLE. CD4+ T cells can recognize antigen through their antigen-specific receptor and they become activated and able to exert effector function upon antigen-specific activation. The role of CD4+ T cells reactive to nuclear antigens in SLE is not known. It is important to address several open questions to investigate the role of autoreactive CD4+ T cells in SLE such as their specificity to autoantigens, their frequency in health and disease, their effector function, their role in autoantibody production, their role in local tissue inflammation, and their suppressive mechanism (Figure 1).

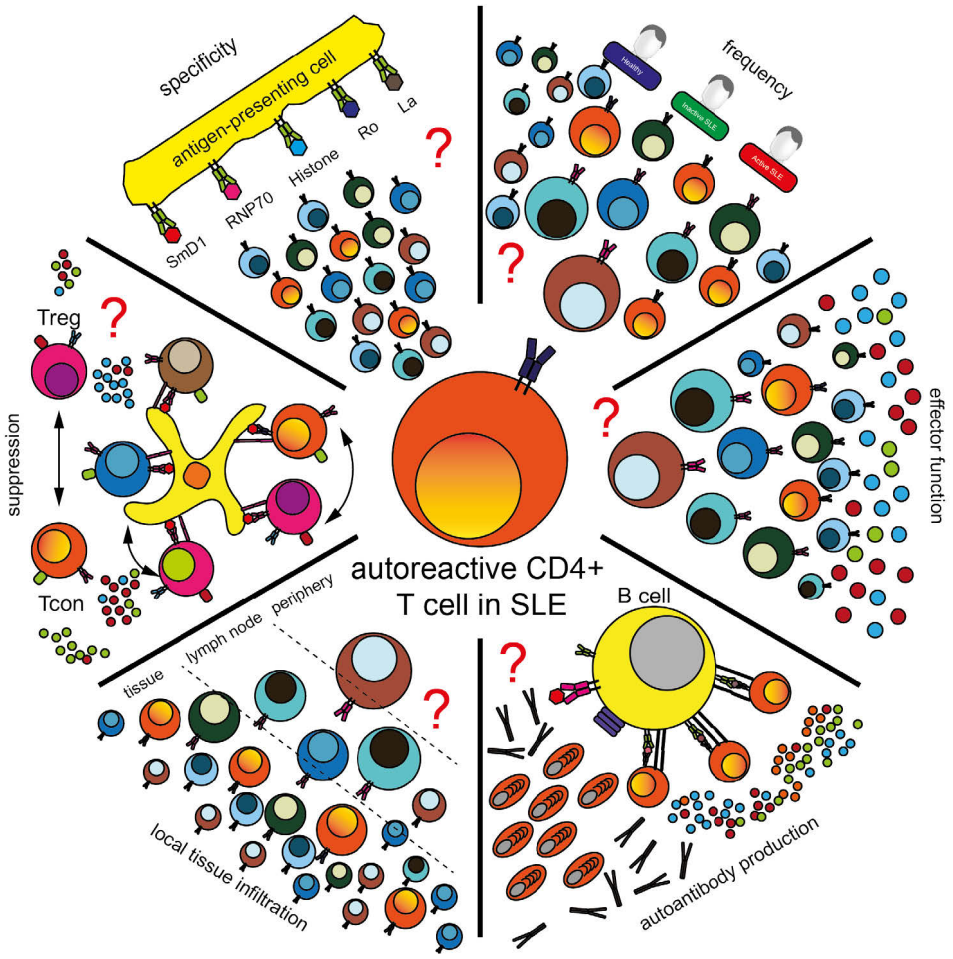


Figure 1: **Investigating the role of autoreactive CD4+ T cells in SLE.** To investigate the role of autoreactive CD4+ T cells in SLE it is necessary to evaluate their antigen-specificity, to enumerate their frequency in health and disease, to identify their effector function through cytokine production, to observe the mechanism of autoantibody production, to determine their infiltration in the inflamed tissue, and to analyse their suppressive mechanisms.

Chapter 2

Assessment of CD154 expression on activated autoantigen-specific CD4+ T cells by *in vitro* stimulation with SmD1$_{(83-119)}$

Abstract

Autoantigen-specific CD4+ T cells play a central role in establishing specific immune responses against presented autoantigens. This suggests a possible origin of T cell-mediated pathogenesis in autoimmune disease. The detection of such cell populations is extremely challenging due to rare frequency and limited available detection technologies. In recent years, several studies have reported both cell-surface and intracellular expressions of CD154 on CD4+ T cells when stimulated with antigen *in vitro*, allowing direct detection of antigen-specific T cells e.g. by multi-parametric flow cytometry. In this study, we performed flow cytometric analysis of the expression of CD154 on activated CD4+ T cells following stimulation with the SLE-associated autoantigen SmD1$_{(83-119)}$ in patients with SLE. We focused on the detection of CD154 on the cell surface as it allows purification of live antigen-specific CD4+ T cells for further characterization and functional analyses. Our results revealed that, when compared with control antigens such as SEB, *Candida albicans* MP65, or PLP, CD4+ T cells stimulated with autoantigen showed similar levels of CD154 expression of negative control. The results were consistent in our optimization experiments e.g. by depleting CD25+ cells and by enriching CD154-expressing cells. Our data delivered information on whether short-fragmented autoantigen SmD1$_{(83-119)}$ displays antigenic epitope to SmD1-specific CD4+ T cells.

Keywords: *SmD1$_{(83-119)}$, CD4+ T cell stimulation, CD154 expression on cell surface*

Contents

1. Introduction

1.1. Background

Disease pathogenesis of SLE is mediated by the development of autoantibodies against nuclear, cytoplasmic, and cell membrane antigens, e.g. anti-Sm antibodies that target Sm antigens of small nuclear ribonucleoproteins [76]. Besides anti-dsDNA antibodies, anti-Sm antibodies are considered to be specific for SLE and have been included as one criterion for diagnosing the disease by the American College of Rheumatology [77, 78]. Riemekasten *et al.* have underlined the importance of a subtype of anti-Sm antibody, anti-SmD1, on the specific amino acid sequence from position 83 to 119. Anti-$SmD1_{(83-119)}$ antibody was specifically found in the majority of sera from patients with SLE [79]. This antibody response is associated with specific HLA-DR3 haplotypes [80], suggesting that CD4+ T cells specific to SmD1 may contribute to SmD1-specific autoimmune responses.

In early studies, we have shown T cells with reactivity to $SmD1_{(83-119)}$ in patients with SLE as well as in lupus prone NZB/NZW F1 female mice [81, 82]. The next questions after these important findings were whether the number of autoantigen-specific T cells can be quantified and whether qualitative analysis of these cells at single-cell level can be performed. Until now it has been a major challenge to assess the rare antigen-specific CD4+ T cell population, and more importantly, autoantigen-specific CD4+ T cells which exist in extremely low frequency in normal individuals [83]. The assessment technologies to evaluate antigen-specific T cells have evolved to the quantification of cytokine-producing cells after *in vitro* antigenic stimulation still lack of the specificity due to inhomogeneity of cytokine-producing antigen-specific T cells and involvement of unspecific T cell activation markers such as CD25, CD69, and CD71 for antigen-specific T cell pre-sorting purpose [84, 85]. The introduction of peptide-MHC multimers in the last years also led to a promising alternative to specifically quantify and analyse antigen-specific T cells. But the availability of usable peptide-MHC II multimers is very limited and epitope-containing peptides and MHC alleles need to be fully characterized prior development of a complicated manufacturing process [86]. Thus cell markers, which are selectively upregulated on antigen-specific T cells after antigenic activation, are required for direct assessment of specific T cell responses.

Frentsch *et al.* have introduced a method for the detection of antigen-specific CD4+ T cells after short-term *in vitro* stimulation with defined antigen by assessing stable CD154 expression both intracellularly and on the cell surface, which was previously only known to be transiently expressed on activated T cells [87]. Stable CD154 expression on the cell membrane is very important as it allows isolation and fast generation of antigen-specific T cell lines or even T cell clones, allowing further characterization of the specificity and function of antigen-specific T cells.

1.2. Objectives

The overall goal of the study presented in this chapter was to purify live $SmD1_{(83-119)}$-specific CD4+ T cells for further characterization of their functions. Thus the objective of this study was to investigate CD154 expression on the cell surface of CD4+ T cells after *in vitro* stimulation with $SmD1_{(83-119)}$. Here, experiments were

performed with the aim to establish a standard operating procedure for the detection of $SmD1_{(83-119)}$-specific CD4+ T cells on the basis of CD154 expression on cell surface with an optimized signal-to-noise ratio. This is the ratio of CD154 expression after antigen-specific T cell activation compared with CD154 expression induced by unspecific bystander T cell activation.

2. Materials and methods

2.1. Blood donors and isolation of peripheral blood mononuclear cells

This protocol serves as general procedure for PBMC isolation from whole blood or buffy coat throughout this thesis. The donors can be healthy individuals, patients with inactive SLE, or patients with active SLE depending on the experiments. In the experiments presented in this chapter, human peripheral blood mononuclear cells (PBMC) were freshly isolated from whole blood taken from Sm-/SmD1-seropositive female and male patients with inactive SLE that gave their informed consent based on the ethical approval obtained by the institutional review board and ethics authorities at Charité Universitätsmedizin Berlin (EA 1/342/12, EA 1/098/07 and EA 1/036/16). For some experiments requiring high cell numbers, PBMC were isolated from buffy coats from healthy individuals, which are a by-product of leukapheretic separation of blood plasma and erythrocytes from whole blood donated by voluntary, and contain concentrated leukocytes and platelets. Buffy coats were provided by Deustches-Rotes-Kreuz Ost, Berlin and Dresden, Germany. The list of SLE patients participated and corresponding data are enlisted in the Table A1 of the appendix.

PBMC from whole blood were isolated by density gradient centrifugation using Ficoll (GE Healthcare UK Ltd., Little Chalfont, United Kingdom) as separation solution. Ficoll has a density of 1.077g/L, which is lower than the density of erythrocytes, granulocytes, and dead cells but higher than the density of PBMC and platelets, which therefore remain above the gradient and can easily be removed as an interphase.

Fresh blood was distributed to 50mL Falcon tubes and filled up to 35mL with PBS/1% (w/v) BSA supplemented with 2mM EDTA (PBS/BSA/EDTA, self-prepared at DRFZ). The whole blood suspension was mixed by inversion and 15mL Ficoll solution was carefully mounted into blood suspension to accumulate at the bottom and centrifuged at 2,000 rpm for 20 minutes without breaks at room temperature. After the centrifugation, plasma, PBMC, and erythrocytes are separated in different layers. The PBMC-containing interphase between Ficoll and blood plasma was collected and washed with up to 50mL PBS/BSA/EDTA and centrifuged at 310xg for 10 minutes at 4°C. Following the centrifugation, the supernatant was carefully removed and the pellet was re-suspended in PBS/BSA/EDTA or in cell culture medium. Cell counts were determined using an automated Casy® cell counter or Guava® easyCyte flow cytometer as described in the following sections and following chapter, respectively.

2.2. Determination of cell numbers

Different to conventional cell counting using a Neubauer hemocytometer, Casy® cell counter (Schärfe System GmbH, Reutlingen, Germany) can determine cell

concentration in a cell suspension and measures cell sizes and the ratio of viable cells by electrical current exclusion. The Casy® cell counter works by applying an electric current to singularized cells and intact cell membranes of viable cells function as electrical insulators. Differences in the electric impedance are recorded and are proportional to cell size. The membranes of dead cells have pores, and only the nucleus influences the electric current. Therefore, only the nucleus is detected and dead cells can be excluded due to a smaller size. Casy® cell counter plots the results on a graphical display which further allows for counting of "gated" populations with distinct cell sizes and displays the cell concentration (cells/mL).

For measurement, 10µL of a cell suspension were diluted 1:1,000 in 10mL Casy® ton in a special Casy® tube. The cells in the Casy® ton were taken up and pulsed with the electrical current. Viable cells per 1mL are recorded. Based on this cell concentration, the total cell number can be calculated by multiplying the cell concentration per 1mL with the total volume of the cell suspension.

2.3. Depletion of CD25+ cells

In the experiments where CD25+ cells, mainly expressed by Treg, were depleted to optimize conventional T cell-stimulation, magnetic-activated cell sorting (MACS) technology was used using anti-human CD25 MicroBeads II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, PBMC were labeled with magnetic beads and after incubation, cells were washed with PBS/BSA/EDTA. Bead-labelled cell suspension was applied onto LS MACS column (Miltenyi Biotec GmbH) which was positioned in the magnetic fields of a MACS Separator. Unlabelled cells were collected by washing the column with PBS/BSA/EDTA. The cell number of both fractions was determined and aliquots of both fractions were stained for purity checks. The procedure was performed according to manufacturer's instructions.

2.4. Antigen-specific T cell stimulation

This protocol serves as general procedure for antigen-specific T cell stimulation throughout this thesis by evaluating CD154 expression on T cells after antigenic activation. In the experiments presented in this chapter, $1x10^7$ PBMC or CD25-depleted cells were resuspended in 1mL RPMI 1640 medium with GlutaMAX™ (Life Technologies Ltd., Paisley, United Kingdom) supplemented with 5% (v/v) heat-inactivated human AB serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 1% (v/v) Penicillin-Streptomycin (Life Technologies Ltd.). 200µL cell suspension was transferred into 96-well plate (Greiner Bio-One AG, Kremsmünster, Austria). Cells were stimulated for 6 or 16 hours at 37°C with 5% CO_2 with following antigens: 20µg/mL $SmD1_{(83-119)}$ (generated and provided by Peptidsynthese AG Henklein, Charité Universitätsmedizin Berlin) or 1µg/mL SEB (Sigma-Aldrich Chemie GmbH) in the presence of 1µg/mL antihuman-CD40 antibody (generated at DRFZ), and 1µg/mL antihuman-CD28 functional grade purified (Life Technologies Europe BV, Bleiswijk, The Netherlands). Antihuman-CD40 antibody was used to block CD40 molecules on the surface of antigen-presenting cells to prevent CD40-CD154 interaction, which can destabilize CD154 expression on the cell surface as the molecule is internalized upon interaction with CD40 [88]. Antihuman-CD28 antibody was used as co-stimulator to facilitate the activation of CD4+ T cells.

2.5. Antigen-reactive T cell enrichment

This protocol serves as general procedure for antigen-reactive T cell-enrichment throughout this thesis. Some amendment is described when necessary. In the experiments presented in this chapter, CD154-expressing CD4+ T cells were enriched from $1x10^8$ PBMC after *in vitro* stimulation with antigen to facilitate detection of antigen-specific CD4+ T cells. We followed the protocol established by Bacher *et al.*, the so-called antigen-reactive T cell-enrichment (ARTE) that utilizes CD154 MicroBead Kit (Miltenyi Biotec GmbH) [83]. Briefly, in total $1x10^8$ PBMC were resuspended in two 5mL RPMI medium used for stimulation as described in the section 2.4. Cells were stimulated in 6-well plate (Greiner Bio-One AG) for 7 hours at 37°C with 5% CO_2 with following antigens: 20µg/mL $SmD1_{(83-119)}$ or 1µg/mL PepTivator® *Candida albicans* MP65 or 1µg/mL PepTivator® PLP (both Miltenyi Biotec GmbH) in the presence of 1µg/mL pure-grade antihuman-CD40 antibody (Miltenyi Biotec GmbH) and 1µg/mL antihuman-CD28 functional grade purified (Life Technologies Europe BV). After 7 hours of stimulation, cells were scratched with 1mL pipette tip and pooled into 15mL Falcon tubes. Cells were washed with PBS/BSA/EDTA and labelled with antihuman-CD154-biotin antibodies for 10 minutes at 4°C. The antibodies are conjugated with biotin and bind specifically on CD154 molecule on the cell surface. Subsequently, anti-biotin-microbeads antibodies were added to the cell suspension for 15 minutes at 4°C. The antibodies are conjugated with microbeads and bind specifically on biotin. After a washing step, cells were loaded onto calibrated MS column (Miltenyi Biotec GmbH) to enrich CD154-expressing cells.

2.6. Antibody staining and flow cytometry analysis

Depending on the experiments, several surface molecules were stained on antigen-stimulated cells in different combinations of the following monoclonal antibodies: antihuman-CD3-PacificBlue (clone UCHT1), antihuman-CD3-PerCP (clone SK7), antihuman-CD14-AlexaFluor488 (clone M5E2), antihuman-CD154-BV421 (clone 24-31) (all BioLegend Inc., San Diego, USA), antihuman-CD4-PE-Cy7 (clone SK3), antihuman-CD4-Allophycocyanin-H7 (clone RPA-T4) (all BD Biosciences, San Jose, USA), antihuman-CD8-VioGreen (clone BW135-80), antihuman-CD14-VioGreen (clone TÜK4), antihuman-CD20-VioGreen (clone LT20), antihuman-CD3-VioBlue (clone BW264/56), antihuman-CD4-Allophycocyanin-Vio770 (clone M-T321), antihuman-CD69-FITC (clone FN50), antihuman-CD25-PE (clone 4E3), antihuman-CD25-Allophycocyanin (clone 4E3), antihuman-Biotin-PE, antihuman-CD154-PE (clone 24-31) (all Miltenyi Biotec GmbH) antibodies. Briefly, cells were washed with PBS/BSA and a cocktail of antibodies was added into the cell suspension. Cells were incubated for 15 minutes at 4°C and washed prior to acquisition on a flow cytometer. In the experiments where CD154-expressing cells were enriched, surface staining was performed on the MS column and cells were washed prior to acquisition on a flow cytometer. To discriminate live and dead cells, LIVE/DEAD® Aqua kit (Life Technologies Europe BV) was applied. This dye binds to cellular proteins (amines), where in dead cells, cell membranes are permeable. Therefore, in dead cells not only surface amines are labelled as in viable cells but also intracellular amines. Hence, the fluorescence is more intense on dead cells than on live cells, and can be clearly distinguished. The procedure to prepare the kit and to label dead cells was according

to manufacturer's instruction. The concentration of the antibodies was titrated accordingly.

Flow cytometry is a useful tool for multi-parametric analysis of individual cells within heterogeneous cell populations. It can analyse single cells for protein expression, ranging from surface molecules to intracellular proteins such as transcription factors and cytokines. In parallel, it can deliver information about cell size and granularity. The principle of flow cytometry has been well described in many textbooks. Briefly, cells are singularized in small droplets by hydrodynamic focusing using a small nozzle and an ultrasound source. The cells then pass the so-called interrogation point. Here, laser light at different wavelengths either is refracted or excites fluorochromes. Refracted light or emitted fluorescence are recorded by photomultipliers. The information conveyed by emitted fluorescence depends on the utilized antibodies or compounds and their specific fluorescence. Flow cytometers can also be equipped with an electrostatic deflection system, and can then even serve as cell sorters. For the so-called fluorescence-activated cell sorting (FACS), separate cell-containing droplets receive an electrical charge after the interrogation point dependent on parameters selected by the operator and later pass the deflection plates where the cells are separated into different containers.

Samples were acquired on a BD FACSCanto®II and BD LSRFortessa® (BD Biosiences) flow cytometer at the Flow Cytometry Core Facility (FCCF) Deutsches Rheuma-Forschungszentrum (DRFZ) Berlin using FACS®Diva software (BD Biosciences), or on a MACSQuant flow cytometer (Mitenyi Biotec GmbH) at the laboratory of Prof. Alexander Scheffold. Flow cytometric data were analysed using FlowJo software (Three Star, Ashland, USA).

2.7. Statistics

Statistical tests were performed with Prism software (GraphPad Software, La Jolla, USA). Normal distribution of the data sets was determined by Kolmogorov-Smirnov-Test. Mann-Whitney U test and Wilcoxon matched-pairs signed rank test were used in the experiments with independent and dependent data sets, respectively. The *P* values <0.05 were considered statistically significant with following indication: * $P<0.05$, ** $P<0.01$.

3. Results and discussion

The expression of CD154 is specifically up-regulated on the cell surface of CD4+ T cells following *in vitro* antigenic stimulation, irrespective of particular peptide-MHC combination, thus facilitating direct detection of antigen-specific CD4+ T cells [87]. The main goal of our work is to understand the role of autoreactive CD4+ T cells in the systemic autoimmune disease SLE with the strategy to isolate live autoreactive CD4+ T cells for further functional characterization by targeting the CD154 molecule expressed on the cell surface of antigen-activated CD4+ T cells. We used short peptide fragments of SmD1 from amino acid position number 83 to 119 as a model of SLE-associated autoantigens, and as described in the section 2.4., antihuman-CD40 blocking antibody was used to stabilize CD154 expression on the cell surface.

3.1. *In vitro* stimulation of CD4+ T cells with $SmD1_{(83-119)}$

To reproduce the data from Frentsch *et al.* and subsequently to validate the stimulation procedure, we analysed CD154 expression on CD4+ T cells stimulated with SEB (*n*=5), a superantigen produced by *Staphylococcus aureus*. Here we showed that after 6 hours of stimulation, 8 – 18.5 % of CD4+ T cells expressed CD154 on the cell surface (*P*=0.0025, by Mann-Whitney test). In contrast, when stimulated with a short peptide of autoantigen $SmD1_{(83-119)}$ (*n*=7), the frequency of detectable autoreactive CD4+ T cells was extremely low (median frequency = 0.30%), thus it elicited no specific CD4+ T-cell response compared with the negative control (median frequency = 0.32%) in this experimental setting (Figure 2A). Moreover, when compared within individual SLE patients, no significant difference of CD154 expression on CD4+ T cells stimulated with $SmD1_{(83-119)}$ was observed towards the control (*P*=0.8438, by Wilcoxon signed-rank test) (Figure 2B).

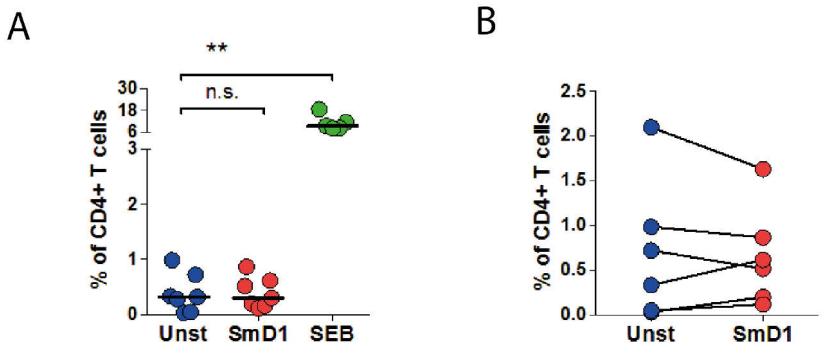


Figure 2: **CD154 expression on the surface of CD4+ T cells of Sm-/SmD1-seropositive SLE patients after *in vitro* antigenic stimulation.** The annotation of Unst and SmD1 in the graph refers to unstimulated and a short peptide in the amino acid sequence from position 83 to 119 ($SmD1_{(83-119)}$), respectively. (**A**) Percentage of CD154-expressing cells within CD4+ T cells in Sm-/SmD1-seropositive, inactive SLE patients. Cells were left unstimulated as negative control, or stimulated with $SmD1_{(83-119)}$ (*n*=7) or SEB (*n*=5). (**B**) CD154+ CD4+ T cell frequencies without and with antigen stimulation within individual SLE patients. Lines indicate median. N.s. is not significant.

Based on our data regarding the frequencies of CD154-expressing CD4+ T cells on the cells stimulated with SEB, we confirmed the specificity of the *in vitro* antigen-specific CD4+ T cell stimulation procedure as described earlier. This protocol can be applied for isolation of antigen-specific CD4+ T cells enabling further functional characterization, which can provide important information to our understanding in the complexity of the human immune system, including autoimmunity. Amino acid sequence on the position number 83 to 119 of ribonucleoprotein SmD1 has been appreciated to be the target of SLE-specific autoantibodies [79]. Thus, it is relevant to address the question whether this epitope is presented by the antigen-presenting cells after antigen uptake and processing, and whether it represents the target of the CD4+ T cells as well, which are considered to mediate disease pathogenesis.

Our data showed that the frequency of CD154-expressing CD4+ T cells within PBMC of Sm-/SmD1-seropositive SLE patients after stimulation with $SmD1_{(83-119)}$ was at the similar level with unstimulated control despite a functional stimulation assay, indicating several possibilities i.e. (1) activation of SLE associated-autoreactive CD4+ T cells may be under physiological steady regulatory T cell (Treg) control, as reported

earlier by our group [89]; (2) CD4+ T cells bearing TCR specific to the presented epitope of $SmD1_{(83-119)}$ are very rare and may be below the detection limit of a conventional flow cytometry method [83], where significant *ex vivo* and/or *in vitro* by-stander CD4+ T-cell activation was observed and altered the quality of the detection of $SmD1_{(83-119)}$-specific CD4+ T cells by CD154 expression; and (3) amino acid sequence of SmD1 in the position from 83 to 119 may be restricted to the context of B cell response or activation for differentiation into antibody-producing plasma cells.

3.2. Depletion of CD25+ cells prior to *in vitro* CD4+ T cell stimulation

As discussed in the section 3.1., it is argued that T cell activation by host antigen in the context of the autoimmune disease SLE is controlled by CD25+ Treg. Humrich *et al.* reported strong evidence of the role of Treg in counteracting lupus autoreactivity and ameliorating disease progression [89]. The low level of CD154 expression among the cells stimulated with the SLE-associated autoantigen $SmD1_{(83-119)}$ may be a result of a suppression mechanism undertaken by Treg to prevent autoreactive T cell activation. Based on this rationale, we aimed to deplete Treg from the cell population prior to antigen stimulation to unmask the CD4+ T cell response demonstrated by CD154 expression. This strategy has been previously explored by our group and we have reported that indeed, depletion of Treg could amplify $SmD1_{(83-119)}$-specific CD4+ T cell responses [90].

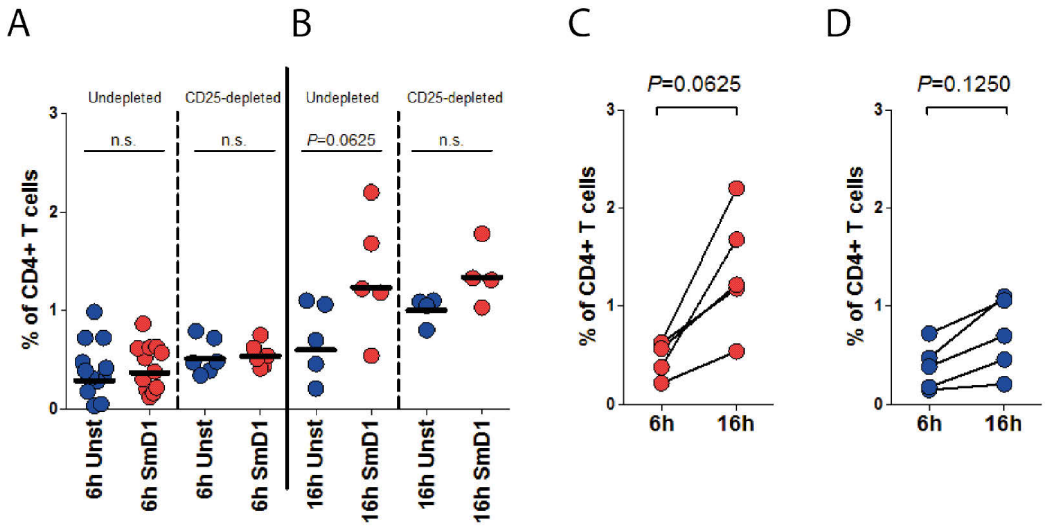


Figure 3: **Impact of CD25+ cell depletion to CD154 expression on CD4+ T cells after stimulation with autoantigen.** The annotation of Unst and SmD1 in the graph refers to unstimulated and a short peptide in the amino acid sequence from position 83 to 119 ($SmD1_{(83-119)}$), respectively. (**A**) Comparison of the CD154 expression among CD4+ T cells, where the cells were stimulated for 6 hours without depletion of CD25+ cells (n=12) and with depletion of CD25+ cells (n=6), as well as when the cells were stimulated for 16 hours without depletion of CD25+ cells (n=5) and with depletion of CD25+ cells (n=4) (**B**). (**C**) Comparison of CD154-expression in 6 and 16 hours of stimulation on $SmD1_{(83-119)}$-stimulated cells and unstimulated cells (**D**) (both n=5) without CD25-depletion. Lines indicate median. Statistical analysis was performed by Wilcoxon signed-rank test, or Mann-Whitney test when two independent data sets were compared. N.s. is not significant.

In our previous study, CD154 expression on CD25 cell-depleted populations has been observed only in the intracellular cell compartment. As the cell surface expression of CD154 was recently of our interest, we tested the strategy to evaluate whether it was

applicable in the setting to detect and to directly isolate autoreactive T cells. Our data revealed that the percentage of cell-surface CD154 expression on CD4+ T cells stimulated with $SmD1_{(83-119)}$ in both undepleted (n=12) and CD25 cell-depleted (n=6) environment was almost similar with the unstimulated control (P=0.8501 for undepleted samples and P=0.9163 for CD25 cell-depleted samples, both by Wilcoxon signed-rank test; median frequencies: 0.36% vs 0.45% and 0.48% vs 0.53%, respectively), indicating no improvement on detection of autoreactive T cells with this strategy (Figure 3A).

As presented previously, depletion of CD25+ cells improved intracellular detection of CD154 expression in CD4+ T cells stimulated with $SmD1_{(83-119)}$ [90]. However, the data collected in this study suggested less detection of cell surface expression of CD154, consistent with the work of Frentsch *et al.* where the difference of intracellular detection of CD154 expression in CD4+ T cells stimulated with SEB was 2.5-fold higher than the detection on the cell surface [87]. Over the last two decades it has been reported that following TCR activation, *de novo* CD154 is synthetized and transported to the cell surface in highly transient nature, which is rapidly internalized after surface expression or is secreted [88, 91, 92]. But the kinetics and duration of the synthesis and transport mechanism of CD154 following TCR activation are unclear. Therefore, we aimed to prolong the duration of *in vitro* stimulation to 16 hours to observe the signal-to-noise ratio of CD4+ T cell activation with $SmD1_{(83-119)}$ in complete PBMC and in CD25-free environment.

When stimulated for 16 hours, signal-to-noise ratio of CD154 expression on CD4+ T cells stimulated with $SmD1_{(83-119)}$ in complete PBMC (n=5) was improved with nearly significant difference (P=0.0625) as analysed by Wilcoxon signed-rank test since the n number was too small to be defined as normally distributed data according to Kolmogorov-Smirnov-Test. Interestingly, in the CD25-depleted cell population (n=4), the detected frequency of CD154 in the stimulated samples was almost similar with the frequency of CD154 of the complete PBMC samples (P=0.9048 by Mann-Whitney test), but the negative control showed elevated CD154 expression tightening its signal-to-noise ratio (P=0.1250, by Wilcoxon signed-rank test) (Figure 3B).

To investigate, whether improved signal-to-noise ratio of CD154 expression in the prolonged duration of stimulation represents antigen-specific T cell activation, we compared CD154 expression of negative control as well as $SmD1_{(83-119)}$-stimulated CD4+ T cells without CD25+ cell-depletion in 6 and 16 hours of stimulation within the same individuals (n=5). The frequency of CD154-expressing CD4+ T cells after stimulation with $SmD1_{(83-119)}$ in 16 hours was around 2.1-fold higher when compared with 6 hours of stimulation (median frequencies: 0.57% for 6 hours and 1.22% for 16 hours, P=0.0625 by Wilcoxon signed-rank test) (Figure 3C). In parallel, background frequency of CD154-expressing cells in the cell culture without antigen has increased for 1.7-fold over additional 10 hours of stimulation (median frequencies: 0.42% for 6 hours and 0.70% for 16 hours, P=0.1250 by Wilcoxon signed-rank test) (Figure 3D). This result indicates that long-time stimulation may facilitate sufficient time for antigen-specific CD4+ T cells to synthetize and transport CD154 molecules to the cell surface allowing an improved detection, but at the same time decreasing detection sensitivity due to increased time-dependent bystander activation that occurs in the absence of antigen. This observation is consistent with previous report stating that CD154 sustains its expression on the cell surface of CD4+ T cells in the absence of

TCR activation but rather it is regulated e.g. by the cytokines, which are present and secreted in a steady-state condition [93, 94].

CD25, the α-chain of the receptor for IL-2, was identified to be highly expressed in a CD4+ T cell subset that can suppress proliferation and inflammatory functions of other T cells following antigen-specific or polyclonal activation *in vitro* and prevent autoreactivity *in vivo* [95, 96]. Furthermore, it was found that the depletion of CD4+CD25+ T cells in mice resulted in the development of systemic autoimmune disease [95]. Thus, depletion of such cells can unmask the response of autoreactive CD4+ T cells upon activation, but at the same time the control mechanisms fail to maintain polyclonal, antigen-independent bystander activation, as demonstrated in our study. Background level of CD154 expression in the CD25-cell depleted environment was elevated and more enhanced in the prolonged cell culture. Taking into consideration that CD25 is also expressed by the *in vivo* pre-activated FoxP3-CD4+ conventional effector T cells that highlights the possibility of the potential loss of autoreactive CD4+ T cells when performing CD25+ cell-depletion assay, findings by Triplett *et al.* described that the population of T cells that responds to antigens associated with chronic immune responses and expands in patients with SLE are CD25 negative [97]. Thus, depletion of CD25+ cells in this study might correspond to the depletion of *bona-fide* Treg, without having to include other Treg-associated marker such as FoxP3.

Taking the data together, depletion of CD25 resulted in a lack of improvement of the cell-surface detection of CD154 after stimulation with autoantigen and it enhanced bystander activation due to the loss of control of polyclonal, antigen-independent T cell activation.

3.3. Enrichment of antigen-stimulated CD154-expressing cells

The challenge in detecting antigen-specific CD4+ T cells is mostly due to their very low frequency in the periphery, where available technologies or methods to detect such cells are still limited. In this study, we used PBMC isolated from Sm-/SmD1-seropositive patients because we argued that these patients should have encountered reactivity against the Sm or SmD1 molecule displayed by humoral response of anti-Sm or anti-SmD1 antibody production and autoreactive T cells specific to Sm or SmD1 are probably involved in the autoantibody production, thus they are available in reasonable, higher frequency than in healthy individuals due to activation and clonal expansion. Nevertheless, the availability of autoreactive CD4+ T cells in the periphery is not restricted to individuals with autoimmune disorders, therefore SmD1-specific CD4+ T cells can also be detected and isolated from healthy individuals, although the frequency is expected to be extraordinarily rare.

Based on this consideration, we performed a strategy to detect $SmD1_{(83-119)}$-specific CD4+ T cells from a high number of immune cells obtained from healthy individuals (as it was logistically not possible to obtain high amounts of blood samples from patients with SLE) by magnetically targeting CD154 expression on the cell surface after stimulation with $SmD1_{(83-119)}$. This method is called ARTE as described in the section 2.5. Figure 4A shows representative gating strategy to detect CD154 expression after antigen stimulation, which was supported by co-staining of CD69, a marker that is expressed on the cell surface following arbitrary T cell activation.

When stimulated with a pool of 15-mer peptides with 11 amino acids overlap covering the sequence of mannoprotein MP65 of *Candida albicans* (MP65) (*n*=2), an airborne antigen derived from fungi of normal mucosal microflora of humans which served here as a positive control [98], less than 20 CD154-expressing cells were detected by flow cytometry in the acquisition limit at $2x10^5$ PBMC. By enrichment of CD154-expressing cells, detection resolution has been successfully improved to 100-fold by acquiring the entire positive fraction with only ~$5x10^4$ total events, allowing quantification of almost 2,000 MP65-reactive CD4+ T cells (Figure 4A). Signal-to-noise ratio in the MP65-stimulated cells was improved in the CD154-enriched fraction, displaying clear detection of antigen-specific CD4+ T cells (median frequency: 12.59% vs 5.25%) (Figure 4A).

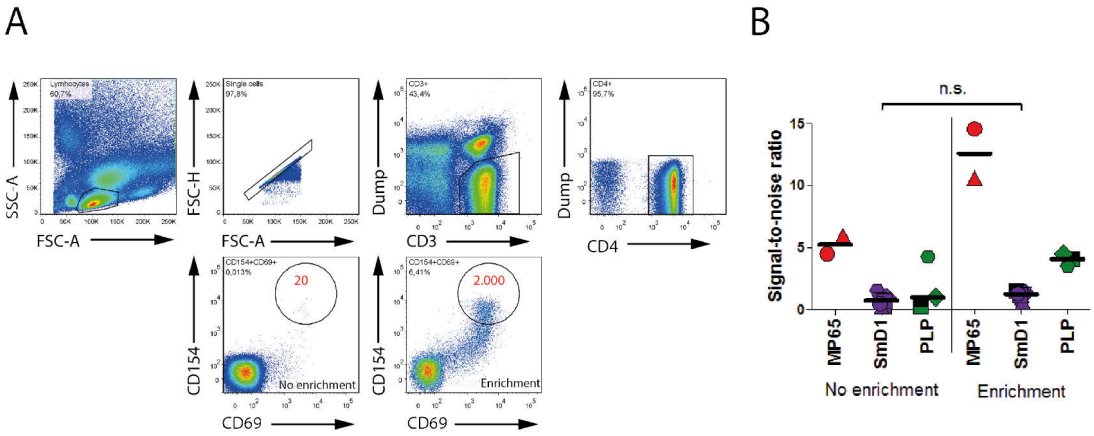


Figure 4: **Enrichment of CD154-expressing CD4+ cells after stimulation with antigen.** The annotation of SmD1 in the graph refers to a short peptide in the amino acid sequence from position 83 to 119 ($SmD1_{(83-119)}$). (**A**) The dot plots are representative of gating strategy in the ARTE experiments. $1x10^8$ cells were stimulated with a pool of 15-mere sequences of 11 amino acids overlap covering the sequences of *Candida albicans* mannoprotein MP65. Lymphocytes were gated based on populations detected in forward and side scatter, then doublets were excluded. CD8+CD14+CD20+ cells as well as dead cells (dump) were excluded. CD3+CD4+ cells were analysed for CD154+CD69+ expression in the original fraction after acquiring $2x10^5$ PBMC (bottom left; no enrichment) and in the CD154-enriched fraction (bottom right; enrichment). Red numbers refer to cell number of CD154+CD69+ CD4+ T cells. (**B**) Signal-to-noise ratio was calculated based on CD154+CD69+ CD4+ T cell numbers in *Candida albicans* MP65- (*n*=2), $SmD1_{(83-119)}$- (*n*=7) as well as PLP-stimulated (*n*=3) and unstimulated PBMC in the no-enrichment assay after acquiring $2x10^5$ PBMC and in the enrichment assay after acquiring complete positive fraction. Symbols represent individual donors. Statistical analysis for signal-to-noise ratio to $SmD1_{(83-119)}$-stimulated samples with and without enrichment was performed by paired Student *t* test. N.s. is not significant.

An improved signal-to-noise ratio was also observed in PBMC stimulated with a pool of 15-mer sequences of 11 amino acids overlap covering the sequences of myelin proteolipid protein (PLP) (*n*=3), an autoantigen expressed in myelin sheaths around the axons in the central nervous system, thus it becomes a target of PLP-specific, autoreactive CD4+ T cells in the context of the autoimmune disease multiple sclerosis [99] (median frequency: 4.091% vs. 1%) (Figure 4B). Our data suggest that CD154-enrichment following stimulation with autoantigen is an important approach to detect extremely rare autoreactive CD4+ T cells. It is very sensitive as these cells were also detectable in a non-disease setting.

However, we still observed almost similar signal-to-noise ratio in the PBMC stimulated with $SmD1_{(83-119)}$, both in CD154-non-enriched and enriched samples (*n*=7) (*P*=0.1706, by Student *t* test; median frequency: 1.252% vs 0.75%) (Figure 4B),

suggesting no detection of $SmD1_{(83-119)}$-specific CD4+ T cells within $1x10^8$ PBMC, as the number of CD154-expressing cells was comparable with the background levels. Taking the data together, the basic principal of CD154-enrichment is to intensify the detection of less-occurring antigen-specific CD4+ T cells in the periphery by processing high-cell numbers, increasing the detection probability of antigen-specific CD4+ T cells, without having to consider the limitation of acquisition capacity in a flow cytometer. On the other hand, bystander activation of T cells either occurring *in vivo*, *ex vivo*, or *in vitro* displays a challenge in the detection of antigen-activated CD4+ T cells. ARTE method has been demonstrated by Bacher *et al.* to overcome this limitation with the improvement of signal-to-noise ratio, even in the detection of neoantigen-specific CD4+ T cells. None of our seven PBMC samples stimulated with $SmD1_{(83-119)}$ showed significant elevation of CD154-detection compared to bystander activation leading to fundamental question of the nature of SLE-autoantigen $SmD1_{(83-119)}$ to trigger $SmD1_{(83-119)}$-specific T cell activation.

4. Conclusion

To elucidate the specificity and function of pathogenic T cells is one of the last major frontiers in our understanding of human systemic autoimmunity. Knowledge in this area would contribute a significant value to the advancement of therapy against chronic inflammatory diseases that affect nearly 10% of the human population. In our work within this doctoral thesis, we aimed to generate a comprehensive study to collect information about the specificity and function of human autoreactive CD4+ T cell subsets in SLE.

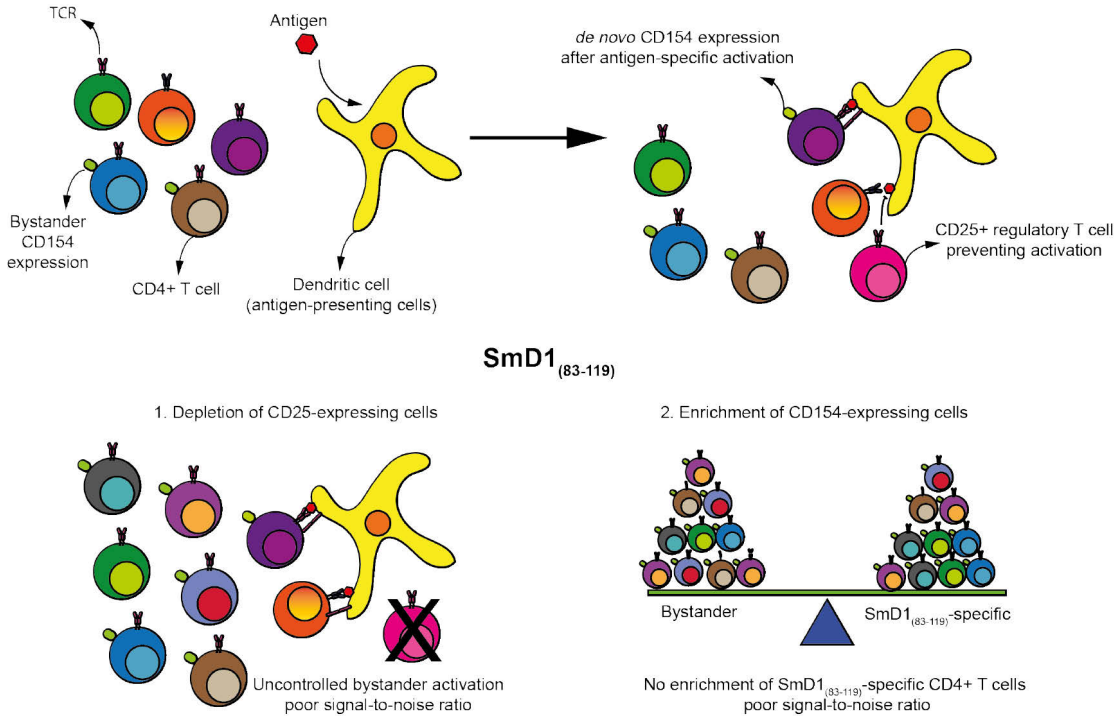


Figure 5: **Stimulation of CD4+ T cells with $SmD1_{(83-119)}$.** CD4+ T cells with unspecific CD154 expression circulate in the periphery. Upon antigen uptake and presentation by antigen-presenting cells, TCR of CD4+ T cells with specificity to cognate antigen are activated and express CD154. CD25+ regulatory T cells control T cell activation. Depletion of CD25-expressing cells prior to stimulation with $SmD1_{(83-119)}$ leads to uncontrolled bystander activation on CD4+ T cells. Enrichment of CD154-

expressing cells after stimulation with $SmD1_{(83-119)}$ results in the same frequency between the background population and "antigen-specific" population, making it difficult to interpret whether CD4+ T cells with reactivity to $SmD1_{(83-119)}$ can be truly detected by ARTE method.

In this chapter, emphasis was given to improving signal-to-noise ratio of CD154 expression in antigen-activated CD4+ T cells against negative controls. It was done by considering the role of Treg that may hamper T cell activation and the rarity of target cell populations that generally limits the detection process. None of the approaches here depicted a promising strategy to purify highly specific $SmD1_{(83-119)}$-responsive CD4+ T cells (Figure 5). Nevertheless, our data suggest important information about the fundamental biology of antigen-dependent T cell activation. Firstly, CD154 was confirmed to be an antigen-specific T cell activation marker, albeit its expression on T cells is also driven by inevitable non-antigen dependent activation. Secondly, excluding the limitation in cell frequency and suppression of activation by Treg, inability of $SmD1_{(83-119)}$ peptide to induce T cell activation might occur because of the limited epitope presented across $SmD1_{(83-119)}$ peptide, which consists of 37 amino acids only. We discontinued the approach to target cell surface expression of CD154 on activated CD4+ T cells to study the role of $SmD1_{(83-119)}$-specific CD4+ T cells in human SLE.

In conclusion, expression of CD154 on activated autoantigen-specific CD4+ T cells after *in vitro* stimulation with $SmD1_{(83-119)}$ was unable to provide direct purification of highly-$SmD1_{(83-119)}$-specific CD4+ T cells, not because of the method to assess CD154 expression on the cell surface, but because short-fragmented autoantigen $SmD1_{(83-119)}$ may not display restricted antigenic epitope to SmD1-specific CD4+ T cells.

Chapter 3

Evaluation of CD4+ T cell response to SmD1$_{(83-119)}$ peptide and to a pool of peptides covering complete SmD1 sequence

Abstract

The CD4+ T cell response to an antigen depends on the presentation of peptide-MHC class II (for human: HLA class II) complex on the surface of antigen-presenting cells, thus antigen sequence, along with TCR and MHC class II molecule, is crucial for the induction of an antigen-specific T cell activation. We have previously shown that CD4+ T cells stimulation with short-fragmented SmD1$_{(83-119)}$ resulted in CD154 expression that was indistinguishable with bystander activation, urging us to evaluate the epitope presented in the SmD1$_{(83-119)}$ sequence. *In silico* analysis using algorithm predicting binding of SmD1 to HLA-DR, HLA-DQ, and HLA-DP demonstrated that SmD1 sequence outside 83-119 region provides more binding possibilities to MHC class II molecules. In addition, we sought to determine the CD4+ T cell response to SmD1$_{(83-119)}$ and a pool of peptides covering the complete SmD1 sequence (SmD1 peptide pool) using libraries of amplified CD4+ T cells. Interestingly, we found that CD4+ T cells recognized more epitopes in SmD1 peptide pool than those in SmD1$_{(83-119)}$. Therefore, native antigen facilitates a broad reactivity to CD4+ T cells, which is useful to study the role of autoreactive CD4+ T cells in the disease where the autoantigens are not yet fully characterized.

Keywords: *SmD1, TCR epitope, peptide-MHC-II binding*

Contents

1. Introduction

1.1. Background

It has been understood for years that T cell activation through its receptor requires an interaction between three parties: a TCR molecule expressed on the surface of T cells, a cognate epitope of targeted antigen, and MHC molecule expressed on the surface of antigen-presenting cells. The adaptive immune response begins with the event where an antigen is engulfed by antigen-presenting cell, processed and presented as a peptide-MHC complex on the cell surface. In addition, antigen in form of short peptide is able to bind directly into unoccupied MHC molecules without having to experience intracellular processing mechanisms. Nevertheless in both scenarios, sufficient binding of antigen into MHC molecules is a prerequisite to facilitate further events in adaptive immunity involving interaction with T cells. Antigen-presenting cells migrate to the secondary lymphoid organs to engage with T cells, where a random T cell senses an abundant amount of presented antigens through its TCR. Only T cells with a suitable TCR sequence to the presented antigen bound to an MHC molecule which is activated and mediates an adaptive immune response [100, 101].

The degree of antigen binding to MHC molecule varies among MHC haplotypes. Thus, MHC polymorphisms may result in different binding properties of an antigen in different individuals. In the context of autoimmunity, an autoantigen may bind with different affinity to MHC molecule among individuals depending on MHC polymorphic alleles, providing an evidence of genetic predisposition with relevant association to certain autoimmune diseases [102]. For example, it has been found that individuals with HLA-DRB1*0301 are susceptible to SLE, HLA-DQA1*0301 to rheumatoid arthritis, and HLA-DRB1*1501 to multiple sclerosis [103].

We hypothesized that one of the fundamental factors driving CD154 expression on the surface of CD4+ T cells after stimulation with $SmD1_{(83-119)}$ is the interaction of the peptide with MHC class II molecules, as well as TCR. Thus, it is critical to investigate and evaluate $SmD1_{(83-119)}$ in terms of its binding with MHC class II molecules and its recognition possibility by the TCR.

1.2. Objectives

The objectives of the study presented in this chapter were to evaluate $SmD1_{(83-119)}$ as a target autoantigen mediating the CD4+ T cell response in SLE. A set of analyses was employed to examine T cell epitopes within the SmD1 sequence in the perspective of T cell and antigen-presenting cell. It is to answer, whether the use of SmD1 full length protein is more preferable than $SmD1_{(83-119)}$ to induce antigen-specific T cell activation, which delivers important information to support our overall goal in understanding the role of SLE-associated CD4+ T cells in the disease.

2. Materials and methods

In the experiments presented in this chapter, the following methods were performed and they were described in the previous chapter: blood donors and isolation of

peripheral blood mononuclear cells (chapter 2, section 2.1.), determination of cell numbers (chapter 2, section 2.2.), and antibody staining and flow cytometry analysis (chapter 2, section 2.6., with some additional information described in this chapter).

2.1. Antigen preparation

Information about antigen concentration and purchasing of $SmD1_{(83-119)}$ and SEB is described in the chapter 2, section 2.4. To study the response of CD4+ T cell to a complete SmD1 sequence which is comparable with $SmD1_{(83-119)}$ peptide, we used a pool of 15-mer peptides with 11 amino acid overlaps spanning an entire protein sequence of SmD1 (SmD1 peptide pool; peptides & elephants GmbH, Potsdam, Germany), which facilitates more probability of CD4+ T cell responses to multiple epitopes, regardless of HLA type. The overlapping peptide mixes have been found to effectively stimulate CD4+ T cell responses by binding directly into unoccupied MHC class II molecule presented on the cell surface of antigen-presenting cells [101]. Table 1 enlists all individual peptide sequences in SmD1 peptide pool. Individual peptides were pooled and resuspended with small volume of dimethyl sulfoxide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and diluted with PBS into a dimethyl sulfoxide's concentration of below 1% (v/v). An amount of 1µg/mL of SmD1 peptide pool was used in the experiments.

Table 1: **SmD1 peptide pool.** The protein sequence of SmD1 is segmented into 15 amino acid sequences with 11 amino acid overlaps.

Index	Start amino acid	Peptide sequence	Peptide length
1	1	MKLVRFLMKLSHETV	15
2	5	RFLMKLSHETVTIEL	15
3	9	KLSHETVTIELKNGT	15
4	13	ETVTIELKNGTQVHG	15
5	17	IELKNGTQVHGTITG	15
6	21	NGTQVHGTITGVDVS	15
7	25	VHGTITGVDVSMNTH	15
8	29	ITGVDVSMNTHLKAV	15
9	33	DVSMNTHLKAVKMTL	15
10	37	NTHLKAVKMTLKNRE	15
11	41	KAVKMTLKNREPVQL	15
12	45	MTLKNREPVQLETLS	15
13	49	NREPVQLETLSIRGN	15
14	53	VQLETLSIRGNNIRY	15
15	57	TLSIRGNNIRYFILP	15
16	61	RGNNIRYFILPDSLP	15
17	65	IRYFILPDSLPLDTL	15
18	69	ILPDSLPLDTLLVDV	15
19	73	SLPLDTLLVDVEPKV	15
20	77	DTLLVDVEPKVKSKK	15
21	81	VDVEPKVKSKKREAV	15
22	85	PKVKSKKREAVAGRG	15
23	89	SKKREAVAGRGRGRG	15
24	93	EAVAGRGRGRGRGRG	15

Index	Start amino acid	Peptide sequence	Peptide length
25	97	GRGRGRGRGRGRG	15
26	101	GRGRGRGRGRGRG	15; double
27	105	GRGRGRGRGGPRR	15

2.2. Cryopreservation of human lymphocytes

Depending on experiments, $5x10^6$ – $1x10^8$ human lymphocytes, either subjected as feeder cells or as antigen-presenting cells, were cryopreserved in 1mL self-prepared freezing medium containing following ingredients: 47.5% (v/v) RPMI 1640 medium with GlutaMAX™ (Life Technologies Ltd., Paisley, United Kingdom), 40% (v/v) heat-inactivated human AB serum, 10% (v/v) dimethyl sulfoxide, and 2.5% (v/v) 1M HEPES solution (all Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Cells were aliquoted in cryotube ampules (Sarstedt AG & Co, Nümbrecht, Germany) and incubated in Nalgene Mr. Frosty™ cryo-freezing container (Thermo Fisher Scientific, Massachusetts, USA) at -80°C overnight prior to preservation in liquid nitrogen. Cell thawing was performed at 37°C. This procedure has been optimized for stable lymphocyte viability and recovery.

2.3. Feeder cell preparation

Long-time *in vitro* culture of CD4+ T cells requires supplements that mimic *in vivo* situation i.e. cytokines and growth factors, which are produced by other immune cells crucial for maintaining cell survival and growth. $1x10^8$ allogeneic human immune cells isolated from buffy coats of two donors were cryopreserved as described in the section 2.2 and thawed prior use. Cells were resuspended in 50mL PBS/BSA and washed by centrifugation at 310xg for 10 minutes at 4°C. Cells were resuspended in 50mL cold PBS/BSA and irradiated at 45Gy on ice to inactivate their proliferation in the cell culture. The cells derived from two donors were pooled and depending on experiments, cells were cultured with CD4+ T cells in the ratio of 100 feeder cells for one CD4+ T cell.

2.4. Antigen-presenting cell preparation

CD3-depleted PBMC contain a number of cells that can process and/or present antigen on their MHC class II molecules such as B cells, monocytes, macrophages, and dendritic cells, thus autologous CD3 negative cells were used as antigen-presenting cells and were preserved for cell stimulation purposes in some experiments. The procedure of antigen-presenting cell preparation is based on MACS technology of CD3 depletion protocol using antihuman-CD3 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), similar to the procedure for depletion of CD25-expressing cells described in the chapter 2, section 2.3. Once prepared, autologous antigen-presenting cells were resuspended in freezing medium and cryopreserved in liquid nitrogen as described in the section 2.2. Depletion purity was regularly checked on flow cytometry using antihuman-CD3-PacificBlue (clone UCHT1) and antihuman-CD4-PE-Cy7 (clone SK3) antibodies. The negative fraction contained almost no CD3+CD4+ cells.

2.5. Amplified CD4+ T cell library

Despite limitation in available technologies for detecting the presence of antigen-specific T cells, the work from Geiger *et al.* in 2009 has offered a promising tool to overcome the challenge. She and her colleagues have established the so-called T cell library assay based on amplification of CD4+ T cells in microcultures and assessment of T cell response through proliferation capacity of CD4+ T cells after stimulation with antigen, so that the detection of antigen-specific CD4+ T cells can be performed simultaneously for a number of antigens, and the frequency of this cell population can be measured [104]. In this thesis, the method has been adopted and adapted for a range of experimental settings presented in this chapter and in the next chapters (Figure 6).

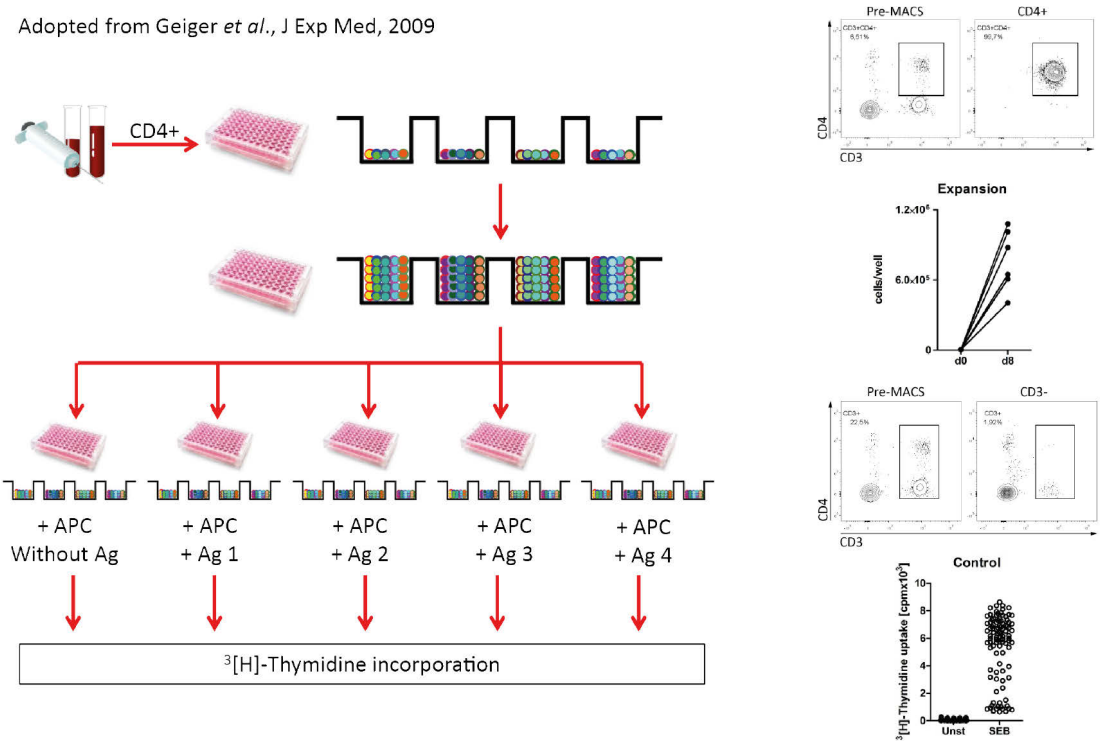


Figure 6: **Scheme of CD4+ T cell library assay.** T cell library assay established by Geiger *et al.* was adopted and adapted for a range of experiments in this thesis. CD4+ T cells were isolated by positive selection using MACS technology. The cells were distributed into 96-well plate in the presence of PHA, IL-2, and non-autologous feeder cells from two donors. CD4+ T cell expansion was monitored and after 1-2 weeks, a fraction of amplified CD4+ T cells were distributed into additional 96-well plates for simultaneous detection of a variety of antigen-specific CD4+ T cells. Prior to stimulation with antigen, amplified CD4+ T cells were rested for several days. To observe antigen-specific CD4+ T cell activation, autologous antigen-presenting cells were cultured with expanded CD4+ T cells in the presence of antigen. The T cell response was evaluated by the cell capacity to proliferate after stimulation with antigen using [^{3}H]-thymidine incorporation assay.

Briefly, 200,000 CD4+ T cells were isolated from PBMC using MACS technology antihuman-CD4 MicroBeads (Miltenyi Biotec GmbH) according to manufacturer's instruction, similar as described in the chapter 2, section 2.3. Different to depletion process, cells from positive fraction were collected. To yield high purity of CD4+ T cells, magnetic sorting was performed using double positive selection setting on AutoMACS Pro (Miltenyi Biotec GmbH). The purity of CD4+ T cell fraction was

routinely checked by flow cytometry based on CD3+CD4+ expression using antihuman-CD3-PacificBlue (clone UCHT1, Biolegend Inc., San Diego, USA) and antihuman-CD4-PE-Cy7 (clone SK3, BD Biosciences, San Jose, USA) antibodies, where the purity always reached >99% (exemplary data on Figure 6). In parallel, antigen-presenting cells were prepared by collecting CD3 negative cells after depletion with antihuman-CD3 MicroBeads and cryo-preserved prior to use as described in the section 2.4. About 2,000 CD4+ T cells per microculture were resuspended in IMDM medium with GlutaMAX^{TM} (Life Technologies Ltd., Paisley, United Kingdom) supplemented with 5% (v/v) heat-inactivated human AB serum, 1% (v/v) Penicillin-Streptomycin, 2µg/mL ciprofloxacin (Sigma-Aldrich Chemie GmbH), and 1% (v/v) MEM non-essential amino acid solutions (Sigma-Aldrich Chemie GmbH), in the presence of 1µg/mL lectin from *Phaseolus vulgaris* (PHA, Sigma-Aldrich Chemie GmbH), 600IU/mL IL-2 (Proleukin/Aldesleukin, Novartis Pharma GmbH, Nuremberg, Germany), and about $2x10^7$ irradiated feeder cells from two non-autologous donors. Medium was replenished periodically after 3-4 days by removing 50% volume of culture medium in each microcultures and adding fresh 1µg/mL PHA and 200IU/mL IL-2. Expansion of cells was optimal with this method (exemplary data on Figure 6). After 1-2 weeks of culture, a fraction of amplified CD4+ T cells were distributed into additional 96-well plates depending on the number of antigens to be analysed. CD4+ T cell libraries for negative control (unstimulated cells) and positive control (cells stimulated with SEB) were always included in the experiments. Prior to stimulation with the antigens, cells were rested in the stimulation medium for at least four days. On the day of stimulation, antigen-presenting cells were thawed and distributed into the T cell culture in a ratio of at least one antigen-presenting cell to 100 CD4+ T cells. CD4+ T cells were stimulated with the antigen for four days, where at least 16 hours before measurement, 1µCi/mL [^{3}H]-thymidine (GE Healthcare UK Ltd., Little Chalfont, United Kingdom) was added into the culture.

2.6. [^{3}H]-thymidine incorporation assay

The [^{3}H]-thymidine incorporation assay is a method to measure the extent of cell division. It is based on the use of a radioactive substitute to hydrogen by tritium, labelled on thymidine ([^{3}H]-thymidine), thus when the cell proliferates and synthesizes new DNA strands, this radiolabelled nucleotide is incorporated into the newly synthesized DNA. The DNA is collected into the filter and the amount of radioactivity, which corresponds to the number of cell divisions during stimulation with antigens, is counted in a scintillation counter, displayed as counts per minute (cpm).

This protocol serves as general procedure for [^{3}H]-thymidine incorporation assay throughout this thesis. In brief, on day four of stimulation culture, cells were harvested through a glass fibre filter membrane (UniFilter-96 GF/B, white 96-well Barex Microplate with GF/B filter; PerkinElmer Inc., Waltham, USA) using cell harvester device (Omnifilter-96 cell harvester; PerkinElmer Inc.). Particles that smaller than 1.5µm can pass through the filter, whereas bigger particles such as intact DNA are attached in the filter. The filter was washed two times with distilled H_2O and subsequently with 70% (v/v) ethanol. The filter was dried at 65°C for 20 minutes. MicroScint-O cocktail liquid (PerkinElmer Inc.) was added into the filter to facilitate scintillation counting. The radioactive counting was performed using MicroBeta^2 Microplate Counters with its corresponding software (PerkinElmer Inc.).

2.7. CD4+ T cell library data analysis

The cpm value delivered information about the proliferation capacity of CD4+ T cells after antigen-stimulation. Here, cpm values of unstimulated microcultures were used to determine the donor-specific *z*-value that represents the significance threshold in order to identify microcultures, in which polyclonal-expanded CD4+ T cells displayed reactivity to the given antigen. The *z*-value was calculated as four standard deviation values above the mean of cpm values of unstimulated microcultures. When the cpm value of an antigen-stimulated microculture was higher than the threshold, it was interpreted that the microculture contained on antigen-reactive CD4+ T cell clone.

To visualize using a graphic illustration about in which microculture the antigen-reactive CD4+ T cells can be detected, statistical significance score for each antigen-stimulated microculture was calculated as a quotient of its cpm value with the donor-specific *z*-value. When the score of a particular antigen-stimulated microculture was higher than one, it was interpreted that the microculture contained antigen-reactive CD4+ T cell clone. The calculation was done using Microsoft Excel (Microsoft Inc., Redmond, USA) and the illustration was developed using GraphPad Prism.

2.8. *In silico* analysis to HLA binding prediction

To predict the binding of peptides to HLA class II molecules (HLA-DR, HLA-DP, and HLA-DQ), an open-source online algorithm NetMHCII (Centre for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark) was used. The algorithm works according to novel artificial neural network-based method that allows for simultaneous identification of the HLA class II binding core and binding affinity [105]. The algorithm provides information about peptide affinity to the HLA molecules, represented by IC_{50} values of particular peptide sequence in nM, which are the peptide amount that prevents binding of 50% of the $1x10^6$ random natural peptides to the 14 HLA-DR, six HLA-DQ, and six HLA-DP haplotypes. Prediction of binding capacity, showed as binding score, was determined by the algorithm using an equation described in the calculation result. Strong binder threshold was defined at IC_{50} value of 50nM of peptide, whereas weak binder threshold was defined at IC_{50} value of 500nM of peptide. Peptide with IC_{50} value >500nM is interpreted as non-binder.

To illustrate the binding capacity of the individual amino acids of the entire protein sequence to 26 representative HLA class II molecules, the binding score for particular weak and strong-binder peptide sequences was applied to their corresponding amino acids. Finally, the total binding score for each individual amino acid was calculated. The visualization was developed using Microsoft Excel and GraphPad Prism.

2.9. HLA-DR and –DP peptide binding assays

$SmD1_{(83-119)}$-peptide binding assays to HLA-DR and HLA-DP was performed in 2006 at the laboratory of Prof. Sylviane Muller at Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France. Generated data from the experiment are included and shown in this PhD thesis under kindly written permission from Prof. Sylviane Muller in March 2017. The quantity of

$SmD1_{(83-119)}$-peptide bound to HLA-DR and –DP molecules was determined in a fluorescent solid-phase assay as described by Monneaux *et al* [106]. HLA-DR and –DP molecules were purified from EBV homozygous cell lines by affinity chromatography. The molecules were incubated with different concentrations of competitor peptides and an appropriate biotinylated reference peptide. The data were presented as the peptide amount that prevented binding of 50% of the biotinylated reference peptide (IC_{50}). As control, purified HLA class II molecules were incubated with biotinylated reference peptides in the absence of competitor, facilitating maximal binding. The data represented average IC_{50} values of at least three independent experiments. Table 2 shows the reference biotinylated peptides and their average IC_{50} values to specific HLA class II molecules. The results obtained from a peptide of the U1A protein, which is an excellent binder, is also shown for comparison.

Table 2: **Average IC_{50} values of the reference biotinylated peptides.** Reference peptides were used to assess the validity of HLA-DR and –DP binding assays.

Reference peptides	Average IC_{50} values
$HA_{(306-318)}$	DR1: 1nM DR4: 10nM DR11: 14nM DRB5: 9nM
$MT_{(2-16)}$	DR3: 421nM
YKL	DR7: 6nM
TER	DRB*1301: 160nM
$A3_{(152-166)}$	DRB1*1501: 78nM
$LOL_{(191-210)}$	DRB3*0101: 38nM
E2/E7	DRB4*0101: 10nM
Oxy	DP401: 4nM DP402: 5nM

3. Results and discussion

Great variability of TCR, unknown sequence of cognate epitope in the target antigen, and complexity of peptide-MHC binding mechanism are the challenge to understand the biology of CD4+ T cell activation through engagement of TCR-Peptide-MHC-II complex. We sought to answer the questions underlying TCR activation in the context of SLE, particularly focusing on the autoantigen SmD1 and its short-fragmented form $SmD1_{(83-119)}$. This chapter emphasizes SmD1 interaction with TCR and MHC class II molecules to draw an evidence of SmD1-specific CD4+ T cell activation. Here, we tried to compare the CD4+ T cell response to complete SmD1 sequence and $SmD1_{(83-119)}$, which can provide us with information whether $SmD1_{(83-119)}$ represents an exclusive antigenic epitope for SmD1-specific CD4+ T cells. Finally, we tried to elucidate the binding of SmD1 to MHC class II molecules using an algorithm and HLA binding assays to build a comprehensive picture about molecular mechanism of TCR-autoantigen-MHC-II interaction in SLE pathogenesis.

3.1. Binding capacity of the SmD1 protein to HLA class II molecules

Knowledge about the binding capacity of the SmD1 protein to a variety of HLA class II molecules is essential to draw a causal relationship between immunogenic antigen epitopes with T cell responses since antigen-specific CD4+ T cell activation is strictly dependent on the engagement of TCR with peptide-MHC-II-complexes. Here we collected data about the binding capacity of the SmD1 protein to 14 different HLA-DR molecules (Figure 7), six HLA-DP molecules (Figure 8) and six HLA-DQ molecules (Figure 9) predicted using NetMHCII algorithm. We have processed the data for delineation of binding affinity of SmD1 protein to particular HLA-DR, -DP and –DQ molecules with a highlight on the SmD1 amino acid region 83-119.

The data show differential binding capacity of regions throughout the SmD1 protein to different HLA class II molecules. Interestingly, binding spots of SmD1 to HLA molecules were not exclusively detected in the region of $SmD1_{(83-119)}$, but mostly outside of this region. Nevertheless, $SmD1_{(83-119)}$ region still has sizeable binding spots to some of HLA class II strains, confirming this fragment as a non-exclusive MHC class II epitope, and therefore also a TCR epitope, in SmD1 protein.

Genetic predisposition has been acknowledged as a possible aetiology of SLE due to association of several HLA alleles to the disease [103, 107]. The effort to characterize risk HLA genes has made important contribution e.g. identification of HLA-DR2 and HLA-DR3 as major susceptibility genes for SLE [108], but also a number of polymorphic alleles such as HLA-DRB1*1501/DQB1*0602 (DR2/DR6), HLA-DRB1*0801/DQB1*0402 (DR8/DQ4), and HLA-DRB1*0301/DQB1*0201 (DR3/DR2) [109]. Five years after this identification, Graham *et al* have reported a positive correlation of SLE-related autoantibody production in individuals with HLA-DR2 and HLA-DR3 genes [110]. The production of Sm/SmD1-antibodies has been linked to HLA-DR3 genes with strong indication of the involvement of SmD1-reactive CD4 + T cells and generation of lupus nephritis [80, 111].

Notably, our *in silico* data confirmed the binding of SmD1 to HLA-DRB1*0301 (HLA-DR3) in almost all amino acids with exception in the last amino acid sequence at the N-terminus of the protein, where the $SmD1_{(83-119)}$-fragment locates (Figure 7). Similar results were indicated for HLA-DRB1*1501 (HLA-DR2). The binding spots spread over the sequence with exception in the last amino acid sequence at the N-terminus of the protein (Figure 7). $SmD1_{(83-119)}$ fragment was predicted to bind on the HLA-DQA1*0501-DQB1*0301 (Figure 9). Thus, autoantibody production as well as SmD1-specific T cell response in individuals with SLE-suspected HLA-DR2 and HLA-DR3 genes are probably promoted by the affinity of SmD1 protein to these HLA molecules in the amino acids that are not exclusively located in the $SmD1_{(83-119)}$ fragment.

Other possible mechanism to connect genetic predisposition on HLA molecules, antigenic epitope, and autoantibody production is the phenomenon called epitope spreading [112]. A B cell receptor (BCR) may recognize SmD1 antigen specifically on the sequence spreading from the amino acid 83 to 119. Once SmD1-specific B cells are activated and differentiated, antibody-secreting plasma cells secrete this BCR as autoantibody that still capable of targeting $SmD1_{(83-119)}$ epitope. In parallel, SmD1 antigen is engulfed and processed into small pieces by B cells in the endoplasmic

reticulum. Different pieces of SmD1 are coupled with HLA molecule depending on their binding affinity. These complexes are transported into the cell surface for presentation to the T cells. These complexes may contain different epitopes of SmD1 antigens, not necessarily the epitope of $SmD1_{(83\text{-}119)}$. Thus, the epitope of antigen recognized by BCR is not necessarily the identical epitope recognized by TCR.

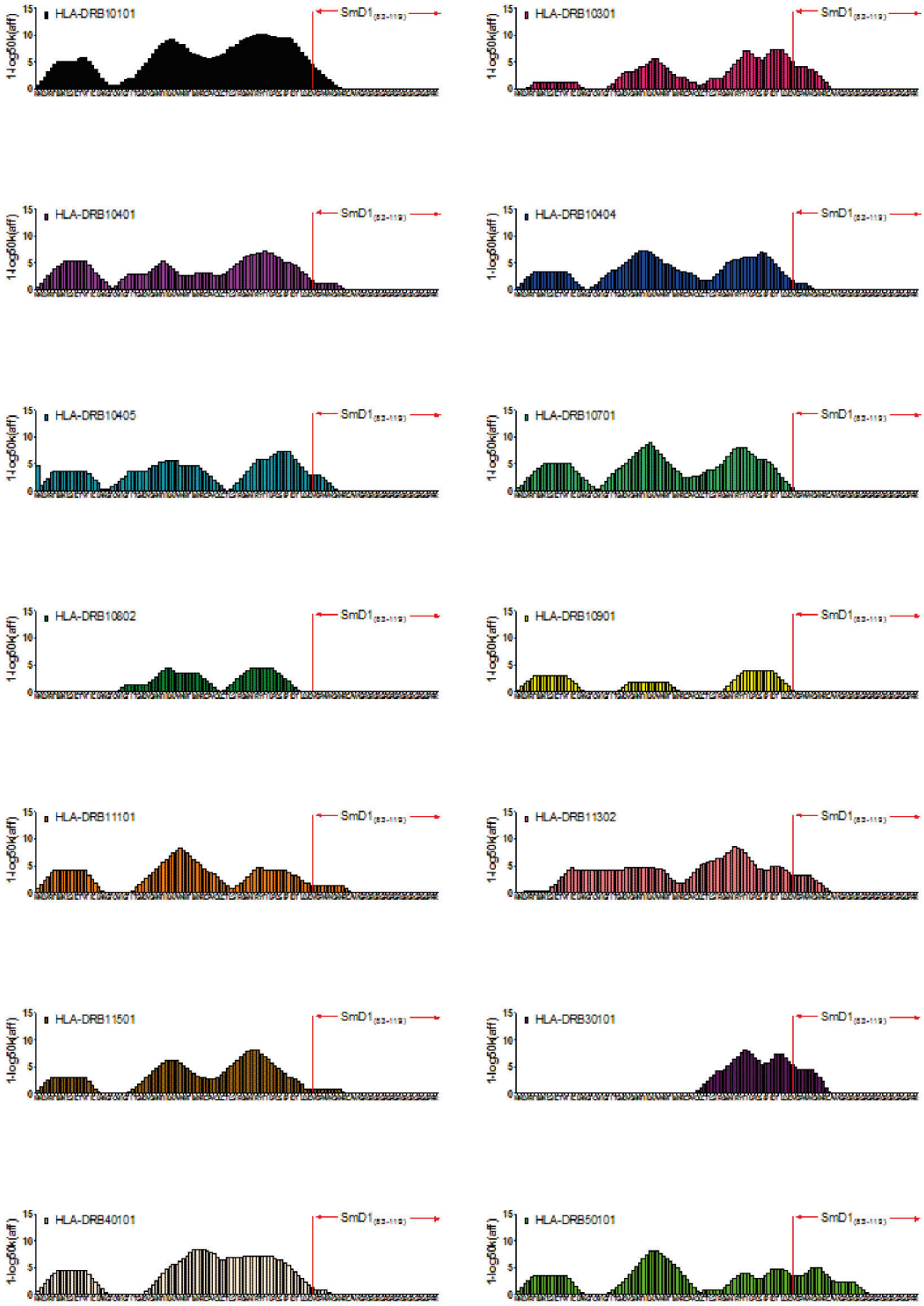


Figure 7: **Binding capacity of SmD1 to a variety of HLA-DR molecules predicted using NetMHCII algorithm.** Binding capacity of the entire amino acids of SmD1 was calculated as a total binding score to 14 different HLA-DR molecules. Binding score is expressed as 1-log50k(aff). The region of $SmD1_{(83\text{-}119)}$ is highlighted with red line/arrows.

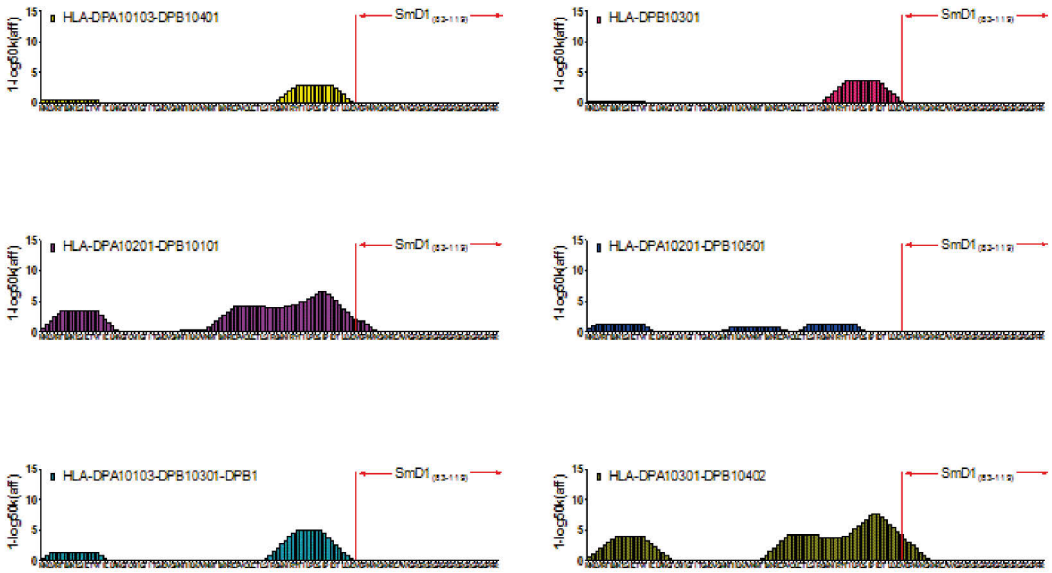


Figure 8: **Binding capacity of SmD1 to a variety of HLA-DP molecules predicted using NetMHCII algorithm.** Binding capacity of the entire amino acids of SmD1 was calculated as a total binding score to six different HLA-DP molecules. Binding score is expressed as 1-log50k(aff). The region of $SmD1_{(83-119)}$ is highlighted with red line/arrows.

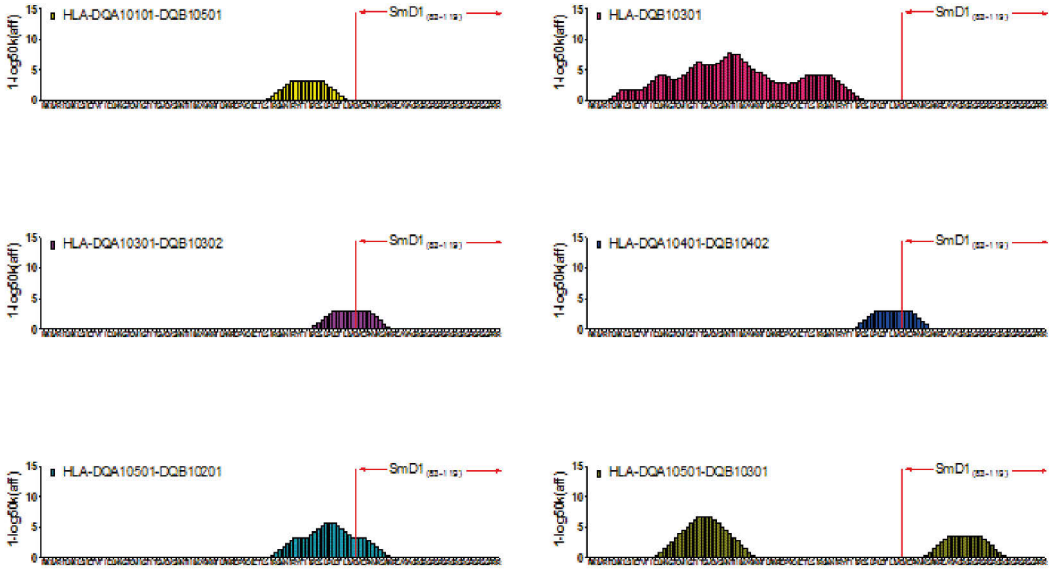


Figure 9: **Binding capacity of SmD1 to a variety of HLA-DQ molecules predicted using NetMHCII algorithm.** Binding capacity of the entire amino acids of SmD1 was calculated as a total binding score to six different HLA-DQ molecules. Binding score is expressed as 1-log50k(aff). The region of $SmD1_{(83-119)}$ is highlighted with red line/arrows.

Our group has performed *in vitro* HLA binding assays of $SmD1_{(83-119)}$ in collaboration with the laboratory of Prof. Sylviane Muller at Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France. It was aimed to measure IC_{50} values of $SmD1_{(83-119)}$ to a set of HLA-DR and –DP molecules. Ten HLA-DR and two HLA-DP molecules were purified from EBV homozygous cell

lines and incubated with different concentration of $SmD1_{(83-119)}$ in the presence of appropriate biotinylated reference peptides for each molecule, to obtain IC_{50} values of $SmD1_{(83-119)}$ to particular HLA-DR and –DP molecules. A ratio of the IC_{50} value of $SmD1_{(83-119)}$ and biotinylated reference peptide was calculated, where the values lower than 20 indicate elevated affinity, values comprised between 20 and 200 indicate intermediate affinity, and the value >200 are considered as no affinity. Confirming our *in silico* analysis, the data revealed that from ten HLA-DR and two HLA-DP screened, only HLA-DR13 showed advanced affinity with $SmD1_{(83-119)}$, whereas the peptide displayed intermediate affinity to HLA-DR15 and no affinity to the rest of the molecules (Table 3). For comparison, the results were obtained in parallel with a peptide of U1A protein that served as an excellent binder.

Table 3: **IC_{50} value of $SmD1_{(83-119)}$ and U1A peptide and their ratio to reference biotinylated peptide measured by HLA binding assay.** The ratio provides evidence of degree of affinity of the peptide to designated HLA-DR and –DP molecules.

Molecule	$SmD1_{(83-119)}$			U1A		
	IC_{50}	Ratio	Affinity	IC_{50}	Ratio	Affinity
DR1	>10,000	>12,250	Non-binder	1	1	Advanced
DR3	>10,000	>238	Non-binder	47	0	Advanced
DR4	>10,000	>1,014	Non-binder	1	0	Advanced
DR7	>10,000	>1,721	Non-binder	6	1	Advanced
DR11	3,642	265	Non-binder	26	2	Advanced
DR13	314	2	Advanced	216	1	Advanced
DR15	9,000	103	Intermediate	8	0	Advanced
DRB3	>10,000	>262	Non-binder	>10,000	>216	Non-binder
DRB4	>10,000	>1,032	Non-binder	828	85	Intermediate
DRB5	>10,000	>1,170	Non-binder	6	1	Advanced
DP401	>10,000	>2,266	Non-binder	931	211	Non-binder
DP402	>10,000	>2,108	Non-binder	31	7	Advanced

When comparing with our *in silico* data, HLA-DR13 and HLA-DR15 are represented by the serotype allele group HLA-DRB1*1302 and HLA-DRB1*1501, respectively, where the binding of $SmD1_{(83-119)}$ was predicted to both molecules, however, binding spots were also identified outside of this fragment (Figure 7). This information is crucial to select not only $SmD1_{(83-119)}$ but also the whole protein sequence as a tool to study CD4+ T cell response in SLE.

3.2. CD4+ T cell response to complete SmD1 compared with $SmD1_{(83-119)}$

Amplified CD4+ T cell clones distributed in nearly 100 microcultures was a powerful tool to display antigen reactivity against SmD1 antigen. It enabled us to answer whether $SmD1_{(83-119)}$ is an exclusive TCR epitope in SmD1 protein in the context of SLE, or whether there is a set of available TCR epitopes outside the amino acid region 83-119. By knowing so, we can modify our approach to study the role of autoreactive CD4+ T cells apart from the boundary of short fragment $SmD1_{(83-119)}$, but in a wider range of possible epitopes spreading the entire protein sequence. Our observation in three Sm/SmD1-seropositive SLE patients with active disease showed detection of CD4+ T cells which were reactive for both SmD1 peptide pool and $SmD1_{(83-119)}$ (Figure 10A), whereas minor detection was found in the control group (Figure 10B), suggesting an elevated frequency of SmD1-reactive CD4+ T cells in the

active phase of SLE pathogenesis. The detection was more observable for SmD1 peptide pool than for $SmD1_{(83-119)}$ leading to an estimation of the availability of various SmD1 epitopes recognized by CD4+ T cell clones derived from active SLE patients.

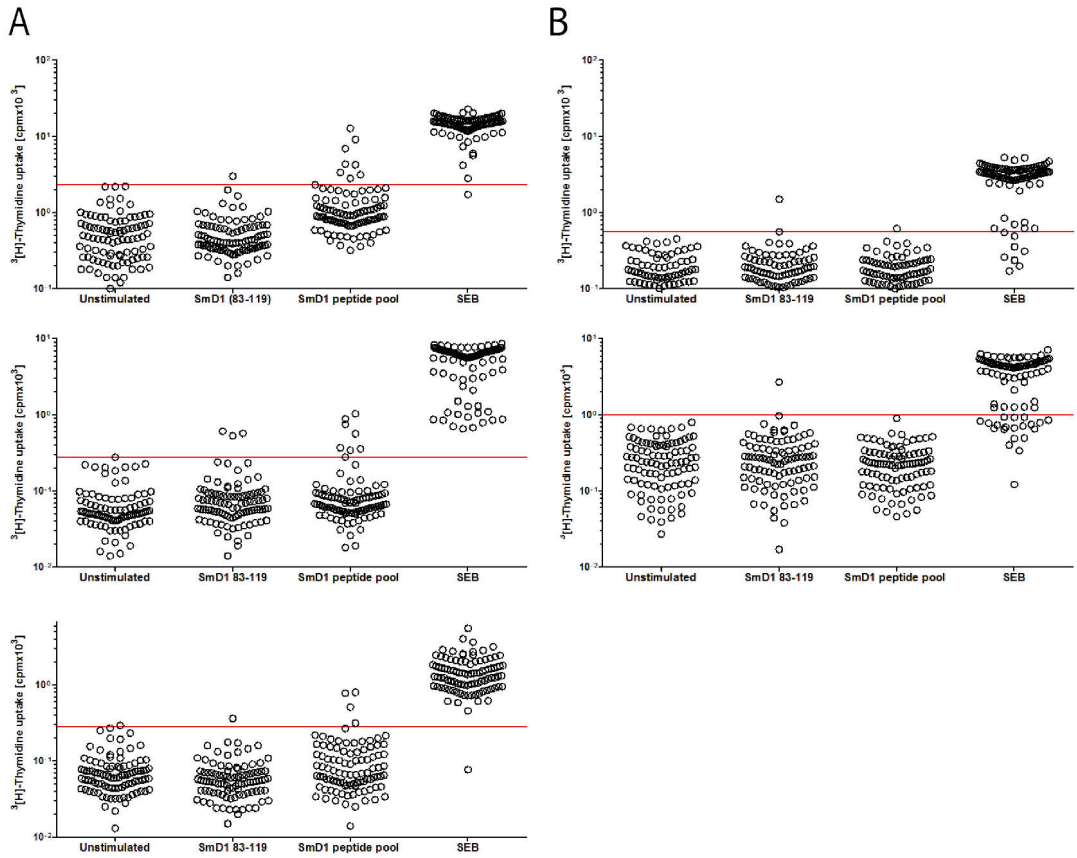


Figure 10: **Detection of SmD1- and $SmD1_{(83-119)}$-specific CD4+ T cells by libraries of amplified CD4+ T cell blasts.** Libraries consisting of 96 polyclonal CD4+ T cell cultures were prepared from Sm/SmD1-seropositive patients with active SLE (*n*=3) (**A**) and healthy individuals (*n*=2) (**B**) and screened for their capacity to proliferate in response to $SmD1_{(83-119)}$, SmD1 peptide pool, and SEB. Unstimulated cells served as negative control to define donor-specific *z*-value calculated as four standard deviations above its mean represented by the red lines. Each dot symbol illustrates one microculture out of 96 measured. Microcultures above the significance threshold were interpreted to contain CD4+ T cell clones reactive to the given antigen.

We then spotlighted the microcultures depicting cpm values above the significance threshold for both SmD1 peptide pool and $SmD1_{(83-119)}$ which indicated the existence of autoreactive CD4+ T cells. Our results revealed that microcultures screened as significant for $SmD1_{(83-119)}$ were also significant for SmD1 peptide pool, but not necessarily vice versa (Figure 11), with an exception for a single microculture in one examined SLE donor (highlighted in blue frame, Figure 11). The data suggested identification of TCR epitope for $SmD1_{(83-119)}$ expressed by some CD4+ T cell clones located in the microcultures with significant cpm value for both SmD1 peptide pool and $SmD1_{(83-119)}$. On the other hand, the data also suggested the existence of other TCR epitopes outside the region of amino acid 83-119 expressed by CD4+ T cell clones located in the microcultures with specificity restricted to SmD1 peptide pool but not to $SmD1_{(83-119)}$, urging us to reconsider that $SmD1_{(83-119)}$ is not exclusively a representative SmD1 epitope in the context of SLE.

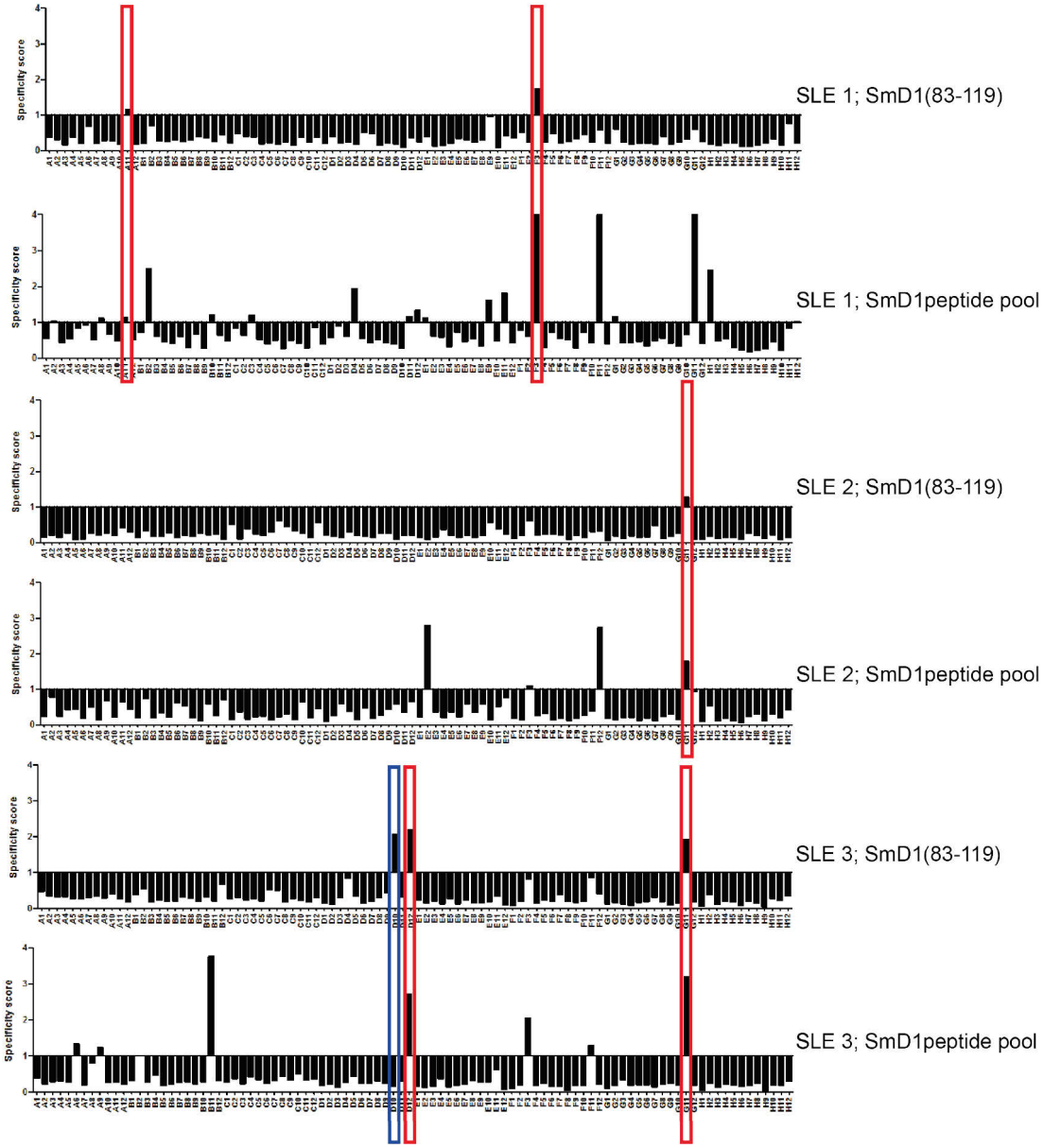


Figure 11: **Indirect identification of SmD1 epitopes recognized by TCR of CD4+ T cell clones.** Specificity score was calculated as a quotient of cpm value of antigen-stimulated microcultures with donor-specific *z*-value derived from Sm/SmD1-seropositive SLE patients with active disease (*n*=3). Microcultures with specificity score higher than one indicates the existence of antigen-reactive CD4+ T cell clones. Red frame highlights microcultures with specificity score higher than one for both $SmD1_{(83-119)}$ and SmD1 peptide pool. Blue frame highlights a single microculture with specificity score higher than one for $SmD1_{(83-119)}$ only but not for SmD1 peptide pool.

Table 4: **Specificity score of microcultures containing CD4+ T cell clone reactive to $SmD1_{(83-119)}$ and SmD1 peptide pool.**

Donor	Microculture	$SmD1_{(83-119)}$	SmD1 peptide pool
SLE 1	A11	1.14	1.14
	F3	1.73	7.35
SLE 2	G11	1.26	1.78
SLE 3	D12	2.21	2.73
	G11	1.93	3.20

In addition, when focusing on the microcultures containing SmD1-reactive CD4+ T cell clones, the specificity scores (stimulation index calculated as quotient of cpm value of antigen-stimulated microculture against cpm value of unstimulated microculture) for SmD1 peptide pool were higher than those observed for $SmD1_{(83-119)}$ in almost all microcultures containing $SmD1_{(83-119)}$-reactive CD4+ T cell clone (Table 4), suggesting an evidence of reactivity against peptide sequence other than $SmD1_{(83-119)}$ alone. Our investigation was true for prediction of T cell epitopes on SmD1 protein without knowledge on the HLA types of the donors, and it was narrowed to comparison of bulk SmD1 versus $SmD1_{(83-119)}$. In the experiments using HLA class II-transgenic mouse model, Jiang *et al.* have identified HLA class II strains with strong correlation to the production of anti-SmD1 autoantibodies, and additionally evaluated recognition of T cell epitopes on SmD1 proteins with knowledge on HLA class II types [80]. After immunization with purified recombinant SmD1 protein, the authors performed T cell epitope mapping by culturing the cells with a pool of 15-mer peptides with 11 amino acid overlaps spanning an entire protein sequence of SmD1 and analysing the proliferation capacity through [^{3}H]-thymidine incorporation assay. Supporting our data, they reported that $SmD1_{(83-119)}$ was not a restricted T cell epitope on SmD1 protein and that the response against $SmD1_{(83-119)}$ was observable only on HLA-DR3-mice, but not on HLA-DQ8-, HLA-DQ0601-, and HLA-DQ0604-mice.

4. Conclusion

We have shown in chapter 2 that CD4+ T cells of Sm/SmD1-seropositive SLE patients have responded insufficiently to $SmD1_{(83-119)}$ illustrated by the expression of CD154 on the cell surface that was at almost similar expression level as observed in the unstimulated control. In this chapter, we evaluated autoantigen $SmD1_{(83-119)}$ as an immunodominant epitope in SLE at molecular level by investigation on TCR-peptide-MHC-II complex interaction. Our T cell library data suggested crucial evidence that $SmD1_{(83-119)}$ is not exclusively an epitope that can mediate CD4+ T cell response, but accompanied with other epitopes spreading within the SmD1 protein. Deshmukh *et al* have performed SmD1 T cell epitope mapping using A/J mouse model, in which the animals were immunized with recombinant SmD1 and isolated lymph node cells were stimulated with a pool of 20-mer peptides with 15 amino acid overlaps [113]. They found that T cell epitopes for SmD1 were localized to $SmD1_{(26-55)}$, $SmD1_{(52-69)}$ and $SmD1_{(86-115)}$, which locates within the fragment $SmD1_{(83-119)}$. The findings were in line with our conclusive information that $SmD1_{(83-119)}$ represents T cell epitope for SmD1, but together with other immunodominant epitopes.

More importantly, we have demonstrated that antibodies directed specifically to the fragment $SmD1_{(83-119)}$ were found in the majority of sera of SLE patients [79], which addressed a specific B cell response to this fragment. The question, whether the antigen fragment recognized by BCR that initiates antibody production necessarily mimics the antigen recognized by TCR, remains speculative. It has been previously known in autoimmune disease such as SLE, that B and/or T cell response to an initial target antigen can be diverse to the immune responses to this antigen targeted at a later time point, a phenomenon described as intermolecular epitope spreading [112]. This may propose a probability that previous recognition of $SmD1_{(83-119)}$ by B cells

results in the presentation of other epitopes to the T cells by the antigen-presenting cells as peptide-MHC-II complex.

T cell epitopes do not only display a binding to particular TCR, but in parallel also to particular MHC class II molecules since otherwise T cell activation is insufficient. We have evaluated the binding of $SmD1_{(83-119)}$ to a variety of HLA class II molecules *in silico* and *in vitro*. Here, we have demonstrated that SLE-related HLA molecules mediate binding possibility not restricted to $SmD1_{(83-119)}$, but also to different SmD1 fragments (Figure 12). Considering the results together, we conclude that the native form of SmD1 consisting of its complete sequence, and the same is true for other autoantigens, should be used to study the response of CD4+ T cells in our effort to unravel the role of autoantigen-specific CD4+ T cells in SLE.

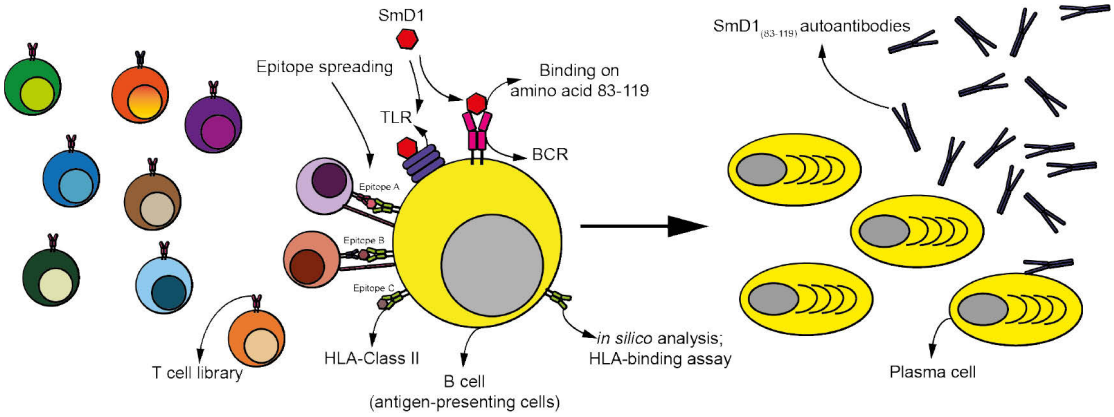


Figure 12: **Epitope spreading.** After analysing possible interaction of $SmD1_{(83-119)}$ as well as SmD1 peptide pool with HLA class II molecule by *in silico* analysis and HLA-binding assay, and with TCR by T cell library, it is possible that SmD1 molecule is recognized by B cell-receptor (BCR) on amino acid 83-119 and therefore differentiated plasma cells secrete anti-SmD1 autoantibody with specificity to SmD1 amino acid sequence 83-119. But B cells have potential to present anything that endocytosed via their BCR or Toll-like receptor (TLR), thus the epitope recognized by T cells can be varied, not necessarily the same epitope recognized by BCR.

Chapter 4

Correlation of the frequency of SLE-associated autoantigen-specific CD4+ T cells with disease activity

Abstract

Autoantibodies directed against nuclear structures such as SmD1, RNP70, Histone, Ro, and La are dominant in SLE, emphasizing these proteins as key autoantigens in the pathogenesis of SLE. We sought to detect the presence of peripheral CD4+ T cells specific to full-length native protein structure of SmD1, RNP70, Histone, Ro, and La and determine their frequency. Full-length antigen was chosen, since it may possess multiple immunodominant epitopes, as described in the previous chapters. We were able to demonstrate the detection of SLE-associated autoantigen-specific CD4+ T cells using libraries containing 13,344 microcultures derived from 18 individuals. The frequency of such autoreactive CD4+ T cells was very low, but still observable, with more frequent detection in SLE patients with active disease as compared with SLE patients with inactive disease and healthy individuals. The presence of circulating autoreactive CD4+ T cells correlated with SLE disease activity, indicating their pivotal role in disease manifestation. This is for the first time the quantity of SmD1-specific, RNP70-specific, Histone-specific, Ro-specific, and La-specific CD4+ T cells was determined in different groups of donors and confirmed with an additional method, providing an advanced resolution for the detection of SLE-associated autoantigen-specific CD4+ T cells.

Keywords: *SmD1, RNP70, Histone, Ro, La, T cell library, ARTE, SLEDAI*

Contents

1. Introduction

1.1. Background

Strong association of several MHC class II molecules with SLE and high prevalence of autoantibody production in patients with SLE constitute an indispensable role of CD4+ T cells at antigen-specific level although the mechanisms underlying antigen-specific T cell-mediated autoimmune responses in SLE is still poorly understood. Nuclear autoantigens such as Sm/SmD1, RNP70, Histone, Ro, and La have been described as targets for autoantibodies that progressively develop preceding the onset of the clinical manifestations of SLE [70]. Studies aimed to investigate CD4+ T cell responses to the aforementioned autoantigens have been conducted in last years. The detection of CD4+ T cells reactive to SmD1 was reported by us, whereas SmB/D-reactive, RNP70-reactive, Histone-reactive, and La-reactive CD4+ T cells isolated from patients with SLE were reported by others [71-74]. Ro-specific T cell hybridomas were generated from HLA-DR3 transgenic mice [75], but the presence of Ro-specific CD4+ T cells in patients with SLE has never been documented.

In other autoimmune diseases such as multiple sclerosis (MS), myelin antigens targeted by CD4+ T cells have been identified and characterized as MS-associated autoantigens. These include proteolipid protein, myelin basic protein and myelin oligodendrocyte glycoprotein [114-116]. T cells reactive with these proteins can be detected both in patients with MS and healthy individuals, however, whether patients with MS have higher frequency of MS-associated antigen-specific CD4+ T cells is still under debate (reviewed in [117]). It is not known if this observation also appears in SLE. Interestingly, differences in the frequency of autoreactive CD4+ T cells in people with and without MS were also observed within the same antigen but across different epitopes (reviewed in [117]). The fact that immunodominant epitopes of MS-associated myelin proteins are highly various, supported our results presented in the chapter 3 and urged us to focus on global T cell response driven by all possible T cell epitopes.

Identification of SLE-associated autoantigen-specific CD4+ T cells is still lacking information about their frequency in health and disease and their contribution to disease pathology. In this chapter, we sought to determine the frequency of CD4+ T cells reactive to SmD1, RNP70, Histone, Ro, and La. Enumeration of these cells provide critical information whether T cell pathogenesis in SLE happens in a “classical” response i.e. it is initiated through antigen-specific T cell activation leading to a clonal expansion of activated T cells. For this purpose, we have learnt from previous chapters that the use of native full-length protein is preferable for the study of autoreactive T cells in a cohort where HLA-types and T cell epitopes of the autoantigen are not known as it will facilitate a global indication about T cell response in a broad and heterogeneous population both in people with or without SLE.

1.2. Objectives

On this chapter, our results about the frequency of SLE-associated autoreactive CD4+ T cells in cohorts of patients with active as well as inactive SLE and in healthy individuals are presented. The detection and calculation of the frequency of SLE-associated autoreactive CD4+ T cells were performed using novel T cell library

assays that enables simultaneous multiple interrogations of T cell reactivity against a series of antigens. The obtained results were compared with the second novel detection method utilizing enrichment of CD154-expressing T cells after short-term stimulation with antigen. Finally, the correlation of the frequency of SLE-associated autoreactive CD4+ T cells with the disease activity was determined.

2. Materials and Methods

In the experiments presented in this chapter, the following methods were performed and they were described in the previous chapters: blood donors and isolation of peripheral blood mononuclear cells (chapter 2, section 2.1.), determination of cell numbers (chapter 2, section 2.2. with additional information, re-described as a new section in this chapter), antigen-reactive T cell enrichment (chapter 2, section 2.5., with additional information, re-described as a new section in this chapter), antibody staining and flow cytometry analysis (chapter 2, section 2.6., with additional information, re-described as a new section in this chapter), statistics (chapter 2, section 2.7., with additional information, re-described as a new section in this chapter), cryopreservation of human lymphocytes (chapter 3, section 2.2.), feeder cell preparation (chapter 3, section 2.3.), antigen-presenting cell preparation (chapter 3, section 2.4.), amplified CD4+ T cell library (chapter 3, section 2.5.), [^{3}H]-thymidine incorporation assay (chapter 3, section 2.6.), and CD4+ T cell library data analysis (chapter 3, section 2.7., with additional information, re-described as a new section in this chapter).

2.1. Antigen and antigen pool preparation

Information about antigen concentration and purchasing of SEB is described in the chapter 2, section 2.4. We used following antigens to stimulate CD4+ T cells in T cell library assay: 0.5µg/mL human SNRPD1 (SmD1) recombinant protein (Biorbyt Ltd., Cambridge, United Kingdom), 50ng/mL human SNRP70 (RNP70) recombinant protein (Abcam Plc., Cambridge, United Kingdom), 0.5µg/mL natural human histone protein (Abcam Plc.), 0.5µg/mL human SS-A/Ro recombinant protein (kindly provided by Euroimmun AG, Lübeck, Germany), 0.5µg/mL human SS-B/La recombinant protein (kindly provided by Orgentec Diagnostika GmbH, Mainz, Germany) and 0.5µg/mL human transthyretin recombinant protein (ATGen, Seongnam, South Korea).

We established a pool containing SLE-associated autoantigens (or “lupus pool”) which was used in the experiments to determine the frequency of SLE-associated autoreactive CD4+ T cells by the ARTE method. We decided to use the lupus pool rather than single antigens to overcome limitations in detecting autoreactive T cells when working with limited amount of blood samples, therefore observation in the T cell response against lupus pool represents pan antigen-specific T cell responses in SLE. The pool was generated as described in the Table 5. We defined the required final concentration of lupus pool for our stimulation experiments as 5µg/mL. Due to variations in protein mass among the antigens, required amount of each protein was taken for lupus pool according to its protein mass and diluted in PBS into concentration of 500µg/mL.

Table 5: **Lupus pool.** Lupus pool was generated from five SLE-associated autoantigens. Required amount of particular antigen was calculated according to its protein mass.

Antigen	Protein mass [kDa]	Amount equivalent with 1nmol [µg]	Amount taken for lupus pool [µg]
SmD1	16	16	10
RNP70	71	70	0.5
Histone	60	60	5
Ro	60	60	5
La	48	48	5
Total	255	255	25.5

2.2. Determination of cell numbers

Enumeration of the frequency of autoreactive CD4+ T cells both using T cell library and ARTE method requires nearly exact viable lymphocyte number. We obtained live cell number using flow cytometry-based technology Guava® easyCyte Single Sample Flow Cytometer (Merck Millipore, Massachusetts, USA). Lymphocytes were diluted with same amount of Guava ViaCount Reagent (Merck Millipore) used for discrimination of viable cells. Cells were incubated with this solution for three minutes at room temperature. Dead cells are attributed with permeabilized cell membrane, thus Guava ViaCount reagent can access inner cell mass and bind on the DNA. Through this process, dead cells have more intense emission signal from the dye and therefore could be excluded from viable cells. At least 1,000 events were acquired and recorded for the calculation.

2.3. CD4+ T cell library data analysis

13,344 microcultures were assessed for their cpm values according to the procedures described in the chapter 3, section 2.6. CD4+ T cell library data were highly heterogeneous among the donors thus statistical data processing was required (we thanked Dörte Huscher, biostatistician at department of epidemiology, DRFZ, for her assistance). First, we set an exclusion criterion that evaluated the cpm-values of SEB-stimulated microcultures. Microcultures with SEB stimulation index (quotient of cpm value in response to stimulation with SEB against cpm value of unstimulated environment) lower than five were excluded. Then, median, 75th percentile, and 25th percentile of cpm values of unstimulated microcultures for each donor were calculated. Finally, donor-specific z-score was calculated as five times the difference of 75th and 25th percentile above the median of unstimulated microcultures. The z-score represents significance threshold in order to identify microcultures, in which polyclonal-expanded CD4+ T cells displayed reactivity to the given antigen. For comparison across different donors, z-score was normalized (Equation 1).

Equation 1: **Calculation of donor-specific z-score.**

$$z - score = median\ cpm\ of\ unstimulated\ microcultures + 5 \times (75th - 25th\ percentile\ of\ cpm\ of\ unstimulated\ microcultures)$$

Enumeration of precursor frequency of antigen-specific CD4+ T cells was calculated based on numbers of negative microcultures according to Poisson distribution and expressed per one million cells [118]. Microcultures with z-score higher than or equal

to one were interpreted to contain at least one antigen-specific CD4+ T cell clone within 2,000 polyclonal CD4+ T cells.

The sensitivity of the method of T cell library was confirmed by selecting cells from microcultures with *z*-score higher than or equal to one for stimulation with the respected autoantigen in the presence of antigen-presenting cells. Stimulation procedure and intracellular staining procedure were performed as described in the chapter 5, section 2.2.

2.4. Antigen-reactive T cell enrichment

The principle of ARTE method was described in the chapter 2 section 2.5. Amendment in the procedure was applied for the experiments in this chapter. Briefly, in total of 1-2x10^7 PBMC were resuspended in 1mL RPMI medium used for T cell stimulation as described in the chapter 2, section 2.4. Cells were stimulated in 12-well plate (Greiner Bio-One AG) for 7 hours at 37°C with 5% CO_2 with following antigens: 5µg/mL lupus pool or 1µg/mL PepTivator® *Candida albicans* MP65 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in the presence of 1µg/mL pure-grade antihuman-CD40 antibody (Miltenyi Biotec GmbH), and 1µg/mL antihuman-CD28 functional grade purified (Life Technologies Europe BV). 1µg/mL brefeldin A (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added for the last two hours of stimulation. After stimulation, cells were scratched with 1mL pipette tip and pooled into 15mL Falcon tubes filled with PBS/BSA/EDTA up to 10mL. At this point, an aliquot of cell suspension was taken for determination of cell number and for staining of original fraction in order to obtain the initial frequency of CD4+ T cells of Treg when necessary. Cells were washed with PBS/BSA/EDTA and labelled with antihuman-CD154-biotin antibodies for 10 minutes at 4°C. The antibodies are conjugated with biotin and bind specifically on CD154 molecule on the cell surface. Subsequently, anti-biotin-microbeads antibodies were added to the cell suspension for 15 minutes at 4°C. The antibodies are conjugated with microbeads and bind specifically on biotin. After a washing step, cells were loaded onto calibrated MS column (Miltenyi Biotec GmbH) to enrich CD154-expressing cells.

2.5. Antibody staining, cell fixation and permeabilization

The following description illustrates procedures of antibody staining, cell fixation, and cell permeabilization belong to the ARTE method. After cells were loaded onto MS column, cells were washed twice with PBS/BSA/EDTA. 60µL antibody master-mix to stain cell surface marker expression was prepared and applied to the cells in the MS column for 15 minutes at room temperature. The master-mix was prepared from following monoclonal antibodies: antihuman-CD3-VioGreen (clone BW264/56), CD4-Allophycocyanin-Vio770 (clone M-T321), antihuman-CD69-VioBlue (clone FN50), antihuman-Biotin-PE (all Miltenyi Biotec GmbH), antihuman-CD69-PerCP (clone FN50, BioLegend Inc., San Diego, USA). In some experiments following human monoclonal antibodies were included: antihuman-CD25-BV650 (clone 4E3, BioLegend Inc.) and antihuman-CD71-Alexa647 (clone L51, DRFZ) antibodies. After 15 minutes incubation time, cells were washed with PBS/BSA/EDTA and eluted into 96-well plates. Cells were fixated with 2% (v/v) paraformaldehyde for 15 minutes at room temperature and permeabilized with BD FACS™ Permeabilizing Solution 2 (BD Biosciences, San Jose, USA) for 10 minutes

at room temperature in the dark. Cells were further stained for intracellular compartment but this step is not relevant for the results presented in this chapter.

Samples were acquired on a BD LSRFortessa® (BD Biosciences) flow cytometer at the FCCF, DRFZ Berlin using FACS®Diva software (BD Biosciences). Flow cytometric data were analysed using FlowJo software (Three Star, Ashland, USA).

2.6. Determination of SLEDAI

The SLE disease activity score according to SLEDAI was retrieved from the medical records of the patients by authorized medical staff at the Charité Universitätsmedizin Berlin and pseudonymized for further analyses. Calculation of SLEDAI score was performed by medical staff who treated and examined the patients around the time when we received blood sample from the patients. SLEDAI is a validated assessment model of SLE clinical manifestations, which is constituted by a weighted metric combining 24 clinical components [119].

2.7. Statistics

Statistical tests were performed with Prism software (GraphPad Software, La Jolla, USA). Normal distribution of the data sets was determined by Kolmogorov-Smirnov-Test. Mann-Whitney U test and Wilcoxon matched-pairs signed rank test were used in the experiments with independent and dependent data set, respectively. Correlation analyses were performed by measurement of linear dependence between two continuous variables using Pearson's correlation coefficients. The P values <0.05 were considered statistically significant with following indication: * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

3. Results and discussion

It has been hypothesized that autoreactivity to nuclear antigens underlies the disease development of SLE. The identification and characterization of nuclear antigen-specific T cells are of fundamental relevance to understanding the immune function of autoreactive CD4+ T cells in SLE pathogenesis. The investigation started with an effort to detect such cells in different groups of donor and to determine their frequency. The results in this chapter focus on the significance contribution of the number of autoreactive T cells to the disease progression.

3.1. Detection of autoreactive CD4+ T cells by T cell library

Given limitations of available methods for analysis of autoreactive CD4+ T cells, it has not been possible to interrogate the T cell reactivity to various numbers of nuclear autoantigens and to quantify their frequencies in health and disease. To overcome this technical challenge, we used advances in T cell libraries as described in the chapter 2. Upon exposure to variety of nuclear antigens, rates of proliferation were measured. Because SLE is characterized by diversity in autoreactive epitopes as described in the chapter 3, we chose to use native full-length recombinant protein for SmD1, RNP70, Histone, Ro, and La. Our data revealed highly heterogeneous proliferation capacity among individuals that participated in our study, thus it was difficult to ascertain

global basal responses which is applicable to all donors (Figure 13A). Therefore, we processed the data by calculating donor-specific *z*-score and normalized the *z*-score for all donors. Normalized *z*-score lower than one represents basal proliferation capacity or no responsiveness to the given antigen (Figure 13B).

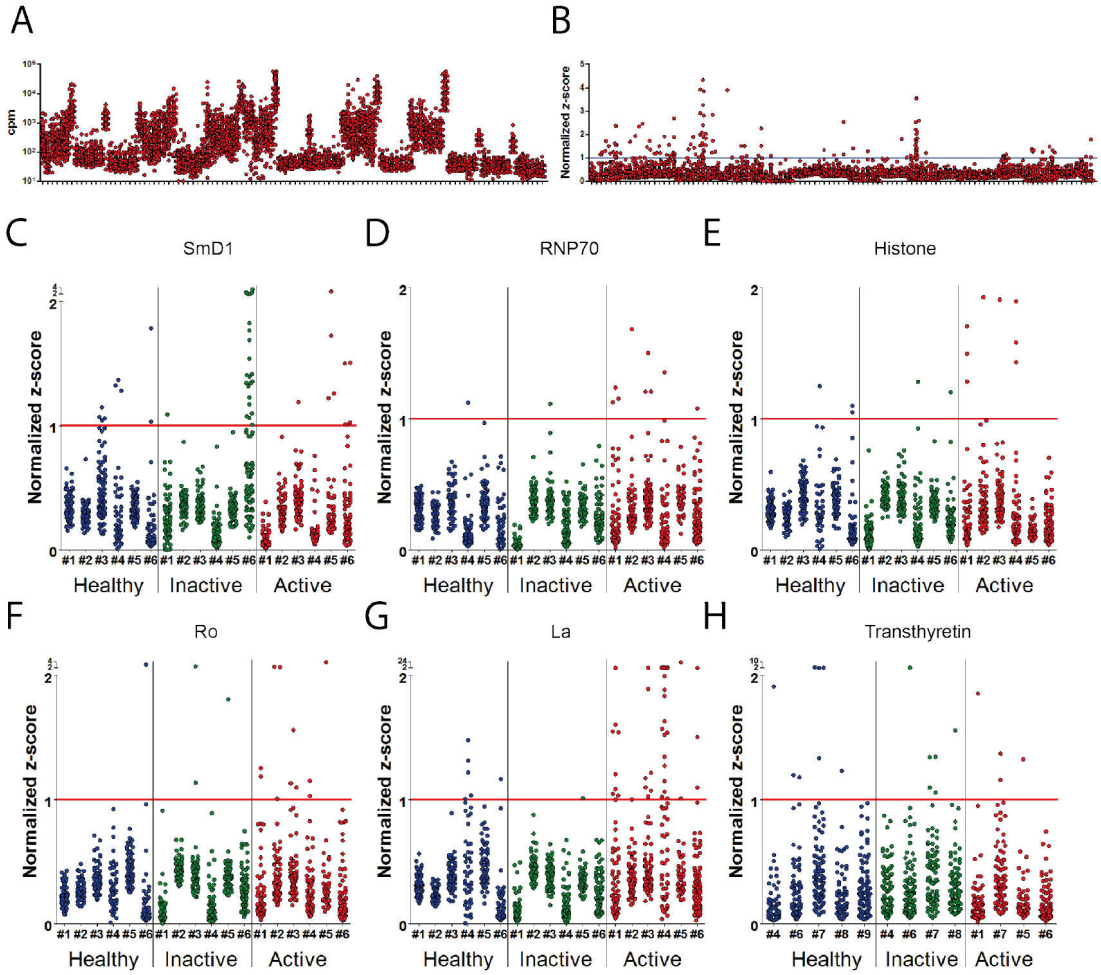


Figure 13: **Detection of autoreactive CD4+ T cells by T cell library.** Libraries consisting of 96 polyclonal CD4+ T cell microcultures were prepared from patients with active SLE (*n*=6, except for transthyretin data *n*=4), patients with inactive SLE (*n*=6, except for transthyretin data *n*=4), and healthy individuals (*n*=6, except for transthyretin data *n*=5). (**A**) Raw data of T cell response measured in cpm was highly heterogeneous thus required data processing. (**B**) Donor-specific *z*-score was determined and normalized. Each dot symbol illustrates one microculture out of 96 measured. Blue line represents the normalized *z*-score that serves as a threshold for proliferation capacity in the absence of antigen. Microcultures above the normalized *z*-score were interpreted to contain CD4+ T cell clones reactive to the given antigen. Detection of autoreactive CD4+ T cells was performed for (**C**) SmD1, (**D**) RNP70, (**E**) Histone, (**F**) Ro, (**G**) La, and (**H**) Transthyretin in three groups (patients with active SLE, patients with inactive SLE and healthy individuals). Red line in (**C**-**H**) represents the threshold.

We then clustered the donors into three cohort groups: patients with active SLE, patients with inactive SLE, and healthy individuals. Patients with active SLE were on disease flare with SLEDAI score more than 10, whereas patients with inactive SLE mostly underwent treatment or in the remission with SLEDAI score up to 10. Healthy subjects were arbitrary assumed to have none of the 24 clinical components relevant for SLEDAI thus they were accounted to have SLEDAI equal to zero. When displayed for particular antigens, microcultures with *z*-score equals to one or higher were found in all three groups indicating the detection of autoreactive CD4+ T cells in

circulating human lymphocytes (Figure 13C-G). Transthyretin, a transport protein for thyroid hormones thyroxine and vitamin A in human plasma [120], served as control autoantigen which is irrelevant for SLE (Figure 13H). Microcultures stimulated with SEB served as positive control to evaluate stimulation procedure and to define exclusion criteria in analysing T cell library data (exemplary data on Figure 6).

The detection of autoreactive CD4+ T cells was unique for each individual. It was observed that an active SLE patient pseudonymized with "Active #4" had an elevated detection of La-specific CD4+ T cells but no detection for SmD1-specific CD4+ T cells. An inactive SLE patient pseudonymized with "Inactive #6" had an intense occurrence of SmD1-specific CD4+ T cells but less detection of other types of autoantigen-specific CD4+ T cells (Figure 13C-G). Unique occurrence of T cells reactive to particular antigen in a single individual may be a consequence of the genetic variability on HLA class II genes. T cell library method enables the measurement of the total repertoire without missing previously unknown antigen epitopes, thus it requires no prior knowledge of donor-HLA type or epitope specificity [104]. The power of this technique includes the ability to perform proteome-wide analysis of the frequency of antigen-specific CD4+ T cells in a variety of T cell subsets and in a HLA diverse population, facilitating inter-donor variation analysis to discover unique HLA and protein specificity among individuals [121]. The advantage of this approach may open possibilities to establish personalized therapy targeting autoreactive T cells.

SLE-associated autoantigen-specific responses, excepts for SmD1, were detected in the circulating CD4+ T cells mostly in patients with active disease when counting the number of positive microcultures within each cohort irrespective of considerable inter-donor variation. Giving the possibility that patients with active SLE were likely responsive to all possible immune agents, our result on CD4+ T cell response to an irrelevant autoantigen transthyretin showed almost equal distribution in all three groups (Figure 13H). However, enumeration of the frequencies of these cells is still required to generate any conclusive knowledge regarding their possible contribution to disease pathology.

3.2. Enumeration of frequency of autoreactive CD4+ T cells

The data from individual microcultures that were screened for proliferation capacity in response to a series of SLE-associated autoantigens can be transformed using statistical approach Poisson distribution to yield quantitative precursor frequency of CD4+ T cells reactive to the autoantigens. The technique is very robust, so that the frequencies of extremely rare T cell populations such as those reactive to neoantigens and those reactive to virus components in the seronegative, unexposed individuals could also be enumerated [104, 121]. However, besides its advantages, the method is highly laborious and time-consuming, in which that human error in practical handling and inhomogeneity of T cell clone expansion in the cell culture could affect the result and accuracy of enumeration of antigen-responsive CD4+ T cells.

The calculation using Poisson distribution showed that the number of RNP70-specific, Ro-specific, and La-specific CD4+ T cells, but not SmD1-specific and Histone-specific, was significantly increased in patients with active disease when compared with patients with inactive disease (SmD1, P=0.7888; RNP70, P=0.0399;

Histone, *P*=0.2306; Ro, *P*=0.0376; La, *P*=0.0062, by Mann-Whitney test) and healthy subjects (SmD1, *P*=1; RNP70, *P*=0.0399; Histone, *P*=0.3472; Ro, *P*=0.0165; La, *P*=0.0416, by Mann-Whitney test) (Figure 14A). Moreover, when focusing on the status of health and disease (active and inactive SLE patients versus healthy individual), the difference in the median number of autoreactive CD4+ T cells was clearly more detectable in patients with active SLE than in patients with inactive SLE and in healthy subjects (SmD1, *P*=0.6781; RNP70, *P*=0.0403; Histone, *P*=0.2122; Ro, *P*=0.0167; La, *P*=0.0416, by Mann-Whitney test). Total number of SLE-associated autoreactive CD4+ T cells varied among individuals (Figure 14B). At least a portion of SLE-associated autoreactive CD4+ T cells was available among one million cells in all patients with active SLE, but only five out of six donors and three out of six donors were found in patients with inactive SLE and healthy subjects, respectively. Interestingly, the occurrence of autoreactive T cells was also heterogeneous among the three groups. In patients with active SLE, at least three types of autoreactive CD4+ T cells were detected, but the variety was reduced in patients with inactive SLE (active SLE: in three out of six donors for SmD1, in four out six donors for RNP70 and Histone, in five out of six donors for Ro and all donors for La; inactive SLE: in one out of six donors for RNP70 and La, and two out of six donors for SmD1, Histone and Ro). The results display broad T cell responses to a variety of SLE autoantigens which is unique for each individual.

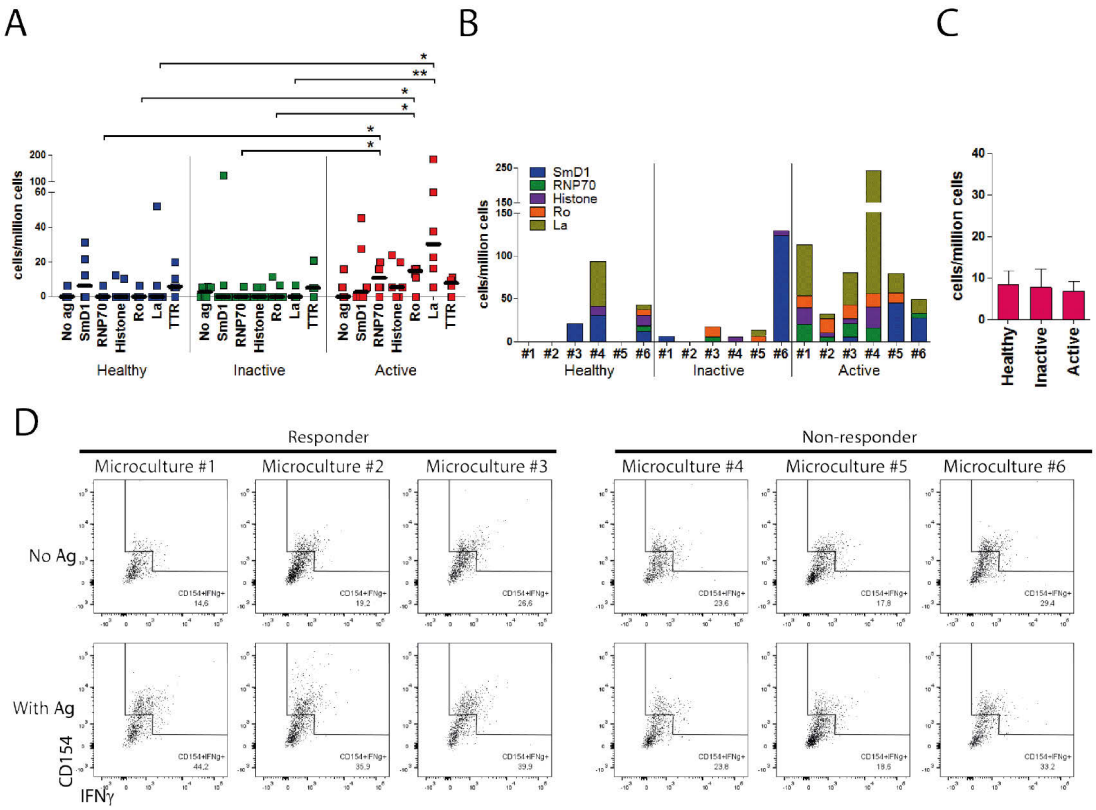


Figure 14: **Enumeration of frequency of autoreactive CD4+ T cells.** The precursor frequencies of autoreactive CD4+ T cells in patients with active SLE (*n*=6, except for transthyretin data *n*=4), patients with inactive SLE (*n*=6, except for transthyretin data *n*=4), and healthy individuals (*n*=6, except for transthyretin data *n*=5) were calculated using Poisson distribution. (**A**) Frequencies of SLE-associated autoantigen-specific CD4+ T cells were shown for cohorts of healthy individuals, patients with inactive SLE, and patients with active SLE. Solid black line indicates median number of each autoantigen. (**B**) Stacked bars show total number and variety of SLE-associated autoantigen-specific CD4+ T cells in all 18 donors participated. (**C**) Frequency of transthyretin-specific CD4+ T cells in healthy individuals, patients with inactive SLE, and patients with active SLE (mean with standard error). (**D**) RNP70

responder and non-responder microcultures were verified by stimulating the cells with RNP70. Dot plots are shown for unstimulated samples and antigen-stimulated samples in upper and lower row, respectively. The number indicates the frequency of CD4+ T cells that express CD154 and co-produce IFN-γ.

Due to the fact of abnormalities in T cell activation signalling observed in patients with SLE characterized by T cell hyperresponsiveness and sustained T cell co-stimulation [122], reactivity to the autoantigens in the patients with active SLE could be a basal phenomenon. To demonstrate that T cell reactivity was restricted to SLE-associated autoantigens, we examined the frequency of CD4+ T cells reactive to an irrelevant autoantigen, transthyretin. Our results imply that the frequency of transthyretin-specific CD4+ T cells was at similar number when comparing the groups (active SLE vs. inactive SLE, $P = 0.7715$; active SLE vs. healthy, $P = 0.6228$; inactive SLE vs. healthy, $P = 1$, by Mann-Whitney test) (Figure 14C) and importantly, when comparing between health and disease (active and inactive SLE patients versus healthy individual) ($P = 0.8253$, by Wilcoxon test). Thus, we exclude the possibility of pan-reactivity against all possible autoantigens in patients with active SLE. The determined frequency of SLE-associated autoreactive CD4+ T cells in the total T cell repertoire was within the range of previous reports for autoreactive CD4+ T cell frequencies but in the naive T cell repertoire [123]. Therefore, it was difficult to ascertain whether the detected autoreactive CD4+ T cells in SLE patients were indeed naive T cells or they were effector T cells that already underwent clonal expansion during disease progression. Based on our data, we assumed that the autoreactive CD4+ T cells in SLE patients have been exposed to primary immune response and proliferate *in vivo* in response to antigen activation. Low number of circulating autoreactive CD4+ T cells is a consequence of T cell migration to the tissue where they contribute to the inflammation. This claim will be further discussed in the chapter 8. An approach to solve this question could be by sorting naive and effector CD4+ T cells prior to expansion with the T cell library technique, so that simultaneous analysis for both T cell compartments can be performed.

Median numbers of particular autoantigen-specific CD4+ T cells showed that the cells exist in the periphery of patients with active SLE at a frequency of 2.69 – 30.18 cells per one million cells, whereas no cell was detected among one million cells in patients with inactive SLE and healthy individuals (except for SmD1). Low occurrence of autoreactive CD4+ T cells in patients with inactive SLE could be due to ongoing treatment with immune suppressive agents. Interestingly, three healthy donors exhibited elevated frequency of some autoreactive CD4+ T cells. Again the question arises, whether these cells are naive or effector T cells. Although it is possible that the cells were effector T cells, we assumed that they were naive autoreactive T cells circulating in the periphery of normal individuals. As discussed in chapter 1, naive autoreactive T cells both with high and low affinity for the autoantigens can escape the negative selection in the thymus where they received thymic education useful for T cell development and magnitude of responsiveness in the periphery [124]. Circulating low-avidity autoreactive naive T cells have potential to loss its anergy and become responsive. But breakdown of peripheral tolerance could activate the cells and cause autoimmunity [125, 126]. Detection of autoreactive CD4+ T cells among one million cells in healthy subjects was possible due to tremendous diversity of the naive T cell repertoire, in which naive T cells specific for any one peptide-MHC class II complex are extremely uncommon [127]. This claim will be supported by our data subjecting cytokine production as a character of effector T cells presented in chapter

6. Nevertheless, our data suggest proliferation of autoreactive CD4+ T cells in patients with active SLE as a consequence of T cell activation by autoantigens which could be an initiation part of disease pathogenesis. Considering that those cells are effector T cells, the frequency in the periphery is however still very low. This fact was in line with the results of our T cell clonal distribution study. In an independent study using next generation sequencing to unravel dominant TCR sequence from blood and urine samples obtained from four patients with active SLE and proliferative lupus nephritis, it was found that almost no occurrence of dominant clones was observed in the peripheral CD4+ T cells, but a number of dominant clones was enriched in the urine, indicating that the autoreactive CD4+ T cells populate more in the inflamed tissue than circulate in the periphery (data shown in chapter 8). This phenomenon will be described comprehensively in chapter 8.

The specificity and sensitivity of the T cell library method was confirmed by an experiment where cells in both representative RNP70-responder and non-responder microcultures were isolated and stimulated with RNP70 autoantigen in the presence of antigen-presenting cells. Responder microcultures were defined as microcultures with normalized RNP70 *z*-score more than or equal as one, whereas non-responder microcultures were defined as microcultures with normalized RNP70 *z*-score lower than one. Responder microcultures contained a sizeable proportion of CD154+IFN-γ+ CD4+ T cells after stimulation with autoantigen indicating reactivity of some cells to RNP70. In contrast, no reactivity against RNP70 was found in the non-responder microcultures (Figure 14D). Although the verification data delivered information of the sensitivity of the method, determination of the number of autoreactive CD4+ T cells in groups of patients with active SLE, patients with inactive SLE, and healthy individuals should be assessed again using an alternative method to comprehensively confirm the sensitivity of the presented results.

3.3. Detection of autoreactive CD4+ T cells by ARTE

We continued our investigation to detect SLE-associated autoreactive CD4+ T cells and calculate their frequency in health and disease by enrichment of CD154-expressing CD4+ T cells, a method known as ARTE which has been described in chapter 2. Comparable with the T cell library technique, the method is useful to obtain proteome-wide analysis of an antigen and direct analysis of the functional phenotype of antigen-specific CD4+ T cells [128]. Limitations in this technique include the requirement of high input cell numbers. This is indispensable as antigen-specific CD4+ T cells are available in extremely rare frequency. Thus, simultaneous interrogation of our five SLE-associated autoantigens was very difficult because it might require up to 500 million cells to be able to detect them in a flow cytometer – a precondition which might be problematic when working with a patient sample. To overcome this limitation, we generated a pool containing all five SLE-associated autoantigens, the so-called lupus pool, and assessed T cell reactivity against this pan-SLE autoantigens. The results will represent the total number of SLE-associated autoreactive CD4+ T cells in each individual.

Precursor frequency of PBMC stimulated with lupus pool was calculated by pre-sorting CD154-expressing cells and counting the number of those cells which co-expressed CD69, a T cell-activation marker which is upregulated after short-term T cell stimulation (Figure 15A). By using combination of both markers, we aimed to

reduce the noise due to unspecific activation. However, unstimulated cells showed sizeable proportion of *in vitro* activated cells in all of our cohorts (median frequency: healthy control = 1,127 cells; inactive SLE = 1,088 cells; active SLE = 681.2 cells/million cells) possibly due to addition of the co-stimulatory agent antihuman-CD28 antibodies to provide an additional stimulation signal to the T cells (Figure 15B). The number of CD4+ T cells reactive to lupus pool was similar to the background in healthy control (P = 0.4375, by Wilcoxon signed-rank test) and inactive SLE (P = 0.5625, by Wilcoxon signed-rank test), but revealed significant difference in patients with active SLE (P = 0.0078, by Wilcoxon signed-rank test). No significant difference was observed in the number of autoreactive CD4+ T cells in patients with active SLE when compared with healthy subjects (P = 0.2721) and patients with inactive SLE (P = 0.1810). The results imply that autoreactive CD4+ T cells were detected in patients with active SLE, whereas the detection in healthy individuals and patients with inactive SLE was not clear because of higher signal-to-noise ratio. In contrast with the previous results in assessment of frequency of particular autoantigen-specific CD4+ T cells by T cell library method, no significant difference in the total number of autoreactive CD4+ T cells was detected among three groups, although the number was increased in the patients with active disease.

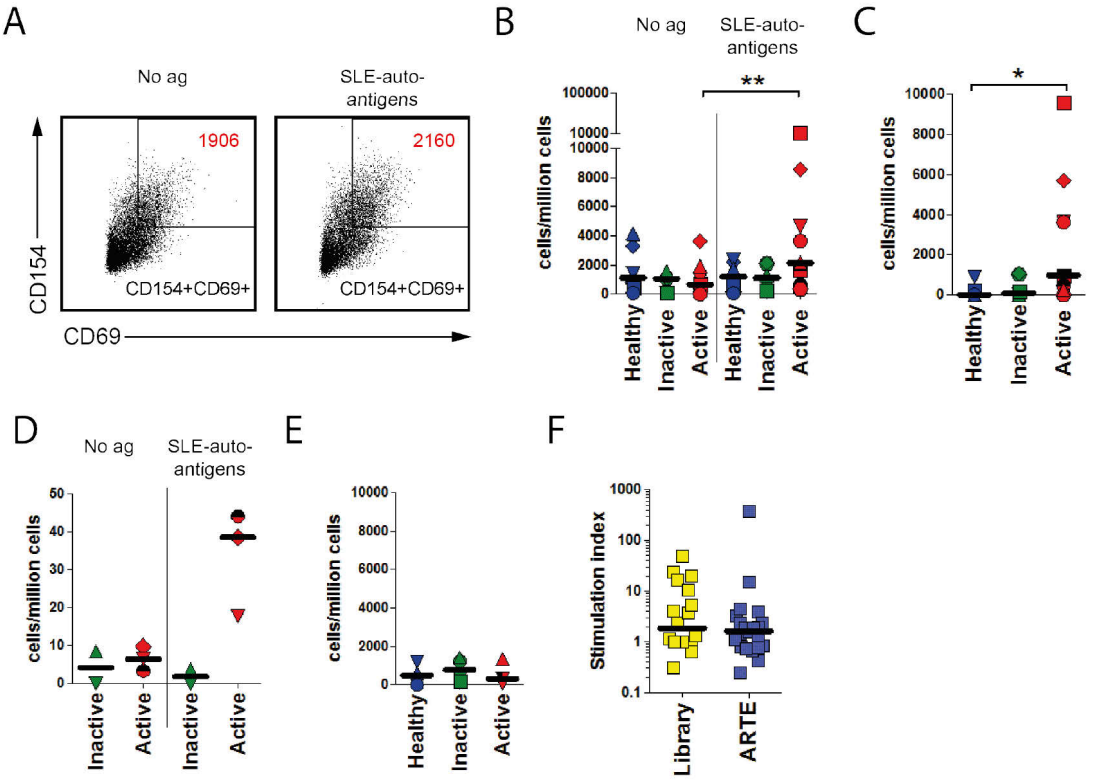


Figure 15: **Detection of autoreactive CD4+ T cells by ARTE.** CD4+ T cells reactive to SLE-associated autoantigens were detected by enrichment of CD154-expressing cells after *in vitro* stimulation. (**A**) Representative dot plots show post enrichment-gating strategy to detect autoreactive CD4+ T cells after *in vitro* culture in absence (no ag) or in presence of SLE-associated autoantigens. Red number indicates counted events in flow cytometry. (**B**) Precursor frequency of CD154+CD69+ CD4+ T cells without (no ag) stimulation or after stimulation with SLE-associated autoantigens determined in healthy controls (healthy, n=6), patients with inactive SLE (inactive, n=6), and patients with active SLE (active, n=9). Each symbol illustrates a distinct individual. (**C**) Precursor frequency of CD154+CD69+ CD4+ T cells after subtraction of background. (**D**) Precursor frequency of CD25+CD69+CD154+ CD4+ T cells with or without autoantigens determined in patients with inactive SLE (n=2) and patients with active SLE (n=3). (**E**) Background-removed frequency of CD154+CD69+ CD4+ T cells after stimulation with *Candida albicans* MP65 in healthy individuals (n=4), patients with

inactive SLE (*n*=4), and patients with active SLE (*n*=3). (**F**) Comparison of the stimulation index from total frequency of autoreactive CD4+ T cells assessed by T cell library (yellow, *n*=18) using Poisson distribution and by ARTE (blue, *n*=21) using calculation of CD154+CD69+ CD4+ T cells. Black line represents median value.

High signal-to-noise ratio hampered the coverage of autoreactive CD4+ T cells in our cohort of healthy individuals and patients with inactive SLE but it is not conclusive that autoreactive CD4+ T cells were really not available in these cohorts among one million PBMC. Thus, we subtracted the number of CD154+CD69+ CD4+ T cells in the samples stimulated with lupus pool with those left unstimulated to yield a number of autoreactive CD4+ T cells without background population (Figure 15C). We found that the number of autoreactive CD4+ T cells ranged from zero to 9,631 cells per one million cells. Indeed, median frequencies of autoreactive CD4+ T cells in healthy individuals, patients with inactive SLE, and patients with active SLE were zero, 100.8, and 983.4 cells in a million cells, respectively. These data were in line with previous results that following progression of disease, the number of autoreactive CD4+ T cells increase. The difference of the frequency of these cells in patients with active SLE was significant when compared with healthy subjects (P = 0.0193, by Mann-Whitney test) but not significant when compared with patients with inactive SLE (P = 0.1103, by Mann-Whitney test). However, to retrieve any conclusions to the correlation of autoreactive CD4+ T cells with disease activity, measurable comparison must be performed, not merely relying on the clustering of the donors to their health and disease condition.

Here we showed that a population of autoreactive CD4+ T cells circulating in patients with active SLE, but not in patients with inactive SLE, was likely to be pre-activated *in vivo* as they also expressed CD25, an activation marker associated with long and sustained T cell stimulation (median number: inactive SLE = 1.879 cells; active SLE = 38.56 cells/million cells) (Figure 15D). A combination of CD25 and CD69 expression may help to distinguish pre-activated T cells to Treg. Because of elevated number of pre-activated CD4+ T cells in active SLE patients, we also checked the frequency of recall-antigen specific CD4+ T cells in all groups of donors (*Candida albicans* MP65) and found that the frequencies were almost similar across the cohorts (median frequency: healthy individuals = 498.5 cells/million cells; inactive SLE = 775.3 cells/million cells; active SLE = 316.5 cells/million cells) (Figure 15E). We also have previously examined the frequency of CD4+ T cells reactive to an irrelevant protein transthyretin using T cell library assay and we found that all three groups showed similar number of such cells.

We next compared the two methods, T cell library and ARTE technique, for measuring SLE associated autoantigen-specific CD4+ T cell repertoire in all donors participated, without differentiating their health and disease status. Because the total number of autoreactive CD4+ T cells determined by T cell library was not comparable with those determined by ARTE, we calculated the stimulation index for all donors as the ratio of the frequency of SLE-associated autoantigen-specific CD4+ T cells against the background frequency. Given that the dataset contain zero value, a log(x+1) transformation was performed. Notably, we found that the median value of the stimulation index derived both from T cell library and ARTE method was comparable (T cell library = 1.857; ARTE = 1.614. P = 0.3306 by Mann-Whitney test) (Figure 15F). Unlike the approach to detect antigen-specific T cells using HLA-multimer technology, both T cell library and ARTE techniques require no prior

knowledge on HLA type of the donors. The T cell library method facilitates broad interrogation of antigen-specific T cells using small numbers of cells, whereas high input cell numbers are necessary for detection by ARTE assays. Nevertheless, T cell library method is highly laborious and provides no information about homogeneity of T cell clonal expansion during cell culture, whereas ARTE enables direct and fast detection of antigen-specific T cells. Obtained cell frequency from both methods may vary, but the detection of autoreactive CD4+ T cells was confirmed to be more prominent in the patients with active SLE. These observations demonstrate that both methods are capable of detecting and calculating extremely rare amounts of autoreactive CD4+ T cells, and importantly, difference in the number of those cells in health and disease can be studied using both techniques.

3.4. Correlation of autoreactive CD4+ T cells with disease activity

Finally, we asked whether the frequency of autoreactive CD4+ T cells circulating in the periphery correlates with SLE disease activity. To depict the degree of disease manifestation in SLE, we chose SLEDAI as a tool to measure SLE disease activity. SLEDAI scores for patients participated in our study were retrieved from medical staff who supervised the patients at the time point when their blood samples were delivered to the laboratory. Association of a clinical parameter with SLEDAI score is widely used to gain information about possible contributions of this parameter to SLE disease pathogenesis.

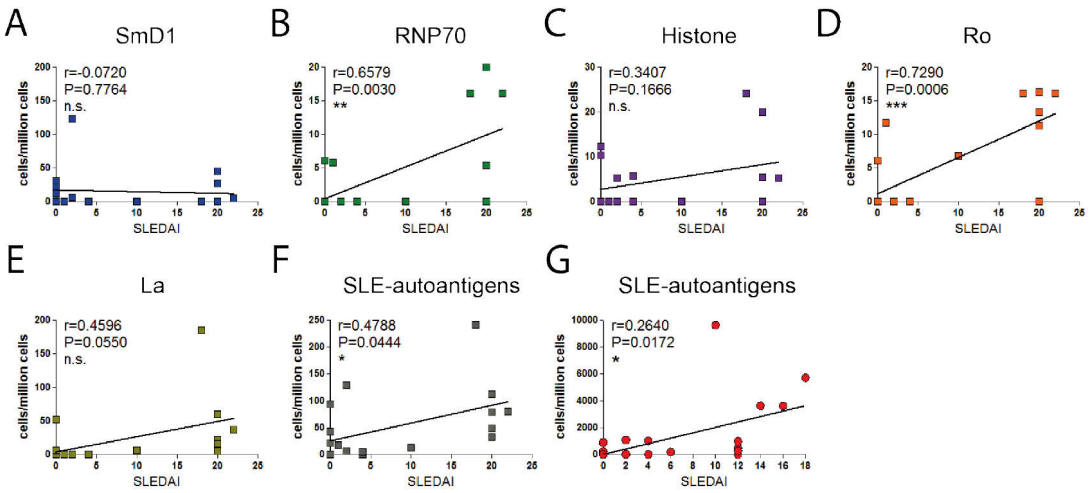


Figure 16: **Correlation of autoreactive CD4+ T cells with disease activity.** The frequencies of autoreactive CD4+ T cells determined by T cell library and ARTE method were compared with SLE disease activity score. Frequency of CD4+ T cells reactive to SmD1 (**A**), RNP70 (**B**), Histone (**C**), Ro (**D**), La (**E**), and to total SLE-associated autoantigens (**F**) derived from T cell library data (n=18) were compared with SLEDAI score. (**G**) Background-subtracted frequency of CD4+ T cells reactive to lupus pool (n=21) shown as number of CD154+CD69+ CD4+ T cells in one million cells was compared with SLEDAI score. R indicates Pearson's correlation coefficient. Each symbol illustrates distinct individual. Line represents linear regression determined using Pearson's correlation coefficient.

Our T cell library results show that frequencies of CD4+ T cells reactive to SLE-associated autoantigens depicted different correlation with SLE disease activity (Figure 16A-E). SmD1-specific CD4+ T cells seemed to occur independent on the activity of disease (P = 0.7764). Histone-specific and La-specific CD4+ T cells were likely found in higher frequency in the patients with increased disease activity (Histone: P = 0.1666; La: P = 0.0550). The frequencies of CD4+ T cells reactive to

RNP70 and Ro were significantly higher following disease progression (RNP70: P = 0.0030; Ro: P = 0.0006). These data suggest possible distinct target autoantigens in T cell-mediated immune pathology in SLE. Given the fact that RNP70 and Ro are not exclusive autoantigen for SLE as antibodies against these proteins are also reported in mixed connective tissue disease and Sjögren's syndrome [70], respectively, the role of these autoantigens as T cell targets in SLE is still unknown.

When the frequency of particular SLE-associated autoantigen-specific CD4+ T cells was calculated together, total number of autoreactive CD4+ T cells assessed by T cell library revealed significant positive correlation with SLE disease activity index (P = 0.0444) (Figure 16F). We have determined the frequency of SLE-associated autoantigen reactive CD4+ T cells by calculating the number of cells expressing CD154 and CD69 after enrichment of CD154-expressing cells. Subtraction of background population uncovered the number of autoreactive CD4+ T cells, in which the frequency between patients with active SLE and healthy control was significantly different. By comparing with SLEDAI score, we provide correlating analysis of the number of autoreactive T cell assessed by ARTE method with disease activity. The number of SLE-associated autoantigen-specific CD4+ T cells correlated significantly with SLEDAI (P = 0.0172) (Figure 16G). These data help explain that the total frequency of autoreactive T cells determined by T cell library and ARTE assays exhibits positive correlation with disease activity indicating pivotal role of autoreactive CD4+ T cell in the complexity of immune orchestra that promote SLE autoimmunity.

SLE is an autoimmune disease characterized with complex molecular and clinical heterogeneity, high risk of failure of clinical trials, and unpredictable disease course that leads to cumulative organ damage over time [49]. Stratification of SLE manifestation that includes a number of molecular and clinical parameters and biomarkers is necessary to facilitate the development of customized and personalized therapies to the patients with SLE. Classification of SLE so far uses scoring systems such as SLEDAI that is limited to certain, but not all, clinical manifestation making reliable disease identification challenging. Banchereau *et al.* recently conducted an elegant study to unravel molecular and clinical complexity in SLE and proposed stratification of SLE patients into seven groups according to their molecular networks, including SLEDAI score [61]. In their study, transcriptional correlation of SLE-associated immunomonitoring data was performed by comparing them with SLEDAI score until they identified clusters of patients with unique molecular and clinical signature, thus enabling possibility of personalized and targeted therapy. However, their study covered limited aspects of T cells, particularly at antigen-specific level. Thus, our study in this thesis would provide critical contribution to information about autoreactive CD4+ T cells in SLE pathogenesis. Our results about the frequency of autoreactive CD4+ T cells needs to be supplemented with additional information about their antigen specificity and functional role to generate a complete picture about the role of autoreactive CD4+ T cells in SLE.

4. Conclusion

The datasets presented in this chapter provides the first comprehensive qualitative analysis of the frequency of autoreactive CD4+ T cells in SLE and their correlation

with disease activity. We report for the first time the frequencies of five SLE-associated autoantigen-specific CD4+ T cells, SmD1, RNP70, Histone, Ro, and La measured by the T cell library method. T cell library has been used as a powerful tool to estimate the number of virus-specific CD4+ T cells [129, 130], recall-antigen-specific CD4+ T cells [131], and also autoantigen-specific CD4+ T cells [132, 133]. We could confirm the detection of autoreactive CD4+ T cells using the second method which relies on the enrichment of CD154-expressing CD4+ T cells after stimulation with a pool of proteins containing all five SLE-associated autoantigens. Furthermore, using the data of detection and enumeration of the frequency of autoreactive CD4+ T cells from T cell library and ARTE method, we suggest that autoreactive CD4+ T cells play a crucial role in the SLE disease pathogenesis as the frequency of these cells positively correlated with SLE disease activity (Figure 17). The correlation with SLEDAI was evaluated for both T cell library and ARTE methods and it revealed a close concordance indicating sensitivity of the analysed data. Our results underline the important role of SLE-associated autoantigens and their autoreactive CD4+ T cell counterparts in the pathogenesis of SLE.

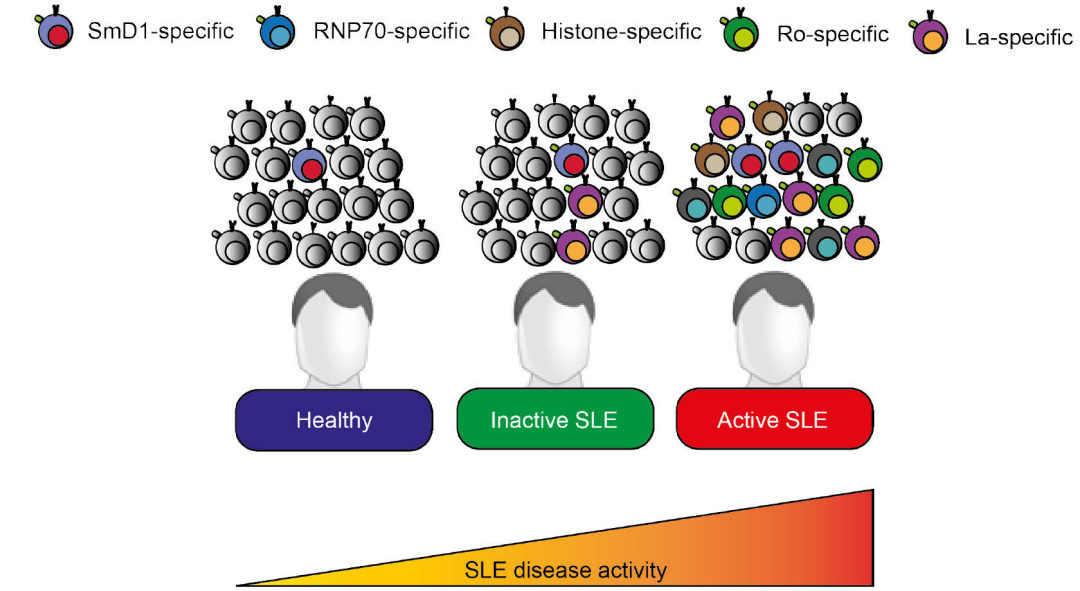


Figure 17: **SLE-associated autoantigen-specific CD4+ T cells are expanded in active SLE.** Using two independent detection methods, we found that SLE-associated autoantigen-specific CD4+ T cells are expanded in patients with active SLE. Their frequency also correlates with SLE disease activity. CD4+ T cells reactive to SLE-associated autoantigens are pathogenic and play an important role in SLE disease pathogenesis.

Chapter 5

Analysis of antigen specificity of SLE-associated autoantigen-specific CD4+ T cells by generation of T cell clones and T cell lines

Abstract

We have demonstrated the detection and enumeration of SLE-associated autoantigen-specific CD4+ T cells by using two novel independent methods, T cell library and ARTE. Autoreactive CD4+ T cells were detected in both healthy subjects and patients with SLE. The frequency of these cells is greater in patients with active disease and correlates with disease activity. Specificity of autoreactive CD4+ T cells to the given autoantigen has been an important issue to validate this observation. In order to further dissect the knowledge of autoantigen specificity, we generated a series of SLE-associated autoantigen-specific CD4+ T cell clones isolated from healthy individuals by ARTE method combined with subsequent single-cell sorting of CD154+CD69+ cells. We could demonstrate that a population of clones generated by our strategy was specific to the given antigens even in the healthy subjects where the frequency was very low and within the background level. In addition, we established lupus-specific CD4+ T cell lines using the same method. Following re-challenge with SLE-associated autoantigens, only small portions of the cells were reactive, confirming our finding on T cell clones. This approach not only assists the verification of autoantigen-specific, *bona-fide* SLE-associated CD4+ T cells, but also enables us to interrogate the sensitivity of the applied methods. It can provide other functional information such as TCR avidity and give the basis of TCR-targeted immune therapy.

Keywords: *Autoantigens, ARTE, T cell clones, T cell lines, TCR, EC_{50}*

Contents

1. Introduction

1.1. Background

A TCR is generated during T cell development by random genetic recombination events of the T cell receptor alpha and beta gene, resulting in an enormously diverse T cell repertoire important for protecting against invading foreign pathogenic structures. A Highly heterogeneous TCR repertoire in the periphery also opens the potential of reactivity against self-structures which may cause pathological autoimmune diseases under certain conditions when homeostatic balance is disturbed, although our body possesses a sophisticated education system in the primary lymphoid organ to eliminate T cells with strong self-reactive TCR specificity [126]. The fact that autoreactive CD4+ T cells are not only found in patients but they are also present in significant numbers in otherwise healthy individuals has been reported previously in a number of publications (reviewed in [117] and [134]).

We have shown the detection of CD4+ T cells with reactivity against nuclear proteins both in lupus patients and in healthy individuals using two independent novel detection methods, T cell library and ARTE. Although useful, the methods have limitation regarding higher detected background frequency. The concern arises when focusing on the cohort of healthy subjects, where the occurrence of autoreactive CD4+ T cells may seem unexpected. Our results revealed that the frequency of autoreactive CD4+ T cells in healthy subjects was within a range of background level. Considering that, it is still unclear whether or not autoreactive CD4+ T cells detected in non-diseased state were *bona-fide*, functional CD4+ T cells that are able to trigger autoimmunity [135].

Cai and colleagues have performed an approach to discovering autoreactive CD4+ T cells in the human repertoire by labelling T cells from healthy individuals with CFSE and stimulating them with autologous antigen-presenting cells containing endogenous self-peptides. They generated single-cell clones of CFSE-low and CFSE-high cells, representing cells with and without reactivity against self-antigens, respectively. The clones were screened against a panel of known autoantigens and microbial recall antigens to evaluate their antigen specificity. Interestingly, CD4+ T cell clones derived from CFSE-low cells exhibited a higher degree of cross-reactivity to multiple antigens, thus they were more multispecific than those derived from CFSE-high cells [136]. These data indicate that T cell specificity to a given autoantigen is an important issue as it relates closely with the possible function of these cells. An approach to elucidate T cell specificity, e.g. by generating single-cell clones or antigen-specific T cell lines, will not only validate our observations regarding the frequency of autoreactive CD4+ T cells in health and disease, but also expand our knowledge about the sensitivity of the applied methods and physiological function of autoreactive CD4+ T cells.

1.2. Objectives

In this chapter, specificity of SLE-associated autoantigen-specific CD4+ T cells was evaluated by generating autoreactive CD4+ T cell clones and CD4+ T cell lines. The study was focused on autoreactive CD4+ T cells isolated from healthy individuals.

The results should also deliver knowledge about sensitivity of the applied methods and possible physiological functions of autoreactive CD4+ T cells.

2. Materials and methods

In the experiments presented in this chapter, the following methods were performed and they were described in the previous chapters: blood donors and isolation of peripheral blood mononuclear cells (chapter 2, section 2.1.), determination of cell numbers (chapter 2, section 2.2. and chapter 4, section 2.2.), antigen-reactive T cell enrichment (chapter 2, section 2.5., and chapter 4, section 2.4.), antibody staining, cell fixation and permeabilization, and flow cytometry analysis (chapter 2, section 2.6. and chapter 4, section 2.5., with additional information, re-described as a new section in this chapter), statistics (chapter 4, section 2.7.), cryopreservation of human lymphocytes (chapter 3, section 2.2.), feeder cell preparation (chapter 3, section 2.3.), antigen-presenting cell preparation (chapter 3, section 2.4.), and antigen pool preparation (chapter 4, section 2.1.).

2.1. Generation of single-cell clones

Limiting-dilution assay has been widely used as a method to generate single-cell clones which relies on the statistical probability that no more than one cell will be distributed in each microcultures i.e. continuous dilution of cells with known concentration into a concentration below the detection limit. This method is very laborious and still cannot provide an exact condition to achieve one single cell in a culture, as many microcultures may contain no T cells, and repeated cloning may be required due to expansion of mixed populations. In our work, we used advancements of single-cell sorting by flow cytometry, in order to select and sort a single cell with particular phenotype into a cell culture. Critical factors to this method include cell cross-contamination during sorting, or during prolonged cell culture.

Briefly, after enrichment of antigen-reactive CD4+ T cells by ARTE method and subsequent extracellular staining with antihuman-CD3-PacificBlue (clone UCHT1, Biolegend Inc., San Diego, USA), antihuman-CD4-PE-Cy7 (clone SK3, BD Biosciences, San Jose, USA), antihuman-CD69-FITC (clone FN50, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), antihuman-Biotin-PE (Miltenyi Biotec GmbH) antibodies, and LIVE/DEAD® Aqua kit (Life Technologies Europe BV, Bleiswijk, The Netherlands), CD154+ cells were filtered with 30µm pre-separation filter (Miltenyi Biotec GmbH) and resuspended in 1mL cold PBS/BSA/EDTA. Cells were single-cell sorted for CD154+CD69+ phenotype after gating on live CD3+CD4+ cells using BD FACSARIA™ II or BD Influx™ cell sorter (all BD Biosciences) at FCCF DRFZ using FACS®Diva software (BD Biosciences). In this ARTE method, anti-CD28 antibody and brefeldin A were not supplemented into the stimulation culture, since no intracellular compartment was analysed and the sorting strategy was performed by targeting CD154+CD69+ expression on the cell surface of CD4+ T cells. One sorted cell was cultured in one well of 96-well plate containing 200µL IMDM medium with GlutaMAX™ (Life Technologies Ltd., Paisley, United Kingdom) supplemented with 5% heat-inactivated human AB serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1% (v/v) Penicillin-Streptomycin (Life Technologies Ltd.), 2µg/mL ciprofloxacin (Sigma-Aldrich Chemie GmbH), and 1% (v/v) MEM non-essential amino acid solutions (Sigma-Aldrich Chemie GmbH), in the

presence of 1µg/mL lectin from *Phaseolus vulgaris* (PHA, Sigma-Aldrich Chemie GmbH), 600IU/mL human recombinant IL-2 (Proleukin/Aldesleukin, Novartis Pharma GmbH, Nuremberg, Germany), 10ng/mL human recombinant IL-7 (PeproTech, Hamburg, Germany), and about $1x10^6$ irradiated feeder cells from two non-autologous donors (further described as cloning medium). The cell cultures were maintained for 3-4 weeks in the incubator at 37°C with 5% CO_2 until several clones grew and could be visually detected under the microscope. Medium was replenished periodically after three days by removing 50% volume of culture medium in each microculture and adding fresh 1µg/mL PHA, 200IU/mL IL-2, and 10ng/mL IL-7. Every seven days, $5x10^5$ irradiated feeder cells from two non-autologous donors were added. Grown clones were transferred into 48 well-plates and cultured in 500µL cloning medium. After one week, cell number was determined and the clones were transferred back into 96-well plate format and rested in the stimulation medium for at least four days prior to restimulation with the antigens.

2.2. Single-cell clone stimulation

Antigen-specific CD4+ T cell clones were re-stimulated with corresponding antigen in a procedure similar to that described in the chapter 2, section 2.4. and chapter 3, section 2.5. Briefly, clones were rested in stimulation medium prior to stimulation with the antigens for at least four days. One day prior to restimulation, antigen-presenting cells were thawed and distributed into the T cell culture in a ratio of at least one antigen-presenting cell to 100 CD4+ T cells in 100µL stimulation medium. On the day of restimulation, depending on their specificity, the clones were re-stimulated with either 1µg/mL human SNRPD1 (SmD1) recombinant protein (Biorbyt Ltd., Cambridge, United Kingdom), or 100ng/mL human SNRP70 (RNP70) recombinant protein (Abcam Plc., Cambridge, United Kingdom), or 1µg/mL natural human histone protein (Abcam Plc.), or 1µg/mL human SS-A/Ro recombinant protein (kindly provided by Euroimmun AG, Lübeck, Germany), or 1µg/mL human SS-B/La recombinant protein (kindly provided by Orgentec Diagnostika GmbH, Mainz, Germany), or 1µg/mL EndoGrade® endotoxin-free ovalbumin (Hyglos GmbH, Bernried am Starnberger See, Germany), or 50µg/mL *Aspergillus fumigatus* lysate (Miltenyi Biotec GmbH), in the presence of 1µg/mL antihuman-CD40 antibody (generated at DRFZ) and 1µg/mL antihuman-CD28 functional grade purified (Life Technologies Europe BV). Unstimulated cells served as negative control. Cells were cultured in 96-well plate (Greiner Bio-One AG) for seven hours at 37°C with 5% CO_2. 1µg/mL brefeldin A (Sigma-Aldrich Chemie GmbH) was added for the last two hours of stimulation. After stimulation, cells were washed with PBS/BSA.

2.3. Generation of antigen-specific CD4+ T cell lines

In order to better understand the antigen-specificity of autoreactive CD4+ T cells and sensitivity of ARTE method, CD4+ T cell line reactive to a pool of lupus antigens (lupus pool) and to *Aspergillus fumigatus* lysate were generated from a healthy individual. Briefly, after enrichment of antigen-reactive CD4+ T cells by ARTE method and subsequent extracellular staining with antihuman-CD3-PacificBlue (clone UCHT1, Biolegend Inc.), antihuman-CD4-PE-Cy7 (clone SK3, BD Biosciences), antihuman-CD69-FITC (clone FN50, Miltenyi Biotec GmbH), antihuman-Biotin-PE (Miltenyi Biotec GmbH) antibodies, and LIVE/DEAD® Aqua kit (Life Technologies Europe BV), CD154+ cells were filtered with 30µm pre-separation filter (Miltenyi

Biotec GmbH) and resuspended in 1mL cold PBS/BSA/EDTA. In this ARTE method, anti-CD28 antibody and brefeldin A were not supplemented into the stimulation culture, since no intracellular compartment was analysed and the sorting strategy was performed by targeting CD154+CD69+ expression on the cell surface of CD4+ T cells. Cells were sorted for CD154+CD69+ phenotype after gating on live CD3+CD4+ cells using BD FACSARIA™ II or BD Influx™ cell sorter (all BD Biosciences) at FCCF DRFZ using FACS®Diva software (BD Biosciences). Sorted CD154+CD69+ cells were cultured into FACS tube (Sarstedt GmbH, Nürmbrecht, Germany) or into falcon round bottom polypropylene (Corning GmbH, Wiesbaden, Germany) when sorting was performed on BD Influx™ cell sorter containing cloning medium without IL-7 and in the presence of about $1x10^7$ irradiated feeder cells from two non-autologous donors. The cell cultures were maintained in 48-well plate (Greiner Bio-One AG) for two weeks in the incubator at 37°C with 5% CO_2. Medium was replenished periodically after three days by removing 50% volume of culture medium in each microculture and adding fresh 1µg/mL PHA, and 200IU/mL IL-2. After one week, $1x10^6$ irradiated feeder cells from two non-autologous donors were added. Cell number was determined and the antigen-specific T cell lines were rested in the stimulation medium for at least four days prior to restimulation with the antigens.

2.4. Antigen-specific CD4+ T cell line stimulation

Antigen-specific CD4+ T cell lines were re-stimulated with corresponding antigen in a procedure similar to that described in the chapter 2, section 2.4., chapter 3, section 2.5., and section 2.2. in this chapter. Briefly, T cell lines were rested in stimulation medium prior to stimulation with the antigens for at least four days. One day prior to restimulation, antigen-presenting cells were thawed and distributed into the T cell line culture in a ratio of at least one antigen-presenting cell to 100 CD4+ T cells in 1mL stimulation medium. On the day of restimulation, each T cell line was distributed into three samples, and re-stimulated with either 5µg/mL lupus pool, or 50µg/mL *Aspergillus fumigatus* lysate (Miltenyi Biotec GmbH), or left unstimulated, in the presence of 1µg/mL antihuman-CD40 antibody (generated at DRFZ) and 1µg/mL antihuman-CD28 functional grade purified (Life Technologies Europe BV). Cells were cultured in 96-well plate (Greiner Bio-One AG) for seven hours at 37°C with 5% CO_2. 1µg/mL brefeldin A (Sigma-Aldrich Chemie GmbH) was added for the last two hours of stimulation. After stimulation, cells were washed with PBS/BSA.

2.5. Determination of autoantigen's EC_{50} value

Autoreactive CD4+ T cell clones derived from healthy individuals can be used as a tool to study TCR avidity against SLE-associated autoantigens, to predict their physiological function in non-diseased state. Autoreactive single-cell clones were generated from up to five healthy individuals or from one patient with active SLE as described in the section 2.1., and re-stimulated with corresponding autoantigen as described in the section 2.2., in 10-fold decreasing antigen concentration starting from 100nM to 10pM (except for RNP70, starting from 10nM to 1pM). Unstimulated cells were served as negative control. The frequency of CD154+IFN-γ+ cells after restimulation were used as a read-out to define EC_{50} value, or the value of half-maximal effective concentration of autoantigens, in which the autoantigen concentration can induce half-maximal response as a consequence of TCR-

autoantigen interaction. The calculation of EC_{50} was performed using Prism software (GraphPad Software, La Jolla, USA). Briefly, the frequency of CD154+IFN-γ+ cells induced by different antigen concentration was normalized by subtracting the background. Antigen-concentration was transformed to logarithmical scale. Using non-linear regression, sigmoidal curve was generated and half-maximal response of the antigen was determined.

2.6. Antibody staining of intracellular compartment

The principal of antibody staining was described in previous chapters. In this thesis, intracellular cytokines and transcription factors were stained using fluorochrome-conjugated antibodies and analysed on a flow cytometer. Prior to intracellular staining, cells were fixated with 2% (v/v) paraformaldehyde for 15 minutes at room temperature and permeabilized with BD FACS™ Permeabilizing Solution 2 (BD Biosciences, San Jose, USA) for 10 minutes at room temperature in the dark. Intracellular staining was performed in 96-well plate (Greiner Bio-One AG). 60µL of mater mix was prepared from following monoclonal antibodies depending on the experiment: antihuman-CD154-BV421 (clone 24-31, Biolegend Inc.), antihuman-CD154-PE (clone 24-31, Miltenyi Biotec GmbH), antihuman-IFN-γ-APC (clone 4S.B3, Biolegend Inc.), antihuman-IFN-γ-PacificBlue (clone 4S.B3, self-prepared at DRFZ), antihuman-IL-2-PE (clone MQ1-17H12, Biolegend Inc.), antihuman-IL-10-BV421 (clone JES3-9D7, Biolegend Inc.), antihuman-IL-10-PECy7 (clone JES3-9D7, Biolegend Inc.), and antihuman-IL-17-BV786 (clone N49-653, BD Biosciences). Cells were incubated with mastermix at 4°C for 30 minutes in the dark. After intracellular staining, cells were washed with PBS/BSA and resuspend with PBS/BSA for acquisition on a flow cytometer.

3. Results and discussion

Using the technique of single-cell cloning of CD154+CD69+ cells after subsequent combination of ARTE method and single-cell sorting, we were able to generate single cell clones to demonstrate that the TCR binding to the antigen was specific. Our observation was focused on healthy individuals to better characterize the sensitivity of the method. Furthermore, the results were confirmed by generation of antigen-specific T cell lines. Finally, the clones were used as a tool to analyse the physiological function of autoreactive CD4+ T cells by determining TCR avidity in healthy individuals.

3.1. Single-cell cloning of autoreactive CD4+ T cells

PBMC of two healthy individuals were stimulated with a series of autoantigens, a recall antigen, a neoantigen, or left unstimulated. We chose *Aspergillus fumigatus* lysate as a recall antigen that served as a positive control, since every individual seems to have undergone exposure against this fungal airborne antigen [128]. We wanted to compare the frequency of autoantigen-specific CD4+ T cells with neoantigen-specific CD4+ T cells, thus we chose ovalbumin protein as a potential tumour neoantigen for healthy individuals [137]. Derived from two donors, PBMC of donor #1 were stimulated with Histone, Ro, and La, whereas PBMC of donor #2 were stimulated with *Aspergillus fumigatus* lysate, SmD1, RNP70, and Ovalbumin. After

stimulation, CD154-expressing CD4+ T cells were enriched and co-stained with CD69 on the cell surface. A population of CD154+CD69+ CD4+ T cells was observed in the $1x10^7$ PBMC of donor #2 stimulated with autoantigen (exemplary shown for SmD1, 29 cells/$1x10^7$ PBMC), with neoantigen (ovalbumin, 24 cells/$1x10^7$ PBMC) and with recall antigen (*Aspergillus fumigatus*, 300 cells/$1x10^7$ PBMC) (Figure 18A). The frequency of autoantigen-specific CD4+ T cells and neoantigen-specific CD4+ T cells was almost at similar levels. The number of cells within background population was five cells out of $1x10^7$ PBMC. The staining of antigen-specific CD4+ T cells demonstrated in this result was somewhat different to the staining of antigen-specific CD4+ T cells presented in chapter 4, where the frequency of CD154+CD69+ CD4+ T cells in the background population was high. This is because in this setting we only targeted antigen-specific CD4+ T cells via their surface expression of CD154 and CD69, therefore additional induction of T cell activation via costimulatory molecule CD28 was not necessary. The use of anti-CD28 antibody is critical when expecting cytokine production as the read-out of TCR stimulation, since our observation in the laboratory revealed that it can enhance intrinsic T cell-activation signalling sufficiently for cytokine production. However, application of anti-CD28 antibody also may increase unspecific, antigen-independent expression of CD154 and CD69, deteriorating the signal-to-noise ratio of the frequency of antigen-specific CD4+ T cells.

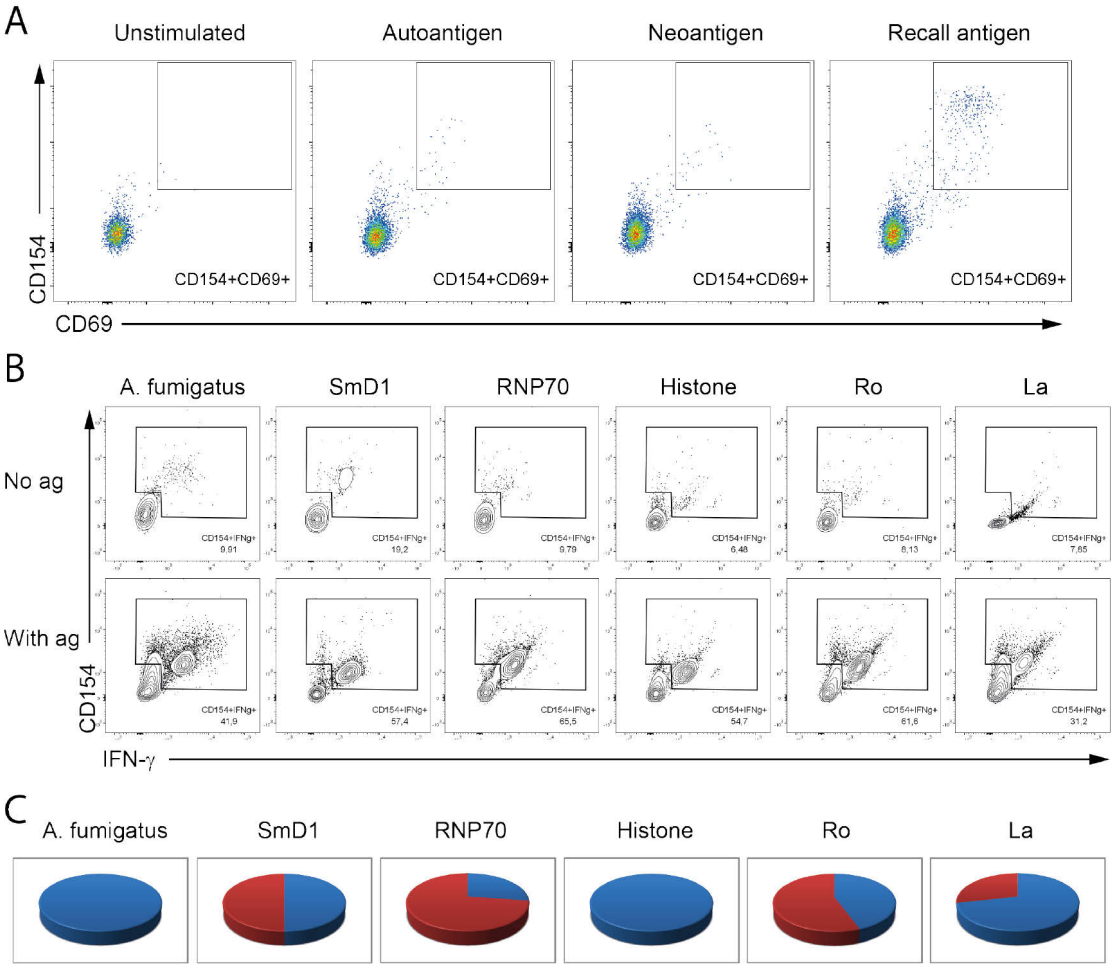


Figure 18: **Single-cell cloning of antigen-specific CD4+ T cells and specificity testing.** PBMC from healthy individuals (*n*=2) were stimulated with antigens and single-cell sorted for CD154+CD69+

expression on the surface of CD4+ T cells to generate single cell clones. (**A**) Gating strategy of CD154+CD69+ CD4+ T cells on the unstimulated sample, sample stimulated with an autoantigen (exemplary shown for SmD1), with a neoantigen (ovalbumin), and with a recall antigen (*Aspergillus fumigatus* lysate). (**B**) Representative clone specific for *Aspergillus fumigatus*, SmD1, RNP70, Histone, Ro, and La was tested for its specificity by restimulation with the corresponding antigen. Upper row shows unstimulated clones (no ag) and lower row shows antigen-stimulated clones (with ag). CD154+IFN-γ+ gating strategy was used to identify antigen-reactive cells within the clones. Number shows the frequency of CD154+IFN-γ+ cells within the clones. (**C**) Pie bars show the distribution of clones which are reactive and not reactive to the corresponding antigens. Blue colour represents reactivity against corresponding antigen and red colour represents non-reactivity against corresponding antigen.

Table 6: **Single-cell cloning.** Antigen-specific CD4+ single-cell clones were established from two healthy individuals by ARTE method and subsequent single-cell sorting of CD154+CD69+ CD4+ T cells. *Asp* refers to *Aspergillus fumigatus*. The table shows the number of sorted cells, the number of established clones, the cloning efficiency, the number of randomly chosen clones, and the number of specific clones assessed by comparing the frequency of CD154-expressing cells producing IFN-γ in the sample stimulated with corresponding antigen against with unstimulated sample.

Donor ID	Antigen	# CD154+ CD69+	# Clones established	Cloning efficiency [%]	# Randomly chosen clones	# Specific clones
#2	*Asp*	96	34	35.41	5	5
#2	SmD1	29	13	44.83	8	4
#2	RNP70	36	12	33.33	11	3
#1	Histone	52	11	21.15	5	5
#1	Ro	54	12	22.22	9	4
#1	La	68	13	19.12	7	5

We then sorted single CD154+CD69+ CD4+ T cell from PBMC stimulated with SmD1, RNP70, Histone, Ro, and La to generate autoreactive CD4+ single-cell clones enabling us to study antigen specificity and functional property of antigen-specific CD4+ T cells [138]. *Aspergillus fumigatus*-specific CD4+ T cells were also single-cell sorted served as a high control. The experiments for the generation of single-cell clones were performed for 23 times in total involving more than 10 individuals. In this thesis, only two experiments with independent datasets aimed for analysis of autoantigen specificity are shown. The experiments provided representative data in terms of cloning efficiency (Table 6). The cloning efficiency in our experiments was between 19.12%-44.83%, which was slightly improved when compared with previous reports describing cloning of autoantigen-specific CD4+ T cells in healthy individuals [139]. The expansion of T cells clones from a single-cell is very challenging, even the novel strategy for generation of antigen-specific T cell clones only revealed 47%-61% cloning efficiency because it is influenced by various factors such as inhomogeneity of expansion potential of individual T cells, non-optimal expansion pre-condition, and possible cell exhaustion [140]. For specificity tests, five to eleven clones were randomly chosen from particular antigen and examined for antigen reactivity as measured by CD154 expression and subsequent IFN-γ production. Notably, we could demonstrate that several T cell clones were specific to the corresponding antigen as shown by CD154-expressing cells within the clones producing IFN-γ (Figure 18B). This was an important observation revealing TCR specificity of autoreactive CD4+ T cells against the autoantigen. Moreover, this data indicated that autoantigen-specific CD4+ T cells circulating in the periphery of healthy subjects are *bona-fide* autoreactive CD4+ T cells, and CD154+CD69+ CD4+ T cells detected in healthy individuals were not necessarily restricted to a background population, although their

frequency was nearly similar. However, we need to consider that not all clones were reactive to the antigens, except for *Aspergillus fumigatus*-specific and Histone-specific CD4+ T cell clones (Figure 18C and Table 6). As expected, *Aspergillus fumigatus*-specific CD4+ T cell clones showed high specificity probably because the population of CD154+CD69+ cells was clearly distinguishable from unstimulated control with higher mean fluorescence intensity for CD154 than those examined in the samples stimulated with autoantigen or neoantigen when assessed by flow cytometry. Whether or not the mean fluorescence intensity of T cell activation markers influences specificity of antigen recognition by CD4+ T cells is unclear. The results demonstrated that CD4+ T cells with undefined specificity were also sorted together with *bona-fide* autoreactive CD4+ T cells using our sorting strategy, questioning the degree of sensitivity of ARTE method when objecting isolation of autoreactive CD4+ T cells.

Another important issue to be discussed is that only a proportion of antigen-specific clones expressed CD154 and co-produced IFN-γ. This seems unexpected because it contradicts with the definition of a clone, where the cells should have the same TCR structure thus a homogenous antigen response was expected. However, the amount of antigen applied to re-stimulate the clone could be critical. It is possible that the clone could possess a higher TCR activation threshold that is different with their *ex vivo* threshold, thus higher antigen concentration than the initial stimulation dose should be considered when stimulating the clone. Nevertheless, our study was still convenient to distinguish the response of antigen-stimulated clones apart to the unstimulated clones and evaluate their antigen specificity. We will address and discuss these issues based on the data we have generated in this chapter.

Besides validation of antigen specificity of SLE-associated autoantigen-specific CD4+ T cells, successful cloning of *bona-fide* autoreactive CD4+ T cells opens several possibilities. First, it is possible to define the antigen dose required for activating autoreactive CD4+ T cells, facilitating the study of physiological function of these cells in terms of TCR avidity. Secondly, the TCR sequence of autoreactive CD4+ T cells can be identified and studying the clonal distribution of this particular cell population will be possible. Thirdly, TCR-transgenic autoantigen-specific CD4+ T cells and Treg can be generated by transducing TCR sequences via retroviral vectors enabling further characterization of pathogenic T cells and their regulatory counterparts. The method for this purpose has been established previously [141]. Fourth, development of tetramer technology to target T cells with specific TCR will provide important contribution to our understanding in human autoimmunity.

3.2. Specificity testing on antigen-specific CD4+ T cell lines

We have shown that the cloning of autoreactive CD4+ T cells through ARTE method via single-cell sorting of CD154+CD69+ cells revealed clones without specificity to the initial antigen, but not in the clones reactive to the recall-antigen *Aspergillus fumigatus*. In an independent experiment using PBMC from other healthy individuals, we could reproduce the result regarding high specificity of *Aspergillus fumigatus* clones (Figure 18C). Evidence of unspecific clones within autoreactive CD4+ T cell clones appeared probably because of possible TCR alteration through somatic mutation or physiological re-calibration of TCR thresholds to autoantigens, and also, it could be because of less-sensitivity of the ARTE method when applied to detect

autoreactive CD4+ T cells. In order to test the sensitivity of the ARTE method, we aimed to generate antigen-specific CD4+ T cell lines by sorting CD154+CD69+ cells after stimulation with antigen and subsequent procedure according to ARTE protocol.

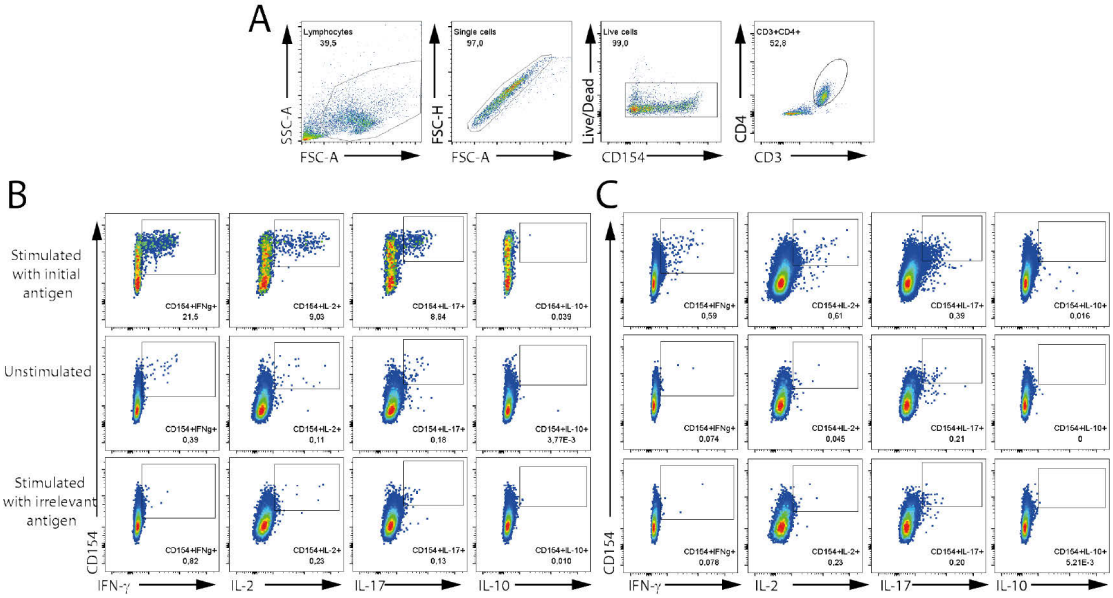


Figure 19: **Specificity testing on antigen-specific CD4+ T cell lines.** 2x10^7 PBMC from one healthy individual were stimulated with lupus pool, 1x10^7 cells were left unstimulated, and additionally 1x10^6 PBMC from the same individual were stimulated with *Aspergillus fumigatus* lysate. After stimulation, CD154+CD69+ CD4+ T cells were sorted and polyclonally expanded with PHA and IL-2 in the presence of irradiated feeder cells. **(A)** Expanded T cell lines were analysed for specificity. Sorting was performed by gating the lymphocytes, excluding the doublets and dead cells and gating CD3+CD4+ cells. Number indicates frequency of gated population within parent population. *Aspergillus fumigatus*-specific CD4+ T cell line **(B)** and lupus antigen-specific T cell line **(C)** were analysed for specificity. Cells were stimulated with initial antigen, (with *Aspergillus fumigatus* for *Aspergillus fumigatus*-specific CD4+ T cells line or with lupus pool for lupus-antigen specific CD4+ T cell line, upper row), left unstimulated (middle row), or cross-stimulated with irrelevant antigen (with lupus pool for *Aspergillus fumigatus*-specific CD4+ T cells line or with *Aspergillus fumigatus* for lupus-antigen specific CD4+ T cell line, lower row). As read-out for specificity, the frequency of CD154-expressing cells producing IFN-γ, IL-2, IL-17, and IL-10 was measured in all samples.

After stimulation of with antigen, 59 *Aspergillus fumigatus*-specific CD4+ T cells from 1x10^6 PBMC and 88 lupus antigen-specific CD4+ T cells from 2x10^7 PBMC were isolated using FACS sorter. *Aspergillus fumigatus*-specific and lupus antigen-specific CD4+ T cells were expanded *in vitro* using irradiated feeder cells, IL-2 and PHA. After two weeks, antigen-specific CD4+ T cell lines were generated. T cell lines were re-stimulated with the initial antigen, or cross-stimulated with irrelevant protein (*Aspergillus fumigatus*-specific CD4+ T cell line was stimulated with lupus pool and lupus antigen-specific CD4+ T cell line was stimulated with *Aspergillus fumigatus* lysate), or left unstimulated. These antigen-specific CD4+ T cell lines were analysed with flow cytometry by gating live CD3+CD4+ single cells (Figure 19A). To demonstrate T cell response to the antigen, the production of IFN-γ, IL-2, IL-17, and IL-10 was measured on CD154-expressing cells. *Aspergillus fumigatus*-specific CD4+ T cell line was responsive to stimulation with *Aspergillus fumigatus*, but not to lupus pool, as a proportion of CD154-expressing cells secreted IFN-γ, IL-2, IL-17, but less IL-10, after stimulation with *Aspergillus fumigatus* lysate (Figure 19B). Background population was detected in the unstimulated sample and sample

stimulated with irrelevant protein, possibly due to the presence of co-stimulator anti-CD28 antibody in the culture. Importantly, the frequency of total CD154+ cells in the sample stimulated with *Aspergillus fumigatus*, without considering cytokine production, were clearly increased when compared with the unstimulated sample as well as the sample stimulated with irrelevant protein (Table 7). This indicates that the capability of individual clones of responding to the same antigen is different. The frequency of single, double, and triple-cytokine producer within CD4+ T cells was also increased in the sample stimulated with initial antigen confirming the specificity of the *Aspergillus fumigatus*-specific CD4+ T cell line. The fact that more than 60% of the cells were specific to *Aspergillus fumigatus* upon restimulation implied that the ARTE method and sorting strategy for isolating *Aspergillus fumigatus*-specific CD4+ T cells were effective.

Table 7: **Antigen-specific CD4+ T cell lines.** The table shows the frequency of CD3+CD4+ cells expressing CD154, as well as CD3+CD4+ cells expressing CD154 and producing one cytokine (single-producer), two cytokines (double-producer), and three cytokines (triple-producer). *Asp*, lupus and no ag refer to *Aspergillus fumigatus*, lupus pool and unstimulated, respectively.

Subset (% of CD3+CD4+)	**Asp-specific CD4+ T cell line**			**Lupus-specific CD4+ T cell line**		
	+ Asp	+ No Ag	+ Lupus	+ Lupus	+ No Ag	+ Asp
CD154+	62.5	10.7	18.1	17.4	15.8	18.9
CD154+IFN-γ+	21.5	0.39	0.82	0.59	0.074	0.078
CD154+IL-2+	9.03	0.11	0.23	0.61	0.045	0.23
CD154+IL-17+	8.84	0.18	0.13	0.39	0.21	0.2
CD154+IL-10+	0.039	0.003	0.01	0.016	0	0.005
CD154+ IFN-γ+IL-2+	2.78	0.015	0.024	0.032	0	0
CD154+ IFN-γ+IL-17+	4.17	0.094	0.059	0.079	0.015	0
CD154+ IFN-γ+IL-2+ IL-17+	3.78	0.019	0.035	0.026	0.005	0.01

The frequency of CD154-expressing CD4+ T cells within lupus the antigen-specific CD4+ T cell line was almost similar in three conditions: simulation with initial antigen, stimulation with irrelevant antigen, and no stimulation (Figure 19C). Although it seemed that the lupus antigen-specific CD4+ T cell line failed to respond to the initial antigen, the frequency of CD154-expressing cells producing IFN-γ and IL-2 was clearly increased in the sample stimulated with initial antigen. Production of IL-17 within activated T cell was also observed but still within background frequency. The frequencies of double- and triple-producers were also elevated in the sample stimulated with lupus pool, providing more evidence of specificity of the T cell line to the corresponding antigen (Table 7). When considering cytokine producing cells as *bona-fide* lupus antigen-specific CD4+ T cells, only less than 1% of the cells were specific. The data highlighted less sensitivity of the procedure for isolating autoreactive CD4+ T cells, but not for isolating recall antigen-specific CD4+ T cells. Three possible explanations arise regarding this observation. Firstly, although no discrimination of naive and memory cells was applied (e.g. by CD45RA or CD45RO co-staining), recall antigen-specific CD4+ T cells were assumed to be memory cells, whereas lupus antigen-specific CD4+ T cells were probably naive autoreactive cells in healthy individuals. It could be possible that the ability to re-respond to the antigen

is different between the cells that originate from effector/memory population and the cells that originate from naive cell repertoire. It is in line with the fact that a secondary immune response is more enhanced than a primary immune response. Secondly, the dot plots showed preferential cytokine production in the lupus antigen-specific CD4+ T cell line when stimulated with lupus pool, but not the upregulation of CD154. This is in contrast with the observation on *Aspergillus fumigatus*-specific CD4+ T cell line when stimulated with *Aspergillus fumigatus*, where upregulation of CD154 and cytokine production was enhanced. The length of stimulation could probably explain the condition in lupus antigen-specific CD4+ T cell lines, that the production of cytokines could be an earlier event followed by upregulation of CD154. Thirdly, TCR threshold of autoreactive naive T cells are likely higher than non-autoreactive memory T cells, so that antigen-stimulation efficiency may be dose-dependent. To determine the affinity of a TCR to respond recall and autoantigens in healthy individuals, we aimed to determine the EC_{50} value of *Aspergillus fumigatus* and SLE-associated autoantigens.

3.3. Determination of autoantigen's EC_{50} value in healthy individuals

In order to describe the phenomenon regarding less sensitivity of autoreactive CD4+ T cell clones to their cognate autoantigen, we studied the functional heterogeneity observed among antigen-specific T cell clones derived from CD154+CD69+ CD4+ T cells of two to four healthy individuals. In our study, expanding T cell clones were analysed for antigen reactivity by re-stimulating the clones with titrated concentration of antigens. Upon clone restimulation, the dose-dependent frequency of CD154-expressing cells that co-produce IFN-γ was measured to calculate the amount of antigen that can promote a half-maximal T cell-response (EC_{50}). EC_{50} value of SLE-associated autoantigens and their difference with the value of a recall antigen in healthy subjects could support the identification of the avidities of T cell clones in non-diseased state and estimate their physiological function.

We could demonstrate that both in healthy individual and in patient with SLE, autoreactive CD4+ T cell clones reactive with their cognate autoantigen in dose-dependent manner. SmD1-specific CD4+ T cell clones were successfully generated from five healthy individuals and one patient with SLE and the titration of SmD1 concentration from 100nM to 10pM revealed a decreased frequency of CD154+IFN-γ+ cells in response to the stimulation (Figure 20A). Due to the technical challenge, we were only able to generate clones from one patient with active SLE with reactivity to *Aspergillus fumigatus* and SmD1. Nevertheless, the generation of clones from one SLE patient was still useful to compare the EC_{50} value of an autoantigen with recall antigen in diseased state, as well as to compare the EC_{50} value of those antigens in health and disease.

In total 95 clones derived from five healthy subjects were generated by ARTE method and single-cell sorting of CD154+CD69+ CD4+ T cells. 10 *Aspergillus fumigatus*-specific clones, 21 SmD1-specific clones, 19 RNP70-specific clones, 20 Histone-specific clones, 20 Ro-specific clones, and 15 La-specific clones were generated from two, four, three, four, three, and three donors, respectively. The clones were stimulated with 10-fold titrated concentration of antigen ranging from 100nM to 10pM, except for RNP70 ranging from 10nM to 1pM. The frequency of CD154+IFN-γ+ cells was measured in response to stimulation with antigen. We found a wide

avidity range among clones within the same individual and/or across different individuals (Figure 20B). The median, minimum and maximum EC_{50} values of all antigens among individuals were summarized (Table 8). The TCR avidity to SmD1, RNP70, Ro, and La seemed to have high degree of diversity. Interestingly, the median EC_{50} value of *Aspergillus fumigatus*, Histone, and La among the donors appeared to be identical (mean EC_{50} value of *Aspergillus fumigatus*, Histone, and La = 1.552±0.12, 0.893±0.24, and 3.029±0.31, respectively). Our results regarding clone specificity revealed that *Aspergillus fumigatus*-specific, Histone-specific, and La-specific clones isolated from healthy individuals were highly reactive to the initial antigens upon restimulation (five out of five specific clones for *Aspergillus fumigatus*, five out five specific clones for Histone, and five out of seven specific clones for La). Higher specificity could be explained by identical EC_{50} value among clones specific for those antigens, whereas inadequate specificity to other antigens such as SmD1, RNP70, and Ro may be a result from highly diverse EC_{50} values among the clones.

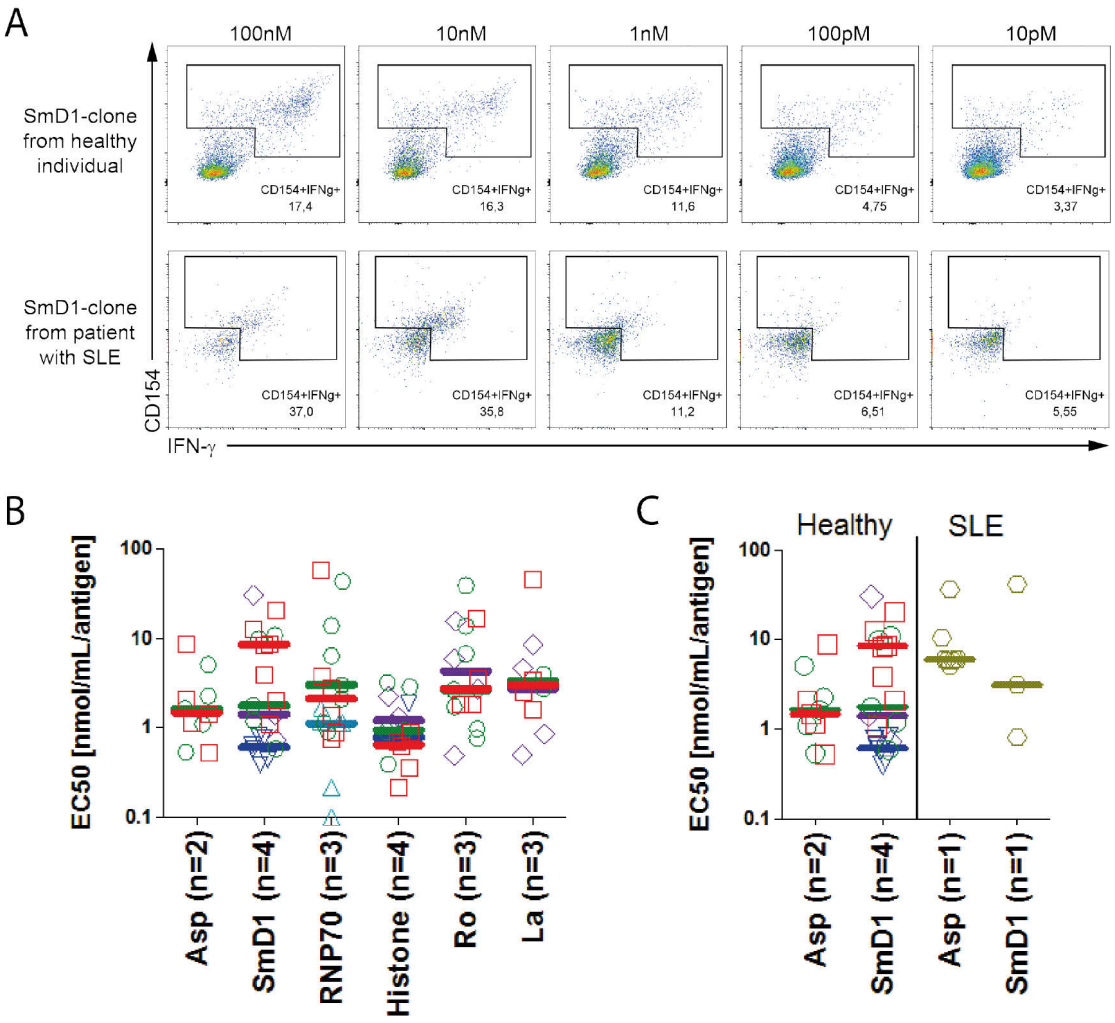


Figure 20: **Avidities of autoreactive CD4+ T cell clones to SLE-associated autoantigens.** In total 95 clones derived from five individuals were generated by ARTE method and single-cell sort of CD154+CD69+ CD4+ T cells after stimulation with antigen. The clones were reactive to *Aspergillus fumigatus* (Asp), SmD1, RNP70, Histone, Ro, and La. Nine clones with reactivity to Asp and SmD1 were also generated using the same approach from a patient with active SLE. The clones were re-stimulated with titrated concentrations of antigen to determine the EC_{50} values of corresponding antigens. **(A)** Representative SmD1-specific CD4+ clones derived from a healthy individual and a patient with active SLE were re-stimulated with SmD1 in antigen concentration ranging from 100nM

to 10pM. Frequency of CD154+IFN-γ+ cells are shown. **(B)** EC_{50} values of 95 clones with different reactivity were measured using non-linear regression by calculating antigen concentration that can induce half-maximal T cell response. **(C)** EC_{50} value of 31 clones reactive to Asp or SmD1 from in total four healthy individuals was determined and compared with EC_{50} value of nine clones that are also reactive to Asp and SmD1 from one patient with active SLE. Each symbol and colour represents one donor. Lines represent median EC_{50} value of each donor.

Table 8: **EC_{50} value of *Aspergillus fumigatus* (Asp) lysate and SLE-associated autoantigens.** The table shows the median, minimum and maximum EC_{50} value of Asp, SmD1, RNP70, Histone, Ro, and La derived from up to five healthy individuals, and median, minimum and maximum EC_{50} value of Asp and SmD1 derived from one patient with active SLE. Donor ID and total number of analysed clones were shown.

Antigen	Donor ID	# Clones	Median EC_{50} value [nmol/mL]	Minimum EC_{50} value [nmol/mL]	Maximum EC_{50} value [nmol/mL]
Asp	HC#1	5	1.468	0.537	8.895
	HC#2	5	1.636	0.531	5.079
SmD1	HC#1	7	8.463	1.101	20.80
	HC#2	5	1.764	0.579	10.80
	HC#3	6	0.607	0.388	0.814
	HC#4	3	1.407	0.722	30.20
RNP70	HC#1	6	2.122	0.752	57.64
	HC#2	7	3.009	0.907	43.23
	HC#3	6	1.118	0.100	1.663
Histone	HC#1	5	0.640	0.218	0.884
	HC#2	5	0.939	0.393	3.211
	HC#4	6	0.784	0.697	1.856
	HC#5	4	1.208	0.757	2.262
Ro	HC#1	4	2.726	1.845	17.13
	HC#2	7	2.641	0.763	38.90
	HC#5	4	4.267	0.494	15.51
La	HC#1	4	3.006	1.610	45.88
	HC#2	2	3.350	2.739	3.960
	HC#5	4	2.731	0.499	8.397
Asp	SLE#1	6	5.974	5.094	35.92
SmD1	SLE#2	3	3.100	0.801	71.47

When comparing the EC_{50} value of *Aspergillus fumigatus* as recall antigen with SLE-associated autoantigens, we found that in healthy individuals, autoantigens except Histone exhibited higher EC_{50} value than *Aspergillus fumigatus*, indicating lower TCR affinity to autoantigens when compared with foreign antigen. Low TCR affinity requires high dose of antigen to initiate TCR stimulation since otherwise the cells are anergic. This description is in line with the definition of autoreactive CD4+ T cells in healthy individuals that they exist but they are attributed with lower TCR affinity to self-antigen [142]. If this phenomenon holds true, the EC_{50} value of autoantigen in patients with SLE should be lower or similar to the EC_{50} value of recall antigens, which means that the cells have lower TCR avidity to respond to self-antigen and consequently, be activated. To demonstrate this, we generated clones specific for SmD1 and for *Aspergillus fumigatus* from a patient with active SLE. We were able to calculate the EC_{50} value of three SmD1-specific clones and six *Aspergillus fumigatus*-specific clones. When compared with the clone data from healthy subjects, the EC_{50} value of the recall antigen in the SLE patient was increased. EC_{50} value of SmD1

varied in three clones. Interestingly, when focusing on individual and comparing the EC_{50} value of recall antigen with autoantigen, the median EC_{50} value of autoantigen in healthy individuals was higher (or slightly higher) than recall antigen, but not in the patient with SLE, where the median EC_{50} value for autoantigen was lower than recall antigen, which would suggest higher TCR affinity in response to self-antigen in the diseased state (Figure 20C). However, our data could only highlight an evidence of possible physiological disease mechanisms rather than a comprehensive hypothesis-driven analysis because of the low clone number and low patient number involved in the study. Future studies aimed to answer TCR avidity to autoreactive CD4+ T cells could consider the staining of CD5 expression on the surface of T cells after stimulation with autoantigen that correlates with the degree of TCR-autoreactivity [124].

4. Conclusion

The concept of reactivity to autoantigens in physiological in contrast to pathological immune responses is not well understood. In this study, we directly examined the autoimmune response to autoantigens in several healthy individuals using a single-cell cloning method that relies on the isolation of autoantigen-specific CD4+ T cells. This chapter provides important information regarding the possible initiation mechanism of autoreactivity mediated by autoreactive CD4+ T cells. First, we could show that *bona-fide* autoreactive CD4+ T cells exist in the periphery of healthy individuals. We have analysed the specificity of these cells, however, a subpopulation within autoreactive CD4+ T cells in healthy individuals showed unresponsiveness to the self-antigen, leading to questions about whether they lost their specificity to the initial antigen and thus have a broad range of antigen cross-specificity [143], or whether they are anergic [144], or whether the ARTE method used to isolate autoantigen-specific CD4+ T cells were less sensitive so that the background cell population was inevitably included in the sorting strategy. Secondly, we could demonstrate an improved recovery of CD4+ T cells specific to a recall antigen, *Aspergillus fumigatus*, after sorting of CD154+CD69+ cells and *in vitro* T cell expansion, but decreased recovery of autoreactive CD4+ T cells using the same approach. The unresponsiveness of CD4+ T cell lines reactive to lupus pool was probably due to physiological factors such as TCR avidity. Thirdly, generation of autoreactive CD4+ T cell clones could provide an insight into their TCR avidity in non-diseased state, which was higher when compared with recall antigen. Finally, we speculated that autoreactive CD4+ T cells escaping the negative selection in the thymus have low-level affinity to autoantigen after receiving peripheral toleration (Figure 21). The TCR avidity among autoreactive CD4+ T cells in non-diseased state seems to be heterogeneous, so that the detection of such cells using a method that relies on targeting cell markers after T cell activation becomes less sensitive.

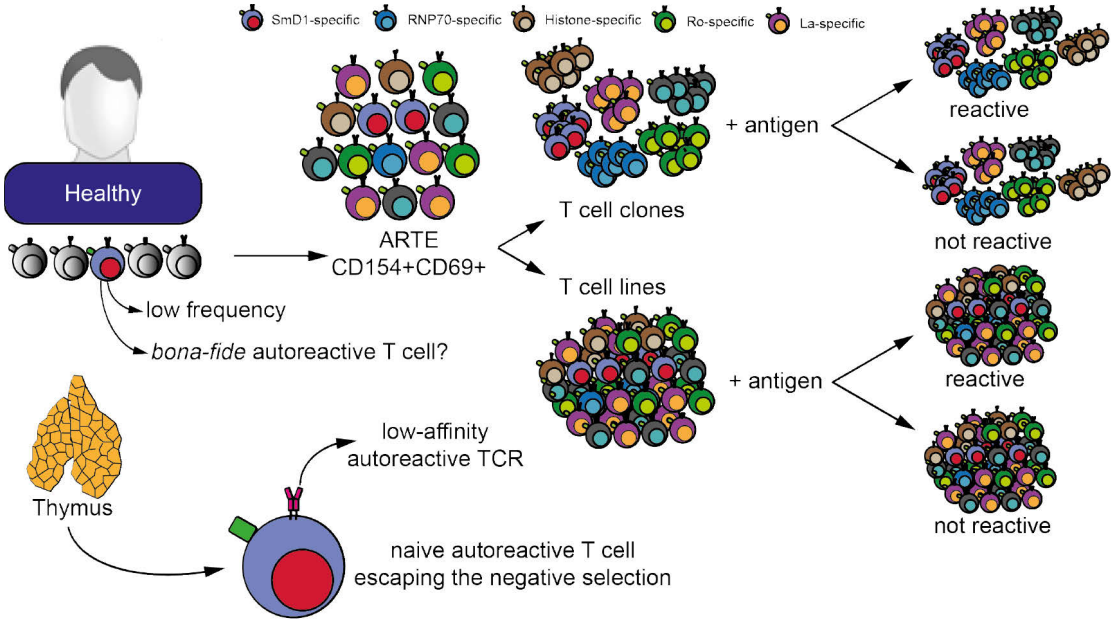


Figure 21: ***Bona-fide* autoreactive CD4+ T cells in healthy individuals.** CD4+ T cell reactive to SLE-associated autoantigens in healthy individuals are *bona-fide* autoreactive CD4+ T cells. They are specific to the autoantigen. Analysis of autoreactive CD4+ T cell clones and T cell lines revealed that these cells are low-affine to the autoantigens in healthy individuals. Probably, these cells are anergic naive cells that escape the negative selection in the thymus.

Chapter 6

Characterization of effector function of SLE-associated autoantigen-specific CD4+ T cells by cytokine production

Abstract

Although SLE-associated autoantigen-specific CD4+ T cells are expanded in patients with active SLE and their frequency in the periphery correlates with disease activity, the effector function responsible for their pathogenicity still remains poorly defined. Using *in vitro* antigenic stimulation combined with the ARTE method and intracellular staining of produced cytokines, we were able to demonstrate that SLE-associated autoantigen-specific CD4+ T cells produced significant levels of IFN-γ, IL-17, and IL-10, but less IL-4. When eliminating the background cell population, the frequencies of cytokine-expressing autoreactive CD4+ T cells were significantly increased in patients with active SLE but remained undetected in patients with inactive SLE and healthy individuals. We also found restricted positive correlation of the frequency of IFN-γ producing autoreactive CD4+ T cells with SLE disease activity, indicating possible pathogenic role of IFN-γ in SLE. Our results suggested that autoreactive circulating CD4+ T cells in SLE were mostly within the Th1 or non-conventional Th1 cell population that produce IL-17 as well as IL-10, rather than within the Th2 subset. This is the first extensive report about cytokine production by autoreactive CD4+ T cells in SLE supporting our findings in the expansion of autoreactive CD4+ T cells in the active phase of the disease. The results also lead to further discussions regarding acquisition of effector functions that modulate direct tissue inflammation and autoantibody production, as well as determine the degree of inflammation.

Keywords: *Cytokines, SLEDAI, Th cell-subsets, effector function*

Contents

1. Introduction

1.1. Background

CD4+ T cells are critical regulators of the immune system both in the host defence mechanism against invading pathogens and also in the pathogenesis of autoimmune diseases, depending on the type of antigens presented to and recognized by CD4+ T cells. Human CD4+ T cells, also known as helper T (Th) cells, have multiple subsets which can be functionally distinguished according to their cytokine producing capacity, the expression of characteristic lineage-defining transcription factors and the combination of the expression of homing receptors on the surface [20]. This information is useful to functionally dissect the complexity of human Th cell subsets i.e. the possibility to analyse effector function by observing cytokine production in response to stimulation with a high number of different antigens.

Th1, Th2, and Th17 are considered main subsets of differentiating CD4+ T cells with specific production of cytokines. Th1 cells are appreciated to produce IFN-γ in a mechanism to defeat intracellular pathogens like bacteria or viruses, while Th2 cells produce IL-4, IL-5, and IL-13 in response to attack extracellular parasites [22], but since they induce IgE production from B cells their role in allergies was also described [145]. Th2 cells have been initially reported to be the primary producer of IL-10 that can promote B cell proliferation [23, 24], but it was later found that all CD4+ T cells can produce IL-10 to limit the magnitude of an immune response, providing a wide possible role of IL-10 in promoting antibody production and supressing excessive immune response [25]. Th17 cells have been identified and characterized by their production of IL-17, IL-22, GM-CSF [26], and they are required for elimination of fungi and extracellular bacteria [27, 28]. Altogether, focusing on cytokine production capacity may help to discern the heterogeneity of CD4+ T cells and predict their effector function.

In autoimmunity, the effector function of CD4+ T cells is still poorly understood. All Th cell subsets are seen as causative agents in autoimmune responses. In the context of the autoimmune disease multiple sclerosis, myelin-reactive CD4+ T cells are reported to produce IFN-γ, IL-17, and GM-CSF in patients, but secrete more IL-10 in healthy individuals [133]. Whether or not Th cells in SLE exhibit similar cytokine production remains unknown. Since the production of autoantibody and B cell hyper-reactivity are hallmarks in SLE, Th2 cells are suggested to play a substantial role in the pathogenesis. Recently, U1-70-reactive CD4+ T cells are found to secrete IL-17 in patients with SLE [146], but no other further evidences are reported since then regarding the cytokine production of autoreactive CD4+ T cells in SLE.

1.2. Objectives

In this chapter, we sought to elucidate the effector function of SLE-associated autoreactive CD4+ T cells from patients with active SLE, inactive SLE, and healthy individuals, identifying key functional differences between these cohort groups. We used ARTE method to enrich SLE-associated autoreactive CD4+ T cells after stimulation with antigens combined with staining of intracellular cytokines that were produced upon antigenic stimulation.

2. Materials and methods.

In the experiments presented in this chapter, the following methods were performed and they were described in the previous chapters: blood donors and isolation of peripheral blood mononuclear cells (chapter 2, section 2.1.), determination of cell numbers (chapter 2, section 2.2. and chapter 4, section 2.2.), antigen pool preparation (chapter 4, section 2.1.), antigen-reactive T cell enrichment (chapter 4, section 2.4.), antibody staining, cell fixation and permeabilization, and flow cytometry analysis (chapter 4, section 2.5.), antibody staining of intracellular compartment (chapter 4, section 2.6., with additional information, re-described as a new section in this chapter), determination of SLEDAI (chapter 4, section 2.6.), and statistics (chapter 4, section 2.7.).

2.1. Antibody staining of the intracellular compartment

The principal of antibody staining was described in previous chapters. In the experiments presented in this chapter, produced cytokines were stained using fluorochrome-conjugated antibodies and analysed on a flow cytometer. After performing the ARTE method to enrich CD154-expressing cells and prior to intracellular staining, cells were fixated with 2% (v/v) paraformaldehyde for 15 minutes at room temperature and permeabilized with BD FACS^{TM} Permeabilizing Solution 2 (BD Biosciences, San Jose, USA) for 10 minutes at room temperature in the dark, according to the protocols described in the chapter 4, section 2.5. Intracellular staining was performed in 96-well plate (Greiner Bio-One AG, Kremsmünster, Austria). 60µL of master mix was prepared from following monoclonal antibodies depending on the experiment: antihuman-CD154-BV421 (clone 24-31, Biolegend Inc., San Diego, USA), antihuman-CD154-PE (clone 24-31, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), antihuman-IFN-γ-APC (clone 4S.B3, Biolegend Inc.), antihuman-IFN-γ-PacificBlue (clone 4S.B3, self-prepared at DRFZ), antihuman-IL-4-FITC (clone BS4, Life Technologies Europe BV, Bleiswijk, The Netherlands), antihuman-IL-10-BV421 (clone JES3-9D7, Biolegend Inc.), antihuman-IL-10-PECy7 (clone JES3-9D7, Biolegend Inc.), antihuman-IL-17-BV786 (clone N49-653, BD Biosciences), and antihuman-IL-17-PerCPCy5.5 (clone eBio64DEC17, Life Technologies Europe BV) antibodies. Cells were incubated with mastermix at 4°C for 30 minutes in the dark. After intracellular staining, cells were washed with PBS/BSA and resuspended with PBS/BSA for acquisition on a flow cytometer.

3. Results and discussion

Cytokines are considered as “language tool” among immune cells to interact and communicate with each other, including performing protective and regulative mechanisms against invading pathogens. Both innate and adaptive immune cells produce cytokines and orchestrate complex communication i.e. to stimulate or inhibit the function of other cells, as well as to attack infected cells or suppress unwanted immune responses. In autoimmunity, when self-particles are seen as pathogenic agents, cytokines can act as crucial mediators of disease pathogenesis (pro-inflammatory cytokines) but also as promising regulatory instrument to suppress the

pathogenesis (anti-inflammatory cytokines). Patients with SLE display highly diverse clinical manifestations including degree and diversity of cytokine production, with type I IFN (IFN-α and IFN-β) as the most prominent type of pro-inflammatory cytokine in SLE, also used as disease biomarker [147]. However, type I IFN signature is associated with innate immune responses being secreted mainly by macrophages and dendritic cells, whereas cytokine production as a result of adaptive immune response (e.g. by T cells) in SLE is still poorly understood.

3.1. Cytokine production of autoreactive CD4+ T cells

Enrichment of CD154-expressing cells enables further characterization of antigen-specific CD4+ T cells. We have determined the number of autoreactive CD4+ T cells in health and disease and demonstrated their antigen specificity. Autoreactive CD4+ T cells are expanded in active SLE and correlate with disease activity. Abundance of autoreactive CD4+ T cells displays an indication of pathogenesis, but the quantity in the periphery alone is not conclusive for pathogenicity without having characterized the effector function of these cells. Thus, we made use of this method to dissect functional features of autoreactive CD4+ T cells by assessing their cytokine production. We selected IFN-γ, IL-17, and IL-4 as representative cytokines that illustrate distinct Th cell responses, which are Th1, Th17, and Th2, respectively. In addition, we included IL-10 in our panel that can be produced by all virtually Th cell subsets.

A mixture of nuclear antigens specific for SLE (SmD1, RNP70, Histone, Ro, and La) was able to induce different type of polarized T cells to secrete different cytokine. After antigenic stimulation, cytokines were produced solely by CD154+CD4+ T cells. Cytokine production by this cell population highlights the antigenic response and re-confirms the specificity of CD154-expressing antigen-specific CD4+ T cells. By considering the representative dot plots from a patient with active SLE, IFN-γ was highly produced by autoreactive CD4+ T cells followed by IL-10 and IL-17, whereas IL-4 production was not detected (Figure 22A). The enrichment method allows calculation of the frequency of cytokine-producing cells based on the absolute number of cytokine-producing CD154-expressing CD4+ T cells in a defined input cell number. We analysed the cytokine production of autoreactive CD4+ T cells in healthy individuals (n=6), patients with inactive SLE (n=6), and patients with active SLE (n=9, except for CD154+IL-4 assessment, n=5) expressed in one million of cells. In parallel, we also analysed the ability of non-antigen-specific CD154-expressing CD4+ T cells in producing the cytokines indicated as background population (Figure 22B). In order to obtain absolute numbers of cytokine-producing autoreactive CD4+ T cells, the number of the background population was subtracted (Figure 22C).

IFN-γ-producing CD154+ CD4+ T cells in unstimulated samples were observed in all cohort groups (median number: healthy controls = 18.17 cells; inactive SLE = 2.97; active SLE = 15.67 cells/million cells). The number of autoreactive CD4+ T cells producing IFN-γ ranges between 0 to 308.5 cells/million cells in the individuals that participated in our study (median number: healthy controls = 35.87; inactive SLE = 5.55; active SLE = 67.10 cells/million cells). The frequency of IFN-γ-producing autoreactive CD4+ T cells was not significant in healthy controls and inactive SLE when compared with the background population, but significantly differed in active SLE (healthy control, P = 1; inactive SLE, P = 0.1875; active SLE, P = 0.0039 by

Wilcoxon signed-rank test). Significant difference of IFN-γ production in stimulated versus unstimulated samples emphasized prominent IFN-γ production by autoreactive CD4+ T cells in patients with active SLE. By subtracting the background population, the absolute number of IFN-γ-producing autoreactive CD4+ T cells was between 0 to 282.2 cells/million cells (median number: healthy controls = 0.42; inactive SLE = 2.09; active SLE = 57.95 cells/million cells). The frequency of this cell population was significantly increased in patients with active SLE when compared with healthy controls and inactive SLE (active SLE vs. healthy control, $P = 0.0026$; active SLE vs. inactive SLE, $P = 0.0018$; healthy control vs. inactive SLE, $P = 0.9338$, by Mann-Whitney test). The minimal frequency of IFN-γ-producing autoreactive CD4+ T cells in active SLE is 20.27 cells/million cells, whereas in other cohort groups, there were individuals with no IFN-γ-production at all within CD154-expressing CD4+ T cells.

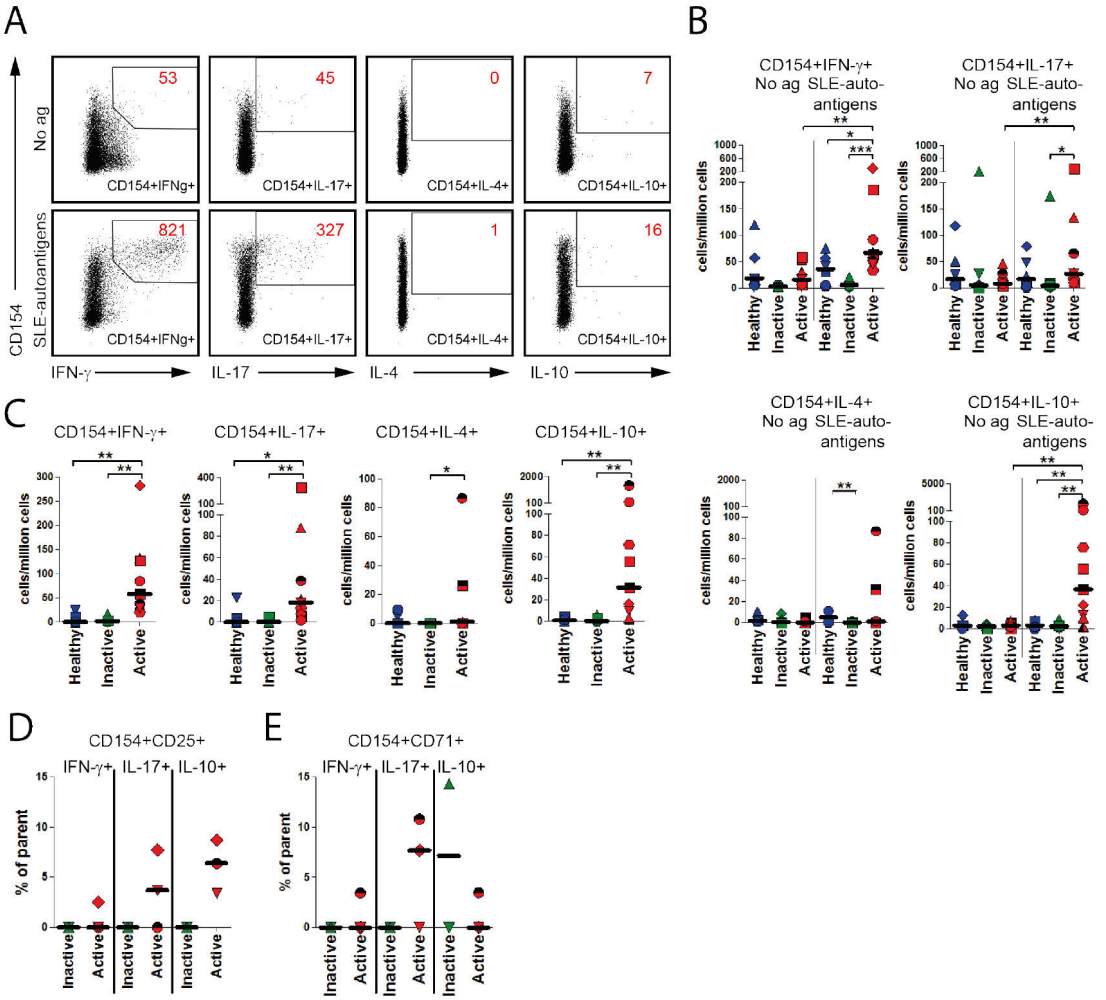


Figure 22: **Cytokine production of autoreactive CD4+ T cells by ARTE.** Cytokine production of CD4+ T cells reactive to SLE-autoantigens were assessed by enrichment of CD154-expressing cells after *in vitro* stimulation with subsequent staining of intracellular cytokines. (**A**) Representative dot plots show post enrichment-gating strategy to quantify the number of autoreactive CD4+ T cells producing IFN-γ, IL-17, IL-4, and IL-10 after *in vitro* culture in absence (no ag, upper row) or in presence of autoantigens (SLE-autoantigens, lower row). Red number indicates counted events in flow cytometry. (**B**) Precursor frequency of cytokine-producing CD154+ CD4+ T cells with (SLE autoantigens) or without (no ag) autoantigens determined in healthy controls (healthy, *n*=6), patients with inactive SLE (inactive, *n*=6) and patients with active SLE (active, *n*=9, except for CD154+IL-4+ assessment, *n*=5). (**C**) Precursor frequency of cytokine-producing CD154+ CD4+ T cells after subtraction of background. Frequency of CD154+CD25+ (**D**) and CD154+CD71+ (**E**) CD4+ T cells

producing IFN-γ, IL-17, and IL-10 in patients with inactive (*n*=2) and active (*n*=3) SLE. Each symbol illustrates a distinct individual. Black line represents median value.

Significant preferential production of IFN-γ by autoreactive CD4+ T cells in response to SLE-associated autoantigens suggests a quantitative increase of T cells exhibiting an activated phenotype in active disease. In another autoimmune disease multiple sclerosis (MS) it was described that the production of IFN-γ is pathogenic [133] and IFN-γ production is increased in MS patients suffering from a relapse compared to heathy controls or patients in remission [148]. In SLE, significant role of IFN-γ in disease pathogenesis is unclear because the role of adaptive immune cells in the disease is still rarely investigated. Besides the production of anti-nuclear protein antibodies including anti-dsDNA antibodies, high levels of type I IFN (IFN-α and IFN-β) in the blood is highly correlated to the disease activity. Some investigations underlined the importance of low-dose type I IFN in priming cells to produce type II IFN (IFN-γ) [149]. In parallel, IFN-γ is able to enhance Toll-like receptors signalling to promote type I IFN secretion [150]. Using sophisticated modular transcriptional analysis, Chiche *et al*. recently emphasized that not only IFN-α, but also IFN-β and IFN-γ contribute to the progression of SLE [151]. In the context of anti-viral immunity, it has been described that IFN-γ is responsible for the effector phase of an immune response, whereas type I IFN are critical in the earlier phase [152]. Elevated production of IFN-γ has been found in T cells of SLE patients, but less in healthy controls, after polyclonal stimulation with anti-CD3 and anti-CD28 antibody [153]. The investigation also suggested the contribution of IFN-γ to the immunopathogenesis by promoting B cell activation and maturation. Taken the information together, our study provides evidence that IFN-γ production is a result of an antigen-specific CD4+ T cell response, underlining the critical role of autoreactive CD4+ T cells in active SLE. Since IFN-γ production is correlated with Th1 responses in SLE [154], it could be possible that autoreactive CD4+ T cells in SLE are skewed towards the Th1 phenotype.

We next analysed IL-17 production in our study groups by observing first the basal cytokine production. Up to 213.3 cells/million cells produced IL-17 in the absence of antigens (median number: healthy controls = 16.30; inactive SLE = 5.19; active SLE = 7.85 cells/million cells). In the presence of antigens, IL-17-producing CD154+ CD4+ T cells ranges from 0.86 cells to 289.5 cells/million cells (median number: healthy controls = 16.59; inactive SLE = 4.17; active SLE = 26.78 cells/million cells). Only active SLE patients showed significant difference of the frequency of IL-17-producing CD154+ CD4+ T cells in the stimulated samples versus unstimulated samples (healthy controls, $P = 0.4375$; inactive SLE, $P = 0.2188$; active SLE, $P = 0.0039$ by Wilcoxon signed-rank test). These data indicate that the production of IL-17 was preferably induced after antigen stimulation in patients with active SLE. The number of IL-17-producing autoreactive CD4+ T cells in patients with active SLE was slightly higher than healthy controls ($P = 0.3277$, by Mann-Whitney test), but significantly higher than the frequency in inactive SLE patients ($P = 0.0256$, by Mann-Whitney test), whereas the frequency in healthy controls versus inactive SLE was not significantly different ($P = 0.3095$, by Mann-Whitney test). However, by subtracting the background cell population, the frequency of IL-17-producing autoreactive CD4+ T cells was noticeably increased in active SLE (median number: healthy controls = 0; inactive SLE = 0; active SLE = 18.10 cells/million cells). Significance tests also revealed preferential IL-17 production in active SLE (active

SLE vs. healthy controls, $P = 0.0204$; active SLE vs. inactive SLE, $P = 0.0024$; inactive SLE vs. healthy controls, $P = 0.9241$, by Mann-Whitney test). These data indicate that IL-17 is also produced by autoreactive CD4+ T cells upon antigenic stimulation in active SLE.

In the last years, IL-17-producing cells have moved into the focus of research in autoimmunity. IL-17 is reported to contribute to tissue inflammation and organ failure in autoimmune diseases by inducing chronic inflammation [155]. In SLE, a pathogenic role of IL-17 was described in a mouse model, where IL-17-deficient mice were more protective from lupus-like manifestations such as development of autoantibodies and glomerulonephritis, than their wild-type counterparts [156]. Patients with SLE have increased serum levels of IL-17 and enhanced frequency of IL-17-producing cells compared with healthy controls [157]. Considering the elevated levels of IL-17 in the serum, several studies have also reported IL-17-producing CD4+ T cells in PBMC of SLE patients when stimulated with PMA/Ionomycin [158]. However, it was not clear which type of CD4+ T cells produce IL-17. To date, only one study showed IL-17 production by U1-70-reactive Th17 cells in SLE when analysed by tetramer technology [146]. Our study here revealed that the inflammatory status of active SLE patients is characterized by production of IL-17 by autoreactive CD4+ T cells. It needs to be defined whether these cells represent Th17 phenotype, since neither staining for RORγt, a transcription factor to Th17 cells, nor staining of other Th17-associated cytokines such as IL-22 was performed. Furthermore, our data also show preferential production of IFN-γ by autoreactive CD4+ T cells in the active course of the disease. Th17 cells are known for their plasticity with the ability to switch completely from IL-17-producing Th17 cells to IFN-γ-producing Th1 cells in chronic inflammation [159]. In our study, autoreactive CD4+ T cells producing IFN-γ did not co-produce IL-17 and vice versa, unlike our observation to the response of CD4+ T cells reactive to *Candida albicans* MP65, where IFN-γ+IL-17+ double producers were identified (data not shown). It is unclear whether IFN-γ-producers and IL-17-producers within autoreactive CD4+ T cells originate from different cell types with different antigen specificity or if they come from the same precursor undergoing different fate and function during a chronic immune response. To answer this, co-staining with CD161 could be useful to distinguish Th17 cells from conventional Th1 cells [160]. Production of IL-17 by autoreactive CD4+ T cells could suggest a Th17 phenotype, but that alone it is not sufficient due to heterogeneity and plasticity of Th17 cell population by definition. Many studies proposed a pathogenic role of IL-17 in SLE such as promoting autoantibody production and direct organ inflammation [161], but since Th17 cells are plastic in the chronic inflammation and they have the ability to transdifferentiate to e.g. Treg and vice versa, Th17 cells can also be protective and might be required for maintaining immune homeostasis [162, 163].

We then focused on the production of IL-4 by autoreactive CD4+ T cells in SLE. First, we measured basal production of IL-4 by CD154-expressing CD4+ T cells in absence of antigens. The frequency of IL-4-producing CD154+ CD4+ T cells was very low in the absence of antigens (median number: healthy controls = 2.10; inactive SLE = 0.52; active SLE = 0 cells/million cells), and the same was true in the presence of antigens (median number: healthy controls = 4.88; inactive SLE = 0; active SLE = 1.28 cells/million cells). Although detectable, extremely low median cell numbers were measured in one million cells indicate no preferential IL-4 production in SLE. When the frequency of IL-4-producing cells in response to stimulation with antigens

was compared with background frequency, no significant difference was observed (healthy controls, $P = 1$; inactive SLE, $P = 0.5$; active SLE, $P = 0.1250$, by Wilcoxon signed-rank test). Among three groups, only the number of IL-4-producing cells in healthy individuals was significant when compared with inactive SLE patients (active SLE vs. healthy controls, $P = 0.9307$; active SLE vs. inactive SLE, $P = 0.0732$; healthy controls vs. inactive SLE, $P = 0.0062$, by Mann-Whitney test). These data provide an open question, whether IL-4 production was initiated due to antigenic stimulation or due to continuous IL-4 production by immune cells. By subtracting the background population, absolute number of IL-4-producing autoreactive CD4+ T cells could be calculated (median number: healthy controls = 0; inactive SLE = 0; active SLE = 1.08 cells/million cells). Only two out of five patients with active SLE exhibited increased frequency of IL-4-producing cells with more than 20 cells/million cells. Although the frequency of IL-4-producing cells in active SLE patients was significantly higher when compared with inactive SLE patients (active SLE vs. healthy controls, $P = 0.2133$; active SLE vs. inactive SLE, $P = 0.0465$; healthy controls vs. inactive SLE, $P = 0.4620$, by Mann-Whitney test), the frequency was still extremely low, thus it was not sufficient to claim IL-4 production by peripheral autoreactive CD4+ T cells as a pivotal characteristics of progressive SLE.

IL-4, along with IL-5 and IL-13 are typical signature cytokine secreted by Th2 cells upon activation to fight extracellular parasites. Th2 cells are also appreciated for their ability to induce IgE antibody production from B cells, thus play a critical role in humoral immunity. Many studies have focused on the B-cell stimulatory and Th2 promoting features of IL-4 in the development of autoantibodies and autoantibody-mediated diseases such as SLE, but the studies have turned out with conflicting results whether IL-4 could enhance autoantibody production or could contradictory protect against autoimmunity [164]. Unlike IFN-γ, our results here suggest minimal contribution of IL-4 in SLE, supporting previous report about decrease of IL-4- but increase of IFN-γ-producing CD4+ T cells in patients with active SLE [165]. Uniquely, Th2 cells do not produce IFN-γ, and both IFN-γ and IL-4 were shown to exhibit inhibitory features to the differentiation of Th1 or Th2 cell population to the opposite differentiation lineage, in which that exclusive capacity to produce either IFN-γ or IL-4 is epigenetically imprinted [166]. Although it has been initially shown that human Th1 clones can acquire IL-4-producing capacities upon TCR stimulation without diminishing IFN-γ-production [138], it was later found that CD4+ T cell clones with the same antigen specificity can actually give rise to multiple helper cell fates [167].

The frequency of IL-10-producing autoreactive CD4+ T cells was also of great interest. Firstly, we measured the production of IL-10 by CD154-expressing CD4+ T cells in absence of antigens. The basal frequency of IL-10-producing CD154+ CD4+ T cells was almost similar when compared across our three cohort groups (median number: healthy controls = 2.76, inactive SLE = 2.31, active SLE = 3.08 cells/million cells). But when PBMC were stimulated with antigens, different frequencies of IL-10-producing autoreactive CD4+ T cells were observed between 0 to 1,370 cells/million cells, with the most production in the patients with active SLE (median number: healthy controls = 3.17, inactive SLE = 2.21, active SLE = 36.86 cells/million cells). These results indicate that IL-10 was produced as a response upon antigen-specific stimulation. The stimulation index illustrated by IL-10 production was clearly increased in the patients with active SLE (healthy controls, $P = 1$; inactive SLE, $P =$

0.8125; active SLE, $P = 0.0039$, by Wilcoxon signed-rank test), and the production of IL-10 in active SLE is superior among healthy individuals and inactive SLE (active SLE vs. healthy controls, $P = 0.0048$; active SLE vs. inactive SLE, $P = 0.0028$; inactive SLE vs. healthy controls, $P = 0.7483$, by Mann-Whitney test). After subtraction of the background, the number of IL-10-producing autoreactive CD4+ T cells in one million cells was almost absent in healthy individuals and patients with inactive SLE, but remained high in patients with active SLE (median number: healthy controls = 0.65, inactive SLE = 0, active SLE = 31.62 cells/million cells). Thus the frequency of IL-10-producing autoreactive CD4+ T cells was significantly increased in patients with active SLE (active SLE vs. healthy controls, $P = 0.0038$; active SLE vs. inactive SLE, $P = 0.0052$; inactive SLE vs. healthy controls, $P = 0.9289$, by Mann-Whitney test).

IL-10 can be produced not only by all CD4+ T cell subsets, but to a wide range of innate and adaptive immune cells such as mast cells, eosinophils, macrophages, dendritic cells, B cells, regulatory T cells and CD8+ T cells [168]. IL-10 is a multifunctional cytokine with immune inhibitory properties, and it plays a functional role in promoting B cell growth and differentiation. There is now compelling evidence that immune cells can acquire regulatory function in parallel with effector function upon chronic inflammation as shown in the Th1-associated infectious intracellular parasite model, where the main producer of host-protective IL-10 are IFN-γ-producing Th1 cells [169]. In the context of the autoimmune disease SLE, IL-10 is elevated in the serum and tissues of patients with SLE and its titre correlates with disease activity [170]. It has also been reported that CD4+ T cells of patients with SLE produce elevated amount of IL-10 [171]. We show here that IL-10 was produced by CD4+ T cells upon stimulation with autoantigens and the frequency was increased in the patients with active SLE. This observation raises two possible functions of IL-10-producing autoreactive CD4+ T cells. First, production of IL-10 exclusively in the stage of disease progression highlights the self-limiting mechanism of IL-10 in chronic inflammation as previously shown. Remarkably, IFN-γ was also produced by autoreactive CD4+ T cells in our study indicating the phenotype of protective CD4+ type 1 T regulatory (Tr1) cells. Nevertheless, additional characterization is necessary to conclude that IL-10-producing autoreactive CD4+ T cells shown here are Tr1 cells e.g. by phenotyping the cells by CD49b and LAG-3 expression [34]. Secondly, due to IL-10 function as mediator for B cell growth, survival, differentiation, proliferation and antibody production, IL-10 production could contribute to disease pathogenesis of SLE. Production of autoantibodies as a result of B cell hyperreactivity in SLE may be mediated through high production of IL-10 by autoreactive CD4+ T cells. Although IL-10 has been proposed as a potential biomarker predictive for disease activity in SLE [172], depleting IL-10 as therapeutic approach may result in loss of immune control despite effective restriction of B cell response thus exacerbating the disease.

Chronic inflammation reflected in patients with active SLE is characterized by *in vivo* activated CD4+ T cells. We sought to predict which cytokine-producing autoreactive CD4+ T cells acquire a recently-activated phenotype. We used expression of surface marker CD25 and CD71 combined with CD154 to detect *in vivo*-activated autoreactive cells. Within CD154+CD25+ (Figure 22D) and CD154+CD71+ (Figure 22E) CD4+ T cells of two inactive and three active SLE patients, we determined the frequency of IFN-γ–, IL-17-, and IL-10-producing cells. By observing the

frequencies, our results provided a descriptive analysis that IL-17- and IL-10-, but not IFN-γ-producing cells, could be activated cells *in vivo* circulating in the periphery. *In vivo*-activated IFN-γ-producing cells were not detected in the periphery but this cell subset occurred at high frequencies, indicating that IFN-γ-producing autoreactive CD4+ T cells acquire effector function in the inflamed organs to mediate local tissue inflammation. A subset of IL-17- and IL-10-producing autoreactive CD4+ T cells was recently activated *in vivo*, emphasizing their role in the CD4+ T cell-mediated pathogenesis in SLE.

3.2. Correlation of cytokine production with disease activity

Production of the cytokines by autoreactive CD4+ T cells was identified mostly in patients with active SLE when compared with healthy individuals and patients with inactive SLE. Cytokines can be secreted by various types of immune cells in the context of the autoimmune disease SLE, but our results highlight cytokine production by CD4+ T cells as a response of antigen-specific activation. We then asked whether cytokine production by autoreactive CD4+ T cells is characteristic for disease progression. Thus, we used SLEDAI information from each individual to obtain correlations between the frequencies of cytokine-producing autoreactive CD4+ T cells and disease activity.

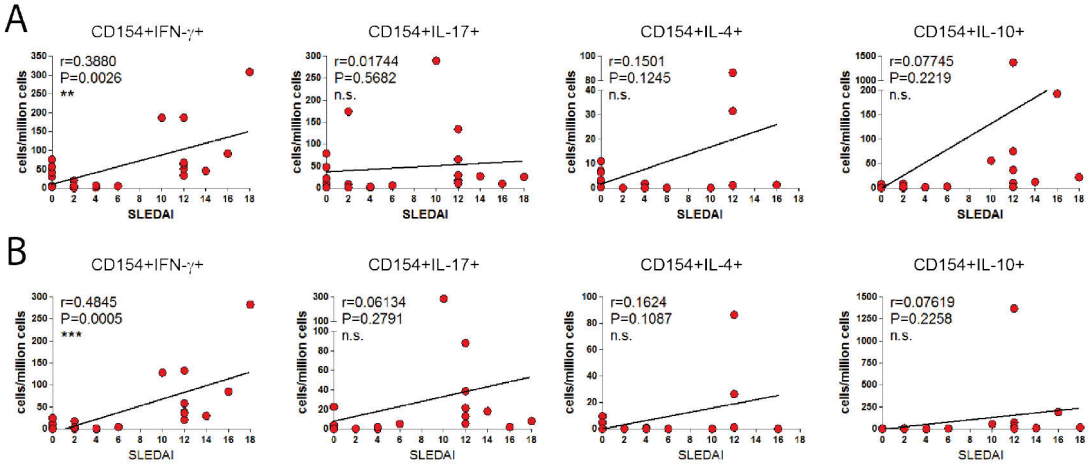


Figure 23: **Correlation of cytokine-producing autoreactive CD4+ T cells with disease activity.** The frequencies of IFN-γ- (*n*=21), IL-17- (*n*=21), IL-4- (*n*=17), IL-10 (*n*=21)- producing autoreactive CD4+ T cells with (**A**) and without (**B**) background population were compared with SLE disease activity score. R indicates Pearson's correlation coefficient. Line represents linear regression determined using Pearson's correlation coefficient.

When comparing the frequencies of cytokine-producing cells with SLEDAI score, we found that only IFN-γ-production by autoreactive CD4+ T cells showed significant positive correlation with the disease progression, whereas IL-17-, IL-4-, and IL-10-production demonstrated no correlation with the disease activity (Figure 23A). The correlation was improved after subtraction of basal cytokine production (Figure 23B), and all cytokine production by autoreactive CD4+ T cells showed a tendency of positive correlation. Our data imply that major pathogenic function of autoreactive CD4+ T cells in SLE could be mediated by secretion of IFN-γ. It has been discussed that patients with active SLE exhibit elevated IL-17 and IL-10 levels in the serum and blood and it correlates with disease activity. Using our data, although the frequencies of IL-17- and IL-10-producing autoreactive CD4+ T cells were increased in patients

with active SLE, we suggest that autoreactive CD4+ T cells may not be primary producers for IL-17 and IL-10 in mediating disease progression. It is of great interest to elucidate the function of IL-17 and IL-10 when produced by autoreactive CD4+ T cells in the pathogenesis, whether their function is different when produced by other immune cells. It could be possible that IL-17 and IL-10 are produced as instruments of self-limitation during chronic inflammation rather than as a pathogenic mediator.

4. Conclusion

Effector function in CD4+ T cells is mediated by three main cell subsets: Th1, Th17, and Th2. These distinct CD4+ T cell populations have unique roles in both host defence against invading pathogens and in immunopathology. Th1, Th17, and Th2 were initially defined on the basis of their cytokine production of IFN-γ, IL-17, or IL-4, respectively. IL-10 was originally described as a cytokine for Th2, but later it was found to be produced in all CD4+ T cell subsets. SLE-associated autoreactive CD4+ T cells produce IFN-γ, IL-17, and IL-10. The frequencies of cytokine-producing autoreactive CD4+ T cells were significantly higher in patients with active SLE when compared with healthy individuals and patients with inactive SLE. Further analysis revealed that a population of IL-17- and IL-10-producing autoreactive CD4+ T cells in the periphery was probably recently-activated cells. Furthermore, we demonstrated that the frequency of IFN-γ-producing CD4+ T cells significantly correlated with SLE disease activity. IFN-γ may support the enhancement of TLR-signalling on innate immune cells that leads to the production of type I IFN. On the other hand, type I IFN can conversely promote IFN-γ production by autoreactive CD4+ T cells. Cytokines produced by autoreactive CD4+ T cells may potentially play a dual role in the autoimmunity of SLE. For instance, high production of IL-10 could ameliorate the inflammation through its suppressive function, but on the other hand could modulate B cell hyper-reactivity through its stimulatory function. Thus, dissecting the function of cytokines in homeostasis becomes one interesting research focus for SLE. Altogether, the results imply effector function of autoreactive CD4+ T cells through cytokine production in SLE i.e. mediating direct tissue inflammation, modulating interaction between innate and adaptive immune cells, promoting autoantibody production, but also self-limiting chronic inflammation. Although not causal, less IL-4 production excludes the possibility that autoreactive CD4+ T cells circulating in the periphery were Th2 cells, but SLE-associated autoantigen-specific Th2 cells may be recruited for another effector function such as promoting autoantibody production in the secondary lymphoid organs thus they are poorly detectable in the peripheral blood. High IFN-γ-production by autoreactive CD4+ T cells indicates that the cells are skewed towards the Th1 subset. Since IL-17 and IL-10 was also produced by autoreactive CD4+ T cells, we propose that in SLE, Th1 and non-conventional Th1 subsets such as Th1/Th17 and Tr1 cells are dominating in the active phase of the disease. Diversity of cytokine production by autoreactive CD4+ T cells could be due to interclonal and intraclonal functional heterogeneity of the human T cell response against SLE-associated autoantigens (Figure 24). Finally, we report here for the first time that autoreactive CD4+ T cells in SLE are not only expanded in terms of their frequency, but also functionally contribute to the disease pathogenesis through their effector functions.

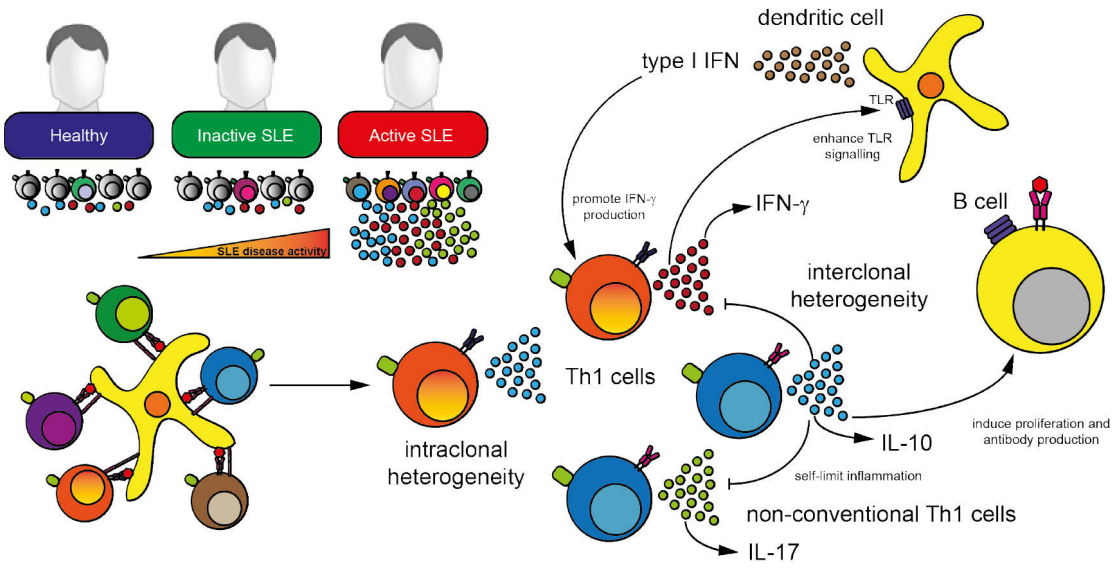


Figure 24: **Cytokine production by autoreactive CD4+ T cells in SLE.** Autoreactive CD4+ T cells are not only expanded in active SLE, they also produce effector cytokines such as mainly IFN-γ, IL-17, and IL-10, but less IL-4. The production of IFN-γ by autoreactive CD4+ T cells correlates with disease activity. Variation of cytokine production is due to intraclonal and interclonal heterogeneity. It means that cells with identical specificity within a clone or among different clones have the capacity to produce different cytokines. Autoreactive CD4+ T cells in active SLE are skewed towards Th1 or non-conventional Th1 subset. IFN-γ production can induce TLR signalling on innate immune cells that leads to the production of type I IFN. In a feedback-loop mechanism, type I IFN can conversely induce IFN-γ production by autoreactive CD4+ T cells. IL-10 production by autoreactive CD4+ T cells in active disease is a consequence of chronic inflammation and acts as self-limiting mediator. But IL-10 can also promote B cell proliferation, differentiation, and autoantibody production, which supports disease pathogenesis.

Chapter 7

Development of autoantibodies in correlation with SLE-associated autoantigen-specific CD4+ T cells

Abstract

SLE is characterized by loss of tolerance towards B cells resulting in B cell hyper-reactivity and development of autoantibodies directed to nuclear antigens such as anti-dsDNA, anti-ANA, anti-Sm/SmD1, anti-RNP70, anti-Ro52, anti-Ro60, and anti-La. Although autoantibody production by differentiated B cells, known as plasma cells, can be driven via T cell-independent mechanisms, autoantigen-specific CD4+ T cells are believed to play key role to autoantibody production in SLE by providing T cell-help important for B cell differentiation, generation of germinal centres, and immunoglobulin class switching. Provision of T cell-help is therefore suggested as a disadvantaged function of autoantigen-specific CD4+ T cells thereby modulating disease pathogenesis of SLE. To elicit the functional impact of autoreactive CD4+ T cells into the production of pathogenic autoantibodies, we performed correlation analysis by comparing the frequency and cytokine production capacity of autoantigen-specific CD4+ T cells with the serum levels of particular autoantibodies targeting the same autoantigen in patients with active and inactive SLE. The frequencies of cytokine-producing CD4+ T cells were additionally compared with the serum concentration of anti-dsDNA and anti-nuclear antigen (anti-ANA) autoantibodies. Interestingly, we found that none of the frequencies of autoreactive CD4+ T cells correlated quantitatively with the production of autoantibodies. However, our data imply that IL-4, along with IL-10, could probably promote the development of anti-ANA autoantibodies. Our results here emphasize the pathogenic role of autoreactive CD4+ T cells in SLE and may explain why therapies targeting B cells alone often result in poor efficacy to ameliorate the disease.

Keywords: *Autoantibodies, B cells, cytokines, Th cell-subsets*

Contents

1. Introduction

1.1. Background

Despite the heterogeneity of clinical manifestations in SLE, the prominent, near-universally biomarker to diagnose the disease is the autoantibodies, which are specific to the components of the cell nucleus such as dsDNA and ribonucleoproteins. The development of autoantibodies appears before the disease onset, while patients are still asymptomatic. Anti-nuclear antibodies (anti-ANA), anti-double-stranded DNA (anti-dsDNA) antibodies, anti-Ro antibodies, anti-La antibodies, anti-Sm antibodies, and anti-nuclear ribonucleoprotein (anti-RNP70) antibodies were present among individuals with SLE in decreasing-frequency [70]. Progressive and sustained production of autoantibodies is characteristic of B cell hyperreactivity, when self-tolerance mechanisms fail to control the immune response. B cells are thought to be the crucial mediator in SLE since memory B cells and its differentiated form plasma cells, are found in increased frequency in SLE (reviewed in [173]). Therapeutic approaches for patients with SLE using monoclonal antibodies to target B cell survival factors (e.g. belimumab, against BAFF also known as BLyS) or to deplete B cells via CD20-expression (e.g. rituxumab) have been approved to the market and undergo clinical trials [174]. However in fact, restricted treatment targeting B cells has resulted in limited therapeutic benefit, because B cell function, survival, proliferation, and differentiation are modulated by a complex interplay between various types of cytokines and immune cells, including T cells.

The role of T cells in the production of autoantibodies relies on the evidences that the autoantibodies identified in SLE are generally of high-affinity, somatically mutated IgG antibodies indicating that they have arisen in germinal centres after provision of T cell-help for class switching. Furthermore, activation of B cells in germinal centres strongly depends on the stimulation of CD40 molecule, the ligand of CD154, which also suggests an antigen-specific T cell role in the process. Up-regulation of HLA class II molecules on activated B cells and genetic predisposition of HLA class II in SLE further suggest antigen-specific mechanism involving T cells in the pathogenesis (reviewed in [173]). Altogether, CD4+ T cells with identical antigen specificity are thought to promote disease progression via their function in T and B cell-interaction.

The mechanisms underlying autoantibody production via effector function of CD4+ T cells in SLE are still poorly investigated. The production of autoantibodies may be an antigen-specific process. Although it is likely that follicular T helper cells (Tfh) are the key player among CD4+ T cell subsets that provide B cell-help in the germinal centres [175], it is not impossible that other T cell subsets may functionally contribute to the development of autoantibodies. Recently, novel Tfh-like CD4+ T cell subset that provides B cell-help outside germinal centre has been identified, defined as peripheral T helper cells (Tph), their role is implicated in the autoimmune disease rheumatoid arthritis [176]. Moreover, as discussed in the previous chapter, cytokines may play specific roles in B cell activation and differentiation. In SLE, it is still unclear which cytokines promote B cell hyperreactivity, and whether these B cell activation-driven cytokines are produced by CD4+ T cells in an antigen-specific manner.

1.2. Objectives

With correlation analysis, we aimed to understand the contribution of autoreactive CD4+ T cells to the development and production of autoantibodies in patients with SLE. We compared the frequencies of autoreactive CD4+ T cells and cytokine-producing autoreactive CD4+ T cells with autoantibody titres and concentration in SLE patients. Correlation analysis may help to elicit evidence about mechanisms of provision of T cell-help in the disease pathogenesis of SLE.

2. Materials and methods.

In the experiments presented in this chapter, the following methods were performed and they were described in the previous chapters: blood donors and isolation of peripheral blood mononuclear cells (chapter 2, section 2.1.), determination of cell numbers (chapter 2, section 2.2. and chapter 4, section 2.2.), antigen pool preparation (chapter 4, section 2.1.), cryopreservation of human lymphocytes (chapter 3, section 2.2.), feeder cell preparation (chapter 3, section 2.3.), antigen-presenting cell preparation (chapter 3, section 2.4.), amplified CD4+ T cell library (chapter 3, section 2.5.), [^{3}H]-thymidine incorporation assay (chapter 3, section 2.6.), CD4+ T cell library data analysis (chapter 4, section 2.3), antigen-reactive T cell enrichment (chapter 4, section 2.4.), antibody staining, cell fixation and permeabilization, and flow cytometry analysis (chapter 4, section 2.5.), antibody staining of intracellular compartment (chapter 6, section 2.1.), and statistics (chapter 4, section 2.7.).

2.1. Determination of autoantibody titres and concentration

Serum of patients with active and inactive SLE was collected at the time when their blood was used for analysis in this study. The collection of serum was performed as part of regular standard operational procedure when a patient visited Charité Universtitätsmedizin Berlin for ambulant or hospital treatment. Serum was collected for laboratory analysis e.g. rheumatologic factors including anti-dsDNA, anti-ANA, anti-Sm, anti-RNP70, anti-Ro52, anti-60, and anti-La autoantibodies. Determination of titres and concentration of autoantibodies was performed by Labor Berlin – Charité Vivantes GmbH, Berlin, Germany, a Charité half-owned laboratory institution. The data were retrieved from laboratory records by authorized medical staff and pseudonymized according to the patient's initial applied in our study. According to information from Labor Berlin, concentration of autoantibodies was measured using AKLIDES® technology (Medipan GmbH, Dahlewitz, Germany), that relies on the use of immunofluorescence screening.

The concentration of autoantibodies was expressed in unit/mL (U/mL). According to Labor Berlin and manufacturer of AKLIDES® technology, the concentration below 10U/mL is defined as negative (antibody seronegative). The concentration of some autoantibodies was determined by calculating their titre value using dilution series. The serum was further diluted and measured until a number of dilutions, in which the autoantibody was not more detectable. Therefore, strong dilution titre means that the antibody is present at a higher concentration.

3. Results and discussion

Although SLE has multiple clinical manifestations, patients are almost universally diagnosed by serological presence of autoantibodies directed against nuclear antigens. This phenomenon generates hypothesis of T cell contribution in B cell-mediated immune pathogenesis, because high affinity pathogenic antibodies are generally produced after engagement of B cells with T cell in the germinal centres. The connection between autoreactive CD4+ T cells with autoantibody production in SLE is presented and discussed here. The results draw a possible disadvantaged function of autoreactive CD4+ T cells in the development of autoantibodies.

3.1. Antigen recognition and autoantibody production

In order to predict whether autoreactive CD4+ T cells recognize the same antigen as B cells for production of autoantibodies, we performed quantitative and qualitative correlation analysis by comparing the frequencies of particular autoantigen-specific CD4+ T cells with the concentration of corresponding autoantibodies. The frequencies of particular autoreactive CD4+ T cells were calculated using Poisson distribution of CD4+ T cell library data as presented in chapter 4. We compared the frequency of SmD1-specific CD4+ T cells with the concentration of anti-Sm antibodies, the frequency of RNP70-specific CD4+ T cells with the concentration of anti-RNP70 antibodies, the frequency of Ro-specific CD4+ T cells with the concentration of two isotypes of anti-Ro antibodies, anti-Ro52 and anti-Ro60 antibody, and the frequency of La-specific CD4+ T cells with the concentration of anti-La antibodies (Figure 25A). Notably, using linear regression determined by Pearson's correlation coefficient, no correlation was observed between the frequencies of autoreactive CD4+ T cells with the serologic concentration of autoantibodies.

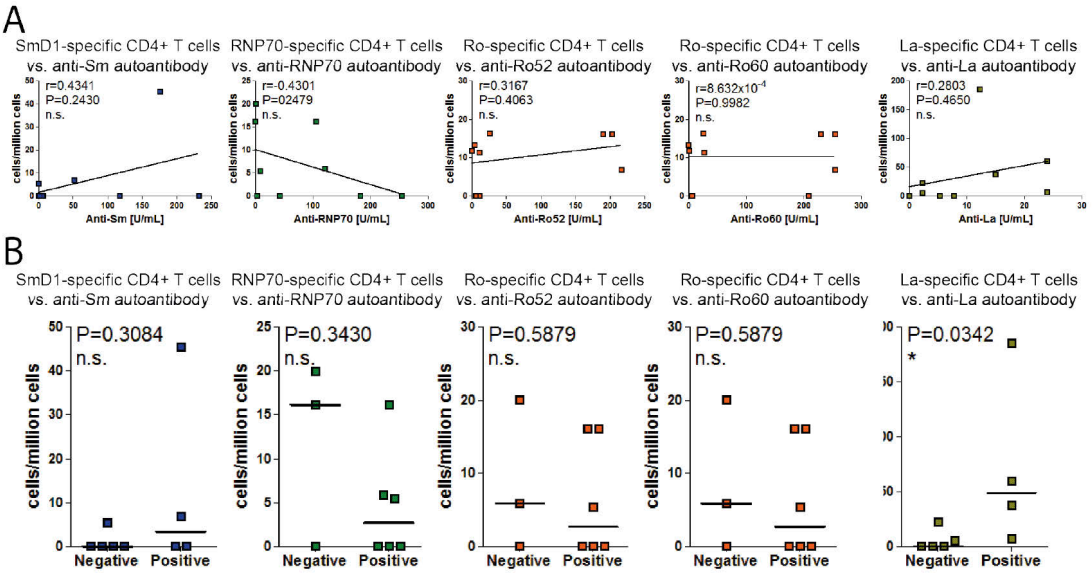


Figure 25: **Correlation of the frequency of autoreactive CD4+ T cells with the production of autoantibody.** The concentration of autoantibodies in the serum of patients with active and inactive SLE (*n*=9) was compared with the frequency of corresponding autoantigen-specific CD4+ T cells determined by CD4+ T cell library method. (**A**) Quantitative correlation analysis of the concentration of autoantibodies with the frequencies of corresponding autoreactive CD4+ T cells. Frequency of SmD1-specific CD4+ T cells was compared with the concentration of anti-Sm antibody, frequency of

RNP70-specific CD4+ T cells was compared with the concentration of anti-RNP70 antibody, frequency of Ro-specific CD4+ T cells was compared with the concentration of anti-Ro52 and anti-Ro60 antibody, and the frequency of La-specific CD4+ T cells was compared with the concentration of anti-La antibody. R indicates Pearson's correlation coefficient. Line represents linear regression determined using Pearson's correlation coefficient. (**B**) Frequencies of autoreactive CD4+ T cells were compared qualitatively with autoantibody production, where the concentration of autoantibody less than 10U/mL was defined as negative. Each symbol illustrates a distinct individual. Black line represents median value.

Then, we classified the production of autoantibodies based on their concentration as suggested by the manufacturer of the detection platform. We defined threshold concentration of antibodies less than 10U/mL as negative, while the concentration more than this value as positive. This threshold enables categorization of SLE patients who had negative autoantibody production in the serum at the time point when the frequencies of autoreactive CD4+ T cells were measured (Figure 25B). Using this approach, we could quantify the frequencies of autoreactive CD4+ T cells in autoantibody-specific seronegative and seropositive patients with SLE (medium number: SmD1-specific CD4+ T cells, Sm-seronegative (n=5) = 0, Sm-seropositive (n=4) = 3.425 cells/million cells; RNP70-specific CD4+ T cells, RNP70-seronegative (n=3) = 16.13, RNP70-seropositive (n=6) = 2.72 cells/million cells; Ro-specific CD4+ T cells, Ro52-seronegative (n=3) = 5.88, Ro52-seropositive (n=6) = 2.72 cells/million cells; Ro-specific CD4+ T cells, Ro60-seronegative (n=3) = 5.88, Ro60-seropositive (n=6) = 2.72 cells/million cells; La-specific CD4+ T cells, La-seronegative (n=5) = 0, La-seropositive (n=4) = 48.82 cells/million cells). When comparing the frequencies of particular autoreactive CD4+ T cells with corresponding antibody-seropositive and seronegative SLE patients, only the frequencies of SmD1-specific and La-specific CD4+ T cells were increased in seropositive patients, where significant increase was observed only for the frequency of La-specific CD4+ T cells in La-seropositive SLE patients (SmD1-specific CD4+ T cells among anti-Sm-seronegative and seropositive donors, P = 0.3084; RNP70-specific CD4+ T cells among anti-RNP70-seronegative and seropositive donors, P = 0.3430; Ro-specific CD4+ T cells among anti-Ro52-seronegative and seropositive donors, P = 0.5879; Ro-specific CD4+ T cells among anti-Ro60-seronegative and seropositive donors, P = 0.5879; La-specific CD4+ T cells among anti-La-seronegative and seropositive donors, P = 0.0342, by Mann-Whitney test). For other autoantigen-specific CD4+ T cells, the correlation with autoantibody concentration was not identified.

It has been hypothesized that the pathogenesis of SLE is initiated by defects of clearance mechanisms of apoptotic nucleus materials such as dsDNA and nucleosomes and persistence of impaired checkpoint mechanism to eliminate self-reactive B cells at various stages of B cell-maturation. Breakdown of immune tolerance leads to increased numbers of autoreactive mature naive B cells [177]. In the milieu where the apoptotic materials are present at a higher concentration, this debris is recognized by autoreactive BCR, internalized and processed by autoreactive B cells for presentation on MHC class-II molecules. On the other hand, only through antigen-mediated activation, naive B cells differentiate into short-lived plasmablasts and long-lived plasma cells or memory B cells for antibody production [178]. At this point, B cells play a role as antigen-presenting cells for T cells, but they also undergo differentiation into antibody-secreting cells. This condition facilitates two possible models of autoantibody production by B cells, which could be a T cell-dependent and/or T cell-independent mechanism [179]. The later however, does not exclude the

possibility that T cells may still be able to modulate B cell activation [180]. Interaction in the T and B cell-axis is however indispensable in the context of SLE due to the fact that (1) there is association of certain HLA alleles with certain autoantibody specificities and this issue is discussed in chapter 3 of this thesis, (2) CD154/CD40 ligation has been suggested to be necessary for activation of B cells but also for autoreactive T cells [181], and (3) SLE-associated autoantibodies are high-affinity, somatically mutated IgG indicating they have developed in germinal centres after provision of T cell-help for class switching [182]. Recent study also showed that antigen-specific interaction between B cells and Tfh cells promotes dopamine secretion that in turn upregulates CD154 on the cell surface of Tfh cells to induce functional antibody production [183].

No concordance between the frequencies of autoantigen-specific CD4+ T cells with autoantibody production may be explained through the theory about somatic hyper-mutation of SLE-associated autoantibodies in the study performed by Mietzner *et al.* [184]. They showed that self-reactive and polyreactive autoantibodies both in healthy individuals and patients with SLE are present in comparable frequency. The antibodies were then cloned and tested for their specificity against SLE-associated autoantigens including SmD, RNP70, Histone, Ro52, Ro60, and La, where they found that only six among 200 autoantibodies derived from patients with SLE were specific, whereas no reactive clones were found among 84 antibodies derived from healthy individuals. These autoantigen-specific autoantibodies were not clonally related with each other and showed specificity against Ro52 and La, or cross-reactivity against both autoantigens. Although the patient was identified as Ro52- and La-seropositive, donor's serum also displayed reactivity against SmD and RNP but reactivity to SmD and RNP was not observed among 200 autoantibodies tested. They later figured out that the number of somatic hypermutation in these six SLE-associated autoantibodies was significantly higher compared with all other autoantibodies. By reverting back the immunoglobulin light and heavy chain into their germline configuration, only one reverted autoantibodies clone retained a low level of reactivity against its corresponding antigen. This observation implied that pathogenic self-reactive autoantibodies are developed from non-reactive or polyreactive precursors that are shaped by somatic hypermutation. Furthermore, this study was supported by the fact that both healthy individuals and patients with SLE display similar frequency of ANA-reactive antibodies in CD27+IgG+ memory B cells, but in SLE, autoantibodies undergo somatic hypermutation to become pathogenic [185].

Taking together, poor correlation between the frequencies of autoantigen-specific CD4+ T cells with the concentration of their corresponding autoantibodies is not necessarily diminish the fact that autoreactive CD4+ T cells provide T cell help for autoantibody production, but in addition, it describes an additional mechanism termed somatic hypermutation. Importantly, the study conducted by Mietzner *et al.* reported that immunoglobulin light chain replacement occurred late, possibly after antigen-mediated stimulation and the onset of somatic hypermutation [184]. After engaging with B cells to support antibody production, Tfh cells could probably already recirculate back in the periphery before the production of pathogenic and functional autoantibodies is initiated. Thus, we argue that the presence of autoantigen-specific CD4+ T cells in circulating blood and their corresponding autoantibodies in serum should be observed at different time point. Furthermore, it is possible that antigen-cross reactivity may play a significant role in T and B cell interaction. For example,

dsDNA-specific B cells might be activated by chromatin, but after antigen-processing, histone instead of chromatin is presented by B cells as antigen to histone-specific CD4+ T cells, resulting in proliferation and differentiation of histone-specific CD4+ T cells, as well as in provision of T cell-help signals to the B cells

3.2. Correlation of cytokines with autoantibody production

Somatic hypermutation has been presumed to occur in germinal centres, where specialized subset of CD4+ T cells, Tfh cells, engage with B cells through CD154-CD40 interaction, but also ICOS-ICOS-L and CD80-CD86 ligation [186]. Tfh cells were characterized by different combination of surface markers and receptors, where the frequency of CXCR5+PD-1-high CD4+ Tfh cells producing IL-21 is increased in SLE [187, 188]. Interestingly, Tfh cells in fact comprise of three different Th cell subsets, such as IFN-γ-producing Th1 cells, IL-4-producing Th2 cells, and IL-17-producing Th17 cells [189]. Th2 and Th17 cells within CXCR5+ cell compartment were able to induce naive B cells to produce immunoglobulins through production of IL-21, whereas Th1 cells lacked the capacity to provide help to B cells [189]. We have shown no concordance between the frequencies of autoantigen-specific CD4+ T cells in the periphery with the production of corresponding autoantibodies, leading to the assumption that the production of autoantibodies may be modulated via helper function e.g. by effector cytokines of Th cells. Indeed, recent publication reported that Tfh cells promote germinal-centre development of memory B cells by cytokine IL-9 secretion [190]. In order to identify which Th cell subsets may have the potential to induce autoantibody production in SLE, we performed correlation analysis to compare the frequencies of cytokine-producing autoreactive CD4+ T cells with the concentration of anti-dsDNA and anti-ANA autoantibodies in patients with both inactive and active SLE.

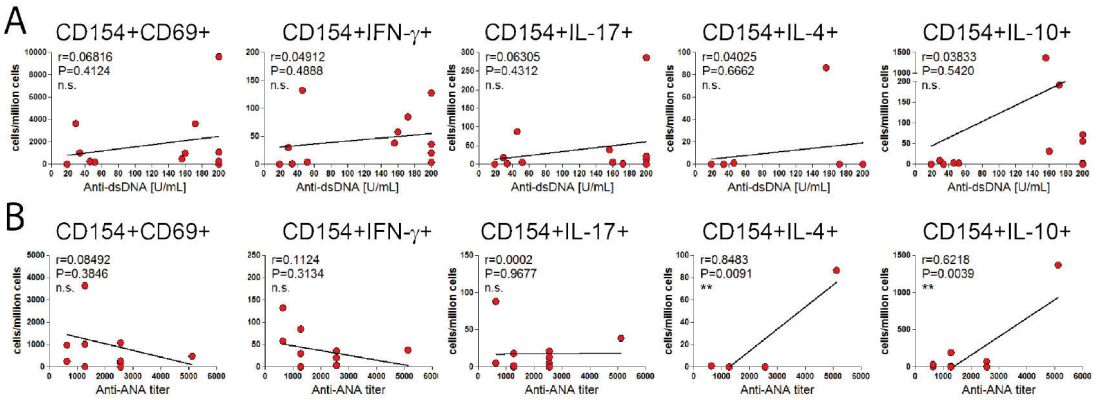


Figure 26: **Correlation of the frequencies of cytokine-producing autoreactive CD4+ T cells with the concentration of anti-dsDNA and anti-ANA autoantibodies.** The correlation of the background-subtracted frequencies of CD154+CD69+ autoreactive CD4+ T cells and cytokine-producing (IFN-γ, IL-17, IL-4, and IL-10) CD154-expressing CD4+ T cells with the concentration of anti-dsDNA autoantibody (*n*=12, except for CD154+IL-4+ CD4+ T cells, *n*=7) (**A**), and with the concentration of anti-ANA autoantibody (*n*=11, except for CD154+IL-4+ CD4+ T cells, *n*=6) (**B**). R indicates Pearson's correlation coefficient. Line represents linear regression determined using Pearson's correlation coefficient

Elevated production of anti-dsDNA and anti-ANA autoantibodies is notable characteristics in SLE with 62% and 84% of patients with SLE developing anti-

dsDNA and anti-ANA autoantibodies, respectively [70]. We found no significant correlation between the concentrations of anti-dsDNA autoantibody with the number of total autoreactive CD4+ T cells displayed by CD154+CD69+ expression and with the number of cytokine-producing CD154-expressing autoreactive CD4+ T cells (Figure 26A). However, we observed two clustered populations of SLE patients regarding IL-10 production by autoreactive CD4+ T cells and anti-dsDNA autoantibody production in the serum. It is likely that patients with low number of IL-10-producing autoreactive CD4+ T cells also had lower levels of anti-dsDNA autoantibody production, whereas patients with high numbers of IL-10-producing autoreactive CD4+ T cells had improved production of anti-dsDNA autoantibodies. However, this clustering was apparent lack of significant difference. Then we compared the correlation between the concentration of anti-ANA autoantibody with the number of CD154+CD69+ and cytokine-producing CD154-expressing CD4+ T cells. Anti-ANA autoantibody recognizes a wide variation of nuclear antigens, which are responsible autoantigens for SLE. Comparing the titre of anti-ANA autoantibodies with the frequencies of autoreactive CD4+ T cells would be relevant to find information about provision of antigen-specific T cell-help. Our data showed that no significant correlation was observed between the serum level of anti-ANA autoantibody with the frequencies of total autoreactive CD4+ T cells displayed by CD154+CD69+ expression, as well as CD154+IFN-γ+ and CD154+IL-17+ autoreactive CD4+ T cells. The frequencies of CD154+CD69+ and CD154+IFN-γ+ CD4+ T cells tended to correlate negatively with the concentration of anti-ANA autoantibody in the serum of SLE patients, whereas the frequency of CD154+IL-17+ CD4+ T cells had no correlation with the serum level of anti-ANA autoantibodies (Figure 26B). In contrast, the frequencies of CD154+IL-4+ and CD154+IL-10+ CD4+ T cells showed significant positive correlation with the serum amount of anti-ANA autoantibody in patients with SLE.

Genome-wide association studies revealed that the HLA region in general is the highest susceptible genetic association with SLE emphasizing an antigen-specific T cell role in the disease [191]. In parallel, excessive isotype-switched autoantibody production against nuclear antigens also underlines interaction between T and B cells. However, since more than half of the patients with SLE develop autoantibodies against dsDNA, a direct T cell-dependent mechanism in this regard is still speculative. A HLA-association with anti-dsDNA autoantibody has not yet been established, leading to hypotheses, whether the plasmablasts producing anti-dsDNA autoantibodies may be less dependent on CD4+ T cells at antigen-specific level, or whether the T cell epitopes that support the production of these antibodies may be indiscriminating, thus an HLA association is not observable, or whether the production of anti-dsDNA autoantibody is an stochastic event driven by T cells through secretion of cytokines. Although cytokines produced by autoreactive CD4+ T cells seem to provide no connection with the production of anti-dsDNA autoantibodies, patients with higher frequency of IL-10-producing autoreactive CD4+ T cells showed elevated serum levels of anti-dsDNA autoantibodies, which is in contrast to the patients with lower frequency of IL-10-producing CD4+ T cells. This observation may lead to a suggestion of unbeneficial role of IL-10 that has been known also to promote pathogenic B cell survival and proliferation as discussed in the chapter 6. Increased production of anti-dsDNA autoantibodies in SLE could be a consequence of cytokine-driven mechanisms of B cell activation, rather than an antigen-specific and HLA-dependent process.

In contrast to the production of anti-dsDNA autoantibodies, production of anti-ANA autoantibodies is linked to HLA class-II gene susceptibility indicating a functional role of antigen-specific CXCR5+ Tfh cells in the process. Whether Tfh cells are antigen-specific remains controversial [192, 193], but a study conducted by Morita *et al.* could show that these cells expressed CD154 upon antigen stimulation, expanded, and produced IL-2 and IFN-γ [189]. Importantly, their study was able to demonstrate the relevance between Tfh cells with other Th subsets, in which they claimed that Tfh cells are skewed towards Th2 and Th17 cells, when dissecting the subsets according to their cytokine production, chemokine receptor, and transcription factor expression. This relation has been suggested due to the fact that Tfh cells are highly plastic and can be converted from Th1 [194], Th2 [195], and Treg cells [196] *in vivo*. We found positive significant correlation between IL-4- and IL-10-producing CD4+ T cells reactive to nuclear antigens with the production of autoantibodies directed to the same target. In line with our results, it has been known that Tfh cells secrete IL-4 and IL-10 that promote growth, differentiation, and class switching of B cells [197, 198]. Moreover, blocking of IL-4 may result in a substantial inhibition of IgE production by Tfh cells. Based on our data and the data from others, we assumed that autoreactive CD4+ Tfh cells in SLE promote autoantibody production against nuclear antigens through secretion of IL-4 and IL-10, thus it could be that these Tfh cells are differentiated to Th2 subsets. Our data, however, did not include information of CXCR5 expression and/or IL-21 production by autoreactive CD4+ T cells, so that whether the cells here are indeed Tfh cells is still speculative. Furthermore, lack of correlation between IL-17-producing autoreactive CD4+ T cells with the autoantibody production is probably because IL-17-producing cells demonstrate a higher degree of plasticity. Tfh and Th17 cells have several similarities in the molecular requirements for differentiation such as expression of ICOS, IRF-4, c-Maf, and STAT3, as well as production of IL-21 and their potential to induce B cell differentiation [199].

4. Conclusion

Therapeutic approaches targeting B cells by using the anti-CD20 antibody rituximab and the BAFF/BLyS neutralizing antibody belimumab often result in poor effectiveness to ameliorate the disease of SLE since remaining B cells may still have pathogenic potential [200, 201], when interacting with other immune cells including T cells. However, to date only polyclonal T cells are targeted for therapy by using mycophenolate mofetil (MMF), blocking of CD154-CD40 interaction, and blocking of CD28-CD80/CD86 interaction (abatacept) [202, 203]. Targeting autoreactive CD4+ T cells has been challenging since very little information is available about the specificity and the function of these cells in promoting disease development such as autoantibody production. In this chapter, we explained our investigation into possible functions and mechanisms of autoreactive CD4+ T cells in the development of clinical-relevant autoantibodies against nuclear antigens. The production of autoantibodies in SLE is most likely an antigen-specific mechanism involving autoreactive CD4+ T cells. Whether autoreactive CD4+ T cells express TCR specific to the same antigen as target for autoantibodies remains speculative because there is possible evidence of epitope spreading, somatic hypermutation, and antigen cross-reactivity. Here, we propose the following scenario about the induction of autoantibodies by autoreactive CD4+ T cells (Figure 27). First, autoantigen-specific

CD4+ T cells recognize autoantigen presented by antigen-presenting cells such as dendritic cells in the secondary lymphoid organs. These autoantigen-specific CD4+ T cells are skewed towards Th2 cells that differentiate to Tfh lineage and enter the germinal centre with the ability to produce IL-4 and IL-10 upon activation by naive or memory B cells. After antigen recognition and receiving T cell help through secretion of IL-4 and IL-10, B cells differentiate into plasmablasts and plasma cells for autoantibody production. In addition, IL-4, IL-10, IL-21, and IL-9 which are produced by autoreactive Tfh cells also provide T cell-help for immunoglobulin class-switching. The autoantibodies undergo extensive receptor editing by somatic hypermutation leading to functionally active autoantibodies that can target the initial antigens but also other antigens (cross-reactive). Thus, frequencies of autoreactive CD4+ T cells specific to particular antigens do not necessarily correlate with the existence of autoantibodies specific to this antigen. But production of IL-4 and IL-10 by CD4+ T cells reactive to a wide variety of nuclear antigens may result in the production of high variable autoantibodies directed to nuclear components (such as anti-ANA) or to non-antigen such as DNA (anti-dsDNA). Since IFN-γ-producing autoreactive CD4+ T cells do not correlate to autoantibody production but to a global disease activity, we assumed that autoreactive CD4+ Th1 and Th2 cells acquire different pathogenic functions in SLE. Autoreactive CD4+ Th1 cells are likely responsible for modulating the disease and also direct inflammation through the secretion of IFN-γ, whereas autoreactive CD4+ Th2 cells are more prone to supporting autoantibody production through secretion of IL-4 and IL-10, which frequency is independent from SLE disease activity as demonstrated in the chapter 6.

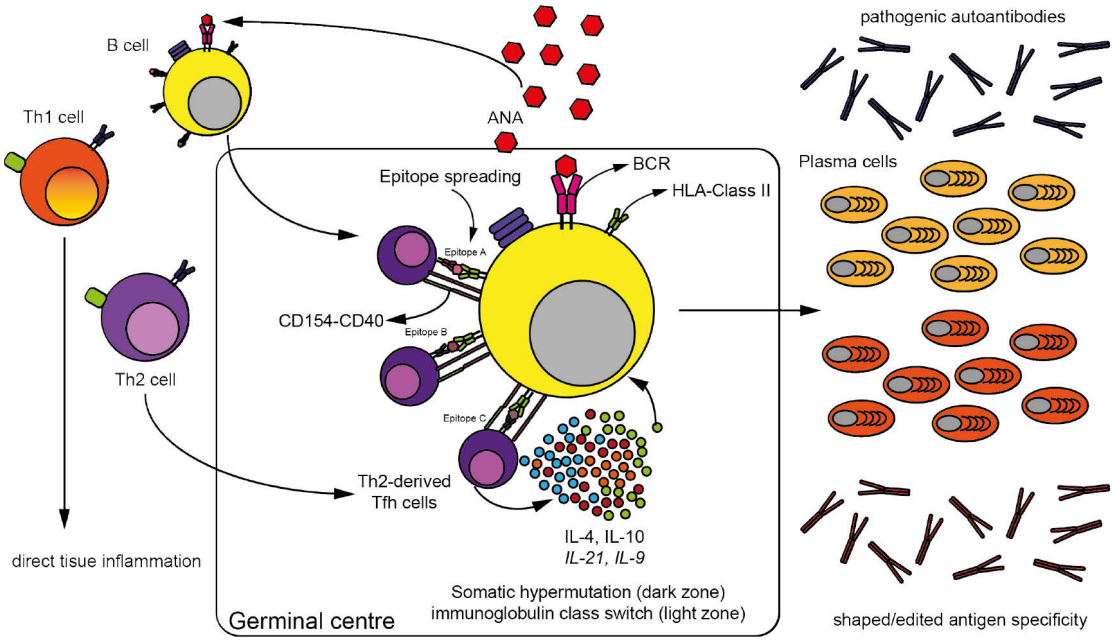


Figure 27: **Pathogenic autoantibody production mediated by autoreactive CD4+ T cells in SLE.** Antigen-activated B cells enter the light zone of germinal centre where they exert somatic hypermutation of immunoglobulin gene segments. Autoreactive Th2-derived Tfh cells recognize autoantigen presented by naive or memory B cells in the dark zone and secrete cytokines such as IL-4 and IL-10, but also IL-21 and IL-9 to help B cells differentiate into antibody-secreting plasma cells and to promote immunoglobulin class switching. The combination of somatic hypermutation and cytokine-induced antibody class-switching results in the development of pathogenic high affinity somatically-mutated autoantibodies of switched-isotypes with shaped antigen-specificities to SLE-autoantigens.

Chapter 8

Detection of SLE-associated autoantigen-specific CD4+ T cells in urine of active SLE patients with lupus nephritis

Abstract

In order to describe the role of SLE-associated autoantigen-specific CD4+ T cells in the disease pathogenesis of SLE, we have determined the frequencies of circulating autoreactive CD4+ T cells, re-assessed their antigen-specificity, characterized their effector cytokine production, and estimated their functional mechanisms in the development of autoantibodies. However, their role in local tissue inflammation remains unclear. SLE is characterized by multiple organ manifestations for example in the kidneys leading to severe lupus nephritis that often results in high mortality among patients. We sought to understand the function of autoreactive CD4+ T cells in mediating organ failure with focus on the kidneys of active SLE patients with lupus nephritis. We developed two approaches by generating libraries of polyclonal, urinary CD4+ T cells, and by labelling urinary CD4+ T cells with CFSE for detection on flow cytometry. We found that the frequencies of autoreactive CD4+ T cells in the urine were higher than in the peripheral blood within individuals with lupus nephritis, indicating an infiltration of such cells into the kidneys at antigen-specific level. Our methods demonstrate a state-of-the-art approach to detect antigen-specific CD4+ T cells in the kidneys without having to perform operative actions such as biopsies. Furthermore, our results provide important findings about localization of pathogenic SLE-associated autoantigen-specific CD4+ T cells in human organs which may be important for development of therapies to protect organ damage.

Keywords: *Lupus nephritis, kidney, urine, detection methods, stimulation index*

Contents

1. Introduction

1.1. Background

SLE is a systemic autoimmune disease with multiple organ manifestations, for example to the kidneys leading to severe lupus nephritis. Between 30-60% of adult patients with SLE develop renal involvement, in which 18% of the patients show five years of mortality and up to 15% of them suffer from kidney failure despite treatment [64-66]. Standard therapies using cyclophosphamid or MMF are of high risk for the patients due to side effects that lead to organ complications and death [204]. Therefore, better understanding of the pathogenesis of lupus nephritis is necessary for development of clinically-relevant targeted therapies.

Immune complexes, complement, and autoantibodies are found in the glomeruli of patients with lupus nephritis and their accumulation induces kidney inflammation. Additionally, infiltration of cells of the innate and the adaptive immune system such as CD4+ T cells, CD8+ T cells, macrophages, B cells, plasmablasts, and plasma cells is also observed in the renal interstitial space, resulting in tubular cell malfunction and fibrosis [205-207]. CD4+ T cells are crucial mediators of autoimmune pathogenesis and related organ damage, e.g. by promoting B cell differentiation and autoantibody production or by directly modulating inflammation against renal tissues [49]. The mechanism of T cell-mediated pathology can be initiated by expansion of autoreactive CD4+ T cell in the periphery that become polarized to produce effector cytokines prior to migration to the kidneys, or by expansion of naive autoreactive CD4+ T cells after encountering autoantigens *in situ*.

The mechanism underlying how lymphocytes provoke kidney failure remains unclear. Renal biopsy taken from SLE patients with lupus nephritis revealed oligoclonality of infiltrating CD4+ T cells in the kidneys based upon TCR usage [208]. Furthermore, a transgenic mouse model that enables expression of antigens in glomerular podocytes demonstrated local tissue inflammation driven by T cells [209]. Thus, the mechanism of T cell-mediated inflammation in the kidneys could be an antigen-specific response. However, to date there is no available method that facilitates direct detection of antigen-specific CD4+ T cells in the kidneys as well as in other tissues. Renal biopsy often causes complications to the organ. A new and safe approach to study tissue-resident antigen-specific CD4+ T cells is therefore necessary for better understanding of the disease.

Our group has established a method to detect cells from the innate and the adaptive immune system from urine of SLE patients with lupus nephritis [210]. The frequency of CD4+ T cells in urine can be used as a biomarker to predict disease progression [211]. These CD4+ T cells infiltrated the kidneys from peripheral blood [212] and showed elevated expression of CD69 and CD154 that emphasizes possible antigen-specific activation on these cells [210]. Whether or not the cells are reactive to lupus-associated autoantigens and accumulate in the kidneys have become key questions. Answer would provide information about the role of autoreactive CD4+ T cells in SLE.

1.2. Objectives

In this thesis, we aimed to establish state-of-the-art methods to detect antigen-specific CD4+ T cells from urine of active SLE patients with lupus nephritis by developing two available antigen-specific CD4+ T-cell detection techniques, T cell library and ARTE. We also sought to determine the frequencies of urinary autoreactive CD4+ T cells and to compare them with the frequencies of autoreactive CD4+ T cells in the periphery within individuals, to predict the deposition and the accumulation of these cells in inflamed kidneys.

2. Materials and methods

In the experiments presented in this chapter, the following methods were performed and they were described in the previous chapters: blood donors and isolation of peripheral blood mononuclear cells (chapter 2, section 2.1.), determination of cell numbers (chapter 2, section 2.2. and chapter 4, section 2.2.), antigen pool preparation (chapter 4, section 2.1.), cryopreservation of human lymphocytes (chapter 3, section 2.2.), feeder cell preparation (chapter 3, section 2.3.), antigen-presenting cell preparation (chapter 3, section 2.4.), amplified CD4+ T cell library (chapter 3, section 2.5.), [^{3}H]-thymidine incorporation assay (chapter 3, section 2.6.), CD4+ T cell library data analysis (chapter 4, section 2.3, with additional information, re-described as a new section in this chapter), antigen-reactive T cell enrichment (chapter 4, section 2.4.), antibody staining, cell fixation and permeabilization, and flow cytometry analysis (chapter 4, section 2.5.), antibody staining of intracellular compartment (chapter 6, section 2.1.), and statistics (chapter 4, section 2.7.).

2.1. Isolation of urinary leukocytes

Urine of SLE patients with lupus nephritis contains a wide variety of cells such as kidney epithelial cells and leukocytes. At least 300mL fresh urine was collected from the donor. Urine was mixed with 20% (v/v) PBS/BSA (self-prepared at DRFZ) supplemented with 1% (v/v) Penicillin/Streptomycin (Life Technologies Ltd., Paisley, United Kingdom). The suspension was centrifuged two times at 310xg for 10 minutes at 4°C. After centrifugation, the supernatant was aspirated and the pellet was resuspended in 10mL cold PBS/BSA. The cell suspension was filtered with 30µm pre-separation filter (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) and resuspended in cold PBS/BSA. In some cases when the cell suspension contained an increased amount of sediment, pellet was resuspended with 35mL PBS/BSA and then 15mL Ficoll solution was carefully mounted into the urine suspension to accumulate at the bottom and centrifuged at 2,000 rpm for 20 minutes without breaks at room temperature. After the centrifugation, urinary leukocytes and urine sediment are separated in different layers, comparable with the separation of PBMC from whole blood using the same technique. The leukocytes in the interphase layer were collected and washed with up to 50mL PBS/BSA and centrifuged at 310xg for 10 minutes at 4°C. Following the centrifugation, the supernatant was carefully removed and the pellet was re-suspended in PBS/BSA. Cell counts were determined using an automated Casy® cell counter or Guava® easyCyte flow cytometer as described in the chapter 2, section 2.2. and chapter 4, section 2.2.

2.2. Isolation of urinary CD4+ T cells

Urinary CD4+ T cells were isolated using antihuman CD4-antibodies coupled with magnetic beads (Miltenyi Biotech GmbH) according to manufacturer's instruction. Briefly, $1x10^7$ urinary cells were resuspended in 80µL PBS/BSA/EDTA. 20µL of antihuman CD4-antibodies coupled with magnetic beads were added to the suspension and incubated for 15 minutes at 4°C. When the cell number was more than $1x10^7$ cells, the buffer volume and volume of magnetic beads were scaled up accordingly. Cell suspension was washed with PBS/BSA/EDTA after incubation with magnetic beads. After calibrating the LS column (Miltenyi Biotech GmbH), cell suspension was loaded onto the column and washed several times with PBS/BSA/EDTA. Aliquots were collected from positive and negative fractions to check the sorting efficiency and to determine the cell number. The purity of CD4+ T cell fraction was routinely checked by flow cytometry based on CD3+CD4+ expression using antihuman-CD3-PacificBlue (clone UCHT1, Biolegend Inc., San Diego, USA) and antihuman-CD4-PE-Cy7 (clone SK3, BD Biosciences, San Jose, USA) antibodies.

2.3. Generation of amplified urinary CD4+ T cell libraries

The protocol to generate amplified urinary CD4+ T cell library was established at our laboratory in parallel with the generation of blood CD4+ T cell libraries from the same donors in order to compare the frequency of antigen-specific CD4+ T cells in blood and urine within each individual. Therefore, to restimulate expanded urinary CD4+ T cells, CD3-depleted PBMC were used as antigen-presenting cells as described in the chapter 3, section 2.4. Briefly, 50,000 – 100,000 isolated urinary CD4+ T cells were resuspended in IMDM medium with GlutaMAX™ (Life Technologies Ltd.) supplemented with 5% (v/v) heat-inactivated human AB serum, 1% (v/v) Penicillin-Streptomycin, 2µg/mL ciprofloxacin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and 1% (v/v) MEM non-essential amino acid solutions (Sigma-Aldrich Chemie GmbH), in the presence of 1µg/mL lectin from *Phaseolus vulgaris* (PHA, Sigma-Aldrich Chemie GmbH), 600IU/mL IL-2 (Proleukin/Aldesleukin, Novartis Pharma GmbH, Nuremberg, Germany), and about $2x10^7$ irradiated feeder cells from two non-autologous donors. To improve cell viability and expansion potential, 10ng/mL homeostatic cytokine IL-7 (PeproTech Germany, Hamburg, Germany) was added into the medium. This step is important since urinary cells were already exhausted due to their localization in the organ and their presence in the urine which is not an optimal environment for leukocytes. Different to the cell culture procedure for blood T cell libraries, medium in urinary T cell libraries was replenished periodically after 2-3 days by removing 50% volume of culture medium in each microculture and adding fresh 1µg/mL PHA, 200IU/mL IL-2 and 10ng/mL IL-7. After 2-4 weeks of culture, a fraction of amplified urinary CD4+ T cells were distributed into additional 96-well plates depending on the number of antigens to be analysed. Urinary CD4+ T cell libraries for negative control (unstimulated cells) and positive control (cells stimulated with SEB) were always included in the experiments. Prior to stimulation with the antigens, cells were rested in the stimulation medium for at least four days. On the day of stimulation, antigen-presenting cells were thawed and distributed into the urinary T cell culture in a ratio

of at least one antigen-presenting cell to 100 urinary CD4+ T cells. Urinary CD4+ T cells were stimulated with the antigen for four days, at least 16 hours before measurement, 1µCi/mL ^{3}[H]-thymidine (GE Healthcare UK Ltd., Little Chalfont, United Kingdom) was added to the culture.

2.4. Urinary CD4+ T cell library data analysis

Microcultures containing CD4+ T cells isolated from urine of active SLE patients with lupus nephritis were assessed for their cpm values according to the procedures described in the chapter 3, section 2.6. Also here in the analysis of urinary CD4+ T cell library data, we set an exclusion criterion that evaluated the cpm-values of SEB-stimulated microcultures. Microcultures with a SEB stimulation index (quotient of cpm value in response to stimulation with SEB against cpm value of unstimulated sample) lower than four were excluded. Then, median, 755th percentile, and 25th percentile of cpm values of unstimulated microcultures for each donor were calculated. Finally, donor-specific *z*-score was calculated as five times the difference of 75th and 25th percentile above the median of unstimulated microcultures. The *z*-score represents the significance threshold in order to identify microcultures, in which polyclonal-expanded urinary CD4+ T cells displayed reactivity to the given antigen. For comparison across different donors, the *z*-score was normalized for all donors as described in the chapter 4, section 2.3.

2.5. CFSE-labelling of urinary leukocytes

Urinary leukocytes were washed twice with cold PBS at 310xg for 10 minutes at 4°C. At maximum 1x10^{7} cells were resuspended in 1mL cold PBS, then followed by addition of 1µM carboxyfluorescein diacetate *N*-succinimidyl ester (CFDA-SE, Sigma-Aldrich Chemie). CFDA-SE is non-fluorescent, but after entering cytoplasm of cells by diffusion, acetate groups in this molecule are cleaved by an intracellular esterase enzyme forming amine-reactive products, called carboxyfluorescein succinimidyl ester (CFSE), which is fluorescent and can bind covalently to intracellular lysine residues. Thus, cells can be fluorescently labelled, enabling the detection e.g. in flow cytometry. Urinary leukocytes were incubated with CFDA-SE for 3.5 minutes at room temperature in the dark. After incubation, cells were washed twice with cold PBS/BSA at 310xg for 10 minutes at 4°C.

2.6. Establishment of antigen-reactive urinary CD4+ T cell-enrichment

In order to detect antigen-specific urinary CD4+ T cells by upregulation of antigen-specific T cell-activation marker CD154, we labelled urinary leukocytes with CFSE and mixed the cells with PBMC of the same individuals. Mixed cells were stimulated with antigens and then enriched via their CD154 expression according to ARTE method as described in the chapter 4, section 2.4. CFSE can be detected by flow cytometry thus enabling discrimination of urinary cells within PBMC. Staining of urinary cells was performed as described in the chapter 4, section 2.5., and chapter 6, section 2.1.

2.7. Calculation of the antigen-specific stimulation index

Because the detection of SLE-associated autoantigen-specific CD4+ T cells in blood and urine was performed by two independent approaches, T cell library and ARTE, the percentage of an antigen-specific stimulation index for blood and urine was calculated, enabling direct comparison of both methods. For the calculation of the stimulation index from T cell library data, the number of both total peripheral and urinary SLE-associated autoantigen-specific CD4+ T cells was divided with the number of autoantigens and the background frequency expressed in percent. For calculation of stimulation index from ARTE data, percentages of autoreactive CD154+CD69+ CD4+ T cells isolated from both blood and urine within the total CD3+CD4+ cells was divided by the percentages of CD154+CD69+ CD4+ T cells within total CD3+CD4+ T cells in unstimulated sample (background) and expressed in percent.

Equation 2 and 3 describe the calculation of the antigen-specific stimulation index for T cell library and ARTE method, respectively. To show the accumulation of SLE-associated autoantigen-specific CD4+ T cells in urine of active SLE patients with lupus nephritis, antigen-specific stimulation index of blood and urine for each donor was compared by Wilcoxon matched-pairs signed rank test.

Equation 2: **Calculation of stimulation index (SI) of T cell library data.**

$$SI = \frac{\Sigma\, CD4 + T\ cells\ in\ response\ to\ all\ antigens}{number\ of\ antigens\ \times\ \Sigma\, CD4 + T\ cells\ in\ unstimulated\ sample} \times 100\%$$

Equation 3: **Calculation of stimulation index (SI) of ARTE data.**

$$SI = \frac{\dfrac{\Sigma\, autoreactive\ CD4 + T\ cells\ (CD154 + CD69 + CD4 + T\ cells\ in\ stimulated\ sample)}{\Sigma\, CD3 + CD4 +\ cells\ of\ stimulated\ sample}}{\dfrac{\Sigma\, autoreactive\ CD4 + T\ cells\ (CD154 + CD69 + CD4 + T\ cells\ in\ unstimulated\ sample)}{\Sigma\, CD3 + CD4 +\ cells\ of\ unstimulated\ sample}} \times 100\%$$

3. Results and discussion

The techniques described here facilitate detection of antigen-specific CD4+ T cells isolated from the circulating blood as well as from urine within the same individuals with lupus nephritis. Therefore, it is possible to compare the frequencies of autoreactive CD4+ T cells in the periphery to their frequencies in the kidneys. Comparison of the frequencies in both tissues can deliver information, whether there is a preferable accumulation of autoreactive CD4+ T cells in the organ, and whether this accumulation is a prerequisite for immunopathology in the organ.

3.1. Characteristics of urinary CD4+ T cells

Abnormally elevated urinary markers such as proteinuria are used to diagnose lupus nephritis in patients with SLE, but this is not sufficient to provide information about the complexity of the autoimmune pathology in the kidneys. Invasive methods such as kidney biopsy are usually applied to monitor deposition of kidney-infiltrated immune cells but it can cause kidney injury and complications [213]. Due to limitations in

renal biopsy and possible assessment inaccuracy when diagnosing autoimmune kidney damage only by the level of proteinuria in the urine, examining the presence of urinary immune cells that can reflect renal inflammation is now of great interest [214]. It has been shown previously that urine of SLE patients with lupus nephritis consists of a number of immune cells including CD4+ T cells that can be used as a biomarker for renal involvement in lupus, and also different types of epithelial cells originating from urinary tract [210]. Moreover, the phenotype of urinary immune cells such as CD4+ T cells is qualitatively reminiscent of renal infiltrating cells rather than the T cells circulating in the peripheral blood, hence it is reasonable to argue that urinary immune cells originate from renal interstitium space before migrating through the epithelium layers into the urinary track [212, 215]. The next question after this finding is whether antigen-specific CD4+ T cells residing in the inflamed kidneys are also reflected by their occurrence in the urine. The fact that there is an accumulation of oligoclonal CD4+ T cells in the kidneys of patients with lupus nephritis has been found by Murata *et al.* using analysis of TCR repertoire isolated from renal biopsy [208], and also by us using an analysis of the urinary TCR repertoire when compared with the TCR repertoire in the circulating blood (Figure 28A, kindly provided by Anna-Sophie Grießbach, data analysis was performed by Dr. Ulrik Stervbo. The data is incorporated in the doctoral thesis of Anna-Sophie Grießbach. The graph has been reworked to fit with the data presentation in this thesis). This oligoclonality also suggests that urinary CD4+ T cells share close characteristic with renal infiltrating CD4+ T cells.

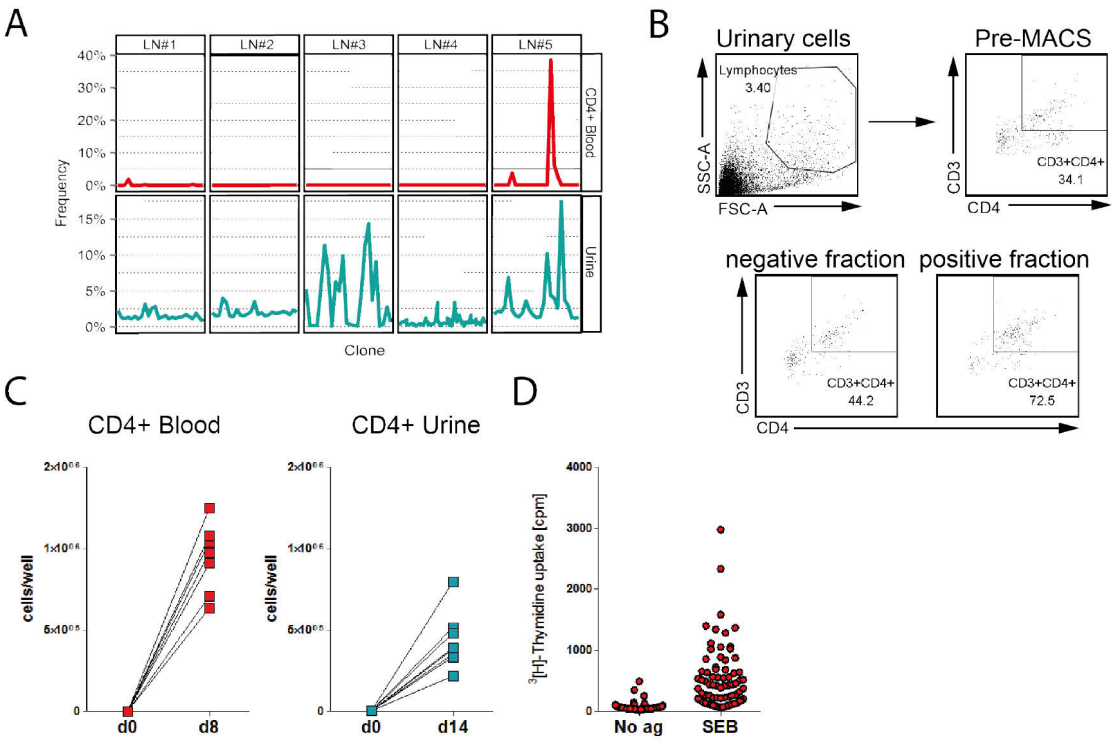


Figure 28: **Characteristics of urinary CD4+ T cells.** (**A**) Analysis of TCR-β repertoire in blood and urine of patients with active SLE and LN (*n*=5). The graphic depicts frequencies of the 20 most abundant TCR clones from CD4+ T cells in peripheral blood (upper diagram, in red) and all viable cells in urine (lower diagram, in turquoise). (**B**) Representative dot plots to the left show the gating strategy to identify lymphocytes from total urinary cells according to their side and forward scatter properties. CD4+ T cells were isolated using CD4 MACS technology. Gating strategy in the right dot plots show CD3+CD4+ cells before sorting (Pre-MACS) and CD4 negative and positive fractions after sorting. (**C**) Expansion of CD4+ T cells derived from blood and from urine using PHA, IL-2, and IL-7.

(**D**) Proliferation capacity of urinary CD4+ T cells without stimulation and when stimulated with SEB reflected by ^{3}[H]-thymidine uptake.

On flow cytometry, leukocytes or lymphocytes could be distinguished from other cell types such as epithelial cells based on their side and forward scatter properties. Additionally, population of CD3+CD4+ cells could also be observed (Figure 28B). By using magnetic cell sorting, urinary CD4+ T cells could be isolated from total urinary cells although the strategy still yielded poorly purified populations. Unlike CD4+ T cells from peripheral blood, urinary CD4+ T cells exhibited reduced expansion capacity *in vitro* even when the length of expansion time was prolonged (Figure 28C). This observation indicated exhaustive properties of urinary CD4+ T cells when compared with CD4+ T cells from the peripheral blood, probably because these cells have experienced multiple activation processes when migrating from the blood or from the secondary lymphoid organs into the kidneys and from the kidneys to the urinary tract. Furthermore, urine might provide an unideal physiological environment to the immune cells. However, urinary CD4+ T cells were still functional in terms of their viability and metabolism, making it possible to characterize them for further analyses *in vitro*. Importantly, expanded urinary CD4+ T cells also displayed antigen-specific proliferation capacity. Libraries of amplified urinary CD4+ T cells were evaluated for their proliferation capacity in response to a superantigen SEB, where microcultures containing urinary CD4+ T cells stimulated with SEB showed increased ^{3}[H]-thymidine uptake when compared with unstimulated microcultures (Figure 28D). These data imply that CD4+ T cells isolated from urine were functional viable cells and can be used to facilitate studies of tissue-resident CD4+ T cells in the kidneys at antigen-specific level.

3.2. Urinary CD4+ T cells library

Methods that enable the detection of human tissue and antigen-specific T cells are to date extremely limited, thus studies of human T cells have primarily focused on circulating blood, representing an incomplete view of the human immune system such as cancer immunity, host defence, allergy, and autoimmunity. Recently, using extensive tissue cell isolation and mass cytometry, a systematic map of T cell phenotypes throughout the human body has been established but without analysis of kidney-specific T cells [216]. In chapter 4 we have shown frequencies of autoreactive CD4+ T cells in peripheral blood using the T cell library technique. In parallel, we had the possibilities to analyse urine samples of three out of six active SLE patients, all of which were patients with lupus nephritis. We extended the protocol for the peripheral blood CD4+ T cell library method to generate a "urinary CD4+ T cell library" technique as described in section 2.3. of this chapter. Simultaneous generation of libraries of amplified CD4+ T cells derived from whole blood and urine facilitated determination of the frequencies of antigen-specific CD4+ T cells in the circulating blood and in the tissue at the same time point, which might be helpful to predict the tissue migration and localization of those cells.

Our urinary CD4+ T cell library data showed that SLE-associated autoantigen-specific CD4+ T cells were detectable in the urine of three active SLE patients with lupus nephritis (Figure 29A). When compared with the autoreactive CD4+ T cells in the blood, urinary autoreactive CD4+ T cells showed more broad specificity against all SLE-associated autoantigens in all three donors. The ability of the urinary CD4+ T

cell library technique to detect autoreactive CD4+ T cells in urine has made this method a promising tool to predictively enumerate the number of such cells in the renal tissue using Poisson distribution (Figure 29B). By calculating the median frequencies of each SLE-associated autoantigen-specific CD4+ T cell population in the peripheral blood and urine, we observed increased numbers of autoreactive CD4+ T cells in the urine compared to circulating blood (SmD1: blood = 0 vs. urine = 64.52 cells/million cells; RNP70: blood = 16.13 vs. urine = 87.72 cells/million cells; Histone: blood = 5.44 vs. urine = 52.63 cells/million cells; Ro: blood = 16.30 vs. urine = 64.52 cells/million cells; La: blood = 37.60 vs. urine = 52.63 cells/million cells). In summary, urine of active SLE patients with lupus nephritis contained three-fold greater frequency of SLE-associated autoantigen-specific CD4+ T cells than the peripheral blood (median number: blood = 96.77 vs. urine = 298.20 cells/million cells).

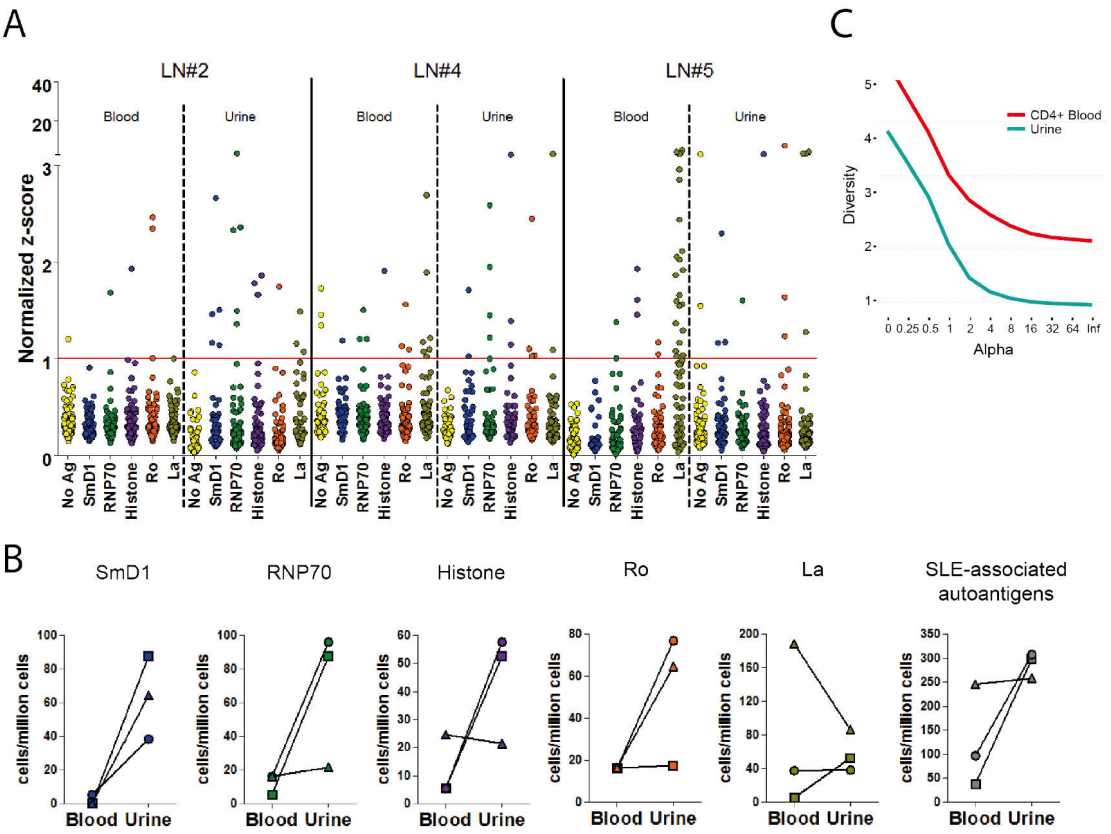


Figure 29: **Urinary CD4+ T cell library.** (**A**) Normalized *z*-score values of microcultures containing amplified CD4+ T cells isolated either from blood or from urine of active SLE patients with lupus nephritis (*n*=3) were calculated by assessing proliferation capacity of CD4+ T cells reflected by ^{3}[H]-thymidine uptake in response to antigen stimulation. Red line represents the proliferation threshold. Microcultures with a normalized z-score value higher than the threshold were predicted to contain at least one antigen-specific CD4+ T cell clone. (**B**) The frequencies of particular and total SLE-associated autoantigen-specific CD4+ T cells were enumerated by Poisson distribution. (**C**) The Rényi profiles of peripheral CD4+ cells and urinary cells from active SLE patients with LN (*n*=5) were calculated by pooling 20 most abundant clones from each patients. The usual alpha-value of 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 were applied to determine the clone's diversity within each population.

Patients with lupus nephritis in active disease flare were characterized with increased numbers of both CD4+ and CD8+ T cells in urine during disease progression as reported by Dolff *et al.*, in which they monitored the number of urinary T cells within donors with lupus nephritis in their inactive and active stage [217]. However, an

elevated T cell number in urine is not accompanied by a wide heterogeneity of the TCR repertoire but in contrast, our urinary TCR sequencing data revealed bottlenecked diversity of the TCR-β repertoire suggesting enrichment of antigen-specific cells in the kidneys (Figure 29C, kindly provided by Anna-Sophie Grießbach, data analysis was performed by Dr. Ulrik Stervbo. The data is incorporated in the doctoral thesis of Anna-Sophie Grießbach. The graph has been reworked to fit with the data presentation in this thesis). This claim is supported by our previous study that CD4+ T cells in urine displayed extensive CD154 and Ki67 expression [210]. Urinary CD4+ T cell library data showed increased median numbers of SLE-associated autoantigen-specific CD4+ T cells in urine when compared with the numbers in the circulating blood indicating SmD1, RNP70, Histone, Ro, and La as potential autoantigens that induce autoimmune kidney inflammation in patients with lupus nephritis. Identification of target autoantigens in lupus nephritis is essential for a better understanding of inflammatory responses in severe lupus nephritis to promote progress in treatment for this disease. However, it is possible that autoantigen diversity in lupus nephritis is not limited to the aforementioned nuclear proteins, because autoantibodies identified in proliferative active nephritis can target up to 50 different autoantigens [218].

3.3. Antigen-reactive urinary CD4+ T cell-enrichment

Our effort to detect autoreactive CD4+ T cells in urine of active SLE patients with lupus nephritis was expanded by the second approach utilizing the concept and procedure of the ARTE method. CD4+ T cells were isolated from urine and labelled with CFSE. These CFSE-labelled urinary CD4+ T cells were then mixed with donor PBMC and stimulated with a mixture of SLE-associated autoantigens or left unstimulated. This technique allows simultaneous stimulation of peripheral blood and urinary CD4+ T cells and their assessment according to CD154 and CD69 expression. Due to elevated background frequencies of peripheral blood autoreactive CD4+ T cells, comparison of autoreactive CD4+ T cells in the blood and urine was performed by calculating an antigen-specific stimulation index.

After stimulation and subsequent antigen-specific enrichment, CD154+CD69+ CFSE-labelled urinary CD4+ T cells could be detected in all donors both in unstimulated and antigen-stimulated samples (Figure 30A). We then aimed to compare the detection of urinary autoreactive CD4+ T cells assessed by urinary CD4+ T cell library and antigen-reactive urinary CD4+ T cell-enrichment method using calculation of an antigen-specific stimulation index percentage. Although the median percentage of the antigen-specific stimulation index of urinary CD4+ T cell assessed by library method was higher than the antigen-specific stimulation index of antigen-reactive urinary CD4+ T cell assessed by enrichment method, the difference was not significant (median percentage: urinary CD4+ T cell library = 17%, antigen-reactive urinary CD4+ T cell-enrichment = 2.79%; $P = 0.4$, by Mann-Whitney test) (Figure 30B). This result demonstrates that both techniques provide a different level of sensitivity for the detection of urinary autoreactive CD4+ T cells. Furthermore, we pooled the antigen-specific stimulation index data from both methods and compared the values of autoreactive CD4+ T cells in blood and urine. Notably, we found that the percentage of antigen-specific stimulation index of urinary cells was significantly higher than the stimulation index of PBMC (median percentage: blood = 2.46%, urine = 4.33%; $P = 0.0156$, by Mann-Whitney test) and all donors showed higher antigen-

specific stimulation indices in urine than in blood (Figure 30C). This observation suggests accumulation of SLE-associated autoantigen-specific CD4+ T cells in the kidneys of active SLE patients with lupus nephritis.

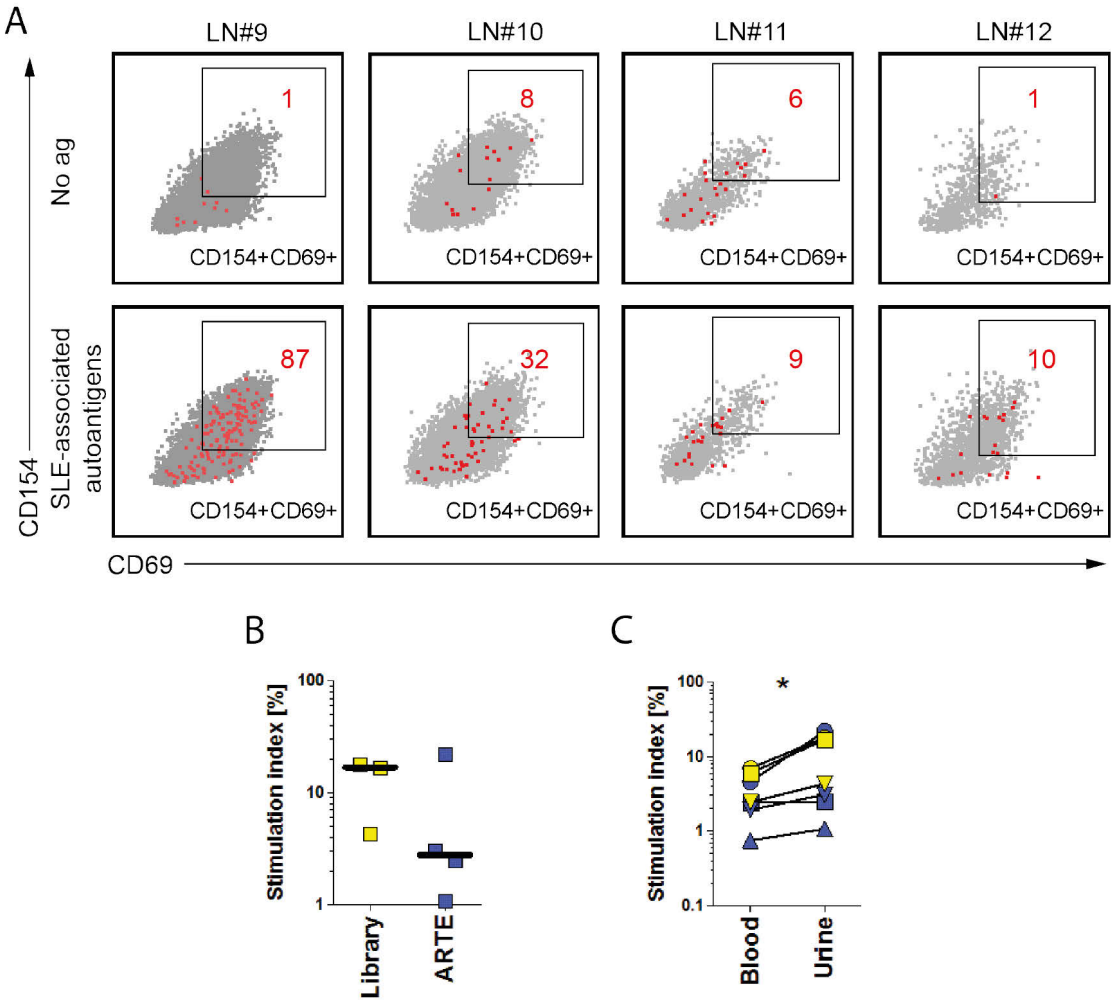


Figure 30: **Antigen-reactive urinary CD4+ T cell-enrichment.** (**A**) Flow cytometry dot plots display gating strategy of CD154+CD69+ CD4+ T cells in the unstimulated sample (top row) and antigen-stimulated sample (bottom row) of active SLE patients with lupus nephritis (*n*=4). Grey background represents CD4+ T cells of peripheral blood, whereas red dots represent CFSE-labelled urinary CD4+ T cells which were mixed with donor PBMC. Red numbers indicate the absolute number of detected CD154+CD69+ CFSE-labelled urinary CD4+ T cells after enrichment. (**B**) Stimulation index of urinary SLE-associated autoantigen-specific CD4+ T cells assessed by urinary CD4+ T cell library (library, *n*=3) and antigen-reactive urinary CD4+ T cell-enrichment (ARTE, *n*=4) method. Horizontal line represents median value. (**C**) Percentage of stimulation index of autoreactive CD4+ T cells in blood and urine of active SLE patients with lupus nephritis (accumulative, *n*=7) was compared for each donor. Yellow and blue symbols represent data measurement from urinary CD4+ T cell library and antigen-reactive urinary CD4+ T cell-enrichment, respectively. Statistical analysis was performed using Mann-Whitney test.

Accumulation of autoreactive CD4+ T cells in the kidneys of active SLE patients with lupus nephritis underlines a pathogenic role of these cells in mediating renal tissue inflammation and organ damage. However, open questions remain in our results, whether this accumulation in the kidneys represents infiltration of autoreactive CD4+ T cells from the circulating blood or whether these cells are tissue-resident memory T cells (Trm) that already exist in the kidneys. The theory of local kidney inflammation mediated by autoreactive Trm cells in lupus nephritis has evolved from the finding

that individual CD8+ T cell clones in renal inflammatory infiltrates persisted for long time in repeated biopsy samples from SLE patients with lupus nephritis [219]. The characteristics of Trm-mediated autoimmunity are displayed by its rapid response to antigens that can occur within hours of antigen exposure, because Trm cells are already on site and do not recirculate back to peripheral blood [220]. This kind of quick T cell response could be contradictive with the development of nephritis in almost all autoimmune kidney diseases, which is often initiated by deposition of immune complexes and autoantibodies in the kidneys prior to autoimmune inflammation triggered by the cells from the adaptive immune system [221]. On the other hand, tissue-specific autoimmune inflammation can be induced by the invasion of autoreactive T cells migrating to the organ where their cognate antigens are present abundantly, such as in the case of type-1 diabetes (T1D). The study conducted by Babon *et al.* visualized direct outgrowth of T cells from handpicked islets of patients with T1D, without T1D, and with type-2 diabetes (T2D), where they found that T cell outgrowth was only observable in patients with T1D, neither in patients without T1D nor in patients with T2D. Thus, their study indicated that autoreactive T cells in the islets of patients with T1D are infiltrating cells rather than lymphocytes that reside in islet capillaries [222]. Using intravital imaging in the study of multiple sclerosis, infiltration of autoreactive CD4+ T cells to the central nervous system has been successfully visualized, demonstrating clear evidence that autoreactive CD4+ T cells are recruited to the tissue to mediate autoimmune pathology [223]. Finally, analysis of adoptive transfer of CD4+ T cells transduced with TCR from T cells isolated from nephritic MRL/lpr mice showed progressive promotion of nephritis-like clinical manifestations in the recipient mice emphasizing infiltration of autoreactive CD4+ T cells from the periphery to the inflamed kidneys in lupus nephritis [224].

In our previous study, we have identified that 60% of cells in renal biopsy tissues were CXCR3+ CD4+ T cells, compared to 50% of urinary cells and 22% of PBMC [212]. The frequency of CXCR3+ CD4+ T cells in urine of patients with lupus nephritis correlated with disease activity and indicated recruitment into the inflamed kidneys before the cells are enriched in the urine [225]. The ligand of CXCR3, CXCL10, is produced in renal tissues making it possible for CXCR3-expressing CD4+ T cells to co-localize *in situ*. CD4+ T cells expressing homing receptor CXCR3 are mostly Th1 cells that express the transcription factor T-bet and can produce IFN-γ upon activation [22]. In line with this, we have characterized that SLE-associated autoantigen-specific CD4+ T cells significantly produced IFN-γ suggesting that these cells are directed to a Th1 phenotype. The disease pathogenesis in local renal tissues could probably be triggered by autoreactive Th1 cells. Autoantigens in form of nuclear proteins are present in the kidneys as free antigens or as part of immune complexes due to altered clearance mechanism of apoptotic materials. Autoreactive Th1 cells recognize the autoantigens presented by antigen-presenting cells and produce effector cytokines such as IFN-γ that promote macrophage activation and results in autoimmune inflammation. Some autoreactive CD4+ T cells as Tfh subset migrate to draining lymph nodes near the kidneys to support autoantibody production by the B cells. Furthermore, it has been reported that IL-17-producing cells or Th17 cells may also play role in the pathogenesis of nephritis [226], consistent with our data that IL-17 production by autoreactive CD4+ T cells in blood was significantly higher in active SLE patients. CFSE-labelled urinary CD4+ T cells in our experiments did not produce any kind of effector cytokines upon antigen stimulation, probably because the cells were already extremely exhausted so that the induction of cytokine

production was no more possible. Nevertheless, by taking our data together it could be that infiltrating Th1 or Th17 cells activate innate effector cells in the kidneys and may also directly affect renal tissues and promote kidney tissue inflammation [227].

4. Conclusion

In this chapter, we demonstrated novel methods to detect SLE-associated autoantigen-specific CD4+ T cells in the urine of active SLE patients with lupus nephritis that can reflect the condition in inflamed kidneys. CD4+ T cells isolated from urine were functionally and metabolically active cells but with exhaustive features probably because the cells have migrated through different tissues until they accumulated in the urine. Urinary immune cells have a selective TCR repertoire diversity that could indicate preferential accumulation of antigen-specific CD4+ T cells in the kidneys. With our established detection method, we could not only detect the presence of CD4+ T cells in urine with reactivity to nuclear proteins associated with SLE, but we could also enumerate their frequency enabling direct comparison with their frequency in the peripheral blood. Calculation of antigen-specific stimulation indices on both peripheral and urinary CD4+ T cells demonstrated increased frequencies of autoreactive CD4+ T cells specific to nuclear antigens in the urine of active SLE patients with lupus nephritis. This evidence confirmed the hypothesis of accumulation of CD4+ T cells with antigen specificity to SLE-associated autoantigens (Figure 31). We suggest circulating autoreactive CD4+ T cells as the origin of accumulated autoreactive CD4+ T cells in the urine that migrate and infiltrate into the kidneys, rather than the possibility that accumulated autoreactive urinary CD4+ T cells are kidney-specific Trm cells. Furthermore, majority of urinary CD4+ T cells express the homing receptor CXCR3, which is suggestive for the Th1 subset. IFN-γ was the primary effector cytokine produced by autoreactive CD4+ T cells following activation with SLE-associated autoantigens, thus Th1 or non-conventional Th1 cells are the predicted candidate of effector T cell subsets promoting antigen-specific inflammation through IFN-γ production that can activate cells from the innate immune system such as macrophages [228]. Since lupus nephritis has been also characterized by elevated IL-17 accumulation and autoantibody deposition, it is possible that Th17 cell subset also play a role in mediating local inflammation. In summary, our study here provides important insight about how to investigate the pathogenic role of autoreactive CD4+ T cells in tissue inflammation and organ failure using the model of lupus nephritis through our established state-of-the-art detection methods, which may have implications for better understanding of human systemic autoimmune diseases and for the design of successful immunotherapies.

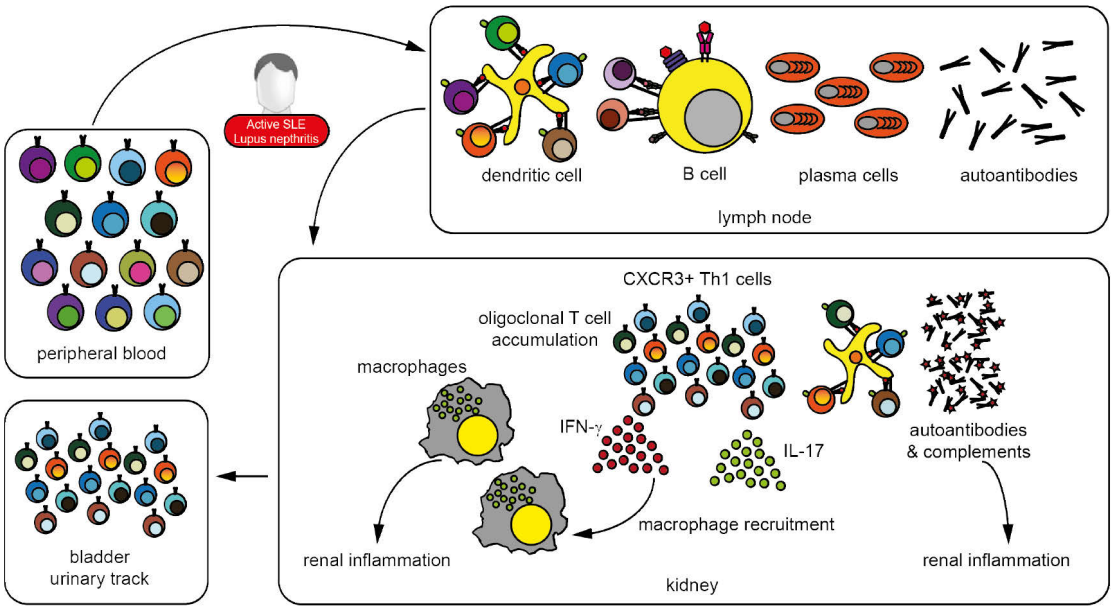


Figure 31: **Renal tissue inflammation in lupus nephritis.** Autoreactive CD4+ T cells migrate from the peripheral blood to the lymph node where they interact with the antigen-presenting cells that activate them to infiltrate renal tissue. Autoantigens, autoantibodies, and complements are present abundantly in the kidneys. Autoreactive CD4+ T cells in the kidney are oligoclonal with selective TCR repertoire and characterized with CXCR3 expression and IFN-γ (and IL-17) secretion. IFN-γ induces macrophage activation that leads to renal tissue inflammation. Autoreactive CD4+ T cells in the kidney migrate from interstitial space into the urinary track, therefore their accumulation is detectable in the urine.

Chapter 9

Identification of SLE-associated autoantigen-specific regulatory CD4+ T cells with their beneficial function in SLE

Abstract

Regulatory T cells (Treg) are a subset of CD4+ T cells with regulatory function to control excessive immune response such as in autoimmunity. Patients with SLE are characterized by altered Treg function and homeostatic imbalance between effector conventional T cells (Tcon) and Treg in terms of their proliferation capacity. The study of Treg in human autoimmune disease such as SLE has primarily focused on polyclonal Treg populations. Here we show for the first time the potential contribution of Tregs at antigen-specific level to the amelioration of disease pathogenesis of SLE. SLE-associated autoantigen-specific Treg were detected using ARTE method by enrichment of CD137, a marker of antigen-specific Treg. The frequency of CD137-expressing FoxP3+CD127low CD4+ T cells was assessed after *in vitro* stimulation with the autoantigens. Here, we found that SLE-associated autoantigen-specific CD4+ T cells outnumbered their regulatory counterparts in patients with active SLE. This imbalance of Tcon/Treg ratio correlated with disease activity and may explain how the autoreactive T cell-mediated autoimmunity in SLE develops. In addition, a representative Treg clone specific to SmD1 showed antigen-specific suppressive capacity against proliferation of a representative SmD1-specific CD4+ T cell clone. Our results imply the relevance and importance of antigen-specific Treg as a promising therapeutic tool for SLE.

Keywords: *Treg, TCR, CD137, Tcon/Treg ratio, SLEDAI, suppressive capacity*

Contents

1. Introduction

1.1. Background

Regulatory T cells (Treg) are a subset of CD4+ T cells with the regulatory capacity to inhibit unwanted immune responses such as autoimmunity. These cells were firstly identified as cells with expression of CD25 on the cell surface [36, 37], and further characterized by their expression of the master transcription factor FoxP3 [38, 39]. Proving their critical function in maintaining immune homeostasis, individuals with FoxP3 mutations suffer from the development of a severe autoimmune disease, the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [40, 41]. Treg are generally produced in the thymus but can also be induced from naive T cells in the periphery, in line with their plasticity characteristics [43, 44]. Thus, nomenclature for Treg has been recommended to distinguish Treg derived from the thymus and those developed in the periphery or induced *in vitro* [45]. The beneficial properties of Treg have become now a great interest for the development of Treg-based immunotherapy to treat patients with autoimmune disorders [46]. Several research groups, including our group, have conducted clinical trials focusing on Treg for treatment of patients with autoimmune diseases such as SLE.

In SLE and other human autoimmune diseases, Treg have been the focus of extensive research covering their function and implication in the pathogenesis such as their induction, maintenance, antigen specificity, trafficking and localization, proliferation, differentiation, and their potential clinical use (reviewed in [229]). Studies of Treg in human SLE demonstrated controversial data about their frequency (reviewed in [230]), which may help to explain the heterogeneity of the disease. Despite of this controversy, most reports on human SLE mainly demonstrate aberrant Treg function reflected by their abnormal phenotype. We and others have identified lack of CD25 expression on Treg of patients with SLE due to deprivation of its ligand, IL-2 [89, 231, 232]. Shortage of IL-2 in SLE results in homeostatic imbalance between conventional effector T cells and Treg and induction of autoimmunity [89]. In accordance with this finding, we also showed that administration of low-dose IL-2 can selectively correct the imbalance between conventional effector T cells and Treg and improves the amelioration of the disease [233]. Also in other autoimmune disease such as multiple sclerosis, impaired proliferation of Treg is attained in the IL-2-CD25 axis [234].

So far, studies of Treg in human SLE, as well as in other diseases such as allergy and cancer, are limited only to general and polyclonal Treg populations. Little is known about antigen-specificity of Treg and their implication in health and disease. This is because the knowledge of antigens that are recognized by Treg in certain diseases such as in SLE is missing. Furthermore, the detection of antigen-specific Treg is very challenging since the cells are present in a very rare frequency and the methods to detect antigen-specific Treg is still lacking. Few years ago, it has been found that activated alloreactive thymus-derived Treg and activated conventional T cells differ in their CD137 and CD154 expression signature, allowing dissection of activated Treg from activated conventional T cells at allospecific level [235]. By targeting CD137 expression on the cell surface of Treg after antigen stimulation, it is now possible to detect antigen-specific Treg [128]. Indeed, the detection of antigen-specific Treg is crucial to understand their contribution to autoimmune pathology.

1.2. Objectives

Given that the knowledge of antigen specificity of Treg is limited, the study presented in this chapter shall provide information about Treg in SLE at antigen-specific level, covering their frequency, antigen-specificity, and suppressive capacity. Using the ARTE method to enrich autoantigen-specific Treg based on their CD137 expression after stimulation with SLE-associated autoantigens, the ratio between autoantigen-specific conventional effector T cells and Treg should be determined. Treg stability in terms of FoxP3 expression during in vitro expansion and antigen-specific proliferation capacity should be assessed using a state-of-the-art method, Treg library. To this end, Treg-clones specific to an autoantigen should be generated to test the function of Treg to suppress proliferation of conventional CD4+ T cell clones specific to the same antigen.

2. Materials and methods

In the experiments presented in this chapter, the following methods were performed and they were described in the previous chapters: blood donors and isolation of peripheral blood mononuclear cells (chapter 2, section 2.1.), determination of cell numbers (chapter 2, section 2.2. and chapter 4, section 2.2.), antigen pool preparation (chapter 4, section 2.1.), antigen-reactive T cell enrichment (chapter 4, section 2.4.), antibody staining, cell fixation and permeabilization, and flow cytometry analysis (chapter 4, section 2.5.), determination of SLEDAI (chapter 4, section 2.6.), and statistics (chapter 4, section 2.7.), and antibody staining of intracellular compartment (chapter 6, section 2.1.).

2.1. Sorting of viable Treg

FoxP3 is widely used as a marker for Treg but FoxP3 is a transcription factor, thus it is expressed intracellular and cannot be used as a target molecule to isolate viable Treg since otherwise the cells need to be fixed and permeabilized to allow targeting the intracellular compartment. Therefore, we used a combination of IL-2 and IL-7 receptor expression on the cell surface, CD25 and CD127, respectively, to isolate viable Treg. Low expression of CD127 has been appreciated as a marker to dissect Treg from conventional CD4+ T cells [236]. In the study presented in this chapter, we isolated three different Treg subsets which are naive Treg, memory Treg, and Treg without discrimination of naive or memory phenotype. Cells were stained with the following fluorochrome-conjugated anti-human antibodies: antihuman-CD3-PacificBlue (clone UCHT1, Biolegend Inc., San Diego, USA), antihuman-CD3-PerCP (clone SK7, Biolegend Inc.), antihuman-CD4-PE-Cy7 (clone SK3, BD Biosciences, San Jose, USA), antihuman-CD4-Allophycocyanin-H7 (clone RPA-T4, BD Biosciences), antihuman-CD25-PE (clone 4E3, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), antihuman-CD25-Allophycocyanin (clone 4E3, Miltenyi Biotec GmbH), antihuman-CD45RO-PE-Cy7 (clone UCHL1, Biolegend Inc.), and antihuman-CD127-eFluor450 (clone eBioRDR5, Life Technologies Europe BV, Bleiswijk, The Netherlands) antibodies. Briefly, cells were washed with PBS/BSA and a cocktail of antibodies was added to the cell suspension. Cells were incubated for 15 minutes at 4°C and washed prior to acquisition on a flow cytometer. To

discriminate live and dead cell, LIVE/DEAD® Aqua kit (Life Technologies Europe BV) was applied. CD25+CD127low cells were sorted after gating on live CD3+CD4+CD45RO- for naive Treg phenotype, CD3+CD4+CD45RO+ for memory Treg phenotype, and on CD3+CD4+ for global Treg phenotype. Cells were sorted using BD FACSARIA™ II or BD Influx™ cell sorter (all BD Biosciences) at FCCF DRFZ using FACS®Diva software (BD Biosciences).

2.2. Intracellular staining of FoxP3

In the study presented in this chapter, staining of FoxP3 was performed to validate Treg sorting purity, to assess FoxP3 expression after in vitro culture, and to detect Treg in a diverse experimental system such as the CD137-enrichment method. The fixation and permeabilization procedure for intracellular staining of FoxP3 were performed using Foxp3 Transcription Factor Staining Buffer Kit (Life Technologies Europe BV) according to manufacturer's instruction. Antihuman-FoxP3-AlexaFluor488 (clone 259D, Biolegend Inc.) and antihuman-FoxP3-APC (clone 3G3, Miltenyi Biotec GmbH) antibodies were used to stain FoxP3.

2.3. Generation of dendritic cells from human monocytes

Stimulation of Treg with antigen requires professional antigen-presenting cells. For the experiment to detect antigen-specific Treg using libraries of amplified Treg, we used monocyte-derived dendritic cells as antigen-presenting cells rather than CD3-depleted PBMC. Since the frequency of dendritic cells is rare in the peripheral blood, we generated dendritic cells by subsequent *in vitro* stimulation and differentiation of human monocytes [237]. Briefly, up to $1x10^8$ PBMC were resuspended in 15mL very low-endotoxin RPMI 1640 medium containing L-Glutamin (VLE RPMI, Biochrom-Merck, Berlin, Germany) supplemented with 1.5% (v/v) human heat-inactivated AB serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The cell suspension was transferred into a T75 culture flask (BD Biosciences) and incubated for 50 minutes at 37°C and 5% CO_2. After 45 minutes, monocytes attached on the plastic surface and were observable as small round cells under the microscope. The supernatant was carefully collected and the attached cells were washed three times with low-endotoxin PBS solution without Ca^{2+} and Mg^{2+} (Biochrom-Merck). Collected cells were resuspended in 15mL of VLE RPMI medium with 1.5% (v/v) human heat-inactivated AB serum supplemented with 100ng/mL and 20ng/mL human recombinant GM-CSF and IL-4, respectively (all PeproTech GmbH, Hamburg, Germany). After 48 hours of incubation, 18mL of VLE RPMI medium with 1.5% (v/v) human heat-inactivated AB serum supplemented with 50ng/mL GM-CSF, 10ng/mL IL-4, 10ng/mL IL-1β, 10ng/mL TNF-α, 15ng/mL IL-6 (all PeproTech GmbH), and 1µg/mL PGE2 (Santa Cruz Biotechnology, Dallas, USA) was added into the culture and the cells were further incubated for 24 hours at 37°C and 5% CO_2. To validate the differentiation of monocytes to dendritic cells, an aliquot of cells was taken prior to as well as after cell culture and stained with following fluorochrome-conjugated antibodies for detection on flow cytometry: antihuman-CD3-PacificBlue (clone UCHT1), antihuman-CD3-PerCP (clone SK7), antihuman-CD83-AlexaFluor488 (clone HB15e) (all Biolegend Inc.), antihuman-CD14-VioGreen (clone TÜK4), antihuman-CD83-APC (clone HB15) (all Miltenyi Biotec GmbH), antihuman-CD80-PE (clone L307.4), and antihuman-CD86-FITC (clone 2331/FUN1) (all BD Biosciences) antibodies.

2.4. Generation of amplified Treg libraries

The protocol to generate amplified Treg libraries was established at our laboratory to detect the presence of antigen-specific Treg and to evaluate antigen-specific Treg response by their proliferation capacity after stimulation with antigen. Treg were sorted according to their phenotype as described in the section 2.1. To restimulate expanded Treg, monocyte-derived dendritic cells were used as antigen-presenting cells as described in the section 2.3. Briefly, ~25,000 isolated Treg were resuspended in IMDM medium with GlutaMAX^{TM} (Life Technologies Ltd.) supplemented with 5% (v/v) heat-inactivated human AB serum, 1% (v/v) Penicillin-Streptomycin, 2µg/mL ciprofloxacin (Sigma-Aldrich Chemie GmbH), and 1% (v/v) MEM non-essential amino acid solutions (Sigma-Aldrich Chemie GmbH), in the presence of each 1µg/mL antihuman-CD3 and antihuman-CD28 functional grade purified (clone OKT3, DRFZ), 1µg/mL antihuman-CD28 antibody (clone OKT3 and CD28.2, respectively, Life Technologies Ltd.) 600IU/mL IL-2 (Proleukin/Aldesleukin, Novartis Pharma GmbH, Nuremberg, Germany), 30ng/mL rapamycin (Sigma-Aldrich Chemie GmbH), and in 4 – 5x10^5 irradiated feeder cells from two non-autologous donors. Cells were distributed into 96-well plates (Greiner Bio-One AG, Kremsmünster, Austria) and incubated at 37°C with 5% CO_2. Medium in Treg library was replenished periodically after 3-4 days by removing 50% volume of culture medium in each microculture and adding fresh 1µg/mL antihuman-CD3 and antihuman-CD28 functional grade antibodies and 200IU/mL IL-2. After 2-4 weeks of culture, a fraction of amplified Treg was distributed into additional 96-well plates depending on the number of antigens to be analysed. Treg libraries for negative control (unstimulated cells) and positive control (cells stimulated with SEB) were always included in the experiments. Prior to stimulation with the antigens, cells were rested in the standard stimulation medium for at least four days. On the day of stimulation, antigen-presenting cells were thawed and distributed into the Treg culture in a ratio of at least one antigen-presenting cell to 100 Treg. Treg were stimulated with the antigen for four days, where at least 16 hours before measurement, 1µCi/mL ^{3}[H]-thymidine (GE Healthcare UK Ltd., Little Chalfont, United Kingdom) was added into the culture. We used following antigens to stimulate Treg in Treg library assay: 1µg/mL SEB (Sigma-Aldrich Chemie GmbH), 50µg/mL Aspergillus fumigatus lysate (Mitenyi Biotec GmbH), 1µg/mL CMV lysate (Acris Antibodies GmbH, Herford, Germany), and 0.5µg/mL SNRPD1 (SmD1) recombinant protein (Biorbyt Ltd., Cambridge, United Kingdom).

The analysis of Treg library data follows the procedures as described in the chapter 4, section 2.3., and chapter 8, section 2.4. The exclusion criterion for this analysis is similar with that of CD4+ T cell library, in which that the microcultures with SEB stimulation index lower than five were excluded. The generation of Treg library was part of our project for a thesis to a master student, Nicole Affinass, under our supervision.

2.5. Antigen-reactive CD137+ T cell-enrichment

The ARTE method that is used to detect antigen-specific CD4+ T cells based on CD154 expression can also be used to detect antigen-specific Treg according to their CD137 expression as shown by Bacher *et al.* [128]. The method is described as “Treg

ARTE" or "CD137-ARTE". After enrichment of CD154-expressing cells (as described in the chapter 4, section 2.4.), the negative fraction of each sample was collected and labelled with antihuman-CD137-APC antibodies (clone 4B4-1, Miltenyi Biotec GmbH) for 15 minutes at 4°C. Subsequently, anti-APC-microbeads antibodies (Miltenyi Biotec GmbH) were added to the cell suspension for 15 minutes at 4°C. After a washing step, cells were loaded onto calibrated MS column (Miltenyi Biotec GmbH) to enrich CD137-expressing cells. 60µL antibody master-mix to stain cell surface marker expression was prepared and applied to the cells in the MS column for 15 minutes at room temperature. The master-mix was prepared from following monoclonal antibodies: antihuman-CD3-VioGreen (clone BW264/56), CD4-Allophycocyanin-Vio770 (clone M-T321), and antihuman-CD127-eFluor450 (clone eBioRDR5, Life Technologies Europe BV, Bleiswijk, The Netherlands) antibodies. After 15 minutes incubation time, cells were washed with PBS/BSA/EDTA and eluted into 96-well plates. CD137-expressing cells were fixed, pemeabilized, and stained for FoxP3 expression as described in the section 2.2.

2.6. Generation of single-Treg clones

To further analyse the characteristics of Treg at antigen-specific level, we generated single-Treg clones reactive to SLE-autoantigen SmD1. Here, $5x10^6$ – $1x10^7$ PBMC were stimulated with 1µg/mL SmD1 or 1µg/mL CMV lysate on in 1mL RPMI 1640 medium with GlutaMAX™ (Life Technologies Ltd.) supplemented with 5% (v/v) heat-inactivated human AB serum and 1% (v/v) Penicillin-Streptomycin. 200µL cell suspension was transferred into 96-well plate. Cells were stimulated for 16 hours at 37°C with 5% CO_2 in the presence of 1µg/mL antihuman-CD40 antibody (generated at DRFZ), 200IU/mL IL-2, 1µg/mL antihuman-CD3 functional grade antibody, and 1µg/mL antihuman-CD28 functional grade antibody. After 16 hours, cells were washed and stained for surface marker expression similar to the procedure as described in the section 2.1. for sorting of viable cells. Single CD137-expressing cells were sorted after gating of CD3+CD4+CD25+CD127low cells using BD FACSARIA™ II or BD Influx™ cell sorter at FCCF DRFZ using FACS®Diva software (BD Biosciences). Single cells were expanded in vitro by using specialized medium similar to that described in the section 2.4. in presence of 2,000 irradiated feeder cells from two non-autologous donors. Medium was replenished periodically after 3-4 days by removing 50% volume of culture medium similar to that described in the section 2.4. Proliferation and FoxP3 expression was monitored periodically by counting on the cell counter and staining of FoxP3 as described previously. In addition to this method, SmD1-specific conventional CD4+ T cell clones were also generated as described in the chapter 5, section 2.2.

2.7. *In vitro* Treg suppression assay

Treg are characterized by their suppressive capacity to control proliferation of conventional CD4+ T cells. In order to understand whether this Treg function can also be observed at antigen-specific level, both polyclonal and antigen-specific Treg suppression assays were performed. First, an antigen-specific CD4+ T cell clone was labelled with CFSE, in the same procedure as described in chapter 8, section 2.5. At maximum $1x10^7$ CD4+ T cell clones were resuspended in 1mL cold PBS, then followed by addition of 1µM carboxyfluorescein diacetate *N*-succinimidyl ester (CFDA-SE, Sigma-Aldrich Chemie) for 3.5 minutes at room temperature in the dark.

After incubation, cells were washed twice with cold PBS/BSA at 310xg for 10 minutes at 4°C. Secondly, labelled antigen-specific CD4+ T cell clones were cultured in RPMI medium supplemented with 5% (v/v) FCS (prepared by DRFZ lab managers) at a concentration of $1x10^4$ cells per well in a 96-well round-bottom plate either in absence of Treg or in presence of varying amounts of unlabelled antigen-specific Treg clones at a Treg clone:CD4+ T cell clone ratio of 0:1, 10:1, 5:1, and 1:1. The clones were either polyclonally stimulated using antihuman-CD3 and CD28 purified grade antibodies or antigen-specific stimulated with 1µg/mL SmD1. After stimulation of four days, cells were fixed, permeabilized, and stained intracellularly with antihuman-FoxP3 antibody, antihuman-Helios antibody (clone: 2296), and antihuman-Bcl-2 antibody (clone: Bcl-2/100) (all BD Biosciences) as described in the section 2.2. to discriminate Treg clone from CD4+ T cell clone. Proliferation of CD4+ T cell clone was assessed by FACS of CFSE dilution, where each CFSE-peak represents a daughter cell population. To visualize the suppression, percentages of CFSEdim cells were shown.

2.8. TCR sequencing

In this chapter, TCR sequence of SmD1-specific CD4+ T cell clone and SmD1-specific Treg clone are shown. TCR sequencing was performed by the laboratory of Professor Wolfgang Uckert, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany. We sent SmD1-specific CD4+ T cell and Treg clones to the group of Professor Wolfgang Uckert for isolation and sequencing of TCR sequences in his laboratory using RACE-PCR-based technique and we received the information of sequenced TCR of the clones in form of CDR3 sequence of TCR-α and TCR-β chain.

3. Results and discussion

Treg play important role in maintaining immune homeostasis to inhibit unwanted immune responses such as autoimmunity. Aberrant Treg function is identified in patients with SLE at polyclonal level. In this thesis, we aimed to understand the role of Treg at autoantigen-specific level in SLE. We performed a broad range of methods and developed novel techniques to answer the question of antigen-specificity of Treg and their implication in the disease pathogenesis of SLE. We demonstrate here the capacity of Treg to proliferate in response to antigen-specific stimulation, the stability of Treg in terms of FoxP3 expression, their frequency in correlation with SLE disease activity, and their suppressive function.

3.1. Detection and characterization of antigen-specific Treg

We sought to understand the antigen-specificity of Treg, whether Treg are activated upon antigen-specific stimulation as displayed by their capacity to proliferate. To date, methods to evaluate antigen specificity for Treg are not widely available. In our work, we developed libraries consisting of amplified Treg (Treg library) to detect the presence of antigen-specific Treg in the periphery. This effort required understanding about Treg physiology such as how Treg recognize antigen presented by antigen-presenting cells, how they expand in response to polyclonal stimulation, and how they maintain their stability. Because for the library method it is necessary to amplify the clones, we needed to establish cell culture procedure enabling efficient expansion of

Treg. Treg expansion *in vitro* is challenging and it is indeed of great interest because Treg are potential immunotherapeutic tool for inflammatory diseases. Treg expansion protocols that comply with GMP standards are currently under deep research.

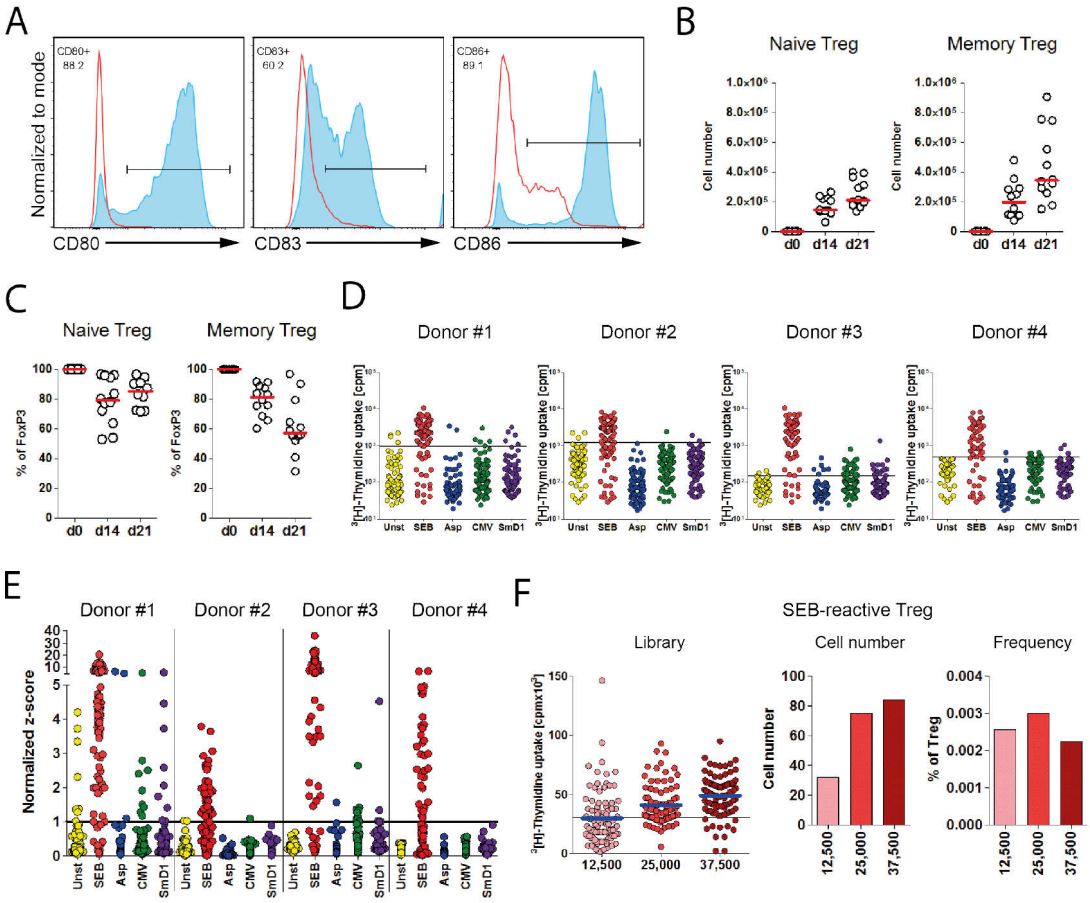


Figure 32: **Detection of antigen-specific Treg by Treg library.** (**A**) Representative histograms show upregulation of cell surface molecules CD80, CD83, and CD86 expressed by human monocyte-derived dendritic cells after *in vitro* differentiation of monocytes to dendritic cells. Open red line represents monocytes before differentiation to dendritic cells and closed blue line represents dendritic cells after differentiation from monocytes. (**B**) Number of CD45RO-FoxP3+ cells (naive Treg) and CD45RO+FoxP3+ (memory Treg) after two and three weeks of *in vitro* expansion culture. Red line represents median cell number. (**C**) Expression of FoxP3 by naive and memory Treg after two and three weeks of *in vitro* expansion culture. Red line represents median percentage of FoxP3 among CD3+CD4+ cells. (**D**) Libraries of Treg from healthy individuals (n=4) were tested for their capacity to proliferate after stimulation with SEB, *Aspergillus fumigatus* (Asp), cytomegalovirus (CMV), and SmD1, or left unstimulated. Proliferation was assessed by ^{3}[H]-thymidine uptake. (**E**) Normalized z-score values of microcultures containing amplified Treg isolated either from blood of healthy individuals (n=4) were calculated by assessing proliferation capacity of Treg reflected by ^{3}[H]-thymidine uptake in response to antigen stimulation. Microcultures which normalized z-score value was higher than the threshold were predicted to contain at least one antigen-specific Treg clone. (**F**) Microcultures containing different number of Treg were pulsed with SEB to validate the technique in providing cell numbers and frequency of antigen-specific Treg. Blue line represents mean cpm value. For (**D**, **E**, and **F**) black line represents the proliferation threshold.

Given the condition requiring stimulation of amplified Treg clones in the microcultures to assess their antigen-specificity, appropriate antigen-presenting cells are necessary. Instead of CD3-depleted PBMC as antigen-presenting cells, we used dendritic cells as these cells were able to induce antigen-specific activation to Treg [238]. It has been shown that *in vivo* antigen-presenting dendritic cells were able to

induce Treg proliferation and to convert naive CD4+ T cells into suppressor FoxP3+ cells [239]. Because the population of human dendritic cells is limited and therefore the number was not sufficient for our study, we generated dendritic cells from human monocytes *in vitro*. Prior to differentiation, monocytes expressed low level of co-stimulatory molecules such as CD80, CD83, and CD86, which were upregulated following induction into dendritic cells (Figure 32A). The phenotype of *in vitro*-generated dendritic cells was similar with native dendritic cells under the microscope. Furthermore, we analysed our expansion system by counting the number of Treg after two and three weeks of culture. Here, Treg subsets were dissected based on CD45RO expression, to distinguish between naive (CD45RO-FoxP3+ CD4+ T cells) and memory Treg (CD45RO+FoxP3+ CD4+ T cells) population. Naive and memory Treg population in our system expanded following two and three weeks of polyclonal stimulation, in which that some memory Treg cultures demonstrated high expansion capacity (Figure 32B). This observation contradicted previous reports about poor proliferation capacity of memory Treg *in vitro* [240], but on the other hand, this observation was important because memory Treg are attributed with high immunosuppresive capacity thus their effective expansion *in vitro* provides promising therapeutic development [241]. However, in line with the finding by Miyara *et al.*, memory Treg in the culture gradually lost their FoxP3 expression, while naive Treg could maintain their FoxP3 expression (Figure 32C), probably due to the methylation status of their *FOXP3* gene [240]. Repetitive *in vitro* stimulation of memory Treg may cause loss of FoxP3 expression and emergence of conversion into effector T cells producing pro-inflammatory cytokines, which is displayed by the increase of CpG methylation in a conserved region within the *FOXP3* gene locus [44].

Treg library opens the possibility to assess antigen-specific proliferation capacity of amplified Treg clones in response to a wide variety of antigens. We tested antigen-specific proliferation of Treg from four donors to superantigen SEB, lysate of *Aspergillus fumigatus* (Asp) and cytomegalovirus (CMV), as well as SLE-associated autoantigen SmD1. We compared their antigen-specific proliferation capacity with their basal proliferation in absence of antigen. Antigen-specific Treg proliferation could be identified in all donors reflected by thymidine uptake that was assessed in counts per minute (Figure 32D). By normalizing the donor-specific *z*-scores, microcultures containing antigen-specific Treg could be identified (Figure 32E). SEB-reactive Treg were found in all donors. Donor #1 showed the most antigen-specific Treg detection but the number of cell proliferation in microcultures without antigen-stimulation was high, hindering conclusive evidence about their antigen-specificity. Donor #2 and #4 showed minimal antigen-specific Treg detection, while donor #3 revealed detection of Treg specific to all antigens tested. To confirm our Treg library assay, we generated libraries consisting different initial numbers of Treg. These libraries were tested for reactivity against SEB and showed, that thymidine uptake was increased in the libraries consisting of higher number of initial cells (Figure 32F), while cpm value in the unstimulated libraries remained similar for all initial cell numbers. Using Poisson distribution we could calculate the number of SEB-reactive Treg, where the higher is the initial Treg number in the library, the more SEB-reactive Treg can be detected, but the frequency remained nearly constant at around 0.002% of total Treg (Figure 32F). Taken together, we could establish a system to detect the presence of antigen-specific Treg and in parallel also evaluate their characteristics in term of their polyclonal expansion *in vitro* and FoxP3 expression stability during cell

culture. Our cell culture method could expand Treg with memory phenotype comparable with naive Treg, although FoxP3 expression was lost over the time.

3.2. Autoreactive Treg and autoreactive CD4+ T cells in SLE

The detection of antigen-specific Treg including Treg with reactivity to SmD1 provides a rationale to study autoreactive Treg in autoimmune diseases such as SLE. Treg-mediated immune suppression in autoimmunity may likely operate in a synergistic and sequential manner including outcompeting of autoreactive Treg with autoreactive conventional effector CD4+ T cells (Tcon) for interaction with antigen-presenting dendritic cells, modulation of the antigen-presenting cell function by supporting down-regulation of costimulatory molecules on dendritic cells (such as CD80, CD83, and CD86), and killing or inactivation of autoreactive Tcon by secreting granzyme or perforin or anti-inflammatory cytokines [229, 242]. Considering these mechanisms, homeostatic balance between the number of autoreactive Tcon and Treg for a particular antigen is critical and must be achieved, importantly at antigen-specific level. Thus, the analysis of the number of autoreactive Tcon and Treg in individuals with autoimmune disease and in healthy individuals could be a powerful approach to identify immune imbalance as potential source of autoimmune pathology. We have shown previously that the ratio of proliferative Tcon and Treg was disturbed in favour of Tcon proliferation at polyclonal level in lupus murine models and in humans with SLE resulting in promotion of disease pathogenesis of SLE [89, 233].

We aimed to investigate the ratio of Tcon and Treg that are reactive to a pool of SLE-associated autoantigens in patients with active SLE, patients with inactive SLE, and healthy individuals. To facilitate direct identification and comparison of autoreactive Tcon and Treg within the same sample from an individual, we used ARTE technique that was developed to enrich CD154 and CD137 expression for targeting Tcon and Treg, respectively [243]. Unlike the original procedure from Bacher *et al.*, we analysed the antigen-reactive Tcon and Treg in a sequential manner after stimulation with antigens, i.e. first by isolating antigen-reactive Tcon, collecting the negative fraction, and isolating autoreactive Treg from the negative fraction of autoreactive Tcon. Using this procedure, we could interrogate the absolute number of autoreactive CD154+ Tcon and CD137+ Treg and avoid cross-contamination between autoreactive Tcon and Treg since it was reported that antigen-specific Treg could also express CD154 after antigen stimulation [244], Tcon can express CD137 after antigenic stimulation [235], and Treg could lose their FoxP3 expression during activation. CD137-ARTE system has been used to simultaneously detect Tcon and Treg with reactivity to food antigens, commensal bacteria, infectious antigens and vaccines, and aeroantigens [245]. The application of this technique for detection of autoantigen-specific Treg has so far never been performed. Our analyses started with the comparison of total FoxP3+ CD4+ T cell frequency (Treg) in population of patients with active SLE, inactive SLE and healthy individuals. As previously reported, the frequency of Treg in all groups was between 5 – 10% of total CD4+ T cells (median frequency among CD4+ T cells: healthy individuals = 7.33%; inactive SLE = 7.99%; active SLE = 7.52%) (Figure 33A). However, patients with active SLE had a broader range of Treg frequency (range of frequency among CD4+ T cells: healthy individuals = 2.33 – 10.00%; inactive SLE = 4.49 – 15.80%; active SLE = 1.50 – 22.30%). This variety of Treg frequency among patients with active SLE may explain

the complexity of clinical parameters in SLE (referred to [230]). Moreover, we found no correlation between Treg frequency and SLE disease activity indicating that total number of Treg may not directly have an implication to the disease.

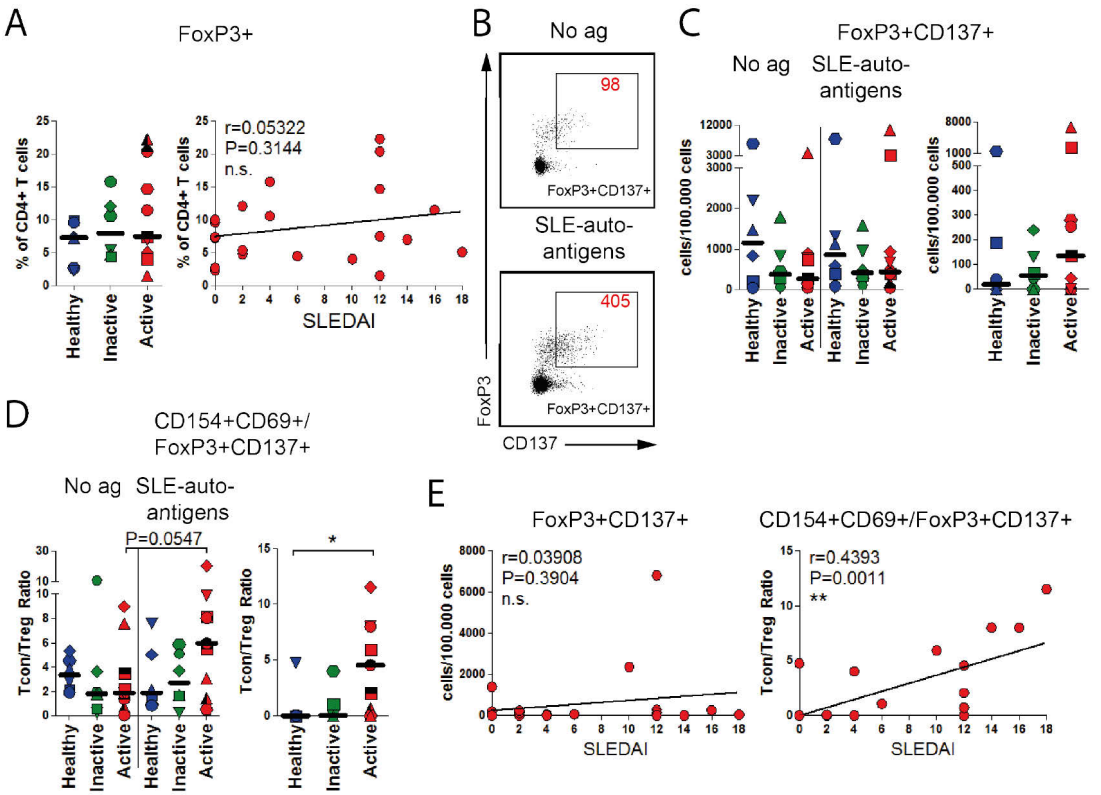


Figure 33: **Autoreactive Treg and autoreactive CD4+ T cells in SLE.** (**A**) Frequency of FoxP3+ cells (Treg) was assessed among CD4+ T cells in healthy individuals (*n*=6), patients with inactive SLE (*n*=6), and patients with active SLE (*n*=9) (left), and the frequency of Treg was compared with SLEDAI (right). (**B**) Dot plots show gating strategy for detection of antigen-reactive Treg based on their CD137 expression without antigen stimulation (no ag) and after stimulation with SLE-associated autoantigens (SLE-autoantigens). Red number indicates absolute number of CD137+FoxP3+ cells (antigen-reactive Treg). (**C**) Frequencies of CD137-expressing Treg without antigen stimulation (no ag, background) and after stimulation with SLE-associated autoantigens (SLE-autoantigens) were determined in healthy individuals, patients with inactive SLE, and patients with active SLE (left) and the frequency of CD137-expressing Treg after subtraction of background (right). (**D**) Ratio of the absolute number of CD154+CD69+ and FoxP3+CD137+ CD4+ T cells for samples without antigen stimulation (no ag, background ratio) and samples after stimulation with SLE-associated autoantigens (SLE-autoantigens) were calculated in healthy individuals, patients with inactive SLE, and patients with active SLE (left) and the ratio of absolute number of CD154+CD69+ and FoxP3+CD137+ CD4+ T cells after subtraction of background ratio (right). (**E**) Background-subtracted frequencies of FoxP3+CD137+ cells after stimulation with SLE-associated autoantigens (left) and background-subtracted ratio of absolute number of CD154+CD69+ and FoxP3+CD137+ CD4+ T cells were compared with SLEDAI. For (**C** & **D**), when the background frequency or background ratio was higher than the antigen-specific frequency and ratio, background-subtracted antigen-specific frequency or ratio was defined as zero. For (**A**, **C**, and **D**), short black line represents the median value. For (**A** & **E**), r indicates Pearson's correlation coefficient. Long black line represents linear regression determined using Pearson's correlation coefficient.

In our experiments, PBMC were stimulated with SLE-associated autoantigens or left unstimulated, then CD154-expressing cells were enriched from each sample, and finally CD137-expressing cells were isolated from the negative fraction of the same sample. Thus, we could calculate the absolute number of CD154+CD69+ CD4+ T cells (which we defined as antigen-specific Tcon) and of FoxP3+CD137+ CD4+ T

cells (which we defined as antigen-specific Treg) (Figure 33B). FoxP3+CD137+ CD4+ T cells in our experiments demonstrated low expression of CD127 (data not shown) expanding definition of Treg as FoxP3+CD127low CD4+ T cells. CD137-expressing FoxP3+ CD4+ T cells in samples without stimulation revealed higher number of non-antigen-specific activated Treg in healthy individuals followed by patients with inactive SLE and patients with active SLE (median frequency: healthy individuals = 1,156 cells/100,000 cells; inactive SLE = 377.4 cells/100,000 cells; active SLE = 271.6 cells/100,000 cells) (Figure 33C). Treg reactive to SLE-associated antigens were observed to be present at similar frequencies as the background population (median frequency: healthy individuals = 861.9 cells/100,000 cells; inactive SLE = 420.1 cells/100,000 cells; active SLE = 445.2 cells/100,000 cells). High background of CD137-expressing Treg was also reported in the initial study using this technique [246]. Therefore, to obtain the absolute number of antigen-reactive Treg, background frequency was subtracted. Surprisingly, after subtraction of background, the frequency of SLE-associated autoantigen-specific Treg was higher in patients with active SLE, followed by patients with inactive SLE and healthy individuals, although the difference was not significant (median frequency: healthy individuals = 20.32 cells/100,000 cells; inactive SLE = 50.97 cells/100,000 cells; active SLE = 134.6 cells/100,000 cells). The results raised a question why higher number of autoreactive Treg could not maintain suppression of autoimmunity in SLE. There are at least two possible explanations. First, higher number of autoreactive Treg in the inflammation could be a mechanism how our body responds to inflammation, which is by expanding the number of Treg specific to pathogenic antigens, but the number of these cells was not sufficient to control inflammation at antigen-specific level. Secondly, it could be that these cells, although existing in higher numbers, fail to mediate immune suppression due to aberrant suppressive capacity.

To answer the question about possible antigen-specific mechanism involving Treg in SLE, we compared the absolute number of antigen-specific Tcon and Treg in unstimulated and antigen-stimulated samples to obtain Tcon/Treg background ratio and antigen-specific ratio, respectively. The number of autoreactive Tcon was retrieved from the absolute number of CD154+CD69+ CD4+ T cells as presented in chapter 4. Healthy individuals had the highest background Tcon/Treg ratio when compared with SLE patients in both inactive and active stage (median ratio: healthy individuals = 3.36; inactive SLE = 1.83; active SLE = 1.90), which could mean that unspecific activated Tcon in healthy individuals appear to be more abundant than activated Treg in healthy individuals (Figure 33D). However, after antigen-specific stimulation with SLE-associated autoantigens, healthy individuals showed lower autoreactive Tcon/Treg ratio followed by patients with inactive SLE and patients with active SLE (median ratio: healthy individuals = 1.90; inactive SLE = 2.72; active SLE = 5.97). The ratio of autoreactive Tcon/Treg was not significantly different among three groups (healthy individuals vs inactive SLE, $P = 0.8182$; healthy individuals vs active SLE, $P = 0.1447$; inactive SLE vs active SLE, $P = 0.1447$, by Mann-Whitney test). Autoreactive Tcon/Treg ratio in patients with active SLE was clearly increased when compared with other groups and it showed nearly significant difference with the background ratio ($P = 0.0547$, by Wilcoxon signed-rank test). To better visualize whether Tcon/Treg ratio in active SLE was elevated, we subtracted antigen-specific Tcon/Treg ratio with the background Tcon/Treg ratio. Our results revealed that healthy individuals and patients with inactive SLE had comparable amount of autoreactive Tcon and Treg, where patients with active SLE showed increase in the

number of autoreactive Tcon when compared with the number of autoreactive Treg (median ratio: healthy individuals = 0; inactive SLE = 0.03; active SLE = 4.56) although the ratio was significant only when compared with the population of healthy individuals (healthy individuals vs inactive SLE, $P = 0.446$; healthy individuals vs active SLE, $P = 0.04$; inactive SLE vs active SLE, $P = 0.082$, by Mann-Whitney test). Our findings emphasize that, although patients with active SLE had a higher number of autoreactive Treg, these cells were not sufficiently abundant to encounter the reactivity of autoreactive Tcon. This imbalance between the number of Tcon and Treg at antigen-specific level may probably explain the development of autoimmunity in SLE. Importantly, when comparing the frequency of background-subtracted CD137-expressing Treg with the disease activity after stimulation with SLE-associated autoantigens, no correlation was identified (Figure 33E). But background-subtracted Tcon/Treg ratio showed a significant correlation with SLE disease activity, indicating that the amount of Tcon and Treg at antigen-specific level is indeed a factor that could compromise disease progression.

Autoantigen is critical in maintaining Tcon/Treg balance in a steady-state since *in vivo* persistent exposure of Tcon and Treg with tissue-specific self-antigen was first reported to enable preservation of self-reactive Treg but depletion of self-reactive Tcon. However, other groups have later argued that the tolerance to the tissue-specific self-antigen is mediated by autoantigen-specific Treg rather than depletion of autoantigen-specific Tcon [247], where autoantigen-specific Treg-mediated tolerance is lost upon repetitive antigen challenges [248]. Abundant amounts of autoantigens as a result of defective clearance mechanism of apoptotic materials from nucleus may reflect the conditions in SLE. Underlying the importance of antigen-specific Treg in maintaining the function of antigen-specific Tcon, Bacher *et al.*, could show using CD137-ARTE technique that antigen-specific Tcon responses can be counterbalanced by a strong antigen-specific Treg response. They also found in model of allergy that patients with cystic fibrosis demonstrated imbalance of *Aspergillus fumigatus*-specific Tcon/Treg ratio in favour of expansion of Th2 cells [128]. Taken together, imbalance in Tcon/Treg ratio could represent a major evidence of breakdown of Treg-mediated tolerance at antigen-specific level.

3.3. Autoreactive Treg and production of effector cytokines in SLE

After identification of antigen-specific Tcon/Treg imbalance, we then aimed to figure out which autoreactive effector T cells are superior in terms of cell number to Treg with identical antigen-recognition in SLE. As described in the chapter 6, autoreactive CD4+ T cells produce effector cytokines such as IFN-γ, IL-17, IL-4, and IL-10 upon stimulation with SLE-associated autoantigens, where prominent production of IFN-γ was observed in patients with active disease. Here, we wanted to know whether the imbalance of Tcon/Treg ratio is also found at the cytokine-production level. In order to analyse this evidence, we compared the frequencies of cytokine-producing autoreactive CD4+ T cells (defined as cytokine-producing autoreactive Tcon) with the frequency of autoreactive Treg. In addition, to identify whether autoreactive Treg produce anti-inflammatory cytokine such as IL-10, we also analysed the frequency of IL-10-producing Treg in our study populations.

Firstly, we found that stimulation of Treg with SLE-associated autoantigens could significantly increase the frequency of IL-10-producing autoreactive Treg in patients

with active SLE, but no in patients with inactive SLE neither in healthy individuals (*P* = 0.313, by Mann-Whitney test) (Figure 34A). Moreover, ratio between IFN-γ-producing Tcon and autoreactive Treg was significantly increased in patients with active SLE (median ratio: healthy individual = 0.084; inactive SLE = 0.015; active SLE = 0.202. Healthy individual vs inactive SLE, *P* = 0.0260; healthy individual vs active SLE, *P* = 0.0176; inactive SLE vs active SLE, *P* = 0.0004, by Mann-Whitney test), with significant increase of ratio when compared with background ratio (*P* = 0.0195, by Wilcoxon signed-rank test). Ratio between IL-17-producing Tcon and autoreactive Treg was also significantly increased in patients with active SLE only when compared with the ratio of patients with inactive SLE (median ratio: healthy individual = 0.035; inactive SLE = 0.0118; active SLE = 0.0567. Healthy individual vs inactive SLE, *P* = 0.3095; healthy individual vs active SLE, *P* = 0.3884; inactive SLE vs active SLE, *P* = 0.0496, by Mann-Whitney test), with a significant increase of ratio when compared with background ratio (*P* = 0.0391, by Wilcoxon signed-rank test). We observed no significant difference of IL-4-producing Tcon/autoreactive Treg ratio in all group populations (median ratio: healthy individual = 0.008; inactive SLE = 0.0013; active SLE = 0.0028. Healthy individual vs inactive SLE, *P* = 0.1031; healthy individual vs active SLE, *P* = 1; inactive SLE vs active SLE, *P* = 0.3501, by Mann-Whitney test). Finally, ratio between IL-10-producing Tcon and autoreactive Treg was also significantly increased in patients with active SLE when compared with the ratio of patients with inactive SLE and healthy individuals (median ratio: healthy individual = 0.015; inactive SLE = 0.0049; active SLE = 0.0464. Healthy individual vs inactive SLE, *P* = 0.3358; healthy individual vs active SLE, *P* = 0.0176; inactive SLE vs active SLE, *P* = 0.0028, by Mann-Whitney test), with significant increase of ratio when compared with background ratio (*P* = 0.0018, by Wilcoxon signed-rank test).

Secondly, we calculated the frequency of IL-10-producing Treg and the ratio between cytokine-producing Tcon and autoreactive Treg when the background frequency and background ratio were subtracted. Despite of qualitative production of IL-10 by Treg after antigenic stimulation, no difference on their frequency among individuals with and without SLE was observed (median frequency: healthy individual = 0.18 cells/100,000 cells; inactive SLE = 0 cells/100,000 cells; active SLE = 0.68 cells/100,000 cells) (Figure 34B). The background-free ratio between IFN-γ-producing Tcon and autoreactive Treg was significantly increased in the patients with active SLE only when compared with patients with inactive SLE (median ratio: healthy individual = 0.0528; inactive SLE = 0.004; active SLE = 0.0939. Healthy individual vs inactive SLE, *P* = 0.9289; healthy individual vs active SLE, *P* = 0.0592; inactive SLE vs active SLE, *P* = 0.0357, by Mann-Whitney test). The background-subtracted ratio between IL-17-producing Tcon and autoreactive Treg was significantly increased in the patients with active SLE among other group populations (median ratio: healthy individual = 0; inactive SLE = 0; active SLE = 0.0149. Healthy individual vs inactive SLE, *P* = 0.7526; healthy individual vs active SLE, *P* = 0.0385; inactive SLE vs active SLE, *P* = 0.0264, by Mann-Whitney test). The ratio between IL-4-producing Tcon and autoreactive Treg remained similar across all groups also when the background ratio was removed (median ratio: healthy individual = 0; inactive SLE = 0; active SLE = 0.0014. Healthy individual vs inactive SLE, *P* = 0.9289; healthy individual vs active SLE, *P* = 0.3703; inactive SLE vs active SLE, *P* = 0.3889, by Mann-Whitney test). Similar with the background-subtracted IL-17-producing Tcon/autoreactive Treg ratio, significant imbalance in the background-

subtracted ratio between IL-10-producing Tcon and autoreactive Treg was observed in patients with active SLE when compared with inactive SLE patients and healthy individuals (median ratio: healthy individual = 0.0017; inactive SLE = 0.0009; active SLE = 0.0131. Healthy individual vs inactive SLE, $P = 1$; healthy individual vs active SLE, $P = 0.0174$; inactive SLE vs active SLE, $P = 0.0174$, by Mann-Whitney test).

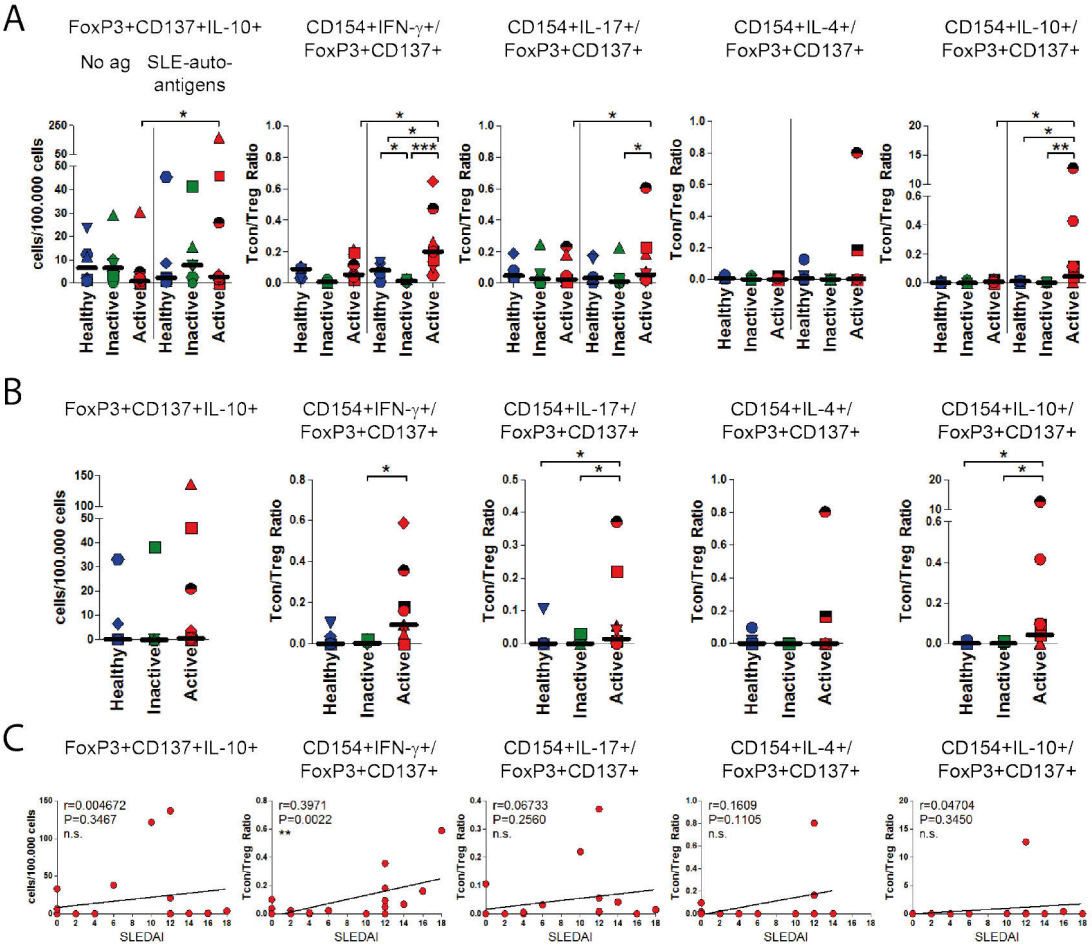


Figure 34: **Autoreactive Treg and production of effector cytokines in SLE.** (**A**) The frequency of IL-10-producing FoxP3+ cells (IL-10 Treg) was assessed among CD4+ T cells in healthy individuals (*n*=6), patients with inactive SLE (*n*=6), and patients with active SLE (*n*=9) without antigen stimulation (no ag) and after stimulation with SLE-associated autoantigens (SLE-autoantigen) (left), as well as ratio between cytokine-producing Tcon (IFN-γ, IL-17, IL-4, IL-10) with CD137-expressing FoxP3+ CD4+ T cells without stimulation (no ag) and after stimulation with SLE-associated autoantigens (SLE autoantigens). (**B**) The frequency of IL-10 Treg and ratio between cytokine-producing Tcon with CD137-expressing FoxP3+ CD4+ T cells were assessed after subtraction of background. (**C**) Background-subtracted frequencies of IL-10 Treg after stimulation with SLE-associated autoantigens (left) and background-subtracted ratio between cytokine-producing Tcon with CD137-expressing FoxP3+ CD4+ T cells were compared with SLEDAI. For (**B** & **C**), when the background frequency or background ratio was higher than the antigen-specific frequency and ratio, background-subtracted antigen-specific frequency or ratio was defined as zero. For (**A** and **B**), black line represents the median value. For (**C**), r indicates Pearson's correlation coefficient and black line represents linear regression determined using Pearson's correlation coefficient.

Thirdly, we analysed the correlation between the frequency of IL-10-producing Treg as well as the ratio between cytokine-producing Tcon and autoreactive Treg after removal of background frequency or ratio with the disease activity of SLE. No correlation was identified between frequency of background-subtracted IL-10-

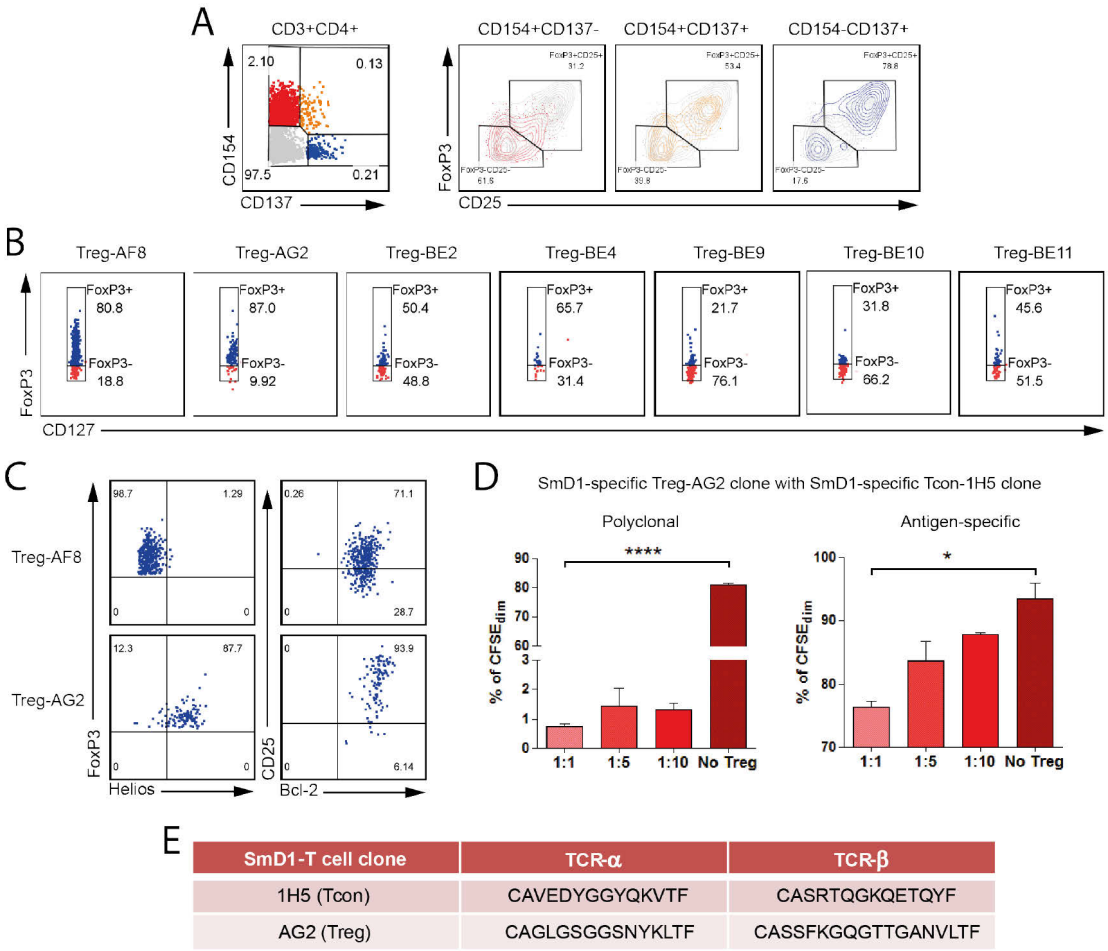
producing Treg and SLEDAI (Figure 34C). The same was true for the cytokine-producing Tcon/autoreactive Treg ratio, where significant correlation with SLEDAI was found on the background-removed ratio between IFN-γ-producing Tcon with autoreactive Treg.

Taken the results together, here we demonstrate that autoreactive Treg of active SLE patients produce IL-10 after stimulation with SLE-associated autoantigens but this IL-10 production seems not to influence disease conditions. Furthermore, our results revealed that imbalance between autoreactive Tcon and Treg also occurred on the Tcon subsets, which were Tcon producing IFN-γ, IL-17, and IL-10, but not IL-4. However, only imbalance in IFN-γ-producing Tcon and autoreactive Treg appeared to have an implication in disease progression. Predictive explanations about why autoimmune imbalance between cytokine-producing Tcon and Treg exists at antigen-specific level have been reported in a series of recent studies, most of which have suggested the evidence of Treg plasticity as the aetiology. In the last years, scientists could examine the expression of effector CD4+ T cell-transcription factors such as T-bet, GATA-3, and RORγt on Treg that regulate type 1-, type 2-, and type 17-immunity [249-251]. In the scope of autoimmunity, Komatsu *et al.* have described a phenomenon of Treg transdfifferentiation into pathogenic Th17 cells that produced IL-17 in mouse model of arthritis and in the synovium of patients with rheumatoid arthritis [163]. In line with Th17-Treg plasticity theory, Gagliani *et al.* reported that during resolution of inflammation, IL-17-producing Th17 cells can transdifferentiate into Tregs [162]. Whether or not the transdifferentiated Th17 to Treg and vice versa originate from the same cells with the same antigen-specificity remains unclear. Recently, Levine *et al.* found evidences of differentiation of T-bet-expressing Treg with unique TCR specificity capable of promoting potent immunosuppression limited to Th1 cells (and also CD8+ T cells), and suggested analogous roles of GATA3- and RORγt-expressing Treg in suppression of Th2 and Th17 responses, respectively [252]. Thus, it could be possible that disrupted immune homeostatic in favour of Tcon happens because autoreactive Treg have transdifferentiated into autoreactive Tcon. But then our question for this theory is about TCR-specificity of these cells. Do autoreactive Tcon and Treg have the same TCR specificity?

3.4. Antigen-specific suppression capacity of autoreactive Treg

It is still unknown whether Treg-mediated tolerance is antigen-specific. This limits our understanding of the role of Treg for human autoimmune pathology that is driven by antigen-specific events and burdens our effort to develop Treg-based treatment strategies. Antigen-specificity of Treg is generated during thymocyte selection. In general, the process lies on education of T cells in recognizing self-peptide-MHC complexes with their TCR. No affinity with the self-peptide-MHC complex results in apoptosis of T cells by neglect, whereas high affinity of TCR with the self-peptide-MHC complex also results in cell death by negative selection. This concept underlines the strength of the interaction of the TCR with self-peptide-MHC complexes as an important determinant of lymphocyte fate. Interestingly, recent studies using transgenic-TCR systems indicated a redirection of autoreactive T cells into Treg cell lineage through interaction of TCR with self-peptide-MHC complexes in high affinity [253], thus it has been proposed that Treg differentiation occurs within a window between positive and negative selection [254]. It is possible that natural repertoires of TCR expressed by naive CD4+ T cells show some overlap with TCR of Treg.

However, this concept is still controversial [255]. In order to obtain information about antigen-specificity of Treg, we performed analysis directly involving the function of Treg at antigen-specific level i.e. their suppression capacity against Tcon with the same antigen-specificity in SLE. First, we needed to establish a system that could facilitate study of the antigen-specific function of Treg, therefore we aimed to generate Treg-clones with a defined TCR specificity to a SLE-associated autoantigen. As previously described, we made use of the combination of CD154 and CD137 expression on CD4+ T cells after stimulation with antigen. In our establishment experiments, we could demonstrate that expression of CD154 and CD137 was useful to dissect antigen-specific Tcon and Treg after stimulation with a recall-antigen CMV lysate in the procedure described in the section 2.6. (Figure 35A). Indeed, CD154+CD137- were mostly FoxP3-CD25- or CD25+ cells, whereas CD154-CD137+ were mostly FoxP3+CD25high cells with some Tcon contamination. CD4+ T cells expressing both CD154 and CD137 were a mixed population consisting antigen-specific Tcon and Treg.



SmD1-T cell clone	TCR-α	TCR-β
1H5 (Tcon)	CAVEDYGGYQKVTF	CASRTQGKQETQYF
AG2 (Treg)	CAGLGSGGSNYKLTF	CASSFKGQGTTGANVLTF

Figure 35: **Antigen-specific suppression capacity of autoreactive Treg.** (**A**) Dot plots show CD4+ T cells after stimulation with CMV lysate gated for CD154+CD137- (red), CD154+CD137+ (orange), CD154-CD137+ (blue) cell subsets (right) and the cell subsets were further gated for FoxP3-CD25- and FoxP3+CD25+ cells (left). Coloured contour plots highlight cell subsets within total CD3+CD4+ T cells (grey contour plot on the background). Numbers represent percentage of parent population. (**B**) Dot plots show SmD1-Treg clones (*n*=7) generated from CD154-CD137+ CD4+ T cells after stimulation with SmD1 derived from two patients with inactive SLE. Gating strategy identified FoxP3+ and FoxP3- cells. All cells were negative for CD127. Numbers represent percentage of FoxP3+ or FoxP3- cells within CD4+ T cell population. (**C**) SmD1-specific Treg-AF8 and Treg-AG2

clones were analysed for their FoxP3, Helios, CD25, and Bcl-2 expression. Numbers represent percentage of the subset within FoxP3+ cell population. (**D**) Analysis of suppressive capacity of SmD1-specific Treg-AG2 clone was done by co-culturing the clone with donor-matched CFSE-labelled SmD1-specific Tcon-1H5 clone after polyclonal stimulation with antihuman-CD3 and antihuman-CD28 antibodies (left) as well as after antigen-specific stimulation with SmD1 in presence of donor-matched dendritic cells (right). Percentage of CFSEdim cell population was measured. X-axis displays Treg/Tcon ratio. Data are shown from two experiments. (**E**) CDR3-region of TCR-α and TCR-β molecules were sequenced from SmD1-specific Tcon-1H5 clone and Treg-AG2 clone.

We generated clones of Treg specific to the SLE-autoantigen SmD1 according to CD154-CD137+ expression on CD4+ T cells after antigenic stimulation. Using single cell sort we were able to establish ~20 Treg clones derived from two individuals with inactive SLE, but only seven clones expanded in the cell culture. Expanded Treg-clones were then stained for FoxP3 expression. Our results revealed that almost all SmD1-specific Treg clones had a decreased expression of FoxP3 after *in vitro* expansion except for SmD1-specific Treg clone AG2 (Figure 35B). SmD1-specific Treg clone AF8 still could maintain high FoxP3 expression, thus we focused on these two Treg clones, AF8 and AG2, for further analysis. Notably, our analysis revealed that Treg clone AG2, but not AF8, was additionally positive for Helios, transcription factor that allows the differentiation of thymus-derived Treg from peripherally induced Treg [256] and high-expression of CD25+ within the anti-apoptotic, Bcl-2-expressing population (Figure 35C). Because Helios was claimed to control certain aspects of Treg-suppressive function [257], we selected SmD1-specific Treg-clone AG8 for polyconal and antigen-specific suppression analysis against donor-matched SmD1-specific Tcon clone 1H5. The generation of autoreactive Tcon clone was described in the chapter 5 of this thesis. SmD1-specific Tcon clone 1H5 was labelled with CFSE and co-cultured with SmD1-specific Treg clone AG2 in the ratio of 1:1, 1:5, and 1:10. A culture consisting of CFSE-labelled SmD1-specific Tcon clone 1H5 alone served as a basis of Tcon proliferation. Here, we used two Tcon proliferation settings, which are first polyclonally, using antihuman-CD3 and antihuman-CD28 functional grade antibodies, and secondly antigen-specific, using donor-matched dendritic cells and SmD1 protein.

SmD1-specific Treg-clone AG2 demonstrated extensive suppression capacity against Tcon proliferation at polyclonal level. In absence of Treg-clone AG2, Tcon-clone 1H5 entered proliferation cycles after stimulation with antihuman-CD3/CD28 but the proliferation could be efficiently abolished when Treg-clone AG2 was present, even in a 10-fold lower amount than Tcon-clone 1H5 (Figure 35D). Interestingly, in the presence of dendritic cells and SmD1 protein, Tcon-clone 1H5 could proliferate, and this proliferation could be minimally limited by Treg-clone AG2. This observation emphasized that Treg-suppressive capacity also applied at antigen-specific level, but why polyclonal suppression by Treg appeared to be superior to antigen-specific suppression still remains unclear. As previously mentioned in this chapter, Sakaguchi *et al.* have proposed three possible mechanisms of Treg-mediated suppression, which are (1) outcompeting Tcon for interaction with dendritic cells, (2) modulation of antigen-presenting cell function, and (3) killing or inactivation of T cells [229]. Given the fact that polyclonal expansion of Tcon could be efficiently inhibited by only low amount of Treg in absence of dendritic cells and antigen, polyclonal Treg-suppression may probably work by inactivation of Tcon through secreted cytokines as a result of polyclonal activation of Treg, in line with the theory (3). During antigen-specific suppression by Treg, theories (1) and (2) may probably be relevant. However, theory

(1) about competition between Tcon and Treg to interact with dendritic cells through TCR-antigen-MHC complex may require a pre-requisite that is overlap of TCR specificity for both Tcon and Treg. Theory (2) that proposed modulation of antigen-presenting cell function e.g. by down-regulation of co-stimulatory molecules on the surface of antigen-presenting cells may probably require no TCR-overlap between Tcon and Treg. Theory (2) additionally represents the strength of antigen-specific suppression, in which that only a specific Tcon population is controlled in an indirect way, giving the possibility to other Tcon populations to remain functional. For Treg-based therapy, this option is preferable because it can reduce side-effect of immune suppression that may lead to complications due to infections.

To visualize whether SmD1-specific Treg-clone AG2 and SmD1-specific Tcon clone 1H5 share the same TCR specificity, we sequenced CDR3 region of TCR-α and TCR-β of both clones using techniques carried out by the laboratory of Prof. Wolfgang Uckert at Max-Delbrück-Centrum für Molekulare Medizin in Berlin, Germany. Despite same antigen-specificity for SmD1 and though antigen-specific suppression, Tcon-clone 1H5 and Treg-clone AG2 had distinct CDR3-sequence on TCR-α and TCR-β molecule, indicating that they recognized different epitope of SmD1 (Figure 35E). The evidence that Treg-TCR repertoire in the thymus is similar with Treg-TCR repertoire in the periphery but not necessarily the same with TCR repertoire of peripheral Tcon has been reported by Hsieh *et al.* [258]. They however described that Treg and potentially pathogenic autoreactive CD4+ T cells could use overlapping pools of autoreactive TCR, because it might be relevant for Treg cells which can transdifferentiate into effector T cells in the onset of inflammation as well as for effector T cells that can transdifferentiate into Treg in the resolution of inflammation. In line with the concept of non-overlapping TCR diversity in peripheral Tcon and Treg, Pacholczyk *et al.* reported that the TCR repertoire of Treg is not similar with the TCR repertoire of naive T cells [259]. Supporting our results and contradicting the finding by Hsieh *et al.*, Liu *et al.* also observed dissimilar TCR repertoire in their CDR3 sequence between Tcon and Treg, in which they claimed that TCR of autoreactive Tcon and Treg are distinct but have convergent autoantigen-specificity [260]. This group has later re-confirmed limited overlap of TCR of Tcon and Treg in mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), and emphasized that their TCR are largely unrelated although they recognize the same autoantigen myelin oligodendrocyte glycoprotein [261]. Dissimilar antigen recognition properties by Treg and Tcon TCR have been then reported by Bacher *et al.* using CD137-ARTE system in an allergic model against aeroantigens in humans. They found that the Th2 response escapes Treg control due to divergent antigen specificities that causes antigen-specific loss of tolerance in human allergy [245]. Recently, interesting findings about protection from HLA-linked autoimmunity by antigen-specific Treg was reported by Ooi *et al.* in autoimmune kidney Goodpasture disease [262]. Different HLA-DR molecules may result in either protection or risk from autoimmunity. They showed that HLA-DR15 and HLA-DR1 can induce autoreactive pathogenic Tcon and autoreactive protecting Treg, respectively. This phenomenon may explain that HLA polymorphisms can shape the abundance of autoreactive Treg that leads to protection or causation of autoimmunity.

Taking our results together, we could show that SLE-associated autoreactive Treg can induce suppression of autoreactive T cells in a mechanism that may be distinct from

polyclonal suppression by Treg. Furthermore, we also supported the findings by other groups that TCR of autoreactive Treg and Tcon are dissimilar and may have divergent antigen-specificities. Suppressive capacity of autoreactive Treg can open strategies of Treg-based immunotherapies for autoimmune diseases. Moreover, in this thesis we showed methods to generate human autoantigen-specific Treg clones, thus completing our broad range of techniques used to study the role of autoreactive CD4+ T cell subsets in disease pathogenesis and as therapeutic tool in SLE.

4. Conclusion

Treg are indispensable in our immune system to maintain immune homeostasis and to prevent unwanted immune responses such as autoimmunity. Implications of Treg in human autoimmune diseases such as SLE have been reported in a number of recent publications, but knowledge about antigen-specific Treg remains missing, mainly because of technical challenges that hamper our ability to investigate antigen-specific Treg. Within our work, we were able to overcome technical challenges by providing evidence of *in vitro* Treg expansion with high expansion rate even for memory Treg and by evaluating Treg response to antigen using Treg library assays. We also used another technique, CD137-ARTE, to interrogate the existence of SLE-associated autoantigen-specific Treg in healthy individuals, patients with inactive SLE, and patients with active SLE. We found that the frequency of Treg among individuals was nearly similar, but patients with active SLE have a broad range of Treg frequency and increased number of autoreactive Treg when compared with inactive SLE patients and healthy individuals. But high amount of autoreactive Treg was not sufficient to control the disease, because Tcon/Treg ratio in patients with active SLE was higher than other group populations and correlated with disease activity indicating an imbalance between the frequency of autoreactive Tcon and Treg in favour of the abundance of autoreactive Tcon during disease progression (Figure 36). This evidence is critical for autoimmune pathology in SLE. Tcon/Treg imbalance was not only found in the total autoreactive cell population but also in the effector cytokine production. We could answer the question about antigen-specific suppression by Treg using antigen-specific Treg clones and we could identify little evidence about distinct TCR repertoires between autoreactive Tcon and Treg that recognize the same antigen. We could demonstrate that TCR stimulation to Tcon leads to cytokine production, and TCR stimulation to Treg is critical for their regulatory capacity in a mechanism involving interaction with antigen-presenting cells, presumably dendritic cells, consistent with previous reports [263]. Our methods to unravel the function of Treg at antigen-specific level open promising strategies of Treg-based immunotherapy. To summarize, we propose a theory that (1) autoreactive Treg are expanded following disease progression and increased chronic inflammation, (2) these autoreactive Treg are functional with potent suppressive capacity, (3) although in the active disease autoreactive Treg proliferate, greater expansion of autoreactive conventional CD4+ T cells outnumbers their regulatory counterparts and leads to antigen-specific Tcon/Treg imbalance in term of cell numbers, and (4) some Treg happen to transdifferentiate into Tcon during chronic inflammation compromising a greater Tcon/Treg imbalance and disease pathogenesis.

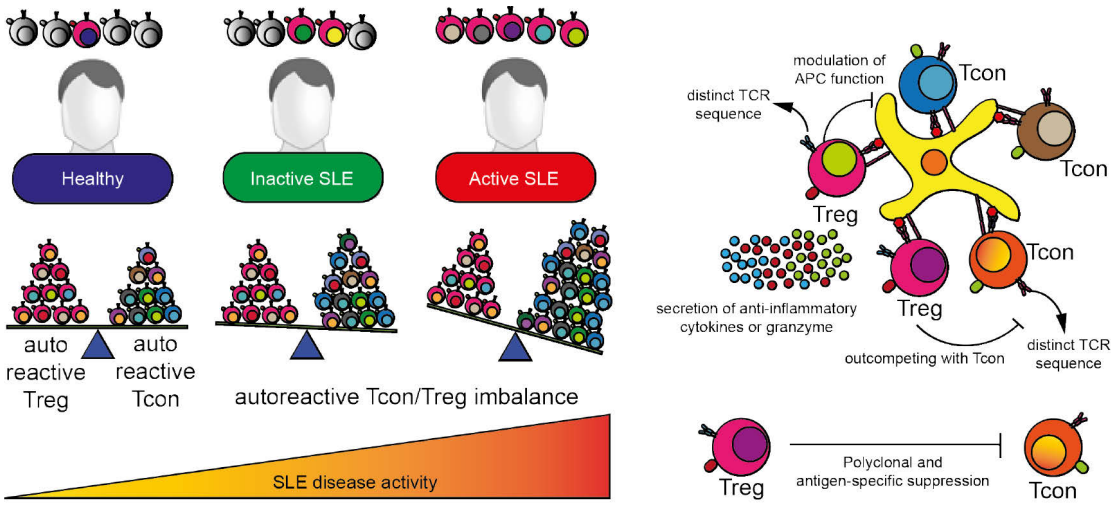


Figure 36: **Autoreactive Treg and disease pathogenesis in SLE.** Patients with active SLE have more autoreactive Treg, but their number is not sufficient to control unwanted immune responses because of the higher frequency of autoreactive Tcon. This antigen-specific Tcon/Treg imbalance correlates with disease activity. Autoreactive Treg suppress Tcon function such as cytokine production and their proliferation by outcompeting in recognizing autoantigen presented by the antigen-presenting cells, modulating the function of autoantigen-presenting cells, or inactivation of Tcon through killing or secretion of anti-inflammatory cytokines. Autoreactive Treg can suppress autoreactive Tcon polyclonally or in an antigen-specific manner. TCR sequence of autoreactive Tcon and Treg are probably distinct, although their TCR recognize identical autoantigen.

Chapter 10

Role of autoantigen-specific CD4+ T cell subsets in SLE: general discussion, concluding remarks, and future perspectives

Abstract

CD4+ T cells are key players in our adaptive and innate immune system. To date, the role of human CD4+ T cells in SLE has been primarily described at polyclonal level. Our understanding in the details of antigen-specific CD4+ T cells has been hampered by technical limitations because these cells exist in extremely low frequencies in the periphery. In this thesis, we were able to provide new knowledge about CD4+ T cell subsets that are specific to SLE-associated autoantigens and their implication in the disease pathogenesis of SLE. Moreover, apart from the main goal of this thesis, we were successful to introduce a set of state-of-the-art methods as tools to study human antigen-specific CD4+ T cell subsets in autoimmune diseases. Our theories, findings, and analyses are reviewed and summarized in this chapter. Based on what we have observed here, there are several advisable further approaches to complete the picture of CD4+ T cell-role in SLE. There are many interesting research areas to be explored in this field and there is a number of promising possibilities to help people with SLE and other autoimmune diseases. Our work is meant to bring contributions to our quality of life by better understanding our immune system in health and disease.

Keywords: *SLE, CD4+ T cells, concluding remarks, future perspectives*

Contents

1. General discussion

To study the role of autoantigen-specific CD4+ T cell subsets in disease pathogenesis and as therapeutic tool in SLE, we employed structural and organized strategies using available facilities, technologies, and social networks. We generated the hypotheses, then discussed the goal of our research and developed approaches to reach the goals. We collected data from our experiments either by observation or by evidence, and we analysed the data to transform them into information. We communicated our analyses with other researchers and scientists to ensure impartiality and quality of our work. We have started with problem identification in the study of human autoreactive CD4+ T cells in SLE, and then we evaluated the scope of this work, whether some changes or improvements were necessary for conducting our study. We observed the indication of T cell-mediated pathogenesis in SLE because of the expansion of autoreactive CD4+ T cells in the patients with active disease. We confirmed this observation through testing of antigen-specificity. We wondered which effector functions these cells exert following antigen-specific activation and what kind of mechanisms of autoantibody production involving autoreactive CD4+ T cells are possible. Given the fact that autoimmune inflammation occurs in the tissue, we were curious about the evidence describing tissue inflammation mediated by autoreactive CD4+ T cells. Finally, autoimmunity is linked with loss of tolerance, therefore we wanted to understand, which regulatory mechanisms are important to maintain immune tolerance. Here we present a general discussion about findings about the role of autoreactive CD4+ T cells in the human systemic autoimmune disease SLE.

1.1. SLE and autoreactive CD4+ T cells

SLE is a prototypic systemic autoimmune disorder with a broad spectrum of clinical manifestations affecting almost all organs and tissues. The extreme heterogeneity of the disease indicates that SLE represents an accumulation of multiple diseases rather than a single disease. The incidence of SLE has nearly tripled in the last 40 years mainly because of advancement in the diagnosis, but still many factors about SLE are not known. The aetiology of SLE is unclear. Some investigators have proposed genetic factors, epigenetic effects, environment, and hormones as components that lead to an irreversible break in immunological tolerance. During our work, we noticed extreme variations in clinical data as well as data generated in the laboratory among the patients with SLE involved in our study. It is difficult therefore to generalize clinical symptoms and molecular evidences in SLE and that is why current therapies seem to be far from being fully effective.

Despite tremendous variability, loss of tolerance to nuclear antigens is until now the universally accepted major determinant of the disease. Patients with SLE develop antibodies directed to nuclear antigens already in the pre-clinical phase. More than two decades ago, it has been reported that 96% of SLE patients develop antinuclear antibodies as a serological feature, followed by 78% for anti-DNA antibodies, 25% for anti-Ro antibodies, 19% for anti-La antibodies, 13% for anti-RNP antibodies, and 10% for anti-Sm antibodies [264]. These autoantigens are released by apoptotic cells, where normally clearance mechanism can avoid the accumulation of apoptotic materials in the body, but in case of SLE this mechanisms seems to be defect. Thus the autoantigens are endocytosed by antigen-presenting cells for further

immunological responses involving immune cells from innate and adaptive immune system.

Some investigators claim that SLE is driven only by innate immunity because of high cellular and molecular prevalence of type-I IFN, complements, and NET formation by neutrophils. However, we believe that adaptive immunity plays a central role in SLE and other autoimmune diseases. CD4+ T cells are a population of heterogeneous cells with unique receptors to recognize pieces of molecules presented by antigen-presenting cells. The receptor, also known as TCR, is generated by a genetic recombination process of VDJ genes that is able to establish a TCR repertoire consisting up to 10^{15} TCR [265]. But our body has only approximately $4x10^{11}$ circulating T cells [266], and due to clonal selection, not all 10^{15} TCR exist in our body. The number of TCR is estimated to be around 10^6 to 10^8 [267]. This number is still high and can provide detection to many possible protein structures. In SLE, it is not impossible that some receptor of CD4+ T cells can recognize an epitope of nuclear antigens presented by antigen-presenting cells that leads to their activation. Although most T cells with self-reactive TCR are deleted in the thymus, some of them can escape the negative selection and circulate in the body. When peripheral tolerance fails to maintain tolerance to these cells, autoreactivity may occur. CD4+ T cells function in such way, that they compose, organize, modulate, induce, and govern other immune cells to orchestrate complex immune responses. Thus, CD4+ T cells are "masterminds", and once they are involved in autoimmunity, the impact can be serious and complicated. It is true that signalling abnormalities in T cells such as impaired TCR-signal transduction are characteristic in SLE, but antigen-specificity of CD4+ T cells to nuclear antigens is one indispensable source where the autoimmune response in SLE begins.

1.2. HLA-linked autoimmunity, epitope spreading, and autoantibodies

Genome-wide association studies have been performed in individuals with SLE and shown to be a powerful way of identifying susceptibility genes in the disease [191]. The results revealed that HLA genes in general are the strongest susceptible genes in SLE. Particular HLA alleles are associated with the risk and protection against human autoimmune diseases, including rheumatoid arthritis, type I diabetes, and multiple sclerosis. In SLE, the mechanisms underpinning HLA-mediated effects and self-tolerance are not yet fully characterized. Recently, data about HLA-linked autoimmunity in Goodpasture disease demonstrated that while HLA-DR15 confers increased disease risk, HLA-DR1 is protective against disease-relevant immunodominant CD4+ T cell-epitopes derived from the α3 chain of type IV collagen, $\alpha3_{135\text{-}145}$ [262]. Genetic predisposition on HLA genes in SLE has been described for correlation with the production of certain autoantibodies, but the correlation between HLA alleles with T cell-epitopes remains unknown.

HLA-DR3 has the strong association with SLE. It was reported that individuals with HLA-DRB1*0301 haplotypes are susceptible to SLE [102]. Furthermore, when focusing on SLE-associated autoantigen SmD1, it was shown that HLA-DR3-transgenic mice could produce antibody responses to SmD1 [80]. Anti-SmD1 autoantibody is the most specific autoantibody for SLE, where other autoantibodies such as anti-RNP70, anti-Ro, and anti-La autoantibodies are partially specific for SLE because they are also found in mixed-connective tissue disease and Sjögren's

syndrome. This interesting observation has linked three important parts in SLE: HLA-DR3, SmD1, and autoantibody against SmD1. Our previous study revealed that a specific region of SmD1 on the amino acid sequence 83-119 is the target of SmD1-autoantibodies in patients with SLE. Since the production of autoantibodies involves antigen-specific CD4+ T cells for germinal centre formation, immunoglobulin class-switching, and somatic hypermutation, we wondered whether $SmD1_{(83-119)}$ represents a cognate epitope for TCR of SmD1-specific CD4+ T cells. Notably, the detection of $SmD1_{(83-119)}$-specific CD4+ T cells is very difficult probably because of their very rare frequency, but SmD1-specific CD4+ T cells recognizing other SmD1 epitopes than $SmD1_{(83-119)}$ could be identified. In parallel, HLA-binding analysis *in vitro* and *in silico* showed limited binding affinity of HLA molecules to $SmD1_{(83-119)}$ but broad binding probability to SmD1 epitope other than $SmD1_{(83-119)}$. These data indicate a mechanism called epitope spreading, in which B cells present a number of various antigen epitopes to the T cells that are not necessarily identical with the epitopes recognized by BCR. This phenomenon has been supported by a study reporting that $SmD1_{(26-55)}$, $SmD1_{(52-69)}$, and $SmD1_{(86-115)}$ are major cognate antigen epitopes for SmD1-specific CD4+ T cells [113].

The production of autoantibodies in SLE is an antigen-specific mechanism involving autoreactive CD4+ T cells. Autoreactive Th2 cells recognize nuclear antigens presented by antigen-presenting cells such as dendritic cells in the secondary lymphoid organs. These cells differentiate into Tfh lineage and enter the germinal centres with the ability to produce IL-4 and IL-10 upon activation by naive or memory B cells. After antigen recognition and receiving T cell help through secretion of IL-4 and IL-10, B cells differentiate into plasmablasts and plasma cells for autoantibody production. In addition, IL-4 and IL-10, along with IL-21 and IL-9 which are produced by autoreactive Tfh cells also mediate T cell-help for immunoglobulin class-switching. The immunoglobulins perform extensive receptor editing by somatic hypermutation leading to functionally active autoantibodies that can target the initial antigens with shaped specificity but also with specificities to other antigens. Because of the event of somatic hypermutation, it is not possible to compare the frequency of CD4+ T cells reactive to an autoantigen with the production of autoantibodies targeted same autoantigen. This phenomenon was supported by our observation at cytokine level that IL-4 and IL-10 production by autoreactive CD4+ T cells reactive to a wide variety of nuclear antigens may cause the production of all possible autoantibodies directed to nuclear components (anti-ANA) or to non-antigen such as DNA (anti-dsDNA).

1.3. Technical limitations in studying antigen-specific T cells

Advancements in technologies such as mouse model, genetic modification, and multiparametrical flow cytometry have contributed significantly to our understanding of the immune system in the last years. However, several unresolved issues remain due to low frequency of certain antigen-specific T cell populations and the complexity of T cell antigen recognition. The limitations in detection of antigen-specific CD4+ T cells are true for both Tcon and Treg cells that play significant and indispensable roles in immune protection and pathology. The detection of autoantigen-specific CD4+ T cells is one of the major challenges because their frequency in the periphery is thought to be extremely rare. Furthermore, the challenge increases when working with patient's samples. Information about the phenotype, frequencies, and functional

capacities of autoantigen-specific CD4+ T cells is essential to gain knowledge related to the pathogenesis.

Antigen-specific CD4+ T cell-reactivity has been evaluated for decades only on the basis of their proliferation upon antigenic stimulation in a bulk T cell population. Using such assays, it is almost impossible to determine the actual frequency of reactive cells in the starting cell sample. A smart approach developed from proliferation assays has been introduced by Geiger *et al.*, which is based on the screening of libraries of polyclonally expanded CD4+ T cells while maintaining the TCR diversity, facilitating the determination of frequency of CD4+ T cells specific for a variety of antigens [104]. Amplified polyclonal libraries of human CD4+ T cells can be interrogated to identify cultures containing diverse types of autoantigen-specific T cell precursors simultaneously and to enumerate their frequencies. Another advantage of this method is that it allows the enumeration of autoantigen-specific CD4+ T cells specific for naturally processed antigens. It becomes important because immunodominat TCR epitopes of a single antigen can be varied. Furthermore, by presorting different T cell subpopulation such as different Th cell subsets or even Treg for the generation of the libraries, autoantigen-specific T cell frequencies from various cell compartments can be predicted and compared. Despites the advantages of the T cell library assay, the technique requires maintaining several hundreds of individual cell cultures for several weeks which is very laborious. This technique also cannot exclude the possibility of selective outgrowth or loss of certain CD4+ T cell clones during the long cell culture. In addition, the detection limit of T cell library assay is restricted to a frequency window from one cell within 2,000 cells to maximally one cell within 100,000 cells enumerated by Poisson distribution thus limiting the detection of very rare CD4+ population e.g. one cell within a million cells.

Flow cytometry-based detection methods open the possibility to identify detailed phenotypical and functional characteristics of single detected cells. The number of possible detected phenotypes can be up to 20 or more different parameters. The use of mass cytometry even significantly increases the possibility to detect a number of different phenotypes up to 100 or more different parameters. The common flow cytometry-based method to detect antigen-specific CD4+ T cells relies on the labelling of the specific TCR using recombinant MHC-peptide multimers. It allows identification of specific CD4+ T cells according to their specific antigen receptor thus enabling unbiased access to the total pool of T cells. Multimerization of peptide-MHC complexes has helped to solve the problem regarding low binding affinity of the TCR to MHC-peptide complexes. This technology however requires knowledge on detailed characteristics of antigenic epitope, which is a defined peptide sequence of the antigen restricted to a particular MHC haplotype [268]. This condition is not feasible in a setting where the antigen is not yet fully characterized such as in our study.

Standard flow cytometry is limited by the number of events that restricts the analysis of samples of a detected population at frequencies more than 0.01%. Our observation using T cell library revealed that the frequency of circulating autoreactive CD4+ T cells for a particular autoantigen in patients with disease flare was around 20 cells in a million cells representing a frequency of 0.002%, therefore the detection of autoantigen-specific CD4+ T cells with standard, non-manipulated flow cytometry is

almost impossible. Bacher *et al.* introduced a pre-enrichment of CD4+ T cells that are reactive to particular antigens before acquisition on flow cytometry, known as the antigen-reactive T cell-enrichment (ARTE) method [243]. Using the cell surface expression of CD154 for conventional CD4+ T cells and CD137 for Treg, autoreactive CD4+ T cell subsets can be enriched allowing detection and further phenotypic characterization of antigen-specific CD4+ T cells such as cytokine expression. This technique requires high specificity of the sorting marker to identify antigen-specific CD4+ T cells within the high number of irrelevant cell populations hence a combination of CD154 or CD137 with a secondary cell marker such as CD69 or FoxP3 is necessary. The major disadvantage of this approach is that it requires a high number of starting cells to obtain good quality and improved specificity of the measurement. This condition is often very difficult when working with patient's samples, where the number of cells is limited because of unavoidable clinical conditions and ethics.

1.4. Autoreactive CD4+ T cell-expansion in disease flares

Despite of limited technologies to determine the frequencies of autoantigen-specific CD4+ T cells, we were able to overcome the challenges and provide information about frequencies of circulating SLE-associated autoantigen-specific CD4+ T cells in healthy individuals, SLE patients with inactive disease, and SLE patients with active disease. Using T cell library assays, we showed that SLE-associated autoantigen-specific CD4+ T cells were more detectable in the patients with active SLE than in the patients with inactive disease and in healthy individuals. We validated the results by comparing the frequency of CD4+ T cells reactive to an irrelevant protein among the groups. Transthyretin-specific CD4+ T cell frequency was observed at the same level across the cohorts indicating that higher frequency of SLE-associated autoantigen-specific CD4+ T cells in the patients with active disease was not due to biased CD4+ T cell reactivity to all possible antigens. The quality of our results was validated using ARTE method, where we observed an increase in frequency of SLE-associated CD4+ T cells in patients with active disease when compared with inactive SLE patients and healthy individuals. Also for ARTE method we validated the results by comparing the frequency of recall-antigens, *Candida albicans* MP65-specific CD4+ T cells across all groups and we found that the frequency of CD4+ T cells reactive to recall-antigens was almost at the same level. Thus, CD4+ T cell reactivity to nuclear antigens is a phenomenon identified in the SLE patients with disease flare.

Furthermore, by analysing the frequencies of autoreactive CD4+ T cells with the disease activity index, we found significant correlation both when we assessed the frequencies using T cell library and using ARTE method. Thus, we suggest that autoreactive CD4+ T cells play a crucial role in the SLE disease pathogenesis. Low frequency of SLE-associated autoantigen-specific CD4+ T cells in healthy individuals represents the collection of the cells within naive T cell repertoire where they become activated following antigen recognition during inflammation. As a result, these cells are expanded and can be detected at a higher frequency. Notably, lower frequency of SLE-associated autoantigen-specific CD4+ T cells in patients with inactive disease may represent a consequence of reduced disease activity due to the treatment. Supporting our theory about naive and anergic circulating SLE-associated CD4+ T cells in healthy individuals, we could show that despite of antigen-specificity in this group, not all SLE-associated autoantigen-specific CD4+ T cells demonstrate

reactivity to the given antigen indicating higher TCR avidity when compared with CD4+ T cells reactive to a recall antigen. To summarize, we propose a theory describing that CD4+ T cells reactive to nuclear antigens escape the central tolerance mechanism in the thymus and circulate in the periphery as naive and anergic CD4+ T cells. These cells are maintained by peripheral tolerance and they never encounter nuclear antigens under normal conditions where nuclear apoptotic material is efficiently cleared. When clearance mechanisms of apoptotic material are disturbed, they are frequently exposed to their cognate antigens presented by the antigen-presenting cells. Autoreactive CD4+ T cells are only activated when peripheral tolerance is disrupted. These autoreactive CD4+ T cells proliferate and expand to exert their effector function thus worsening the disease characterized by the increase of the disease activity.

1.5. Effector cytokine-mediated autoimmunity

CD4+ T cells are also defined as helper T cells characterized by their ability to provide help to other immune cells during the course of an immune response. While CD8+ T cells can directly attack the target by releasing cytolytic materials such as granzymes and perforin, CD4+ T cells mainly exert their function through secretion of effector cytokines. The cytokines are important to modulate the degree of immune responses. CD4+ T cells are subdivided into three main subsets, Th1, Th2, and Th17 cells. Each subset acquires unique effector function such as the production of IFN-γ by Th1 cells, IL-4 by Th2 cells, IL-17 by Th17 cells, or IL-10 by virtually all Th cell subsets upon activation. SLE-associated autoantigen-specific CD4+ T cells produce effector cytokines such as IFN-γ, IL-4, IL-17, and IL-10. However, only the production of IFN-γ, IL-17, and IL-10 was significantly observed in patients with active SLE, whereas the production of those cytokines by autoreactive CD4+ T cells is almost absent in inactive SLE patients and healthy individuals. Only the frequency of CD4+ T cells producing IFN-γ correlate with progression of the disease.

Based on these results we propose a theory describing that IFN-γ produced by autoreactive CD4+ T cells supports the enhancement of TLR-signalling on innate immune cells such as dendritic cells and macrophages that leads to the production of type I IFN. GWAS results have identified type I IFN signalling as one of the most apparent triggers in SLE. In addition, type I IFN can conversely promote IFN-γ production by autoreactive CD4+ T cells in such a feedback-loop mechanism. Our proposed theory is supported by the finding describing that not only IFN-α, but also IFN-β and IFN-γ contribute to the progression of SLE [151]. Since IFN-γ production is correlated with Th1 response in SLE [154], it is possible that autoreactive CD4+ T cells in SLE are skewed towards a Th1 phenotype.

Cytokines produced by autoreactive CD4+ T cells may potentially play a dual role in the autoimmune reaction in SLE. We observed high production of IL-10 in patients with active SLE. IL-10 production can be a consequence of chronic inflammation where our immune system acquires anti-inflammatory responses to limit the chronic inflammation, thus IL-10 could theoretically ameliorate the inflammation through its suppressive function. However, evidence showing the function of IL-10 in a pro-inflammatory setting such as promoting B cell hyper-reactivity demonstrates its disadvantaged function. Our results imply effector function of autoreactive CD4+ T cells through cytokine production in SLE i.e. promoting direct tissue inflammation,

mediating interaction between innate and adaptive immune cells, supporting autoantibody production, but also self-limiting chronic inflammation. Since IL-17 and IL-10 was also produced by autoreactive CD4+ T cells, we proposed a theory that in SLE, Th1 and non-conventional Th1 subsets such as Th1/Th17 cells are dominating in the active phase of the disease. Taking the data together, we showed here that autoreactive CD4+ T cells in SLE are not only expanded in terms of their frequency, but also functionally contribute to the disease pathogenesis through their various effector functions highlighted by the production of effector cytokines such as IFN-γ, IL-17, and IL-10.

1.6. Pathogenic T cell-infiltration in the tissue

Expansion of circulating SLE-associated autoreactive CD4+ T cells and cytokine-producing autoreactive CD4+ T cells is indicative of disease pathogenesis. However, pathogenic CD4+ T cells infiltrate and accumulate in the tissue to perform their functions. Detection of autoreactive CD4+ T cells in the tissue can reflect the nature of the T cell-mediated pathogenesis. Since it is required to isolate and process tissue samples such as biopsies in order to detect tissue-resident pathogenic CD4+ T cells, this burden has forced investigation of autoreactive CD4+ T cells in human mainly to focus on blood samples. In order to overcome this limitation, we have demonstrated novel methods to identify autoreactive CD4+ T cells in the kidneys of active SLE patients with lupus nephritis. No biopsy is necessary for this approach replaced by the use of urine as a mediator in order to monitor the presence of immune cells in the inflamed kidneys. Our protocol has been validated to reflect the condition in the kidney, even at antigen-specific level. We found a restricted TCR variability in the urinary CD4+ T cells in line with the observations in kidney biopsies [208]. Although CD4+ T cells isolated from urine are functionally and metabolically active cells, they show signs of exhaustion probably because these cells have migrated through different tissues from the peripheral blood into the secondary lymphoid organs, before they arrive in the glomerulus of the kidney and accumulate in the urine. Selective TCR repertoire diversity exhibits signs of preferential accumulation of antigen-specific CD4+ T cells.

We have developed two state-of-the-art detection methods developed from T cell library and ARTE techniques. Using our protocol, we could not only detect the presence of CD4+ T cells in urine with reactivity to nuclear proteins, but we could also enumerate their frequency enabling the direct comparison with their frequency in the peripheral blood. By calculating the stimulation index both peripheral and urinary in CD4+ T cells, we could demonstrate increases in the frequencies of autoreactive CD4+ T cells specific to nuclear antigens in the urine of active SLE patients with lupus nephritis when compared with the frequencies in peripheral blood. We propose a theory about infiltration and accumulation of autoreactive CD4+ T cells with antigen specificity to nuclear antigens. Autoreactive CD4+ T cells are activated upon antigen recognition in the secondary lymphoid organs. These cells are expanded and can exert effector function. Some of these cells support antibody production in the lymph nodes where some other cells recirculate back in the periphery to migrate into site of inflammation. In the inflamed kidneys, the accumulation of autoantibody and complement is found, leading to activity of cells from the innate immune system such as macrophages and neutrophils. Autoreactive CD4+ T cells that infiltrate the kidneys express the homing receptor CXCR3 [212], indicating that these cells are Th1 cells

that produce IFN-γ upon activation. IFN-γ is required to activate macrophages to induce renal inflammation [228] and the source of IFN-γ in the kidney can be kidney-infiltrating autoreactive Th1 cells. In summary, our study here provides important knowledge about how to investigate the pathogenic function of autoreactive CD4+ T cells in tissue inflammation and organ failure using the model of lupus nephritis through our established state-of-the-art detection methods, which may have implications for a better understanding of SLE that affects almost all tissues and organs.

1.7. Homeostatic imbalance and loss of tolerance

Loss of peripheral tolerance in SLE is a consequence of dysbalanced proportions of autoreactive conventional T (Tcon) cells and autoreactive Treg. Our results revealed that patients with active SLE displayed a significant imbalance between the frequency of autoreactive Tcon and Treg in favour of autoreactive Tcon. Autoreactive Tcon are pathogenic and their function and proliferation need to be controlled by peripheral tolerance mechanisms. Whether polyclonal or antigen-specific Treg are more powerful to control Tcon autoreactivity remains an unsolved question. By generating Treg library and using CD137-ARTE method we could identify that the frequency of autoreactive Treg was actually increased in the patients with active SLE. However, high amounts of autoreactive Treg seem to insufficient to control the disease since autoreactive Tcon outnumber the Treg counterparts.

Tcon/Treg imbalance was not only observed in the total autoreactive cell population but also in the effector cytokine production. It leads to the assumption that the imbalance in the frequencies of Tcon and Treg can be within the same cell population which reciprocally transdifferentiates to other lineages. Very recent data published by Xu *et al.* suggest selective targeting of a glutamate-dependent metabolic pathway can inhibit hypermethylation of *FOXP3* gene locus leading to amelioration of experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis, by regulating the balance between Th17 and Treg cells in favour of Treg [269]. The questions arise whether induced Treg cells have the same TCR repertoire with the pathogenic Tcon. Hsieh *et al.* have described that Treg and potentially pathogenic autoreactive CD4+ T cells could use overlapping pools of autoreactive TCR [258]. This might be relevant for Treg cells with the ability to transdifferentiate into effector T cells during the inflammation and for effector T cells that can transdifferentiate into Treg in the resolution of inflammation. But as mentioned previously, recent data published by Ooi *et al*. showed HLA-linked autoimmunity in autoimmune kidney Goodpasture disease, where people with HLA-DR15 and HLA-DR1 can induce autoreactive pathogenic Tcon and autoreactive protecting Treg, respectively. This phenomenon may explain that both autoreactive Tcon and Treg could actually have a distinct TCR repertoire [262].

We propose a theory describing that in the periphery both thymus-derived and peripheral-induced Treg exist to establish peripheral tolerance. During the progression of the disease, the number of autoreactive Treg increases, but the number of autoreactive Tcon exceeds their Treg counterparts possibly due to transdifferentiation of Treg into pathogenic Tcon. Treg suppression capacity can be initiated both polyclonally and at antigen-specific level. It was shown by our results that a SmD1-specific Treg clone could suppress the proliferation of a SmD1-specific Tcon clone in

the absence of antigen but in the presence of polyclonal stimulator anti-CD3 and anti-CD28 antibodies, as well as in the presence of antigen. Importantly, our SmD1-specific Treg clone expressed transcription factor Helios as a marker for natural thymus-derived Treg and the cells had distinct CDR3 sequences on TCR-α and TCR-β chain when compared with SmD1-specific Tcon clone. Polyclonal suppression can be reached by the secretion of cytolytic molecules or anti-inflammatory cytokines, whereas antigen-specific suppression can be established by modulating the function of antigen-presenting cells or by outcompeting with antigen-specific Tcon. In summary, maintaining the homeostasis between Tcon and Treg at antigen-specific level is indispensable to prevent loss of tolerance that results in autoimmunity.

2. Concluding remarks

Our work within this doctoral thesis can provide data and analyses about the role of autoantigen-specific CD4+ T cell subsets in disease pathogenesis and as therapeutic tool in SLE. We have successfully demonstrated the specificity and frequency of CD4+ T cells reactive to nuclear antigens and analysed their functions in mediating immune responses, antibody production, and tissue inflammation. We were also able to show the function of autoreactive Treg in the disease pathogenesis of SLE. In summary, Table 9 shows the main objectives and corresponding results presented in the thesis. We also show additional results that have been obtained apart from the main objectives.

Table 9: **Summary of main objectives, corresponding results, and additional results in the thesis.** The thesis aimed to answer the main objectives in order to understand the role of SLE-associated autoantigen-specific CD4+ T cells in disease pathogenesis of SLE. By knowing their pathogenic role, therapeutic tool targeting these cells can be developed.

Main objectives	Corresponding results	Additional results
Detection	CD4+ T cells reactive to SmD1, RNP70, Histone, Ro, and La were detected by T cell library method.	Detection of CD4+ T cells reactive to the nuclear antigens was additionally verified by the second method ARTE.
Antigen-specificity	SmD1-, RNP70-, Histone-, Ro-, and La-specific CD4+ T clones confirmed antigen specificity.	The EC_{50} value describing TCR affinity to the nuclear antigens in healthy donors was additionally determined.
Frequency	By T cell library method, patients with active SLE had the highest frequencies of CD4+ T cells reactive to RNP70, Ro, and La.	ARTE method verified that patients with active SLE had the highest frequencies of CD4+ T cells reactive to the nuclear antigens. In addition, the frequencies correlated with disease activity.
Effector function	CD4+ T cells reactive to nuclear antigens produce IFN-γ, IL-17, IL-10, but less IL-4 in patients with active SLE.	The production of IFN-γ by CD4+ T cells reactive to nuclear antigens correlated additionally with disease activity of SLE.

Main objectives	Corresponding results	Additional results
Autoantibody production	Production of IL-4 and IL-10, but not IFN-γ and IL-17, by CD4+ T cells reactive to nuclear antigens correlated with the production of anti-ANA autoantibodies.	TCR-specificity and MHC-binding for $SmD1_{(83-119)}$, a target epitope for anti-SmD1 autoantibodies in SLE, were additionally determined and analysed.
Detection in the tissue	CD4+ T cells reactive to SmD1, RNP70, Histone, Ro, and La accumulated in urine of active SLE patients with lupus nephritis.	Generation of state-of-the-art methods to detect urinary autoantigen-specific CD4+ T cells termed urinary T cell library and CFSE-labelling of urinary cells for detection with ARTE technique.
Detection of Treg	Regulatory T cells reactive to nuclear antigens could be detected. Patents with active SLE have the highest Tcon/Treg ratio, correlated with disease activity.	Generation of methods to detect antigen-specific Treg termed Treg library and generation of Treg clone to study antigen-specific Treg suppression capacity.

Finally, we explain our concluding remarks into two categories. First, the role of autoantigen-specific CD4+ T cells in the pathogenesis and secondly, their role as therapeutic tool to help people with SLE.

2.1. The role of autoreactive CD4+ T cell subsets in the pathogenesis

The scenario of autoreactive CD4+ T cell-mediated SLE pathogenesis starts with disturbed immune homeostasis because of multiple factors such as infection, drugs, ultraviolet exposure, metabolism, stress and burn-out, dysregulated hormone production, and diet. In some individuals, the risk of SLE is higher than other individuals depending on their HLA types. These factors lead to aberrant clearance mechanisms of apoptotic materials such as nuclear antigens thus there is an accumulation of apoptotic waste in the body. The presence of dsDNA can activate innate immune cells through sensing on TLR9 that can attach to nucleic acid structure. Activation of innate immune cells results in the production of cytokines such as type I IFN. At the same time, neutrophils are also activated to exert effector function characterized by the generation of NET which is mainly composed from DNA and histone molecules. Thus, in SLE there is an excessive accumulation of material from the cell nucleus. Nuclear antigens are recognized by autoreactive BCR leading to the activation of autoreactive B cells. Some of the activated B cells differentiate into memory B cells and some of them circulate in the periphery to the secondary lymphoid organs such as lymph nodes in order to interact with CD4+ T cells. B cells process the antigen and present it in different pieces to the CD4+ T cells in a phenomenon called epitope spreading. Dendritic cells also engulf and process the antigen for presentation into autoreactive CD4+ T cells. Immune disturbance in SLE results in hyperreactivity of TCR signalling in T cells so that autoreactive CD4+ T cells become reactive. It is characterized by calibration of TCR thresholds that result in increased affinity of autoreactive TCR to autoantigen. Autoreactive CD4+ T cells recognize nuclear antigen presented by dendritic cells, so that they are activated and

expanded. Some activated autoreactive CD4+ T cells in form of Th2 cells enter the germinal centres and differentiate into Tfh cells to provide T cell-help for B cell differentiation into antibody-producing plasma cells, immunoglobulin isotype-switch, and antibody somatic hypermutation through the secretion of IL-4, IL-10, IL-21, and IL-9. Pathogenic functionally-active autoantibodies are released and bind into nuclear antigens promoting more enhanced autoimmune response and recruiting other immune cells to exert effector function. Some of activated autoreactive CD4+ T cells in form of Th1 cells or non-conventional Th1 cells circulate in the periphery to infiltrate inflamed tissue where apoptotic materials, autoantibodies, complements, and innate immune cells accumulate. Activated autoreactive CD4+ T cells produce effector cytokines such as IFN-γ, IL-17, and IL-10. The secreted cytokines promote progression of inflammation but they can also limit chronic inflammation. Autoreactive Treg are activated and expanded upon antigen recognition by the antigen-presenting cells, but the number of autoreactive Treg is not sufficient to control the pathogenic function exerted by autoreactive Tcon. Imbalance in Tcon and Treg number is apparent as a result of transdifferentiation phenomenon from polyclonal Treg into Tcon, but also as a result of disproportional expansion rate between autoreactive Tcon and Treg. Autoreactive Treg are able to suppress the proliferation of autoreactive Tcon both at polyclonal and antigen-specific level, thus imbalance in Tcon/Treg ratio promotes disease progression and autoimmunity (Figure 37). In summary, we could demonstrate in this doctoral thesis that CD4+ T cells reactive to nuclear antigens are central and play an indispensable role in the disease pathogenesis of SLE.

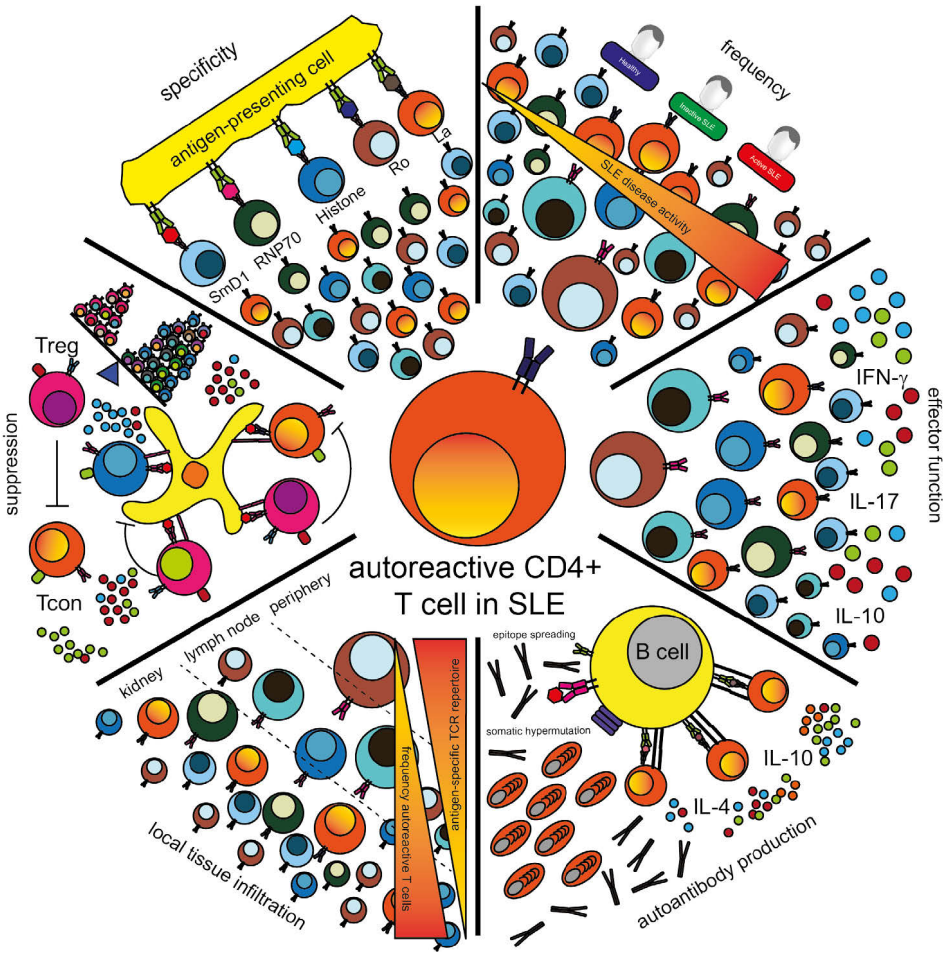


Figure 37: **Role of autoreactive CD4+ T cell subsets in SLE disease pathogenesis.** The role of autoreactive CD4+ T cells is diverse. They can specifically recognize nuclear antigens and get activated upon antigen stimulation. They are expanded in the disease flare and produce effector cytokines such as IFN-γ, IL-17, and IL-10. They also support autoantibody production through the secretion of IL-4 and IL-10, along with IL-21, and IL-9. They infiltrate the inflamed tissue and exert effector function to mediate organ failure. They also outnumber their regulatory counterparts to promote the imbalance between autoreactive Tcon and Treg resulting in loss of tolerance.

2.2. The role of autoreactive CD4+ T cell subsets as therapeutic tool

After identifying the role of autoreactive CD4+ T cells in the pathogenesis of SLE, it is now possible to expand our ideas to developing new therapeutic targets in SLE. Current therapeutic strategies mainly focus on B cells such as B cell depletion through anti-CD20 rituximab, reset of B cell-signalling through anti-CD22 epratuzumab, inactivation of autoreactive BCR through abetimus, blocking of B cell growth factors BLyS and APRIL by belimumab and atacicept, blocking of CD154-CD40 interaction through anti-CD154 antibody, and blocking of CTLA-4-CD28 interaction through abatacept (Figure 38). Novel therapeutic approaches could focus on the autoreactive CD4+ T cells and autoreactive Treg. Information about TCR sequence of autoreactive CD4+ T cells may help to develop multimers that can bind specifically to the TCR thus preventing its interaction with antigen presented by antigen-presenting cells. Combinatorial approach by blocking autoreactive TCR with CXCR3 homing receptor could prevent migration and infiltration of autoreactive CD4+ T cells to the inflamed tissue. Targeting cytokines produced by autoreactive CD4+ T cells may also provide therapeutic potential to prevent their effector functions but this strategy requires deep understanding of the biology of cytokine production so that the therapy will not interfere with the protective immune response. Alternatively, blocking of cytokine receptors e.g. on B cells could be advantageous. In order to selectively target the cytokine receptor on specific cell population, blocking antibodies against cytokine receptors can be coupled with another antibody against surface marker expression. For example, the monoclonal antibody targeting IL-10R coupled with rituximab can selectively prevent IL-10 binding to induce B cell proliferation.

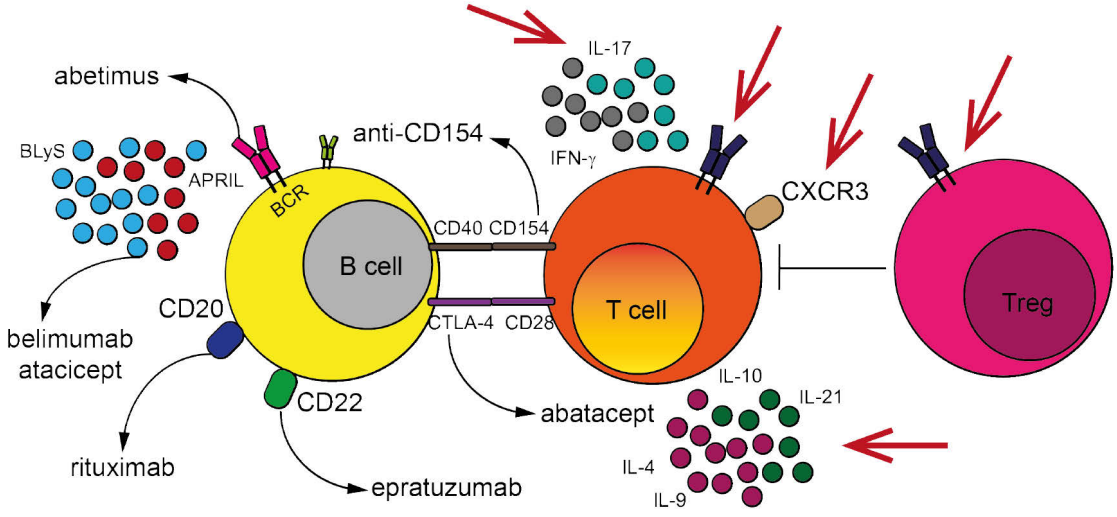


Figure 38: **Role of autoreactive CD4+ T cell subsets as therapeutic tool for SLE.** Current therapies in SLE focus on targeting B cells. After identifying the pathogenic functions of autoreactive CD4+ T cells, it is possible now to develop novel therapeutic strategies focusing on autoreactive CD4+ T cells

and autoreactive Treg. Sequencing of TCR molecules can facilitate development of multimer molecule that can bind specifically on the autoreactive TCR, thus autoreactive TCR can be depleted or the function of autoreactive CD4+ T cells can be blocked. Identification of TCR of autoreactive Treg allows isolation of these cells for *in vitro* expansion. Autoreactive Treg can be used for adoptive Treg therapy to restore Tcon/Treg imbalance and enhance their function in maintaining immune tolerance.

Furthermore, application of autoreactive Treg for therapeutic purposes is promising. We could show that these Treg acquire suppressive function both at polyclonal and antigen-specific level. Identification of the TCR sequence of autoreactive Treg allows isolation of these cells for *in vitro* expansion. We have established the protocol for efficient and stable Treg expansion *in vitro*. Expanded Treg can be used for adoptive Treg therapy to restore Tcon/Treg imbalance and enhance their function to maintain peripheral tolerance. Because Treg are plastic and they can transdifferentiate into effector T cells, combining adoptive Treg therapy with drugs that maintain stability of FoxP3 expression in Treg is of great interest [269].

3. Future perspectives

Although this work can provide information about the role of autoreactive CD4+ T cells in SLE pathogenesis and as therapeutic tool, there are still many unsolved questions remaining. In this part, we highlight which further approaches are needed and advisable to answer the remaining open questions in SLE. We also underline several interesting research areas which can be explored to improve our understanding and therefore to open new perspectives for our aim to help patients with autoimmune disorders. Epidemiologic research reveals that the socio-economic impact of SLE is tremendous. The variety of the disease not only relies on its nature, but it is also attributable to a variety of factors, including demography, income level, education, health insurance status, medication compliance, and occupation. Not only the costs for treatment are high, but also the costs due to the loss of economic productivity. Patients with SLE have reduced health-related quality of life and productivity throughout their life [270].

3.1. Big-data analysis and stratification of patients with SLE

SLE is a multifactorial disease and has many faces, different symptoms, various clinical manifestations, and heterogeneous organ involvements. Clinical features among patients with SLE can be extremely variable so that treatment of patients still often results in poor efficacy. Personalized therapy for patients with SLE is beneficial since the therapy can be adjusted and developed according to the patient's clinical records. Great variability in SLE has driven investigators to cluster SLE patients into different subgroup depending on their clinical features. Banchereau *et al.* have assessed the molecular heterogeneity of SLE by longitudinally profiling the blood transcriptome of more than 150 patients with SLE and by considering the demography of the patients, treatments, disease activity, and nephritic class [61]. By combining the data, they could identify enrichment of neutrophil transcripts in disease progression and distinct signatures in response to treatment. They were able to stratify SLE patients into seven groups uncovering molecular heterogeneity and providing an explanation for the failures of clinical trials. However, their data lack information regarding CD4+ T cells reactive to nuclear antigens. Therefore it is now interesting to develop research with the similar direction aimed to collect data for stratification of

patients with SLE. Correlation of the frequency of autoreactive CD4+ T cells, IFN-γ production, and Tcon/Treg imbalance with disease activity may contribute to the improvement of current stratification strategies. Big data analysis could be an interesting approach, for example by collecting transcriptome or genome sequencing data using next generation sequencing from patients with SLE and healthy individuals as well as phenotypic analysis of autoreactive CD4+ T cells using a combination of multimer technology and mass cytometry. Combination of epidemiological and clinical data with biological data can facilitate an in-depth analysis promoting better understanding of the disease.

3.2. Urine as a window to study renal involvement in SLE

Urine of active SLE patients with lupus nephritis contains a number of immune cells from the innate and adaptive immune system and also epithelial cells. Our idea is to model renal inflammation *in vitro*. First, epithelial cells are collected from urine and they are cultured in culture medium containing all supplements needed for epithelial cell growth. Additionally, autoreactive CD4+ T cells are isolated from urine and expanded *in vitro*. Epithelial cells are pulsed with recombinant nuclear antigens thus the antigens are presented by the epithelial cells on MHC class II molecules. A nuclear antigen-reactive CD4+ T cell line is then added into epithelial cell culture and their activation as well as cytokine production is observed. Furthermore, addition of autoreactive Treg line into the culture may also provide an approach to study suppressive function of Treg in the inflamed tissue.

3.3. Diversity of microbiome and SLE

The microbiome represents the collection of bacteria, viruses, and fungi that live on and in the human body. Microbial cells extremely outnumber human cells within the body and today they are no longer acknowledged as silent passenger as previously thought after we know that some can modulate the immune system [271]. Research about the microbiome has grown exponentially, as it now represents a novel and promising target for therapy. Evidence about the connection of microbiome population and SLE has been reported in some publications. Female SLE patients bear a lower ratio of *Firmicutes* to *Bacteroidetes* than healthy individuals and patients in the remission [272]. The urinary tract is residence of several microbiomes and their presence is detectable in the urine [273]. Analysing the collection of microbiome in urine of patients with active SLE and lupus nephritis compared with healthy individuals may provide new information about the role of microbiome diversity and SLE. The effect of microbiome is not fully investigated, but certain bacteria in the gut were observed to foster the development of Treg [274, 275]. Although investigation to develop therapeutic strategies using the microbiome in humans has been limited to the setting of infection and inflammatory bowel disease, research in this field represents promising alternatives to understand the pathogenesis of SLE and to find novel therapeutic strategies against SLE.

3.4. Novel T cell-associated lupus-mouse model

In biomedical research including in SLE, the mouse model is widely used to study the disease for a better understanding of the genetic and cellular factors involved in the development of systemic autoimmunity and SLE-associated clinical symptoms.

Although useful to demonstrate the clinical relevance of the disease *in vivo*, mouse models cannot fully represent the conditions in humans. Developing a humanized mouse model to study the role of autoreactive CD4+ T cell subsets including Treg becomes important and advisable. Our idea to use a humanized mouse model to study T cell-mediated pathogenesis in SLE has been inspired by the study conducted by Andrade *et al.* [276]. They have shown that PBMC from SLE patients efficiently engraft in BALB-Rag-2$^{-/-}$IL2-R$\gamma^{-/-}$ mice. Such humanized mice show typical features of human SLE, such as proteinuria and human IgG deposits in the kidneys, and a shorter life span compared to mice engrafted with PBMC from healthy donors. We can use this model to evaluate the function of isolated or TCR-transgenic SLE-associated autoantigen-specific CD4+ T cells *in vivo*. BALB-Rag-2$^{-/-}$IL2-R$\gamma^{-/-}$ mice can be reconstituted with T cell-depleted PBMC from HLA-DR3+ donors together with autoreactive or polyclonal CD4+ T cells. Transfer of the following combinations of PBMC and T cells will determine the function of CD4+ T cells in general and of SLE-associated autoantigen-specific CD4+ T cells in particular in a healthy or diseased immune setting. In addition, induction of lupus in BALB-Rag-2$^{-/-}$IL2-R$\gamma^{-/-}$ mice by autoreactive CD4+ T cell transfer can provide us with a model to test the ability of polyclonal versus antigen-specific Treg to control the lupus autoimmune reaction, which could be the basis for cellular therapies in SLE.

3.5. Antigen-specific Treg-based therapy in SLE

Essential progress in understandig the biology of Treg and their fuction in health and disease has led to an increasing interest in the possibility of using Treg as a biological therapy to restore tolerance to autoantigens in the course of an autoimmune diseases such as SLE. Adoptive transfer of Treg becomes a promising immunotherapy that may have several advantages over conventional therapies [46]. We could show that antigen-specific Treg can suppress the proliferation of autoreactive CD4+ T cells both at polyclonal and antigen-specific level. Although it is still not known whether antigen-specific Treg are superior to polyclonal Treg in the suppression of an antigen-specific T cell response, we believe that application of antigen-specific Treg for therapeutical purposes provides improved safety because antigen-specific Treg will not interfere with other Treg that may induce ignorance in case of malignancy or immune deficiency in case of infection. The research should start with the isolation of antigen-specific Treg for isolation of their TCR sequence. It should be followed by the generation of TCR-transgenic Treg for adoptive therapy by using retroviral transduction. TCR-transgenic Treg can be expanded *in vitro* to reach sufficient cell numbers before adoptively transfering them into patients. Current therapy by depletion of immune cells and injection of donor-matched haematopoietic stem cells into SLE patients seems to be promising, but it can only target cells circulating in the periphery. Memory pathogenic cells residing in the tissue are therefore not targeted and they can be a source of new reccurring pathology. Combination of stem cell therapy with adoptive antigen-specific Treg therapy may result in improved clinical outcome in the future.

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Appendix

Table A1: **SLE patient data.** Due to data protection, only following information is shown. Patient data were retrieved from the record at Charité Universitätsmedizin Berlin. LN: Lupus nephritis. SLEDAI: SLE Disease Activity Index. Sex f: female. Sex m: male. N/A: data not available.

Initial	Age	SLEDAI	Sex	LN class
N5	31	20	f	IV
N10	23	20	f	IV
N11	31	22	f	IV
N4	27	18	f	IV
N7	31	20	f	IV
N8	38	20	f	IV
L6	33	2	f	N/A
L10	40	4	f	N/A
L11	28	1	f	V
L20	23	4	f	II/V
L12	57	10	f	III
L23	31	2	f	IV
L301	20	6	f	N/A
L303	40	2	f	N/A
L304	36	2	f	IV
N300	57	16	f	IV
L300	53	10	f	N/A
L302	31	12	f	IV
N304	65	14	f	N/A
N305	41	18	f	IV
N307	57	12	f	IV/V
L305	20	12	f	IV
L306	24	12	f	IV
N308	34	12	m	V
L307	42	4	f	N/A
L309	38	2	f	N/A
L310	40	4	f	N/A
A30	46	2	f	N/A
A31	37	4	f	N/A
A32	33	14	f	N/A
A33	30	7	f	N/A
A34	23	2	f	N/A
A35	34	4	f	N/A