View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repositório da Universidade Nova de Lisboa

RICARDO LUÍS CARVALHO MONTEIRO
Licenciado em Química, Minor em Biologia pela Faculdade de Ciências da Universidade de Lisboa

LINK BETWEEN VIRAL INFECTION AND AUTOIMMUNITY IN PRIMARY SJÖGREN'S SYNDROME: CLUES FROM LYMPHOCYTIC PROFILE AND EBV ANTIBODIES

DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE EM BIOQUÍMICA PARA A SAÚDE

Orientador: Catarina Gregório Martins, PhD, Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Co-orientador: Luís Miguel Borrego, MD, PhD, Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Setembro 2018

Ricardo Luís Carvalho Monteiro
Licenciado em Química, Minor em Biologia pela Faculdade de Ciências da Universidade de Lisboa

Link between viral infection and autoimmunity in primary Sjögren's Syndrome: clues from lymphocytic profile and EBV antibodies

Dissertação para obtenção do grau de Mestre em Bioquímica para a Saúde

Orientador: Catarina Gregório Martins, PhD, Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Co-orientador: Luís Miguel Borrego, MD, PhD, Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Júri:

Presidente: Prof. Doutor António Sebastião Rodrigues – Coordenador do Mestrado, Professor Auxiliar da Faculdade de Ciências Médicas | NOVA Medical School da Universidade NOVA de Lisboa

Arguente: Prof. Doutor Artur Augusto Paiva – Professor Adjunto da Escola Superior de Tecnologia da Saúde de Coimbra

Vogal: Profª. Doutora Ana Catarina Silva Gregório da Costa Martins – Professora Auxiliar Convidada da Faculdade de Ciências Médicas | NOVA Medical School da Universidade NOVA de Lisboa

Vogal: Profª. Doutora Maria Teresa Nunes Mangas Catarino - Professora Auxiliar da Faculdade de Ciências e Tecnologias da Universidade NOVA de Lisboa

Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Setembro 2018

‘*An intelligent heart acquires knowledge, and the ear of the wise seeks knowledge*.’

Book of Proverbs 18.15

Bibliographic elements resulting from this dissertation

Poster

Filipe Barcelos, Catarina Martins, **Ricardo Monteiro**, Glória Nunes, Teresa Lopes, Luis Miguel Borrego **Follicular CD8 T cells: correlation with circulating B cells and disease activity in primary Sjögren's Syndrome**, XX Congresso Português de Reumatologia, May 2018 **poster presentation.**

Ricardo Monteiro, Catarina Martins, Filipe Barcelos, Glória Nunes, Teresa Lopes, Luís Miguel Borrego. **Follicular CD8 T cells in primary Sjögren's Syndrome: clues for viral aetiology?** SPI'44 XLIV Anual Meeting - Sociedade Portuguesa de Imunologia, June 2018, **poster presentation.**

Oral communications

Ricardo Monteiro, Catarina Martins, Filipe Barcelos, Glória Nunes, Teresa Lopes, Luís Miguel Borrego. **Follicular helper and follicular cytotoxic T cells in primary Sjögren's Syndrome: clues for an abnormal antiviral response as a pathogenic mechanism**, 3rd CiiEM International Congress, June 2018 **oral communication.**

Published extended abstracts

Ricardo Monteiro, Catarina Martins, Filipe Barcelos, Glória Nunes, Teresa Lopes, Luís Miguel Borrego. **Follicular helper and follicular cytotoxic T cells in primary Sjögren's Syndrome: clues for an abnormal antiviral response as a pathogenic mechanism**, Annals of Medicine, June 2018, **extended abstract accepted for publication.**

Agradecimentos

Quero demonstrar o meu sincero agradecimento a todos os que tornaram possível a escrita desta tese e todo o trabalho que possibilitou a elaboração deste pedaço de conhecimento científico que, embora talvez seja uma pequena contribuição, pode ser um passo importante na compreensão de uma síndrome que afeta milhares de pessoas por todo o mundo.

Em primeiro lugar, é a essas pessoas, as pacientes de Síndrome de Sjögren, a quem quero dirigir o meu primeiro agradecimento e às quais dedico este trabalho. A todas as mulheres que se ofereceram para participar neste estudo e, dessa forma, contribuíram para o avanço do conhecimento nesta área.

Quero agradecer a minha orientadora, a Profª Doutora Catarina Martins, cuja dedicação, competência, conhecimento, amabilidade e disponibilidade foram indispensáveis para que este trabalho acontecesse.
Agradeço ao Prof. Doutor Luís Miguel Borrego pelo seu sempre pertinente *insight* e pela paixão contagiante pela imunologia.
Ao Dr. Filipe Barcelos os meus agradecimentos pelo apoio prestado e pela perseverante curiosidade e vontade de descobrir.
Uma palavra especial para as companheiras de laboratório, a Ionela, a Teresa e a Glória cuja companhia e auxílio na bancada não foram apenas trabalho, mas muitas vezes partilha de experiências e histórias que ficarão comigo.

Quero deixar um agradecimento muito especial à minha noiva, Diana Carvalho. Sem o teu encorajamento, esta tese não seria escrita. Ouviste-me muitas vezes quando te falava sobre imunologia, citometria de fluxo, autoimunidade, virologia e tantos outros temas que fui ganhando paixão. Ouviste muitos "…hoje não posso…" para que este trabalho acontecesse. Estiveste ao meu lado a cada passo, e é por isso e muito mais que te amo.

Ao meu pai e ao meu irmão, que sempre estão lá para me apoiar e encorajar- Obrigado! Também pelas perguntas que faziam sobre o que estava a fazer que me obrigavam a elaborar sobre o trabalho e, consequentemente, aumentavam a minha compreensão do trabalho.

Quero agradecer aos coordenadores do curso de mestrado em Bioquímica para a Saúde que estiveram mais presentes durante este processo - a Prof. Doutora Teresa Catarino e ao Prof. Doutor António Sebastião Rodrigues, pela constante disponibilidade e auxílio.

Por último, quero agradecer a Deus. Por me dar este privilégio de estudar e aprender. Pela oportunidade e alegria que é produzir ciência para benefício da vida de muitos. Pelo prazer da curiosidade e da descoberta, do qual muito pude experimentar durante a elaboração desta tese.

Obrigado.

Abstract

Primary Sjögren's Syndrome (pSS) is a chronic systemic autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, predominantly the salivary and lachrymal glands, leading to damage and secretory impairment. It occurs mainly in women, with ratios of incidence female/male are between 10:1 and 20:1. Women over 50 years of age are the most affected. The main symptoms of this condition are xerostomia (dry mouth), *keratoconjunctivitis sicca* (ocular dryness with corneal damage), and dryness of other mucosal surfaces (skin, vagina and respiratory epithelium). Extraglandular manifestations frequently occur, especially inflammatory articular and cutaneous manifestations, as well as less common and more serious involvement such as neuropathy, nephritis, vasculitis or lymphoma. Several viruses have been associated with pSS. Epstein-Barr virus (EBV) has been a strong candidate for the triggering of autoimmune epithelitis occurring in pSS. There isn't, however, an established viral trigger for SS autoimmune epithelitis. Our study revealed a lymphocytic profile indicating B cell hyperactivity, Breg impairment and T cell alterations, namely in circulating T cells with follicular-like phenotype. These changes can be attributed to an underlying viral agent triggering an unregulated response. Indeed, pSS patients presented a higher percentage of positive serum for anti-EBV EA-D IgG, which might point towards a role of EBV in pSS. Still, more work is required to establish a definite viral trigger for pSS. Adding to this study, more antiviral Abs can be assessed, namely, against CMV, human T-lymphotropic virus (HTLV), HCV and others. We call the attention towards follicular T cell subsets for a possible link between viral infection and pSS. More studies using animal models can be paramount in the understanding of follicular T cells roles in both contexts and identify similar mechanisms and differentiation patterns and a breakdown of tolerance mechanisms.

Resumo

A Síndrome de Sjögren primária (pSS) é uma doença sistémica autoimune caracterizada pela presença de infiltrados linfocitários nas glândulas exócrinas, predominantemente nas glândulas salivares e lacrimais. Ocorre principalmente em mulheres, onde os rácios de incidência feminino/masculino estão entre 10:1 e 20:1. Mulheres com mais de 50 anos de idade são mais afetadas. Os sintomas principais desta condição são: xerostomia (boca seca), *keratoconjuctivitis sicca* (secura dos olhos com inflamação), e secura de outras superfícies mucosas (pele, vagina e epitélio do aparelho respiratório). Vários vírus têm sido associados à pSS. O vírus Epstein-Barr (EBV) tem sido um forte candidato para o despoletar da epitelite autoimune presente na pSS. No entanto, não existe um causador viral estabelecido da epitelite autoimune em pSS. O presente estudo demonstrou um perfil linfocítico que indica hiperatividade da célula B, limitação da célula Breg e alterações na célula T, nomeadamente nas células T circulantes que possuem um fenótipo caracteristicamente folicular. Estas alterações podem apontar para a existência de um agente viral subjacente causador de uma resposta imune desregulada, uma vez que alterações semelhantes estão presentes num contexto de infeção viral. De facto, pacientes com pSS apresentam uma percentagem aumentada de soros positivos de IgG anti-EBV EA-D, que poderá apontar para o envolvimento do EBV na pSS. Assim, é necessário mais trabalho para estabelecer um causador viral em pSS. Para acrescentar a este estudo, mais anticorpos para antivirais podem ser avaliados, nomeadamente, anti-citomegalovirus, anti-vírus T linfotrópico humano, anti-hepatite C entre outros. Chamamos a atenção para as células T foliculares para uma possível ligação entre infeção viral e pSS. Estudos adicionais com modelos animais podem ser cruciais na compreensão do papel destas células em ambos os contextos e identificação de mecanismos e padrões de diferenciação similares e a falha dos mecanismos de tolerância.

Table of Contents

BIBLIOGRAPHIC ELEMENTS RESULTING FROM THIS DISSERTATION........II

AGRADECIMENTOS III

ABSTRACT.. V

RESUMO VI

TABLE OF CONTENTS VII

FIGURES INDEX... IX

TABLES INDEX.. XI

ABBREVIATIONS XII

1. CHAPTER 1 – INTRODUCTION...1

1.1. Immunology overview 1

1.1.1. Innate and adaptive immunity...1

1.1.2. Lymphocytes and membrane markers 2

1.1.1. Germinal centres 5

1.1.2. Cytokines and antibodies 6

1.2. Sjögren's Syndrome (SS)...8

1.1.3. General features 8

1.1.4. Lymphocytic distribution in pSS 10

1.1.5. Pathophysiology..11

1.1.6. Etiopathogenesis 11

1.1.7. Viral infections and Sjögren's Syndrome 12

1.3. Objectives 14

2. CHAPTER 2 – MATERIALS AND METHODS...15

2.1 Patients...15

2.2 Flow cytometry 16

2.3 T cell characterization...17

2.4 T cell functional assay 18
2.5 B cell characterization 22
2.6 Anti-Epstein-Barr virus Abs 26
2.7 Statistics 28

3. CHAPTER 3 - RESULTS AND DISCUSSION 29

3.1 Demographic and clinical data of patient groups and HC assessed for B and T cell subsets 29
3.2 T cell subsets 30
3.2.1 Percentages and absolute values of T cell subsets 30
3.2.2 CD4 T cell subsets in pSS patients 32
3.2.3 Positive correlation between IL-21^{+} CD4 and CXCR5^{+} CD4 T cells 33
3.2.4 CD8 T cell subsets in pSS 35
3.3 B cell subsets 39
3.3.1 Percentages and absolute values of B cell subsets 39
3.3.2 Alterations in B cell subsets in pSS patients: the IgD/CD27 classification......41
3.3.3 Alterations in B cell subsets in pSS: the Bm1-5 classification 44
3.3.4 Plasmablasts and Breg subsets in pSS patients 48
3.4 EBV serologic markers in pSS patients 52
3.4.1 Demographic data 52
3.4.2 Anti-EBV EA-D IgG 53
3.4.3 Anti-EBV EA-D IgA 54
3.4.4 Anti-EBV CA IgG 54
3.4.5 Anti-EBV CA IgA 55
3.4.6 Anti-EBV-CA IgM 55
3.4.7 Anti-EBNA IgG 56
3.4.8 Summary 57

4. CHAPTER 4 - DISCUSSION AND CONCLUSIONS 59

5. BIBLIOGRAPHIC REFERENCES 66

Figures Index

Figure 1. **B cell development with highlighted subsets that might include regulatory B cells.** 5
Figure 2. **Immunoglobulin general structure, showing L and H chains.** 7
Figure 3. **Main symptoms of Sjögren's Syndrome** 9
Figure 4. **Images representative of gating strategies used to assess CD4 and CD8 T cell subsets** 20
Figure 5. **Images representative of the gating strategy for the evaluation of IL-21 and IL-17 expression by CD4 and CD8 T cells**. 21
Figure 6. **Gating strategies used to assess B cell subsets**. 24
Figure 7. **Circulating CD4 T cells in the groups of patients studied** 32
Figure 8. **Circulating CXCR5^{+} CD4 T cells in the groups of patients studied** 33
Figure 9. **IL-21-secreting cells in pSS patients** 34
Figure 10. **Correlation of IL-21^{+} CD4 T cells with CXCR5^{+} CCR7$^{dim/-}$ CD4 T cells in pSS**. 35
Figure 11. **Circulating CD8 T cells in the groups of patients studied** 36
Figure 12. Percentages of CXCR5^{-} CCR7^{hi} and CXCR5^{-} CCR7^{+} CD8 T cells 36
Figure 13. **Circulating IL-21^{+} CD8 T cells in the groups of patients studied** 37
Figure 14. **cTfc cells and disease activity in pSS** 38
Figure 15. **Percentages and absolute values of naïve B cells** 41
Figure 16. **Percentages and absolute values of memory B cells** 42
Figure 17. Percentages and absolute values of memory unswitched B cells 43
Figure 18. **Percentages and absolute values of memory switched B cells.** 44
Figure 19. **Percentages and absolute value of Bm1 cells** 45
Figure 20. **Percentages and absolute value of Bm2 cells** 46
Figure 21. Percentages and absolute values of Bm2' cells. 47
Figure 22. **Percentages and absolute values of eBm5 cells.** 47
Figure 23. Percentages and absolute values of Bm5 cells 48

Figure 24. Percentages and absolute values of IgM$^{+/-}$ CD38^{hi} plasmablasts..................49
Figure 25. **Percentages and absolute value of CD24^{+} CD27^{+} Breg cells.** 50
Figure 26. **Percentages and absolute values of CD24^{+} CD38^{hi} Breg.** 51
Figure 27. **Calibration curve for anti-EBV EA-D IgG**..53
Figure 28. **Calibration curve for anti-EBV CA IgG**..54
Figure 29. **Calibration curve for anti-EBNA IgG**..56

Tables Index

Table 1. **Surface molecules expressed in T cells subsets**..3
Table 2. **Stages of B cell development and membrane markers**.....................................4
Table 3. **EBV serology used to determine stage of infection.**..13
Table 4. **Monoclonal Abs used in this study** 17
Table 5. **Tube panel 1 for follicular T cell characterization** 18
Table 6. **Tube panel for T cell IL-21 and IL-17 expression** 18
Table 7. **Tube panel for B cell characterization** 22
Table 8. **Abs assessed with ELISA** 26
Table 9. **Patient groups and HC demographic data**. 29
Table 10. **Percentages and absolute values of CD4 T cell subsets**. 30
Table 11. **Percentages and absolute values of CD8 T cell subsets**. 31
Table 12. **Percentages and absolute values of B cell subset**. 39
Table 13. **Percentages (relative to total B cells) and absolute values of Bm1-Bm5 cells**. 40
Table 14. **pSS, RA patients and HC assessed for EBV Ab demographic data**.............52
Table 15. Results for Anti-EBV EA-D IgG...53
Table 16. **Results obtained for anti-EBV EA-D IgA**..54
Table 17. Results for Anti-EBV CA IgG...55
Table 18. **Results obtained for anti-EBV CA IgA**..55
Table 19. **Results obtained for anti-EBV CA IgM**...55
Table 20. **Results obtained for anti-EBNA IgG** 56
Table 21. **Summary of results obtain for all positive anti-EBV Ab and p-values for differences among groups.** 57

Abbreviations

ºC - Celsius

µL – microlitre

Ab – antibody

ADCC – antibody dependent cell-mediated cytotoxicity

AECG - American-European Consensus Group

Ag - antigen

APC – allophycocyanin

BAFF – B cell activating factor

BCR – B cell receptor

Breg – regulatory B cells

CA – capsid antigen

CCL – chemokine ligand

CCR – CC chemokine receptor

CD – cluster of differentiation

CNS - central nervous system

Cy5.5 - cyanine5.5

cTfc – circulating follicular cytotoxic T cell

cTfh – circulating follicular helper T cells

CXCR – CXC chemokine receptor

DC – dendritic cell

DN – double-negative

EA-D – diffuse early antigen

EBNA – Epstein-Barr natural antigen

EBV – Epstein-Barr virus

ELISA – enzyme-linked immunosorbent assay

eSS – secondary Sjögren's Syndrome

ESSDAI – European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index

EULAR – European League Against Rheumatism

FACS – Fluorescence Activator Cell Sorter

FBS – foetal bovine serum

FITC - fluorescein isothiocyanate

FL – fluorescence channel

FMO – fluorescence minus one

FoxP3 – forkhead box protein 3

FSC – forward scatter

GC – germinal centre

HC – healthy controls

IFN – interferon

Ig – immunoglobulin

IL – interleukin

LPS – lipopolysaccharide

MHC – major histocompatibility complex

mL – millilitre

mm – millimetre

NA – not applicable

NK – natural killer

PAMP – pathogen associated molecular patterns

PB – peripheral blood

PCR – polymerase chain reaction

PE – phycoerythrin

PerCP - peridinin chlorophyll protein complex

PMA – phorbol 12-myristate 13-acetate

PNS – peripheral nervous system

PRR – pattern recognition receptors

pSS – primary Sjögren’s Syndrome

RA – rheumatoid arthritis

RNA – ribonucleic acid

RU – relative units

SD – standard deviation

SLE – systemic lupus erythematosus

SLO – secondary lymphoid organs

SS – Sjögren's syndrome

SSA - Sjögren's-syndrome-related antigen A

SSB - Sjögren's-syndrome-related antigen B

SSC – side scatter

TCR – T cell receptor

Tfc – cytotoxic follicular T cell

Th – helper T cell

Tfh – follicular helper T cell

Treg – regulatory T cell

UCTD – undifferentiated connective tissue disease

1. Chapter 1 – Introduction

1.1. Immunology overview

1.1.1. Innate and adaptive immunity

The human immune system is responsible for defending the organism from harmful invaders such as viruses, bacteria and macromolecules.[44] It can also protect the organism from internal threats such as cancer cells.[1] Human immunity is divided into innate (also called non-specific) and adaptive (specific) immunity.[36] Innate immunity is the first barriers, such as the skin and the mucosal epithelia. It prevents microbes from establishing infections, as well as providing an immediate response if the microbes can penetrate the first barriers of defence (skin, stomach high acidity, mucosal secretions, etc). Innate responses are mediated by cells like the granulocytes (basophils, neutrophils, eosinophils and mast cells), phagocytes (monocytes/macrophages and also neutrophils) and natural killer (NK).[44] Pathogen associated molecular patterns (PAMP) from the invading microorganisms are detected by pathogen recognition receptors (PRR) present in immune cells and in soluble form in the serum and other body fluids.[3] PAMPs are essential molecules of the microorganism, like double-stranded RNA, lipopolysaccharide (LPS), lipoproteins and carbohydrates. Innate immunity acts by recognizing these general molecular structures common to a group of related microbes and is unable to distinguish between different types of microbes.[2] The time of response and efficacy of threat elimination is practically unchanged, even with repeated invasion.

Adaptive immunity responds to microbial invaders in a specific manner and the time and effectiveness of response improves each time a specific invader is encountered. This type of immunity is capable of defending the organism against a large number of microbes and establishing immunological memory, which allows for a more effective response in a second exposure to a given pathogen.[14] The adaptive immune response also adapts to better respond to an invading pathogen, increasing its recognition and elimination effectiveness. Adaptive immunity can be divided into humoral immunity and cell-mediated immunity. The cells

responsible for humoral immunity are the B cells, which are produced and matured in the bone marrow. These cells that produce and release antibodies which are the mediators of humoral immunity recognize microbial antigens (Ags) and opsonize them for posterior destruction by various mechanisms.[27]

These cells, when activated become plasmablasts, that produce and release the antibodies (Abs) in humoral responses.[27] Cell-mediated immunity aims for the destruction of cells infected with intracellular microbes, such as virus and some bacteria. This is carried out by cytotoxic T cells which are lymphocytic cells produced in the bone marrow, but their maturation occurs in the thymus. Also, another type of T cells are the helper T cells, whose function is to provide stimuli to B cells.[44]

Though distinct in terms of mechanisms and effector cells, innate and adaptive immune responses establish constant and diverse interactions. For instance, innate immune cells play a significant role in messaging adaptive response cells, and promote the recruitment of lymphocytes to sites of infection.

1.1.2. Lymphocytes and membrane markers

In immune responses, B and T cells interact with other immune players through membrane receptors. The B cell receptor (BCR) and the T cell receptor (TCR) are surface complexes present in B cells and T cells, respectively. In T cells, the TCR function is to bind an Ag presented by the major histocompatibility complex (MHC) molecules, expressed in the surface of Antigen-Presenting cells (APC).[38] However, this interaction between the TCR and the Ag:MHC complex is weak, and the binding affinity is enhanced by co-receptors.[54] Two major subsets of T cells are distinguished within T cells, according to the type of co-receptor they express: $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells - the helper T cells (Th) - are responsible for stimulating differentiation and proliferation of antibody-producing B cells, binding to the Ag presented by the MHC class II molecules.[72] Also, they engage in cytotoxic T cell activation and help macrophages in their phagocytic activity.[44,96] Th cells can be further divided in specific subgroups such as Th_1, Th_2, Th_{17}, Th_{17}/Th_1-like, Treg (regulatory T cells)

and Tfh (follicular helper T cells), which are distinguished by the type of cytokines and surface markers that they express.[8]

$CD8^+$ T cells are cytotoxic cells responsible for cell-mediated immunity and bind to the Ag presented in MHC class I.[72] Both types of T cells express yet another receptor, CD28, that works as a co-stimulator.[13,91] The interaction between CD28 present on T cells and CD80/86 (B7.1 and B7.2) on APCs is essential for T cell activation and survival.[41,46] T cells originate in a bone marrow cellular precursor that migrates to the thymus where development to mature T cells occurs. [26,63]

Table 1. Surface molecules expressed in T cells subsets.

T cell subsets	CCR7	CXCR5	CD4	CD8
Naïve	+	-	+	-
Effector	+	-	+	-
Central memory	+	-	-	-
Peripheral memory	+	-	-	-
Follicular T cells	-	+	+/-	+/-
T helper 1	-	-	+	-
T helper 17	-	-	+	-

During maturation, T cells go through several stages of development in the thymic cortex. After the thymic maturation, T cells can be categorized in different subsets: naïve, effector and memory. T memory cells can be further subdivided in central memory cells and peripheral memory cells. Follicular T cells express specific B cell follicle-homing chemokine receptors that induce their migration to B cell sites in secondary lymphoid organs (SLO). There, these T cells will provide B cell help by inducing specific antibody produced by plasma cells. The different T cell subsets differ in the expression of membrane markers according to the table above.[82]

Another important subset is the regulatory T cell (Treg). This subset is important in suppressing autoreactive T cells and maintaining immune homeostasis. These cells are distinct from other T cells by expressing either CD4 or CD8 along with CD25 with the absence or very low expression of CD127. Also, they express the X-chromosome-encoded transcription factor Foxp3 with required signalling from IL-10 in order to supress inflammation.[18]

On the other hand, B cell development starts in the bone marrow, beginning in stem cells originating lymphoid precursor cells and finishes in the periphery. B cell distinct stages of maturation and differentiation may be identified in peripheral blood (PB) by specific membrane markers expressed on the surface according to the following table.[25]

Table 2. Stages of B cell development and membrane markers.

B cell development stage	CD19	CD20	CD38	CD24	CD27	CXCR5	IgM	IgD
Immature	+	+	-	-	-	-	+	-
Mature naïve B cell	+	+	+/-	-		-	+	-
Naïve activated B cell	+	+	+	+	-	-	+	+
Germinal centre (GC) B cell	+	+	++	-	-	+	+	+/-
Post GC B cell	+	+	+	-	-	+/-	+	+/-
Memory B cell	+	+	-	-	+	-	+/-	+/-
Memory unswitched B cells	+	+	-	-	+	-	+/-	+
Memory switched B cells	+	+	-	-	+	-	+/-	-
Plasma blast	+	-	++	-	++	-	-	-
Plasma cell	+/-	-	+++	-	++	-	-	-

Like Tregs, B cells also have a regulatory subset. The origin and development of this subset are still in discovery, but recent results point toward a role in supressing exaggerated immune responses. Expression of CD24 and IL-10 is a phenotypic marker of Breg.

As for the BCR, it consists of a membrane immunoglobulin complex whose function is to bind to an Ag, leading to the initiation of a cascade of intracellular signalling. Then, the B cell internalizes the Ag and further processes it to be presented in the membrane through MHC class II and bind to the TCR in helper T cell. This binding of the Ag presented by the B cell to the TCR of helper Th cells TCR will stimulate the differentiation and proliferation of B cells. Like the T cells, the BCR also requires co-receptors, namely CD19, CD21 and CD81, that participate in the signal transduction cascade.

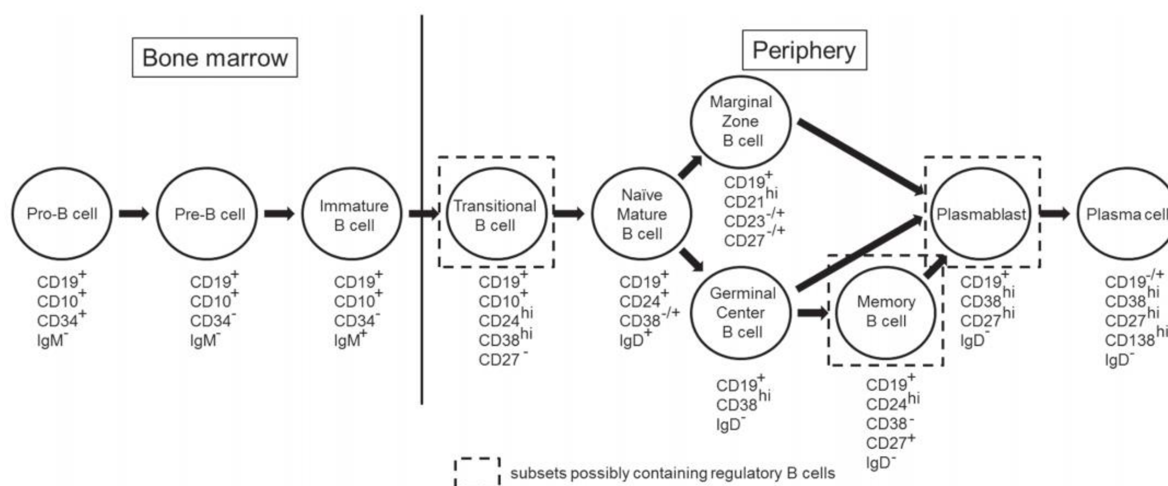


Figure 1. **B cell development with highlighted subsets that might include regulatory B cells.**

Adapted from Miyagaki et al. (2015).

Furthermore, subsets with regulatory functions have been identified in B cells.[64] These regulatory B cells (Bregs) are supposed to play a role in the suppression exaggerated immune responses.[49] A consensual phenotype/marker for Bregs has not been identified so far, thus their origin and development are still unclear. Expression of CD24 and CD38 are markers of Bregs.[32] The production of IL-10 is also considered an important functional marker of Breg.[23]

1.1.2. Cytokines and antibodies

Communication between immune cells is essential for an effective response against invading agents. Cytokines are small molecules that participate in these processes. These molecules potentially secreted by almost all types of cells and bind to specific receptors in target cells, leading to an alteration of enzyme activity and gene expression of that cell. Cytokines can regulate the duration and intensity of an immune response. Many physiological processes require the action of cytokines like wound healing, haematopoiesis, induction of inflammatory response and cellular and humoral immunity.

Antibodies (Abs) are another important set group of immunological proteins are the Abs, also designated as immunoglobulins (Igs). These glycoproteins, which are present in serum, are secreted by plasma cells and are responsible for the neutralization and opsonization for posterior phagocytosis of foreign microbial agents. Also, they participate in complement system activation and antibody-dependent cell-mediated cytotoxicity (ADCC).[44]

As to structures, Abs are heterodimeric, with two light (L) chains and two heavy (H) chains. In the amino-terminal region, there is a highly variable (V) region of about 100-110 amino acids. The rest of the Ab macromolecule has limited variation and is designated as the constant (C) region. The V region binds to Ags present in microbial agents while the C region interacts with immune cells, mediating effector functions (figure 2).[71]

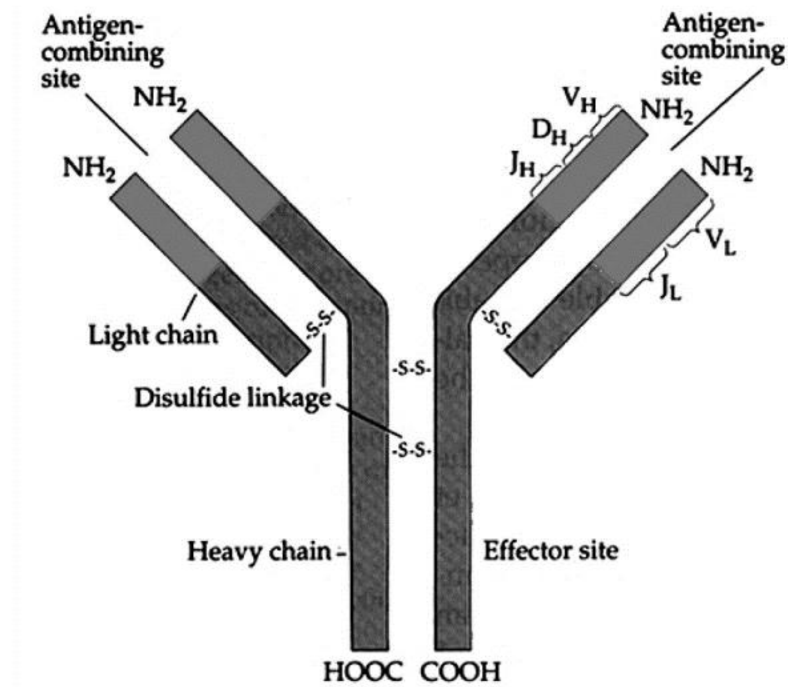


Figure 2. Immunoglobulin general structure, showing L and H chains.

Adapted from Navarro et al. (2010).

Abs can be divided in five different isotypes differing in their heavy chain constant (C_H) region: IgG, IgM, IgA, IgE and IgD. IgG is the most abundant class of immunoglobulins present in serum and has a variety of immunobiological activities such as complement activator (IgG3, IgG1 and IgG2), opsonization (IgG1, IgG3 and IgG2) and placenta crossing for providing foetal immunity (IgG1, IgG3 and IgG4). IgM composes around 5%-10% of serum immunoglobulins and can be present as a monomeric membrane immunoglobulin in B cells or can be secreted as a pentamer. IgM is the first immunoglobulin produced in a first contact with an Ag as well as the first immunoglobulin produced by the foetus. Due to its pentameric structure and subsequent higher valency, IgM is more efficient in antiviral responses, since viruses have a great number of repeating epitopes. IgA is the main immunoglobulin present in external secretions. Although it exists primarily in a monomeric

form, it can present itself in dimers, trimers and tetramers. Secretory IgA is present in dimeric or trimeric form. Mucous membranes are the main sites of IgA production.[44]

1.2. Sjögren's Syndrome (SS)

1.1.3. General features

Sjögren's Syndrome, a condition described initially by a Swedish ophthalmologist Henrik Sjögren in 1933, is classified as a chronic systemic autoimmune disease characterized by the presence of infiltrated lymphocytes in the exocrine glands, predominantly the salivary and lacrimal glands[15]. It occurs mainly in women, where ratios of incidence female/male are between 10:1[74] and 20:1[4]. Women with over 50 years of age are the most affected population.[74,76,78] The main symptoms of this condition are xerostomia (dry mouth), *keratoconjunctivitis sicca* (eye inflammation associates with dryness), and dryness of other mucosal surfaces (nose, larynx, pharynx, tracheo-bronchial epithelium, vagina) and of the skin. Due to the mucosal surface dryness, there is increased susceptibility to intraoral and intraocular infections. Other symptoms include dry skin, fatigue, arthralgia with oedema, parotid gland enlargement (most common in children), Jaccoud's arthropathy (hand deformities which can include swan neck deformity, Z deformity of the thumb, and ulnar drifts)[48], Raynaud's phenomenon (cold fingers accompanied by changes in skin colour and numbness)[11] and hepatomegaly (figure 3).[15] SS patients have a 6.5-fold increased risk of developing non-Hodgkin's lymphoma and a 1000-fold increased risk of parotid gland marginal zone lymphoma.[83] There are neurological manifestations in 20% of SS patients the most common being sensory ganglionopathy, painful small fibre neuropathy and transverse myelitis.[10] These neurological symptoms might even present themselves earlier than the dryness symptoms. Psychopathological features like depression, neuroticism and psychoticism are also a not rare feature in SS patients.[65] The distinction between primary SS (pSS) and secondary SS (eSS) can be made, where secondary SS is used to describe SS symptomology associated with other autoimmune diseases such as rheumatoid arthritis,

systematic lupus erythematosus and scleroderma. However, in a clinical context, this distinction is not useful.[15] SS activity and severity are measured using the EULAR SS Disease Activity Index (ESSDAI), which is the main standard for clinical studies for classification purposes. It evaluates 3 or 4 levels of activity in 12 organ systems (cutaneous, respiratory, renal, articular, muscular, peripheral nervous system (PNS), central nervous system (CNS), haematological, glandular, constitutional, lymphadenopathic and biological).[81]

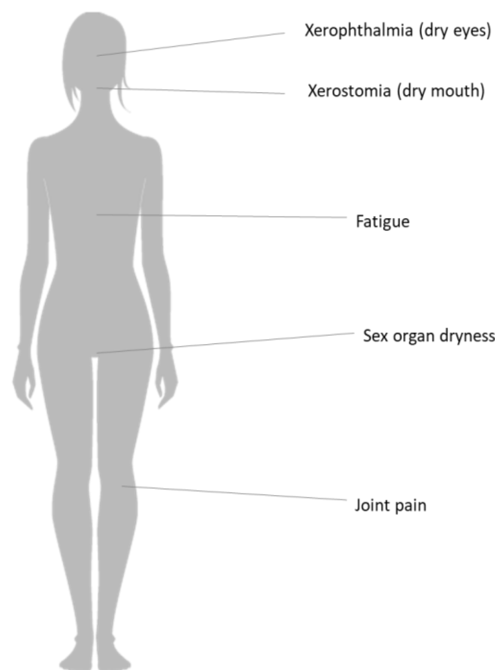


Figure 3. Main symptoms of Sjögren's Syndrome

Due to the presence of many non-specific symptoms like dryness, fatigue and arthralgia, which may to be ignored by the patient or attending physician for some time, diagnosis time can be delayed an average of four years.[24] Several criteria for SS classification diagnosis have been proposed over time, none of which being specifically developed for the diagnosis. The presence of anti-Ro/SSA (70-100% of patients) and anti-La/SSB (35-70% of patients)

Abs in serum and focal lymphocytic sialadenitis (at least 1 focus per 4 mm2 surface at a 4-lobule minimum) in a labial gland biopsy are the most useful criteria for diagnoses. However, since the biopsy is an invasive procedure, it is not suitable for reassessment and some many patients even refuse to do it.[19]

1.1.4. Lymphocytic distribution in pSS

Lymphocytes are central in the pathogenesis of pSS and, therefore, the levels of the different populations of lymphocytes in peripheral blood (PB) usually exhibit changes.[17] Starting with T cells, it is known that a considerable number of these cells infiltrate the organs most affected in pSS, like the salivary glands. Systemic T cell lymphopenia has been observed in pSS patients[7], but different subsets of T cells have been studied and evaluated as to their specific prevalence in SS patients' blood. $CD4^+$ Th cells are decreased in PB[57]. A study as also determined that the Th_1/Th_2 ratio is changed in pSS patients' salivary gland biopsy.[68] Furthermore, it has been demonstrated through cytokine production that the major difference in Th cells is an increased presence of Th_{17}/Th_1-like cells, capable of secreting both IL-17A and IFN-γ.[85] Also, one study demonstrated an increased number of a small subtype of Th_{17} cells, designated DN Th_{17}, which lacks the expression of both CD4 and CD8. These cells have been demonstrated as spontaneous producers of IL-17 in SS, and are present in increased levels in the peripheral blood and infiltrated salivary glands.[6]

Tfh cells might also important in pSS pathogenesis due to its ability to an exacerbated promotion the B cell survival and activity when ectopic-GC formation occurs in pSS affected organs. Compared to healthy individuals, pSS patients have greater amounts of Tfh cells in peripheral blood.[52] Treg cells may also be involved in pSS pathogenesis. These cells can be categorized in two distinct groups: IL-10 producing Treg and $FoxP3^+$ Treg. Evaluation of IL-10 production and $Foxp3^+$ Tregs has shown contradicting results.[34,51] $FoxP3^+$ expressing Treg cells measurements have even shown that there is an inversely correlation between its infiltrated quantity in salivary glands and in the peripheral blood.[20]

As for B cells, increasing evidence supports their active and central role in SS pathogenesis. B-lymphocytes leave the bone marrow as transitional B cell and proceed to the SLO, where their maturation continues under modulation of T cells.

Still, further insight is required in pSS lymphocytic profile. This could be useful for diagnosis and establish a differentiation pattern resembling antiviral responses, giving clue as to how viral pathogens could induce autoimmune epithelitis.

1.1.5. Pathophysiology

Exocrinopathy associated with SS is caused by the presence of activated B and T cells, as well as macrophages and dendritic cells (DC), in the parotid glands.[58,70] B and T cells exhibit an abnormal response to the ribonucleoproteins Ro/SSA and La/SSB. Because of this abnormal response to self-antigens, B and T cells produce increased levels of cytokines and chemokines, which further promote the autoimmune response, aggravating the inflammation that progressively destroys salivary gland epithelial cells. This vicious cycle of inflammation has been called autoimmune epithelitis, considering the role of the epithelial cells as Ag presenters, being central to the accumulation, activation and differentiation of immune cells in the autoimmune process of pSS.[15] The central role of parotid gland epithelial cells in the pathophysiology of SS is further suggested by the greater accumulation of lymphocytic infiltrates close and inside ductal epithelia and signs of in-situ activation demonstrated by histochemical studies that report the increased expression of immune competent molecules by epithelial cells. B-cell activating factor (BAFF) – a macromolecule essential for B cell survival[55] - expression was increased in epithelial cell that presented lesions associated with autoimmunity, suggesting that the B cell alterations described in pSS as well as the ectopic germinal centres (GCs) that are a hallmark of the syndrome.[59]

1.1.6. Etiopathogenesis

Many triggers of the dysregulated autoimmune response in pSS have been proposed, and they include genetic, hormonal and environmental components.[9] However, there is special

interest in the study of environmental triggers of SS, namely viral infections.[37] There are at least four proposed mechanism for the viral triggering of autoimmunity. One is molecular mimicry, where the pathogen, whether viral or bacterial, expresses proteins with similar amino acid sequence and structure to self-proteins. When these Ags are presented by APC through the MHC I and II, immune cells promptly respond to the aggression and start reacting with the self-Ags that are structurally and chemically similar to the pathogen Ags, resulting in an autoimmune response. There is also the mechanism of epitope spreading where the immune response to a persisting bacteria or virus causes damage to self-tissue by inducing lysis. Ags released by these lysed cells are taken by APC and presented as Ags, creating an immune response to self-Ags. The bystander activation mechanism is the indirect killing of non-infected cells during the immune response mediated by cytotoxic T cells and helper T cells. When T lymphocytes are recruited to a focus of infection, the lytic enzymes and cytokines released can damage and kill non-infected cells nearby, inducing additional immunopathology in these sites. The final mechanism of viral triggered autoimmunity in the processing and presentation of cryptic Ags. Cryptic Ags are products of self-Ag processing in which inflammatory and other immunostimulatory conditions cause upregulation of Ag processing events that can lead to enhanced presentation of the previously cryptic epitope by the APCs. This in turn might lead to priming of the cryptic epitope-specific T cells and cause autoimmunity.[29] Taking this in consideration and knowing that autoantibodies and hypergammaglobulinemia play and important role in SS pathogenesis, it is plausible to think that a viral agent is behind the autoimmune mechanism observed in SS.

1.1.7. Viral infections and Sjögren's Syndrome

Several viruses have been associated with pSS. Epstein-Barr virus (EBV) has been a strong candidate for the triggering of autoimmune epithelitis occurring in pSS pathology.[45] EBV is a B-lymphotropic γ-herpesvirus that causes lifelong infection, in most cases, asymptomatic. In spite of being present in around 90% of human adult population, its relationship with

several diseases as been established.[31] The life cycle of EBV can be divided in active lytic and latent phase. In the active lytic phase, the virus replicates and propagates, while in the latent phase, the virus remains inactive in B cells. Considering the main pathway of EBV transmission is through oral mucosa and preference for B cell infection, it is plausible that this virus may be involved in pSS aetiology and/or pathogenesis. Several studies demonstrate that the expression of EBV proteins is increased in SS patients, both in peripheral blood and salivary or lacrimal gland biopsies.[56,61,69,80,92] Also, polyclonal activation of B cells in pSS is greater that in RA and SLE patients.[86] Molecular mimicry has been pointed out as a possible mechanism for EBV-induced autoimmunity. Anti-Ro/La autoantibodies – found frequently in pSS patients – precipitates protein antigens associated with small RNA expressed by EBV.[50] Also, an EBV transcriptional activator, EBNA1 has caused cross reactivity with autoantibodies found in SLE.[75] There isn't, however, an established viral trigger for SS autoimmune epithelitis.

EBV serology can provide clues to the state of EBV infection in humans. The profile of anti-EBV Abs is used to establish the stage on which the infection is and can be used to predict EBV associated diseases.[73,84]

Table 3. EBV serology used to determine stage of infection.

Infection stage	EA-D		CA			EBNA
	IgG	**IgA**	**IgG**	**IgA**	**IgM**	**IgG**
Uninftected	-	-	-	-	-	-
Infectious mononucleosis	+	-	+	+/-	+	-
Convalescent	-	-			-	
Past infection	-	+/-	+	-	-	-
Chronic active infection	+	-	+++	+/-	-	-

Adapted from Straus, S. E. et al (1993)

For instance, in the beginning of an EBV infection, CA Abs are produced, followed by a production of finally EA-D Abs and EBNA Abs later on.

1.3. Objectives

Our aim with this work was to further elucidate the role of viral infection as an etiological agent of the autoimmune epithelitis observed in SS patients by 1) characterizing T and B cell population subsets, assess differences between patients groups and evaluate their correlation with disease activity, clinical manifestation and other immune parameters that might indicate a dysregulated antiviral response, 2) assess the viral background of patients, namely evaluating the presence and quantity of antibodies anti-EBV Ags.

2. Chapter 2 – Materials and methods

2.1 Patients

For this study, we included 57 adult patients (aged 18-75 years) with pSS were recruited from the rheumatology outpatient clinic in two hospitals in Lisbon - CUF Descobertas and Instituto Português de Reumatologia. pSS patients were classified according to the pSS classification criteria proposed by the American-European Consensus Group (AECG).[88] Recruitment and evaluation was performed between July 2014 and March 2017. Also, for comparison, 4 other groups were considered in this study: Sicca syndrome patients with (n=38) and without (n=30) criteria for undifferentiated connective tissue disease (UCTD), patients with rheumatoid arthritis (RA; n=20) and healthy controls (HC; n=24). The Sicca syndrome group as composed of patients with oral and ocular dryness (sicca syndrome) not fulfilling AECG criteria. The exclusion criteria from the AECG criteria were also applied to all sicca patients, and additional exclusion factors were considered for all patients: IgG4-related disease, history of other corneal diseases and refractive surgery. Sicca patients were classified as undifferentiated connective tissue disease (UCTD) if the presented ANA in a titre equal or greater than 1/320 and at least one additional clinical feature of autoimmune rheumatic disease, such as arthritis or inflammatory joint pain, cutaneous rash, Raynaud's phenomenon or cytopenia of autoimmune origin. Sicca patients without those features were considered non-UCTD. RA patients fulfilled the *American College Against Rheumatology/ European League Against Rheumatism (EULAR)* classification criteria.[5] All the participants in these groups were recruited from CUF Descobertas hospital. The control group was composed by healthy individuals without sicca syndrome recruited from the Ophthalmology outpatient clinic of CUF Descobertas hospital.

In pSS patients, disease activity was determined using the (EULAR) Sjögren's syndrome disease activity index (ESSDAI) validated scale.[81] pSS patients were stratified according to the disease activity in two subgroups: low disease activity (ESSDAI<5) and moderate or high disease activity (ESSDAI≥5).

A demographic and anthropometric characterization of patient groups and HC was made.
This study was approved by the Ethics committee of Hospital Cuf Descobertas, Ethics committee of Instituto Português de Reumatologia and NOVA Medical School Ethics (no. 17/2016/CEFCM). In addition, all patient groups involved gave written consent to participate in this study and all procedures were performed according to the guidelines of the Declaration of Helsinki.[93]

2.2 Flow cytometry

Flow cytometry was used for B and T cell compartment characterization. The cytometer used was the BD FACS Calibur™ cytometer (*BD Biosciences, San Jose, California, USA*). This cytometer is equipped with a 488 nm blue light laser emitter, and a red diode laser with wavelength emission of 647 nm, allowing a 4-color multiparametric analysis with 4 different fluorescence channels (FL1-4). A total of 3 ml of whole peripheral blood was collected into EDTA containing tubes and 3 to 5 mL were collected for tubes containing heparin. All samples were processed and analysed within 24 h after collection.
A pre-validated panel of monoclonal Abs was used for the characterization of the cell subsets, using the reagents shown in table 4.

Table 4. Monoclonal Abs used in this study

Marker	Clone	Fluorochrome	Manufacturer
CD3	SK7	FITC	BD Biosciences
CD4	SK3	PerCP Cy5.5	Biolegend
CD8	SK1	PE Cy7	Biolegend
CD8	SK1	APC	Biolegend
CD19	HIB19	PerCP Cy5.5	Biolegend
CD24	ML5	APC	Biolegend
CD27	APC	PE	Biolegend
CD38	HIT2	FITC	Biolegend
CCR7	G043H7	AlexaFluor647	Biolegend
CXCR5	J252D4C	PE	Biolegend
IgD	IA6-2	APC	BD Biosciences
IgM	G20-127	APC	BD Biosciences
IL-17	BL168	PE	Biolegend
IL-21	3A3-N2	APC	Biolegend

Cell Quest Pro™ (*BD Biosciences*) software was used for both acquisition and analysis and Infinicyt™ 2.0 (*Cytognos S.L., Salamanca, Spain*) software was also used for analysis purposes.

Fluorescence minus one (FMO) control tubes were considered for the evaluation of positivity in the panel markers. For functional assays, unstimulated tubes were used to access the basal levels of cytokine expression, acting as biological controls.

Each subset characterized was evaluated in absolute counts (cells/µL) and percentages relative to the respective mother-populations. To obtain absolute counts, a single-platform strategy was used, with BD Trucount™ (*BD Biosciences*) tubes.

2.3 T cell characterization

For T cell subset characterization, a panel with 3 tubes was prepared, according to table 5. In each tube, the described monoclonal antibodies were added to an aliquot of 50 µl of PB. After an incubation of 15 minutes in the dark, a lyse-wash protocol was used with BD FACS Lysing and BD FACS Flow solutions (*BD Biosciences*), respectively. In brief, 2 mL of BD FACS lysing solution were added to each tube; and cells were vortexed and left for incubation in the darkness at room temperature for 10 minutes. After a centrifugation at 400g, for 5 minutes

(*Centrifuge Heraeus Labofuge 400*), supernatants were discarded. Finally, cells were washed with 2 mL of BD FACS Flow washing solution, centrifuged at 400g for 5 minutes ( ), supernatants were removed, and 300 µL of BD CellFix solution was added to the pellet. After vortexing, samples were acquired in the cytometer. A minimum of 10 000 $CD4^+$ T cells were acquired per tube.

Table 5. Tube panel 1 for follicular T cell characterization

Panel 1	FL1	FL2	FL3	FL4
Tube 1	CD3 FITC (2,5 µL)	CXCR5 PE (2,0 µL)	CD4 PerCPCy5.5 (1 µL)	CCR7 Alexa647 (2,5 µL)
Tube 2	CD3 FITC (2.5 µL	-	CD4 PerCPCy5.5 (1 µL)	CCR7 Alexa647 (2.5 µL)
Tube 3	CD3 FITC (2.5 µL)	CXCR5 PE (2,0 µL)	CD4 PerCPCy5.5 (1 µL)	-

2.4 T cell functional assay

The expression of IL-17 and IL-21 was also assessed in T cells. For cytokine expression assessment, the panel of tubes described in table 6 was used.

Table 6. Tube panel for T cell IL-21 and IL-17 expression

Cytokine expression panel	FL1	FL2	FL3	FL4
Tube 1 (stimulated)	CD3 FITC (2,5 µL)	IL-17 PE (2,5 µL)	CD8 PE Cy7 (2,5 µL)	IL-21 APC (2,5 µL)
Tube 2 (unstimulated)	CD3 FITC (2,5 µL)	IL-17 PE (2,5 µL)	CD8 PE Cy7 (2.5 µL)	IL-21 APC (2,5 µL)

T cells need to be stimulated to express cytokines, such as IL-21 and IL-17. In this study, cell stimulation was made with phorbol 12-myristate 13-acetate (PMA) (50ng/mL; *Sigma Aldrich, St. Louis, MO, USA*) and calcium ionophore (1µg/mL, *Sigma Aldrich*) in the presence of Brefeldin A (1.0µg/ml, BD Pharmingen). Incubation was performed in a

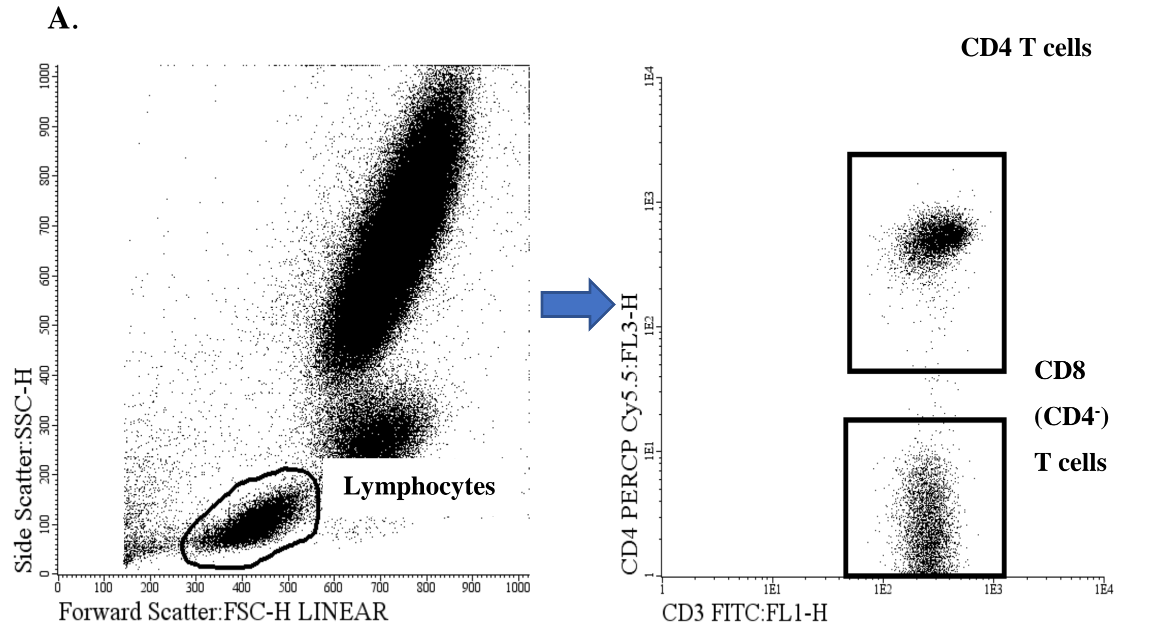
Heraeus™ Heracell incubator, and lasted for 5 hours at 37ºC, in a 5% CO_2 atmosphere. For each patient, stimulated and unstimulated tubes were run in parallel, to assure proper stimulation and staining controls. After incubation, the initial surface staining step was performed with CD3 FITC and CD8 APC in both stimulated and unstimulated tubes.

After this, fixation and permeabilization of cells was performed with the *BD* (BD Cytofix/Cytoperm™ Plus). Cells were thoroughly resuspended and incubated for 20 minutes with 500 µL of Fixation/Permeabilization solution. Cells were then washed twice in a buffer containing a cell permeabilizing agent – saponin (BD Perm/Wash™ buffer). Finally, cells were incubated for 20 minutes in the dark with anti-17 PE and anti-IL21 APC monoclonal antibodies. Before acquisition cells were washed again with BD Perm/Wash™ buffer and resuspended in 300uL of BD Cell Fix. A minimum of 10 000 CD4 ($CD8^-$) T cells were acquired per tube.

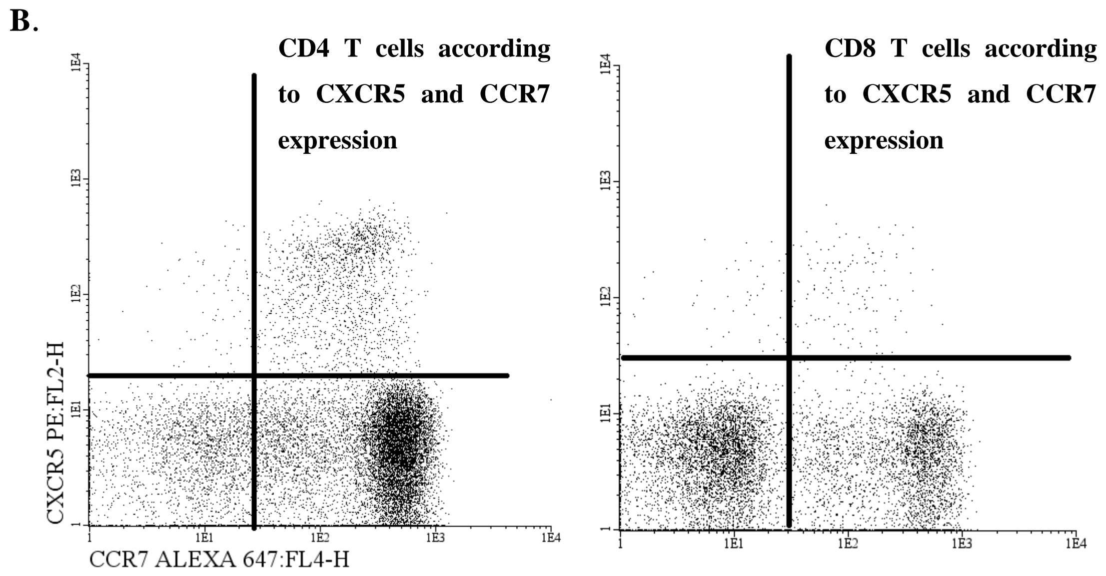
T cell subsets for both immunophenotyping and functional evaluation were defined according to the gating strategies presented in figure 4 and 5.

Figure 4. **Images representative of gating strategies used to assess CD4 and CD8 T cell subsets**

A.



B.

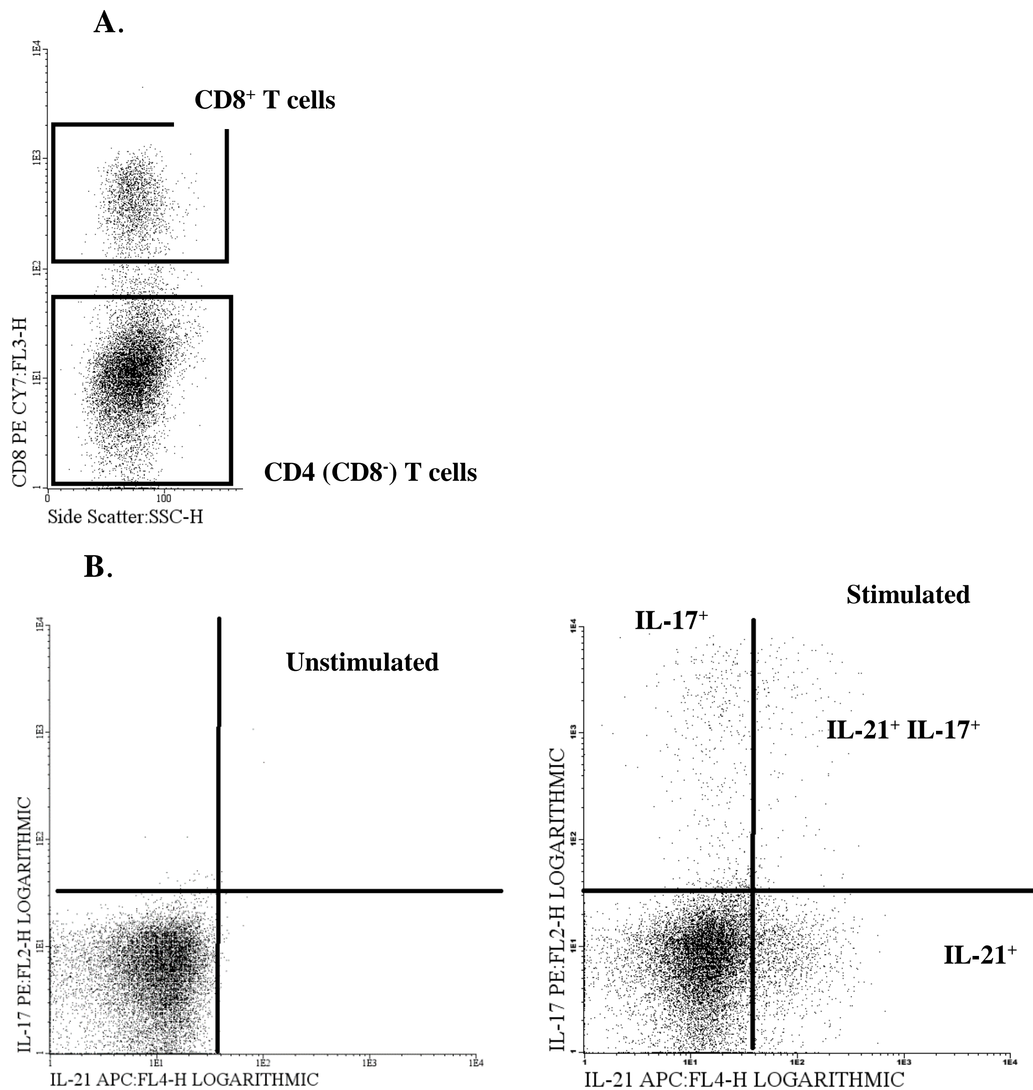


A. **Gating of lymphocytes in a SSC vs FSC dot plot, followed by the identification of CD4 T and CD8 T cells**.

B. **Follicular T cells were identified within CD4 and CD8 T cells according to the expression of CXCR5 (Tfh and Tfc) and were further divided in CCR7^{+} and CCR7$^{dim/-}$.**

Software: Infinicyt 2.0

Figure 5. **Images representative of the gating strategy for the evaluation of IL-21 and IL-17 expression by CD4 and CD8 T cells**.



A. **CD8 and CD4 (CD8-) T cells were identified within CD3+ lymphocytes, according to the expression of CD8. B**. **Identification of IL-21^{+}, IL-21^{+} IL-17^{+} and IL-17^{+} within CD4 and CD8 T cells**. Software: Infinicyt 2.0

2.5 B cell characterization

The panel of monoclonal Abs used to mark specific B cell surface-expressed molecules is described in table 7. As recommended for surface immunoglobulin evaluation in flow cytometry protocols, samples were washed twice with BD FACS Flow before staining. For this purpose, 500 µl of PB were added to a haemolysis tube along with 2mL of BD FACS Flow. A subsequent 400 g centrifugation for 5 minutes (*Centrifuge Labofuge 400*) was performed and followed by the removal of supernatant. After the second wash, the remaining pellet was resuspended with 300 µL of FACSFlow solution. Then, 100 µL of the resuspended cells was added to each tube of the panel, along with the respective monoclonal Abs.

Table 7. Tube panel for B cell characterization

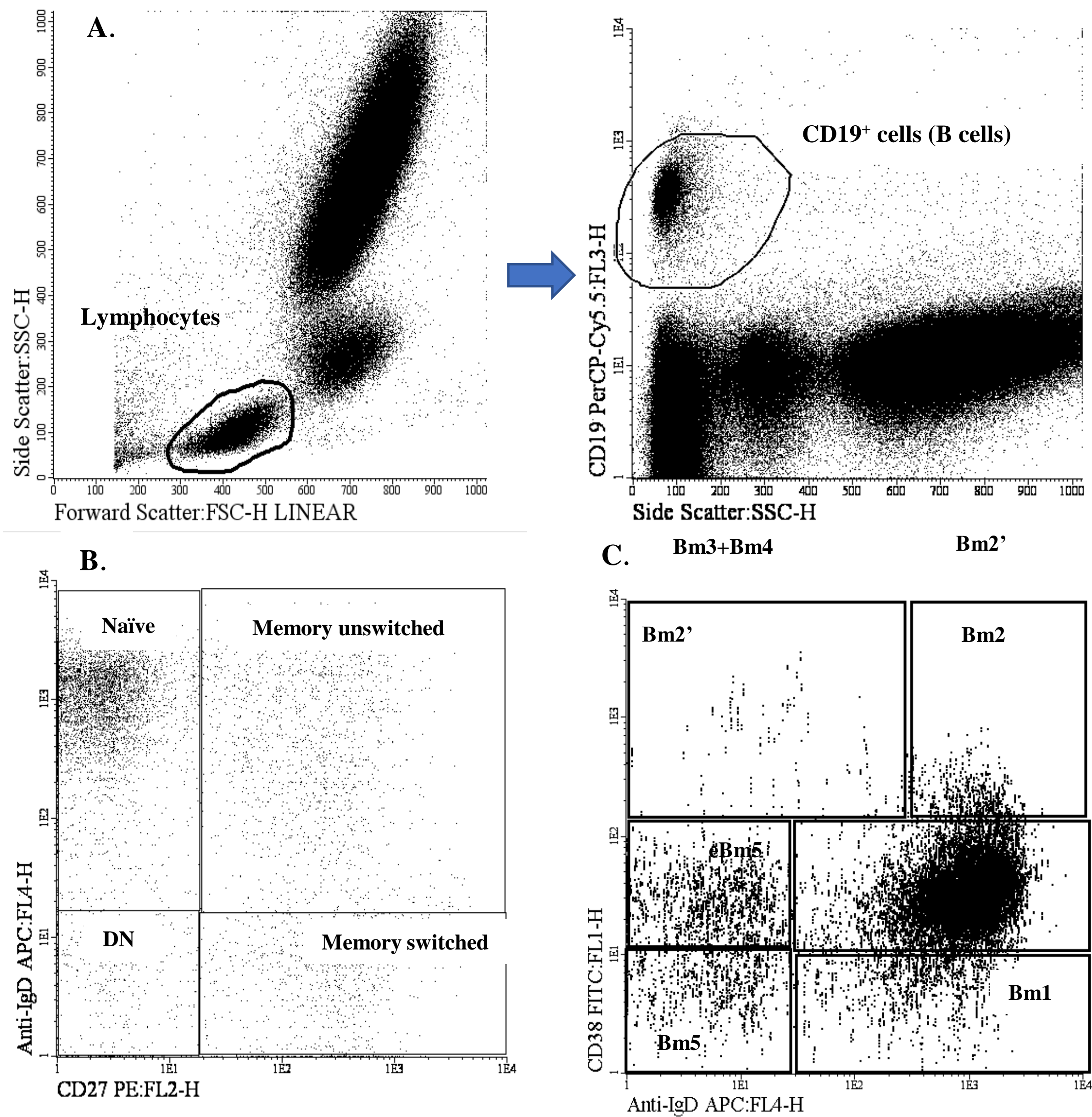
B cell panel	FL1	FL2	FL3	FL4
Tube 1	CD38 FITC (4 µl)	CD27 PE (2 µl)	CD19 PerCPCy5.5 (1 µl)	IgD APC (2,5 µl)
Tube 2	CD38 FITC (4 µl)	CD27 PE (2 µl)	CD19 PerCPCy5.5 (1 µl)	CD24 APC (5 µl)
Tube 3	CD38 FITC (4 µl)	-	CD19 PerCPCy5.5 (1 µl)	IgM APC (10 µl)

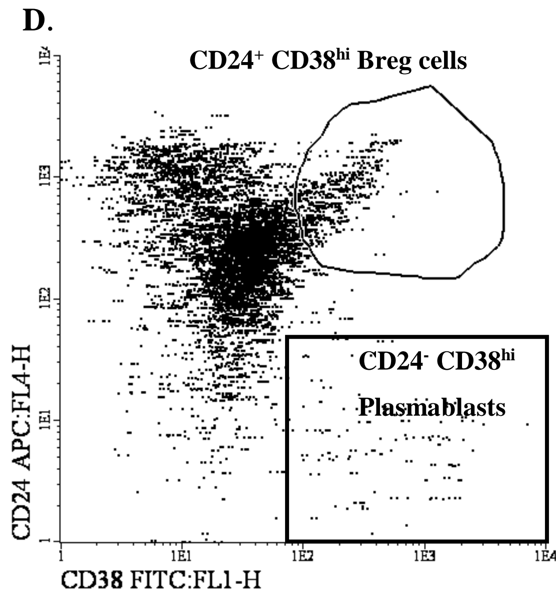
After the monoclonal Abs were added, the mixture was vortexed and left for incubation for 15 minutes in the darkness at room temperature. After the incubation, 2 mL of FACS lysing solution previously prepared were added, the mixture was vortexed and left for incubation in darkness and room temperature for 10 minutes. A subsequent centrifugation at 400g, for 5 minutes (*Centrifuge Heraeus Labofuge 400*) was done, and the supernatant discarded. The cells were washed by adding 2 mL of BD FACS Flow washing solution in each tube, followed by 400g centrifugation for 5 minutes (*Labofuge 400*) and

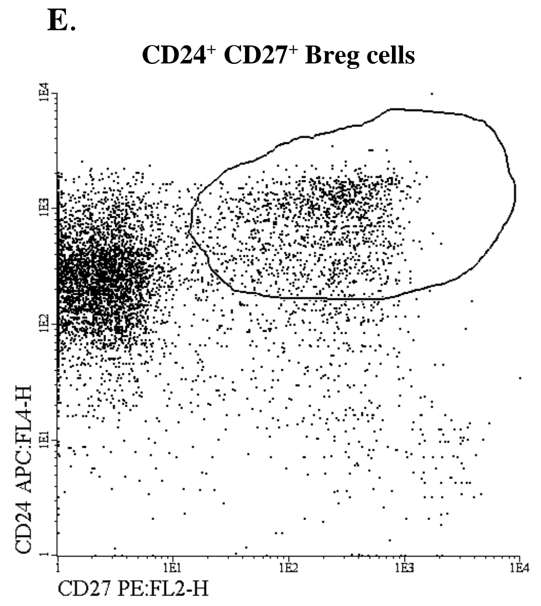
supernatant removal. Finally, 300 μL of BD CellFix solution was added to the pellet, the tubes vortexed and the samples acquired in the cytometer.

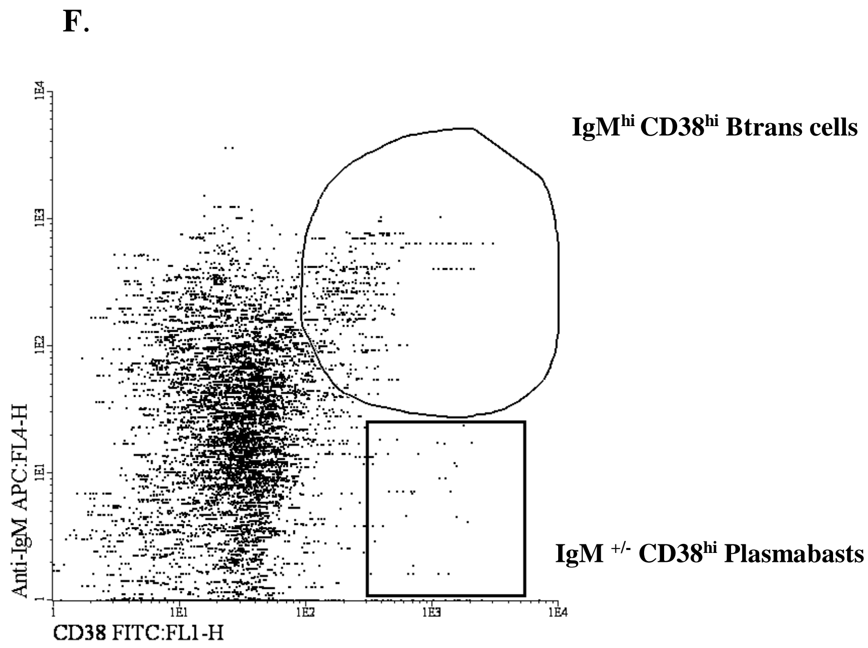
Using Infinicyt™ 2.0, B cells were analysed using CD19, CD27, CD38, IgD, and classified as naïve, unswitched and switched memory and plasmablasts according to the gating strategies displayed in Figure 4 for the classical IgD/CD27 classification and for the Bm1-Bm5 classification using IgD/CD38 expression. Moreover, B cell regulatory subsets were analysed: transitional $CD24^{Hi}$ $CD38^{Hi}$ and memory $CD24^{hi}$ $CD27^{+}$.

Figure 6. Gating strategies used to assess B cell subsets.




D.
CD24+ CD38hi Breg cells
CD24- CD38hi
Plasmablasts
CD24 APC:FL4-H
CD38 FITC:FL1-H
1
1E1
1E2
1E3
1E4


E.
CD24+ CD27+ Breg cells
CD24 APC:FL4-H
CD27 PE:FL2-H
1
1E1
1E2
1E3
1E4


F.
IgMhi CD38hi Btrans cells
IgM +/- CD38hi Plasmabasts
Anti-IgM APC:FL4-H
CD38 FITC:FL1-H
1
1E1
1E2
1E3
1E4

A. Gating of lymphocytes according to SSC and FSC characteristics, followed by the identification of $CD19^+$ cells (B cells) within the lymphocyte gate. B. Identification of naïve, memory switched and memory unswitched B cells ($CD19^+$) according to IgD and CD27. C. Identification of B cell subsets according to the Bm1-Bm5 classification (IgD and CD38). D and E. Identification of regulatory B cell subsets: $CD24^+$ $CD38^{hi}$ and $CD24^+$ $CD27^+$. F. Identification of $CD24^-$ $CD38^{hi}$ plasmablasts. Gating IgM^{hi} $CD38^{hi}$ Btrans cells and $IgM^{+/-}$ $CD38^{hi}$ plasmablasts.

Software: Infinicyt 2.0

2.6 Anti-Epstein-Barr virus Abs

Enzyme-linked immunosorbent assays (ELISA) were used for the assessment of IgG, IgA and IgM Abs against EBV Ags. All ELISA kits were obtained from *Euroimmun* (*Euroimmun, Luebeck, Germany*) and used according to the manufacturers' instructions. The following Abs for EBV Ags were determined: IgG for diffuse early Ag (EA-D), IgG for capsid Ag (CA), IgG for nuclear Ag 1 (EBNA1), IgA for EA-D, IgA for CA and IgM for CA. Tests for IgG Abs were quantitative while the tests for IgA and IgM were semiquantitative, being able to distinguish between positive and negative presence of the immunoglobulin (Table 8).

Table 8. Abs assessed with ELISA

Immunoglobulin	Ag	Type of assay
IgG	EA-D CA EBNA	Quantitative
IgA	CA EA-D	Semiquantitative
IgM	CA	Semiquantitative

For quantitative tests, a calibration curve was constructed using the three calibrator sera included in the kit. Since there are no international reference sera for EBVCA, EBNA and EBV EA-D IgG, relative units (RU) were used for concentration according to the

manufacturers' instructions. The concentrations of the calibrators used were 2 RU/ml, 20 RU/ml and 200 RU/ml. For semiquantitative assessment, a single calibrator serum was determined in triplicate, as recommended in the protocol. Also, a positive and a negative control were used in each assay.

Briefly, in each assay, 100 µl of calibrators, positive and negative controls and sample sera were incubated in individual wells of the pre-coated microplates for 30 minutes at room temperature. After this, the plate was manually washed with 300 µl of washing buffer for 3 times. At the end of the third wash, 100 µl of the proper enzyme-conjugated antibody (anti-human IgG conjugated with peroxidase) were added to the wells and left for incubation during 30 minutes in the dark. Again, three washes were performed as above-mentioned. After this, 100 µl of the chromogen/substrate solution were added to the wells and incubated for 15 minutes protected from light and at room temperature. Photometric reading was performed in an automatic photometric reader (Awareness Technology™ Stat Fax 2100) at 450 nm and 630 nm immediately after.

For quantitative assays, the calibration curve obtained with the calibrators sera was used to determine que antibody concentration in RU. Whenever concentration values for patient's samples were above the highest calibrator, the calibrator value (200 RU/mL) was considered for calculation purposes. In quantitative assays, Ab concentration levels were used to establish positive (≥22 UR/ml), negative (<16 UR/ml) and borderline (≥16 to <22 RU/ml results.

In semi-quantitative assays, a ratio between the sample/control absorbance and average of calibrator absorbances was determined and used to establish a positive or negative result as well as borderline values (ratio <0.8, negative; ratio >0.8 and <1.1; borderline; ratio >1.1, positive).

Sample concentration was determined using calibration curves constructed with ELISA-Logit-V24May2017 free software, available at https://ednieuw.home.xs4all.nl/Calibration/Logit/Logit.htm.

2.7 Statistics

GraphPadPrism™ 6.0 (*Graph Pad Software, San Diego, CA, USA*) was used for statistical analysis. Demographic and clinical data such as age, age of onset, time of disease evolution, age of diagnostic and ESSDAI scores were presented as median (maximum – minimum). Normality of data sets was assessed using D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests. ANOVA/Kruskal-Wallis was made for multiple analysis among groups. When a significant difference was found,each two groups were compared using the Unpaired Student's t test with Welch's correction whenever variables passed both normality tests, while Mann-Whitney test was used for non-normal variables. Results in demographic data tables are presented in median (minimum – maximum). In cell subset tables, percentage values are presented in mean ± SD while absolute values are presented in median (25th – 75th percentile). Fischer's test, Chi-square and odds ratio (OR) were applied to assess differences in positive cases among each patient group (pSS, HC and RA) studied in IgG anti-EBV detection and quantification. Statistical significance was considered for p-values <0,05.

3. Chapter 3 - Results and discussion

3.1 Demographic and clinical data of patient groups and HC assessed for B and T cell subsets

Each patient was registered in a local database and demographic analysis was performed regarding age, age of pSS onset, estimated time of disease progression, age of diagnostic and ESSDAI scores. All these sets of data were assessed and registered by the local clinician.

Table 9. Patient groups and HC demographic data.

	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC
Number	57	38	30	20	24
Gender (female:male)	56:1	38:1	30:0	16:4	24:0
Age, years	60,60 (28,6 – 78,3)	63,95 (33,2 – 75,6)	61,70 (36,8 – 76,2)	56,70 (30,30 – 78,0)	51,25 (38,9 – 66,2)
Age of onset, years	47,10 (24,5 – 68,3)	51,05 (20,5 – 66,3)	53,50 (30,0 – 63,8)	38,55 (22,2 – 61,9)	NA
Time of disease evolution, years	11,30 (1,00 – 29,50)	10,50 (4,1 – 26,0)	7,450 (1,9 -18,0)	10,20 (1,5 – 32,2)	NA
Age of diagnostic, years	53,80 (26,7 – 72,2)	57,70 (28,1 – 72,7)	60,60 (36,7 – 73,5)	42,50 (22,9 – 64,0)	NA
ESSDAI	2,0 (0,0 – 14,0)	NA	NA	NA	NA

Results are presented as median (minimum–maximum).

3.2 T cell subsets

3.2.1 Percentages and absolute values of T cell subsets

Tables 10 and 11 show the values obtained, both in relative percentages and absolute numbers of all T cell subset characterized. Percentages values are shown in mean ± SD while absolute values are presented in cells/µL - median (25th – 75th percentile).

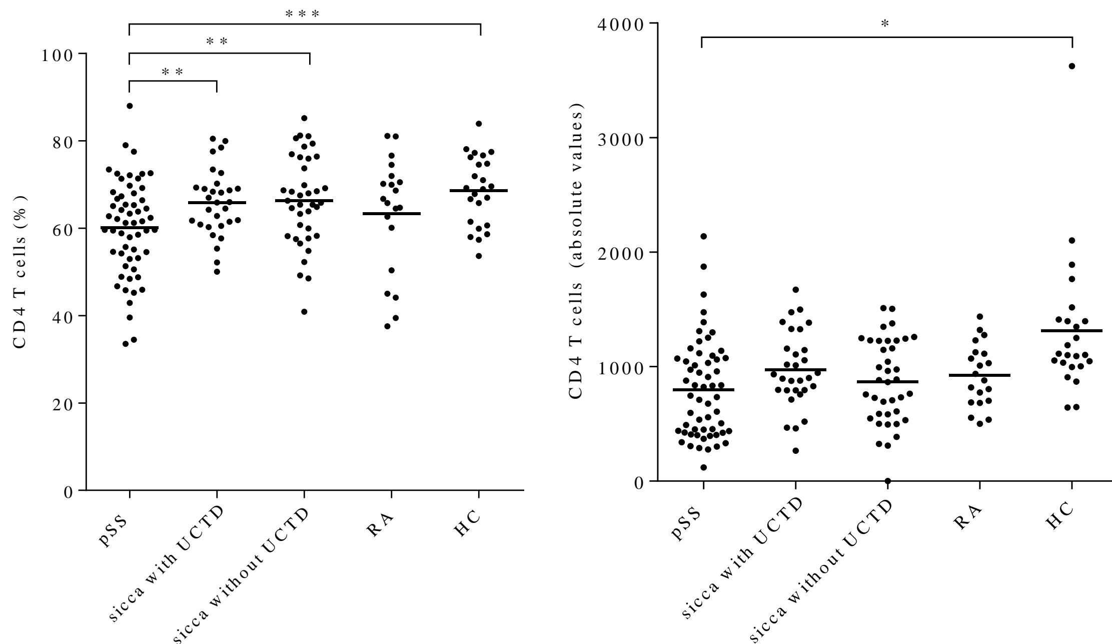
CD4 T cell subsets	%, mean ± SD					Absolute counts, cells/µl (25th – 75th percentile)				
	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC
CD4[a]	60.09 ± 11.00	66.31 ± 10.25	65.88 ± 7.673	63.28 ± 13.21	68.6 1 ± 7.972	746,0 (430,5 – 1073)	873,0 (575,0 - 1227)	917,0 (792,8 - 1200)	907,5 (691,0 - 1122)	1108 (1010 - 1408)
$CD4^+$ $IL\text{-}21^+$	12.38 ± 5.549	10.16 ± 2.910	9.665 ± 3.571	9.707 ± 4.120	9.729 ± 6.171	84,00 (51,50 - 121,5)	81,50 (58,75 - 121,5)	88,00 (57,25- 108,8)	91,00 (63,25 - 105,0)	113,0 (71,00 - 167,3)
$CD4^+$ $IL\text{-}21^+$ $IL\text{-}17^+$	0.7826 ± 0.4388	0.7287 ± 0.3442	0.8047 ± 0.4022	0.698 ± 0.422	0.7238 ± 0.467	5,0 (3,0 - 8,0)	5,0 (4,0 - 7,250)	7,0 (5,0- 8,5)	6,0 (3,0- 8,0)	7,0 (5,0 - 12,75)
$CD4^+$ $IL\text{-}17^+$	2.389 ± 1.182	2.493 ± 1.179	2.772 1.125 ±	2.826 ± 1.994	2.620 ± 1.280	13,0 (8,0 - 25,50)	20,00 (13,00 - 26,25)	25,0 (13,50 - 36,0)	21,50 (12,25 - 33,50)	30,00 (21,00 - 45,75)
$CD4^+$ $CXCR5^+$	18.96 ± 5.92	19.34 ± 4.62	18.05 ± 4.81	20.11 ± 6.10	20.02 ± 4.33	134,0 (82,0 - 203,0)	154,0 (105,0 - 220,5)	172,5 (134,8 - 200,3)	181,0 (136,8 - 218,5)	241,0 (221,0 - 294,8)
$CD4^+$ $CXCR5^+$ $CCR7^+$	18.38 ± 5.79	18.97 ± 4.49	17.66 ± 4.67	19.62 ± 5.99	19.51 ± 4.13	130,0 (78,00 - 195,5)	152,0 (102,0- 217,0)	171,0 (132,0 - 195,3)	179,0 (132,3 - 216,5)	233,0 (215,8 - 283,5)
$CD4^+$ $CXCR5^+$ $CCR7^{dim/-}$	0.58 ± 0.47	0.37 ± 0.22	0.39 ± 0.39	0.49 ± 0.37	0.51 ± 0.34	3,0 (2,0 - 5,0)	3,0 (1,0 - 4,0)	3,0 (2,0 - 5,0)	3,50 (2,0 -5,750)	5,0 (3,250 - 9,0)

CD8 T cell subsets	%, mean ± SD					Absolute counts, cells/µl, median (25th – 75th percentile)				
	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC
CD8^{+a}	39.35 ± 11.51	33,69 ± 10,24	34,13 ± 7,671	36,72 ± 13,21	31,39 ± 7,973	434,0 (320,5 - 643,0)	367,0 (270,8 - 513,0)	507,0 (370,8 - 587,0)	528,0 (336,0 - 667,3)	532,5 (394,0 -752,5)
CD8^{+} IL-21^{+}	5.510 ± 8.499	3,676 ± 2,374	3,235 ± 1,379	2,234 ± 1,328	4,585 ± 9,166	18,0 (10,0- 26,50)	12,0 (5,0 - 21,50)	15,00 (8,750 - 22,25)	9,0 (4,50 - 15,25)	12,50 (5,50 -25,50)
CD8^{+} IL-21^{+} IL-17^{+}	0.3437 ± 0.2862	0,3305 ± 0,3227	0,4540 ± 0,3853	0,3580 ± 0,3440	0,4779 ± 0,3983	1,0 (1,0 - 2,0)	1,0 (0,75 - 1,0)	1,500 (1,0 - 2,250)	1,50 (1,0 - 2,0)	1,50 (1,0 - 4,0)
CD8^{+}IL-17^{+}	1.065 ± 0.7268	1,057 ± 0,6403	1,398 ± 0,9198	1,270 ± 0,9068	1,552 ± 1,204	3,0 (2,0 - 6,5)	3,0 (2,0 - 6,0)	5,50 (2,0 - 9,0)	5,0 (3,0 -8,750)	6,0 (4,0 -11,75)
CD8^{+} CXCR5^{+} CCR7^{+}	2,301 ± 1,627	2,380 ± 1,282	2,103 ± 0,9843	1,719 ± 1,155	2,265 ± 0,9279	9,0 (5,0 - 14,0)	8,0 (4,750 - 12,00)	9,0 (6,750 - 13,75)	6,0 (4,250 - 13,0)	11,00 (7,250 - 19,50)
CD8^{+} CXCR5^{+} CCR7^{-}	0,9418 ± 0,9009	0,9416 ± 0,6836	0,6421 ± 0,3079	0,7020 ± 0,5349	0,8329 ± 0,4180	3,0 (2,0 - 5,0)	3,0 (2,0 - 5,0)	3,0 (1,750 - 4,250)	2,50 (1,0 - 6,750)	5,0 (3,0 -6,0)
CD8^{+} CXCR5^{-} CCR7$^{hi/+}$	16,98 ± 15,51	16,73 ± 15,13	20,18 ± 11,90	17,23 ± 13,01	24,96 ± 15,17	57,00 (23,50 - 112,0)	35,50 (25,00 - 83,50)	69,00 (34,75 - 158,3)	51,00 (24,50 - 130,5)	101,0 (73,25 - 190,0)
CD8^{+} CXCR5^{-} CCR7^{-}	18,78 ± 10,84	21,98 ± 13,68	21,87 ± 10,23	15,17 ± 9,436	24,78 ± 10,46	79,00 (34,50 - 121,5)	82,00 (39,00 - 124,3)	86,0 (55,50 - 144,0)	70,50 (43,50 - 87,50)	107,5 (95,25 - 176,0)

3.2.2 CD4 T cell subsets in pSS patients

Firstly, the values of CD4 T cells were significantly diminished in pSS when compared with sicca with UCTD (p=0,0054), sicca without UCTD (p=0,0061) and HC (p=0,0002) (figure 7).

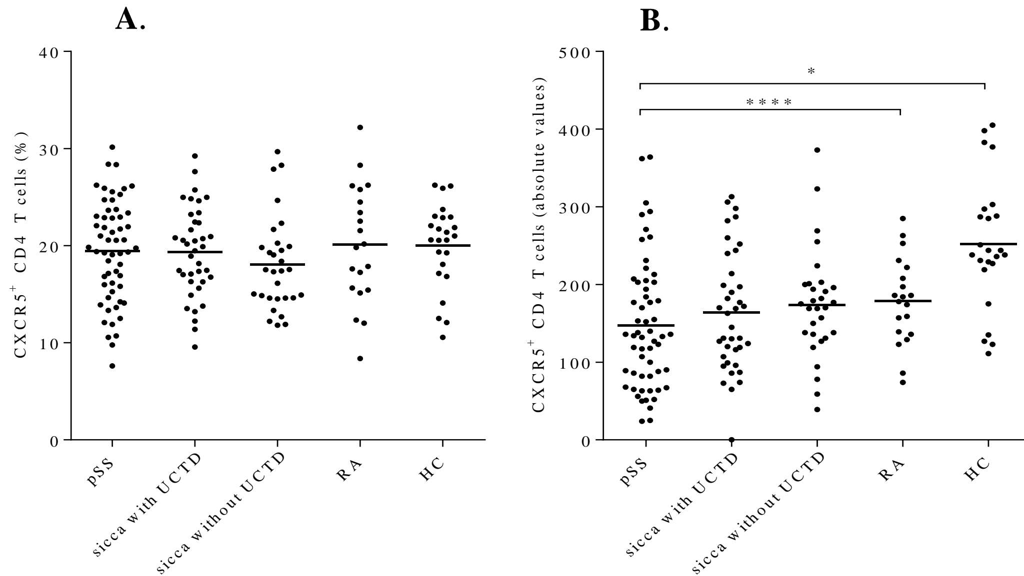
Figure 7. **Circulating CD4 T cells in the groups of patients studied**



A. **Scatter-plots with the distribution of percentage values of CD4 cells within T lymphocytes cells. pSS patients presented lower values when compared with sicca with UCTD, without UCTD and HC (***, p<0,01; **, p<0,001)**. **B. Scatter-plots with the distribution of absolute values of CD4 T cells. Lower CD4 T cells in pSS compared with HC (*, p<0,0001).**

Our results presented no significant differences in the percentages of $CXCR5^{+}$ CD4 T cells among the groups. However, lower absolute values of these cells appeared in pSS patients when compared with all groups, though statistical significance was only obtained when comparing pSS patients to HC (p<0,0001) and RA patients (p=0,0382) (figure 8).

Figure 8. **Circulating CXCR5⁺ CD4 T cells in the groups of patients studied**



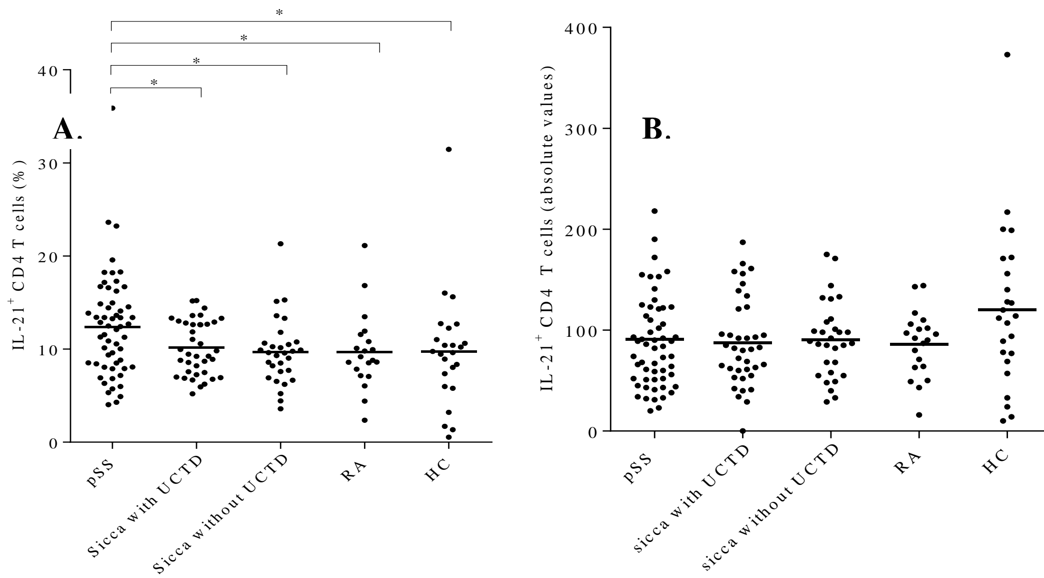
A. Scatter-plots with the distribution of percentage values of $CXCR5^+$ cells within CD4 T cells. All groups presented similar values. B. Scatter-plots with the distribution of absolute values of $CXCR5^+$ CD4 T cells. Lower $CXCR5^+$ CD4 T cell absolute counts in pSS patients (*, p<0,0001; ****, p<0,05).

On the other hand, percentages of IL-21-secreting CD4 T cells were increased in pSS patients compared to sicca patients with UCTD (p=0,02671), sicca patients without UCTD (p=0,04556), RA patients (p=0.04549) and HC (p=0.01581). There were no differences in absolute values of these cells between groups; in fact, HC presented increased values, but no statistical significance was obtained (figure 9).

3.2.3 Positive correlation between $IL\text{-}21^+$ CD4 and $CXCR5^+$ CD4 T cells

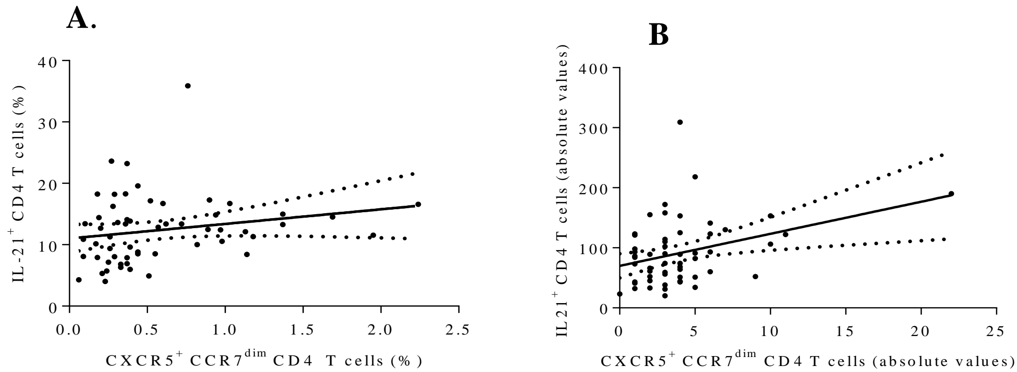
Another interesting finding was the correlation of $IL\text{-}21^+$ CD4 T cells with $CXCR5^+$ CD4 T cells (p=0.034, r=0.281, figure 10). This correlation might support that production of IL-21 by CD4 T cells is substantially assumed by the $CXCR5^+$ subset.

Figure 9. IL-21-secreting CD4 T cells in pSS patients



A. Percentages of IL-21⁺ CD4 T cells in pSS when compared with sicca (with and without UCTD), RA, and HC (*, p<0,0001). B. Comparison of absolute numbers of IL-21⁺ CD4 T cells for all patients' groups.

Figure 10. Correlation of IL-21$^+$ CD4 T cells with CXCR5$^+$ CCR7$^{dim/-}$ CD4 T cells in pSS.

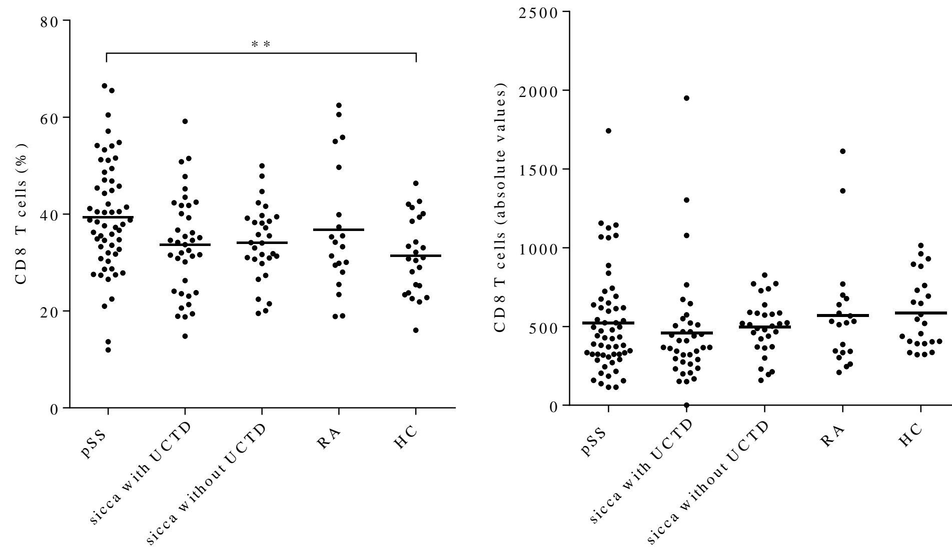


A. Percentage values (p=0.034, r=0.281); B. Absolute values (p=0,028, r=0,291).

3.2.4 CD8 T cell subsets in pSS

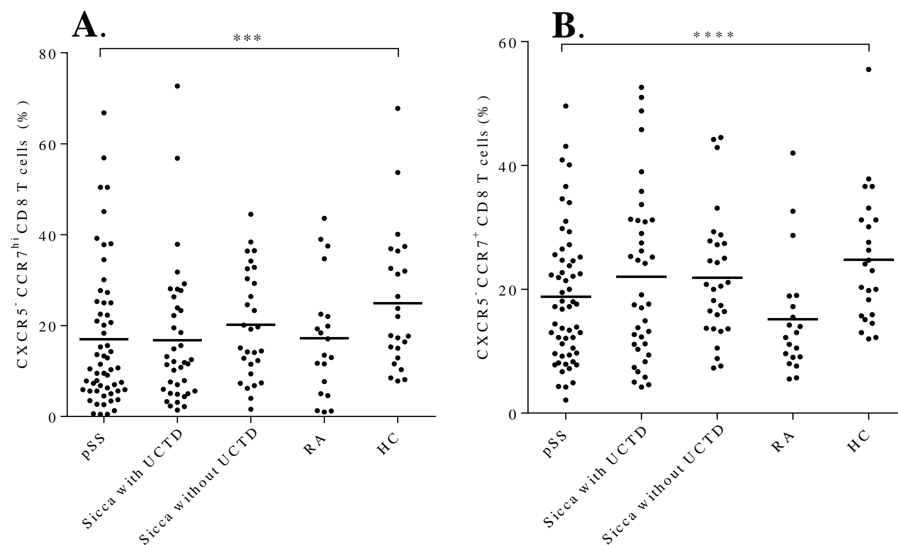
Regarding CD8 T cells, percentages were increased in pSS comparing with all other groups, but only when comparing with HC there was a statistically significative difference (p=0,0007) (figure 11). Regarding CXCR5$^+$ CD8 T cells, no alterations were found. Percentages of CXCR5$^-$ CCR7^{hi} (p=0,0056) and CXCR5$^-$ CCR7$^+$ (p=0,0128) within CD8 T cells were lower in pSS patients when compared with HC (figure 12). This might indicate a tendency towards differentiation. Still, IL-21-secreting CD8 T cells were also higher in pSS patients when compared to HC (p=0.02897) and RA patients (p<0,001). However, there were no differences when comparing pSS and both sicca groups. Nevertheless, absolute values of these cells appeared in lower numbers in pSS patients when compared with all groups, but only when compared with healthy controls, there was a statistically significative difference (p=0,029) (figure 13).

Figure 11. **Circulating CD8 T cells in the groups of patients studied**



A. **Scatter-plots with the distribution of percentage values of CD8 T cells within T cells. There was a statistically significant increase in pSS (**, p<0,001) vs HC B. Scatter-plots with the distribution of absolute values of CD8 T cells. There were no significant differences.**

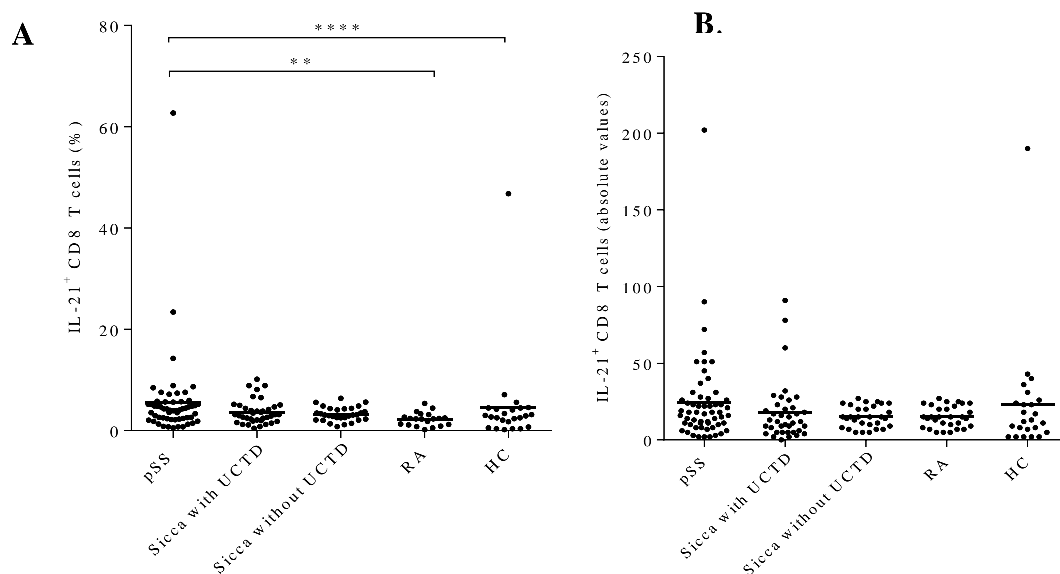
Figure 12. Percentages of CXCR5^{-} CCR7^{hi} and CXCR5^{-} CCR7^{+} CD8 T cells



A. Scatter-plots showing lower percentages of CXCR5^{-} CCR7^{hi} in pSS vs HC (*, p<0,01). B. Scatter-plots showing lower absolute values of CXCR5^{-} CCR7^{+} CD4 T cells (****, p<0,05).**

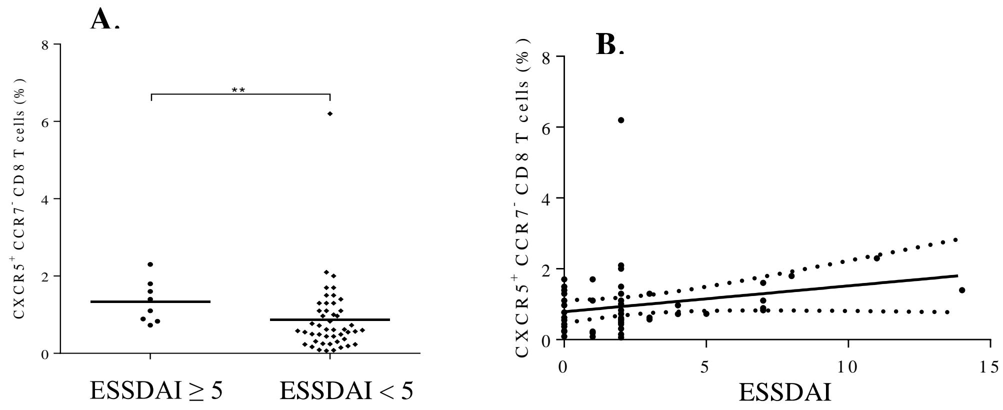
To assess the relation between immune subsets and disease activity, pSS patients were further divided according to ESSDAI scores. Interestingly, pSS patients with ESSDAI ≥5 (n=8) presented a significantly increased percentage of circulating cTfc cells (p=0.0103) compared to pSS patients with ESSDAI <5. No further differences were identified in any other T cell related parameter. cTfc cells were also positively correlated with ESSDAI scores (p=0.029, r=0.430).

Figure 13. **Circulating IL-21⁺ CD8 T cells in the groups of patients studied**



A. **Scatter-plots with the distribution of percentage values of IL-21⁺ CD8 T cells within T cells. There was a statistically significant increase in pSS vs HC and RA (**, p<0,001; **** pz0,05). B. Scatter-plots with the distribution of absolute values of CD8 T cells. There were no significant differences.**

Figure 14. **Tfc cells and disease activity in pSS**



A. Increase of cTfc in pSS patients with ESSDAI (, p<0.001) ≥ 5. B. Correlation with cTfc cells and ESSDAI scores (p=0.029, r=0.430).**

3.3 B cell subsets

3.3.1 Percentages and absolute values of B cell subsets

The following tables represent the percentages and absolute values for B cells subsets in each group.

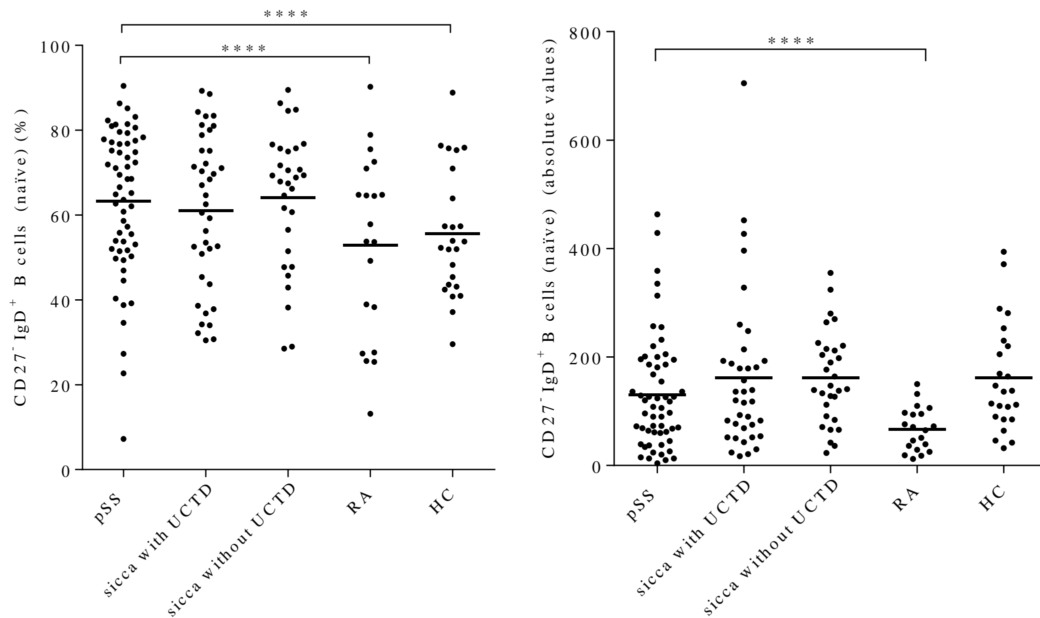
B cell subset	%, mean ± SD					Absolute values, median (25th – 75th percentile)				
	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC
CD19+ B cells	10,79 ± 5,632	12,63 ± 5,960	11,80 ± 3,402	6,410 ± 2,161	11,15 ± 4,322	97,00 (177,0 - 261,0)	219,0 (139,5 - 304,0	170,8 (255,0 - 313,8)	132,5 (82,75 - 153,0)	251,5 (173,0 - 393,3)
CD19+ CD27- IgD+ (naïve)	63,27 ± 17,45	61,03 ± 18,23	64,06 ± 16,26	52,88 ± 21,36	55,58 ± 15,02	108,0 (60,50 - 190,5	120,0 (61,50 - 193,0)	68,37 (50,55 - 75,68)	68,0 (30,75 – 96,50)	53,01 (43,22 - 69,21)
CD19+ D27+ (total memory)	33,52 ± 16,86	36,42 ± 17,47	33,51 ± 15,16	39,56 ± 19,97	42,22 ± 14,74	47,0 (28,50 - 75,00)	65,0 (43,50 - 93,50)	29,70 (22,20 - 44,03)	46,00 (24,00 - 57,25)	105,5 (65,00 - 141,3)
CD19+ CD27+ IgD+ IgM+ (unswitched)	17,32 ± 12,99	37,32 ± 27,40	18,29 ± 8,642	18,06 ± 10,53	22,16 ± 9,250	22,0 (11,50 -37,50)	33,00 (19,50 - 44,00)	18,21 (11,05 - 23,28)	17,50 (12,50 - 29,50	57,00 (31,75 - 80,50)
CD19+ CD27+ IgD- IgM- (switched)	16,20 ± 8,03	17,49 ± 8,317	15,22 ± 8,799	21,50 ± 12,12	20,06 ± 8,481	22,00 (15,00 - 36,50)	30,00 (19,50 - 48,00)	12,55 (9,215 – 18,06	20,00 (13,00 - 35,50	44,50 (36,75 - 7575
CD24+ CD27+ (Bregs)	21,54 ± 15,54	25,74 ± 15,21	25,18 ± 13,32	26,31 ± 17,44	31,70 ± 12,89	28,00 (15,50 - 48,50)	27,50 (44,00 - 74,50)	61,0 (26,75 - 85,25	24,00 (14,50 - 40,75)	81,0 (50,50 - 103,0)
CD24+ CD38hi (Bregs)	6,199± 4,313	4,094 ± 2,092	4,578 ± 3,138	3,945 ± 4,270	4,514 ± 3,447	8,0 (3,5 - 18,0)	4,0 (8,0 - 14,0)	9,5 (4,0 - 13,00)	3,0 (1,0 – 5,75)	7,500 (5,250 - 17,75)

Bm cell subsets	%, mean ± SD,					Absolute values, median (25th – 75th percentile)				
	pSS	Sicca with UCTD	Sicca with UCTD	RA	HC	pSS	Sicca with UCTD	Sicca without UCTD	RA	HC
Bm1	12,48 ± 11,09	14,54 ± 9,067	15,34 ± 6,900	17,76 ± 9,909	15,75 ± 7,215	18,00 (9,500 - 27,00)	27,00 (13,00 - 39,00)	34,50 (18,00 - 51.25)	18,50 (11,25 - 25,25)	38,00 (18,50 - 61,5)
Bm2	57,71 ± 13,43	57,86 ± 14,20	59,76 ± 12,61	45,57 ± 18,80	54,80 ± 10,63	101,0 (57,00 - 163,5)	115,0 (67,50 - 174,0)	143,5 (88,00 - 200,0)	58,50 (21,50 - 83,75)	130,0 (97,25 - 213,5)
Bm2’	8,822 ± 5,988	5,694 ± 3,248	6,083 ± 3,459	6,111 ± 6,342	5,995 ± 3,441	11,00 (6,0 - 29,00)	5,0 (10,00 - 18,50)	13,00 (7,750 - 22,25)	4,0 (2,0 - 10,0)	11,50 (7,00 - 30,25)
Bm3+Bm4	2,859 ± 3,381	2,599 ± 4,245	1,580 ± 1,584	2,110 ± 1,427	2,070 ± 2,777	3,0 (1,0 - 5,0)	3,0 (2,0 - 5,0)	3,0 (2,0 - 4,0)	2,00 (1,00 - 3,00)	3,50 (2,00 - 4,750)
eBm5	9,726 ± 4,745	10,55 ± 5,287	9,633 ± 6,374	11,66 ± 5,886	11,89 ± 4,390	15,00 (9,0 - 23,00)	18,00 (11,50 - 30,50)	18,00 (11,00 - 28,75)	10,50 (7,000 - 17,00)	27,00 (19,25 - 44,50)
Bm5	8,640 ± 5,107	8,915 ± 4,947	7,709 ± 4,824	17,07 ± 11,23	9,528 ± 4,247	11,00 (8,0 - 19,0)	16,00 (9,50 - 22,00)	15,00 (9,500 - 24,50)	14,00 (9,250 - 28,25)	23,50 (14,25 - 35,25)
eBm5+Bm5	66,54 ± 15,70	63,56 ± 15,84	65,84 ± 14,39	51,68 ± 21,60	60,79 ± 12,59	25,00 (17,00 - 41,00)	33,00 (21,00 - 52,00)	35,00 (21,25 - 53,25)	29,00 (17,25 - 43,25)	51,50 (34,75 - 77,75)

3.3.2 Alterations in B cell subsets in pSS patients: the IgD/CD27 classification

Percentages of IgD+ CD27- B cells (naïve) were higher in pSS when compared to HC (p=0,0277) and RA patients (p=0,0427). Absolute values presented higher in pSS vs RA (figure 15).

Figure 15. **Percentages and absolute values of naïve B cells**

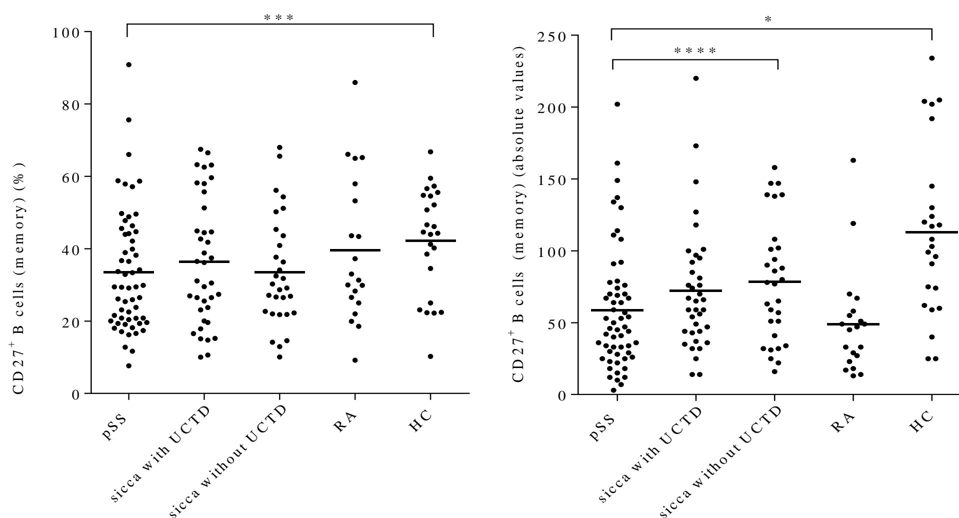


A. Scatter-plots with the distribution of naïve B cells. Higher absolute values of naïve B cells were found in pSS (**, p<0,05). B. Scatter-plots with absolute values of Higher in naïve B cells absolute, with higher values in pSS vs RA (****, p<0,05).**

Percentages of memory B cells were lower in pSS, but only significantly decreased in pSS vs HC (p=0,0099). In absolute values, memory B cells were also decreased (except in pSS vs RA, where RA presented a slight decrease), with a significant decrease when pSS was compared with HC (p<0,0001) and sicca without UCTD (p=0,0245) (figure 16). Regarding IgD+ CD27+ B cells (unswitched memory), though pSS patients presented a tendency for lower percentages when compared to all other groups, only when compared to HC lower

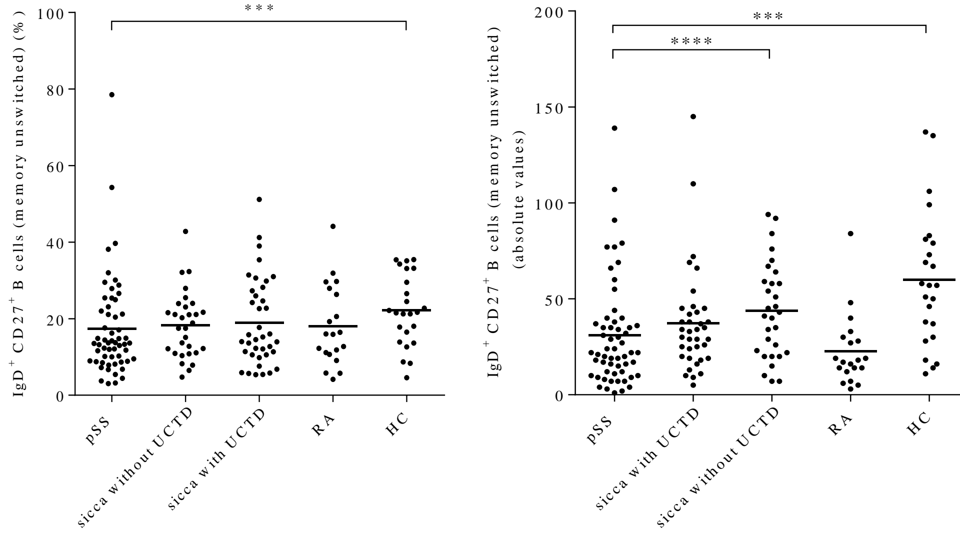
percentages were encountered (p=0,0084). Absolute values were lower in pSS compared to HC (p=0,0001) and to sicca patients without UCTD (p=0,0076) (figure 17).

Figure 16. **Percentages and absolute values of memory B cells**



A. Scatter-plots with the distribution of percentages memory B cells. Higher values were found in pSS compared with HC (*, p<0,01) B. Scatter-plots of memory B cells absolute values. Lower values were observed in pSS (*, p<0,0001; ****, p<0,05)**

Figure 17. Percentages and absolute values of memory unswitched B cells

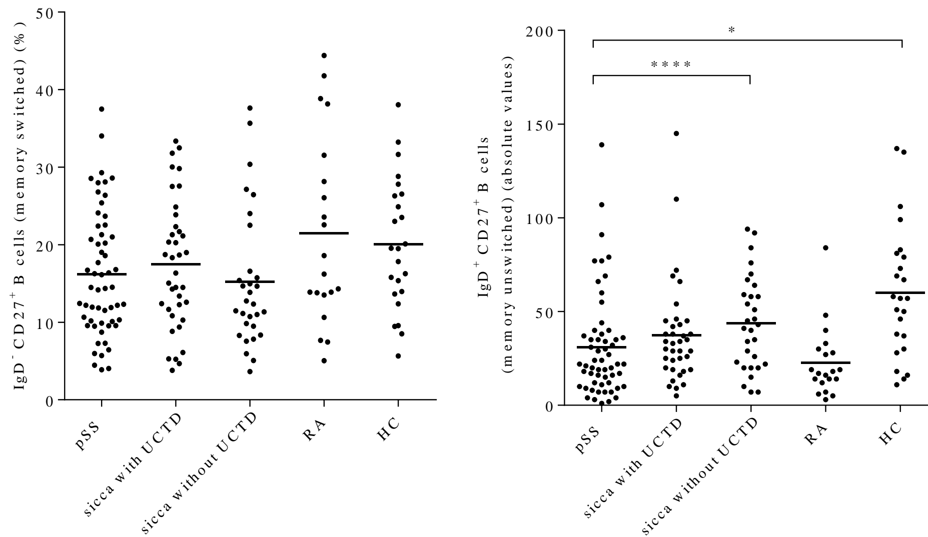


A. Scatter-plots of percentages of unswitched memory B cells percentages (*, p<0,01).**

B. Scatter-plots of unswitched memory B cells absolute values. Lower values were found in unswitched memory B cells absolute values (, p<0,001; ***, p<0,01).**

As for IgD$^-$ CD27$^+$ B cells (switched memory), only in absolute values differences were found, with lower values obtained in pSS patients (pSS vs HC (p<0,0001) and pSS vs sicca with UCTD (p=0,0427)). No significant differences were found in percentage values for switched memory B cells (figure 18).

Figure 18. **Percentages and absolute values of memory switched B cells.**

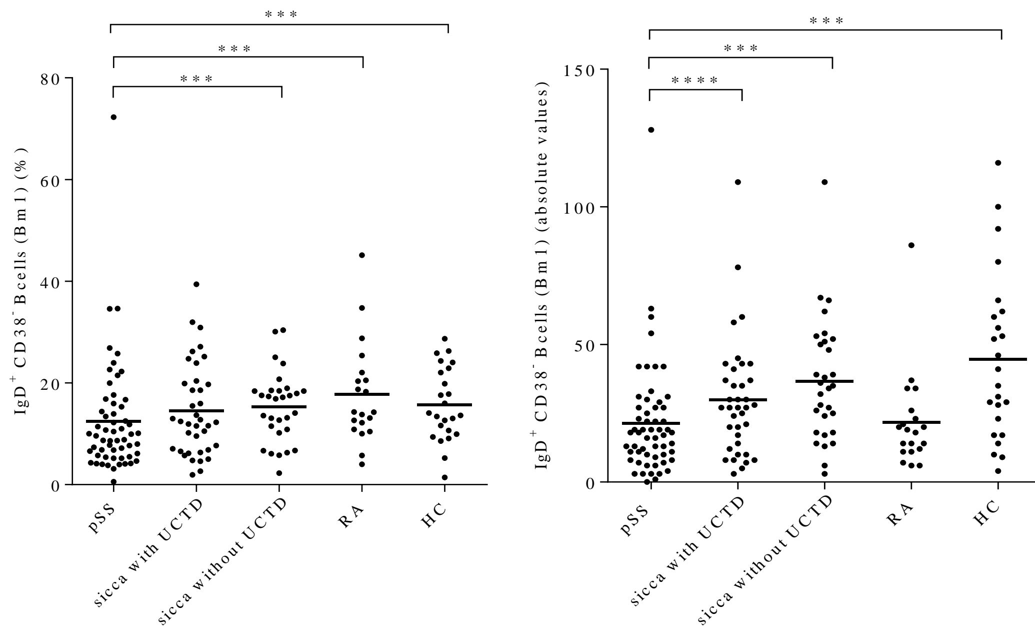


A. **Scatter-plot showing percentages of memory switched B cells. There were no alterations. B. Scatter-plot showing lower switched memory B cells absolute values (****, p<0,01; *, p<0,001).**

3.3.3 Alterations in B cell subsets in pSS: the Bm1-5 classification

Bm1 cells, identified as IgD$^+$ CD38$^+$ B cells are lower in pSS relative to all other groups, but only when compared with sicca without UCTD, RA and HC the difference was statistically significant (p=0,0075, p=0,0048, p=0,0082). As to absolute values, there is a significant decrease in pSS when comparing with both sicca groups (with UCTD, without UCTD p=0,0126 and p=0,0002 and HC (p=0,0002) (figure 19).

Figure 19. Percentages and absolute value of Bm1 cells



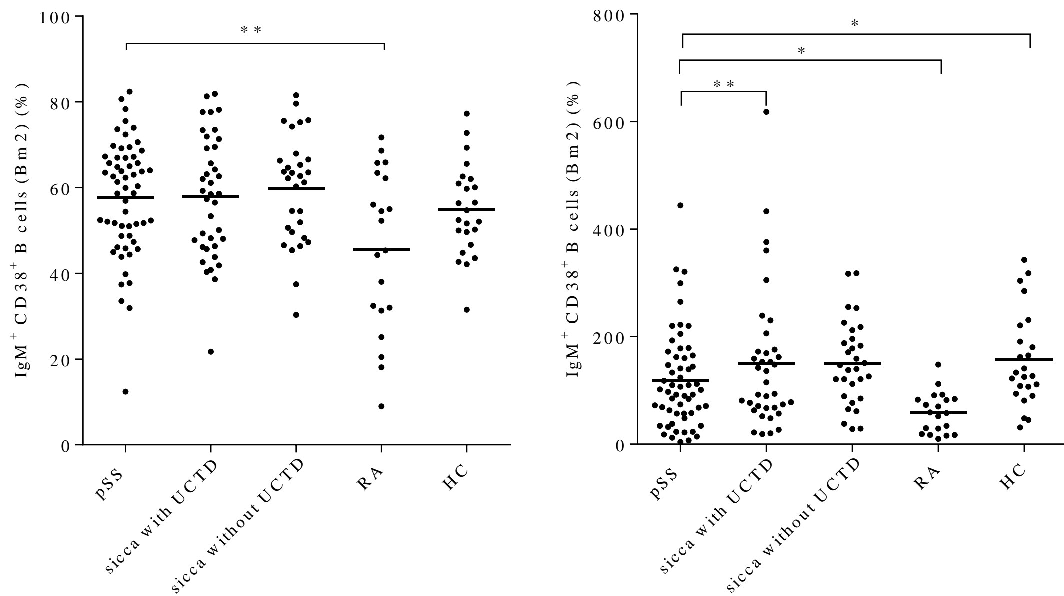
A. Scatter-plot with percentages of Bm1 cells, showing lower in Bm1 cells (*, p<0,01). B. Scatter-plot with absolute values for Bm1 cells. Lower in absolute values of Bm1 cells (***, p<0,01, ****, p<0,05).**

Percentages of Bm2 cells were higher in pSS when comparing with HC, sicca with UCTD and RA, but only vs RA the increase was statistically significant (p=0,0149). Sicca without UCTD group presented a slightly higher. Regarding absolute values, a decrease was found when comparing with all groups, but only in pSS vs HC, sicca without UCTD the difference was statistically significant. However, pSS patients presented an increased presence of Bm2 cells when compared to RA patients (figure 20).

Regarding Bm2' cells, a higher was found in pSS when compared with all groups, although statistically significant differences were only found in pSS vs sicca with UCTD (p=0,021) and RA (p=0,0406). As to Bm2' absolute values, a significant increase was found comparing pSS with RA (p=0,0032) (figure 21).

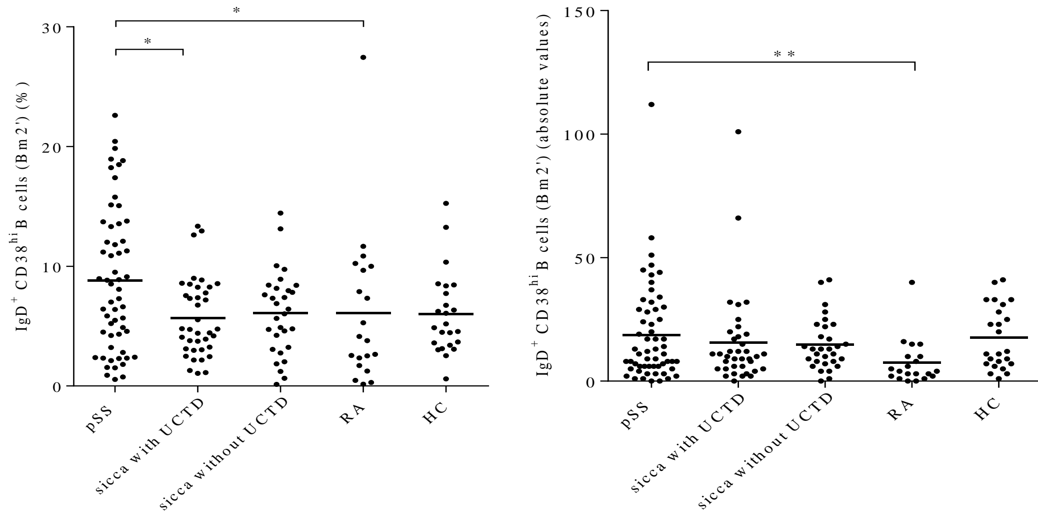
Lower values in pSS when compared with HC was found in percentages (p=0,0373) and absolute values (p<0,0001) of eBm5 cells. (figure 22). Percentages Bm5 were not altered in pSS. Instead, cells higher values in RA vs HC (p=0,011), RA vs sicca with UCTD (p=0,0164) and RA vs sicca without UCTD (p=0,001). Still, a decrease in pSS vs HC in absolute values (p=0,0005) (figure 23).

Figure 20. **Percentages and absolute value of Bm2 cells**



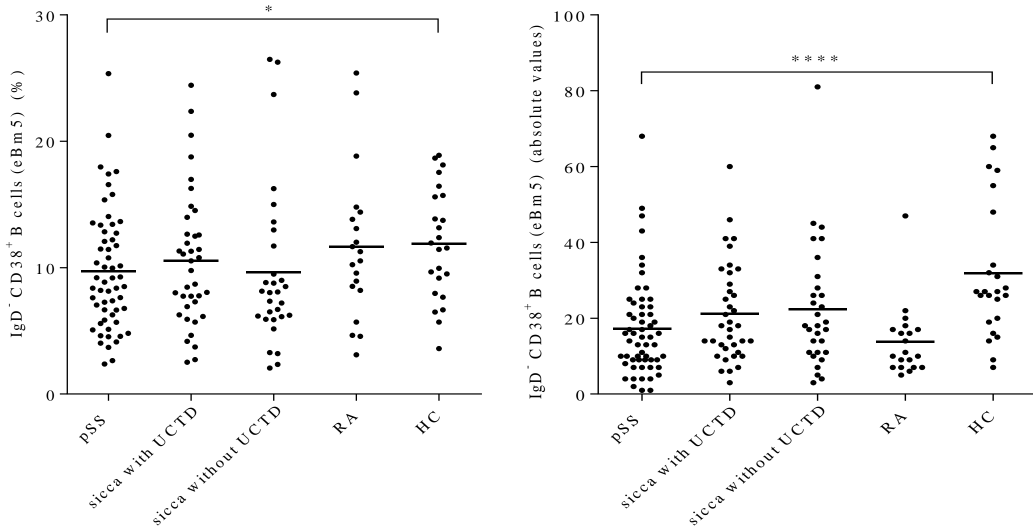
A. Scatter-plot showing lower percentages of Bm2' cells in RA patients (, p<0,01). B. Scatter-plot showing higher in Bm2 cells absolute values in pSS vs RA and decrease in pSS vs sicca without UCTD and HC (*, p<0,05; **, p<0,01).**

Figure 21. Percentages and absolute values of Bm2’ cells.



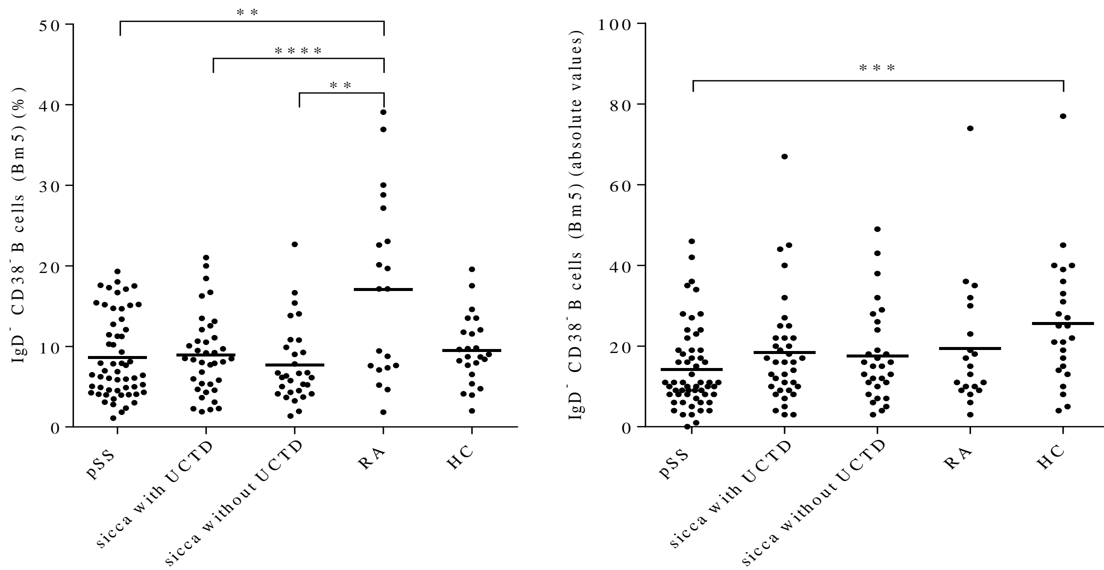
A. Scatter-plots of Bm2’ cells percentages. There were higher values in Bm2’ cells percentages (, p<0,01). B. Scatter-plots of Bm2’ cells absolute values, showing higher in Bm2’ absolute values (**, p<0,01)**

Figure 22. Percentages and absolute values of eBm5 cells.



A. Scatter-plot of eBm5 cell percentages. Higher values of Bm5 percentages in pSS vs HC patients (*, p<0,0001). B. Scatter-plot of Bm5 cell absolute values. Higher values in pSS (**, p<0,05).**

Figure 23. Percentages and absolute values of Bm5 cells



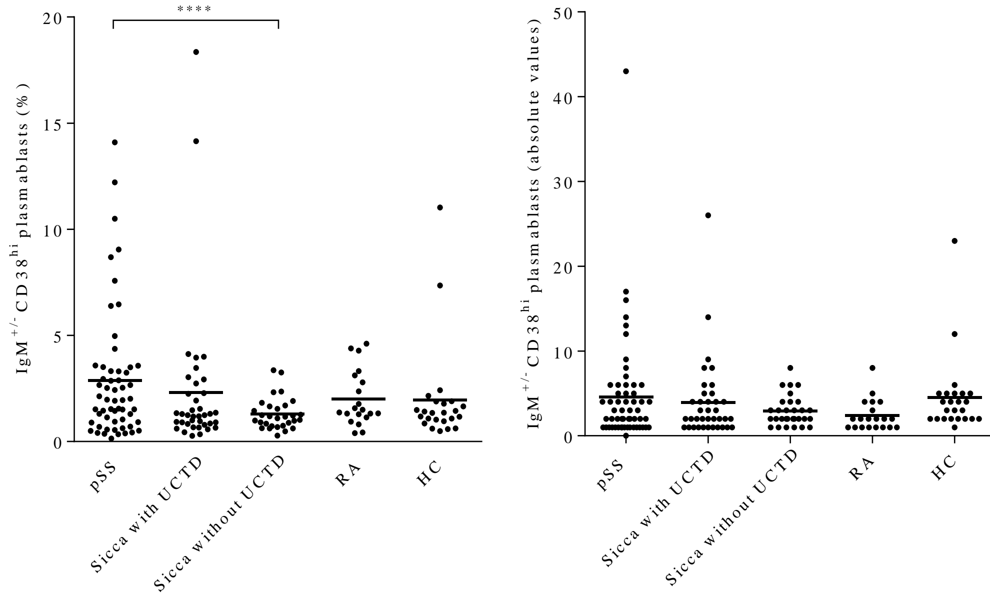
A. Scatter-plot of Bm5 cell percentages. Higher values of Bm5 percentages in RA patients (, p<0,001). B. Scatter-plot of Bm5 cell absolute values. Higher values of Bm5 percentages vs RA (***, p<0,01).**

3.3.4 Plasmablasts and Breg subsets in pSS patients

Higher percentages of $IgM^{+/-}$ $CD38^{hi}$ plasmablasts were observed ($p<0,05$) and no alterations in absolute values (figure 24).

Comparison of $CD24^{+}$ $CD27^{+}$ Breg among groups revealed lower values where observed in pSS when comparing with all groups but only when comparing with HC the difference was statistically significant ($p=0,0006$) (figure 25).

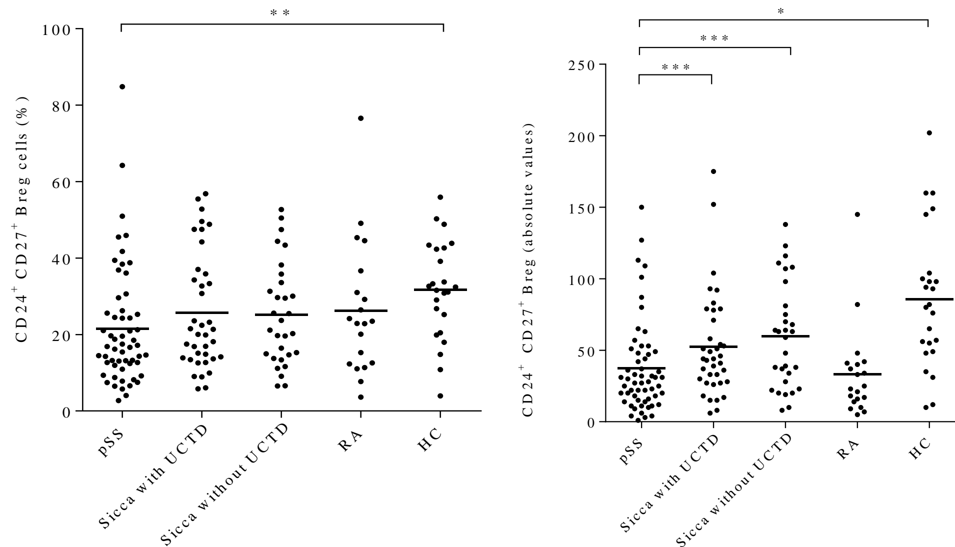
Figure 24. Percentages and absolute values of IgM$^{+/-}$ CD38^{hi} plasmablasts



A. Scatter-plots showing higher percentages of IgM$^{+/-}$ CD38^{hi} plasmablasts in pSS vs sicca without UCTD (**, p<0,05). B. Scatter-plots showing Higher in absolute values of IgM$^{+/-}$ CD38^{hi} plasmablasts in pSS but with no significance.**

Figure 25. **Percentages and absolute value of CD24⁺ CD27⁺ Breg cells.**

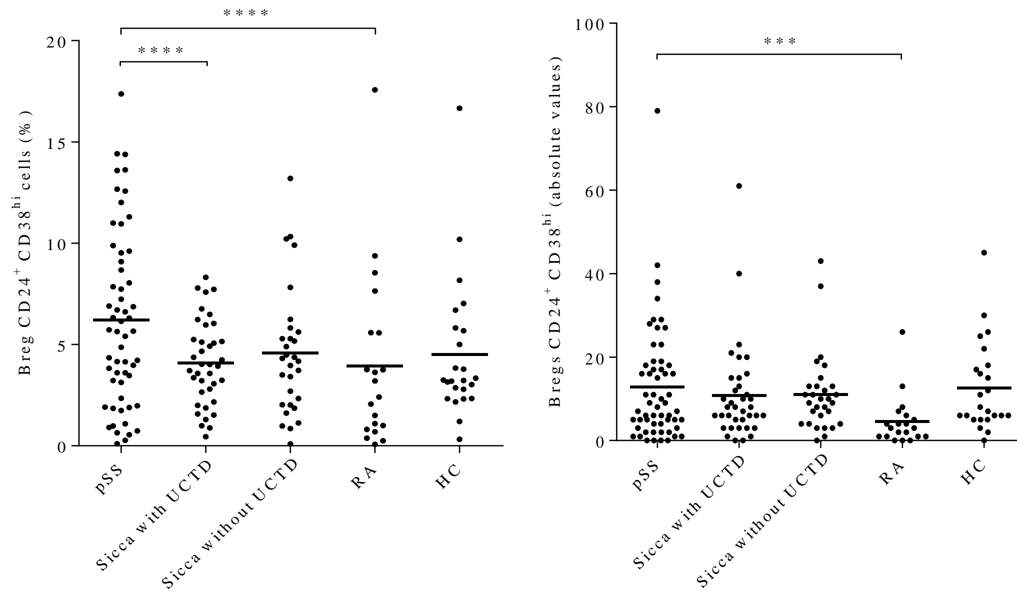
A. Scatter-plots showing percentages of CD24⁺ CD27⁺ Breg cells. Higher percentages of CD24⁺ CD27⁺ Breg cells (, p<0,001)**. **B. Decreased absolute values of CD24⁺ CD27⁺ Breg cells (*, p<0,0001; ***, p<0,01)**.



Regarding absolute values of these cells, there were lower when comparing pSS with both sicca groups (vs sicca with UCTD, p=0,0094; vs sicca without UCTD, p=0,0021) and HC (p<0,0001) (figure 25). Breg cells with elevated expression of CD38 (CD24⁺ CD38^{hi} Breg) were higher in pSS when comparing with sicca with UCTD (p=0,0295) and RA (p=0,0176). As to absolute values of these cells, higher values were found only when comparing pSS with RA (p=0,0015) (figure 26).

Figure 26. Percentages and absolute values of CD24+ CD38hi Breg.

A. Scatter-plots showing percentages of CD24+ CD38hi Breg. Higher values of CD24+ CD38hi Breg cells (**, p<0,05) were found. B. Scatter-plots showing absolute values of CD24+ CD38hi Breg. Higher absolute values of CD24+ CD38hi Breg cells (***, p<0,01).**



3.4 EBV serologic markers in pSS patients

3.4.1 Demographic data

For EBV Abs assessment, sera from pSS, RA patients and HC were assessed. Demographic data as female/male incidence ratio, age, age of onset, time of disease evolution, age of diagnostic and ESSAI scores were determined and presented in table 14. The groups represent a subgroup of the patients analysed previously for the lymphocytic profiling.

Table 14. pSS, RA patients and HC assessed for EBV Ab demographic data.

	pSS	**RA**	**HC**
Number	35	20	20
Sex (female:male)	49:1	16:4	20:0
Age, years	60,60 (28,60 – 74,80)	56,70 (30,30 – 78,0)	50,40 (38,9 – 61,1)
Age of onset median, years	45,00 (24,5 - 68,30)	38,55 (22,2 – 61,9)	NA
Time of disease evolution, years	11,90 (1,0 – 29,50)	10,20 (1,5 – 38,20)	NA
Age of diagnostic median, years	50,30 (26,70 – 71,70)	42,50 (22,9 – 64,09)	NA
ESSDAI	2,0 (0,0 – 14,0)	NA	NA

Results are presented as median (minimum–maximum).

3.4.2 Anti-EBV EA-D IgG

A standard curve was obtained using calibration solutions (R^2=1,0; figure 27). Absorbance values were used to determine sample concentration. Data regarding positive, borderline and negative results, along with mean concentration of EA-D IgG in RU/ml are presented in table 15. Comparing with HC and RA, although there were no differences when considering positive and borderline cases separately, when positive and borderline cases are combined, pSS patients had significantly more cases for EA-D IgG (p=0,0378).

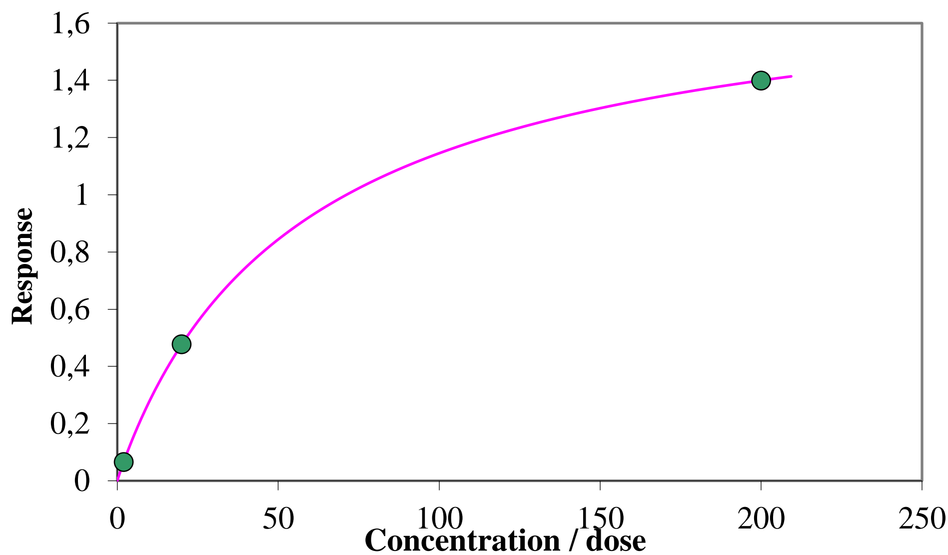


Figure 27. Calibration curve for anti-EBV EA-D IgG

Table 15. Results for Anti-EBV EA-D IgG

Anti-EBV EA-D IgG	**pSS (n=35)**	**RA (n=20)**	**HC (n=20)**
Positive, n (%)	8 (22,86)	4 (20)	1 (5)
Borderline, n (%)	3 (8,57)	0 (0)	0 (0)
Negative, n (%)	24 (68,57)	16 (80)	19 (95)
Concentration (RU/ml), mean ± SD	20,54 ±39,81	10,76 ± 15,96	8,381 ± 8,762

3.4.3 Anti-EBV EA-D IgA

The assay for Anti-EBV EA-D IgA was similar to the previous one. The results for Anti-EBV EA-D IgA A are presented in the following table. No significant differences were found for Anti-EBV EA-D IgA between pSS patients, RA patients and HC (table 16).

Table 16. Results obtained for anti-EBV EA-D IgA

Anti-EBV-EA-D IgA	pSS (n=35)	RA (n=20)	HC (n=20)
Positive, n (%)	0 (0)	0 (0)	0 (0)
Limit, n (%)	0 (0)	0 (0)	1 (5)
Negative, n (%)	35 (100)	20 (100)	19 (95)

3.4.4 Anti-EBV CA IgG

Assessment and quantification of anti-EBV CA IgG was performed. A calibration curve was constructed (R^2=1,0, figure 28). Table 15 presents values results for anti-EBV CA IgG assessment. Although the concentration was slightly increased, there was no significant difference between patient groups regarding positive cases.

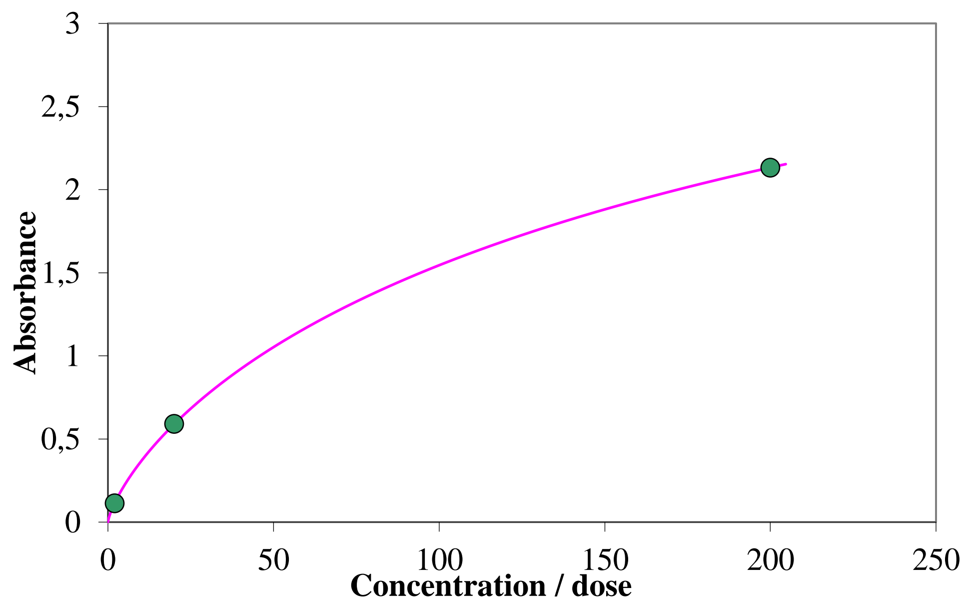


Figure 28. Calibration curve for anti-EBV CA IgG

Table 17. Results for Anti-EBV CA IgG

Anti-EBV CA IgG	pSS (n=35)	RA (n=20)	HC (n=20)
Positive, n (%)	32 (91,43)	20 (100)	20 (100)
Limit, n (%)	1 (2,86)	0 (0)	0 (0)
Negative, n (%)	2 (5,71)	0 (0)	0 (0)
Concentration, mean ± SD	126,8 ± 66,04	125,1 ± 56,68	112,8 ± 43,66

3.4.5 Anti-EBV CA IgA

The assays for Anti-EBV CA IgA were semi-quantitative. Ratios of absorbance were used to determine positive, negative or borderline results for each serum sample as previously described.

The results for anti-EBV-CA IgA are presented in table 18.

Table 18. Results obtained for anti-EBV CA IgA

Anti-EBV-CA IgA	pSS (n=35)	RA (n=20)	HC (n=20)
Positive, n (%)	0 (0)	2 (10)	3 (15)
Borderline, n (%)	11 (31,43)	3 (15)	1 (5)
Negative, n (%)	24 (68,57)	15 (75)	16 (80)

3.4.6 Anti-EBV-CA IgM

The results for anti-EBV-CA IgM are presented in table 19. Again, a semi-quantitative assay was performed, and results refer the evaluation of positive, borderline and negative ratios. No significant differences were found between the three groups evaluated.

Table 19. Results obtained for anti-EBV CA IgM

Anti-EBV-CA IgM	pSS (n=35)	RA (n=20)	HC (n=20)
Positive, n (%)	0 (0)	0 (0)	0 (0)
Borderline, n (%)	0 (0)	0 (0)	0 (0)
Negative, n (%)	35 (100)	20 (100)	20 (100)

3.4.7 Anti-EBNA IgG

Anti-EBNA IgG was also quantified in serum of pSS, RA and HC. The calibration curve was constructed (R^2=1,0, figure 29). Percentages of positive cases were high, and similar in all three groups. This demonstrates that anti-EBNA IgG in ubiquitous in human adults. However, it is interesting to note an increase in concentration in pSS patients. This can lead us to postulate that the stage of anti-EBV immune response of pSS patients might be different from HC and RA. Still, no significant differences in the number of positive and borderline cases were found between groups (table 20).

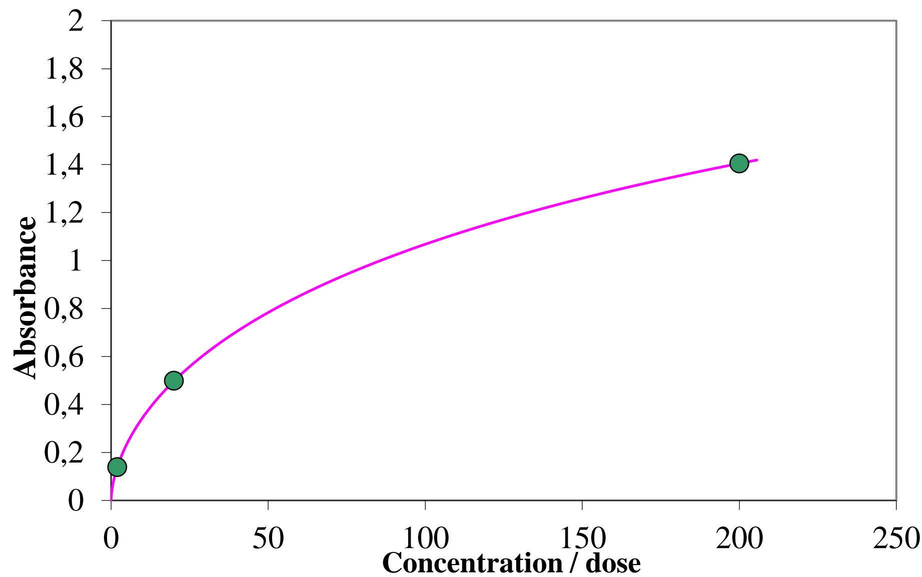


Figure 29. Calibration curve for anti-EBNA IgG

Table 20. Results obtained for anti-EBNA IgG

Anti-EBNA IgG	pSS (n=35)	RA (n=20)	HC (n=20)
Positive, n (%)	25 (71,42)	16 (80)	13 (65)
Limit, n (%)	2 (5,71)	0 (0)	4 (20)
Negative, n (%)	8 (22,86)	4 (20)	3 (15)
Concentration, mean ± SD	64,59 ± 62,62	74,97 ± 52,92	77,45 ± 64,62

3.4.8 Summary

Anti-EBV humoral response phases can be distinguished by the different Abs produced, as presented in Figure 4. For instance, in the beginning of an EBV infection, CA Abs are produced, followed by a production of finally EA-D Abs and EBNA Abs later on.

In table 21 we present the total number of subjects that were positive for a given anti-EBV Ab. The most significant differences are in anti-EBV EA-D IgG, more prevalent and with higher concentrations in pSS patients. Since these Abs are produced in late phases of acute infections, the maintenance of higher levels is a strong indicative of a chronic active humoral immune response against EBV in pSS patients that might be associated with pSS pathology. These data corroborate previous studies made regarding anti-EBV antibodies[45] and shed light on the role of anti-EBV humoral response due to differences in Ig presence and concentration.

Table 21. Summary of results obtain for all positive anti-EBV Ab and *p*-values for differences among groups.

Abs	p value[a] (OR)		
	pSS vs HC	RA vs HC	pSS vs RA
EA D IgG	**0,0378 (8,708)**	0,3416 (4,750)	0,5308 (1,833)
CA IgG	0,5286 (0,3268)	1,0 (1,0)	0,5286 (0,3268)
EBNA IgG	0,7277 (0,5956)	1,0 (0,7059)	1,0 (0,8438)
CA IgA	0,5308 (1,833)	1,0 (1,333)	0,7608 (1,375
EA-D IgA	-b	-b	-b
CA IgM	-b	-b	-b

a. *p*-values using Fischer's t test considering positive+borderline cases

b. unable to determine *p*-value (0 positive+borderline cases)

OR (Odds- Ratio)

The higher levels of EA-D IgG in pSS patients might indicate a humoral response similar to late acute/chronic active infection. In addition, it is possible to establish a serological profile associated with subsequent EBV-associated disease according to that profile. Strikingly, our

study demonstrates that pSS patients fit into the chronic infection profile described in the literature.[73] This is a clue that, in fact, an underlying humoral response characteristic of chronic infection is present in pSS.

However, these results do not assure an active EBV infection is taking place and directly inducing autoimmunity. In fact, we observed negative results for Anti-EBV-CA antigen IgM in all pSS patients evaluated, despite we recognize that the presence of antibodies directed to EBV-CA antigens are mostly restricted to an initial phase post-acute infection. Nevertheless, to determine if an actual active infection is taking place, viral load of EBV should be assessed by polymerase chain reaction (PCR). The quantification of viral DNA in PB is considered the best method for the assessment and confirmation of an EBV active infection.[43] Still, ELISA serological measurements are useful for diagnosing EBV-associated diseases, moreover, they may be informative on the assessment of humoral responses and the comprehension of the interplay between anti-viral and autoimmune mechanisms.

4. Chapter 4 - Discussion and conclusions

The link between viral infection and autoimmunity in Sjogren's Syndrome has been widely suggested and somewhat explored but remains highly controversial. With this project, our aim was to assess the lymphocytic profile of pSS patients and assess the presence and quantity of anti-EBV Abs in pSS patients' serum.

The first conclusion to draw is the importance of B cell action in pSS pathophysiology also associated with the presence of EBV Abs. Regarding naïve B cells, they are mature B cells that leave the bone marrow and express both membrane-bound IgM and IgD and have not yet encountered an antigen. These cells will be activated in SLO, where they will contact the antigen. They will undergo proliferation, along with other important events such as affinity maturation and somatic hypermutation.[27] In the context of autoimmunity, an increase of naïve B cells in peripheral blood from RA patients was found in a previous study.[90] However, this was not observed in pSS. The higher percentages of naïve B cells in pSS when compared with RA and HC and the lower absolute counts when comparing with HC alone, is in line with the hypothesis that memory B cells are being retained in affected organs in pSS – parotid and lachrymal glands. Also, an underlying antigen stimulation may induce the migration of naïve B cells from lymph tissues to circulation. Moreover, it is noteworthy to remark the increase of self-reactive naïve B cells that include $CD19^{+}$ IgD^{+} $CD27^{-}$ found in pSS.[22] Although specific self-reactive B cells were not assessed in this study, they might be contributing to the increase of naïve B cells we observed in pSS patients evaluated in our study. In fact, the levels of naïve and memory B cells might offer clues about differentiation and migration patterns of these cells. An increase in naïve B cells associated with a decrease in memory B cells would possibly indicate a differentiation tendency towards memory B cells with a subsequent retention of these cells in exocrine glands. This would be plausible if a low count of circulating memory B cells was verified, which was the case in our study. Percentages and absolute numbers of memory B cells were significantly decreased in pSS when compared with HC, but not other groups. These observations are corroborated by previous studies that reported the accumulation of memory B cells in pSS patients' exocrine

glands.[12,35] Despite not having performed histological observations or biopsies for local lymphocyte aggregation in pSS patients exocrine glands, we may infer that the increase of naïve B cells and the decrease in memory B cells are possible indicators of memory B cell accumulation in exocrine glands. In fact, this particular B cell profile is observed in such scenarios.[35]

The population of memory B cells is important in establishing immunological memory due to their role in generating and replenishing the plasma cell compartment responsible for Ab production. Memory B cells can be further divided into the switched and unswitched subsets according to the expression of IgD. The significant reduction in the absolute numbers and percentages of unswitched (IgD^+) memory B-cells in pSS patients compared to controls and sicca without UCTD are a strong indicative of a tolerance checkpoint breakdown in B cell generation, where autoreactive B cells escape tolerance mechanisms and survive in the periphery. The changes observed in switched B cells were not so exuberant as the ones observed for total memory B cells; decreases were only observed in absolute values when compared with sicca with UCTD and HC. These results are in line with other studies and may indicate an impairment of B cell tolerance checkpoints. Probably there is also a tendency for a B-cell differentiation towards plasma cells, which is implied by the increase of $IgM^{+/-}$ $CD38^{hi}$ plasmablasts and this was corroborated also by previous studies.[67]

As to the Bm1-Bm5 subset assessment, a clear tendency towards Bm2' cells is noted due to the increase of this population and decrease of others. These cells are known as GC-founder B cells, for their role in the GC structures and participation in immune responses were GC interactions are paramount.

Regarding Breg cells, the decrease of Breg in a memory state (determined by the expression of CD27) might indicate that an immunosuppressive action, albeit insufficient, is taking place. The increase of $CD24^+$ $CD38^{hi}$ Breg can point towards this same hypothesis. However, the values of $CD24^+$ $CD38^{hi}$ Breg are higher only when comparing pSS with sicca with UCTD and RA. This interesting observation might indicate a distinct failure of immune regulation by these cells in a functional basis, rather than a differentiation problem. Functional assays would be important to clear these observations.

The immunosuppressive role of Breg cells was identified in several studies.[23,32] In the context of autoimmunity, a study demonstrated the regulatory function and subsequent functional impairment in a SLE model, using Breg cells defined as $CD24^{hi}$ $CD38^{hi}$ $CD19^{+}$ cells.[60] Higher values for Breg cells have been observed in SLE patients.[32] Furthermore, a close relationship between Tfh and Breg was observed in autoimmune context.[94] In our study, however, there was an increase of circulating Bregs, defined as $CD24^{+}$ $CD38^{hi}$ CD19 cells, in pSS patients. This Breg profile is similar to those observed for naïve and memory B cells. The decrease of Breg with memory phenotype ($CD27^{+}$) and increase of $CD24^{+}$ $CD38^{hi}$ Breg cells (transitional) are consistent with B cell migration towards the affected organ, which, if Breg are functionally operative, would regulate the immune response and attenuate the autoimmune epithelitis. However, this does not seem to occur. Interestingly, regarding the functionality of Bregs, a study associated the production of IL-10 by $CD24^{+}$ $CD38^{+}$ Breg cells as paramount for regulatory action by these cells.[66] The fact that $CD24^{+}$ $CD38^{+}$ Breg cells are increased and still autoimmunity prevails might indicate a functional impairment related to the expression of IL-10. Since we did not evaluate IL-10-producing B cells, we are not able to confirm this assumption.

pSS patients demonstrate biological signals that indicate B cell hyperactivation, including serum polyclonal hypergammaglobulinemia, increased free light chains and autoantibodies that can be present even before symptomatic manifestation.[16,39] Our study is in line with these data because the alterations found in B cells were characteristic of B cell activation. Therefore, it is plausible that B cells are involved in pSS disease mechanisms.

The T cell profile of pSS demonstrates remarkable changes in the cells with follicular phenotype ($IL\text{-}21^{+}$ CD4 and CD8 T cells). This observation might signal a dysfunction regarding GC reactions and the mechanisms of tolerance and B cell affinity maturation in GC. In addition, it might point out the formation and dysregulation of ectopic GC-like structures, common in pSS. In fact, this is supported by the lower numbers of $CXCR5^{+}$ CD4 T cells in the periphery, that might be retained in affected organs and mediate B cell activation in autoimmune inflammation site.

The distinct increase of IL-21^+ CD4 T cells in pSS corroborates the study made by Kang et al.[42], which established an implication of IL-21 $CD4^+$ T cells in pSS pathogenesis. IL-21 has been considered a characteristic cytokine expressed by Tfh cells.[21] IL-21^+ CD4 T cells have been demonstrated to be essential in the maintenance of GC and GC-like structures.[53,89] The affinity maturation process of B cells that occurs in GC's is greatly enhanced by IL-21 secreted by CD4 T cells.[47,53] The secretion of IL-21 by CD4 T cells has also been associated with T cell activation and plasma cell differentiation with subsequent increase in IgG and IgM secretion by plasma cells.[47] These observations support the crucial role of IL-21^+ CD4 T cells in humoral responses. Due to the B cell hyperactivity and hypergammaglobulinemia already described in literature, the increase of CD4 T cells expressing IL-21 does not come as a surprise. Furthermore, the correlation of IL-21^+ CD4 T cells with $CXCR5^+$ CD4 T cells might indicate that a great part of IL-21-producing CD4 T cells have a follicular phenotype, with the expression of the follicle-homing chemokine.

As to viral infections, it has been demonstrated that mice infected with lymphocytic choriomeningitis virus (LCMV) generate IL-21^+ CD4 T cells. Also, the production of IL-21 by CD4 T cells has been demonstrated to be essential for the control of viral infection in the same scenario.[95] The immunological context is a great influence on the differentiation of CD4 T cells. Antigenic persistence directs CD4 T cells differentiation into Tfh. These cells maintain their function during the persistence of the antigen and are paramount compartments in the control of viral replication.[30] Furthermore, the production of IL-21 by Tfh is induced in the context of HCV infection.[79] Additionally, the production of IL-21 by CD4 T cells and the presence of IL-21R in T cells are essential for the control of chronic infection.[28,33,95] The clear increase of IL-21^+ CD4 T cells in pSS points towards a role of these cells in the pathogenesis of the disease.

On the other hand, the cytotoxic counterpart of Tfh has been poorly explored in previous studies. Our work revealed the increased presence of circulating CD8 T cells with follicular phenotype cells (expression of CXCR5 and lack of CCR7 expression) in patients with higher

disease activity measured by the ESSDAI scores. To our knowledge, this is the first time that circulating $CXCR5^{+}$ $CCR7^{-}$ CD8 T cells are characterized in pSS and associated with disease activity. In fact, only recently studies have been addressing this subset of CD8 T cells, which may justify the lack of information on this subject in pSS. $CXCR5^{+}$ cells have been initially described as early effector memory CD8 T cells present in B cell follicles of human tonsils.[77] In fact, this chemokine receptor, CXCR5, directs the homing of cells to B cell sites in secondary lymphoid organs. In line with these data, recently, the CD8 T cell subset that expresses CXCR5 was shown to participate in B cell help.[40]

Tfc cells were recently described as essential in controlling viral replication in lymphoid follicles, besides assuming a supporting role in B cell differentiation and isotype switching.[87] Most studies regarding these cells are exploring their role in limiting replication of viral agents that have preference for B cell follicle residence. However, their role in autoimmune diseases has been scarcely investigated. In this study, we describe, for the first time, an association between the levels of circulating Tfc cells and pSS disease activity. The association of a particular subtype of cells with ESSDAI scores is a strong indication that for their involvement in pathophysiology. The increased presence of Tfc in the peripheral blood of patients with moderate to high disease activity opens the hypothesis that these cells might be involved in an antiviral response that has caused follicle dysfunction or exacerbated activity in ectopic GC-like structures, common in pSS. Still, further investigation regarding Tfc cells and their role in autoimmunity is needed.

Animal models of viral infections and autoimmune diseases could prove rather useful in studying Tfc cells as a possible player in the connection between viral infection and autoimmunity. In humans, due to limit access to samples and biopsies, it would be harder to gain direct insight of Tfc function. Our study provides an entrance gate to study these cells, making use of more accessible models and other experimental tools that allow the direct observation of circulating, but most importantly, tissue Tfc cells, and the assessment of their function and activation.

Regarding the presence of anti-EBV Abs, the increased presence of anti-EBV EA-D IgG found in pSS patients points towards a role of a humoral response against EBV in pSS pathogenesis. Our results agree with the study made by Kivity et al., even approaching similar percentages that their study demonstrated.[45] Additionally, average concentration was higher in pSS. Anti-EA-D IgG is produced in late acute phase of EBV infection and usually decrease after three to six months until undetectable levels. The high levels of anti-EA-D IgG might point towards a presence of unregulated active chronic infection or an acute infection in late phase.

Since EBV is a very ubiquitous viral agent, anti-CA IgG is the most prevalent anti-EBV IgG in human adults. Therefore, it was expectable to find high percentages of positive sera for this Ab in all groups. We can conclude that no major differences exist regarding EBV CA IgG. CA Abs arise in early phases of EBV infection and remain during all adult life of the infected person.

Regarding IgA, it is the major Ig isotype present in secretions such as milk, tears and saliva. Anti-EBV IgA has been associated with increased throat inflammation in EBV-related disease.[62] In fact, mucosal secretion in affected organs could indicate a local humoral response and contribute to local inflammation. An assessment of oral and ocular mucosa for IgA could shed light on the role of IgA in local inflammation in pSS. Regarding IgM, it is usually the first Ig isotype produced in a viral infection. Anti-EBV CA IgM has been found associated in other clinical contexts and has been associated with exacerbated IgG autoantibodies in autoimmune diseases. However, we did not find any presence of this Ig in the serum of our patient groups, concluding that no evidence of acute infection/reinfection was present.

More work is required to establish a definite viral trigger for pSS. In addition to this study, other viral agents should be assessed, namely, CMV, human T-lymphotropic virus (HTLV), or HCV. We highlight that follicular T cell subsets may reveal a link between viral infection and pSS, also considering their role in the regulation of B cell responses. Future studies assessing lymphocytic profile differences in pSS patients were antiviral Abs are more

prevalent could offer insight into the lymphocytic subsets participating in immune pathogenesis. Additionally, the development of animal models can be paramount in the understanding of follicular T cells roles in both antiviral and autoimmune contexts, allowing the identification of similar mechanisms and differentiation patterns and elucidating the breakdown of tolerance mechanisms in GC and GC-like structures.

5. Bibliographic references

1. ACTOR, Jeffrey K. - Cancer Immunology. **Introductory Immunology**. . ISSN 1533-4406. 2014) 116–124. doi: 10.1016/B978-0-12-420030-2.00010-X.
2. AKIRA, Shizuo; HEMMI, Hiroaki - Recognition of pathogen-associated molecular patterns by TLR family. **Immunology Letters**. . ISSN 01652478. 85:2 (2003) 85–95. doi: 10.1016/S0165-2478(02)00228-6.
3. AKIRA, Shizuo; UEMATSU, Satoshi; TAKEUCHI, Osamu - Pathogen recognition and innate immunity. **Cell**. . ISSN 00928674. 124:4 (2006) 783–801. doi: 10.1016/j.cell.2006.02.015.
4. ALAMANOS, Y. *et al.* - Epidemiology of primary Sjögren's syndrome in north-west Greece, 1982-2003. **Rheumatology**. . ISSN 14620324. 45:2 (2006) 187–191. doi: 10.1093/rheumatology/kei107.
5. ALETAHA, Daniel *et al.* - 2010 Rheumatoid Arthritis Classification Criteria. 62:9 (2010) 2569–2581. doi: 10.1002/art.27584.
6. ALUNNO, Alessia *et al.* - IL-17-producing CD4-CD8- T cells are expanded in the peripheral blood, infiltrate salivary glands and are resistant to corticosteroids in patients with primary Sj??gren's syndrome. **Annals of the Rheumatic Diseases**. . ISSN 00034967. 72:2 (2013) 286–292. doi: 10.1136/annrheumdis-2012-201511.
7. BAKHSHI, A. *et al.* - Lymphocyte subsets in Sjogren's syndrome: a quantitative analysis using monoclonal antibodies and the fluorescence-activated cell sorter. **J.Clin.Lab.Immunol.** 10:2 (1983) 63–69.
8. BARANOVSKI, Boris M. *et al.* - T Helper Subsets, Peripheral Plasticity, and the Acute Phase Protein, α 1-Antitrypsin. **BioMed Research International**. . ISSN 23146141. 2015:2015). doi: 10.1155/2015/184574.
9. BAYETTO, K.; LOGAN, RM - Sjögren's syndrome: a review of aetiology, pathogenesis, diagnosis and management. **Australian Dental Journal**. . ISSN 00450421. 55:2010) 39–47. doi: 10.1111/j.1834-7819.2010.01197.x.
10. BERKOWITZ, Aaron L.; SAMUELS, Martin A. - The neurology of Sjögren's syndrome

and the rheumatology of peripheral neuropathy and myelitis. **Practical Neurology**. . ISSN 14747758. 14:1 (2014) 14–22. doi: 10.1136/practneurol-2013-000651.

11. BLOCK, J. A; SEQUEIRA, W. - Raynaud's phenomenon. **Lancet**. . ISSN 0140-6736. 357:9273 (2001) 2042–2048. doi: 10.1016/S0140-6736(00)05118-7.
12. BOHNHORST, J. Ø. *et al.* - Significantly depressed percentage of CD27+ (memory) B cells among peripheral blood B cells in patients with primary Sjogren's syndrome. **Scandinavian Journal of Immunology**. . ISSN 0300-9475 (Print). 54:4 (2001) 421–427. doi: 10.1046/j.1365-3083.2001.00989.x.
13. BOISE, Lawrence H. *et al.* - CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-xL. **Immunity**. . ISSN 10747613. 3:1 (1995) 87–98. doi: 10.1016/1074-7613(95)90161-2.
14. BONILLA, Francisco A.; OETTGEN, Hans C. - Adaptive immunity. **Journal of Allergy and Clinical Immunology**. . ISSN 00916749. 125:2 SUPPL. 2 (2010) S33–S40. doi: 10.1016/j.jaci.2009.09.017.
15. BRITO-ZERÓN, Pilar *et al.* - Sjögren syndrome. **Nature Reviews Disease Primers**. . ISSN 2056676X. 2:July (2016) 1–20. doi: 10.1038/nrdp.2016.47.
16. BROKSTAD, Karl *et al.* - Prediction of Sjögren's Syndrome Years Before Diagnosis and Identification of Patients With Early Onset and Severe Disease Course by Autoantibody Profiling. 67:9 (2015) 2427–2436. doi: 10.1002/art.39214.
17. CARVAJAL ALEGRIA, Guillermo *et al.* - Could Lymphocyte Profiling be Useful to Diagnose Systemic Autoimmune Diseases? **Clinical Reviews in Allergy and Immunology**. . ISSN 15590267. 53:2 (2017) 219–236. doi: 10.1007/s12016-017-8608-5.
18. CHAUDHRY, Ashutosh *et al.* - Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. **Immunity**. 34:4 (2011) 566–578. doi: 10.1016/j.immuni.2011.03.018.Interleukin-10.
19. CHEN, Weiqian *et al.* - Biomarkers for Primary Sjögren's Syndrome. **Genomics, Proteomics and Bioinformatics**. . ISSN 22103244. 13:4 (2015) 219–223. doi: 10.1016/j.gpb.2015.06.002.
20. CHRISTODOULOU, Maria I. *et al.* - Foxp3+T-regulatory cells in Sjögren's syndrome:

Correlation with the grade of the autoimmune lesion and certain adverse prognostic factors. **American Journal of Pathology**. . ISSN 15252191. 173:5 (2008) 1389–1396. doi: 10.2353/ajpath.2008.080246.

21. CHTANOVA, T. *et al.* - T Follicular Helper Cells Express a Distinctive Transcriptional Profile, Reflecting Their Role as Non-Th1/Th2 Effector Cells That Provide Help for B Cells. **The Journal of Immunology**. . ISSN 0022-1767. 173:1 (2004) 68–78. doi: 10.4049/jimmunol.173.1.68.
22. CORSIERO, Elisa *et al.* - Accumulation of self-reactive naïve and memory B cell reveals sequential defects in B cell tolerance checkpoints in Sjögren's Syndrome. **PLoS ONE**. . ISSN 19326203. 9:12 (2014) 1–20. doi: 10.1371/journal.pone.0114575.
23. DAS, Abhishek *et al.* - IL-10 − Producing Regulatory B Cells in the Pathogenesis of Chronic Hepatitis B Virus Infection. 2013). doi: 10.4049/jimmunol.1103139.
24. DOUGLAS, Linda - CPD : Facilitating timely diagnosis of Sjögren ' s syndrome. **Nature Publishing Group**. 2:1 (2018) 1–4. doi: 10.1038/bdjteam.2018.26.
25. EDWARDS, Jonathan C. W.; CAMBRIDGE, Geraldine - B-cell targeting in rheumatoid arthritis and other autoimmune diseases. **Nature Reviews Immunology**. . ISSN 14741733. 6:5 (2006) 394–403. doi: 10.1038/nri1838.
26. EGERTON, Mark; SCOLLAY, Roland; SHORTMAN, Ken - Kinetics of mature T-cell development in the thymus. **Immunology**. . ISSN 0027-8424. 87:April (1990) 2579–2582. doi: 10.1073/pnas.87.7.2579.
27. EIBEL, Hermann *et al.* - B cell Biology: An Overview. **Current Allergy and Asthma Reports**. . ISSN 1529-7322. 14:5 (2014) 434. doi: 10.1007/s11882-014-0434-8.
28. ELSAESSER, Heidi; SAUER, Karsten; BROOKS, David G. - IL-21 is required to control chronic viral infection. **Science**. . ISSN 00368075. 324:5934 (2009) 1569–1572. doi: 10.1126/science.1174182.
29. ERCOLINI, A. M.; MILLER, S. D. - The role of infections in autoimmune disease. **Clinical and Experimental Immunology**. . ISSN 00099104. 155:1 (2009) 1–15. doi: 10.1111/j.1365-2249.2008.03834.x.
30. FAHEY, Laura M. *et al.* - Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. **The Journal of Experimental Medicine**. . ISSN 0022-1007.

208:5 (2011) 987–999. doi: 10.1084/jem.20101773.

31. FAULKNER, Glenda C.; KRAJEWSKI, Andrew S.; CRAWFORD, Dorothy H. - The ins and outs of EBV infection. **Trends in Microbiology**. . ISSN 0966842X. 8:4 (2000) 185–189. doi: 10.1016/S0966-842X(00)01742-X.
32. FLORES-BORJA, Fabian *et al.* - CD19+CD24hiCD38hi B Cells Exhibit Regulatory Capacity in Healthy Individuals but Are Functionally Impaired in Systemic Lupus Erythematosus Patients. **Cell**. 32 (2010) 129–140. doi: 10.1016/j.immuni.2009.11.009.
33. FRÖHLICH, Anja *et al.* - IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. **Science**. . ISSN 00368075. 324:5934 (2009) 1576–1580. doi: 10.1126/science.1172815.
34. GOTTENBERG, Jacques Eric *et al.* - CD4 CD25highregulatory T cells are not impaired in patients with primary Sjögren's syndrome. **Journal of Autoimmunity**. . ISSN 08968411. 24:3 (2005) 235–242. doi: 10.1016/j.jaut.2005.01.015.
35. HANSEN, Arne *et al.* - Diminished peripheral blood memory B cells and accumulation of memory B cells in the salivary glands of patients with Sjögren's syndrome. **Arthritis and Rheumatism**. . ISSN 00043591. 46:8 (2002) 2160–2171. doi: 10.1002/art.10445.
36. HOEBE, K.; JANSSEN, E.; BEUTLER, B. - The interface between innate and adaptive immunity. **Nat Immunol**. . ISSN 1529-2908. 5:10 (2004) 971–974. doi: 10.1038/ni1004-971.
37. IGOE, Ann; SCOFIELD, R. Hal - Autoimmunity and infection in Sjögren's syndrome. **Current Opinion in Rheumatology**. . ISSN 10408711. 25:4 (2013) 480–487. doi: 10.1097/BOR.0b013e32836200d2.
38. JANNIE BORST, Heinz Jacobst And Gaby Brouns - Composition and function of T-cell receptor and B-cell receptor complexes on precursor lymphocytes. **Current Opinion in Immunology**. [s.d.]).
39. JONSSON, R. - Autoantibodies Present Before Symptom Onset in Primary Sjögren Syndrome. **JAMA**. 310:17 (2013) 1854–1855.
40. JUAN SHEN, XI LUO, QIONGLI WU, JUN HUANG, GUANYING XIAO, LIANTANG WANG, BINYAN YANG, Li Huabin And Chang You Wu - A subset of CXCR5+CD8+ T cells in the germinal centers from human tonsils and lymph nodes help

B cells produce immunoglobulins. **Frontiers in Immunology**. 9:2018) 2287.

41. JUNE, Carl H. *et al.* - The B7 and CD28 receptor families. **Immunology Today**. . ISSN 01675699. 15:7 (1994) 321–331. doi: 10.1016/0167-5699(94)90080-9.
42. KANG, Kwi *et al.* - Impact of interleukin-21 in the pathogenesis of primary Sjogren's syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. **Arthritis Research & Therapy**. . ISSN 1478-6354. 13:5 (2011) R179. doi: 10.1186/ar3504.
43. KIMURA, Hiroshi *et al.* - Quantitative Analysis of Epstein-Barr Virus Load by Using a Real-Time PCR Assay. 37:1 (1999) 132–136.
44. KINDT, T. J., GOLDSBY, R. A., OSBORNE, B. A., & KUBY, J. - **Kuby immunology**. 7th. ed. [S.l.] : Macmillan Publishers Limited, 2007
45. KIVITY, Shaye *et al.* - Infection and autoimmunity in Sjogren's syndrome: A clinical study and comprehensive review. **Journal of Autoimmunity**. . ISSN 10959157. 51:2014) 17–22. doi: 10.1016/j.jaut.2014.02.008.
46. KOULOVA, Lydia *et al.* - The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. **J Exp Med**. . ISSN 00221007. 173:3 (1991) 759–762. doi: 10.1084/jem.173.3.759.
47. KUCHEN, S. *et al.* - Essential Role of IL-21 in B Cell Activation, Expansion, and Plasma Cell Generation during CD4+ T Cell-B Cell Collaboration. **The Journal of Immunology**. . ISSN 0022-1767. 179:9 (2007) 5886–5896. doi: 10.4049/jimmunol.179.9.5886.
48. L. SKARE, Thelma; LIMA GODOI, Andressa De; O. FERREIRA, Valéria - Jaccoud arthropathy in systemic lupus erythematosus: clinical and serological findings. **Revista da Associação Médica Brasileira**. . ISSN 01044230. 58:4 (2012) 489–492. doi: 10.1590/S0104-42302012000400022.
49. LEMOINE, Sébastien *et al.* - Regulatory B cells in autoimmune diseases: How do they work. **Annals of the New York Academy of Sciences**. . ISSN 17496632. 1173:33 (2009) 260–267. doi: 10.1111/j.1749-6632.2009.04651.x.
50. LERNER, M. R. *et al.* - Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus

erythematosus. **Proceedings of the National Academy of Sciences**. . ISSN 0027-8424. 78:2 (1981) 805–809. doi: 10.1073/pnas.78.2.805.

51. LI X, QIAN L, WANG G, ZHANG H, WANG X, CHEN K, ZHAI Z, LI Q, WANG Y, Harris DC. - T regulatory cells are markedly diminished in diseased salivary glands of patients with primary Sjögren's syndrome. **The Journal of rheumatology**. 34:12 (2007) 2438–45.
52. LI, Xue Yi *et al.* - Role of the frequency of blood CD4 + CXCR5 + CCR6 + T cells in autoimmunity in patients with Sj??gren's syndrome. **Biochemical and Biophysical Research Communications**. . ISSN 0006291X. 422:2 (2012) 238–244. doi: 10.1016/j.bbrc.2012.04.133.
53. LINTERMAN, Michelle A. *et al.* - IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. **The Journal of Experimental Medicine**. . ISSN 0022-1007. 207:2 (2010) 353–363. doi: 10.1084/jem.20091738.
54. LUSTGARTEN, Joseph; WAKS, Tova; ESHHAR, Zelig - CD4 and CD8 accessory molecules function through interactions with major histocompatibility complex molecules which are not directly associated with the T cell receptor-antigen complex. **European Journal of Immunology**. . ISSN 15214141. 21:10 (1991) 2507–2515. doi: 10.1002/eji.1830211030.
55. MACKAY, Fabienne; BROWNING, Jeffrey L. - BAFF: A fundamental survival factor for B cells. **Nature Reviews Immunology**. . ISSN 14741741. 2:7 (2002) 465–475. doi: 10.1038/nri844.
56. MAITLAND, Norman *et al.* - Detection of cytomegalovirus and Epstein-Barr virus in labial salivary glands in Sjogren's syndrome and non-specific sialadenitis. **Journal of Oral Pathology & Medicine**. . ISSN 16000714. 24:7 (1995) 293–298. doi: 10.1111/j.1600-0714.1995.tb01187.x.
57. MANDL T , BREDBERG A , JACOBSSON LT , MANTHORPE R, Henriksson G. - CD4+ T-lymphocytopenia--a frequent finding in anti-SSA antibody seropositive patients with primary Sjögren's syndrome. **The Journal of Rheumatology**. 31:4 (2004) 726–728.
58. MANOUSSAKIS, M. N. *et al.* - Rates of infiltration by macrophages and dendritic cells

and expression of interleukin-18 and interleukin-12 in the chronic inflammatory lesions of Sjögren’s syndrome: Correlation with certain features of immune hyperactivity and factors associated with h. **Arthritis and Rheumatism**. . ISSN 00043591. 56:12 (2007) 3977–3988. doi: 10.1002/art.23073.

59. MANOUSSAKIS, Menelaos N.; KAPSOGEORGOU, Efstathia K. - The role of intrinsic epithelial activation in the pathogenesis of Sjögren’s syndrome. **Journal of Autoimmunity**. . ISSN 08968411. 35:3 (2010) 219–224. doi: 10.1016/j.jaut.2010.06.011.
60. MANUSCRIPT, Author; NANOBIOMATERIALS, Biodegradable - NIH Public Access. . ISSN 1053-8119. 6:8 (2013) 1111–1119. doi: 10.1021/nn300902w.Release.
61. MARIETTE, Xavier *et al.* - Detection of Epstein-Barr virus DNA by in Situ hybridization and polymerase chain reaction in salivary gland biopsy specimens from patients with Sjögren’s syndrome. **The American Journal of Medicine**. . ISSN 00029343. 90:3 (1991) 286–294. doi: 10.1016/0002-9343(91)80007-9.
62. MARKLUND, Gunnar *et al.* - IgA antibodies to Epstein-Barr virus in infectious mononucleosis. **Scandinavian Journal of Infectious Diseases**. . ISSN 00365548. 18:2 (1986) 111–119. doi: 10.3109/00365548609032316.
63. MARRACK, Philippa *et al.* - The effect of thymus environment on T cell development and tolerance. **Cell**. . ISSN 00928674. 53:4 (1988) 627–634. doi: 10.1016/0092-8674(88)90578-8.
64. MAURI, Claudia *et al.* - Human regulatory B cells in health and disease : therapeutic potential Find the latest version : Human regulatory B cells in health and disease : therapeutic potential. 127:3 (2017) 772–779.
65. MAVRAGANI, Clio P.; MOUTSOPOULOS, Haralampos M. - Sjögren syndrome Review. . ISSN 0820-3946. 186:15 (2014) 579–586. doi: 10.1503/cmaj.122037.
66. MICHEL, Laure *et al.* - Loss of IL-10 secretion by regulatory B lymphocytes in multiple sclerosis patients. **Journal of Translational Medicine**. . ISSN 1479-5876. 9:Suppl 2 (2011) P25. doi: 10.1186/1479-5876-9-S2-P25.
67. MINGUENEAU, Michael *et al.* - Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren’s signature correlating with disease activity and glandular

inflammation. **Journal of Allergy and Clinical Immunology**. . ISSN 10976825. 137:6 (2016) 1809–1821e12. doi: 10.1016/j.jaci.2016.01.024.

68. MITSIAS, D. I. *et al.* - The Th1/Th2 cytokine balance changes with the progress of the immunopathological lesion of Sjogren's syndrome. **Clinical and Experimental Immunology**. . ISSN 00099104. 128:3 (2002) 562–568. doi: 10.1046/j.1365-2249.2002.01869.x.
69. MIYASAKA, Nobuyuki; YAMAOKA, Kunio; TATEISHI, Mutsuto - Possible Involvement of Epstein-Barr Virus (EBV) in Polyclonal B-cell Activation in S jagren ' s Syndrome. 1989) 427–432.
70. MOUTSOPOULUS, H. M. - Autoimmune epithelitis. **Clinical Immunology & Immunopathology**. 72:2 (1994) 132–165.
71. NAVARRO, M. *et al.* - Intravenous Immune Globulin: Clinical Applications in the Newborn. **NeoReviews**. . ISSN 1526-9906. 11:7 (2010) e370–e378. doi: 10.1542/neo.11-7-e370.
72. PARNES, JR - Molecular-Biology and Function of Cd4 and Cd8. **Advances in Immunology**. . ISSN 0065-2776. 44:1989) 265–311.
73. PASCHALE, Massimo DE - Serological diagnosis of Epstein-Barr virus infection: Problems and solutions. **World Journal of Virology**. . ISSN 2220-3249. 1:1 (2012) 31. doi: 10.5501/wjv.v1.i1.31.
74. PLEŠIVČNIK NOVLJAN, M. *et al.* - Incidence of primary Sjögren's syndrome in Slovenia. **Annals of the Rheumatic Diseases**. . ISSN 00034967. 63:7 (2004) 874–876. doi: 10.1136/ard.2003.014027.
75. POOLE, Brian D. *et al.* - Epstein-Barr virus and molecular mimicry in systemic lupus erythematosus. **Autoimmunity**. . ISSN 08916934. 39:1 (2006) 63–70. doi: 10.1080/08916930500484849.
76. QIN, Baodong *et al.* - Epidemiology of primary Sjögren's syndrome: A systematic review and meta-analysis. **Annals of the Rheumatic Diseases**. . ISSN 14682060. 74:11 (2015) 1983–1989. doi: 10.1136/annrheumdis-2014-205375.
77. QUIGLEY, Máire F. *et al.* - CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. **European Journal of Immunology**. . ISSN

00142980. 37:12 (2007) 3352–3362. doi: 10.1002/eji.200636746.

78. RAMOS-CASALS, Manuel *et al.* - Primary Sjögren syndrome in Spain: Clinical and immunologic expression in 1010 patients. **Medicine**. . ISSN 00257974. 87:4 (2008) 210–219. doi: 10.1097/MD.0b013e318181e6af.
79. RAZIORROUH, Bijan *et al.* - Virus-Specific CD4+ T Cells Have Functional and Phenotypic Characteristics of Follicular T-Helper Cells in Patients with Acute and Chronic HCV Infections. **Gastroenterology**. . ISSN 15280012. 150:3 (2016) 696–706e3. doi: 10.1053/j.gastro.2015.11.005.
80. SAITO, I. *et al.* - Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. **The Journal of experimental medicine**. . ISSN 0022-1007. 169:6 (1989) 2191–2198.
81. SEROR, Raphaèle *et al.* - EULAR Sjögren's syndrome disease activity index (ESSDAI): A user guide. **RMD Open**. . ISSN 20565933. 1:1 (2015) 1–9. doi: 10.1136/rmdopen-2014-000022.
82. SHEARER, W. T. *et al.* - Lymphocyte subsets in healthy children from birth through 18 years of age: The pediatric AIDS clinical trials group P1009 study. **Journal Of Allergy And Clinical Immunology**. . ISSN 0091-6749. 112:5 (2003) 973–980. doi: 10.1067/mai.2003.1778.
83. SMEDBY, Karin Ekström *et al.* - Autoimmune disorders and risk of non-Hodgkin lymphoma subtypes: A pooled analysis within the InterLymph Consortium. **Blood**. . ISSN 00064971. 111:8 (2008) 4029–4038. doi: 10.1182/blood-2007-10-119974.
84. STRAUS, Moderator Stephen E.; COHEN, Discussants Jeffrey I. - Epstein-Barr Virus Infections : Biology , Pathogenesis , and Management. **Annals of Internal Medicine.** 118:1 (1993) 45–58.
85. SUDZIUS, Gintaras *et al.* - Activity of T-Helper Cells in Patients with Primary Sjögren's Syndrome. **in Vivo**. . ISSN 1791-7549. 27:2013) 263–268.
86. TATEISHI, M. *et al.* - Spontaneous production of Epstein-Barr virus by B lymphoblastoid cell lines obtained from patients with Sjogren's syndrome. Possible involvement of a novel strain of Epstein-Barr virus in disease pathogenesis. **Arthritis Rheum**. . ISSN 00043591. 36:6 (1993) 827–835.

87. VALENTINE, Kristen M. *et al.* - CD8 Follicular T Cells Promote B Cell Antibody Class Switch in Autoimmune Disease. **The Journal of Immunology**. . ISSN 0022-1767. 201:1 (2018) 31–40. doi: 10.4049/jimmunol.1701079.
88. VITALI C, BOMBARDIERI S, JONSSON R, MOUTSOPOULOS HM, Alexander; AL., EL Et - Classification criteria for Sjögren's syndrome: a revised version of the Erupean criteria proposed by the American- European Consensus Group. **Ann Rheum Dis**. 61:2002) 554–8.
89. VOGELZANG, Alexis *et al.* - A Fundamental Role for Interleukin-21 in the Generation of T Follicular Helper Cells. **Immunity**. . ISSN 10747613. 29:1 (2008) 127–137. doi: 10.1016/j.immuni.2008.06.001.
90. WANG, J. *et al.* - High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis. **Clinical and Experimental Immunology**. . ISSN 00099104. 174:2 (2013) 212–220. doi: 10.1111/cei.12162.
91. WATTS, T. H. - Staying Alive: T Cell Costimulation, CD28, and Bcl-xL. **The Journal of Immunology**. . ISSN 0022-1767. 185:7 (2010) 3785–3787. doi: 10.4049/jimmunol.1090085.
92. WEN, Shumin; SHIMIZU, Norio - Association of Epstein-Barr Virus (EBV) with Sjogren ' s Syndrome Differential EBV Expression between Epithelial Cells and Lymphocytes in Salivary Glands. **American Journal of Pathology**. 149:5 (1996) 1511–1517.
93. WORLD MEDICAL ASSOCIATION - World Medical Association Declaration of Helsinki. **Bulletin of the world health organization.** . ISSN 0042-9686. 79:4 (2001) 373–374. doi: S0042-96862001000400016 [pii].
94. YANG, Xue *et al.* - T follicular helper cells and regulatory B cells dynamics in systemic lupus erythematosus. **PLoS ONE**. . ISSN 19326203. 9:2 (2014) 1–9. doi: 10.1371/journal.pone.0088441.
95. YI, John S.; DU, Ming; ZAJAC, Allan J. - A vital role for Interleukin-21 in the control of a chronic viral infection. **Science**. . ISSN 00368075. 324:5934 (2009) 1572–1576. doi: 10.1126/science.1175194.

96. ZHANG, Sheng; ZHANG, Hongjun; ZHAO, Jiandong - The role of CD4 T cell help for CD8 CTL activation. **Biochemical and Biophysical Research Communications**. . ISSN 0006291X. 384:4 (2009) 405–408. doi: 10.1016/j.bbrc.2009.04.134.