"I was taught that the way of progress was
neither swift nor easy."
Marie Curie (1867 – 1934)

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List of Abbreviations

α-CD3	Anti-CD3 antibody
α-CD28	Anti-CD28 antibody
Ab	Antibodies
AID	Autoimmune disease
APC	Antigen presenting cell
ATP	Adenosine triphosphate
Bcl-6	B-cell lymphoma 6 protein
BBB	Blood brain barrier
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CIS	Clinically isolated syndrome
CSF	Cerebrospinal fluid
CNS	Central nervous system
CP-MS	Chronic progressive multiple sclerosis
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DMF	Dimethyl fumarate
DMT	Disease modifying therapies
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
EDTA	Ethylenediaminetetraacetic acid
F	Female
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate

Foxp3	Forkhead box protein 3
GA	Glatiramer acetate
GARP	Glycoprotein A Repetitions Predominant
GC	Germinal center
GITR	Glucocorticoid-induced TNF receptor-related protein
GWAS	Genome wide association study
HC	Healthy controls
HLA	Human leukocyte antigen
ICOS	Inducible T cell co-stimulator
IL	Interleukin
IL-2Rα	Interleuking-2 receptor alpha chain
IFN	Interferon
Ig	Immunoglobulin
IMSGC	International multiple sclerosis genetics consortium
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
iTreg	Inducible regulatory T cells
KO	Knockout
M	Male
mAb	Monoclonal antibody
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MRI	Magnetic resonance imaging
mTconv	Memory conventional T cell
MTX	Mitoxantrone
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NS	Not significant
nTreg	Naive regulatory T cell
OCB	Oligoclonal bands
OR	Odds ratio
OVA	Ovalbumine

PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PE	R-Phycoerythrin
PE-Cy5	R-Phycoerythrin-Cyanine5
PE-Cy7	R-Phycoerythrin-Cyanine7
PerCP	Phytohaemagglutinin
PML	Progressive multifocal leukoencephalopathy
RAF	Risk allele frequency
RNA	Ribonucleic acid
RRMS	Relapsing remitting multiple sclerosis
RPMI	Roswell park memorial institute medium
RTE	Recent thymic emigrant
SAP	Signaling lymphocytic activation molecule associated protein
S1P	Sphingosine-1-phosphate
SD	Standard deviation
SEM	Standard error of the mean
SLO	Secondary lymphoid organs
SNPs	Single nucleotide polymorphisms
SPMS	Secondary progressive multiple sclerosis
STAT	Signal transducer and activator of transcription
TGFβ	Transforming growth factor β
Tbet	T-box expressed in T cells
Tconv	Conventional T cells
TCR	T cell receptor
Teff	Effector T cells
T_{FR}	Follicular regulatory T cells
T_{FH}	Follicular helper T cells
T_{H}	Helper T cell
TNF	Tumor necrosis factor
tTreg	Thymic-derived T reg
Treg	Regulatory T cell

Tresp	Responder T cells
TSDR	Treg-specific demethylated region
WTCCC	Welcome trust case control consortium

1

INTRODUCTION AND AIMS

1.1 Multiple sclerosis

1.1.1 Discovery and history of multiple sclerosis

In 1868, Jean-Martin Charcot, examined a young woman in Paris with a specific tremor, abnormal eye movements and slurred speech. He tried to cure her with treatments available in the 19th century such as the poisonous strychnine, but failed. He examined her post-mortem brain and defined for the first time the characteristic scars or "plaques" of multiple sclerosis (MS) [1]. In the 1870's, Dr. Walter Moxon recognized MS in England and Dr. Edward Seguin in the United States. By just observing patients with MS, clinicians in that century defined much of what is still known about MS such as the increased risk in woman, the lack of direct heritability and the presence of various neurological symptoms [2].
In 1916, Dr. James Dawson was the first to accurately and precisely describe the typical inflammation around the blood vessels of the brain and the destruction of myelin [3]. The animal model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), was introduced in 1935, by Dr. Thomas Rivers at the Rockefeller Institute in New York City. He demonstrated that immune cells were the cause of this MS-like disease [4]. After the discovery of oligoclonal bands (OCB) and the genetic involvement that accounts for part of the disease, the complexity of MS became more and more apparent [2].

1.1.2 Prevalence, clinical course and etiology

Although the prevalence varies between different countries, ethnical groups and geographical locations, it is thought that between 2 and 150 per 100.000 individuals suffer from MS [5]. Worldwide, more than 2.5 million people have MS [6]. Onset of disease usually takes place between 20 and 50 years of age and females have about a two-threefold higher risk of developing MS compared to males [7]. The clinical development of MS is typical for autoimmune disorders rather than for neurological disorders and different disease courses have been described [8].
Approximately 90% of the patients initially show a relapse-remitting disease course (RRMS), which in about 65% of the cases will evolve after 5-25 years into a secondary progressive course (SPMS).

The relapses are typified by abrupt clinical episodes followed by a recovery. SPMS is characterized by a slow and steady increase of symptom severity without acute relapses. The remaining 10% of the patients do not experience relapses and commence with a primary progressive course (PPMS) [9, 10]. Some evidence exists that patients with PPMS do have a relapsing-remitting phase at onset but they do not display symptoms since their lesions are localized in clinically silent regions. Furthermore, no genetic, imaging or pathological features can distinguish PPMS from SPMS [11-14]. Patients that die within the first year of the disease, are diagnosed with acute multiple sclerosis [15].

While the etiology of MS is unknown, it is thought to be multifactorial by nature where the immune system is chiefly involved but other factors such as (epi)genetics (see 1.1.2.1) and environmental factors also contribute to the risk of developing the disorder. A proposed immunological mechanism, that could potentiate the development of MS, includes cross-reactivity by molecular mimicry caused by a viral infection. Antibodies (Ab) directed against Epstein-Barr virus (EBV) nucleic antigens are shown to have cross-reactivity with myelin antigens and thereby elicit an unspecific neuroinflammatory immune response [16, 17].

With the present knowledge, MS seems to arise when genetically susceptible persons confront environmental triggers that contribute to the initiation of an inflammatory reaction directed against self-antigens in the central nervous system (CNS) [18]. These observations, including the large variability in clinical manifestation, severity and prognosis combined with the great difficulty of understanding the underlying pathology of the disease, demonstrate that MS is a complex and heterogeneous disorder.

1.1.2.1 Genetic susceptibility and evidence for immune driven pathology in multiple sclerosis

The association of MS with variations in the genes encoding human leukocyte antigens (HLAs) within the major histocompatibility complex (MHC) has been known for decades. The major and most important risk allele is HLA-DRB1*15:01 (odds ratio (OR) 3) although other risk alleles such as HLA-DRB1*03:01, HLA-DRB1*13:03 and HLA-DPB1*03:01 were also reported [19].

The identification of HLA risk alleles was mainly due to linkage studies and SNP-based studies (candidate-gene-based studies). Although these can be relevant, they lack high power and are based on modest numbers of cases and controls.
The introduction of genome wide association studies (GWAS) revolutionized the MS genetics field. To date, 14 GWAS have been performed in MS. In 2011, the largest GWAS was published as a result of a collaboration between the international multiple sclerosis genetics consortium and the welcome trust case control consortium (WTCC) to identify risk alleles apart from the HLA genes. This last GWAS identified 34 new associated variants and confirmed the 23 already known associations. Of interest, the genes that lie in proximity of these 57 identified single nucleotide polymorphisms (SNPs) are mostly immune-related (lymphocyte proliferation and T cell activation) adding additional proof to the T cell autoimmune hypothesis that drives MS (Table 1.1) [20].
Linkage disequilibrium, although typical for the human genome, is responsible for the difficult interpretation of the results of GWAS. Therefore, an additional genotyping study is usually necessary to further unravel and reveal the genuine associated SNPs. The ImmunoChip is therefore designed to perform deep replication and thus additional genotyping of major autoimmune diseases and fine-mapping of established GWAS significant loci. The chip contains all known SNPs from loci associated with autoimmune diseases, created by the Genomes project and other initiatives [21]. Besides replication and fine mapping of the previously identified SNPs, this ImmunoChip genotyping array revealed 48 new susceptibility variants, putting the total known risk variations of MS on 110 [22]. It thus seems that not a single gene defect but several genes together explain susceptibility to disease [19, 23]. A large proportion of these identified genes are associated with the immune system, especially with T cell activation [20]. The identification of MS susceptibility genes essential for the immune system, together with the observation that a large portion of the SNPs related to MS are also identified in other autoimmune diseases, highlights the involvement of the immune system [24].
In addition, certain environmental factors play a pivotal role in the development of MS. Both EBV and smoking seem to contribute to the disease development since they lead to sustained activation of the immune system [25-27].

Table 1.1: Function of genes related to the immune system associated with MS at genome-wide significance based on results from International Multiple sclerosis Genetics 2011 and 2013

Chr	Gene	SNP	RA	Function in immune system
1	**CD58**	rs12044852	C	Adhesion molecule for activation of T cells
1	BCL10	rs12087340	A	Protein responsible for B cell and NFκB activation
1	EVI5	rs10735781	G	Protein involved in cytokinesis
1	VCAM1	rs11581062	G	Adhesion molecule for cell-cell recognition and transduction
1	RGS1	rs1323292	A	Signaling protein for regulation of G-protein signal transduction
2	**STAT4**	rs9967792	G	Transcription factor for T cell differentiation
2	CCR4	rs4679081	G	Chemokine receptor
3	**EOMES**	rs11129295	A	Transcription factor for cytotoxic T cells
3	**CBLB**	rs12487066	T	Protein ligase that downregulates T and B cell activation
3	**CD86**	rs9282641	G	Co-stimulatory molecule involved in T and B cell activation
3	**IL12A**	rs2243123	G	Cytokine for T cell independent induction of IFN-Y, differentiation of both T_H1 and T_H2 cells
4	NFKB1	rs228614	G	Transcription factor for immune activation
5	**IL7RA**	rs6897932	C	Cytokine receptor for development and function of T cells
5	**TCF7**	rs756699	A	Transcriptional activator for T cell differentiation
5	**PTGER4**	rs4613763	G	Prostaglandin receptor for T cell activation
5	**IL12B**	rs2546890	A	Cytokine that acts as a growth factor on T and NK cells
6	THEMIS	rs802734	A	Regulatory protein involved in T cell development (T cell selection)
6	IL22RA	rs17066096	G	Cytokine receptor for immune activation
6	**BACH2**	rs72928038	A	Transcription factor involved in Treg development
6	TNFAIP3	rs67297943	A	Ubiquitin-editing enzyme involved in cytokine pathways
6	**HLA-DRB1*1501**	rs3135388	A	Antigen presenting protein for recognition by CD4 T cells
7	CARD11	rs1843938	A	Intracellular protein that activates NFκB
7	IKZF1	rs201847125	G	DNA-binding protein that regulates lymphocyte differentiation
8	**IL7**	rs1520333	G	Cytokine for T cell development
10	**IL2RA**	rs12722489	C	Cytokine receptor for IL2 T cell activation

11	**CD6**	rs650258	G	Cell surface protein for in T cell activation
11	**CXCR5**	rs630923	C	Chemokine receptor for T and B cells
12	**KLRB1**	rs4763655	A	Cell surface protein that inhibits NK cytotoxicity and activates T cells
12	LTBR	rs12296430	C	TNF-receptor for immune signaling and development
12	TNFRSF1A	rs1800693	G	TNF-receptor that regulates infammation
12	**CD69**	rs11052877	G	Cell surface protein for T cell activation and cell transduction
12	**CLECL1**	rs10466829	A	Co-stimulatory molecule for T cells
14	TRAF3	rs12148050	A	TNF-receptor associated factor responsible for signal transduction and immune activation
16	CLEC16A	rs648169	G	C-Type lectin domain family member, expressed on B and NK cells
16	IRF8	rs13333054	A	Transcription factor for IFN- type 1 signaling
16	MAPK3	rs7204270e	G	Protein kinase involved in signal transduction
16	**MAF**	rs7196953	A	Transcription factor that increases T cell susceptibility to death
17	STAT3	rs9891119	C	Transcription factor that binds to the IL-6-responsive elements
17	IKZF3	rs12946510	a	Zinc-finger protein involved in B cell proliferation and differentiation
18	MALT1	rs7238078	A	Caspase involved in NFKB activation
19	**TNFSF14**	rs1077667	G	Co-stimulatory factor to stimulate T cell proliferation
19	TYK2	rs8112449	G	Tyrosine kinase involved in anti-viral immunity
20	CD40	rs2425752	A	Co-stimulatory molecule for immune cells
20	CYP24A1	rs2248359	G	Monooxygenase involved in calcium and vitamin D homeostasis
22	MAPK1	rs2283792	C	Protein kinase involved in signal transduction

Chr: chromosome; SNP: single nucleotide polymorphism; RA: risk allele. **T cell related genes are highlighted.**

Furthermore, there is a global latitude gradient in the risk of getting MS, suggesting a potential role of the vitamin D metabolism in the pathophysiology of MS [28]. Vitamin D has anti-inflammatory effects and low serum concentration of vitamin D modules the differentiation of T cell subsets [29, 30] again emphasizing the role of the immune system in MS pathogenesis.

1.1.3 Immunopathogenesis of multiple sclerosis

MS is a chronic inflammatory disorder affecting the CNS and is characterized by demyelination and axonal degeneration resulting in neurological impairment. Evidence from immunological, genetic, animal and histopathological studies shows that the immune system plays a vital role in the development of MS. Despite the CNS being an immuneprivileged tissue site with restricted access of immune cells, patients with MS have perivascular infiltrates of T lymphocytes, macrophages, B lymphocytes and plasma cells, suggesting that MS is an autoimmune mediated disease [31]. The disease course of MS is heterogeneous, with a period of relapses and recovery in the first phase, followed by a progressive decrease in the second phase. Current treatment options show that the peripheral immune system is the main player in the initial disease phase, while immune reactions within the CNS dominate the progressive phase [6].

1.1.3.1 Immunology during induction and early phase of multiple sclerosis

Although no direct evidence is available, it is thought that the early phase of the disease is initiated by the adaptive immune response and further mediated by the innate immune response (active phagocytes). A dominant hypothesis is that the "first hit" occurs in the periphery where a CNS antigen specific immune activation (priming) occurs by cross reactivity, molecular mimicry or bystander activation and leads to the migration of a few antigen specific T cells from the periphery into an unaffected CNS [32, 33]. Next, these CNS-specific T cells will be reactivated and release cytokines (IFN-γ and IL-17) in the perivascular space and affect oligodendrocytes, astrocytes and activate microglia [34]. Furthermore, plasma cells can arise in the periphery and accumulate in the CNS, releasing antibodies that destruct myelin sheaths and glial cells.
In addition, the tight structures of the blood-brain barrier (BBB) will be damaged and monocytes and additional lymphocytes can intrude leading to the formation of phagocytic lesions [35].

Adaptive immune system

Various $CD4^+$ T helper subtypes (T_H1 and T_H17) have been linked to MS [36-39]. Nonetheless, $CD8^+$ T cells outnumber $CD4^+$ T cells in inflamed lesions and display characteristics of expansion in the cerebrospinal fluid (CSF) and peripheral blood [40, 41]. Still both $CD4^+$ T cells and $CD8^+$ T cells isolated from MS lesions and the CSF were clonally expanded [42, 43], indicating that antigen specific T cells drive the pathogenesis. Although a few antigen candidates such as myelin and heat shock proteins are suggested (reviewed in [44]), it remains difficult to pinpoint one specific T cell antigen, as it likely varies from patient to patientc [45-48].

In addition, various findings have led researchers to gain interest in the role of B cells in MS pathogenesis. B cells can account for up to 25% of the CNS-infiltrating cells during neuroinflammation and presence of oligoclonal immunoglobulins in the CSF, suggests a significant role for B cells in MS pathology [49, 50]. Maybe the most striking evidence for B cells being a part of MS pathology is the therapeutic efficacy seen with administration of Rituximab (depletion of $CD20^+$ cells) or with plasma exchange [51, 52].

Various mechanisms for the contribution of B cells in autoimmunity are suggested, including B cells as a source of autoantibodies, as an immunomodulator by secretion of cytokines or antigen presentation [53]. Nonetheless, despite extensive research target antigens of autoantibodies found in MS are unknown and remain to be identified. Increased levels of autoantibodies directed against myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), sperm associated antigen 16 (SPAG16) and neurofascin have been observed in CSF and serum of subgroups of MS patients. However these findings are still under debate, since titers of these Abs are observed to be similar between MS patients and healthy controls [54, 55].

Innate immune system

Microglia and macrophages have a dual role in CNS pathogenesis since they can directly cause neuronal damage but are also vital in tissue repair and clearance of myelin debris [56]. Different studies support the notion that phagocytic function is dependent on origin.

Phagocytes from the periphery, namely monocyte-derived phagocytes, mostly initiate demyelination while phagocytes within the CNS, microglia-derived phagocytes, clear the cellular debris and enhance tissue recovery [57].

1.1.3.2 Immunology during progressive phase of MS

As MS disease progresses it changes clinically and pathologically leading to assume that a different immunopathological process underlies this second phase. It is thought that the pathology arises from within the CNS, and two main hypotheses exist. One possible mechanism is that the compartmentalized inflammation further drives the disease [58] while another plausible mechanism is that primary neurodegeneration accounts for further disease progression [6, 59]. Of notice, it is also possible that these two mechanisms act together where the compartmentalized inflammation can drive the constant insult that could be worsened by the decreased repair capacity of the damaged CNS [60]. Taken together, the clinical heterogeneity and the volatile disease behavior seen in MS, is a reflection of the balance in which different subsets of autoimmune cells contribute to different stages in the MS pathogenesis.

1.1.4 Diagnosis, clinical features and disability score

To date, no single diagnostic test exists for the identification of MS. When patients experience symptoms typical for the early phases of the disease such as visual disturbances, fatigue and numbness in their limbs, the McDonald criteria are implemented [61]. These include an array of tests such as a dissemination in time and space using MRI (magnetic resonance imaging) scan to visualize plaques, laboratory test to identify OCB in the CSF, medical history assessment, counting relapses and a neurological examination. Since early MS manifestations occur predominantly in the CNS, no blood test is available to diagnose MS.
However, a blood test is important to exclude other diseases that mimic MS such as vitamin B12 deficiency, systemic lupus erythematosus and neuromyelitis optica [62]. Recent evidence shows that early peripheral indicators in the cervical lymph nodes may be used to diagnose MS in the future [63].
The clinical presentation of MS manifests in fatigue, numbness or tingling in the extremities of the body, muscle weakness, vision problems, paralysis, tremor and bladder problems, among others.

Next to these symptoms some patients also experience cognitive deterioration, signs of depression and emotional changes [64].
To score the disability in patients with MS, various scoring scales are available depending on the usage. For an overall representation of disability outcomes the Expanded Disability Status Scale (EDSS) score (scale from 0-10) is usually implemented. The Multiple Sclerosis Severity Score (MSSS) indicates disease severity since it correlates EDSS with disease duration (in years) [65] and was proven fruitful for epidemiological and genetic studies [66].
The Patient-derived MS Severity Scores (P-MSSS) was introduced more recently. First, a Disability Expectancy Table (DET) was made based on 36,000-patient-disability data from a validated Patient-Determined Disease Steps (PDDS) scale. DET displays maximum ranks (cumulative frequencies) of PDDS scores for disease durations of 0–45 years. DET allows easy determination of how a patient's disability compares to others with the same disease duration.
Next, the mean ranks of PDDS scores for each year of disease was determined to assess the P-MSSS [66].

1.1.5 Therapies for multiple sclerosis: the past, the present and the future

At present, an arsenal of disease-modifying therapies (DMT) are available to prevent exacerbations in this heterogeneous and complex disease. Of these therapies, a substantial amount is given for the treatment of RRMS, while only one exists to treat the progressive phase of the disease. First-line therapies discovered and approved 30 years ago are still administered in the beginning of the disease but more recently various drugs were found to be more specific and effective and are a good alternative when first -line treatment fails (Figure 1.1).

1.1.5.1 The past

In 1993, IFN-β1b was approved by the food and drug administration (FDA) for the treatment of RRMS. From that moment onward various formulations of IFN-β1b, IFN-β1a (Avonex®, Betaseron®, Rebif®, Extavia®) and glatiramer acetate (GA, Copaxone®) were introduced and approved.

Although from that time MS was considered a treatable disease, this treatment was expensive, inconvenient (self-injection) and had various adverse effect. Furthermore, albeit IFN-β treatment showed a clear survival benefit and delay in disease progression and symptoms quite well it was found to worsen the disease in a minority of the patients. Today, both interferon and GA are widely used as fist-line treatments in RRMS. While their mode of action is still not fully elucidated, it is agreed that they modulate the immune system, working primarily anti-inflammatory (T_{H1} to a T_{H2} shift) [62, 67, 68].

1.1.5.2 The present

Monoclonal antibodies

A new era in the treatment of MS began with the introduction of natalizumab. Natalizumab (Tysabri®) is a monoclonal antibody (mAb) directed against intergrin-α4 on leukocytes which blocks their migration across the BBB. Despite the fact that natalizumab decreased the amount of relapses and new MRI lesions significantly, some patients develop progressive multifocal leukoencephalopathy (PML) due to infection with a polyoma virus (John Cunningham virus or JCV). The mechanism that leads to this induction is not known [67]. Natalizumab is considered the most effective drug available for the treatment of MS, but risk for subsequent development of PML in some patients must be assessed via presence of JCV antibodies [62, 69, 70].

Another mAb, alemtuzumab (Campath®), is directed against CD52 and leads to the depletion of leukoctyes. Treatment with alemtuzumab leads to a decreased relapse rate and EDSS score and is approved by the FDA for the treatment of refractory MS. However, a major adverse effect in patients treated with alemtuzumab is the development of secondary autoimmunity [67, 69, 71].

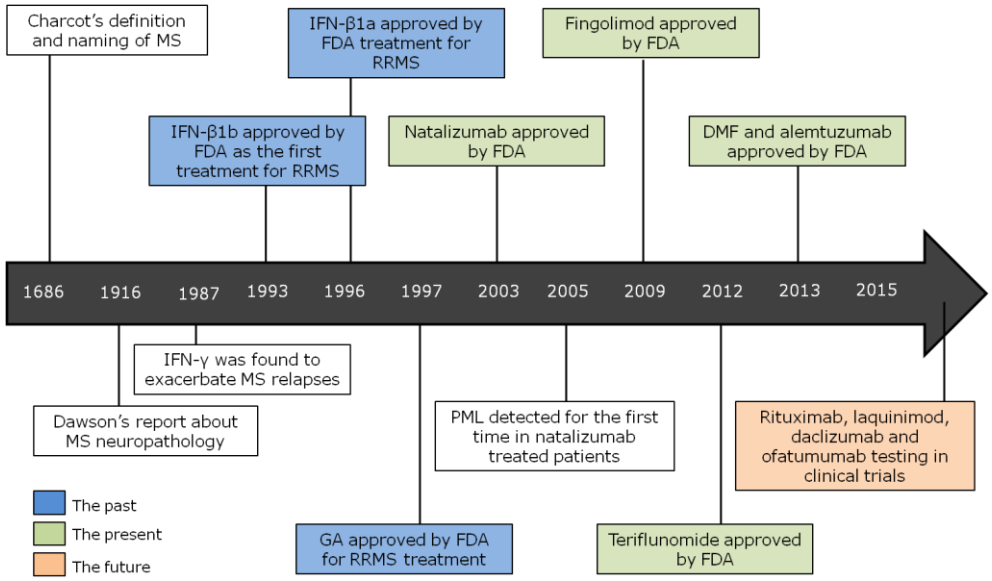

Figure 1.1: Timeline with the development of treatments for MS. MS: multiple sclerosis, FDA: food and drug administration, PML: progressive multifocal leukoencephalopathy, RRMS: relapsing remitting MS, DMF: dimethyl fumarate, IFN: interferon. Adapted from Ransohoff et al. [62].

Oral treatment options

The first oral drug that was introduced for the treatment of MS was fingolimod (Gilenya®). After ingestion, it is converted to a sphingosine-1-phosphate (S1P) analogue. Subsequently, it downregulates S1P receptor on leukocytes leading to the entrapment of activated leukocytes in the lymph nodes. Studies have shown that naive and central memory T cells are mainly captured (both $CCR7^+$) while effector memory T cells ($CCR7^-$) can still recirculate. S1P signaling normally overrides the CCR7 dependent retention in the lymph nodes but fingolimod inhibits S1P signaling trapping $CCR7^+$ cells in lymph nodes [72]. RRMS patients treated with fingolimod have a decreased relapse rate and reduction of new lesions hence a suppression of disease activity. Since vascular endothelium also expresses S1P receptor and fingolimod causes lymphopenia, the most pronounced side-effects are cardiovascular problems and increased risk of opportunistic infections, respectively [69, 73].

Other approved oral drugs are teriflunomide and dimethyl fumarate. Teriflunomide (Aubagio®) is administered for the treatment of RRMS.

It inhibits DNA pyrimidine bases synthesis, more specifically the enzyme dihydroorotate dehydrogenase, in rapidly proliferating T and B cells leading to a reduction in inflammation [74]. Due to its reduction in new lesions with a minimum of side effects it was FDA approved in 2012 [75]. Dimethyl fumarate (DMF, BG-12, Tecfidera®) was approved in 2013 for the treatment of RRMS. Results from two independent clinical trials showed a decreased number of relapses in patients [76]. It resembles fumaric acid, used for treatment of psoriasis, is anti-inflammatory and neuroprotective [62, 69]. More specifically, DMF promotes the anti-oxidative stress cell machinery by activation of the nuclear factor (erythroid derived 2)-like2 (NRF2) [67]. DMF exposure increases the cytosol concentrations of NRF2, which besides immune regulatory effects, has the potential for cytoprotection on glial cells, oligodendrocytes and neurons [77, 78].

Cytotoxic drug

Treatment with mitoxantrone (Novantrone®), a cytotoxic drug that interferes with DNA synthesis, repair and cell replication, is FDA approved but mainly given to patients with unresponsive RRMS and progressive MS (SPMS and PPMS) [67]. Its proposed mechanism of action is the inhibition of rapidly proliferating lymphocytes leading to a decrease in relapses and disease progression [79].

1.1.5.3 The future

Monoclonal antibodies

Daclizumab is directed against the IL2Rα chain present on T cells, inhibiting T cell proliferation. Various clinical trials showed beneficial effects for the treatment of RRMS. A long-term efficacy and safety multicenter randomized controlled Phase IIb study is currently pending [80]. Rituximab is a chimeric mAb against the CD20 antigen, targeting pre-B cells and B cells, resulting in the depletion of $CD20^+$ B cells. In RRMS patients, treatment leads to reduction of relapse and improvement in the EDSS score [81]. In addition, Ofatumumab a completely humanized anti-CD20 antibody is currently being tested for RRMS treatment [67, 69].

Oral treatment options

Laquinimod is a derivate of roquinimex and was shown to be effective in a rat model of EAE. Although the exact mechanism of action is not clear it seems that a downregulation of major histocompatibility complex II and T cell chemokines contributes to the effectiveness. Two Phase III trials are currently ongoing to determine the safety and efficacy. Laquinimod is administered for the treatment of RRMS and SPMS [67, 69, 82].

Experimental studies

Currently, different possible future therapies for MS are being investigated such as immune therapy to boost regulatory T cells [83], inducible pluripotent stem cells (iPSCs) therapy [84] and antigen-specific tolerance induction [85]. These studies are still in a preliminary phase but promise exiting prospects for the future.

1.2 Regulatory T cells in multiple sclerosis

1.2.1 Basic concepts of regulatory T cells

Regulatory T cells (Treg), a suppressive subset of $CD4^+$ T cells, are central in controlling the peripheral immune tolerance. The best characterized Tregs are defined by expression of the transcription factor forkhead box protein 3 (FOXP3) in mice [86] and demethylation of the Treg-specific demethylated region (TSDR) in the FOXP3 locus in humans [87]. Deletion of foxp3 in mice [88] or mutation of the FOXP3 gene in humans [89] leads to multiorgan autoimmunity, inflammatory disease and allergy [86] highlighting their prominent involvement in balancing normal immunity. Since intracellular Foxp3 is selective for murine Tregs but not for humans, other markers have been identified to characterize and subdivide human Tregs such as constitutive expression of the high-affinity IL-2Rα chain (CD25) along with low expression of the IL-7Rα chain (CD127), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), CD39 and more recently glucocorticoid-induced TNFR-related protein (GITR), Glycoprotein A Repetitions Predominant (GARP), TIGIT and Helios [90-95].

Tregs are classified according to their origin [96]. Thymus-derived Tregs (tTreg) originate in the thymus and migrate to secondary lymphoid organs and to inflamed tissue sites, like other $CD4^+$ T cells do. Other Tregs, known as peripherally derived Treg (pTreg), are induced in the periphery from $CD4^+$ T cells and mainly control mucosal inflammation. Nevertheless, both are thought to have an essential role in immune regulation [97].

Human regulatory T cells have various suggested methods of actions in order to limit collateral tissue damage and autoimmunity (Figure 1.2). First, Tregs secrete anti-inflammatory cytokines such as TGFβ and IL-10. Second, Tregs can directly kill target cells by secreting cytolytic molecules such as perforin and granzym A and B. Third, expression of CD39, an ectonucleotidase, by Tregs catalyses the degradation of adenosine triphosphate (ATP), pro-inflammatory molecule, into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) leading to metabolic disruption of the effector cells [98-111]. In mice, Tregs can capture IL-2 by their high expression of the IL-2R (CD25), thereby limiting proliferation of surrounding T cells [112]. It remains to be seen if human Tregs have this capacity.

Lastly, Tregs express CTLA-4 and can bind to CD80, a costimulatory molecule on APC, and subsequently downregulate CD80 expression leading to a decrease in function of APC.

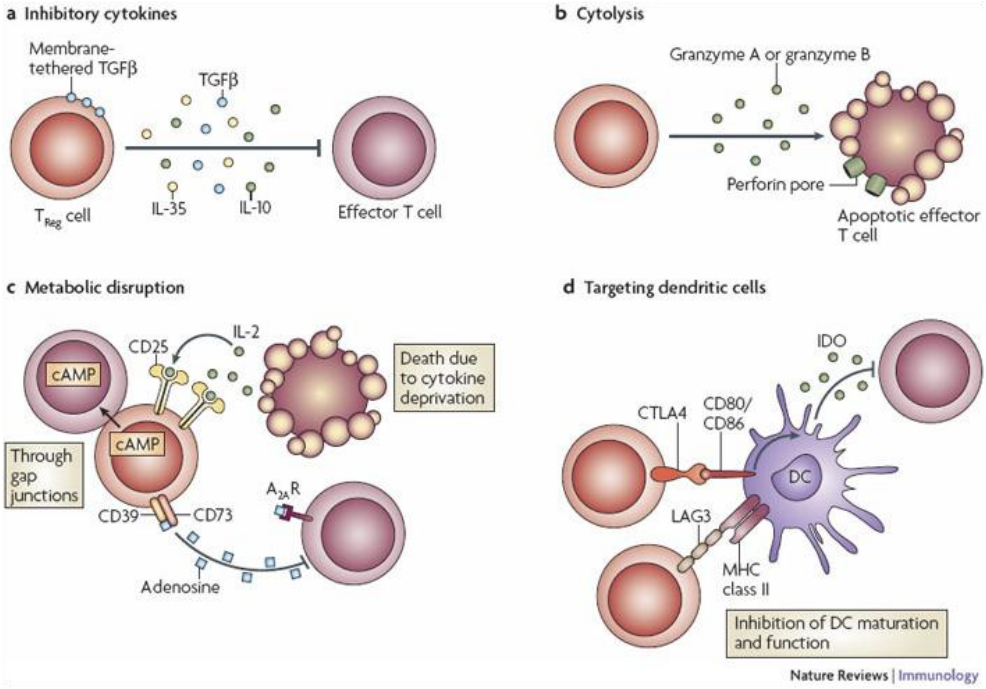

Figure 1.2: Basic mechanisms of suppression by regulatory T cells. (a) Inhibitory cytokines such as interleukin-10 (IL-10), IL-35 and transforming growth factor-β (TGF-β). **(b)** Killing of cells using granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. **(c)** Metabolic disruption with cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A2AR)-mediated immunosuppression. **(d)** Targeting dendritic cells (DCs) with cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), and MHC-class-II mediated suppression of DC maturation. Reprinted with permission from the Nature Publishing Group [99].

1.2.2 Demethylation of FOXP3 in Tregs

There are several indications showing that Foxp3 expression alone is insufficient to maintain a stable lineage of suppressive and functional Tregs. Activated human conventional T cells also express FOXP3, albeit at low levels but have no suppressive capacity [113]. Human circulating $CD4^+$ T cells contain a subpopulation of $FOXP3^+$ T cells that have pro-inflammatory but no regulatory features [114]. In addition, Foxp3 expression is not necessary for Treg signature gene expression since $Foxp3^{gfpko}$ mice can express CTLA-4 and IL2RA genes [115, 116]. Taken together, determination of Treg cell fate and function seems dependent on more than Foxp3 expression alone [117].

The epigenetic status of Treg seems to be crucial for the maintenance, function and phenotype [118-120]. DNA methylation is a reversible epigenetic modification that leads to decreased accessibility of transcription factors and this pattern was found to be different in several genomic regions in conventional T cells compared to Tregs [118, 120]. A Treg-specific demethylation region (TSDR) is the demethylation of a CpG island located in the regulatory elements in a conserved non-coding sequence 2 (CNS2) of the FOXP3 locus intron 1 that leads to highly stable thymic Tregs after activation [118, 121, 122]. Demethylation of this CNS2 region causes persistent FOXP3 gene transcription during lifespan and division thus suggesting that demethylation stabilizes gene expression [123]. Treg that express Foxp3 and have a hypomethylation of the CNS2 region are considered a genuine functional and phenotypically stable cell population of Treg [87, 124].

1.2.3 Regulatory T cells plasticity: a double edged sword

Tregs are shown to maintain immune homeostasis, inhibit autoimmune disease and control infection. To properly exert these wide range of functions, Tregs can adapt to their anatomical and inflammatory surroundings so they can enhance their specific environmental activity.

Effector T cells (Teff) have the ability to adapt a functional fate according to the type of antigen and cytokines that are present while they are activated. Although these functional commitments are essential for a good immune response, the pro-inflammatory response needs to be regulated or even terminated when the pathogen is removed or when tissue destruction is present.

Indeed, Tregs can selectively express transcriptional regulators, much like the Teff, which drives them to a phenotypical and functional specialization to specifically adapt to their surroundings and the Teff they suppress. T-bet and CXCR3 can be expressed by Tregs and is required for T_H1 type inflammatory response [125], IFR4 and CCR4/8 expressed by Tregs regulate a T_H2-type inflammatory response [126], STAT3 and CCR6 are expressed by Tregs to limit a T_H17 mediated response [127] and Bcl-6 and CXCR5 are expressed by a subset of Tregs, called follicular regulatory T cells (T_{FR}) to suppress follicular helper T cells (T_{FH}) [128].

The ability of Tregs to adapt based on their surroundings enhances their functional capacities but several lines of evidence suggest that Tregs can also become pro-inflammatory effector cells in inflammatory conditions. Tregs can secrete effector cytokines, lose their suppressive capacity, downregulate or lose Foxp3 expression and become 'ex-Tregs' [129-131]. To counterbalance this capacity various research groups sought to identify the regulatory mechanisms behind this phenomenon. Recently, a transcription factor (BACH2) was identified as a key regulator in stabilizing immunoregulatory capacity while repressing the differentiation within T_H1, T_H2 and T_H17 cell lineages [132]. Furthermore, the expression of semaphorin-4a on immune cells and the expression of neuropilin-1 on Tregs can maintain Treg stability and function at inflammatory sites [133, 134].

1.2.4 Regulatory T cells in multiple sclerosis

Myelin-specific T cells can be found in MS patients as well as in healthy individuals [135]. Tregs from healthy persons have the ability to prevent activation and function of these auto-reactive T cells [136]. These regulatory mechanisms are impaired MS patients. Mechanisms by which a Treg deficit could result in autoimmune disease include decreased numbers, disturbed homeostasis, impaired suppressive function or resistance of auto-reactive T cells against Treg mediated immunosuppression [137].

Differences in frequencies reported in MS patients and healthy donors is dependent on the usage of Treg markers [98]. Although the majority of researcher found that MS patients showed no differences or an increase in numbers compared to healthy controls when using $CD25^{high}$ [138-140], other research groups found a decreased Treg frequencies when adding CD39 [110, 141] or CD127 [142] to the phenotype. In contrast, MS patients have an enrichment of Tregs in their CSF compared to peripheral blood [139, 143].

An impairment of immunosuppressive capacity of Treg cells derived from RRMS patients *in vitro* has been demonstrated by various groups including ours [140, 142-146]. Of notice, this effect was not seen in patients with SPMS [145]. In addition, different groups found that the loss of Treg function in MS patients was linked to a decrease in Foxp3 expression and CTLA-4 expression [143, 147, 148]. A subpopulation of human Tregs, $CD39^{+}$ Tregs essential for controlling pathogenic T_H17 responses in human MS, are also functionally impaired in MS patients [141, 149].

Plausible explanations for a decreased function and altered frequencies of Tregs in MS are: an impairment in generation of naive tTregs leading to the exhaustion of memory Tregs [142, 150], enhanced plasticity of Tregs in MS [151] and a genetic defect in MS-related Treg genes such as IL2RA, IL7RA and CD58 [152]. In addition, the proliferation of Treg cells after TCR stimulation is impaired in RRMS patients and correlates with the clinical state of the subject, where increasing disease severity is associated with a decline in Treg cell expansion. These results suggest a previously unrecognized mechanism that may account for the progressive loss of Treg cells in autoimmune disease [153].

1.3 Aims of the study

T_{FR} and T_{FH} are a specialized subtype of Tregs and Teff respectively, present in the germinal centers (GC) of lymphoid organs. A normal homeostasis of both T_{FR} and T_{FH} is essential to orchestrate normal humoral immunity [128, 154]. MS is characterized by disturbances in the Treg and humoral cell compartment. In addition, Tregs have been demonstrated to modulate B cell immunity **(Chapter 2)**. In this study, we therefore aim to identify possible disturbances in follicular T cell populations that could contribute to the pathogenesis of MS.

Since blood is our main source to investigate immune cells we focus on the circulating counterparts of these cells. We first investigate the relevance and reflection of blood circulating follicular T cell populations compared to those present in secondary lymphoid organs (SLO). Next, we assess whether a numeral and/or functional impairment could be detected in MS patients. Finally, we analyze whether treatment or genetic predisposition affects the follicular T cell compartment. To determine the involvement of follicular T cells subsets in the pathogenesis of MS, the following aims were put forward:

1.3.1 Determine if circulating follicular T cell populations are a good reflection of genuine follicular T cells in humans

Follicular T cell populations develop and predominantly exert their function in the follicles of GC in SLO [128, 155]. Still, peripheral blood is the most accessible source to investigate auto(immunity) in humans. In **Chapter 3,** we therefore determine whether blood derived follicular T cells can be used as an alternative source to study GC derived follicular T cells. To assess if circulating T_{FR} are genuine 'bona fide' T_{FR} and to give insight into their functional nature we compare circulating T_{FR} with tonsil-derived T_{FR}. In addition, we measure the impact of vaccination on the circulating compartment to investigate whether they can reflect GC processes. T_{FH} cells were included as comparative control in all analyses. Results from these experiments determine if circulating T_{FR} can be used as an alternative source to represent SLO-derived T_{FR}.

1.3.2 Determine disturbances in circulating follicular T cell populations of multiple sclerosis patients

Patients who suffer from MS, are characterized by an increase in autoreactive B cells and autoantibodies [156-158] and a decrease in Treg functionality [142, 143, 145]. We hypothesize that follicular T cell populations can contribute to disease pathogenesis since dysfunctions in T_{FH} and regulatory T cell subsets were shown to contribute to autoimmunity **(Chapter 2)**. We first investigate alterations in the amount and the effector phenotype of circulating T_{FR} and T_{FH} by comparing MS patients and healthy controls (HC) **(Chapter 3)**. In addition, we compare the suppressive capacity of circulating T_{FR} from RRMS patients and HC in a robust co-culture suppression assay to detect a possible functional deficit. Lastly, we take clinical features such as disease duration, type of MS and EDSS into account to correlate T_{FR} frequency and disease course. Results from this aim are indicative for a possible contribution of a T_{FR} and/or T_{FH} defect in MS disease course.

1.3.3 Investigate the effect of treatment on circulating follicular T cell populations in multiple sclerosis

To date, MS cannot be cured. Current treatment strategies can be subdivided into DMT, which have a global anti-inflammatory effect, and specific treatments, that directly target a defined part of the immune system [159]. Considering that MS treatment mainly works by sequestering the immune response, we analyze the effect of treatment with DMT (such as interferon) and specific therapies (such as natalizumab) on the percentage of circulating T_{FR} and T_{FH} **(Chapter 3)** by comparing untreated versus treated MS patients. In addition, we investigated the long-term effects of fingolimod, a FDA approved MS treatment that sequesters various lymphocyte subsets within the SLO. We determined whether this therapy had an effect on follicular T cell subsets since their main site of activity resides within the lymphoid tissues **(Chapter 4)**.

1.3.4 Investigate the influence of genetic predisposition on circulating follicular T cell populations and expression of key molecules

While MS is not a heritable disease, part of the susceptibility can be explained by genetic predisposition [19]. Almost all of the genes identified and confirmed in the GWAS and the Immunochip study, reveal a primary role for the immune system. Polymorphisms within CXCR5, IL2RA and CD58, all T cell related genes, were found to be associated with an increased risk for developing MS [152]. Since these genes are involved in T cell function and homeostasis, a polymorphisms (MS related SNPs) within these genes could contribute to an altered frequency and/or phenotype of regulatory T cells subsets. To analyze this, we investigate the effect of genetic variation on T_{FR} and Treg frequency and expression of CXCR5 and IL2RA on the surface of these cells in MS patients and HC **(Chapter 5)**. This part of the study identifies the functional consequences of carrying a genetic polymorphism in T cell related genes, explaining a potential impaired follicular T cell homeostasis.

2

HUMORAL AUTOIMMUNITY: A FAILURE OF REGULATORY T CELLS?

This chapter is based on:
Dhaeze, T, Stinissen, P, Liston, A, and Hellings, N.
Humoral autoimmunity: A failure of regulatory T cells?
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Abstract

Regulatory T cells (Tregs) are essential in maintaining tolerance to self. Several lines of evidence indicate that Tregs are functionally impaired in a variety of autoimmune diseases, leading to inefficient regulation of autoimmune T cells. Recent findings also suggest that Tregs are essential in controlling autoreactive B cells. The recently identified follicular regulatory T cell subset (T_{FR}) is thought to regulate the production of autoantibodies in the germinal center (GC) response. Here we provide an update on the role of Tregs in controlling the GC response, and whether defective control over B cell tolerance contributes to autoimmunity.

2.1 Introduction

The immune system is dependent on various checkpoints to both ensure an optimal recognition and elimination of foreign antigens and to maintain tolerance against self-antigens. While negative selection of self-specific immune cells occurs during early differentiation, additional peripheral control mechanisms are in place to further ensure tolerance to self. Regulatory T cells (Tregs) are central in controlling peripheral tolerance and are characterized by the expression of the transcription factor Foxheadbox3P (Foxp3). Deletion of *Foxp3* in mice [88] or mutations in the *FOXP3* gene in humans [89] lead to multi-organ autoimmunity, inflammatory disease and allergy [86]. Tregs can be classified according to their origin [96]. Thymus-derived Tregs (tTreg) originate in the thymus and migrate to secondary lymphoid organs (SLO) and inflamed tissue sites, as conventional $CD4^+$ T cells do. Other Tregs, knows as peripherally-derived Treg (pTreg), are induced in the periphery, where the functional evidence is strongest for control over mucosal immunity. Accumulating evidence suggests the induction by commensal bacteria in the gut is the major source of pTregs [91, 92]. Intracellular Foxp3 expression identifies Tregs, but given the obvious limitation other markers have been used to characterize Tregs such as $CD25^{hi}CD127^{lo}$, CD39, cytotoxic T lymphocyte antigen-4 (CTLA-4) [160], and more recently Glycoprotein A Repetitions Predominant (GARP), TIGIT and Helios [93-95, 161] (for an elaborate overview of Treg markers see [161]).

The maturation and differentiation of the humoral immune response is T cell-dependent and occurs in the germinal centers (GC) of SLO. The GC response is mainly orchestrated by three types of cells: B cells, follicular helper T cells (T_{FH}) and follicular dendritic cells (FDC). After naive T and B cells bind their cognate antigen, both cell types migrate towards the border of the T-B cell zone as a result of upregulation and downregulation of certain chemokine receptors (e.g. CXCR5 and CCR7, respectively) [162, 163]. The T cell differentiates into a T_{FH} and interacts with the antigen-activated B cell, leading to the formation of an extrafollicular focus, where short-lived plasmablasts thrive, or the development of a GC. T_{FH} and B cells migrate to the center of the follicle where T_{FH} cells further stimulate B cell survival and maturation, including B cell expansion, isotype switching, immunoglobulin affinity maturation and selection [164-166].

In turn, B cells stimulate T_{FH} cells (by presenting antigen) to expand and further develop, as well as conversely, negatively regulating T cells to keep the response in check [167, 168]. Eventually, the GC response results in the formation of long-lived plasmablasts and memory B cells [169]. The GC response requires exquisite regulation to ensure the production of antibody-producing and memory B cells and to minimize unwanted auto-reactive or low affinity antibodies. The cellular characteristics of Tregs identify them as ideal candidates for the regulation of this complex and delicate process, with the ability to migrate to sites of T and B cell accumulation and a suppressive effect on both cell types. A specialized subset of regulatory T cells, named the follicular regulatory T cells (T_{FR}) was recently found to be indispensible for the regulation of the GC response [128, 155, 170, 171].

In this review, we describe how Tregs control the humoral immune response and prevent humoral autoimmune responses and disease. First, we provide an overview of Tregs involvement in controlling B cell immunity. Specific attention is paid to the T_{FR} specialized subset of Tregs. Second, we review recent data on the role of T_{FR} in autoimmunity. Finally, we consider the immunological function of circulating T_{FR} in light of recently published data on human circulating T_{FH}.

2.2 Regulatory T cells control the maturation of B cells

The primary physiological function of Tregs is the regulation of T effector cells (Teff). Nonetheless, Tregs are also capable of controlling other immune cells, such as B cells (Figure 2.1). Early evidence showed that murine Tregs were able to directly inhibit B cell proliferation, proving their ability to modulate the maturation of B cells *in vivo* [172]. This suppression of proliferation was explained by an increase in cell death of antigen-presenting B cells, thereby contributing to a reduction in T helper (T_H) cell activity. The mechanisms that induced B cell apoptosis were dependent on the secretion of perforin and granzymes [109]. In humans, Tregs were shown to suppress IgG and IgA production by B cells in a T cell-independent manner. Furthermore, Tregs suppressed class-switching and affinity maturation of B cells, independent of the presence of T_H cells [173]. Of note, this suppression was dependent on cell-cell contact, and blockade of TGF-β and CTLA-4 abolished suppression.

It is worth mentioning that regulatory B cells can also, in return, regulate T cells (reviewed in [174]), thus constituting an elaborate regulatory network.

2.2.1 Regulatory T cells control the germinal center response

During B cell maturation an enormous number of somatically mutated B cells emerge in the GC. To maintain self-tolerance, regulatory pathways need to be employed (Figure 2.1). A regulatory T cell subtype with capacities to migrate towards the T cell zone in SLO in humans was discovered 10 years ago [175]. Lim et al. found that highly suppressive $CD4^+CD25^+CD69^-$ Tregs are able to regulate the GC-T_H cell-dependent IgG synthesis. These cells were considered to be regulatory based on expression of TGF-β, CD62L, GITR and FOXP3. They also showed that Tregs in human tonsils are able to suppress B cells in a T_{FH}-independent manner, regulating class-switching [173].
Several research groups have added or depleted Tregs (or essential Treg genes) *in vivo* to demonstrate the effect on the GC response. Fields *et al.* used adoptive transfer of labeled Tregs *in vivo* to assess the time point of Treg intervention in the GC response. They showed that Tregs block the maturation but not the initiation of autoantibodies and did not interfere with the initial follicular entry or activation of T_H or B cells [176]. In the reciprocal experiment, depletion of Tregs by anti-glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) monoclonal antibodies lead to increased GC B cells with an increased proportion of class-switched B cells [177]. In addition, Treg-deficient mice show expansion of GC-T_{FH} cells [178]. Furthermore, blocking TGF-β or IL-10 results in enhanced GCs [177]. Constitutive signaling of TGF-β inhibits T_{FH} cell accumulation and B cell autoreactivity, confirming the latter [179]. Recently, the phosphatase PTEN was identified as a crucial element in governing the stability of Tregs in suppressing T_{FH} responses in mice [180]. In summary, Tregs are essential to regulate the GC response during B cell maturation to obtain an optimal humoral response.

2.2.2 Follicular regulatory T cells

The GC response is a highly sensitive and delicate temporary process where a high number of dying cells are present, thereby provisioning an arsenal of potential self-antigens. Selection mechanisms, such as competition between maturing B cells for crucial T_{FH} support, have been demonstrated in the GC [128, 170]. During this process T_{FH} cells select B cells depending on their ability to bind and present specific antigen [181].

A limiting number of T_{FH} cells are required to enable competition in a 'survival of the fittest'-like mechanism and thereby eliminating undesirable (self-reactive) B cells. Yet, the factors that are responsible for limiting the availability of T_{FH} cells remained to be elucidated. In 2012, three research groups independently identified and characterized a new subset of regulatory T cells, the follicular regulatory T cells (T_{FR}) (Figure 2.1). The T_{FR} control the normal GC response and prevent emergence of auto-reactive B cells. T_{FR} are characterized by phenotypic overlaps with the surface profile of T_{FH} cells ($CD4^+CXCR5^+PD\text{-}1^+ICOS^+Bcl\text{-}6^+$), but also express Foxp3, CD25, CTLA-4, GITR and IL-10, which are characteristic markers for activated Tregs [128, 170, 171].

To address the question of the origin of T_{FR}, adoptive transfer models and Helios expression was used [128, 170]. These studies showed that T_{FR} develop from thymic-derived Foxp3+ T cells, indicating that T_{FR} cells are induced in the periphery from $CXCR5^-Foxp3^+$ Tregs.

T_{FR} development and differentiation depends on CD28, Bcl6, SAP and B cells, as is the case for T_{FH} cells. Recently, the transcription factor Nuclear Factor of Activated T cells 2 (NFAT2) has been identified as a crucial component for T_{FR} development, as it is essential for CXCR5 upregulation in T_{FR} cells [182]. Additionally, transcriptional regulators Id2 and Id3, surface expression of PD-1 and intracellular expression of TNF receptor (TNFR)–associated factor 3 (TRAF3) have been identified as essential checkpoints for differentiation from Treg to T_{FR} cell [183-185].

Further research revealed important roles for programmed death 1 (PD-1) and Blimp-1 in the homeostasis of T_{FR} cells. PD-1 is an inhibitory molecule that influences the decision between tolerance and activation, while Blimp-1 is a repressor of Bcl-6 activity [128, 186].

PD-1-deficient mice (*Pdcd1*$^{-/-}$, knockout of the gene encoding PD-1) and Blimp-1 deficient mice (*Prdm1*$^{gfp/gfp}$, knockout of the gene encoding Blimp-1) demonstrated a significant increase in the percentage of T_{FR} compared to wild-type controls, leading to the conclusion that both PD-1 and Blimp-1 are essential to regulate the number of T_{FR} *in vivo.* [128, 184]. PD-1 also seems to influence functionality, as *Pdcd1*$^{-/-}$ deficient T_{FR} cells more potently inhibit the humoral immune response [184].

The most important functional factor for both T_{FR} and Tregs in controlling the GC reaction is likely to be CTLA-4. In the absence of CTLA-4, T_{FR} cells are unable to suppress T_{FH} cells or the formation of GC [187]. Furthermore, expression of CTLA-4 on both Tregs and T_{FR} is necessary to control the GC reaction in the T cell zone and in the follicle respectively (reviewed by [188]). Sage et al. further highlighted the role of CTLA-4 in regulating the GC response by demonstrating that CTLA-4 expression regulates T_{FH} cell number while also increasing the suppressive capacity of T_{FR} cells [189]. While other effector molecules are likely to play important roles, especially in specialized contexts, the function of CTLA-4 is likely to remain dominant.

In normal human immunity, the role of T_{FR} is still under investigation. Various studies suggest a detrimental role for T_{FR} in human lymphomas, since they attenuate the immune response against tumors (reviewed by [155]). The physiological impact of these cells in other contexts, e.g. vaccination, can be extrapolated from mouse studies, but direct experimental evidence in humans is sorely lacking. In one human study the effect of treatment with a monoclonal anti-CD20 antibody (Rituximab, RTX) on T_{FH} and T_{FR} cells was studied. RTX treatment resulted in a lack of GC B cells in human lymph nodes without affecting the T_{FH} or T_{FR} cell populations. These data demonstrated that human T_{FH} and T_{FR} do not require an ongoing GC response for their maintenance [190]. Other differences and similarities between human and mouse T_{FR} require further study, but it is likely that, as in mouse, human T_{FR} constitute a distinct population of regulatory cells with overlap between Treg and T_{FH} and important functions in regulating humoral responses.

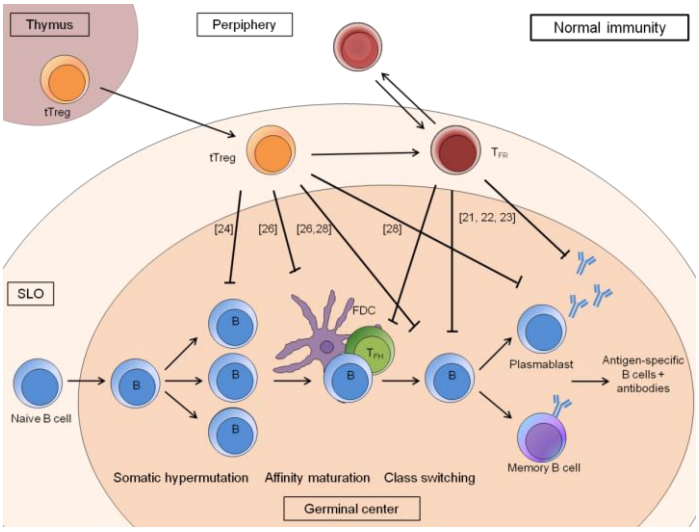

Figure 2.1: Regulatory T cells regulate B cells in normal immunity. Naive B cells develop during the germinal center response into antibody producing plasmablast or memory B cells to provide an arsenal of antigen specific B cells and antibodies. This process was shown to be regulated by Tregs and by T_{FR}. Abbreviations: tTreg: thymic derived regulatory T cells, T_{FR}: follicular regulatory T cells, FDC: follicular regulatory T cells, T_{FH}: follicular helper T cells, SLO: secondary lymphoid organs, [X] reference number of an article in the text.

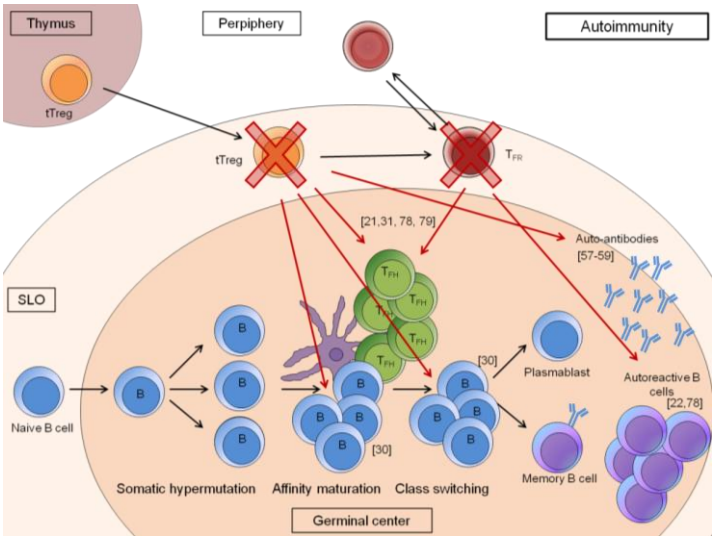

Figure 2.2: Depletion of regulatory T cells results in humoral autoimmunity. Depletion of Tregs or T_{FR} (X) leads to (→) an increase in germinal center B cells, classs-switched B cells, T_{FH}, auto-antibodies and autoreactive B cells. Abbreviations: tTreg: thymic derived regulatory T cells, T_{FR}: follicular regulatory T cells, FDC: follicular regulatory T cells, T_{FH}: follicular helper T cells, SLO: secondary lymphoid organs, [X] reference to an article.

2.3 Involvement of follicular regulatory T cells in autoimmune disease

It is well established that impairment of regulatory T cells can contribute to the pathology of T cell mediated autoimmune diseases [161, 174, 191-200]. Since Tregs also suppress B cells, they are also likely able to control humoral autoimmunity (Figure 2.2).

2.3.1 Regulatory T cells and B cells in autoimmune disease

The regulation of B cells by Tregs is essential to govern a normal humoral immune response. Early evidence of a role of Tregs in the control of B cell autoimmunity came from scurfy mice (*Foxp3*^{sf} mice), which develop B cell-driven autoimmunity accompanied by spontaneous germinal center responses and high serum autoantibody levels [201, 202]. Furthermore, thymectomy was shown to result in an increased production of autoantibodies and multi-organ autoimmunity [203-205]. Moreover, adoptive transfer of Tregs is essential and sufficient to suppress autoreactive B cells and to prevent them from producing autoantibodies in transgenic systems [206-208]. In one study, this suppression was dependent on the inhibitory molecule PD-1 [209]. Various mouse models have demonstrated that a defect in Tregs has a major impact on the production of autoantibodies (reviewed by [210]). For instance, when an autoantibody-dependent mouse model was set up on a Treg-deficient background, there was an accumulation of long-lived plasma cells, a worsening of disease course, and an increased GC response [211].

Initial insights into the importance of Tregs regulating B cell autoimmunity in humans came from patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX). IPEX is caused by a mutation in the human *FOXP3* gene. To determine the impact and time-point of the Treg effect on B cell tolerance, antibodies from single B cells from IPEX patients were cloned and their reactivity was compared to those derived from healthy donors. Transitional B cells (IgM^{hi}), which depend on central tolerance, and mature naive B cells (IgM^{lo}), dependent on peripheral tolerance to ensure no reactivity to self, were isolated.

Reactivity of antibodies expressed by transitional B cells from IPEX patients were similar to those from healthy donors, but mature naive B cells from IPEX patients expressed autoreactive antibodies. This suggests an important role for Tregs in maintaining peripheral B cell tolerance while no effect on central B cell tolerance was found. Furthermore, T cells from IPEX patients displayed an activated phenotype and showed upregulation of CD40L, PD-1, and ICOS, which may favor the accumulation of autoreactive B cells [212]. Other immune diseases such as multiple sclerosis (MS) are characterized by a functional impairment in Tregs and the presence of autoreactive B cells, consistent with a link between both compartments [213, 214]. Of note, patients with systemic lupus erythematosus (SLE) have a decreased percentage of Tregs, but with a high functionality, which can directly suppress autoantibody-producing autoreactive B cells, showing that a numeric deficiency of Tregs is sufficient to induce B cell autoimmunity [215, 216].

To conclude, regulatory T cells stand central in the regulation and control of autoimmune B and T cells and are therefore directly linked to maintaining humoral tolerance to self in humans and mice.

2.3.2 Follicular regulatory T cells in autoimmune disease

Since the regulation of the maturation of autoantibodies primarily takes place in the GC, it is probable that an aberrant GC response can contribute to autoimmunity (Figure 2.1). In mouse models, T_{FR}-deficiency led to increased numbers of T_{FH} [128] and an overwhelming outgrowth of non-specific B cells [170]. T_{FR} numbers and functionality is therefore believed to play an important role in limiting T_{FH} cell support to B cells during the GC response, and thereby sustaining self-tolerance. Following this line of reasoning, an impaired T_{FR} compartment could enhance T_{FH} activity, thereby giving autoreactive B cells the opportunity to expand and partake in autoimmune pathology [128]. Various mouse [217] and human [218-223] studies have emphasized the involvement of this overt T_{FH} reactivity in driving autoimmunity.

2.3.2.1 Tolerance-inducing T_{FR} molecules

As noted above, the optimal development and functioning of T_{FR} is dependent on expression of various molecules. In this part, we discuss the importance of expression of these molecules on T_{FR} in tipping the balance from tolerance to autoimmunity.

It has been suggested by various groups that PD-1-PD-L1 axis is decisive for T cell tolerance versus activation (reviewed in [224]). PD-1 is considered an inhibitory receptor, as it regulates the level of T cell activity and cytokine production. Contradictory results have shown that PD-1 can also activate T cells. Sage et al also used *Pdcd1*$^{-/-}$ mice and showed a higher number of T_{FR} cells in the lymph nodes and these cells had a higher suppressive capacity, indicating the involvement of PD-1-PD-L pathway in controlling T_{FR} immunity [184]. Activated follicular T cells express high amounts of NFAT. In NFAT2-deficient mice an increased GC reaction was found together with a decreased number of T_{FR} cells, along with a reduced migratory capacity into the B cell follicles. Furthermore, the expression of CXCR5 was lower when Foxp3^{+} Tregs were deficient for NFAT2. Induction of lupus in NFAT2-deficient mice, using chromatin from lymphocytes, revealed an increase in the amount of T_{FH} and GC B cells, but also a decrease in T_{FR} frequency. Furthermore, NFAT2-deficient T_{FR} cells fail to control lupus-like disease manifestation, supporting the proposition that T_{FR} cells play a role in maintaining self-tolerance [182].Treg-specific TFARF3 knock-outs (*Traf3*$^{Treg\text{-}KO}$*)* displayed an increased GC reaction together with an aberrant induction of antibodies. To explain these results the effect of *Traf3*$^{Treg\text{-}KO}$ on T_{FR} induction were assessed. It seemed that TRAF3 is indispensible for the development of T_{FR}, explaining the previous results and highlighting the importance of T_{FR} to prevent loss of tolerance [185]. While many more genes will have important functions in the capacity of T_{FR} to prevent autoimmunity, functional studies are currently lacking.

2.3.2.2 T_{FR} in autoimmune mouse models

Autoimmune mouse models can give insight into involvement of T_{FR} and essential T_{FR} molecules, in disease development. So far, only two studies have used autoimmune mouse models to determine the contribution and relevance of T_{FR}. The BXD2 mouse model, derived from an intercross between C57BL/6J (B6) and DBA/2J (D2) strains, is characterized by an increased number of autoantibody producing B cells, an accumulation of T_{FH} cells in the spleen, and development of spontaneous GCs [225], and can be used as a model for general autoimmunity [226]. It was first shown that interleukin-21 (IL-21) promotes T_{FH} cell differentiation in BXD2. Thus, to investigate the role of T_{FR} in this autoimmune mouse model, BXD2 mice were made deficient for IL-21 (BXD2-*Il21*$^{-/-}$). BXD2-*Il21*$^{-/-}$ mice have an increased percentage of T_{FR} cells compared to wild-type BXD2 mice. When IL-21 was administered, the number of T_{FR} decreased. In addition, BXD2 mice that received T_{FR} from BXD2-*Il21*$^{-/-}$ mice had a decreased number of GC and a decrease in their autoantibody-producing B cells. This mouse model thus showed that IL-21-dependent autoimmunity (typical for instance in SLE) can enhance the T_{FH} cells to promote autoreactive GC reaction and inhibit the regulatory role of T_{FR} cells [227].

The mouse model for human myasthenia gravis (MG) is experimental autoimmune myasthenia gravis (EAMG). Both MG and EAMG development are defined by a B cell-mediated, T cell dependent autoimmune disease where acetylcholine receptor (AChR)-specific T_H cells are important. A key study in using the EAMG mouse model demonstrated that disease development was associated with an increase in T_{FH} and a decrease in T_{FR}. Treatment with a vitamin A metabolite restored the T_{FH}:T_{FR} ratio and led to a reduction in potentially harmful cells (T_H1/T_H17/T_{FH}) and increased the number of Treg and T_{FR} [228].

Although these studies focused on T_{FR}:T_{FH} ratio in autoimmunity and not on T_{FR}:autoantibodies, these observations suggest a prominent role for T_{FR} cells in autoimmunity.

2.4 Circulating follicular regulatory T cells

2.4.1 What can we learn from circulating follicular helper T cells?

Our current understanding on T_{FR} cell biology and function comes predominantly from investigations performed on T_{FR} isolated from SLO of mice. Since blood is the most convenient source for diagnostics and research in human health and disease, investigating follicular populations from peripheral blood to understand the phenotype and function is of high importance. Although circulating T_{FH} are present in the blood (2-6% within the conventional T cells) of healthy donors and patients with autoimmune disease, their origin and homeostasis is unclear. One hypothesis is that circulating T_{FH} are a population of 'precursor T_{FH}', developing in an early phase prior to GC formation. As these cells leave before a crucial developmental time point they become memory-like, since they experienced part of the response, without being bona fide T_{FH}, since they are not fully differentiated. This hypothesis is supported by the observation that circulating T_{FH} are Bcl-6 negative, as well as studies done on SAP-deficient animal models and humans with SAP-deficient X-linked lymphoproliferative diseases, in which circulating T_{FH} are present, despite the absence of the B cell interaction crucial for full T_{FH} differentiation [229].

Another hypothesis is that they are a population of bona fide memory cells that leave the GC after the response is terminated. This is supported by research showing that they can migrate back to GC and respond quickly to a second antigen exposure [229, 230].

At a functional level, different groups independently demonstrated that the circulating subsets of follicular helper T cells are highly functional, correlate with antibody responses, are indicative for T_{FH} cell activity in the GC and can promote antibody responses [229, 231-233]. They are more potent than lymph node T_{FH} and produce more cytokines than T_{FH} cell from the lymph node in mice [230]. Furthermore, phenotyical analysis of these circulating counterparts indicates a memory phenotype that can be subdivided in effecter cell types ($T_H1/2/17$) using CXCR3 and CCR6 [234]. The circulating T_H2 and T_H17 effector T_{FH} can induce B cell differentiation in humans. In addition, these effector follicular phenotypes are disturbed in autoimmune diseases such as MS [223] and juvenile dermatomyositis [234].

While these circulating counterparts seem a distinct population from the follicular cells primarily located in SLO, they do have a related effector phenotype, and based on availability they provide a viable option for investigating the T_{FH} participation in normal immune homeostasis and autoimmunity in humans.

2.4.2 Circulating follicular regulatory T cells

As illustrated above, T_{FH} from lymph nodes can recirculate into the peripheral blood and exist as a central memory pool that responds quickly to a secondary immune response. But, is this also the case for circulating T_{FR}? Sharpe *et al.* found that in mice a small population of T_{FR} can be found in the blood after immunization (0,5 % within the $CD4^+$ population) [184].
They found that circulating T_{FR} had a lower expression of CXCR5, ICOS, and Ki67 compared to T_{FR} from the lymph nodes and were positive for Ki67, CD62L and CD44, indicating a central memory homing phenotype [230]. Furthermore, the suppressive function seemed to be lower compared to T_{FR} from lymph nodes, although they are able to suppress T_{FH} and B cell activation, antibody production and class-switching. In addition, blood T_{FR} can circulate in the body for long periods of time while they patrol for antigen, and can migrate towards lymph nodes and tissues. From this mouse study, it seems that circulating T_{FR} are a long-lived functional memory population that recirculates to the LN. While direct comparison of circulating T_{FR} and follicular T_{FR} will need to be performed in humans, it is likely that the majority of research will rely on the relative access available to circulating T_{FR} to investigate alteration in human autoimmunity.

2.5 Conclusions

Tregs form an essential population with high plasticity providing them the capacity to control various immune responses, including the humoral immune response. With the identification of T_{FR}, specialized in the regulation of B cell maturation, this central role is becoming even more prominent. The study of circulating T_{FR} is allowing the elucidation of the role of T_{FR} in the controlling human humoral autoimmunity.

Conflict of interest

The authors declare no conflict of interest.

Take-home messages

- Regulatory T cells are involved in the homeostasis of humoral immunity and an impairment in Tregs can be directly linked to B cell-mediated autoimmunity in both humans and mice
- Follicular regulatory T cells stand central in the regulation of a primary GC response, and are also involved in peripheral regulation of ongoing antibody selection
- Circulating follicular T cells provide a key tool for unraveling the role of follicular cells in autoimmunity in humans
- Future studies need to define the relative contribution of T_{FR} in autoimmunity in humans

3

CIRCULATING FOLLICULAR REGULATORY T CELLS ARE DEFECTIVE IN MULTIPLE SCLEROSIS

This chapter is based on:

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Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis.

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Abstract

Follicular regulatory T cells (T_{FR}) have been extensively characterized in mice and participate in germinal center (GC) responses by regulating the maturation of B cells and production of (auto)antibodies. We report that circulating T_{FR} are phenotypically distinct from tonsil-derived T_{FR} in humans. They have a lower expression of follicular markers, display a memory phenotype and lack of high expression of Bcl-6 and ICOS. However, the suppressive function, expression of regulatory markers and the FOXP3 methylation status of blood T_{FR} is comparable with tonsil-derived T_{FR}. Moreover, we show that circulating T_{FR} frequencies increase after influenza vaccination and correlate with anti-flu antibody responses, indicating a fully functional population.

Multiple sclerosis (MS) was used as a model for autoimmune disease to investigate alterations in circulating T_{FR}. MS patients had a significantly lower frequency of circulating T_{FR} compared to healthy controls. Furthermore, the circulating T_{FR} compartment of MS patients displayed an increased proportion of T_H17-like T_{FR}. Finally, T_{FR} of MS patients had a strongly reduced suppressive function compared to healthy controls.

We conclude that circulating T_{FR} are a circulating memory population derived from lymphoid resident T_{FR} making them a valid alternative to investigate alterations in GC responses in the context of autoimmune diseases and T_{FR} impairment is prominent in MS.

3.1 Introduction

Given the prompt emergence of somatically mutated B cells during B cell maturation in the germinal center (GC) response, it seems evident that regulatory pathways need to be engaged to secure the maintenance of self-tolerance [169, 235]. In this GC process follicular helper T cells (T_{FH}), are believed to select B cells based on their capacity to bind and present the specific antigen [181]. This 'survival of the fittest' mechanism requires a limited amount of T_{FH} cells to ensure competition and thereby elimination of undesirable (auto-reactive) B cells. The recently identified follicular regulatory T cells (T_{FR}) propose themselves as ideal candidates for regulating the normal GC response and preventing emergence of auto-reactive B cells [128, 170, 171]. T_{FR} cells comprise 5-25% of the GC T cells in mice and originate from forkhead box P3 positive ($Foxp3^+$) thymic-derived regulatory T cell (Treg) precursors [128]. Like T_{FH} cells, T_{FR} differentiation depends on CD28, B-cell lymphoma 6 (Bcl-6), signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) and B cells [128]. Phenotypic analysis of T_{FR} cells overlaps with the surface profile of T_{FH} cells ($CD4^+CXCR5^+PD\text{-}1^+ICOS^+$) and both cell types also express Bcl-6. In contrast to T_{FH}, T_{FR} do not express IL-20, IL-4 or CD40L. Besides sharing surface markers with T_{FH} cells, T_{FR} cells also express regulatory markers such as Foxp3, CD25, cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNFR-related protein (GITR) and IL-10 [128, 170, 171]. The transcription factor nuclear factor of activated T cells 2 (NFAT2) has been identified as crucial for T_{FR} development in mice, since it is essential for the induction of CXCR5 in these cells [236]. CXCR5 directs the migration of T_{FR} into the GC where they suppress the magnitude of the GC response by regulating the number of T_{FH}, B cells and antibodies. Taken together, T_{FR} cells are a distinct population of Tregs located primarily in the GC, showing overlap with both Tregs and $T_{FH.}$

Since regulation of (auto)antibody production primarily takes place in the GC, it is probable that an aberrant GC response can contribute to autoimmunity. In the absence of T_{FR} overwhelming outgrowth of non-specific B cells leads to lower amounts of antigen-specific B cells [237]. Furthermore, a lack of $CXCR5^+$ Tregs in mice results in increased GC activity, including affinity maturation and differentiation towards plasma cells.

Additionally, PD-1 was found to be crucial for the homeostasis of T_{FR}, since PD-1 deficient mice show increased numbers of T_{FR} in lymph nodes. Also, the PD-1 pathway seems to impair T_{FR} function, considering PD-1 deficiency results in an increased T_{FR} suppressive capacity [184]. NFAT2 deficient T_{FR} fail to control lupus-like disease manifestations in mice, supporting the proposition that T_{FR} play a role in maintaining self-tolerance [238].

In this study, we sought to identify the involvement of T_{FR} in human disease by using multiple sclerosis (MS) as a model for an autoimmune disease (AID) with Treg disturbances [239] and elevated autoantibody levels [240, 241]. Peripheral blood (PB) is the most accessible source to analyze immune responses in healthy controls (HC) and patients with AID. Therefore, we first determined whether blood T_{FR} are a good representation to investigate T_{FR} responses ongoing in secondary lymph nodes. To do so, a pairwise comparison was made between T_{FR} derived from blood and tonsils of HC. Next, circulating T_{FR} were monitored after influenza vaccination to define GC-induced changes in this subset. Finally, circulating T_{FR} alterations were investigated in MS patients and compared to HC to evaluate their involvement in AID.

3.2 Materials and Methods

3.2.1 Human samples

Ethics approvals were obtained from each institute's human ethics committee. Tonsils and blood were obtained from adult patients without autoimmune diseases who were undergoing routine tonsillectomies at ZOL hospital (Genk, Belgium) (Table 3.1). Adult healthy volunteers were recruited for the vaccination of inactivated influenza vaccine Influvac S® 2013/2014 (ABBOTT BIOLOGICALS B.V., Brussels, Belgium) (Table 3.1). MS patients were recruited from the Rehabilitation and MS-center (Overpelt, Belgium). Detailed clinical characteristics are shown in Table 3.1.

3.2.2 PBMC purification and flow cytometric analysis

PB was collected in heparin-coated tubes (Venosafe plastic tubes, Terumo Europe N.V., Leuven, Belgium). Tonsils were cut into small pieces and singles cells were obtained using a cell strainer (EASYstrainer™ 70µM, Greiner Bio-One BVBA/SPRL, Wemmel, Belgium). After collection of the plasma, density centrifugation was used to isolate the (PB) mononuclear cells ((PB)MC) (Lympholyte®; Cedarlane® Laboratories, SanBio B.V., Uden, the Netherlands). In line with recent publications, specific flow cyctometric markers were used to identify circulating T_{FR} and T_{FH} in human blood [242, 243]. For flow cytometric analysis of the T cell subsets following antibodies were used: CD4 APC, CD4 PE-CF594, CD4 FITC, CD45RO PE-CF594, CXCR5 Alexa Fluor 488, CXCR3 PE-CF594, CCR6-PerCP-cy5.5, Bcl-6 PE-CF594, Foxp3 PE-CF594 (all from BD Biosciences, Erembodegem, Belgium), CD25 PerCP-Cy5.5, CD127 PE, PD-1 PE-Cy7, CD45RA APC-H7, CD31 APC, CCR7 PE, CD62L APC-Cy7, SAP-PE, Helios APC (all from eBioscience, San Diego, USA), CD25 APC-Cy7 and CD27 APC (from Biolegend, ImTec Diagnostics N.V., Antwerp, Belgium). B cell analysis was performed with: CD19 PerCP-Cy5.5, IgD APC-Cy7, CD27 PE-Cy7 (all from BD Biosciences, Erembodegem, Belgium). Appropriate isotype controls were used to establish the proper gating strategies (all from BD Biosciences, Erembodegem, Belgium). All flow cytometric analyzes were performed on a FACSAriaII flow cytometer and analyzed with FACS Diva software (BD Biosciences).

For Foxp3 intranuclear staining the eBioscience kit was used, for other intracellular stainings the BD Cytofix/Cytoperm kit (BD) was used according to the manufacturers' guidelines.

3.2.3 Purification of CD4 T cell subsets

$CD4^+$ T cells were purified using CD4 negative selection (STEMCELL Technologies SARL, Grenoble, France). CD25 positive selection (STEMCELL Technologies SARL) was used to obtain a $CD25^+$ enriched population and a $CD25^-$ population. $CD4^+CD25^-CD127^+$ responder T cells (Tresp) and $CD4^+CD25^-CD127^+CXCR5^+PD^-1^+$ T_{FH} cells were sorted from the $CD25^-$ population, $CD4^+CD25^{hi}CD127^{lo}$ Tregs and $CD4^+CD25^{hi}CD127^{lo}CXCR5^+PD\text{-}1^+$ T_{FR} cells were sorted from the $CD25^+$ population using following antibodies CD4 FITC (BD), CD25 PerCP-cy5.5 (eBioscience), CD127 PE (eBioscience), CXCR5 Alexa Fluor 647 (BD), PD-1 PE-Cy7 (eBioscience) using a FACS Aria II (BD). Purity of the isolated cells was confirmed. Flow cytometric analysis was performed using FACS Diva software (BD Biosciences) and FlowJo V10.

3.2.4 Suppression assays

A 96-well round bottom plate (Nunc, Roskilde, Denmark) was coated for 2 hours on 37°C with 0.01µg/ml anti-CD3 (HIT3, BD) and washed with PBS. $CD4^+CD25^-CD127^+$ Tresp (labeled with 4µM CFSE (Invitrogen)) were cultured at 1×10^4 cells/well with 1×10^5 irradiated autologous PBMC (feeder cells) in the presence or absence of the same number of Tregs or T_{FR} in duplo. The isolated Treg population thus also includes the $CXCR5^+PD\text{-}1^+$ T_{FR} population. Cell cultures were also stimulated with soluble anti-CD28 (BD) for 4 days. The following controls were used: (1) a non-labeled stimulated control to serve as reference for setting the regulatory T cell gate, (2) a labeled non-stimulated control to serve as reference for setting the non-proliferated gate and (3) a labeled stimulated control with double amount of responder cells to exclude possible nutrient deprivation effects. Cocultures were analyzed on a FACS AriaII on day 4. The suppressive capacity (percentage) of regulatory T cells towards Tresp in coculture was calculated relative to the maximal proliferation of the Tresp alone: [100 - (% proliferation Tresp alone/ % proliferation Tresp + Treg)]*100.

3.2.5 Foxp3 methylation assay

$CD4^+CD25^+$ cells (purified as described above) were sorted to obtain $CD4^+CD25^{hi}CD127^{lo}$ Tregs and $CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$ T_{FR} cells and $CD4^+CD25^-$ cells were sorted to obtain $CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$ T_{FH} cells using FACS Aria II (BD). Purity of the obtained cells was confirmed. Purified cells were pelleted and frozen at -80°C. Next, the proportions of cells with a demethylated FOXP3 intron 1 allele were quantified by qPCR on bisulfite-treated genomic DNA, as described previously [244].

3.2.6 Haemagglutination assay

Plasma samples from HC were obtained before and 21 days after vaccination, for antibody titration against the 2013-2014 influenza H1N1pdm vaccine strain A/California/7/2009 (A/California/7/2009 NYMC X-179A, Cat. No. FR-1184, IRR/CDC) and the 2013-2014 influenza H3N2 vaccine strain A/Texas/50/2012 (A/Texas/50/2012 X-223, Cat. No. FR-1185, IRR/CDC), using a HAI assay. Briefly, 1:2 serial dilutions of inactivated human plasma samples were pre-incubated with a standardized amount of virus prior to the addition of 1% RBC (Glutaraldehyde-stabilized freshly prepared Guinea Pig Red Blood Cells, Cat. No. 88R-P001, Bio-connect). After incubation, HAI titers are recorded. Controls samples were included in all analyzes. Samples were tested in duplicate, and assays were independently repeated. The titer analyzed was the geometric mean (GM) of these test results. Patients who showed non-specific agglutination of RBCs were excluded.

3.2.7 Statistics

Statistical analyzes were done using SAS 9.3, SAS Jump and Graphpad prism 6. Graphics were made using Graphpad prim 6. Data sets were checked for normality. Data sets were checked for effect of age and gender. Analyzes of the vaccination study was done using a mixed model (multiple measurements SAS 9.3). Analysis of multiple groups was done using ANOVA, non parametric testing (Krukal-Wallis) or linear correlations using SAS Jump. A Mann-Whitney test was used for non-parametric unpaired data.

Wilcoxon matched pair test was used for non-parametric paired data. Tests were considered significant when p-value was below 0.05 (two-sided tests).

3.2.8 Study approval

All human blood samples and tonsils were obtained with ethical approval of each institute's human ethics committee, the 'Commissie Medische Ethiek UZ Leuven' and 'comité medisch Ethiek ZOL', respectively. Written informed consent was obtained from all study subjects.

3.3 Results

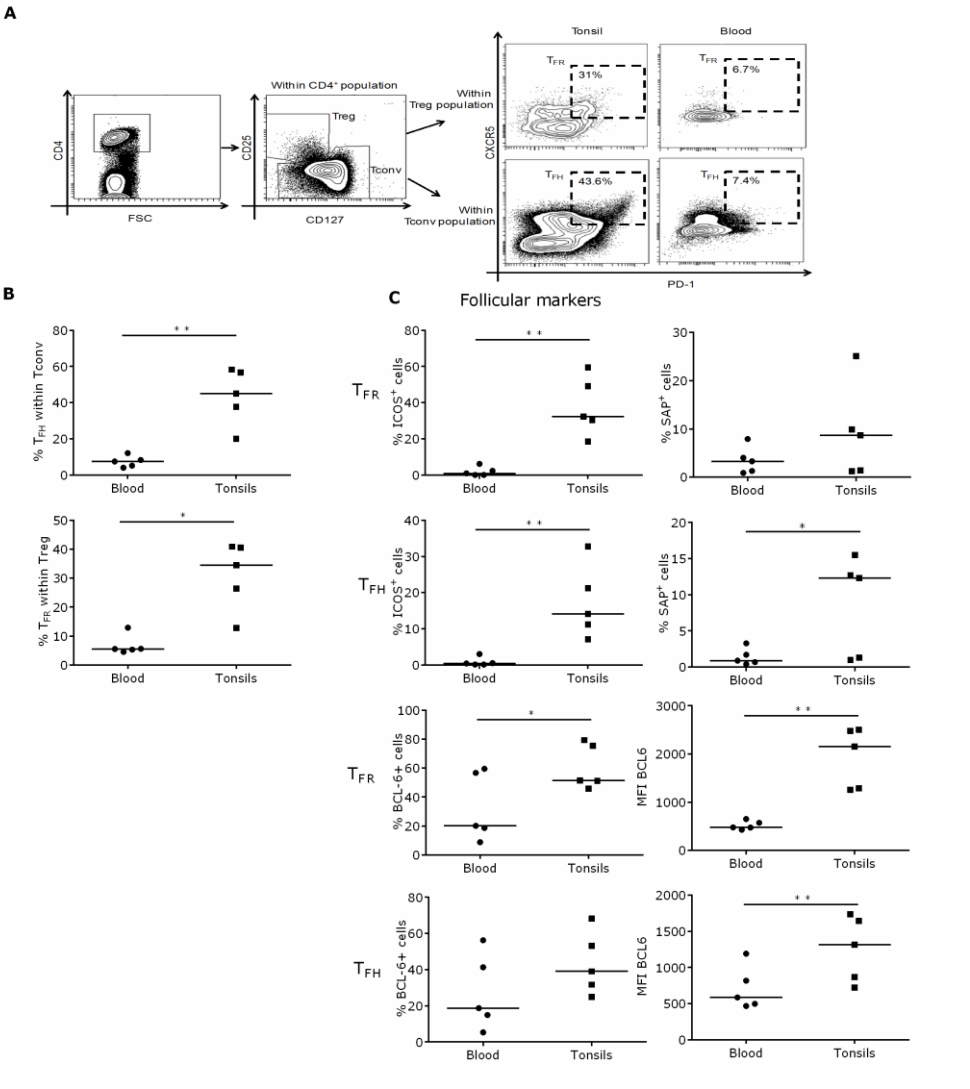
3.3.1 Human circulating follicular regulatory T cells comprise a phenotypically distinct population

The presence and origin of genuine follicular subsets in the circulation remains controversial [169, 229]. This led us to the question whether circulating T_{FR} are phenotypically bona fide T_{FR} or rather represent a distinct population.

First, circulating T_{FR} were phenotypically characterized in detail comparing blood and tonsils of HC (for more information see Table 3.1). Distinct subpopulations of Treg ($CD4^+CD25^+CD127^-$) and conventional T cells (Tconv, $CD4^+CD25^-CD127^+$), were found to express the follicular markers CXCR5 and PD-1 in blood and in tonsils (Figure 3.1). Based on that, $CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$ and $CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$ were defined as (circulating) counterparts of respectively T_{FR} and T_{FH}. In tonsils, T_{FR} and T_{FH} comprise a much larger population compared to the blood (Figure 3.1A and Figure 3.1).

We next analyzed whether circulating follicular cells are phenotypically similar to those derived from tonsils using both follicular (inducible T-cell co-stimulator (ICOS), SAP and Bcl-6) and regulatory markers (Foxp3, Helios and CD31) markers (Figure 3.1C-E). Circulating T_{FR} and T_{FH} did not express ICOS while tonsil-derived cell subsets did. SAP, essential for T-B cell interaction, was significantly lower expressed on circulating compared to tonsil-derived T_{FH}, while no significant differences were found for T_{FR}. A significant higher proportion of T_{FR} from tonsil express Bcl-6 compared to circulating T_{FR}, while T_{FH} did not significantly differ in percentage of $Bcl\text{-}6^+$ cells. In contrast, the mean fluorescent intensity (MFI) of Bcl-6 is significantly decreased on both blood T_{FR} and T_{FH} compared to their tonsil derived counterparts. In addition, the expression levels of essential follicular markers (CXCR5, PD-1, Bcl-6 and ICOS) are shown in Figure 3.1D, again highlighting the phenotypical difference in follicular expression on blood T_{FH} and T_{FR}. The regulatory markers Foxp3 and Helios were equally expressed by T_{FR} in tonsils and blood. No expression of regulatory markers was seen in T_{FH} from any source. CD31 is a key molecule for the regulation of T cell homeostasis, effector function and trafficking [245-247]. We found an increased expression of CD31 on the surface of circulating follicular cells whereas the tonsil-derived counterparts did not express this marker.

Lastly, we determined the differentiation stage and effector phenotype of human circulating T_{FR}. As a negative and positive control we compared these cells with naive T cells (T_N) and memory T cells (T_M) respectively, based on their CD45RO expression. Tonsil-derived T_{FR} and T_{FH} cells have a $CD45RO^+$ and $CD45RO^-$ cell population unlike the circulating follicular cell subsets which are all $CD45RO^+$ indicating a memory phenotype (Figure 3.2A). To further characterize this memory phenotype, CCR7 and CD62L were used to distinguish effector memory (T_{EM}) (Figure 3.2B) from central memory cells (T_{CM}) (Figure 3.2C). While all tonsil-derived follicular T cells have a T_{EM} phenotype ($CCR7^-CD62L^-$) only half of the circulating T_{FR} have a T_{EM} and a minority is T_{CM}. We next examined the effector phenotype based on the expression of chemokine receptors CXCR3 and CCR6. On follicular helper T cells ($CD4^+CXCR5^+$), CXCR3 expression is reported to represent a T_H1 phenotype, while CCR6 indicates a T_H17 phenotype [234]. Combining both markers gives a more elaborate view on the effector phenotype (T_H2; $CXCR3^-CCR6^-$, T_H17; $CXCR3^-CCR6^+$ and T_H1; $CXCR3^+CCR6^-$). We found that circulating T_{FR} have a significantly higher percentage of $CXCR3^+CCR6^-$ cells (T_H1-like phenotype) compared to tonsil-derived T_{FR} (Figure 3.2D, $p=0.03$). An trend towards an increase in the percentage of $CXCR3^-CCR6^+$ cells (T_H17-like phenotype) was found (Figure 3.2E), while the percentage of $CXCR3^-CCR6^-$ cell (T_H2-like phenotype) was significantly decreased (Figure 3.2F, $p=0.0079$). Together, these data suggest that human circulating T_{FR} are phenotypically distinct from their counterparts in the secondary lymphoid organs (tonsils were used as a model) since they express lower levels of follicular markers, are a memory population and have a pro-inflammatory effector phenotype.


A
CD4
FSC
Within CD4+ population
Treg
Tconv
CD25
CD127
Within Treg population
Within Tconv population
Tonsil
Blood
TFR
31%
6.7%
TFH
43.6%
7.4%
CXCR5
PD-1
B
% TFH within Tconv
% TFR within Treg
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C
Follicular markers
TFR
TFH
% ICOS+ cells
% SAP+ cells
% BCL-6+ cells
MFI BCL6

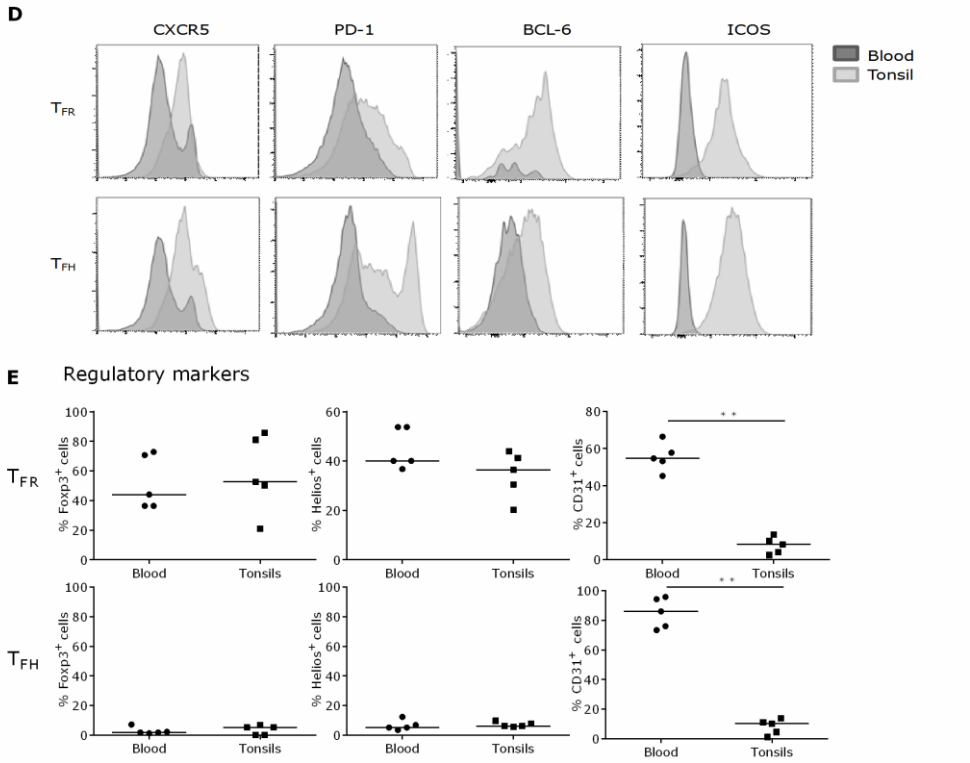


Figure 3.1: Circulating follicular regulatory T cells comprise a distinct phenotypical population compared to tonsil-derived T_{FR}. (A) Gating strategy to identify circulating follicular regulatory (T_{FR}, $CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$) and helper ($T_{FH}$, $CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$) T cells within the regulatory T cells (Treg) and conventional T cells (Tconv) gate respectively in tonsils and blood. **(B)** Percentage of follicular regulatory T cells (T_{FR}) and follicular helper T cells (T_{FH}) in blood and in tonsils **(C)** Percentage of follicular markers (ICOS, SAP and Bcl-6) on both T_{FR} and T_{FH} from blood and tonsils. The mean fluorescent intensity (MFI) of Bcl-6 is shown **(D)** Expression levels of follicular markers CXCR5, PD-1, Bcl-6 and ICOS on T_{FR} and T_{FH} in blood (dark gray) and tonsils (light gray). **(E)** Percentage of regulatory markers (Foxp3, Helios and CD31) on both T_{FR} and T_{FH} from blood and tonsils. Data are shown as median for n= 5 HC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Wilcoxon matched-pair test.

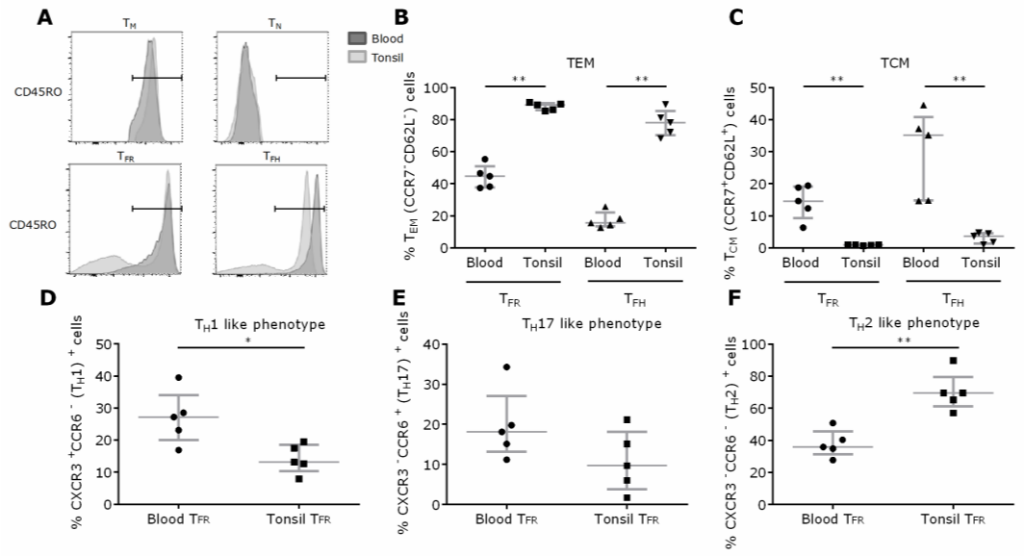


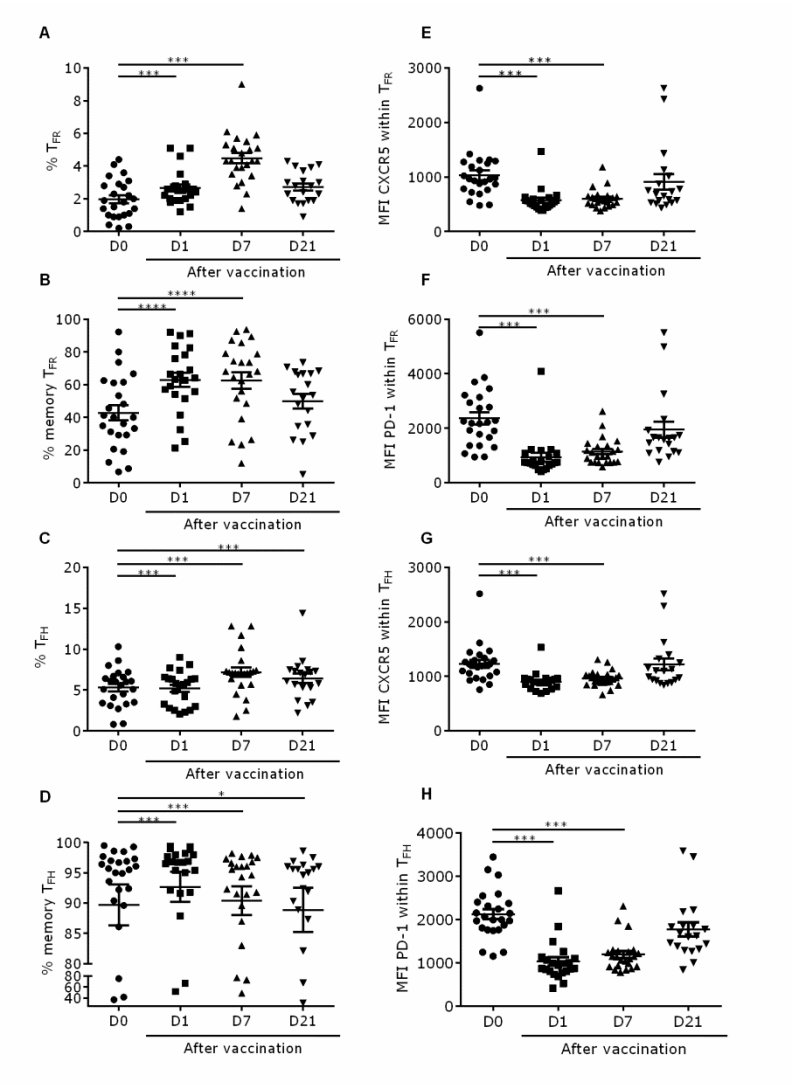
Figure 3.2: **Circulating follicular regulatory T cells comprise a distinct population compared to tonsil-derived T_{FR} (continued). (A)** Flow cytometric histogram and gating strategy of CD45RO expression on blood (dark gray) derived and tonsil (light gray) naive T cells (T_N), memory T cells (T_M), T_{FR} and T_{FH}. **(B and C)** Percentages of T_{EM} and T_{CM} (T_{EM}: effector memory $CD62L^-CCR7^-$, T_{CM} : central memory cells $CD62L^+CCR7^+$, n=5) in the T_{FR} and T_{FH} from blood and tonsils. **(D-F)** Expression of CXCR3 and CCR6 is used to distinguish various effector phenotypes in blood and tonsils of 5 HC (T_H2; $CXCR3^-CCR6^-$, T_H17; $CXCR3^-CCR6^+$ and T_H1; $CXCR3^+CCR6^-$). Data are median with interquartile range with n=5 HC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Wilcoxon matched-pair test.

3.3.2 Influenza vaccination boosts the number of circulating follicular regulatory T cells

To investigate whether follicular cell activity is measurable in the blood, we next assessed the effect of vaccination on circulating follicular and B cell subsets. PB from HCs (n=24, detailed information see Table 3.1) was taken before (D0), 1 day (D1), 1 week (D7) and 3 weeks (D21) after influenza vaccination. The percentage of circulating T_{FR} cells significantly increased after 24 hours ($p<0.001$) and 7 days ($p<0.001$) after vaccination, and returned back to baseline after 3 weeks (Figure 3.3A). Memory T_{FR} ($CD45RO^+CD45RA^-$ T_{FR}) significantly increased (D1 and D7, $p<0.0001$) after vaccination (Figure 3B) while the percentage of naïve T_{FR} ($CD45RO^-CD45RA^+$) decreased significantly after 1 and 7 days ($p=0.01$, data not shown). In line with other groups [231, 232], we confirmed a significant increase in circulating T_{FH} after vaccination (all time points, $p<0.001$) (Figure 3.3C). Similar to memory T_{FR}, memory T_{FH} increased significantly after influenza vaccination (Figure 3.3D, D1 day and D7, $p<0.001$, D21 $p<0.05$). CXCR5 and PD-1 levels of circulating T_{FR} and T_{FH} decreased after vaccination (D1 and D7, $p<0.001$) and returned to baseline after 3 weeks (Figure 3.3E-H).

Next to circulating T cells, we also evaluated various B cells subtypes after vaccination. Similar to a previous report [232], we show that percentage of plasmablasts ($CD19^+CD27^+CD138^+$) increased after vaccination, although it did not reach statistical significance (D7, $p=0.07$, Figure 3.3I). While no difference in the percentage of class switched B cells ($CD19^+IgD^-CD27^+$) was found (data not shown), the ratio of non-class switched B cells ($CD19^+IgD^+CD27^+$) over class switched B cells was significantly increased after 21 days compared to D1 ($p<0.001$) and compared to D7 ($p=0.03$) (Figure 3.3J).

To investigate whether the changes in the circulating T_{FR} compartment are linked with the production of protective antibody responses, hemagglutination inhibition (HAI) assays were performed on plasma samples collected at baseline and D21 using two vaccine virus strains (A/California/7/2009 (H_1N_{1pdm}) and A/Texas/50/2012 (H_3N_2). For both strains, the percentage of circulating T_{FR} at D7 was positively correlated with post-vaccination (D21) geometric mean titers (GMTs) ($p=0.05$ and $p=0.03$, Figure 3.3 K and L).


A
% T_{FR}
B
% memory T_{FR}
C
% T_{FH}
D
% memory T_{FH}
E
MFI CXCR5 within T_{FR}
F
MFI PD-1 within T_{FR}
G
MFI CXCR5 within T_{FH}
H
MFI PD-1 within T_{FH}
D0
D1
D7
D21
After vaccination

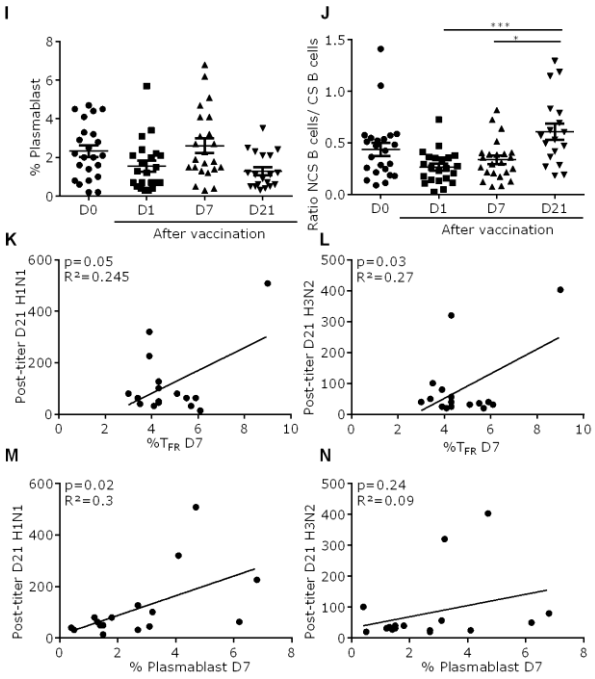


Figure 3.3: Circulating follicular regulatory T cells increase after influenza vaccination and correlate with anti-influenza antibodies. PBMC (peripheral blood mononuclear cells) from HC (n=24) were collected before (D0) and after influenza vaccination (Influvac S 2013/2014) at different timepoints (after 1 day (D1), after 1 week (D7) and after 3 weeks (D21) Circulating T_{FR} and T_{FH} were identified as $CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$ and $CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$ respectively, gated as shown in Figure 1A. **(A en C)**. The percentage of both circulating T_{FR} and T_{FH} cells after vaccination was assessed. **(B and D)** The percentage of $CD45RO^+$ subpopulation in the T_{FR} and T_{FH} cells before and after vaccination. **(E-H)** The MFI (mean fluorescent intensity) of both CXCR5 and PD-1 in the circulating follicular cells. **(I)** The percentage of plasmablast ($CD19^+CD27^+CD138^+$) at different time points. **(J)** The ratio of non-class switched B cells (NCS B cells, $CD19^+IgD^+CD27^+$) on class switched B cells (CS B cells, $CD19^+IgD^-CD27^+$) at different time points. **(K- N)** The percentage of both circulating T_{FR} and plasmablast at D7 was correlated with the plasma titers for H1N1 strain and H3N2 strain after D21. Data are mean ± SEM. Analyzes were done using a mixed model (multiple measurements SAS 9.3) with * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Correlation analyzes were done using a linear regression model.

Moreover, the percentage of plasmablasts significantly correlated with post-vaccination H1N1pdm GMTs titers (Figure 3.3 M, p=0.02) but not with post-vaccination H3N2 GMTs (Figure 3.3 N).

In summary, we show that seasonal influenza vaccination in HC leads to a significant increase in circulating (memory) T_{FR} and T_{FH} which significantly correlate with titers of anti-vaccine antibodies. We therefore conclude that circulating T_{FR} are a relevant source to measure T_{FR} activity and regulation of antibody responses in response to GC reactions.

3.3.3 Human circulating and tonsil-derived follicular regulatory T cells are equally suppressive *in vitro*

To assess the functionality of circulating $T_{FR,}$ a pairwise comparison of the suppressive capacity of sorted blood- and tonsil-derived T_{FR} and total Treg of HC (n=7, Table 3.1) was made using an *in vitro* co-culture suppression assay. Briefly, CFSE labeled responder T cells (Tresp) were co-cultured in a 1:1 ratio with T_{FR} , total Tregs or no regulatory T cells and this in absence or presence of CD3/CD28 stimulation for 4 days [248-250]. Figure 3.4A shows a representative example with the various conditions that were incorporated in the assay. Blood-derived T_{FR} and Tregs were equally suppressive as their tonsil-derived counterparts indicative of an equal functional activity (Figure 3.4B). In addition, the methylation status of the FOXP3 gene was assessed as previously described [244]. No significant difference in demethylated FOXP3 was found between T_{FR} from blood compared to tonsils, indicating equal differentiation into Treg lineage (Figure 3.4C). Sorted total Tregs and T_{FH} were used as a positive and negative control respectively. Together, these results show that circulating T_{FR} are equally suppressive and have the same level of demethylated FOXP3 as T_{FR} of secondary lymphoid organs, governing them as a good alternative for functional analyzes in the context of (auto)immunity.

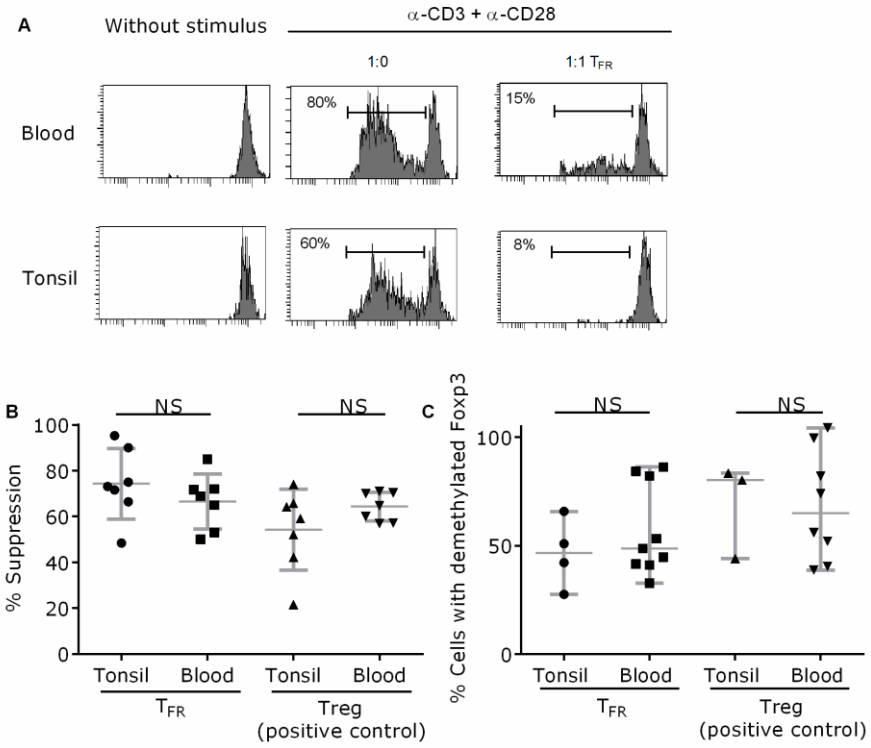


Figure 3.4: Human circulating follicular regulatory T cells are equally suppressive *in vitro* as their tonsil-derived follicular regulatory T cells counterparts. Follicular helper T cells (T_{FH}), responder T cells (Tresp) and follicular regulatory T cells (T_{FR}) were sorted. Tresp were CFSE-labeled and cultured with or without anti-CD3 and anti-CD28 stimulation. Stimulated Tresp were cultured with (1:1) or without (1:0) Treg or T_{FR} to assess their proliferation suppressive capacity *in vitro*. **(A)** Representative flow cytometric Figure of suppressive capacity of circulating T_{FR} and tonsil-derived T_{FR} from the same donor using a CFSE-based coculture assay *in vitro***.** The shown percentages are proliferation of responder T cells with or without T_{FR}. **(B)** T_{FR} from blood and tonsil of HC (n=7) were sorted and a CFSE-based coculture was used to determine the suppressive capacity *in vitro*. Tregs were incorporated as a positive control for suppressive capacity. **(C)** Demethylation status of FOXP3 of sorted T_{FR}, Treg and T_{FH} from HC (n=4 for tonsil, n=9 for blood). Tregs and T_{FH} were incorporated as a positive control and negative control respectively. Data are median with range. Differences were assessed using a Wilcoxon matched-pair test.

3.3.4 Circulating follicular regulatory T cell frequencies are decreased in patients with multiple sclerosis

Various groups, including ours, have reported an impairment in the Treg compartment of MS patients [140, 143, 150, 239]. Moreover, elevated levels of autoantibodies have been reported in MS [55, 251]. In light of these findings, we investigated whether the T_{FR} compartment known to regulate humoral immunity is disturbed in MS. We show a decreased frequency of circulating T_{FR} in MS patients (n=172, Table 3.1) compared to HC (n=107, Table 3.1) (Figure 3.5A, $p<0.0001$). In addition, when separating the MS patients based on treatment a significant decrease can still be found in the untreated MS patients (Figure 3.5B, $p=0.0036$). Moreover, T_{FH}/T_{FR} ratio was significantly increased in blood of MS patients ($p<0.0001$, Figure 3.5C) indicating a relative imbalance between these interacting T cell populations. Age and gender had no effect on the frequency of circulating T_{FR} in MS patients. We also looked into the effect of treatment but did not observe any effect. Moreover, in a one year follow-up study of patients treated with fingolimod, we did not detect an effect of treatment on percentage of blood T_{FR} (Chapter 4, Figure 4.4D [252]). In addition, no significant association could be found between T_{FR} frequencies and EDSS, MS disease type and disease duration (Figure 3.5D-F).

Next, we assessed the T_{FR} effector phenotypes in MS. T_{FR} with a T_H17-like phenotype are increased in MS patients (n=14 for MS patients and n=16 for HC, $p=0.0033$, Figure 3.5G, Table 3.1), while other effector phenotypes did not differ. In contrast to T_{FR}, no alterations were found in the T_{FH} compartment of MS (Figure 3.6).

Taken together, our results show that the frequency of circulating T_{FR} is significantly decreased in patients with MS, and that the T_H17 effector subpopulation of MS-derived T_{FR} is increased.

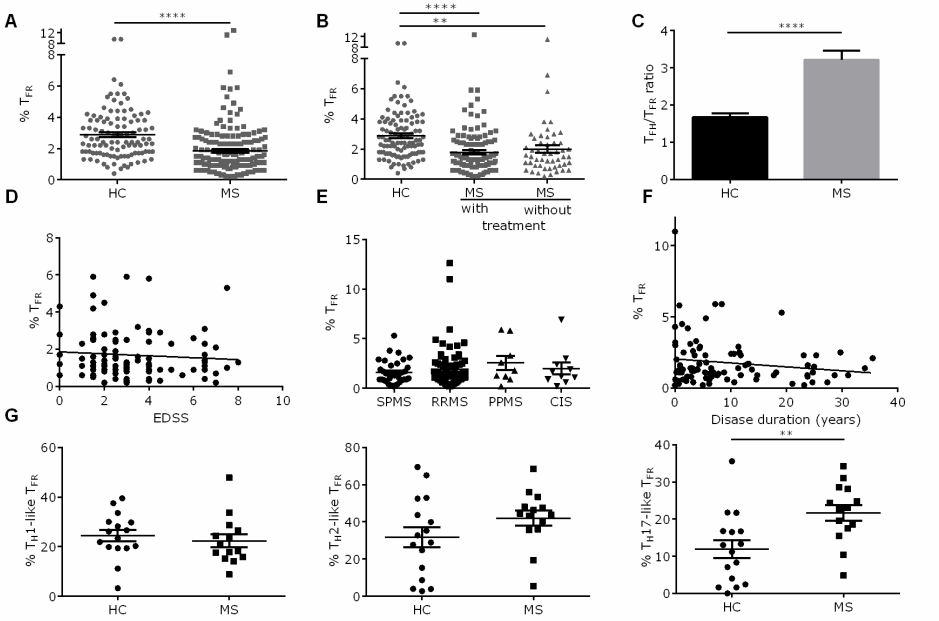


Figure 3.5: Frequency of circulating T_{FR} in MS patients and HC. **(A)** The percentage of circulating T_{FR} in HC and MS patients compared to HC ($p<0.0001$, n=172 for MS patients and n=107 for HC) **(B)** The percentage of circulating T_{FR} ratio in MS patients with treatment or without treatment and HC ($p<0.0001$ for MS patients with treatment and $p=0.0036$ for MS patients without treatment, n=172 for MS patients and n=107 for HC). **(C)** The T_{FH}/T_{FR} ratio MS patients and HC ($p<0.0001$, n=120 for MS patients with treatment and n=52 for MS patients without treatment and n=107 for HC). **(D)** Correlation of clinical scores (EDSS) and percentage T_{FR}. **(E)** The percentage of T_{FR} in different MS disease types (One-way ANOVA). **(F)** Correlation between the percentage of circulating T_{FR} and the disease duration in patients with MS. **(G)** Within the circulating T_{FR} the percentage of effector cells was investigated using CXCR3 and CCR6: T_H2-like; $CXCR3^-CCR6^-$, T_H17-like; $CXCR3^-CCR6^+$ and T_H1-like; $CXCR3^+CCR6^-$ in MS patients and HC (n=14 for MS patients and n=16 for HC, $p=0.0033$, Man-Whitney test). Data are mean ± SEM. Correlation were made using a standard linear regression model * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

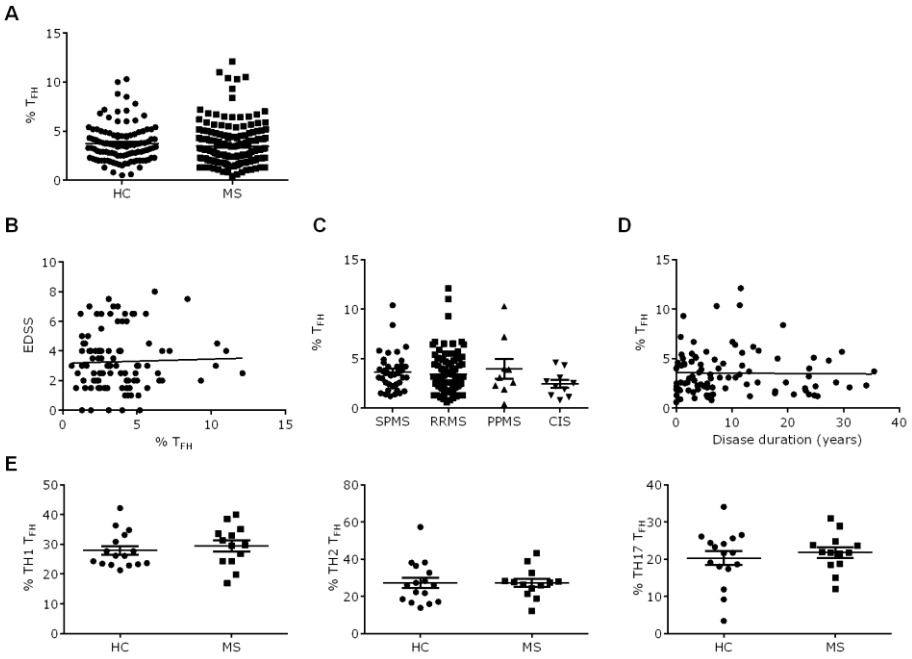


Figure 3.6: **Percentage of circulating T_{FH} in MS patients and HC. (A)** The percentage of circulating T_{FH} in MS patients (unpaired t-test**,** n=172, 3.48% ± 2.08) compared to healthy controls (n=107, 3.7 % ± 1.8) **(B)** Correlation between EDSS and T_{FH} percentage. **(C)** The percentage of T_{FH} in various types of MS. **(D)** Correlation between disease duration (in years) and percentage circulating T_{FH}. **(E)** Within the circulating T_{FH} the percentage of effector cells (T_H2; $CXCR3^-CCR6^-$, T_H17; $CXCR3^-CCR6^+$ and T_H1; $CXCR3^+CCR6^-$) was investigated (Man-Whitney test). Data are mean ± SEM. Correlation were made using a standard linear regression model.

3.3.5 Circulating follicular regulatory T cells are functionally impaired in patients with multiple sclerosis

The functionality of MS-derived circulating T_{FR} was tested using the *in vitro* co-culture suppression assay described above (Figure 3.7A). MS-derived T_{FR} displayed a strongly impaired suppression compared to HC (HC n=15, MS n=12, $p<0.0001$, Figure 3.7B, Table 3.1). We further confirmed that conventional Tregs from these MS patients were also significantly impaired ($p<0.0001$, Figure 3.7B). When correlating the suppressive function of both Tregs and T_{FR} from the same donor a significant positive association was found for both MS patients and HC (Figure 3.7C). For one MS patient (RRMS, no treatment), the suppressive function was assessed at 2 time points one month apart. The suppressive capacity of Tregs and T_{FR} (at a 1:1 ratio) at both time points was in the same range (data not shown), confirming the reproducibility and stability of the assay as well as the constant nature of Treg/T_{FR} function.

To conclude, we found that both Tregs and T_{FR} isolated from MS patients had a reduced capacity to suppress the proliferation of responder T cells showing for the first time a functional impairment of circulating T_{FR} in MS patients.

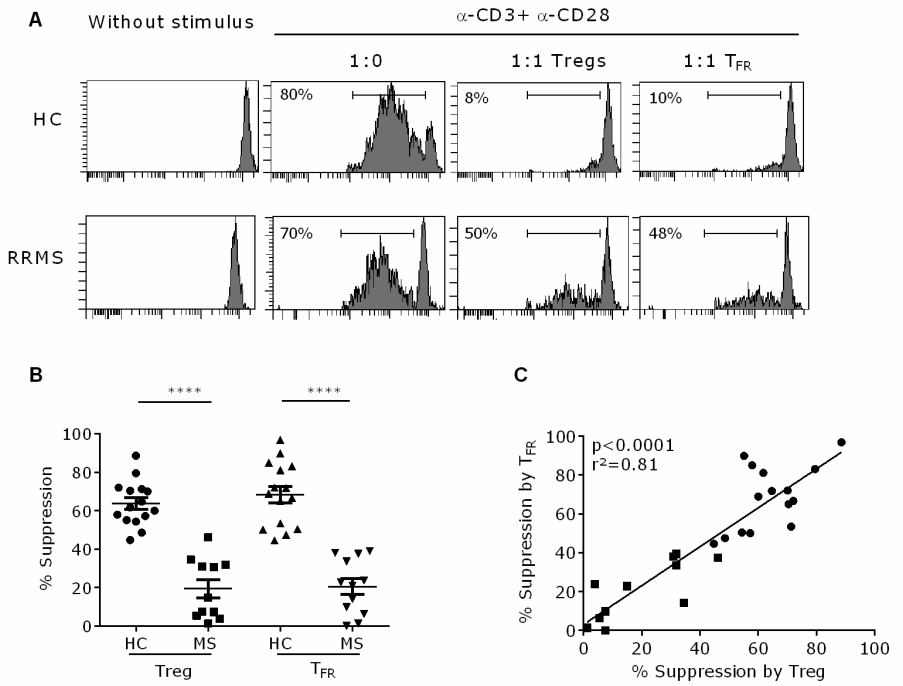


Figure 3.7: Circulating T_{FR} are functionally impaired in patients with MS. (A) Representative flow cytometric histogram of CFSE-labeled responder T cells ($CD4^+CD25^-CD127^+$) from a healthy control (HC) and a RRMS patient that were incubated with or without autologous regulatory T cells (regulatory T cells (Tregs) and follicular regulatory T cells (T_{FR}), isolated from the same donor) in a 1:1 ratio. The percentages indicate the proliferation of responder T cells with or without T_{FR} or Tregs. **(B)** The percentage suppression of Tregs and T_{FR} from HC and MS patients was measured using a CFSE-based co-culture assay. **(C)** Correlation between the suppressive function of Tregs and T_{FR} in the same HC (●) and MS patients (■). Data are represented as mean ± SEM with 15 HC and 12 MS patients. A unpaired t-test was used to asses differences in donors. Correlation was analyzed with a linear regression mode and ***p<0.001.

Table 3.1: Clinical and immunological characteristics of donors used in this study

Healthy donors undergoing routine tonsillectomies	
T_{FR} (%) in tonsils	31,0 ± 11,7
T_{FR} (%) in blood	6,8 ±3,5
T_{FH} (%) tonsils	43,6 ± 15,6
T_{FH} (%) in blood	11,4 ±5,2
Mean age	24,5 ± 6,0
M/F	4/1
Percentage methylated Foxp3 within T_{FR} from tonsils	46,6 ±8,0
Percentage methylated Foxp3 within T_{FR} from blood	71,1 ± 12,8
Percentage methylated Foxp3 within Treg from tonsils	69,5 ± 12,8
Percentage methylated Foxp3 within Treg from blood	85,3±16,6
Mean age	28,8 ± 6,3
M/F	1/8
Suppressive capacity (%) T_{FR} from tonsils	66,6 ±11,9
Suppressive capacity (%) T_{FR} from blood	74,5±15,5
Suppressive capacity (%) Treg from tonsils	64,5±6,1
Suppressive capacity (%) Treg from blood	54,2±17,6
Mean age	25,8 ± 7,6
M/F	1/6
Healthy donors enrolled in influenza study	
T_{FR} (%) before vaccination	1,9 ± 1,1
T_{FR} (%) 7 days after vaccination	4,4 ±1,5
T_{FH} (%) before vaccination	5,3 ± 2,2
T_{FH} (%) 7 days after vaccination	7,1 ± 2,7
Mean age	37,6 ± 11,5
M/F	11/13

Analysis of circulating follicular T cells		
	Healthy controls	MS patients
T_{FR} (%)	2,9 % ± 0,14	1,8% ± 0,17
T_{FH} (%)	3,7 % ± 1,8	3,4% ± 2,0
Mean age	37,4 ± 14,7	46,4 ± 13,4
M/F	39/62	43/125
T_H1 T_{FR} (%)	24,3 ± 8,9	22,3 ± 9,7
T_H2 T_{FR} (%)	31,7 ± 21,4	42 ±15,03
T_H17 T_{FR} (%)	11,9 ± 9,2	21,6 ± 8
T_H1 T_{FH} (%)	27,9 ± 5,7	29,4 ±6,8
T_H2 T_{FH} (%)	27,2 ± 11,3	27,3 ± 7,9
T_H17 T_{FH} (%)	20,3 ± 7,4	21,8 ± 5,11
Mean age	30,2 ±8,4	46,9 ± 12,19
M/F	7/9	7/7
Type MS	/	RRMS=11, SPMS=1, Missing= 2

Clinical information of MS patients	
EDSS	3,2 ± 1,9
Treatment	Avonex= 15, Betaferon= 15, Copaxone= 9 Extavia= 2, Gilenya= 8, Medrol= 2, MTX= 8, No treatment= 52, Rebif= 10, Start Alemtuzumab= 1, Start gilenya= 28, Tecfidera= 1 and Tysabri= 12
Type MS	CIS n=10, PPMS n=9, SPMS n= 37, RRMS n= 86, Missing data = 30

Analysis of suppressive capacity of T_{FR}		
	Healthy controls	MS patients
Suppressive capacity T_{FR}	68,4±16,5	20,6 ± 14,4
Suppressive capacity Treg	63,7±11,7	19,5 ± 15,6
Mean age, M/F	29,0 ± 8,0, 3/12	45,2 ± 7,12/9
Type MS	NA	RRMS
Treatment	NA	No treatment n = 9 IFNβ_1 treatment n=3

Values are shown as mean in % ±SD, M=male F=female

3.4 Discussion

In this study, we provide an insight into the phenotype and function of circulating T_{FR} in humans. Although circulating T_{FR} represent a population which is phenotypically distinct from their tonsil-derived counterparts, they are an eligible source to measure GC responses ongoing in secondary lymphoid organs. We showed a decreased frequency and impaired functionality of circulating T_{FR} in MS patients, indicating their involvement in breakdown of self-tolerance in human AID. These conclusions lead us to two main questions. What is the origin and fate of these cells? And, what explains the impairment of circulating T_{FR} population in MS?

Circulating T_{FR} are a not considered bona fide T_{FR} since they lack high expression of essential follicular markers, such as Bcl-6 and ICOS. These results are consistent with findings in circulating T_{FH} and likely indicate that follicular markers only upregulate after homing to the GC [229]. This notion is further supported by the decreased expression of CXCR5 and PD-1 on circulating T_{FR} after influenza vaccination, which could reflect homing of $CXCR5^{hi}PD\text{-}1^{hi}$ T_{FR} to the GC. In mice, T_{FR} were shown to originate from thymic-derived Tregs [128]. In line with these findings, we showed that human blood T_{FR} express Helios and demethylated FOXP3 to a similar extend as tonsil-derived T_{FR} and are fully functional. Furthermore, we characterized these cells as central memory with a higher expression of both CCR7 and CD62L compared to tonsil-derived T_{FR}, allowing recirculation to the lymph nodes. He at al. showed that circulating T_{FH} also have a higher expression of these markers [229]. In addition, we found a significant increase in the percentage of memory T_{FR} after seasonal influenza vaccination. It is therefore possible that they originate from GC T_{FR}, migrate to the circulation after a GC response and become a central effector memory population that is long-lived and has the capacity to recirculate. Another theory proposed by He at al. suggested that circulating T_{FH} cells are a population of cells that leave the germinal center response in its early phase, before developing in mature T_{FH} cells, governing a 'precursor memory' population [229].

We showed that while the expression of Foxp3 and Helios was the same in T_{FR}, blood-derived follicular cells are $CD31^+$ while the tonsil-derived follicular cells are negative. It has been reported that CD31 expression regulates T cell activation and can thus prevent hyperactivation [245, 247]. In addition, recent evidence showed that the loss of CD31 after activation leads to a stable interaction of T cells with B cells [246]. This could indicate that circulating T_{FR} are indeed a central memory population which is quiescent by nature and will lose CD31 expression upon activation. In line with this, a recent mouse study [253] reported that circulating T_{FR} are a longlived memory population that homes to GC after reactivation. A human study showed that follicular T cell population do not need an ongoing GC response for their maintenance as treatment with rituximab, known to eliminate GC B cells had no effect on the follicular T cell compartment [190]. Together all these data indicate that circulating T_{FR} are a distinct effector memory population that persists for a long time and is able to recirculate to the lymph nodes when needed.

Multiple sclerosis, an AID of the central nervous system, was used as a model to investigate the role of circulating T_{FR} in autoimmunity. A functional impairment of Tregs in MS was shown by various groups [140, 142, 143, 145, 150]. We confirmed an impairment of Tregs in MS patients and found that T_{FR} from the same patients are also defective in their capacity to suppress Tresp. Also, a decreased percentage of blood T_{FR} in MS patients was found compared to HC. Based on the chemokine markers suggested by Morita et al. [234], we show that the amount of T_H17-like T_{FR} is increased in MS patients. A more pro-inflammatory phenotype could explain the decreased suppressive function of circulating T_{FR}. Furthermore, this impairment could originate from a defect in CTLA-4 signaling as CTLA-4 is essential in T_{FR} function [188]. An alternative explanation for the reduced frequency and suppressive activity in the circulating T_{FR} compartment could be that the most potent T_{FR} homed to the lymph nodes to suppress the ongoing GC response. Sage et al. showed that circulating T_{FR} in mice require dendritic cells (DC) for their development and cytokine production [253]. Circulating DC in MS were shown to have a decreased regulatory character and could in this way contribute to an impairment of functional circulating T_{FR} [254].

To conclude, we believe that follicular populations in the blood form a source of responsive memory cells that quickly reacts when encountering an antigen again. Impairment of circulating T_{FR} and T_{FH} could contribute to the pathogenesis of various AID, including MS, highlighting their importance in conserving normal immunity.

4

COMPOSITIONAL CHANGES OF B AND T CELL SUBTYPES DURING FINGOLIMOD TREATMENT IN MULTIPLE SCLEROSIS PATIENTS: A 12 MONTH FOLLOW-UP STUDY

Part of this chapter is based on:

Dhaeze T*, Claes N*, Fraussen J, Broux B, Van Wijmeersch B, Stinissen P, Hellings N, and Somers V.

Compositional changes of B and T cell subtypes during fingolimod treatment in multiple sclerosis patients: a 12-month follow-up study.

PloS one 2014;9(10):e111115

* Equally contributing authors

Abstract

Background and objective: The long term effects of fingolimod, an oral treatment for relapsing-remitting (RR) multiple sclerosis (MS), on blood circulating B and T cell subtypes in MS patients are not completely understood. This study describes for the first time the longitudinal effects of fingolimod treatment on B and T cell subtypes. Furthermore, expression of surface molecules involved in antigen presentation and costimulation during fingolimod treatment are assessed in MS patients in a 12 month follow-up study.

Methods: Using flow cytometry, B and T cell subtypes, and their expression of antigen presentation, costimulation and migration markers were measured during a 12 month follow-up in the peripheral blood of MS patients. Data of fingolimod-treated MS patients (n=49) were compared to those from treatment-naive (n=47) and interferon-treated (n=27) MS patients.

Results: In the B cell population, we observed a decrease in the proportion of non class-switched and class-switched memory B cells ($p<0.001$), both implicated in MS pathogenesis, while the proportion of naive B cells was increased during fingolimod treatment in the peripheral blood (PB) of MS patients ($p<0.05$). The remaining T cell population, in contrast, showed elevated proportions of memory conventional and regulatory T cells ($p<0.01$) and declined proportions of naive conventional and regulatory cells ($p<0.05$). These naive T cell subtypes are main drivers of MS pathogenesis. B cell expression of CD80 and CD86 and programmed death (PD)-1 expression on circulating follicular helper T cells was increased during fingolimod follow-up ($p<0.05$) pointing to a potentially compensatory mechanism of the remaining circulating lymphocyte subtypes that could provide additional help during normal immune responses.

Conclusions: MS patients treated with fingolimod showed a change in PB lymphocyte subtype proportions and expression of functional molecules on T and B cells, suggesting an association with the therapeutic efficacy of fingolimod.

4.1 Introduction

A complex interplay between T and B cells drives the disease course of multiple sclerosis (MS). Thereby, non class-switched ($CD19^+IgD^+CD27^+$) and class-switched ($CD19^+IgD^-CD27^+$) memory B cells are generally considered to be the main pathogenic B cell subtypes, whereas, conventional (autoreactive) T cells ($CD4^+CD25^-CD127^+$) can drive the disease and regulatory T cells ($CD4^+CD25^{hi}CD127^{lo}$) control immune homeostasis [241, 255, 256]. Both within the conventional and regulatory T cell populations, naive ($CD45RA^+CD45RO^-$) and memory ($CD45RA^-CD45RO^+$) subtypes can be discriminated. The role of other peripheral blood (PB) immune cells in MS pathogenesis, such as naive B cells ($CD19^+IgD^+CD27^-$), double negative B cells ($CD19^+IgD^-CD27^-$), follicular regulatory T cells (T_{FR}; $CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$) and follicular helper T cells (T_{FH}; $CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$), is still unclear. B and T cells interact via surface molecules e.g. human leukocyte antigen (HLA)-DR/DP/DQ, CD80 and CD86 on B cells and programmed death (PD)-1 on T cells. Furthermore, migration of B and T cells is partly mediated via chemokine (C-X-C motif) receptor 5 (CXCR5) [257].

Fingolimod is the FDA approved oral treatment for MS and has shown efficacy in relapsing remitting (RR) MS [258-261]. Fingolimod is an immunomodulator that interferes with the signaling of the sphingosine-1-phospate receptor 1 (S1PR1), present on lymphocytes, and causes the internalization and degradation of this receptor [73]. Consequently lymphocytes cannot exit the lymph nodes into the circulation, leading to the entrapment of lymphocytes in lymphatic systems, causing lymphopenia in peripheral blood (PB) of treated patients, thereby reducing the number of inflammatory cells migrating to the central nervous system (CNS) [72, 73, 262, 263].

Limited information is available concerning the effects of fingolimod on different T and B cell subtypes and on the interplay between these lymphocyte populations in the PB of MS patients [264-266]. To understand the longitudinal immunological effects of fingolimod treatment, we investigated the effect of this treatment on B and T cell subtypes and antigen presentation, costimulation and migration molecules expressed on these cells in PB of MS patients in a 12 months follow-up study.

4.2 Materials and methods

4.2.1 Study population

PB was collected from MS patients in both the Orbis Medical Center (Sittard, the Netherlands) and Rehabilitation and MS-center (Overpelt, Belgium). For PB collection in the Orbis Medical Center, written informed consent was obtained from all participants after approval by the Medical ethical Committee Atrium-Orbis-Zuyd (12-N-56). Furthermore, PB was collected by the Rehabilitation and MS-center in Overpelt after written informed consent from all participants and approval by the UZ Leuven and Hasselt University Commissions of Medical Ethics (S54362 and S54363). A total of 123 MS patients were involved in the study, including 47 treatment-naive MS patients, 27 MS patients on interferon-β (IFN-β) treatment (together referred to as controls) and 49 MS patients on fingolimod treatment (0.5mg/day). All MS patients were diagnosed according to the revised McDonald criteria [267]. Treatment-naive MS patients never received any MS related treatment. PB of the fingolimod-treated group was collected after wash-out of the previous treatment (minimally 2 months) and before the first dose of fingolimod (baseline). MS patients were then followed over time: PB was collected after 1 month (1m), 3 months (3m) and every 3 consecutive months of treatment for a period of up to 12 months (6m, 9m, 12m). Clinical non-responders to fingolimod treatment were characterized by an increase in EDSS score, a relapse or a new magnetic resonance imaging (MRI) lesion after a minimum of 3 months of fingolimod treatment and were excluded from the study.

4.2.2 Flow cytometry

PB was collected in heparin-coated tubes (Venosafe plastic tubes, Terumo Europe N.V., Leuven, Belgium) and PB mononuclear cells (PBMC) were isolated using high density centrifugation (Lympholyte®; Cedarlane® Laboratories, SanBio B.V., Uden, the Netherlands). PBMC (0.5×10^6 cells) were stained using anti-human CD19 PerCP-Cy5.5 and CD4 APC to discriminate between B and T cells, respectively (BD Biosciences, Erembodegem, Belgium).

B cell subpopulations and surface molecules were defined using following anti-human antibodies: IgD APC-Cy7, CD27 PE-Cy7, HLA-DR/DP/DQ (major histocompatibility complex (MHC)-II) FITC, CD80 PE and CD86 PE-CF594 (all from BD Biosciences, Erembodegem, Belgium). Following anti-human monoclonal antibodies were used for T cell analysis: CD45RA APC-H7, CD45RO PE-CF594, CXCR5 Alexa Fluor 488 and PD-1 PE-Cy7 (all from BD Biosciences, Erembodegem, Belgium), CD25 PerCP-Cy5.5 and CD127 PE (eBioscience, San Diego, USA). Following isotype controls were used: mouse IgG1 PerCP-Cy5.5, IgG1 PE, IgG1 Pe-Cy7, IgG2aκ PE-CF594, IgG2bκ APC-H7, IgG1 APC, IgG2aκ FITC, IgG1κ PE-CF594, IgG1 Pe-Cy7, IgG2aκ APC-H7 and rat IgG2b Alexa Fluor 488 (all from BD Biosciences, Erembodegem, Belgium). All flow cytometric analyses were performed on a FACSAriaII flow cytometer and analyzed with FACS Diva software (BD Biosciences).

4.2.3 Statistical analysis

Data analysis was performed using Prism software version 5.01 (Graphpad) and SAS 9.3 software. Appropriate One-way ANOVA analysis was used with Dunn's multiple comparison post-hoc test for comparison of treatment controls and baseline fingolimod-treated patients after normality check (Kolmogorov-Smirnov). A mixed model was used for data analysis of treatment follow-up compared to baseline fingolimod. A p-value of <0.05 was considered statistically significant.

4.3 Results

4.3.1 Reduction of total PB lymphocyte, B and T cell counts after fingolimod treatment

In total, PB of 49 fingolimod-treated MS patients was collected at different time points up to 12 months of treatment. The cohort of fingolimod-treated MS patients was compared at baseline with 47 treatment-naive and 27 IFN-β treated MS patients (together referred to as controls). Fingolimod-treated MS patients at baseline and controls were comparable in terms of age, gender distribution and median EDSS score (Table 4.1). Furthermore, no significant difference was observed in numbers of total lymphocytes, B cells or T cells (Figure 4.1) between baseline fingolimod treatment and controls. For the MS patients receiving fingolimod treatment, pre-treatment (baseline) values were used as reference to assess the effects of treatment. Five of 49 fingolimod-treated MS patients did not finish the study due to side effects caused by the treatment. Seven MS patients were excluded from the study as clinical non-responders, although no differences in T and B cell subtype proportions between non-responders and responders were found (data not shown).

Total lymphocyte numbers were decreased after 1 month (1m) of fingolimod treatment compared with baseline and both control groups for the total duration of the study (12m) ($p<0.001$, Figure 4.1). Furthermore, total $CD19^+$ B cell and $CD4^+$ T cell numbers were decreased at 1m and reached a steady state at 3m ($p<0.001$, Figure 4.1). Similar results were observed for the percentage of $CD19^+$ and $CD4^+$ cells within the lymphocyte population ($p<0.001$, Table 4.2A and B).

Table 4.1 Study population

	Age[a] (range)	Gender F/M	Classification[b]		EDSS[c] (range)
			RR	CP	
Total (n=123)	44 (17-79)	90/33	92	23	2.5 (0.0-7.0)
Treatment naive (n=47)	48 (17-79)	33/14	29	12	2.5 (0.0-7.0)
Interferon (n=27)	42 (17-66)	19/8	22	5	2.5 (1.0-6.5)
Fingolimod (n=49)	44 (18-69)	38/11	43	6	2.5 (0.0-6.5)
Non-responders (n=7)	49 (34-54)	5/2	4	3	4.0 (1.0-6.5)
Drop outs (n=5)	41 (32-56)	5/0	5	0	2.0 (1.5-6.0)
0 m (n=28)	43 (18-67)	21/7	25	3	2.5 (0.0-6.0)
1 m (n=24)	41(18-67)	18/6	22	2	2.5 (0.0-6.0)
3 m (n=29)	43 (18-67)	22/7	26	3	2.5 (0.0-6.0)
6 m (n=26)	43 (18-69)	20/6	24	2	2.5 (0.0-6.0)
9 m (n=27)	45 (18-69)	23/4	24	3	2.5 (0.0-6.0)
12 m (n=13)	45 (29-69)	11/2	12	1	2.5 (0.0-5.0)

a. Mean age in years

b. For 6 treatment-naive patients, MS type was not specified

c. Median EDSS score; this information was not available for 7 treatment-naive patients and 6 IFN-β-treated patients

Abbreviations: F = female; M = male; RR = relapsing-remitting MS; CP = chronic progressive MS; EDSS = expanded disability status scale, m = month

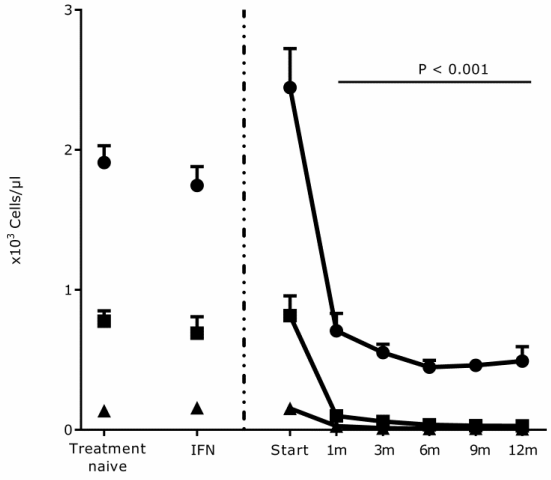


Figure 4.1: Total number of lymphocytes, CD4$^+$ T cells and CD19$^+$ B cells in the PB. Total number (x10^3 cells/µl blood) of lymphocytes, T cells and B cells in treatment-naive, IFN-β treated MS patients at baseline and fingolimod-treated MS patients during 12 months follow-up. Mean and standard error of the mean are presented. ● lymphocytes; ■ CD4$^+$ T cells; ▲ CD19$^+$ B cells.

4.3.2 Fingolimod affects B cell subtype distribution in MS patients

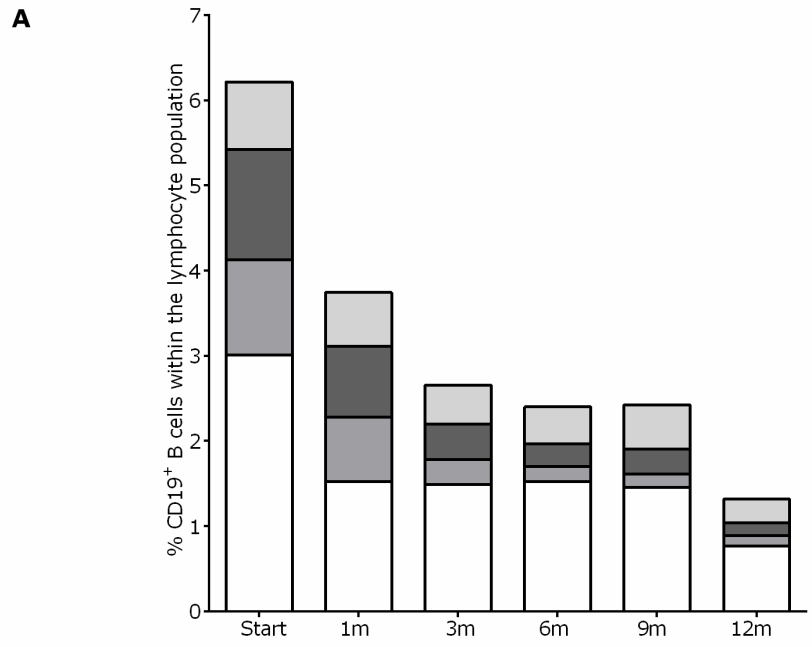
During immune responses, B cells produce antibodies after maturation into plasma cells, function as antigen presenting cells, provide costimulation for T cells and play a role in immune memory. In MS, memory B cells and plasma cells may contribute to the pathogenesis by production of autoantibodies and cytokines [268].

Although B cell numbers were reduced in the PB after fingolimod treatment, we investigated the effects of fingolimod treatment on the remaining B cell population in the PB of treated patients. Both non class-switched (CD19$^+$IgD$^+$CD27$^+$) and class-switched memory B cells (CD19$^+$IgD$^-$CD27$^+$) were significantly decreased in the peripheral B cell population from 3m until end of follow-up ($p<0.001$, Figure 4.2A, Table 4.2B).

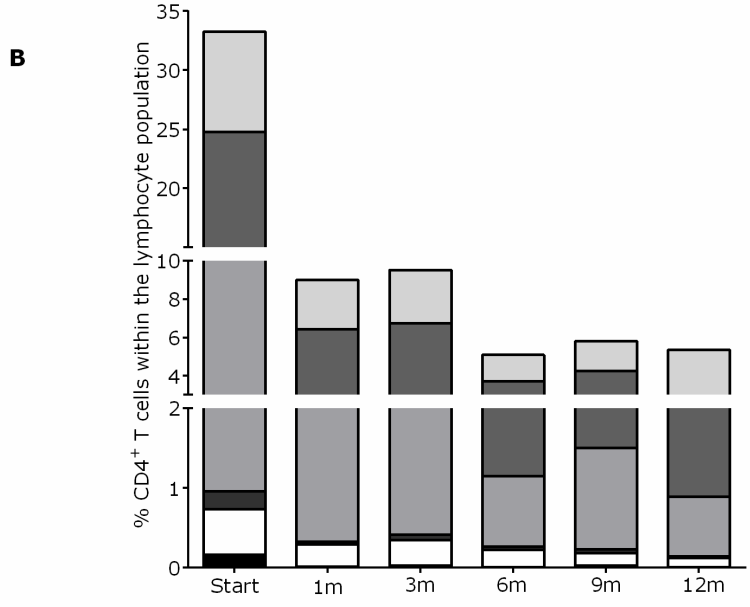
In contrast, the percentage of CD19$^+$IgD$^-$CD27$^-$ cells (double negative B cells) was significantly increased within the B cell population at 1m up to 12m ($p<0.05$ at 1m, $p<0.001$ at 3-12m, Figure 4.2A, Table 4.2B).

Naive B cells (CD19$^+$IgD$^+$CD27$^-$) made up about 50% of the remaining peripheral B cells and the proportion of these cells was increased after 3m fingolimod until end of follow-up ($p<0.03$, Figure 4.2A, Table 4.2B).

Distribution of B cell subtypes at start of fingolimod treatment was the same as in treatment-naive and IFN-β-treated MS patients (Table 4.2A). In general, fingolimod treatment caused a decline in memory B cell subpopulations while naive and double negative B cell proportions were increased in the PB of MS patients.



n =	23	24	25	25	27	12
DN B cells	13.7	**19.4**	**19.7**	**21.6**	**25.7**	**30.3**
CSM B cells	20.1	19.9	**13.8**	**12.5**	**11.9**	**12.2**
NCSM B cells	17.6	15.4	**9.1**	**6.5**	**6.7**	**7.2**
Naive B cells	48.6	45.8	**57.5**	**59.4**	**55.7**	**50.3**



		Start	1m	3m	6m	9m	12m
	n =	28	24	29	26	26	13
	Trans Tconv	24.0	22.9	24.1	22.5	20.3	27.9
	mTconv	42.3	**51.9**	**54.7**	**56.0**	**55.1**	**54.4**
	nTconv	33.5	**18.8**	**16.8**	**14.8**	**18.5**	**13.0**
	Trans Treg	0.7	0.5	0.7	0.6	0.7	0.8
	mTreg	1.8	**5.5**	**5.1**	**6.0**	**4.9**	3.4
	nTreg	0.5	**0.2**	**0.2**	**0.2**	0.3	0.4

Figure 4.2: Proportional B cell and T cell subtype changes in MS patients during fingolimod treatment. **(A)** $CD19^+$ B cell subtype proportion and **(B)** $CD4^+$ T cell subtype proportion within the PB of treatment-naive, IFN-β and fingolimod-treated MS patients. Results are presented as relative values within the $CD19^+$ B cell or $CD4^+$ T cell population. Subtype proportions were calculated as follows: (% subtype/100) x % $CD19^+$ or $CD4^+$ within the total lymphocyte population. Statistically significant differences compared to 0m are shown in bold. For B cells: naive B cells; NCSM B cells = non class-switched memory B cells; CSM B cells = class-switched memory B cells and DN B cells = double negative B cells. For T cells: nTreg = naive Treg; mTreg = memory Treg; TransTreg = transitional Treg; nTconv = naive Tconv; mTconv = memory Tconv; TransTconv = transitional Tconv.

4.3.3 Change in surface expression of molecules involved in B cell antigen presentation and costimulation under fingolimod

B cells are potent antigen presenting cells via the surface molecule HLA-DR/DP/DQ (MHC-II) and are important to provide co-stimulation to T cells via the surface molecules CD80 and CD86 [269].

During fingolimod treatment, both the percentage of HLA-DR/DP/DQ, CD80 and CD86 positive cells and the expression of these surface markers on $CD19^+$ B cells was assessed using flow cytometric analysis. The percentage of HLA-$DR/DP/DQ^+$ B cells (data not shown) and the expression of HLA-DR/DP/DQ (MFI) on B cells was significantly decreased after 3m and 1m of fingolimod treatment, respectively, in comparison with baseline ($p<0.05$, Figure 4.3A, Table 4.3B). Fingolimod treatment resulted in an increased expression of both CD86 (after 1m) and CD80 (after 3m and 12m) on B cells (MFI) compared with baseline ($p<0.05$, Figure 4.3B and C, Table 4.3B). The percentages of $CD80^+$ and $CD86^+$ B cells remained stable during the follow-up period (Table 4.3B). Expression of antigen presentation and co-stimulation markers on B cells was comparable between baseline fingolimod and both of the control groups (Table 4.3A and B). Thus, the expression of HLA-DR/DP/DQ on PB B cells was decreased (both percentage of positive cells and MFI), while the expression of the co-stimulation molecules CD80 and CD86 (MFI) was increased during fingolimod treatment in MS patients.

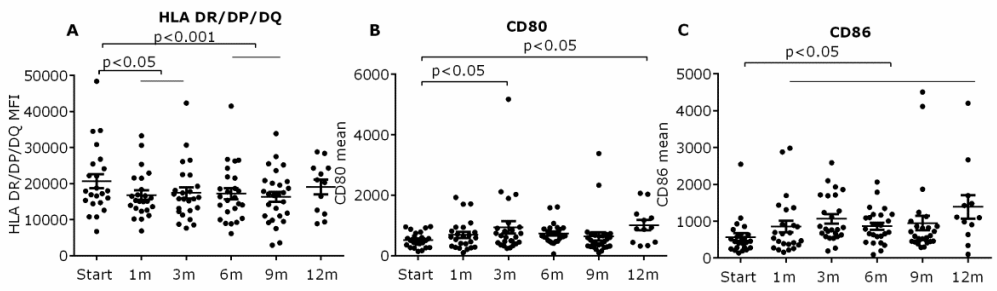


Figure 4.3: B cell expression levels of antigen presentation and costimulation molecules during fingolimod treatment. Mean fluorescence intensity (MFI) of **(A)** HLA-DR/DP/DQ, **(B)** CD80 and **(C)** CD86 expression within the B cell population from fingolimod-treated MS patients during follow-up.

4.3.4 Fingolimod affects conventional and regulatory T cell subtype distribution in the PB of MS patients

Conventional T cells (Tconv, $CD4^+CD25^-CD127^+$) are considered to be main players in maintaining a normal immune response and exert autoreactivity in autoimmune diseases like MS [241]. Regulatory T cells (Treg, $CD4^+CD25^{hi}CD127^{lo}$) are essential for immune homeostasis and were shown to be functionally impaired in MS pathogenesis [241].

The longitudinal effects of fingolimod treatment on different $CD4^+$ T cell subtypes including naive ($CD45RA^+CD45RO^-$), memory ($CD45RA^-CD45RO^+$) and transitional ($CD45RA^+CD45RO^+$) cells within both Tconv and Treg populations were assessed using flow cytometry. For the Tconv population, the proportion of naive cells was decreased in fingolimod-treated patients at all timepoints measured, when compared with baseline ($p<0.001$, Figure 4.2B, Table 4.2B). In contrast, a significant increase in the percentage of memory Tconv was observed after 1m until 12m in comparison with baseline ($p<0.001$, Figure 4.2B, Table 4.2B).

The percentage of naive cells within the Treg subtypes displayed a significant decrease after 1m, 3m and 6m ($p<0.05$, Figure 4.2B, Table 4.2B) while a significant increase was observed in the proportion of memory Tregs after 1m until 9m ($p<0.001$), as observed for the Tconv population.

Interestingly, the transitional T cells ($CD45RA^+CD45RO^+$), both in the regulatory and conventional T cell population, remained stable throughout the 12 month follow-up period. Of note, baseline levels of the fingolimod-treated groups differed significantly compared with treatment-naive patients for Tconv and Treg ($p<0.05$, Table 4.2A and B). Similar changes were observed when comparing IFN-β-treated MS patients to the treatment-naive group. Together, these results show that fingolimod treatment caused a decrease in the proportions of naïve Tconv and Treg cells in the PB, together with an increase in the proportion of memory Tconv and Treg cells.

4.3.5 PD-1 expression increases on circulating follicular helper T cells during fingolimod treatment

Circulating follicular helper T cells (T_{FH}) have the capacity to recirculate in SLO where they can interact with B cells and influence the GC response [169]. Follicular regulatory T cells (T_{FR}) are essential for the regulation of the GC response in SLO [128].

The percentage of circulating T_{FH} ($CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$) and circulating T_{FR} ($CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$) remained stable during fingolimod treatment (Figure 4.4A and D, Table 4.2B).

To assess the effect of fingolimod on the expression of molecules involved in cell migration towards the germinal center and molecules involved in the functionality of the germinal center response, CXCR5 and PD-1 expression levels were determined on T_{FH} cells and T_{FR} cells [155, 169, 170, 235]. While the expression of CXCR5 on T_{FH} and T_{FR} did not change in fingolimod-treated MS patients (Figure 4.4B and 4.4E, Table 4.3B), a significant increase of the expression of PD-1 on PB T_{FH} cells was observed during follow-up ($p<0.05$, Figure 4.4C, Table 4.3B). PD-1 expression on PB T_{FR} remained stable (Figure 4.4F). These results shows that the frequency of circulating T_{FH} and T_{FR} cells that egress from the lymph nodes was not affected by treatment with fingolimod while expression of PD-1 on the T_{FH} cells in the PB of MS patients was increased.

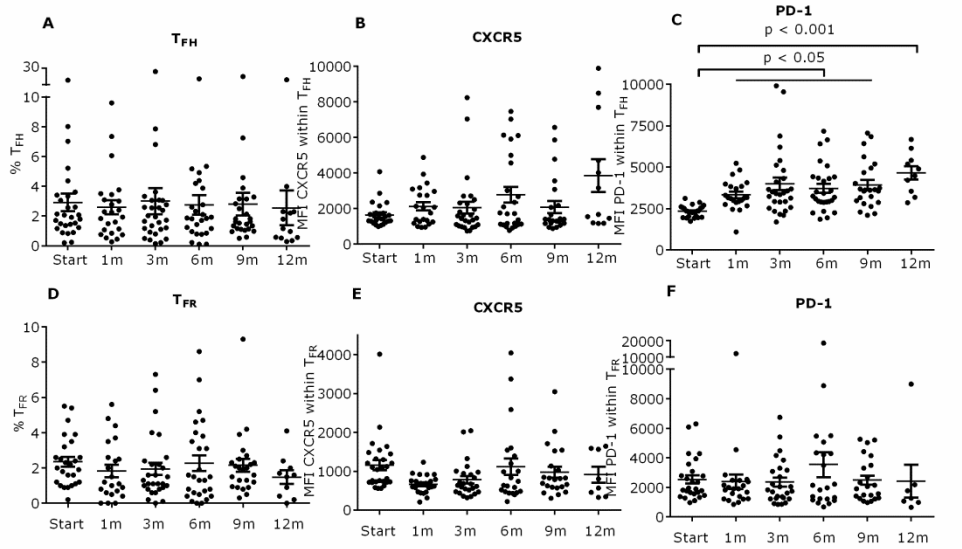


Figure 4.4: Percentage of T_{FR} and T_{FH} and expression of CXCR5 and PD-1 during fingolimod treatment in MS patients. (A) Percentage of PB follicular helper T cells (T_{FH}) in MS patients treated with fingolimod. **(B)** Expression of CXCR5 and **(C)** expression of PD-1 within T_{FH} cell population. **(D)** Percentage of PB follicular helper T cells (T_{FR}) in MS patients treated with fingolimod. **(E)** Expression of CXCR5 within the T_{FR} population **(F)** Expression of PD-1 within the T_{FR} population.

Table 4.2A: Mean percentages of different B and T cell subtypes

Subtype	Treatment-naïve			IFN-β		
	Mean	Sem	n	Mean	Sem	n
CD4$^+$	40.81	1.84	40	48.60	2.02	21
Tconv	98.60	0.14	40	96.9*	0.30	21
mTconv	45.08	2.24	40	36.77	2.64	21
nTconv	31.47	1.98	40	34.39	2.57	21
Trans Tconv	22.05	1.53	40	25.79	2.56	21
T_{FH}	2.52	0.37	37	2.22	0.34	20
Treg	1.37	0.14	40	2.79	0.27	21
mTreg	1.18	0.13	40	1.61	0.19	21
nTreg	0.05	0.01	40	0.40*	0.07	21
M	0.05	0.01		0.32*	0.05	
F	0.05	0.01		0.42*	0.09	
Trans Treg	0.14	0.03	40	0.78*	0.16	21
T_{FR}	1.99	0.25	51	1.4	0.35	20
CD19$^+$	7.06	0.63	47	8.30	0.59	27
Naive	50.43	2.42	47	56.86	3.39	27
NCS memory	13.39	0.96	47	11.24	1.23	27
CS memory	21.77	1.64	47	18.16	2.37	27
Double negative	14.41	1.24	47	13.78	2.32	27

* $p < 0.05$ versus treatment naive; Baseline fingolimod treatment was compared to treatment-naive and IFN-β-treated patients using appropriate one-way ANOVA (SAS 9.3). Interaction between treatment and sex was assessed and only observed for nTreg cells. Baseline differences were subdivided for males (M) and females (F). A mixed model was used for comparison of follow-up data with baseline. Tconv = conventional T cells; mTconv = memory conventional T cells; nTconv = naive conventional T cells; Trans Tconv = transitional conventional T cells; T_{FH} = follicular helper Tcells; Treg = regulatory T cells; mTreg = memory regulatory T cells; nTreg = naive regulatory T cells; trans Treg = transitional regulatory T cells; SEM = standard error of the mean; n = number of samples; NCS = non class-switched; CS = class-switched; M = male; F = female

Table 4.2B: Mean percentages of different B and T cell subtypes

Subtype	Fingolimod (Months)																	
	0			1			3			6			9			12		
	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n
$CD4^+$	33.2	2.77	28	**9.01¥**	**1.77**	**24**	**9.49¥**	**1.71**	**29**	**5.09¥**	**0.88**	**26**	**5.82¥**	**1.46**	**26**	**5.35¥**	**2.07**	**13**
Tconv	**97***	**0.33**	**28**	**93.62¥**	**1.15**	**24**	**93.83¥**	**0.91**	**29**	**93.27¥**	**0.98**	**26**	**93.94£**	**0.91**	**26**	**95.34**	**1.16**	**13**
mTconv	42.3	2.42	27	**51.93¥**	**2.96**	**24**	**54.67¥**	**3.95**	**28**	**55.97¥**	**2.93**	**26**	**55.12¥**	**4.44**	**26**	**54.42¥**	**6.69**	**13**
nTconv	33.5	2.34	27	**18.78¥**	**1.76**	**24**	**16.84¥**	**2.01**	**29**	**14.77¥**	**1.81**	**26**	**18.50¥**	**2.99**	**26**	**13.01¥**	**2.60**	**13**
trTconv	23.9	3.19	28	22.91	2.42	24	24.05	2.95	29	22.53	2.38	26	20.32	3.08	26	27.91	5.70	13
T_{FH}	2.92	0.59	28	2.59	0.46	24	2.99	0.89	29	2.76	0.66	26	2.80	0.75	26	2.54	1.17	13
Treg	**2.9***	**0.34**	**28**	**6.28¥**	**1.14**	**24**	**6.00¥**	**0.91**	**29**	**6.77¥**	**0.99**	**26**	**5.93£**	**0.93**	**26**	4.49	1.14	13
mTreg	1.79	0.27	28	**5.51¥**	**1.08**	**24**	**5.12¥**	**0.91**	**29**	**6.01¥**	**1.00**	**26**	**4.95¥**	**0.89**	**26**	3.38	0.75	13
nTreg	**0.5***	**0.09**	**28**	**0.23$**	**0.05**	**24**	**0.24£**	**0.05**	**29**	**0.17£**	**0.03**	**26**	0.32	0.12	26	0.35	0.15	13
M	**0.8***	0.27																
F	**0.4***	0.07																
trTreg	**0.7***	0.11	28	0.54	0.01	24	0.65	0.12	29	0.60	0.13	26	0.67	0.19	26	0.77	0.36	13
T_{FR}	2.35	0.2	26	1.82	0.35	22	1.95	0.35	28	2.26	0.43	27	2.15	0.36	24	1.48	0.28	10
$CD19^+$	6.21	0.66	23	**3.74¥**	**0.67**	**24**	**2.65¥**	**0.39**	**25**	**2.40¥**	**0.47**	**25**	**2.42¥**	**0.33**	**27**	**1.32¥**	**0.32**	**12**
Naive	48.6	3.82	23	45.82	4.20	24	**57.49$**	**2.65**	**25**	**59.35£**	**3.04**	**25**	**55.71$**	**3.80**	**27**	**50.25¥**	**5.66**	**12**
NCS memory	17.6	2.53	23	15.36	3.42	24	**9.05¥**	**1.48**	**25**	**6.49¥**	**0.88**	**25**	**6.70¥**	**0.85**	**27**	**7.21¥**	**1.62**	**12**
CS memory	20.1	2.01	23	19.89	2.41	24	**13.77¥**	**1.51**	**25**	**12.54¥**	**1.57**	**25**	**11.94¥**	**1.66**	**27**	**12.24¥**	**2.36**	**12**
Double negative	13.7	1.90	23	**19.35$**	**2.93**	**24**	**19.70¥**	**1.81**	**25**	**21.64¥**	**2.30**	**25**	**25.66¥**	**3.54**	**27**	**30.32¥**	**5.97**	**12**

* $p < 0.05$ versus treatment naive; # $p < 0.05$ versus interferon; $ $p < 0.05$ versus 0 months (SAS 9.3); £ $p < 0.01$ versus 0 months; ¥ $p < 0.001$ versus 0 months. Baseline fingolimod treatment was compared to treatment-naive and IFN-β-treated patients using appropriate one-way ANOVA (SAS 9.3). Interaction between treatment and sex was assessed and only observed for nTreg cells. Therefore, baseline differences were subdivided for males (M) and females (F). A mixed model was used for comparison of follow-up data with baseline. Abbreviations: Tconv = conventional T cells; mTconv = memory conventional T cells; nTconv = naive conventional T cells; Trans Tconv = transitional conventional T cells; T_{FH} = follicular helper T cells; Treg = regulatory T cells; mTreg = memory regulatory T cells; nTreg = naive regulatory T cells; trans Treg = transitional regulatory T cells; SEM = standard error of the mean; n = number of samples; NCS = non class-switched; CS = class-switched; M = male; F = female

Table 4.3A*:* Mean fluorescence intensity and percentage positive cells of different surface markers on B and T cells

Subtype	Treatment naive			IFN-β		
	Mean	Sem	n	Mean	Sem	n
MFI of T_{FH}						
PD-1	3080	207.7	37	3634	425.7	20
CXCR5	2669	328.7	37	2001	328.4	20
T_{FR}						
PD-1	2767	175	51	3919	459	20
CXCR5	1076	294.5	51	1262	101.8	20
% of $CD19^+$						
MHC II	98.30	0.294	47	98.36	0.391	27
CD80	17.90	1.889	47	17.59	2.412	27
CD86	15.31	2.034	47	17.60	2.178	27
MFI of $CD19^+$						
MHC II	17393	1143	47	16829	1742	27
CD80	501.4	48.54	47	477.7	44.78	27
CD86	474.0	47.26	47	566.7	62.00	27

* $p < 0.05$ versus treatment naive; # $p < 0.05$ versus interferon; $ $p < 0.05$ versus 0 months; £ $p < 0.01$ versus 0 months; ¥ $p < 0.001$ versus 0 months.Abbreviations: PD-1 = programmed cell death 1; MFI = mean fluorescence intensity; CXCR5 = CXC motif receptor 5; TFH = follicular helper cells; MHC II = major histocompatibility II = HLA-DR/DP/DQ; CD = cluster of differentiation; Sem = standard error of the mean; n = number of samples

Table 4.3B: Mean fluorescence intensity and percentage positive cells of different surface markers on B and T cells

Subtype	Fingolimod (Months)																	
	0			1			3			6			9			12		
	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n	Mean	Sem	n
MFI T_{FH}																		
PD-1	**2340#**	**65**	**26**	**3326$**	**193**	**22**	**3994$**	**384**	**28**	**3726$**	**272**	**27**	**3939$**	**289**	**24**	**4660¥**	**289**	**10**
CXCR5	1644	136	25	2126	235	22	2053	341	27	2770	441	25	2081	335	23	3847	919	12
T_{FR}																		
PD-1	2540	281	26	2391	495	22	2365	298	26	3544	844	22	2492	313	22	2418	1114	7
CXCR5	1155	131	26	663	50	22	780	91	26	1116	214	22	974	138	22	911	208	8
% CD19																		
MHC II	98	0.3	23	98	0.44	24	**95$**	1	25	**94$**	1.9	25	**93$**	1.6	27	**87$**	5	12
CD80	20	2.4	23	20	2.56	24	18	2	25	20	2.1	25	16	2.0	27	22	3	12
CD86	17	2.8	23	20	2.8	24	**24$**	2.7	25	18	2.1	25	20.27	2.0	27	**29$**	4	12
MFI CD19																		
MHC II	20691	1978	23	**16837$**	**1290**	**24**	**17453$**	**1557**	**25**	**17228£**	**1551**	**25**	**16304£**	**1400**	**27**	19059	2053	12
CD80	524	523	23	695	101	24	**939$**	**205**	**25**	730	68	25	639	131	27	**1013$**	**174**	**12**
CD86	569	104	23	**854$**	**157**	**24**	**1065£**	**125**	**25**	**864£**	**95**	**25**	**947$**	**198**	**27**	**1386£**	**320**	**12**

* p < 0.05 versus treatment naive; # p < 0.05 versus interferon; $ p < 0.05 versus 0 months; £ p < 0.01 versus 0 months; ¥ p < 0.001 versus 0 months.Abbreviations: PD-1 = programmed cell death 1; MFI = mean fluorescence intensity; CXCR5 = CXC motif receptor 5; TFH = follicular helper cells; MHC II = major histocompatibility II = HLA-DR/DP/DQ; CD = cluster of differentiation; Sem = standard error of the mean; n = number of samples

4.4 Discussion

In this study, we elucidate the effects of fingolimod, approved as therapy for RR-MS, on different B and T cell subtypes and expression of surface molecules involved in antigen presentation, costimulation and migration during a 12 month follow-up study. Under fingolimod treatment, the B cell subtype distribution changed, resulting in a decreased proportion of memory B cells and an increased proportion of naive and double negative B cells in the PB. In contrast, the proportions of T cell subtypes changed towards less naive Tconv and naive Treg in the PB, while the proportions of memory Tconv and memory Treg increased. Finally, expression of CD86 and CD80 costimulatory molecules on B cells as well as the expression of PD-1 on circulating T_{FH} were changed during fingolimod treatment.

We confirmed, as shown by others, that fingolimod reduced total lymphocyte, B and T cell numbers in the PB of MS patients [263, 270, 271]. For a comprehensive overview of the effects of fingolimod treatment on different cell types, we refer to the review of Brinkmann et al. [73].

The beneficial effects of fingolimod as MS treatment with minimal side effects could be attributed to different mechanisms of action. Fingolimod could entrap lymphocyte subtypes involved in MS pathogenesis in the lymph nodes by directly influencing migration of these lymphocytes from the lymph nodes into the circulation. As already reported by others, we show a decrease in peripheral memory B cells (both non class-switched and class-switched) while the naive B cell proportion increases [265, 266]. Although we do not report functional data, this finding could contribute to the beneficial effect of fingolimod treatment in MS patients. Memory B cells are largely implicated in MS pathogenesis as they are able to produce specific (auto)antibodies and are able to migrate to the CNS to enhance the ongoing immune response [256, 268, 272]. Entrapment of memory B cells in the lymph nodes could be a direct consequence of fingolimods' agonist activity on S1PR1 since egression of these cells could be mediated by S1PR1 signaling [265]. Additional proof of memory B cell entrapment in the lymph nodes comes from mice studies that showed a decrease in high-affinity class-switched antibodies by fingolimod, produced by memory B cells in the serum [273].

Furthermore, vaccination studies in fingolimod-treated healthy volunteers have demonstrated a mild to moderate decrease in immunoglobulin (Ig)G and IgM antibody levels towards some antigens [274].

B cells are important antigen presenting cells and recent evidence from mice studies has indicated that fingolimod can influence antigen handling [275-277]. In our study, B cell expression of the antigen presentation marker HLA-DR/DP/DQ and the percentage of HLA-DR/DP/DQ^{+} B cells was decreased in the PB during fingolimod treatment, which could be beneficial for MS pathogenesis since less antigen presentation occurs. However, this effect could also be attributed to a change in B cell subtype proportions in the PB.

Next to changes in B cells proportions, fingolimod treatment led to a decrease in the proportion of peripheral naive Tconv and an increase in the memory Tconv. Our results are in agreement with previous studies showing that effector memory T cells (TEM), lacking expression of C-C chemokine receptor type 7 (CCR7), were increased in the PB of fingolimod-treated MS patients [278]. It is thought that these circulating TEM have a suppressor function and downregulate the autoimmune response [72].

Furthermore, the homeostasis and function of Treg is disturbed in MS [143, 160]. In addition to the previously described increase in percentage of Tregs in the PB under fingolimod treatment, we show that the increase in Tregs is mostly attributed to an expansion in the memory population while a decrease in naive Treg cells was observed.[264, 279]. Of note, an increase in memory Tregs could be responsible for recovery of Treg suppressive activity under fingolimod treatment as previously illustrated by our group for patients with SPMS [160]. It was already speculated that treatment with fingolimod works by both sequestering autoreactive B and T cells in the secondary lymphoid organs and by enhancing the functionality and frequency of circulating Treg [280].

As circulating T_{FH} and T_{FR} are important for a normal germinal center response [281], we investigated whether these cells are affected by fingolimod treatment in the PB of MS patients and found that fingolimod treatment did not change the percentage of circulating T_{FH} or T_{FR} cells. Recent evidence indicated that circulating T_{FH} cells consist of a CCR7^{lo}PD-1^{hi} subpopulation with an effector phenotype. Therefore these cells could be less responsive to fingolimod as observed for memory Treg and Tconv cells [282].

Although fingolimod causes entrapment of lymphocytes in the lymph nodes, expression of surface molecules involved in costimulation was increased, which could point to a gain of functionality of the remaining circulating lymphocyte subtypes, although functional assessment is needed using both in vitro and animal studies to confirm this argument. During fingolimod treatment expression of CD86 and CD80 costimulatory molecules on B cells was increased and furthermore, an increase in PD-1 expression on T_{FH} cells was observed. The percentage of $CD86^+$ B cells was increased as well during fingolimod treatment, which could be attributed to a change in B cell subtype distribution. Expression of CXCR5 on T_{FH} cells was unchanged during treatment, indicative of normal migration of these cells from the marginal zone to the follicles in the lymph nodes. Considering the beneficial clinical effects of fingolimod, we hypothesize that this increase in B and T cell costimulation and no change in migration capacity is a rescue mechanism to augment functionality of the remaining B and T cells, thereby warranting normal immunity. An additional proof of normal immune function is that vaccine specific production of IgM and IgG towards influenza A and B in fingolimod-treated individuals was not impaired when compared to levels in healthy controls [283].

Due to technical limitations and low cell numbers available for analysis, $CD8^+$ and natural killer (NK) cells were not assessed. Further limitations of the study are the lack of functional data, although we provide evidence that during fingolimod treatment expression of functionally relevant markers on the remaining B and T cell subtypes in the peripheral blood of MS patients can change.

To conclude, this study shows that fingolimod induces compositional changes of B and T cell subtypes that are potentially implicated in MS pathogenesis and may explain the therapeutic efficacy of the treatment, while altered surface expression of functional molecules on B and T cells during fingolimod treatment suggests that normal immune function may prevail, functional evidence for this has still to be provided during future research. With this descriptive study we provide additional longitudinal immunological proof for the diverse mechanisms of action of fingolimod in MS patients.

5

EFFECT OF MULTIPLE SCLEROSIS-ASSOCIATED POLYMORPHISMS IN CXCR5, IL2RA AND CD58 ON FOLLICULAR AND REGULATORY T CELL DISTURBANCES IN MULTIPLE SCLEROSIS

Abstract

Multiple sclerosis (MS) is a chronic neuroinflammatory T cell mediated autoimmune disease that leads to disability in young adults. We previously reported that the regulatory T cell (Treg) compartment is disturbed in MS (see also chapter 3). The cues responsible for these alterations are currently unclear, and could be immunological, environmental or genetic in origin. Various genetic risk variants (single nucleotide polymorphisms) were found to increase susceptibility to MS of which many are related to T cell immunity. We therefore hypothesized that genetic predisposition in genes important for the maintenance of Treg subsets (CXCR5, IL2RA and CD58) could account for disturbances seen in MS. In this study, we confirmed our previously reported findings of a decreased percentage of circulating follicular regulatory T cells (T_{FR}) in MS. MS patients in addition displayed an altered expression of CXCR5 and IL2RA on Treg subsets. Next, we assessed MS-related polymorphisms in our study population and correlated these results with the observed Treg alterations. Although we found that our research population had enough power and participants, we were unable to show a direct association between genetic predisposition and T cell homeostasis in the whole population. When focusing on healthy donors alone, preliminary results show that the percentage of circulating T_{FR} increases with expression of the CXCR5 risk allele, while the percentages decreases with IL2RA risk allele expression. Further confirmation of these initial indications is needed. Together, our study suggests that genetic defects in MS related polymorphisms important for Treg subsets cannot solely explain the immunological differences seen in MS patients.

5.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS). The precise etiology of MS is unknown but various epidemiological studies, the variability in disease frequency worldwide and the substantial increase of frequency of MS seen in relatives of patients attribute a possible role of genetics in susceptibility to MS [152, 284]. The most important risk alleles associated with MS lie within genes encoding the major histocompatibility complex (MHC) of which HLA-DRB1*15:01 has the strongest association. The identification of HLA risk alleles was based on candidate-gene-based studies. With the introduction of genome wide associations studies (GWAS), risk alleles apart from the HLA genes were identified. The GWAS and the ImmunoChip study identified 110 SNPs of which the majority lies in proximity of immune-related genes important for lymphocyte proliferation and T cell activation, adding additional proof to the hypothesis that autoimmune T cells drive MS [21]. Furthermore, recent evidence shows that genetic burden is associated with disease progression [285]. Insight into the functional contribution of MS risk-associated SNPs to immune biology is limited. So far, a few studies were performed that detected a direct link between genetic predisposition and functional changes in T cells. IL-7 receptor alterations were found to be directly linked to a MS-associated SNPs and a MS-specific SNPs in IL-2RA leads to changes in T cell homeostasis [286-290].

Among the identified genes that enhance susceptibility to MS, three can be directly linked to regulatory T cells (Tregs) and follicular regulatory T cells (T_{FR}) properties. Tregs are a specialized subsets of $CD4^+$ T cells that regulate self-tolerance while T_{FR} are a subset of Tregs that specialize in regulating B cell tolerance. CXCR5 is involved in follicular T cell migration and function, IL2RA is essential for the suppressive capacities of Tregs and CD58 costimulates and enhances T cell receptor signaling. Our group previously showed that Tregs and T_{FR} are impaired in patients with MS [145],(Chapter 3). Carriers of MS-related SNPs CXCR5 rs630923, IL2RA rs2104286 and CD58 rs1335532 could therefore have direct immunological changes in their Treg subtypes. Considering these facts, the aim of the study was to assess whether genetic predisposition of known MS-related SNPs in patients with MS could account for observed phenotypical and numeral changes seen in Tregs and T_{FR}.

5.2 Materials and methods

5.2.1 Study subjects

Peripheral blood samples were collected from healthy controls (HC) and MS patients. Clinical information is shown in Table 5.1. This study was approved by the local Medical ethical committee and written informed consents were obtained from all study subjects.

Table 5.1: Summarized clinical data of healthy controls and MS patients

		CXCR5 (n = 161)	IL2RA (n = 158)	CD58 (n = 126)
HC (n=)		60	54	31
M/F		23/37	21/33	21/10
Age (mean ± SD)		38.8 ± 15.8	39.1 ± 17.0	48.2 ± 11.0
MS (n=)		101	104	95
M/F		29/72	30/74	66/92
Age (mean ± SD)		47.5 ± 13.1	47.6 ± 11.1	48.8 ± 12.6
Type MS (n=)	CIS	2	2	1
	PP-MS	5	5	5
	RR-MS	52	54	51
	SP-MS	22	23	19
Treatment (n=)	Avonex	11	11	10
	Betaferon	3	3	3
	Copaxone	5	6	6
	Medrol	2	1	0
	MTX	3	3	3
	No treatment	53	53	43
	Rebif	5	6	6
	Tecfidera	1	1	1
	Tsyabri	10	11	11
EDSS (mean ± SD)		3.1 ± 2.0	3.1 ± 1.7	3.1 ± 1.5

HC = healthy controls, MS = multiple sclerosis, M = male, F = female, SD= standard deviation, EDSS = Expanded Disability Status Scale, CIS = clinically isolated syndrome, PP = primary progressive, RR= relapsing remitting, SP= secondary progressive, MTX = mitoxantrone

5.2.2 Cell isolation and flow cytometry

Peripheral blood was collected in heparin-coated tubes (Venosafe plastic tubes, Terumo Europe N.V., Belgium). After collection of the plasma, high density centrifugation was used to isolate peripheral blood mononuclear cells (PBMC) (Lympholyte®; Cedarlane® Laboratories, Uden, the Netherlands). For flow cytometric analysis of the T cell subsets different antibodies were used: CD4 APC, CD45RO PE-CF594, CXCR5 Alexa Fluor 488 (all from BD Biosciences, Erembodegem, Belgium), CD25 PerCP-Cy5.5, CD127 PE, PD-1 PE-Cy7, CD45RA APC-H7 (all from eBioscience, San Diego, USA). Appropriate isotypes were included for all antibodies (all from BD Biosciences, Erembodegem, Belgium). All flow cytometric analyses were performed on a FACSAriaII flow cytometer and analyzed with FACS Diva software (BD Biosciences). Of each donor a PBMC pellet of 3 million cells was made and stored at -80°C for genetic analysis.

5.2.3 Genomic DNA extraction

The ArchivePure™ DNA purification Blood Kit (5PRIME, VWR International) was used for DNA extraction. Instructions were according to the manufacturer's guidelines. The quantification, concentration and purity of the isolated DNA was determined using NanoDrop 2000 UV-Vis Spectrophotometer and NanoDrop 2000/2000c software (Fisher Scientific Belgium, Belgium).

5.2.4 TaqMan PCR

Genomic DNA diluted in TE buffer (Tris, 10 mM, VWR international; EDTA, 1 mM, VWR international, Belgium; MiliQ) was added to a MicroAmp® Optical 96-well reaction plate (Applied Biosystems, USA). After drying of the DNA, Genotyping Master Mix (Applied Biosystems), TaqMan Assay (Applied Biosystems) and MiliQ was added. Negative controls contained consisted of TE-buffer only. After preparation, the reaction plate was covered with an optical adhesive film (Applied Biosystems). PCR reaction and detection was performed by a 7300 Real-Time PCR system (Applied Biosystems) Detectors "FAM" and "VIC" were used to perform Allelic Discrimination. Background fluorescence was measured prior to the launch of PCR reaction. Data-analysis was carried out with 7300 System Software (Applied Biosystems).

5.2.5 Statistical analysis

The data set was analysed using SAS Jump and Graphpad Prims 6.0 (SAS Institute, USA, version 9.3, Graphpad software Inc, USA, version 6). After normality checks (d'Agostino-Pearson), a student's t-test was used to compare two populations. An One-way Anova test was used to compare three groups. A chi-square or fisher exact test was used to investigate the significance of association between factors and risk allele frequency. A p-value <0.05 was considered significant. Figures were plotted with Graphpad Prism.

5.2.6 Power calculation and sample size calculation

To assess whether our study population had enough power and enough participants to detect a genetic effect and achieve a power of 0.8 respectively, we performed both a power calculation and a sample size calculation for the three selected SNPs using the Quanto software (version 1.2 [291]). Briefly, we used a continuous design with independent individuals and hypothesized a gene only effect (additive) for the different SNPs. We used a type I error rate of 0.05 and a two-sided test.

5.3 Results

5.3.1 Frequencies of the candidate genes in a population of Belgian multiple sclerosis patients and healthy controls

We compared the risk allele frequencies (RAF) and genotype frequencies from our study population (healthy controls with MS patients) with the frequencies found in the European population, derived from Ensemble, to see if our population is a reflection of the general European population (Table 5.2).

Table 5.2: The risk allele frequencies (RAF) and genotype frequencies of CXCR5, IL2RA and CD58 genes in the European population (Ensemble) and our study population

Gene of interest	CXCR5	IL2RA	CD58
Chromosome	11	10	1
SNP rs number	rs630923	rs2104286	rs1335532
Alleles	A/C	C/T	A/G
Risk allele	C	T	A
RAF in European population (%)	84.0	78.0	86.0
RAF in this study population (%)	87.5	77.6	86.2
Genotype frequencies in European population (%)	AA: 2.6 AC: 26.9 CC:71.0	CC:6.9 CT:30.3 TT:62.8	AA: 74.7 AG: 23.0 GG:2.4
Genotype frequencies in this study population (%)	AA:1.9 AC:21.2 CC:76.9	CC:3.8 CT:37.2 TT:59.0	AA: 74.0 AG: 24.4 GG: 1.6

SNP = single nucleotide polymorphism, rs = Reference SNP, RAF = risk allele frequency

For all the genes of interest, we found that our population is characterized by a similar, if not identical, percentage of RAF and genotype frequencies as seen in the overall European population.

Next, we assessed whether we could detect a difference in the risk allele frequencies between HC and MS patients within our population (Table 5.3). Within our population we observed that no difference in the genotype frequencies or allele frequency could be detected between HC or MS patients for these genes. In addition, no effect in age, gender or treatment was found (p (p=0.8, p=0.13 and p=0.18 respectively). Furthermore, all polymorphisms were distributed according to the Hardy-Weinberg equilibrium and the genotype success rate was 99% for all the genes.

Table 5.3: Genotype and allele frequencies of CXCR5, IL2RA and CD58 polymorphisms in MS patients and healthy controls

CXCR5		
	HC	MS
AA	3 (5.0%)	0 (0.0%)
AC	14(23.3%)	19(19.8%)
CC	43(71.7%)	77 (80.2%)
A	20(16.7%)	19 (9.9%)
C[b]	**100(83.3%)**	**173(90.1%)**
Hardy-Weinberg equilibrium p value	0.21	0.28
Allelic p value	0.11	
OR (95% CI)	1.82 (0.92-3.57)	
IL2RA		
	HC	MS
CC	2 (3.7%)	4 (3.9%)
CT	22(40.7%)	36(35.3%)
TT	30(55.6%)	62(60.8%)
C	26(24.1%)	44(21.6%)
T	**82(75.9%)**	**160(78.4%)**
Hardy-Weinberg equilibrium p value	0.40	0.66
Allelic p value	0.66	
OR (95% CI)	1.15 (0.66-2.05)	
CD58		
	HC	MS
AA	21(67.7%)	70(76.1%)
AG	9 (29%)	21(22.8%)
GG	1 (3.2 %)	1 (1.1 %)
A	**51(82.3%)**	**161(87.5%)**
G	11(17.7%)	23 (12.5%)
Hardy-Weinberg equilibrium p value	0.97	0.67
Allelic p value	0.29	
OR (95% CI)	1.5 (0.68-3.30)	

[a]Number of cases shown with percentage of allele or genotype frequencies between brackets
[b]**Risk allele frequency depicted in bold**
HC: healthy controls, MS: multiple sclerosis, OR: odds ratio

5.3.2 Multiple sclerosis patients display a decreased number of circulating T_{FR} and an altered expression of CXCR5 and IL2RA

We previously described a decreased amount of circulating T_{FR} in MS patients (chapter 3). A subpopulation of this study population was used and we first determined whether within this subpopulation of patients and healthy donors the same is observed. Flowcytometric analysis was performed as previously shown, with T_{FR} phenotyped as $CD4^+CD25^{hi}CD127^{lo}$ $CXCR5^+PD\text{-}1^+$ and Treg as $CD4^+CD25^{hi}CD127^{lo}$ (chapter 3, Figure 3.1). We confirm a decreased percentage of circulating T_{FR} in MS patients (Figure 5.1, $p<0.0001$). Next, we assessed the CXCR5 and IL2RA expression on different regulatory T cells subsets: T_{FR} (naive $CD45RO^-CD45RA^+$ and memory $CD45RO^+CD45RA^-$) and Tregs (naive $CD45RO^-$ $CD45RA^+$ and memory $CD45RO^+CD45RA^-$). Both CXCR5 and IL2RA are essential molecules for the function and homeostasis of regulatory T cells. While no difference in CXCR5 expression (mean fluorescent intensity, MFI) was found on circulating T_{FR} (Figure 5.2A), we did find a decreased expression of CXCR5 on the Tregs of MS patients (Figure 5.2D, $p=0.001$). Furthermore, this decrease was mainly found in the memory Tregs (Figure 5.2E, $p=0.048$). In addition, IL2RA expression was increased on both Tregs and T_{FR} of MS patients compared to HC (Figure 5.3A for T_{FR} $p=0.01$ and Figure 5.3D for Tregs $p<0.001$). For Tregs this increased IL2RA expression was seen in both the memory (Figure 5.3E, $p<0.001$) and the naive compartment (Figure 5.3F, $p<0.001$) as shown previously [286] while T_{FR} only displayed this increase in the memory (Figure 5.3B, $p<0.019$) and not the naive compartment (Figure 5.3C).

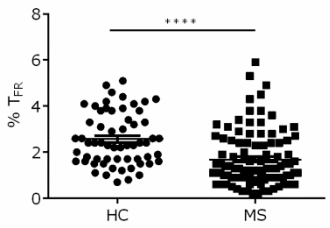


Figure 5.1: Percentage of circulating T_{FR} in healthy controls and MS patients. Percentage of circulating follicular regulatory T cells (T_{FR}) in HC and MS. Values are represented as mean ± SEM, with 60 healthy controls and 104 MS patients. Unpaired t-test was used and ****$p<0.0001$.

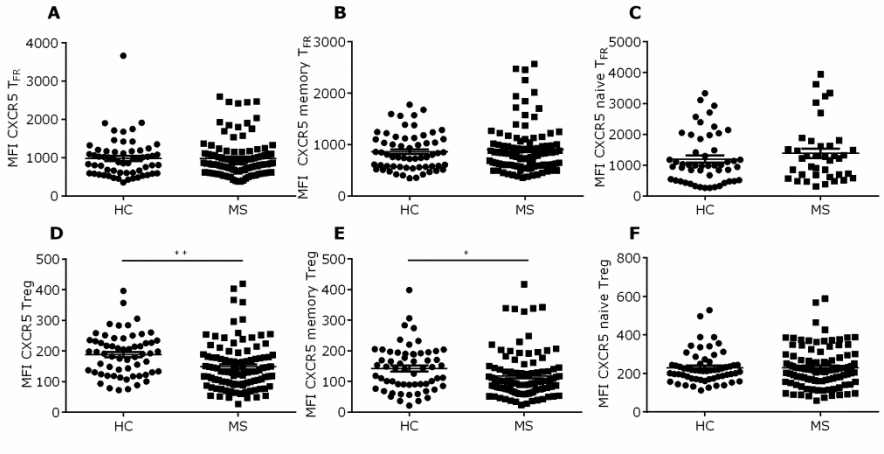


Figure 5.2: Expression of CXCR5 on circulating regulatory T cell subsets in healthy controls and MS patients. (A) Mean fluorescent intensity (MFI) of CXCR5 on circulating T_{FR} in HC and MS. **(B)** MFI of CXCR5 on circulating memory $CD45RO^{+}CD45RA^{-}$ T_{FR} in HC and MS **(C)** MFI of CXCR5 on circulating naive $CD45RO^{-}CD45RA^{+}$ Treg in HC and MS. **(D)** MFI of CXCR5 on Treg in HC and MS. **(E)** MFI of CXCR5 on circulating memory Treg in HC and MS. **(F)** MFI of CXCR5 on circulating naive Treg. Values are represented as mean ± SEM, with 60 healthy controls and 104 MS patients. Unpaired t-test was used and * $p<0.05$, ** $p<0.01$.

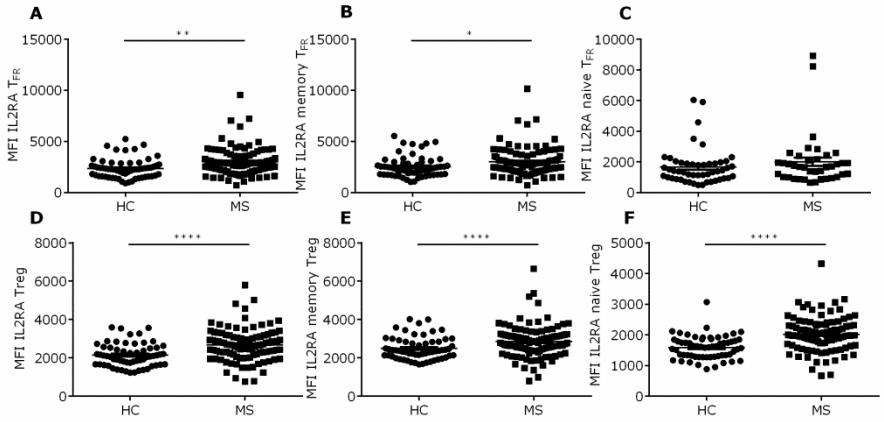


Figure 5.3: Expression of IL2RA on circulating regulatory T cell subsets in healthy controls and MS patients. (A) Mean fluorescent intensity (MFI) of IL2RA on circulating T_{FR} in HC and MS. **(B)** MFI of IL2RA on circulating memory $CD45RO^{+}CD45RA^{-}$ T_{FR} in HC and MS **(C)** MFI of IL2RA on circulating naive $CD45RO^{-}CD45RA^{+}$ Treg in HC and MS. **(D)** MFI of IL2RA on Treg in HC and MS. **(E)** MFI of IL2RA on circulating memory Treg in HC and MS. **(F)** MFI of IL2RA on circulating naive Treg. Values are represented as mean ± SEM, with 60 healthy controls and 104 MS patients. Unpaired t-test was used and * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

5.3.3 Power and sample size calculation

Using the Quanto software, we determined the sample size and power for all relevant populations (Table 5.4) based on the RAF and the observed biological effects (as shown above). We found that our population had a high power (β>0.9) and enough participants to detect a possible genetic contribution in the immunological disturbances. For the detection of a genetic effect on the MFI we found that IL2RA had a high power (β>0.8) within the T_{FR} and CXCR5 had a high power (β>0.9) within the Treg population.

Table 5.4: Sample size and power calculation[a] (β=0.80 and α=0.05) based on the risk allele frequency in this study population and the observed biological main effects

	CXCR5			IL2RA		CD58
	% T_{FR}	MFI T_{FR}	MFI Treg	% T_{FR}	MFI T_{FR}	%T_{FR}
Population (n)		161		158		126
Risk allele frequency		0.88		0.78		0.86
Power calculation	0.99	0.05	0.91	0.99	0.86	0.99
Participants needed to obtain a power of 0.8 (n=)	58	258218	116	35	133	53

[a] Sample size and power calculation performed with Quanto [291]. T_{FR}: follicular regulatory T cells, MFI: mean fluorescent intensity, Treg: regulatory T cells, n: number

5.3.4 The effect of genetic predisposition on regulatory T cell subtypes

5.3.4.1 CXCR5 risk allele leads to an increased percentage of circulating T_{FR} and a decreased CXCR5 expression in healthy donors

Since the CXCR5 rs630923 risk allele is associated with MS susceptibility [152, 292] and homeostatic alterations in T_{FR} in MS patients are present, we wondered whether genetic susceptibility could contribute to this observation. We stratified our donors based on their genotype and analyzed the percentage of T_{FR} and Tregs (Figure 5.4 A-D) and the expression of CXCR5 on T_{FR} and Tregs (Figure 5.4 E-H). For healthy donors, we found that homozygous expression of the risk allele leads to a slight increase in the percentage of circulating T_{FR} compared to heterogeneous expression (Figure 5.4 A, p=0.015). This could not be found in MS patients (Figure 5.4B). A significant effect in the percentage of total Tregs based on genotype could not be detected (Figure 5.4C-D). Moreover, we detected a significant decrease in CXCR5 expression in HC carrying the risk allele (Figure 5.4E, p=0.0029 for AC and CC). A lack of AA genotype kept us from making conclusions in MS patients (Figure 5.4F). CXCR5 expression at the total Treg level, also decreases with CXCR5 risk allele presence in HC, but not in MS patients (Figure 5.4H), although it did not reach statistical significance (Figure 5.4G).

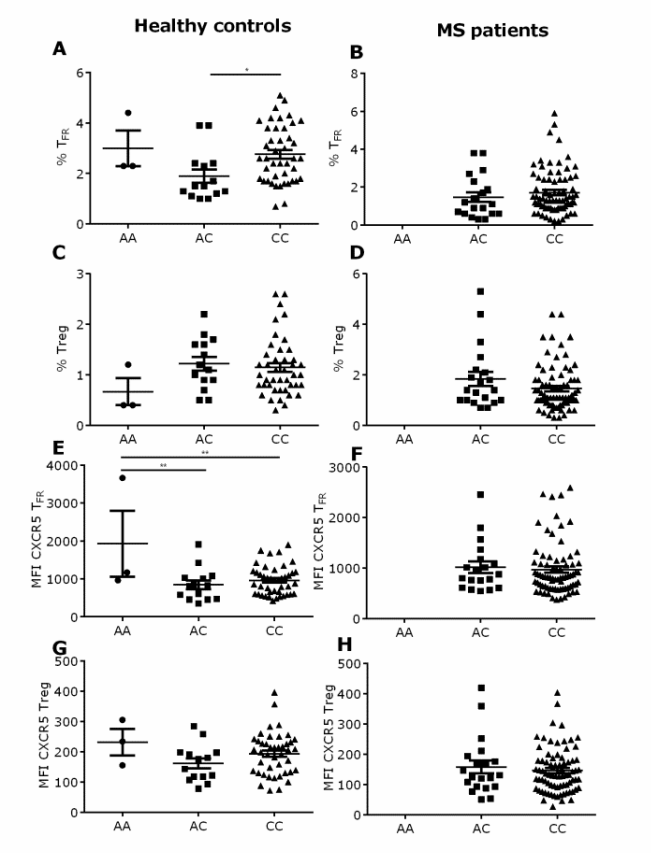


Figure 5.4 Genetic effect of CXCR5 rs630923 SNPs on percentage regulatory T cell subsets in healthy controls and MS patients with risk allele C. (A and B) Percentage of circulating follicular regulatory T cells (T_{FR}) based on genotype in healthy controls (HC) and MS patients **(C and D)** Percentage of regulatory T cells (Tregs) based on genotypes in HC and MS patients. **(E and F)** Mean fluorescent intensity (MFI) of CXCR5 on T_{FR} based on genotype in HC and MS patients. **(G and H)** MFI of CXCR5 on Tregs based on genotype in HC and MS patients. Values are represented as mean ± SEM, with AA n=3, AC n=14, CC n=43 for HC and AC n=19, CC n=77 for MS patients. One-way Anova was used and * $p<0.05$, ** $p<0.01$.

5.3.4.2 IL2RA rs210486 risk allele leads to a decreased percentage of circulating T_{FR} in healthy donors

A IL2RA rs2104286 risk phenotype is associated with various autoimmune diseases, including MS [293, 294]. We assessed the effect of this MS-related genetic variation on circulating T_{FR} (Figure 5.5 A-F) and on the expression level of IL2RA on regulatory T cells subsets (Figure 5.5 G-L). We found a decreased percentage of circulating T_{FR} in HC with homozygous expression of the T risk allele (Figure 5.5A, p=0.02). This effect was not detected in MS patients (Figure 5.5B). Furthermore, healthy carriers of the risk allele, but not MS patients (Figure 5.5 D and F respectively), displayed an increased percentage of memory T_{FR} (Figure 5.3C, p=0.016 for CT and TT) together with a decreased percentage of naive T_{FR} (Figure 5.5E p=0.022 for CT and TT). In addition, we found a significant increase in IL2RA expression of naive Tregs as shown previously [286], but only in HC (Figure 5.5K, p=0.043 and Figure 5.5L). For circulating T_{FR} and naïve T_{FR} of HC, no effect was detected (Figure 5.5G and I).

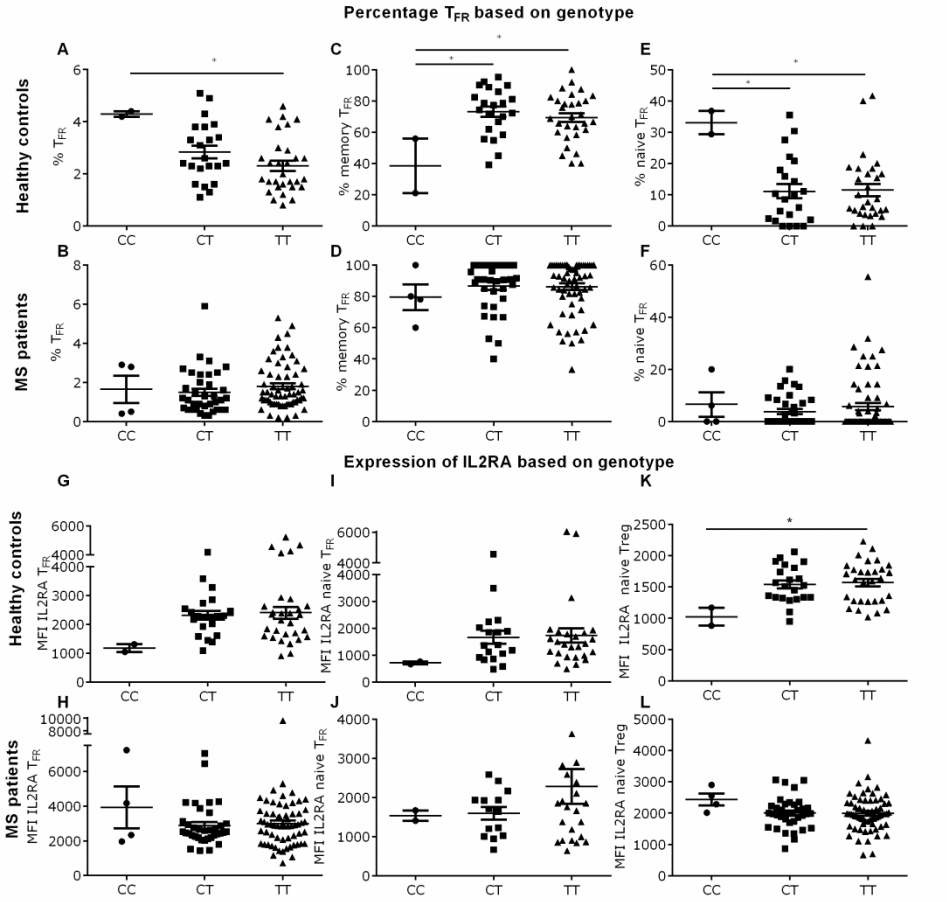


Figure 5.5: Genetic effect of IL2RA rs2104286 SNPs on percentage regulatory T cell subsets with risk allele T. (A and B) Percentage of circulating follicular regulatory T cells (T_{FR}) based on genotype in healthy controls (HC) and MS patients **(C and D)** Percentage of memory $CD45RO^{+}CD45RA^{-}$ T_{FR} based on genotype in HC and MS patients **(E and F)** Percentage of naive $CD45RO^{-}CD45RA^{+}$ T_{FR} based on genotype in HC and MS patients. **(G and H)** Mean fluorescent intensity (MFI) of IL2RA on T_{FR} based on genotype in MS patients and HC. **(I and J)** MFI of IL2RA on naive T_{FR} based on genotype in MS patients and HC. **(K and L)** MFI of IL2RA on naive regulatory T cells (Tregs) based on genotype in MS patients and HC. Values are represented as mean, with CC n=2, CT n=22, TT n=30 for HC and CC n=4, CT n=36, TT n=62 for MS patients. One-way Anova was used to asses differences with * $p<0.05$.

5.3.4.3 CD58 rs1335532 genotype does not affect T_{FR} nor Treg percentages

A SNPs rs1335532 in the CD58 gene was found to be associated to MS disease [294]. Furthermore, an increased expression of CD58 in carriers of the protective allele was reported to enhance Treg function [295]. In our study we investigated the effect of the risk genotype on the percentage of T_{FR} (Figure 5.6A and B) and total Tregs (Figure 5.6C and D) in HC an MS patients but did not observe any effect.

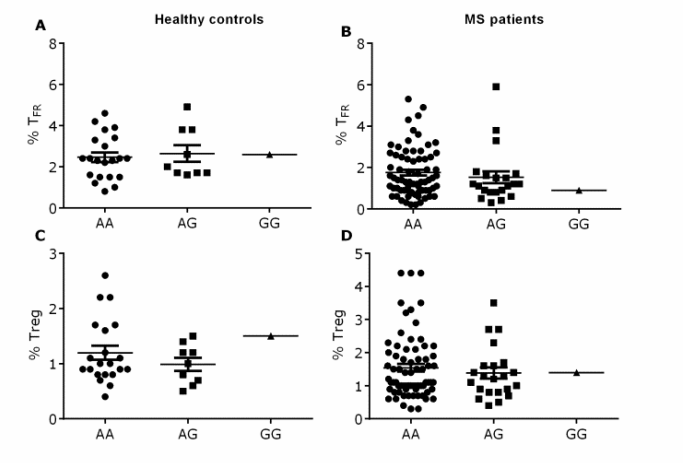


Figure 5.6: Genetic effect of CD58 rs1335532 SNPs on percentage regulatory T cell subsets. (A and B) Percentage of circulating follicular regulatory T cells (T_{FR}) based on genotype in healthy controls (HC) and MS patients **(C and D)** Percentage of circulating Treg based on genotype in HC and MS patients**.** Values are represented as mean, with n=21, n=9 and n=1 for HC and n=70, n=21 and n=1 for MS patients for AA,AG and GG respectively. One-way Anova was used.

5.4 Discussion

Although susceptibility to MS cannot be explained by one genetic defect, a wide range of epidemiology studies support genetic predisposition in MS through the contribution of various genes [19]. This fact, together with the observation that a very large proportion of these genes are essential in T cell immune homeostasis suggests that genetic factors can modulate the immune response and contribute to the development of autoimmunity in MS.

Considering this hypothesis, the present study was carried out to determine whether genetic variants within immune-related genes may have a functional role in T cell homeostasis in patients with MS. Since our previous study detected an impairment in T_{FR} in patients with MS we aimed to assess whether differences seen in regulatory T cell subsets could be explained by the presence of MS related SNPs in T cell related genes. We selected SNPs in CXCR5, IL2RA and CD58 and assessed whether they contribute to disturbances seen in T_{FR} and Tregs of MS patients and in addition also in HC.

Before interpreting genetic effects on frequency and phenotype of lymphocytes, we thoroughly analyzed our study population. First, we showed that the RAF and genotype frequencies of the three SNPS were in agreement with the general European population. Next, we compared the RAF of HC with MS patients and found no significant difference for CXCR5, IL2RA and CD58. Cavanillas et al. showed that the frequency of the three identified risk alleles for IL2RA is increased in MS patients. We confirmed the decreased percentage of the minor allele frequency of IL2RA rs rs2104286 in HC compared to MS, as shown by Cavanillas et al., but it did not reach significance [293]. Third, we used sample size determination and power calculations to show that our study had a high power. Lastly, we showed that the decreased T_{FR} frequency found in a large cohort of patients could also be detected in this sample population. Together, all these findings indicate that our study group was a good representation of the general population, included enough persons and had the same immunological disturbances making it a competent sample population to test our hypothesis.

CXCR5 is a chemokine receptor essential for the migration of follicular T cells and B cells toward the germinal centers in secondary lymphoid organs (SLO).

In the GWAS study of 2011, strong evidence was found for an association of the SNP (rs630923) in proximity of the CXCR5 gene region MS ($p=2.8x10^{-07}$) but there was no sub-genome-wide significance [152, 296].

Although this SNP was not included in the ImmunoChip [292], the International Multiple Sclerosis Genetics Consortium (IMSGC) further investigated this SNP in another large and independent data set and found statistically significant evidence for association with MS confirming it as a genuine MS susceptibility locus [152, 297, 298]. T_{FR}, characterized by high expression of CXCR5, are essential during B cell maturation in the germinal center (GC) response [128, 190]. Since CXCR5 is essential for migration of T_{FR} to the GC a SNP in the CXCR5 gene may thus affect T_{FR} homeostasis [299]. We have indications that HC homozygous for the CXCR5 risk allele displayed a reduced CXCR5 expression on T_{FR} paralleled with an increased frequency of circulating $T_{FR.}$ Possibly, the reduced CXCR5 expression limits T_{FR} migration to the GC leading to a rise in circulating T_{FR}. This effect was no longer seen in MS patients, suggesting that during auto-immune inflammation genetic effects on T cell homeostasis are masked by non-genetic disease factors. Alternatively, compensatory mechanisms may be active during MS disease that restore Treg imbalances as evidenced by the increased proportion of memory Treg cells in MS, in line with a previous report by our group [160].

The alpha chain of the CD25 receptor, or IL2RA, is essential for an optimal functioning of Tregs and is upregulated on activated $CD4^+$ T cells. Tregs and therefore also the T_{FR} phenotype is among others defined by high CD25 expression levels. Moreover, capture of IL2 by this receptor is one mechanism used by Treg to control effector T cells. Association of the IL2RA SNP rs2104286 with MS was first established by the WTCC in 2007 [300] and subsequently confirmed in various GWAS studies [298, 301-304]. Also, in the recent ImmunoChip study the association with this risk locus was again confirmed. Functionally, this SNP causes alterations in membrane expression [290] and in the soluble form of the receptor in patients with MS [305-307]. In addition, the risk allele of this SNP correlated with a decreased IL-2 responsiveness of $CD4^+CD25^{hi}$ T cells. A low number of minor allele homozygotes thoroughly limits the conclusions that can be drawn from this study.

However, we show that HC homozygous for the risk allele displayed reduced frequencies of circulating T_{FR} and have an increased expression of IL2RA. On the other hand, we confirm an increasing trend in the percentage of naive Tregs associated with the risk allele in HC as previously described [290] (p=0.3, data not shown). These results could suggest that Treg numbers and expression of IL2RA increases in risk allele carriers to contemplate for the reduction seen for T_{FR}. We did not detect any effect in MS patients, again indicating that compensatory mechanisms may mask genetic influences on T cell homeostasis in patients with MS.

CD58 or lymphocyte function-associated antigen 3 (LFA-3) binds to CD2 on T cells and subsequently induces activation and adhesion of T cells. Functionally, CD58 expression leads to an increased expression of Foxp3 on Tregs and this enhances their function [295]. In 2009, the rs2300747 in the CD58 locus was shown to be the most associated genetic variant to MS susceptibility, with the protective allele G leading to a higher expression of CD58 RNA in mononuclear cells of RRMS patients [295]. In the GWAS, the SNP, rs1335532, (who tags the known association in the CD58 locus rs2300747[295])[294] was found to be associated with MS ($p=2.00x10^{-09}$). The ImmunoChip study confirmed the association of this SNP with MS. A previous study concerning MS-related CD58 SNPs reported a change in Foxp3 expression and CD58 mRNA levels in Tregs leading to functional changes [21]. In our study, we analyzed the effect of the CD58 SNP on percentage of circulating Tregs and $T_{FR,}$ but did not observe any effect. Possibly this SNP mainly has functional effects without influencing percentages of Tregs and T_{FR}. Future studies should assess this hypothesis.

To conclude, this study provides a first step towards the functional interpretation of MS associated SNPs in T cell related genes and its effect on Treg biology in health and disease. While this pilot study showed that the studied risk variants can affect Treg frequency and phenotype, this could not be confirmed in MS. Nevertheless, it is interesting that the major genetic effects are seen within the HC group, indicating a possible heralding of the immunological effect of genetic predisposition. Based on our study, we therefore propose that genetic influences are not the sole cause of Treg alterations in MS.

This study holds two main weaknesses. First, the lack of high number minor allele homozygotes preludes us from making any hard conclusions. Second, it lacks functional *in vitro* data to investigate whether genetic risk variants affect migration and suppressive capacity instead of numbers of regulatory T cell subsets. Indeed functional Treg impairment patients is evident in MS [308, 309], future studies need to combine *ex vivo* measurement with *in vitro* functional assays to identify a possible impact of genetic MS risk variants on immune cell function in MS.

6

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

6.1 Summary

The immune system is considered to be the main player in the pathogenesis of MS [6]. Alterations in Tregs of MS patients, that normally maintain self-tolerance, have been widely described [142, 143]. In this thesis, we investigated disturbances of follicular helper and regulatory T cells in MS, together with the effect of treatment and genetic predisposition. In the following paragraphs, our main results are summarized (see also Figure 6.1).

6.1.1 Circulating follicular T cells are a memory population relevant to investigate follicular T cell immunity in humans

In humans, blood is the most accessible source to study follicular T cells. Since genuine follicular T cells largely reside in the GC, we first determined the relevance of using circulating follicular T cells to study follicular T cell immunity.
In **Chapter 3** we compared the phenotype and function of blood derived follicular T cells with follicular T cells from SLO (tonsils) of healthy donors. We identified T_{FR} as $CD4^+CD25^{hi}CD127^{lo}CXCR5^+PD\text{-}1^+$ within the Treg compartment and T_{FH} as $CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$ within the Tconv compartment.
First, we showed that circulating T_{FH} and T_{FR} have lower expression levels of the follicular markers CXCR5, PD-1 and SAP. Moreover, they lack expression of ICOS and Bcl-6. In contrast, we found that blood T_{FR} express similar levels of regulatory markers, Foxp3 and Helios, compared to their GC counterparts. In addition, we found that the methylation status of Foxp3 within Tregs, T_{FH} and T_{FR} is similar across the two compartments. Different effector phenotypes can be distinguished in blood derived follicular T cells based on the chemokine receptors CXCR3 and CCR6 [234]. In our study, we showed that blood derived T_{FR} have increased percentages of T_H1 ($CXCR3^+CCR6^-$) and T_H17 ($CXCR3^-CCR6^-$) like cells, while T_H2 ($CXCR3^-CCR6^+$) cells were decreased compared to T_{FR} derived from the tonsils. All follicular T cells in human blood are $CD45RO^+$, indicating a memory phenotype. Furthermore, we show that follicular T cells in tonsils are an effector memory ($CCR7^-CD62L^-$) population, while blood comprises a more heterogeneous population of both effector memory and central memory ($CCR7^+CD62L^+$) cells.

We further demonstrated that human T_{FR} from blood and tonsils displayed the same suppressive capacity towards Tconv. Seasonal influenza vaccination boosted the percentage of blood follicular T cells.

Together, these results show that while blood follicular T cells are phenotypically distinct from GC derived follicular T cells, they are equally suppressive, have a memory phenotype and increase after a GC response making them a relevant population to investigate follicular T cell alterations in humans.

6.1.2 Circulating follicular regulatory T cells are impaired in multiple sclerosis

Due to technical and ethical considerations, it is difficult to assess the implications of GC T_{FR} in human autoimmunity. Therefore, looking into the blood compartment of T_{FR} opens up new and promising prospects. Various autoimmune diseases are characterized by an increase in pathogenic autoantibodies. Since B cell autoreactivity can result from aberrant GC responses (reviewed in **Chapter 2** [310]), it is of interest to study changes in circulating T_{FH} and T_{FR} in humoral autoimmune diseases.

In our study, we defined the frequency, phenotype and function of circulating T_{FR} and T_{FH} in MS patients and compared them with healthy controls. In **Chapter 3** we report a significant decrease in frequency of circulating T_{FR} in patients with MS. Moreover, we showed that $CXCR3^{-}CCR6^{+}$ T_{FR} (T_H17-like cell) and $CXCR3^{-}CCR6^{-}$ (T_H2-like cells) blood T_{FR}, are increased in MS patients. In addition, we described a significant functional *in vitro* impairment of circulating T_{FR} in patients with MS. This impairment was positively associated with a Treg dysfunction. Lastly, we compared the percentage and effector phenotype of circulating T_{FH} between HC and MS patients but could not find a difference.

Taken together, our results show that patients with MS have a numeral and functional impairment in their circulating T_{FR} suggesting that their ability to suppress autoreactive B cell responses is impaired resulting in breakdown of B cell tolerance.

6.1.3 Genetic polymorphisms in the MS-associated genes CXCR5, IL2RA and CD58 do not explain frequency or phenotype changes of follicular T cell subsets in MS

While MS is not a heritable disease, susceptibility can in part be explained by genetic predisposition. Various GWAS studies together with replication studies of the ImmunoChip study identified and confirmed MS-related polymorphisms in the genes of CXCR5, IL2RA and CD58. These genes all directly link to phenotype and/or function of Tregs and T_{FR}. We investigated whether immunological changes in Tregs and T_{FR} frequency and phenotype can be explained by a MS-linked genetic predisposition of CXCR5, IL2RA and CD58.

In **Chapter 5** we confirmed the decreased percentage of circulating T_{FR} and genotyped this population for the known MS SNPs rs630923 (CXCR5), rs2104286 (IL2RA) and rs1335532 (CD58). We have indications that HC homozygous for the CXCR5 risk allele displayed a reduced CXCR5 expression on T_{FR} paralleled with an increased frequency of circulating $T_{FR.}$ The reduced CXCR5 expression could decrease T_{FR} migration to the GC resulting in a rise of circulating T_{FR}. However, these observations were no longer present in MS patients. This could indicate that possibly compensatory mechanisms restore Treg imbalances, as evidenced by the increased proportion of memory T_{FR} cells in MS, thus concealing the genetic effect. HC homozygous for the risk allele of IL2RA had indications of a reduced frequencies of circulating T_{FR} and Tregs, together with an increased percentage of memory T_{FR} and a decreased percentage of naive $T_{FR.}$ This effect was again not evident in the MS group, recurrently showing that compensatory mechanisms could mask genetic influences on T cell homeostasis in patients with MS. No effect of the CD58 SNP on percentage of circulating Tregs and $T_{FR,}$ was found, possibly due to the lack of functional association of Tregs and T_{FR} next to numeral assessments.

6.1.4 Treatment of MS patients does not alter the frequency of follicular T cell subsets

Untreated MS patients had a significant decrease in circulating T_{FR}. To see whether treatment can boost or restore the T_{FR}/T_{FH} balance, we compared the effect of various current MS treatments, both DMT and specific therapies, on T_{FR} and T_{FH} numbers.

In **Chapter 3**, we found that treatment did not have a significant effect on the frequency or phenotype of blood follicular T cells. In addition, in **Chapter 4** we longitudinally monitored T_{FR} and T_{FH} numbers in patients on fingolimod over 1 year but did not observe any effect. We did find an increase in PD-1 expression on blood T_{FH} during treatment. These results show that current treatment strategies have no effect on follicular T cell numbers in MS.

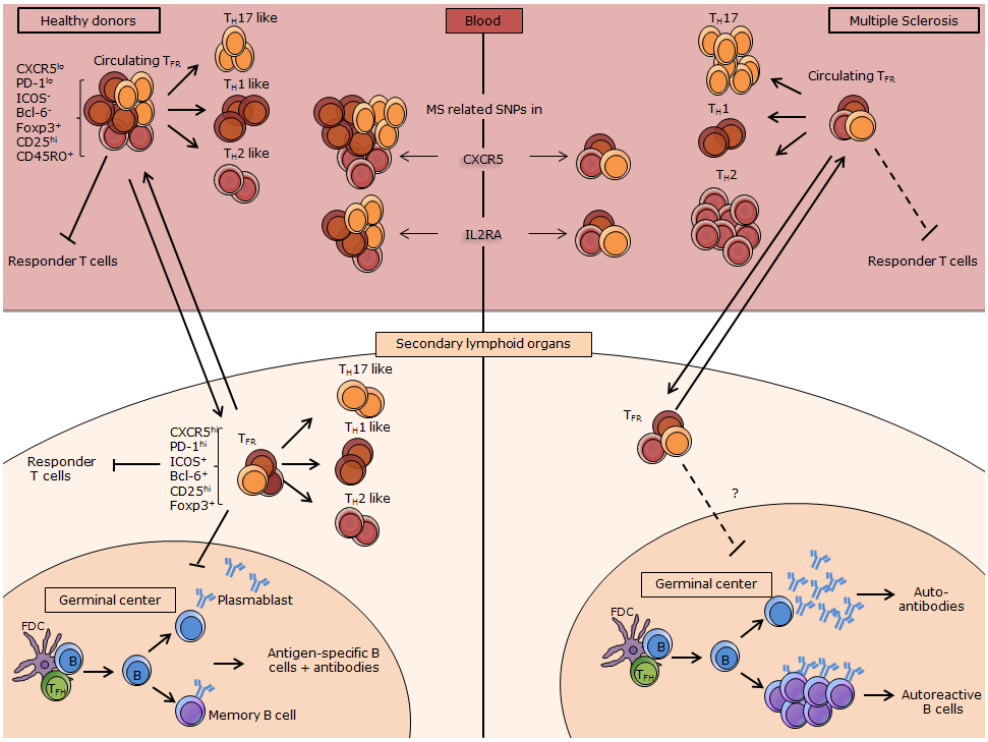


Figure 6.1: Schematic representation of T_{FR} and circulating T_{FR} in healthy donors (left) and MS patients (right). In healthy donors **(left panel)** the T_{FR} from tonsils (secondary lymphoid organs, SLO) were compared with T_{FR} from blood, based on phenotype and function. T_{FR} from blood have a decreased follicular phenotype and are predominantly memory cells. Both tonsil derived T_{FR} and blood derived T_{FR} comprise different effector populations based on CXCR3 and CCR6 expression. T_H1-like cells are CXCR3$^+$CCR6 (dark orange), T_H17-like are CXCR3$^-$CCR6$^-$ (orange) and T_H2-like are CXCR3$^-$CCR6$^+$ (red) cells. Circulating T_{FR} have a larger proportion of T_H17-like and T_H1-like cells compared to T_{FR} from SLO. Both population have an equal suppressive capacity to suppress responder T cells. T_{FR} from healthy donors are capable of regulating the germinal center (GC) response leading to antigen-specific B cells. In MS patients **(right panel)** the frequency of circulating T_{FR} is decreased compared to HC. Furthermore, MS patients have a larger percentage of T_H17- and T_H2-like cells compared to healthy donors. Circulating T_{FR} from MS patients a not capable of suppressing responder T cells (dotted line). A dysfunctional T_{FR} homeostasis in MS could contribute [?] to an altered GC response (dotted line) leading to autoantibodies and autoreactive B cells. A MS-related SNP **(middle)** in CXCR5 (rs630923) leads to a increased percentage of circulating T_{FR} while a SNP in IL2RA (rs2104286) leads to a decreasedpercentage in HC. The percentage of circulating T_{FR} in MS patients is not affected by genetic predisposition. Abbreviations: T_{FR} : follicular regulatory T cells, T_H: T helper cells, T_{FH}: follicular helper T cell.

6.2 General discussion and future perspectives

In this paragraph, we address four prominent issues related to T_{FR}. We highlight our contribution to these questions and relate these to literature. Finally, future experiments are proposed for outstanding uncertainties.

6.2.1 What is the origin of circulating T_{FR} and are they functionally competent?

Since 1994, when $CD4^+CXCR5^+$ T cells were first described in human blood [311], much research has focused on the characterization of circulating T_{FH} in humans. We were the first to address the features of human circulating T_{FR}.
One relevant aspect to address is where these circulating T_{FR} originate from. In a normal GC response, thymic-derived Tregs migrate towards the T cell zone and receive the necessary stimuli to upregulate essential follicular molecules such as Bcl-6, SAP and CXCR5 expression resulting in a differentiation into a follicular regulatory phenotype capable of suppressing the GC response. But what happens to these cells after the GC? Do they migrate to the circulation and hence represent a circulating memory population derived from GC-experienced T_{FR}? Alternatively, circulating T_{FR} may originate directly from thymic-derived Tregs or derive from precursor follicular cells that have not yet entered the GC. Although these three options (Figure 6.2) are plausible, results from our study point to a memory GC-experienced phenotype that is long-lived and can recirculate towards the SLO. Indeed, we found that almost all circulating T_{FR} have a memory phenotype ($CD45RO^+$). Furthermore, we found no difference in the percentage of demethylated Foxp3 or Helios expression between the circulating and lymphoid resident populations. Our results thus indicate that blood T_{FR} are a memory population that is in origin thymic-derived and derives from the same lineage commitment as tonsil-resident T_{FR}. For circulating T_{FH} we found that that they are also predominantly memory cells, in line with other reports [229, 312, 313]. Studies in patients with follicular deficiencies provide further insight into the possible origin of circulating follicular T cells. Patients that suffer from CD40L or ICOS deficiencies have a decreased amount of blood T_{FH} indicative that these cells come from GC experienced cells [314], while patients with SAP deficiency do not have a decreased percentage of blood T_{FH}.

The latter study suggests that blood T_{FH} cells do not originate from bona fide T_{FH}, but from cells that are generated before a GC response is established [229]. Of note, it is also possible that both options are valid and that the origin of blood follicular T cells is manifold. We found that T_{FR} express higher levels of CCR7 and CD62L compared to GC derived follicular T cells and comprise both effector memory and central memory cells governing them the possibility to recirculate towards the SLO. In mice, blood T_{FR} were demonstrated to recirculate towards the germinal centers to interact with GC B cells [230]. In addition, it was shown that T_{FR} in mice are long-lived and human $CD4^+CXCR5^+$ T cells have a stable follicular phenotype and remain antigen-specific for several years [230, 233, 315, 316]. We conclude that circulating T_{FR} are most likely a memory population generated from GC-experienced T_{FR} that are stable and can recirculate towards the SLO.

The next logical question is whether this long-lived population is functionally competent. Our results indicate that blood T_{FR} are a quiescent but fully functional population that quickly reacts when a GC response is present. Indeed, we showed that blood T_{FR} are positive for CD31 and negative for ICOS, in contrast to GC-derived T_{FR}. Since CD31 expression inhibits T cell activation and the interaction with B cells and ICOS is a activation marker, these findings thus indicate that blood T_{FR} are a memory population at rest [246]. We found that the same is true for blood T_{FH} and these results are supported by others [317, 318]. We also showed that human T_{FR} from blood and tonsils displayed the same suppressive capacity towards T responders. In mice, Sage et al. showed that circulating T_{FR} suppressed B cells, but to a lesser extent then LN T_{FR}. This is peculiar since they also found that blood T_{FH} were more potent effector cells then LN T_{FH}. Still, the total $CXCR5^+$ T cell population from blood was more competent to enhance B cell responses compared to LN $CXCR5^+$ T cells. In addition, we found that circulating and tonsil-derived human T_{FR} express the same amount of Foxp3. These results are in line with those reported in mice [230]. In addition, we found a significant increase in blood T_{FR} after vaccination, next to a increase in blood T_{FH} as demonstrated previously [229, 232] underlining the functional and responsive feature of blood follicular T cells. Moreover, we found a positive association between influenza-specific antibodies and the percentage T_{FR}.

Of note, both within T_{FR} and T_{FH} we found a major increase in the memory compartment after vaccination, indicating that blood follicular cells derive from effector follicular cells and become a memory population that circulates the body. Together, it seems that T_{FR} from the blood are as functional as genuine effector $CXCR5^+$ T cells.

The notion that blood circulating follicular T cells are not identical to lymphoid resident follicular T cells comes mainly from their phenotypical differences. Since deficiencies in essential follicular molecules lead to a decrease in blood follicular cells and based on their antigen-experienced memory phenotype we can conclude that blood follicular T cells originate from GC-experienced follicular T cells and survive as long-lasting quiescent fully functional memory cells.

Future research should focus on a more detailed characterization of human blood T_{FR} to gain more insight into their phenotype and function. For instance, CCR7, and ICOS should be included and combined to make a distinction between resting central memory cells and circulating T_{FR} with a partial follicular phenotype. Second, the cytokine profiles of the T helper effector subtypes (T_H1,T_H17 and T_H2) based on CXCR3 and CCR6 should be assessed in circulating T_{FR} to confirm if these markers are indeed associated with a specific effector phenotype [229, 242, 243, 319]. Third, it would be interesting to further investigate the functional properties of circulating T_{FR}. Although we showed that circulating T_{FR} are functional *in vitro*, the exact mechanism of suppression remains to be elucidated. More specifically, surface molecules such as CTLA-4 and the cytokine IL-10 which were proven to be essential for T_{FR} function in mice should be assessed [179, 188]. In addition, assessing the suppressive capacity of T_{FR} toward T_{FH} and B cells would highlight whether T_{FR} mainly function via regulating T_{FH} or can also suppress B cells directly. One of the most striking findings was the expression of CD31 on circulating T_{FR}. Since CD31 is generally known as a marker for recent thymic migrants it is puzzling why these memory cells are positive, while follicular T cells in the SLO are negative. To confirm that circulating T_{FR} are a mature and quiescent population instead of cells directly coming from the thymus, measurements of T-cell receptor excision circles (TRECs) in these cells together with measurement of CD31 expression levels after activation could provide solid evidence on these aspects.

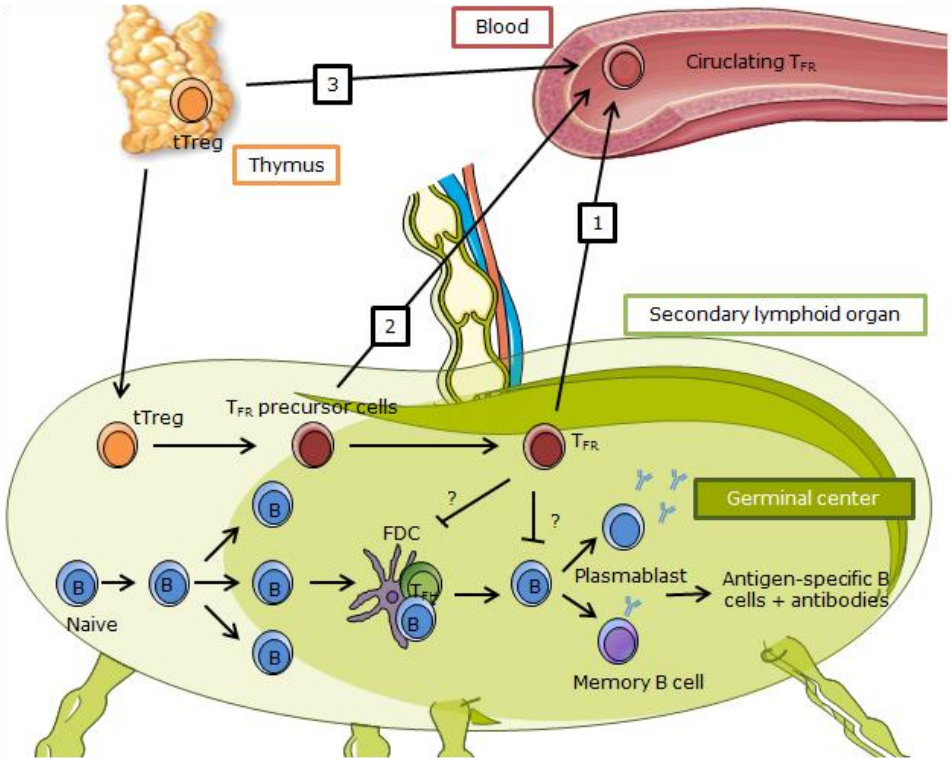


Figure 6.2: Schematic representation of possible origins of circulating follicular regulatory T cells. Follicular regulatory T cells (T_{FR}) originate from thymic-derived Tregs (tTregs) that differentiate in secondary lymphoid organs (SLO) during a GC response. T_{FR} suppress the germinal center response either by suppressing follicular helper T cells (T_{FH}) or B cells or both leading to antigen-specific B cells and antibodies. Currently, the origin of circulating T_{FR} is unknown. We suggest three possible mechanisms. (1) Circulating T_{FR} are an antigen-specific memory population derived from fully differentiated effector T_{FR} that migrates to the blood after the GC is terminated but capable of re-entering the SLO upon a second exposure. (2) Circulating T_{FR} are a precursor population derived from T_{FR} cells that existed the GC response prior to full development into genuine T_{FR}. (3) Circulating T_{FR} originate and differentiate directly from tTregs and obtain their follicular phenotype in the periphery.

6.2.2 What is the functional consequence of an altered circulating follicular T cell homeostasis in MS?

While MS is not a typical autoantibody driven diseases, several lines of evidence indicate a crucial role for B cells in the disease process. First, depleting B cells with anti-CD20 antibodies reduces the number of brain lesions [320]. Second, ectopic B cell follicles are present in the MS brain lesions and their development is linked to diseases severity [158].

Using mouse models, it became apparent that specific deficiencies in T_{FR} [128] or essential T_{FR} molecules [185, 236] are associated with the development of B cell driven autoimmunity. In humans, a disturbed homeostasis of circulating follicular T cells was shown to drive various humoral autoimmune diseases [15, 219, 221, 229, 234, 321-327]. In this study, we investigated if follicular T cells are disturbed in MS.

We describe for the first time that circulating T_{FR} are decreased in patients with MS. This decrease could originate from a reduced migration of T_{FR} towards the blood or reflect an overall decrease. Either way, a decreased amount of T_{FR} may reduce the regulation of non-antigen specific outgrowth of B cells. Furthermore, we showed that $CXCR3^{-}CCR6^{+}$ blood T_{FR} (T_H17-like cell) and $CXCR3^{-}CCR6^{-}$ blood T_{FR} (T_H2-like cells), are increased in MS patients. Previous studies on T_{FH} cells showed that $T_{FH}2$ and $T_{FH}17$ cells are also increased in human autoimmunity [326, 327]. T_H2 cells stimulate humoral immune response, while T_H17 cells induce a pro-inflammatory environment and are linked with autoimmune diseases. These results thus support the hypothesis that T_{FR} from MS patients are phenotypically directed towards humoral autoimmunity. In addition, we described a significant functional *in vitro* impairment of circulating T_{FR} in patients with MS. No difference in percentage nor effector phenotype of circulating T_{FH} was found between HC and MS patients. So far, only one group has also focused on circulating T_{FH} in MS. They found an overall increase in $ICOS^{+}$ blood T_{FH}, a decrease in $T_{FH}1$ cells, and an increase in $T_{FH}17$ cells [223]. These inconsistent results could be explained by the usage of ICOS as a marker. Since ICOS is only expressed by a minor part of blood T_{FH} cells and is correlated with an activated phenotype [229] it seems that only recently activated circulating T_{FH} are increased in patients with MS.

To find out whether T_{FR} disturbances affect B cell immunity in MS, we associated our follicular T cell results with the percentage of relevant B cell subtypes, namely plasma blasts, plasma cells and class-switched B cells, but could not detect a clear association (data not shown). While there was no effect on the amount of B cells, T_{FR} impairment may impact the nature of the B cell response and boost the production of autoantibodies. These issues should be further investigate using *in vivo* mouse models to support direct causality. Knocking out T_{FR} or essential T_{FR} molecules in EAE will provide direct *in vivo* evidence on the involvement of T_{FR} in humoral disease related processes. Of interest, a recent study showed that treatment with CXCL13 antibodies improves disease course of EAE by interfering with B cell immunity [328]. An alternative approach to illustrate a direct involvement of T_{FR} in humoral autoimmunity is to correlate functional and numeral impairment of T_{FR} with the levels of circulating autoantibodies in patients with MS. Lastly, post-mortem analyzes of MS brain tissue could provide additional proof.

Many autoimmune diseases, including MS, exhibit inflammatory GC-like structures at the disease site called ectopic GC. Ectopic GC in the brain contain B cells, and CXCL13 producing follicular dendritic cells and are thought to be the major source of autoantibodies in MS [158, 329]. Examining *ex vivo* post-mortem tissues with ectopic follicles will illustrate whether follicular T cells migrate towards the brain to dampen autoantibody responses.

So far, no study has been performed to link blood follicular T cells with follicular T cells in lymphoid organs and/or ectopic GC in MS. Although technically and ethically challenging, these studies could provide definitive and conclusive evidence of a role of both T cell compartments in MS.

6.2.3 What could cause the impaired T_{FR} functionality in MS?

Our group and others identified a functional defect in Tregs in RRMS patients. In this study, we confirmed this defect and found that T_{FR} are also functionally impaired. Various plausible explanations have been suggested that could cause this functional defect in MS [239], some of which are also highly applicable for T_{FR} dysfunction. First, a loss of function of Tregs in MS is correlated with a decrease in Foxp3 and CTLA-4 expression [143, 148].

Since both molecules are essential for T_{FR} function [128, 188] a decreased expression could explain this defect.

Second, a functional exhaustion of regulatory function due to chronic inflammation could lead to an impaired suppression. This hypothesis is supported by the presence of a higher frequency of memory Tregs next to an impaired generation of naive Tregs in MS patients [142, 150, 160]. We confirm this increase of memory Tregs and decrease of naïve Tregs and also found this altered distribution in T_{FR}. Moreover, human Tregs can interfere with $Ca2^{+}$ signaling during cell-contact dependent suppression of effector T cells and this is mainly mediated by naive CD45RA Tregs. A lower frequency of naive Tregs and naive T_{FR} as reported in our study could thus explain the loss of suppressive function [330]. Third, Treg plasticity can also account for the observed defects [151]. We found that patients with MS had more T_{FR} with a T_H17- and a T_H2-like phenotype compared to healthy donors. More recently, a fourth unrecognized mechanism was suggested that could explain Treg impairment in MS. It was shown that the proliferation of Tregs after TCR stimulation is defective in RRMS patients as a result of altered STAT5 signaling [153]. Fifth, genetic factors could also be responsible for an impairment of Tregs and T_{FR} thus revealing developmental issues. Previous studies already showed a functional link between a genetic polymporphism in CD58 and Treg function in MS [295]. In our study, we found a alteration in T_{FR} percentages in healthy carriers of genetic risk alleles in IL2RA and CXCR5 indicating that genetic predisposition leads to homeostatic changes in T_{FR}. Future genetic studies may include other MS-related genes implicated in follicular T cells homeostasis such as IL12A, IL12B, and IRF8 together with functional suppressive and migration assays.

Lastly, environmental factors such as vitamin D metabolism and sodium chloride have been found to correlate with Treg function in MS [331, 332]. This is of high importance in the context of microbiota and autoimmunity [333]. T_{FR} were found to sustain microbiota diversity through IgA regulation providing a link between environment and T_{FR} function [334, 335].

6.2.4 Why are follicular T cells unaffected by current MS treatment strategies?

The cause of MS is unknown but the immune system is a vital player in the pathogenesis. Therefore, the majority of the current treatments focus on tempering the immune system to dampen the number of relapses and new lesions.

Treatment with DMT such as IFN-β1b, IFN-β1a (Avonex®, Betaferon®, Rebif®, Extavia®) or glatiramer acetate (Copaxone®), did not affect the percentage of circulating follicular T cells in MS patients. It was shown that IFN-β and GA can directly affect T cell metabolism, function, reactivity against MBP and effector distribution but not the amount of various T cells [336-340]. Therefore, it seems that DMT do not have a numeral but rather functional effect, explaining the lack of effect on percentage of follicular T cells seen in this study.

Both natalizumab (Tysabri®) and fingolimod (Gilenya®) target specific immunological processes in humans. Treatment with natalizumab limits migration of activated leukocytes to the CNS by blockage of VLA-4, consequently increasing the amount of circulating immune cells [341, 342]. VLA-4 expression is highest on B cells and NK cells leading to a numeral effect on these subtypes and only minor effects on T cells [342]. However, various groups did show that natalizumab causes a change in T cell receptor heterogeneity and T cell responsiveness in MS patients [343-345]. Since we did not observe any effect on percentage of circulating follicular T cells, it is plausible that these cells do not express VLA-4 rendering them unresponsive to treatment. Fingolimod is an oral antagonist of the S1P1 receptor inhibiting S1P/S1P1-dependent lymphocyte egress from SLO. So far, its general effects on T cell phenotype and function have been well described. Next to a substantial drop in the amount of CD4 and CD8 T cells in the blood, it seems that preferentially central memory and naive T cells ($CCR7^+$) are captured in the SLO leading to a relative increase in effector memory cells ($CCR7^-$). In our study, we confirmed a major decrease in the percentage of circulating lymphocytes. We also showed that naive Tregs and naive Tconv percentage decrease while the proportion of memory Tconv and memory Treg increases. These results are in line with previous findings that show beneficial effect on disease course are paralleled with an increases in Tregs and decrease in T_H17 cells [346, 347].

In addition, we found that both T_{FH} and T_{FR} are not affected significantly by treatment with fingolimod. We showed that both circulating T_{FR} and T_{FH} are a heterogenous population of both T_{EM} en T_{CM} cells partly explaining their steady state during treatment. Since there is a drop in potential harmful T cells together with a proportional increase of both Tregs and T_{FR} it seems beneficial for MS patients to have memory cells with regulatory capacities circulating the body.

In our study, we did not observe an effect of current treatment regimens on $T_{FR.}$ Enhancing the number or functionality of T_{FR} or Tregs by future treatment strategies could be beneficial for patient outcome (reviewed in [348]). Tregs can be used as a therapeutic option in two ways, either by adoptive transfer or by *in vivo* targeting. For the adoptive transfer, naive T cells or Tregs are isolated from the donor and differentiated *in vitro* to be re-injected. For *in vivo* targeting, a recent study showed that TGF-β signaling prevented T_{FH} cell accumulation, autoreactive B cells activation and autoantibody production [179]. These results pave the way for future therapies to enhance the functionally of TGF-β producing cells such as Tregs to dampen humoral autoimmune responses in MS. In addition, RNA sequencing of impaired and functional T_{FR} could reveal new targets and pathways for future treatment strategies that enhance frequency and function of T_{FR}.

7

NEDERLANDSE SAMENVATTING

In deze studie werd er onderzocht of **folliculaire T-cellen** betrokken zijn bij het ziekteproces van **multiple sclerose**.

Wat is multiple sclerose?

Multiple sclerose of MS is een aandoening van het centrale zenuwstelsel waarbij het myeline beschadigd geraakt. Myeline is het geleidend omhulsel rond de zenuwbanen en is essentieel voor het doorgeven van zenuwprikkels. Hierdoor ervaren personen met MS vaak problemen met zicht, vermoeidheid, verminderd gevoel in hun extremiteiten en uiteindelijk verlamming. Het merendeel van de MS-patiënten vertonen periodes van opstoten gevolgd door periodes van herstel (RRMS). De rest kent een progressief ziekteverloop (PPMS). Hoewel de exacte oorzaak van MS niet gekend is, wordt algemeen gesteld dat MS auto-immuun van oorspong is. Hierbij beschadigen immuuncellen (autoreactieve T- en B-cellen) het lichaamseigen myeline. Heel wat onderzoek richt zich bijgevolg naar de rol van het immuunsysteem bij MS-patiënten en het blootleggen van eventuele defecten.

Wat zijn folliculaire T-cellen?

De cellen van het verworven immuunsysteem kunnen onderverdeeld worden in T-cellen en B-cellen. Heel wat onderzoek wijst erop dat auto-reactieve T-cellen een voorname rol spelen in de pathogenese van MS. Folliculaire T-cellen zijn een subpopulatie van T-cellen en omvatten enerzijds de folliculaire regulatoire T cellen (T_{FR}) en anderzijds de folliculaire helper T-cellen (T_{FH}). Deze cellen bevinden zich voornamelijk in lymfoïde organen zoals de milt en de lymfeknopen. In deze organen vormen zich tijdelijke structuren, kiemcentra, waar pas gevormde B-cellen rijpen tot antistofproducerende B-cellen. Dit proces wordt bevorderd door T_{FH} enerzijds en getemperd door T_{FR} anderzijds. Folliculaire T-cellen zijn daarom essentieel voor het onderhouden van de normale B-celimmuniteit.

Waarom is het relevant om folliculaire T cellen te onderzoeken in MS?

MS wordt gekarakteriseerd door immunologische stoornissen zoals de aanwezigheid van auto-reactieve B-cellen die antistoffen produceren gericht tegen het myeline in de hersenen en het ruggenmerg.

De aanwezigheid van deze myelinereactieve B-cellen zou veroorzaakt kunnen worden door afwijkingen in de aantallen en de functionaliteit van folliculaire T-cellen, aangezien deze T-cellen een voorname controle functie uitoefenen op de activiteit van B-cellen.

Zijn folliculaire T-cellen betrokken bij de MS-pathogenese?

Folliculaire T-cellen bevinden zich voornamelijk in de lymfoïde organen. Omwille van ethische en praktische redenen is onderzoek naar normale immuniteit en auto-immuniteit in mensen vaak beperkt tot het gebruiken van bloed. Daarom werd in een eerste deel van dit doctoraat gekeken naar de aanwezigheid van folliculaire T-cellen in het bloed en in welke mate ze verschillen van deze aanwezig lymfoïd weefsel. Praktisch werden T_{FH} en T_{FR} geïsoleerd uit het bloed en de amandelen van gezonde personen met behulp van flowcytometrie. Amandelen zijn het meest toegankelijke lymfoïd orgaan bij mens. Het fenotype en de functionele activiteit van folliculaire T-cellen werden vergeleken. Folliculaire T-cellen uit het bloed hebben een aantal opvallende verschillen in vergelijking met deze uit de amandelen. Ze vertonen een verlaagde expressie van essentiële folliculaire oppervlakte moleculen en brengen geen essentiële folliculaire transcriptiefactor tot expressie. Desalniettemin vertonen ze een volledig gedifferentieerd fenotype en kunnen ze omschreven worden als geheugencellen die de capaciteit hebben om te hercirculeren naar de lymfoïde organen. Op vlak van functionele activiteit hebben we aangetoond dat deze circulerende folliculaire T-cellen even actief zijn als folliculaire T-cellen van de amandelen. Verder hebben we aanwijzingen dat deze cellen de processen in de kiemcentra weerspiegelen aangezien gezonde donoren een significante stijging van folliculaire T-cellen in hun bloed vertonen na een griep vaccinatie. Uit deze voorbereidende studie kunnen we concluderen dat bloed folliculaire T-cellen fenotypisch verschillen van de folliculaire T-cellen in lymfoïde organen, maar volledig functioneel zijn, geheugencellen zijn en de kiemcentra reactie reflecteren in het bloed. Deze eigenschappen maken dat bloedafgeleide folliculaire T-cellen een representatieve populatie is om adaptieve auto-immuniteit te bestuderen in mensen.

In een volgende studie hebben we folliculaire T-cellen onderzocht in het bloed van MS-patiënten. We hebben meer specifiek de aantallen, het fenotype en de functie van circulerende T_{FR} en T_{FH} vergeleken tussen MS-patiënten en gezonde donoren. We constateerden een significante daling in het aantal T_{FR} in het bloed van MS-patiënten. Daarnaast vonden we dat T_{FR} van MS-patiënten een meer pro-inflammatoir fenotype hebben. Tot slot vonden we dat T_{FR} van MS-patiënten functioneel minder in staat zijn om de activiteit van andere T-cellen te onderdrukken. De circulerende T_{FH} verschillen niet op basis van fenotype noch frequentie met gezonde donoren. We kunnen uit deze resultaten besluiten dat T_{FR} van MS-patiënten verlaagd zijn in aantal en functionaliteit en verder een schadelijker fenotype vertonen.

Kunnen variaties in MS-genen de waargenomen folliculaire T-celdefecten verklaren?

MS is geen overerfbare ziekte. Wel zijn er in totaal 110 DNA variaties, "polymorfismen" geïdentificeerd die leiden tot een verhoogde vatbaarheid voor het ontwikkelen van MS. Sommige van deze polymporfismen liggen in genen die essentieel zijn voor de functie van folliculaire T-cellen. We onderzochten of de wijzigingen in T_{FR}-aantallen verklaard konden worden door genetische variaties in T-cel relevante genen (CXCR5, IL2RA en CD58) maar vonden geen oorzakelijk verband. We zagen wel dat gezonde donoren met deze genetische variaties een gewijzigd folliculaire T-celprofiel hadden. Hieruit kan verondersteld worden dat genetische effecten op T-celhomeostase door de auto-immune processen gemaskeerd worden, zodat er geen geneffect meer zichtbaar is.

Kan behandeling de folliculaire T-celaantallen verhogen?

Onbehandelde MS-patiënten vertonen verlaagde aantallen T_{FR} in hun bloed. De beschikbare behandelingen voor MS focussen vooral op het reguleren van de immuuncellen zodat ze minder actief zijn en niet meer kunnen migreren naar de hersenen. We vergeleken het percentage van circulerende T_{FR} in MS-patiënten die onder behandeling ware,, maar vonden we geen verschil met niet behandelde patiënten. Daarnaast hebben we een opvolgstudie van een jaar uitgevoerd bij MS-patiënten die behandeld werden met de eerste orale MS-therapie, Fingolimod.

Fingolimod verhindert de circulatie van potentieel schadelijke immuuncellen doorheen het lichaam. Het percentage circulerende folliculaire T-cellen werd elke 3 maanden gemeten maar er werd geen effect van behandeling gevonden. De huidige behandelingen zijn bijgevolg niet in staat om de verminderde T_{FR} activiteit bij MS te herstellen.

Wat kan er geconcludeerd worden en wat brengt de toekomst?

In dit doctoraat werd voor het eerst een defect in folliculaire T-cellen in MS-patiënten aangetoond. Deze cellen vormen een essentiële link tussen autoreactieve B-cellen en T-cellen, beide belangrijk bij de pathogenese van MS. Dit onderzoek levert zo een belangrijke bijdrage aan verdere inzichten in het ontstaan van de neurologische ziekte. Een aantal sleutelelementen dienen verder uitgewerkt te worden in toekomstig onderzoek om deze hypothese verder te ondersteunen. In de eerste plaats dient er gekeken te worden naar een direct effect op autoreactieve B-cellen en antistoffen. Daarnaast dient de oorzaak van deze defecten verder onderzocht worden. Tot slot kan het gebruik van het muismodel voor MS, EAE, een duidelijker beeld verschaffen over de *in vivo* relevantie van T_{FR} in de pathogenese van MS. Op langere termijn kunnen strategieën ontwikkeld worden om de verminderde activiteit van T_{FR} in MS te herstellen.

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Curriculum Vitae

Tessa Dhaeze werd geboren op 14 oktober 1988 te Messancy (Aarlen, België). In 2006 behaalde ze haar diploma Algemeen Secundair Onderwijs (ASO) in Wetenschappen-Wiskunde aan het Heilig-Graf Instituut te Bilzen. Vervolgens startte ze aan de opleiding Biomedische Wetenschappen aan de Universiteit Hasselt/transnationale Universiteit Limburg om vervolgens haar diploma Bachelor in de Biomedische Wetenschappen te behalen in 2009. Haar bachelor stage, getiteld "Cellular and molecular mechanisms in autoimmune diseases. Markers for regulatory T cells LAG 3 expression: role in T cell regulation" liep ze aan de Universiteit Hasselt (Biomedical research institute, Diepenbeek, Belgium) in de groep van prof. dr. Piet Stinissen en prof. dr. Niels Hellings. In september 2009 begon ze aan haar masteropleiding Biomedische Wetenschappen aan de Universiteit Gent. Haar masterstage "Investigation of the toxic effect of cigarette smoke on pulmonary proteins" aan de Universiteit Gent (VIB, Proteomics Department of Medical Protein Research) werd begeleid door dr. Bart Ghesquière en prof. dr. Kris Gevaert. In 2011 behaalde ze haar masterdiploma in de Biomedische Wetenschappen met onderscheiding samen met de certificaten proefdierkunde I en proefdierkunde II aan de Universiteit Gent. Daaropvolgend startte ze haar doctoraatsopleiding in de Biomedische Wetenschappen aan de Universiteit Hasselt onder het promotorschap van prof. dr. Piet Stinissen en prof. dr. Niels Hellings. Gedurende haar doctoraat focuste ze zich op de rol van folliculaire T-cellen in de pathogenese van multiple sclerose (MS). Tijdens haar doctoraat schreef ze een IWT beurs en heeft ze actief deelgenomen aan het schrijven van een FWO-project. Daarnaast nam ze deel aan verschillende ondersteunende cursussen (*project management, scientific writing and oral presentation, good scientific conduct and lab book taking* en *flow cytometry*) en symposia (ECTRIMS summer school, ISNI en ICI). Tot slot heeft ze reisbeurzen ontvangen om deel te nemen aan de ECTRIMS Summer school (Estland, Tallin, 2014) en het WIRM (Zwitserland, Davos,2015) en won ze de posterprijs op het WIRM (Zwitserland, Davos,2015).

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Publications

Compositional changes of B and T cell subtypes during fingolimod treatment in multiple sclerosis patients: a 12 month follow-up study.
Claes N*, **Dhaeze T***, Fraussen J, Broux B, Van Wijmeersch B, Stinissen P, Hupperts R, Hellings N, Somers V.
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Published abstracts

Follicular Regulatory T cells in Multiple Sclerosis: New Kids on the Block?
Dhaeze Tessa, Broux Bieke, Van Wijmeersch Bart, Stinissen Piet, Hellings Niels
Frontiers in Immunology Conference Abstract: 15th International Congress of Immunology (ICI). August 2013, Milan Belgium

Compositional changes of B and T cell subtypes during fingolimod treatment in multiple sclerosis patients: a 12 month follow-up study
Nele Claes*, **Tessa Dhaeze***, Judith Fraussen, Bieke Broux, Bart Van Wijmeersch, Piet Stinissen, Raymond Hupperts, Niels Hellings, Veerle Somers
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Oral Presentations

Role of follicular regulatory T cells in multiple sclerosis

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- *IUAP meeting (T-time)*, September 4th 2013, Leuven, Belgium
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Compositional changes of B and T cell subtypes during fingolimod treatment in multiple sclerosis patients: a 12 month follow-up studyRole of Follicular Regulatory T cells in Multiple Sclerosis.

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Follicular regulatory T cells are defective in multiple sclerosis
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Compositional changes of B and T cell subtypes during fingolimod treatment in multiple sclerosis patients: a 12 month follow-up studyRole of Follicular Regulatory T cells in Multiple Sclerosis.
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Best Poster Award
WIRM, Davos (Switzerland), Circulating follicular regulatory T cells are defective in multiple sclerosis, 18-21 March 2015

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Tess Dhaeze
September 2015

With ideas it is like with dizzy heights you climb: At first they cause you discomfort and you are anxious to get down, distrustful of your own powers; but soon the remoteness of the turmoil of life and the inspiring influence of the altitude calm your blood; your step gets firm and sure and you begin to look - for dizzier heights.

Nikola Tesla (1856 -1943)

"Life isn't about waiting for the storm to pass
It's about learning to dance in the rain."