Characterization of T Follicular Helper Cells and Follicular Regulatory T Cells in Patient with Multiple Sclerosis

Razaul Haque

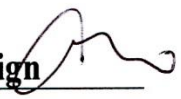
July, 2019

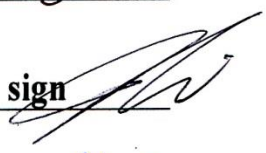
Graduate School of Cancer Science and Policy

Professor Ho Jin Kim (Supervisor)

We hereby approve the M.S. thesis of
[Razaul Haque].

[July 2019]

Eun Jung Park **sign** 
Chairman of Thesis Committee

Ho Jin Kim **sign** 
Thesis Committee Member

Ho Lee **sign** 
Thesis Committee Member

NATIONAL CANCER CENTER
GRADUATE SCHOOL OF CANCER SCIENCE AND POLICY

ABSTRACT

Characterization of T Follicular Helper Cells and Follicular Regulatory T Cells in Patient with Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating autoimmune disease of central nervous system. Follicular T helper (Tfh) cells are distinct subsets of $CD4^+$ T lymphocytes which express $CXCR5^+$ $PD\text{-}1^+$ in their surface. They are specially recognized for helping B cell and antibody mediated immune response in germinal center. Accumulating evidences suggest that over representation of Tfh cells are associated with autoimmunity. This study focused on the phenotypic as well as functional analysis of circulating Tfh (cTfh) cells and regulatory Tfh (Tfreg) cells in MS patient. Multicolor flow cytometry was used to define the T cell subsets through their surface markers and respective cytokine production through intracellular cytokine staining (ICS) using peripheral blood mononuclear cell from healthy controls (HC) and MS patients. MS patients had a higher frequency of cTfh cells compare to HC. Conversely, Tfreg were down regulated in MS patients compare to HC. The overall production of IL-21 was much higher in MS patients than in HC whereas the IL-10 found to be produced lower in MS patient. Comparative analysis of follicular versus non-follicular T helper (non-Tfh) cells showed that cTfh are the main producer of IL-21 in each group where the

expression level is even higher in MS patient. Contrarily, IL-10 secretion by Tfreg cells were decreased in MS patient compared to HC. Interestingly, Th17 related chemokine receptor ($CCR6^{+}CXCR3^{-}$) and T helper-1 like Th17 termed as Th17.1 chemokine receptor ($CCR6^{+}CXCR3^{+}$) were abundantly expressed among Tfh population in MS patient compared to HC. These populations also involved in overproduction of IL-21 in MS patient. Collectively, these findings demonstrated that imbalanced distribution between cTfh and Tfreg exist in multiple sclerosis which contributes to differential production of IL-21 and IL-10 by their major subtypes.

Key Words: Circulating T Follicular Helper Cell, Regulatory T Follicular Cell, Multiple Sclerosis, IL-21 and IL-10.

Copyright by

Razaul Haque

2019

Table of Contents

List of Table

List of Figures

List of Abbreviations and Acronyms

Abbreviation	Full Form
APC	Antigen Presenting Cells
Ascl2	Achaete-Scute Homologue-2
BBB	Blood Brain Barrier
BCL6	B Cell Lymphoma 6
BSA	Bovine Serum Albumin
CCR	Chemokine Receptor
CIS	Clinically Isolated Syndrome
cMAF	Musculoaponeurotic Fibrosarcoma
CNS	Central Nervous System
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
cTfh	Circulating Tfh
DIS	Dissemination of Space
DIT	Dissemination of Time
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein bar virus
EDTA	Ethylenediaminetetraacetic Acid
FoxP3	Forkhead Box P3
FVS	Fetal Bovine Serum
GC	Germinal Center
GM-CSF	Granulocyte–Macrophage Colony Stimulating Factor
GWAS	Genome Wide Association Studies
HLA	Human Leukocyte Antigen
ICOS	Inducible T cell Co-Stimulator
ICS	Intracellular Cytokine Staining
IFR4	Induced Transcription Factor 4

iTfreg	Inducible T Follicular Regulatory
MBP	Myelin Basic Protein
MG	Myasthenia Gravis
MOBP	Myelin Associated Oligodendrocytic Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
nTfreg	Natural T Follicular Regulatory
OCB	Oligoclonal Bands
PBMC	Peripheral Blood Mononuclear Cells
PD-1	Program Death 1
PLP	Proteolipid Protein
PMA	Phorbol Myristate Acetate
PPZMS	Primary Progressive Multiple Sclerosis
PRMS	Progressive Relapsing Multiple Sclerosis
RA	Rheumatoid Arthritis
RRMS	Relapsing Remitting Multiple Sclerosis
Sap	Signaling Lymphocytic Activation Molecule SLAM-Associate Protein
SLE	Systemic Lupus Erythematous
SNP	Single Nucleotide Polymorphism
SPMS	Secondary Progressive Multiple Sclerosis
STAT	Signal Transducer And Activator Of Transcription
TFG-β	Transforming Growth Factor β
Tfh	T Follicular Helper
Tfreg	T Follicular Regulatory
Th	T Helper Cell
Th17.1	T Helper-1 Like Th17
Treg	T Regulatory

1. Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune demyelinating disease of central nervous system (CNS) characterized by the destruction of myelin sheath and nerve axon (*1*). It's a chronic inflammatory neurological disorder which considered as the leading non- traumatic cause of disability in young adults ranging 20 to 40 years of age (*2, 3*). The clinical manifestations of MS are very heterogeneous that include optic neuritis, diplopia, gait ataxia, sensory loss, and loss of bladder control, pain, depression and cognitive dysfunction, sexual and sphincter problems (*4, 5*). This disease has now become a global burden affecting over 2.2 million people throughout the world (*5*). Although the prevalence in South Korea is not so prominent but the rate has been increasing over time. Currently, a total of 7012 MS patients have recorded in South Korea which increased by six fold since last 10 years (*5, 6*).

1.1.1. Subtypes of Multiple Sclerosis

Based on clinical features through magnetic resonance imaging (MRI), MS can be categorized into relapsing remitting (RRMS), secondary progressive (SPMS), primary progressive (PPZMS) and progressive relapsing (PRMS) groups. RRMS is the most common form of MS that covers almost 85~90% of total MS patients. It's typically defined by acute or increasing neurological disorder after partial or full recovery followed by a period of remission (*7*). Majority of CNS

inflammatory activities of MS take place at this stage where recovery is variable depending on age and duration of the disease (*8*). The frequency of RRMS is observed higher in young adults (mean age 30 years) where the degree of prevalence is 3 times larger in women than men (*9*). Conversely, secondary progressive MS is characterized by worsening of neurological function with or without relapse. The disease status is more exacerbated in SPMS as it is defined after suffering from RRMS for a decade or more and the disease modifying treatment no longer works in this condition. It normally appears in older age (≥50 years) and common clinical symptoms observed here are neurodegeneration, irreversible axonal and neuronal loss and brain atrophy (*10, 11*). PPMS is defined by gradual neurological disability from the beginning of symptom without any early relapse or remission occupying 10 % of total MS patients (*12*). SPMS and PPMS are studied together as they share more common entities (*13*). Finally, very little percentage (5%) of progressive MS turned into PRMS, which is characterized by steady worsening of disease from the beginning with acute relapse but no remission. In this case, the disease condition can be recovered or remain stable (*14*). Collectively, the inflammatory insults in CNS for progressive MS and RRMS are relatively similar that differs mainly by the degree and durability of inflammation (*15*). The mysteries behind the clinical diversification of MS are not completely understood. Further clinical investigations are needed for distinctive characterization of MS.

It is well-known that inflammatory lesions describe the pathological status of MS patients. These lesions are not static which changes over time (progress/slow down) either by disease modifiers or immunomodulating phenomena in our body. However, based on the inflammatory lesions MS can be classified as 'active MS' or 'inactive MS. Active MS is regarded as clinical relapse when new lesions are found followed by partial or full recovery but without pseudo-relapse. The study through MRI revealed that active MS patient developed new T2 hypertinase lesions or gadolinium lesions (*16, 17*). On the other hand, inactive MS can be termed as phenotype modifier with relapsing course but not relapse or any other MRI lesions (*16*).

1.1.2. Diagnosis of Multiple Sclerosis

MS is a complex unpredictable disease where inflammatory, progressive and non-progressive events take place. Because of inharmonious clinical profile, diagnosis of MS is still a challenging task and until now, the diagnosis procedures are limited to MRI and cerebrospinal fluid (CSF) oligoclonal bands (OCB) (*18, 19*). The Mcdonald criteria revised in 2017 are widely used method for diagnosing MS using MRI findings (*19*). In this criterion, the clinical features are determined by the dissemination of disease in space and time. Dissemination of space (DIS) describes the anatomical location of CNS in MS whereas dissemination of time (DIT) measure time points that helps to distinguish RRMS from clinically isolated syndrome (CIS), a single symptomatic episode of inflammatory demyelination of CNS lasts for 24 hours but not develops into MS

(*20*). DIS and DIT shows different magnitude in definite MS. For example, in RRMS, MRI evidence for DIS requires minumum one T2 lesion in at least two of four sites, periventricular, juxtacortical, infratentorial regions and the spinal cord while DIT requires asymptomatic gadolinium-enhancing and non-enhancing lesions or a new lesion on a follow-up scan. On the other hand, for progressive MS. Mcdonald criteria provide separate recommendations for testing CSF abnormalities along with MRI to increase the sensitivity. In progressive MS, DIS requires at least one T2 lesion of three sites periventricular, juxtacortical, infratentorial and minimum two T2 lesions in spinal cord. Normally, DIT records the worsening of 12 months period (*21*). But because of unavailability, cost and contra-indication it might not possible to run all procedure at times.

On the other hand, oligoclonal bands are immunoglobulins that produce in serum and CSF when encounter with antigen. Their presences suggest the existence of inflammation in CNS. In MS, the OCBs in CSF are disparate from serum. Around 95% MS-CSF showed the presence of abnormal synthesis of OCB (*22*, *23*). However, it is not clear where OCB represent pathogenic CNS-antigens or not. Moreover acquisition of CSF needs lumber puncture which limits clinical utility of CSF marker for monitoring MS.

As mentioned earlier, MS is an autoimmune disease, therefore immunological characterization provides prominant way to study this disease. But the sources of immune cells are variable that are very challenging task to access them in human. In this sense, peripheral blood is relatively easy source of material for

immunophentyping of MS. Although connection between circulating immune milieu and MS-CNS inflammation is still questionable but the evidences of peripheral blood immmnopathology in MS showed a strong correlation of circulating immune system in the disease context (*24*, *25*). Multiple studies have been proclaiming that changes in circulating immune cells contribute a lot to the initiation, progression and exacerbation of MS (*26-29*). As for example, the frequency as well as functional loss of $FoxP3^+$ Treg cells leads to the aberrant activation of T effector cells that causes the disruption of immune homeostasis resulting autoreactive immune response in MS (*24*, *30*, *31*). Although immunophenotyping open a promising windows to study MS, but immune cells are highly miscellaneous and conserve multidimensional properties requiring a better strategy to study them conveniently. Recently, multicolor flow cytometry paves the way to study bunch of immune cells under one single study panel. The flow cytometry will be discussed further in section 3.3.1

1.1.3. Etiology of Multiple Sclerosis

MS is conceptualized as a multifocal complex disease. The precise cause and risk factors to delineate this disease are poorly understood. It is presumed that genetical, environmental and immunological events act together to decipher the complexity.

1.1.3.1 Genetic Factors

The roles of genetic risk factors in MS have been getting importance since last decades. Heritability study of MS showed a high risk for the familial recurrence among siblings especially twins. Several studies have confirmed that monozygotic twins have 5 fold (25%) higher risk than the dizygotic twins (2~5%) (*32-34*). The discovery of human leukocyte antigen *(HLA)* allelic variant provides a concrete genetic architecture to study MS. HLA class II & I are considered as disease modifiers. But the variant of class II gene product present antigen to $CD4^{+}$T cells whereas Class I gene product alteration present antigens to $CD8^{+}$ T cells (*35, 36*). Interestingly, the variant of class II genes HLA-DRB1 showed striking association with MS by increasing the risk (*37*). Recently, genome wide association studies (GWAS) becomes a powerful tool to study genetic association of autoimmune disease. GWAS have detected around 110 non HLA single nucleotide polymorphisms (SNPs) that coffer the risk for MS (*38*).

1.1.3.2 Environmental Factors and Lifestyle

Despite the importance of genetic factor, environmental and individual life style has been considering most crucial factors for MS. Evidences include Epstein bar virus (EBV) infection, geographical location, smoking, sun exposure/ vitamin D and adolescent obesity demonstrated their influential impact on MS. A strong latitudinal gradient have been observed in MS patients where increased rate of MS found in polar regions compared to equators (*39*). The environmental

factors also correlate with migration studies. Migration to higher risk county from lower risk country in childhood increased the risk of developing MS (*40-42*). On the other hand, there is a convincing relation exist between MS and vitamin D. Vitamin D is considered inversely proportional to the risk associated with MS. Sunlight is the potent source of vitamin D and sunlight exposure are very deem away from the equator, has higher risk for MS (*43*). Furthermore, dietary consumption of vitamin D-rich food and slow regulation of vitamin D metabolism correlate with MS (*44*). Several cohort and meta-analysis studies report that smoking (both active and passive) and oral tobacco intakes are highly correlated in MS regarding disease severity and disability. Elevated cotinine level in serum and plasma (≥10ng/ml) from smoking possibly reflect the high risk for MS (*45-47*). The infectious agent for MS is not yet fully proved, but it is believed that EBV (an infection agent responsible for infectious mononucleosis) has a strong correlation with MS risk where EBV negative individuals showed extremely low recurrence of MS (*48, 49*). Recently, a new candidate named *Chlamydia pneumonia* has found to correlate with MS although the evidence was weak (*50, 51*). Apart from these infectious candidates, the compositions of gut microbiome found to associate with MS risk (*52, 53*). The key strategy of infectious agents in MS is to provide an immunogenic condition that influence activated immune cells against our own protein by a mechanism called 'molecular mimicry'.

1.1.4. Immunology of Multiple Sclerosis

Plaques of inflammatory demyelination within the CNS are the key hallmarks for MS. Inflammation is present in every stage of MS although it is more prominent in acute stage than chronic stage. Early lesions show that peripheral immune cells and the leakage of blood brain barrier (BBB) as a starting point for immunopathology in MS. The leakage of BBB allows monocytes, T cell, B cell to enter in the CNS and reactivate again by interacting with CNS antigen presenting cells (APCs) following the activation of microglia and astrocyte (*54, 55*). Demyelination and axonal injuries are directly correlated with glial reactivity, particularly astrocytic hypertrophy (*56*). One of the classical descriptions of MS is the infiltration of autoreactive immune cells to the CNS. These autoreactive immune cells target self-antigen (auto-antigen) and showed the subsequent immunogenic activity. But how these auto-antigens generated are still remains unmask. The most widely accepted theory is 'molecular mimicry' where antigenic determinant share the same structural and phonotypical attributes and hence activate autoreactive T and B cells to initiate autoimmunity (*57*). Alternatively, primary CNS cytodegeneration can unleashes normally sequestered CNS antigens (both in CNS and periphery) that showed up secondary autoimmunity (*58*). Moreover, a variety of myelin related proteins including myelin basic protein (MBP) (*59*), proteolipid protein (PLP) (*60*), myelin oligodendrocyte glycoprotein (MOG) (*61*), myelin associated oligodendrocytic basic protein (MOBP) (*62*) are found in the periphery which

may associated with the activation of autoreactive immune cells. Additionally, non-myelin protein like αB crystalline, neural protein like contactine-2 are also abundantly identified at periphery which may trigger autoreactive immune response in MS(*63, 64*).

Historically, MS has been considering T cell mediated autoimmune disease. Generation of autoreactive T cells and their inframed network to other immune cells become one of key study of interest to explore MS. Evidences from animal model (EAE-experimental autoimmune encephalomyelitis), clinical and pathological studies confirmed that large number of autoreactive $CD4^+$T cells and $CD8^+$ T cells are highly correlated with MS lesions. Recently, GWAS studies also supported the hypothesis the involvement of T cell in MS (*65, 66*). Interestingly, autoreactive T cells are found in both healthy and MS patients, but the frequency of myelin reactive T cells, their avidity and activation profiles are distinctly elevated in MS patients (*61*). However, generations of autoreactive T cells are not merely capable of initiating autoimmune disease as they are tightly controlled by immune checkpoint. Autoreactive T cells become hyper activated and target auto-antigen when the self-tolerance mechanism provided by immune-checkpoint is disturbed in an immune system (*67*). But how T cell bypass the immune checkpoint is still a mystery. Most widely accepted hypothesis is that abnormal distribution of effector T cells and regulatory T cells (Treg) play crucial roles in the breakdown of immune checkpoint (*31, 68*). Importantly, autoreactive T cells are also capable of infiltrating BBB to become reactivate by

APC and therefore accelerate the process of demyelination. This hypothesis was also confirmed in EAE model, where adoptive transfer of myelin reactive T cell cross the BBB from periphery and reactivate in CNS by APCs.

1.1.5. $CD4^+$ T Cell in Multiple Sclerosis

Conventionally, multiple sclerosis were viewed as $CD4^+$ T helper cells (Th) mediated autoimmune disease for several reasons including evidence from the EAE model, slowdown of disease by depleting $CD4^+$ T cell and confirmation from the genetic study of $CD4^+$ T cell determinant major histocompatibility complex II in MS (*25, 35, 69, 70*). $CD4^+$ T cell can be functionally divided into several subtypes in MS which are independent of each other in terms of their individual transcription factors and cytokines (Fig.1) (*71*). The basic commitment of $CD4^+$ T cell subsets are very specific and but not irreversible. They can change their plasticity and acquire the properties of other subsets under the influence of epigenetic modifications, cytokines and other environmental factors. These switching behaviors influence the abnormal secretion of pro-inflammatory and aniti-inflammatory cytokines that may accelerate autoimmune diseases like MS (*72, 73*). Historically MS pathogenesis showed interferon gamma (IFN-γ) mediated Th-1 cells (Figure 1.1) were responsible for MS which initially described by Th1/Th2 model, proposed by Mossmann and colleagues in 1986 (*74*). But this model had fallen down into weakness when protective role of IFN-γ had found in EAE (*75*). The discovery of Interleukin 17 (IL-17) producing Th17 cells expressing CCR6 (Figure 1.1) provides us a new platform to study

MS. Along with IL-17; Th17 cells are also engaged with IL-22, IL-21 production (*76*). Substantial evidence showed that Th17 bias occurs in MS and other autoimmune diseases. IL-17 found to play role in increasing BBB permeability and induction of several inflammatory mediators by CNS microglia and astrocytes (*77, 78*). The role of $CD4^+$ T cells in MS got a new attraction of scientists when a third factor named granulocyte–macrophage colony stimulating factor (GM-CSF) hypothesis become popular. Multiple evidences suggested that an increased level of GM-CSF has been found in MS pathogenesis (*79, 80*). GM-CSF performs several functions in neuroinflammation in MS. For example, recruitment of peripheral immune cells in CNS, activation and proliferation of myeloid cells are mostly governed by GM-CSF (*81*). But the source of GM-CSF possesses a great controversy where in mice, Th-17 cells were the main producer of GM-CSF but they are dominantly produced by Th1 cells rather than Th17 in human (*82*).

Collectively, considering the controversy it can be assumed that both Th1 and Th17 cells has connection in MS regarding the initiation and severity of the disease. The prevalent difference in Th1 and Th17 between patients describes the heterogeneity of the disease. Recently, IFN-γ as well as IL-17 producing Th cells termed as Th17.1 has been found to increase in MS although functional attributes are not clear enough (*83*).

1.1.6. $CD4^+$ T Regulatory Cells in Multiple Sclerosis

One of the critical features of immune system is to maintain the homeostasis. This mechanism is typically initiated by the activation of effector T cells after pathogenic encounter followed by the parallel regulatory action of special $CD4^+$ T cells subtypes to control them from becoming hyperactive. These special subtypes are known as regulatory T cells (Treg) (*84*). Initially, Treg cells were defined by the expression of CD25 on the surface of $CD4^+$T cells (*85*). But later on, the discovery of forkhead box P3 (FoxP3) created more promising way to define and phenotype Treg cells ($FoxP3^+$ Treg) (Figure 1.1) (*86*). Tregs are now defined by the expression of $FoxP3^+CD25^+$ T cells that predominabtly produce IL-10, TFG-β and IL-2. However,$CD4^+$ Treg can be divided into natural Treg (nTeg) and inducible Treg (iTreg) cells where nTreg cells contains $FoxP3^+CD25^+$ phenotypes and iTreg as $FoxP3^+CD25^-$ phenotypes only. But all of them commonly produce IL-10 (*87, 88*). Surprisingly, there are some regulatory T cells have been observed (Tr1 and Th3) that don't necessarily express FoxP3 or CD25 (*84, 89*).

Functionally, Tregs are widely known as the inhibitor of T cells. They suppress the differentiation, activation and proliferation of T cells to restrain them from cytokine production and preventing autoimmunity in MS (*90, 91*). It has already been established that reduction of functioning Tregs increase severity of EAE where Treg deficient mice or Treg competent ameliorates the disease severity (*92, 93*). However, the role regulatory T cell in MS is controversial. Some reports

claimed that Tregs numbers are not reduced in MS compared to healthy control (*25*, *26*). But a large body of evidences suggested that suppression capability of CD25^{hi}Treg were significantly reduced in MS patient followed by decreased level of FoxP3 in Tregs in MS patients (*94-97*).

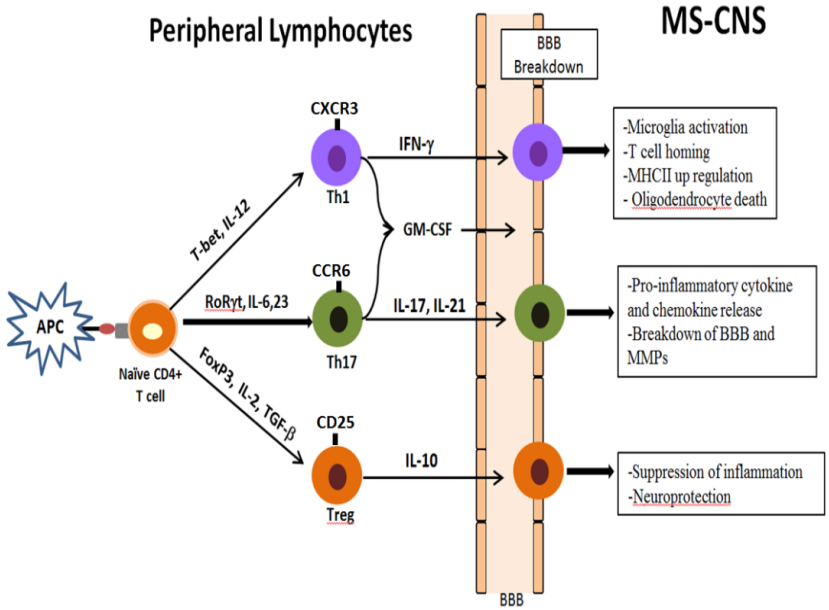


Figure 1.1 Major CD4^{+} T helper cell subsets and their role in MS. Naïve Th cells are activated by APC which further differentiate into several subsets. The key cytokines and transcription factors driving CD4 naïve T cell in functionally into Th1, Th17, Treg cells (left panel). After crossing the BBB they play distinct role during the pathogenesis of MS (right panel) *(71)*.

1.2. T Follicular Helper Cells

1.2.1 Differentiation and Functional Attributes of Tfh Cells

T follicular helper (Tfh) cells were first described in human tonsilar cells as a new subtypes of $CD4^+$ T cells that express chemokine receptor 5 (CXCR5) and Inducible T cell co-stimumulator (ICOS) in their surface (*98, 99*). They mainly reside in the germinal center (GC) where they constantly expose to foreign antigens resulting expansion, affinity maturation of B cells. Anatomically, The GC is a distinct lymphoid structure present in secondary lymphoid organs like tonsils, lymph node, spleen etc. This lymphoid structure is the host ground for taking place of multiple immunological events like clonal expansion, somatic hypermutation, B-cell memory development and generation of long lived plasma cells and thus plays a key role in the protective immunity against pathogens (*100*). By definition, Tfh cell migrates to follicles and interacts with antigen specific B cells to differentiate and produce antibody. However, Tfh cells are very heterogeneous and it is very difficult to detailed analysis of Tfh cells with conventional definition. For this reason Tfh cells are studied on the basis of their surface phenotypes that express CXCR5, program cell death 1 (PD-1), ICOS and transcription factor B cell lymphoma 6 (BCL6), cMAF and cytokine IL-21 (fig. 1.2) (*101, 102*).

The ontogeny of Tfh cells is still a mystery to the scientific world. It is not clear whether they are developed shortly after T cell priming or other Th subtypes like Th1, Th2 or Th17 has role to differentiate Tfh cell through cytokine interaction.

Cytokine such as IL-6, IL-21 (autocrine manner) and IL-12 showed promising role to differentiate Tfh cells in human in STAT3 dependent manner. But in mice, IL-6, IL-23, IL-27 also play role to differentiate these cells (Figure 1.2) (*103-105*). It is believed that Tfh cells differentiation starts when naïve $CD4^{+}$T cells interact with APCs through MHCII-TCR and ICOS-ICOSL (Figure 1.2). After that, they interact with different cytokines and give rise to the expression of transcription factor BCL6 and cMAF. Transcription factor BCL-6 is mainly responsible for CXCR5 expression as well as down regulation of CCR7 in the surface (Figure 1.2). cMAF maintain the Tfh stability by promoting IL-21 production and BCL-6 expression. Thereafter, PD-1expression fulfilled the molecular attributes of Tfh cells. But the source of PD-1 in Tfh surface is unknown. PD-1 expression is also highly observable in blood Tfh cells (*106*). Despite PD-1 literally a negative signaling molecule, it plays important function in Tfh. Shi et al., have proved that PD-1 is essential for positioning and functioning Tfh cells (*107*). Moreover, PD-1 induces memory B cell differentiation and boost up high affinity maturation, plasma cells generation (*108*). One of the key studies of interest about Tfh cells is their migration mechanism to the B cell follicles and GC from T cell zone. CXCL13 (the ligand for CXCR5 also known as B cell chemoattractrant-1) is believed to play as a chemo-attractant role to drive Tfh into GC. Additionally, the APCs such as follicular DCs may play role to attract Tfh toward GC. In GC, Tfh cells undergo multifactorial changes like expression of CD40L, signaling lymphocytic activation molecule SLAM-associate protein (Sap), induced transcription factor 4

(IFR4) and achaete-scute homologue-2 (Ascl2) while interact with B cells. CD40L-CD40 interaction helps Tfh differentiation and maintenance (Figure 1.2) (*109*). IRF4 and Ascl2 positively regulate BCL-6, CXCR5 and follicular migration and maintenance of Tfh cells (*110*). So it is clear that Tfh cells undergo a multistage and multifactorial process during their differentiation where varieties of cytokines, surface molecules and transcription factors involve.

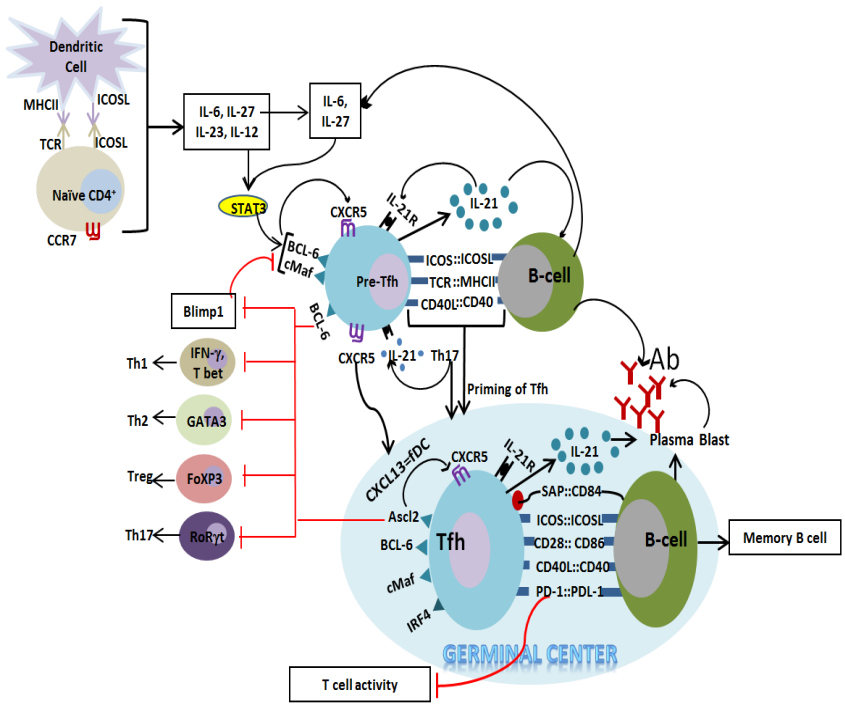


Figure 1.2 Differentiation of Tfh cells. The ontogeny of Tfh is a multistage and multifactorial process. Different signaling molecules, co stimulatory factors and transcriptional profile merged together to shape Tfh cells. BCL-6 is the key regulator which not only differentiate Tfh cell but also repress other Th phenotypes.

The key question arises on Tfh about their lineage difference with other Th cells specially Th 17. Tfh and Th17 cells share some common properties like IL-6 is their common starting point of differentiation and both of them also produce IL-21. Multiple evidences have shown that Tfh cells possess distinct phenotypic characteristics than other Th cells (Th1, Th2, and Th17). Analysis of transcription profiles and signature cytokines showed the key pathway to differentiate the lineage of immune cells. The main transcription factors for Th1, Th2 and Th17 are T-bet, GATA3 and RoRγt respectively (Figure 1.2) whereas BCL-6 is considered as the master transcription factor of Tfh cells. BCL-6 also distinguishes Tfh from others by suppressing transcription factors for other Th cell phenotypes (Figure 1.2) (*111, 112*). Moreover, BCL-6 deficient mice showed defective B cell response in T cells (*113*). Additionally, cMAF also independently express in Tfh cells that reduce the expansion of other Th1, Th2 and Th17 cell lineage (*114*). On the hand, Tfh signaturely produce IL-21 which is also distinctive entity over other Th cells. Th1, Th2 and Th17 cells mainly produce IFN-γ, IL-4 and IL-17 respectively (*104*). Although, both Tfh and Th17 cells produce IL-21, but Tfh cells found to produce only IL-21 but not IL-17A &F or other cytokines produced by Th17.

Functionally, Tfh cells are widely known to help B cells activation and differentiation, promote affinity maturation of plasma cells that are readily produce different immunoglobulins (Igs). $CXCR5^{+}$ compartment of Tfh cells possess functional ability to produce IgG and IgA and undergo class switching

antibody response after co-culturing with B cells (*98*). On the other hand, IL-21 production by Tfh cells is directly involved in plasma cell differentiation and antibody (Igs) production (*115*).

1.2.2. Fate of Tfh Cell after Germinal Center Interaction

One of the unmask feature about Tfh cells is their cellular fate after antigenic interaction in GC. Normally majority Tfh cells undergo apoptosis after pathogenic encounter. Tfh cells can be converted into memory Tfh cells and circulates in human blood or they can respond to the recall mechanism and differentiate back into Tfh or other Th cell types. It has been reported that memory Tfh cells occupy around (15-25) % of total memory $CD4^+$ T cells (*116*). Importantly, memory Tfh cells contain some important features which are very distinct from GC-Tfh. Memory Tfh cells express CXCR5, CCR7, CD62L and folate receptor 4 (FR4) on their surfaces. Contrarily, BCL-6, cMAF, ICOS and PD-1 are found be expressed low in memory Tfh cells (*117*). Currently, memory Tfh cells are drawing attention to many scientists because of their pluripotent, heterogeneous and uncommitted behaviors upon antigenic re-challenge. Memory Tfh can provide quick antigen-specific response to pathogenic re-challenge as they remember the previous events. Importantly, memory Tfh cells can reside not only in peripheral blood and secondary lymphoid organs, but also able to enter non-lymphoid organs. Additionally, they are long lived cells which can further undergo homeostatic proliferation without even antigenic reaction. Finally, these cells take part in cell intrinsic reprogramming to recall their effector function and

differentiate into Tfh or other Th lineage (*118*). But how memory Tfh cells reconverted into different effector cells is yet to be explored. Transcription factor reactivation, epigenetic regulation, complement dependent reactivation and activation of different adhesion molecules are proposed to take part in these events.

1.2.3. Peripheral Blood Tfh Cells

In human blood, Tfh cells were first described in 1994 (*119*). Normally these cells circulate in the blood known as circulating Tfh (cTfh) cells. In human blood, Tfh cells express CD4, CXCR5, and PD-1 but low level of BCL-6 from their counterpart in GC-Tfh. One explanation may be BCL-6 doesn't require any more once it accomplished Tfh generating pathway. The phenotypic source of these cells are unraveled, but it is widely accepted that after GC reactions memory Tfh cells reprogram to differentiate back into Tfh cells and circulate in blood (*118*). Although, cTfh is not as efficient B cell helper as GC-Tfhs, they can able to help B cell. Recently, it has been reported that cTfh produce higher cytokine (IL-21) level than GC-Tfh (*120*). Moreover, cTfh ($CD4^+CXCR5^+$) cells can produce more IL-21 and show higher affinity maturation than. They also help B cells to produce large amount of immunoglobulins and undergo class switching Igs production (*121*). Recently, it has been proposed that cTfh cells show diversified phenotypic attributes in their surface. They share some common characteristics with other $CD4^+$ Th cells specially Th1, Th2, Th17 suggesting go further insight. Recently Morita et al., divide cTfh into three distinct subsets on the basis of Th1

related surface marker CXCR3 and Th17 related surface marker (Table 1.2). The three subtypes are: cTfh1 ($CXCR5^+CXCR3^+CCR6^-$), cTfh2 ($CXCR5^+CXCR3^-CCR6^-$) and cTfh17 ($CXCR5^+CXCR3^-CCR6^+$) (Table 1.1). These sub classes distinctively produce IFN-γ, IL-4 and IL-17 respectively but their main cytokine production is IL-21. These additional features increased helper capacity of Tfh cells even more robust and committed toward B cells (*121*).

Table 1.1 Basic Characteristics of Different Subtypes of Tfh Cells

Subtypes	Surface markers	Cytokine release	B cell help and Ig production	Helping capacity
cTfh1	$CXCR5^+CXCR3^+$ $CCR6^-$	IL-21, IFN-γ	Not proficient helper of naïve B cells	cTfh1 <cTfh2<cTfh17
cTfh2	$CXCR5^+CXCR3^-$ $CCR6^-$	IL-21, IL-4	IgG, IgE, IgA, IgM Proficiently help naïve and memory B cells	
cTfh17	$CXCR5^+CXCR3^-$ $CCR6^+$	IL-21, IL-17	IgG, IgA, IgM Proficiently help naïve and memory B cells	

1.2.4. T follicular Regulatory (Tfreg) Cells

Recently, Follicular regulatory (Tfreg) cells are identified in GC expressing $FoxP3^+$ where they control GC reactions (*122*). After infection and immunization, Tfreg cells differentiate and express canonical Treg surface markers including FoxP3, CD25 along with CXCR5, PD-1, ICOS and BCL-6 (*123, 124*). Similar to Tfh, Tfreg cells also differentiate from $CD4^+$ T expressing FoxP3 cell precursors and migrate to germinal center. Tfreg cells can also derive from $FoxP3^-$ cells (*125*). The exact function of Tfreg cells is still unknown. But they are recognized for limiting Tfh-mediated B cell activation and antibody response (*123*). However, peripheral Tfreg cells are not well characterized yet in human because of accessing difficulties of GC; therefore scientists are focusing their studies on human blood circulating Tfreg cells.

1.3. Tfh/Tfreg Hypothesis in Multiple Sclerosis

It is well-known that avoidance of autoimmunity largely depends on immune tolerance. Progressive breakdown of immune tolerance facilitate the way to accumulate varieties of autoreactive immune cells like T and B cells which ultimately leads to autoantibody production. Emerging evidence claimed that Tfh cells should be controlled tightly to prevent them from overexpressed and initiate autoimmunity. An increased frequency of Tfh cells and their role in autoimmunity has been reported for the first time in mice in 2009 (*126*). Overactive Tfh cells found to play role in several autoimmune disease like

systemic lupus erythematous (SLE) (*105*), Sjogren's syndrome (*127*), rheumatoid arthritis (RA) (*128*), autoimmune thyroid disease (*129*) and myasthenia gravis (MG) (*130*). Moreover, abnormal Tfh polarization toward Tfh subtypes has been reported to produce autoantibody juvenile dermatomyositis, SLE, IgG4-related disease, and rheumatoid arthritis (*131-133*).

On the other hand, lack of Tfreg functioning is responsible for greater GC response. Dysfunction in Tfreg cells in GC facilitates large amount of high-affinity guided autoantibody production by B cells that leads to variety of autoimmune diseases (*102, 134, 135*). A large number of evidences reported that disruption of immune homeostasis by Tfh-Tfreg persist in the peripheral blood of different autoimmune disease. Tfreg dysregulation has been observed in multiple autoimmune diseases like MG, SLE and Child immune thrombocytopenia etc. (*136-138*).

Although multiple sclerosis (MS) comprehensively studied as $CD4^{+}$T cell mediated autoimmune disease, very few and limited reports have been published about the involvement of Tfh and Tfreg cells in MS. But Tfh related markers like CD40L, CXCL13, IL-6, have been found in the CNS of MS patients that eventually made us curious to explore the role of Tfh in MS. Moreover, result EAE model claimed that Tfh cells are positively correlated disease progression and severity (*139*) which was further supported by increasing disease severity in Tfreg defective mice. **Therefore the hypothesis of this study;-**

"Dysregulated T follicular helper cells and regulatory T follicular cells will be present in patients with multiple sclerosis."

2. Objectives

The objectives of this research are as follow;-

- To define the frequency and functional characteristic of Tfh cells in multiple sclerosis (MS).
- To investigate the proportion of Tfreg cells and its relation with Tfh cells in MS.
- To observe how the frequency distortion affect corresponding cytokines production in MS.

3. Materials and Methods

3.1. Participant and Study Design

Ten MS patients (7 female, 3 male) with RRMS, mean age 37 years were considered from the hospital of National Cancer Center. Patients were selected according to revised Mcdonald criteria 2017 *(19)*. Patients were treated with different immunomodifiers. 3 patients were treated with dymethyl fumarate BG12, 4 of them undergone β-IFN treatment, 2 patients were receiving copaxone and one was treatment-naive. The patient baseline characteristics are described in Table 3.1. Ten healthy controls (HC) were recruited for comparative studies. HC were selected by confirming the absent of previous autoimmune history. All participants were provided written informed consent.

3.2. Blood Sample Processing

Whole blood was collected by venipuncture into 10 ml tubes containing sodium heparin and inverted to mix. Samples were place on shaking reck and processed immediately after collection. Peripheral blood mononuclear cells (PBMC) were isolated and used for the experimental purpose.

Table 3.1 The Characteristics of Participants Considered for Experiment

Baseline characteristics	Healthy Controls	MS Patient
No. Of subjects	10	10
Age	34.36±7.35	37.54±6.72
Sex (Male/Female)	4/6	3/7

3.2.1. PBMC Isolation

PBMC were isolated from the fresh blood by density gradient centrifugation over Ficoll-Paque. 1% phosphate-buffered saline (PBS) containing 2mM ethylenediaminetetraacetic acid (EDTA) were used for whole procedure. Collected bloods were mixed with 2mM EDTA-PBS into 1:1 ratio into 50 ml conical centrifuge (Falcon) tube. Then the blood solution was transferred on the top of 15 ml Ficoll-Paque solution in a 50 ml falcon tube. Then the tubes were centrifuged at 1680 rotation per minute (rpm) for 30 minutes at room temperature. The accelerating setting 9 and decelerating setting (brakes off) 0 were considered during centrifugation. After that, buffy coat layer containing PBMC were

separated and transferred in 50 ml falcon tube by plastic Pasteur pipette. Then the PBMC were mixed with 45 ml 2mM EDTA-PBS and centrifuge at 1680 rpm for 15 minutes at room temperature. This-time, both acceleration and deceleration brakes were set 9. This step was repeated two times and finally the pellet was resuspended with 1 ml 2mM EDTA-PBS. The cells were counted by haemocytometer. Some portion of freshly isolated PBMC was processed for experimental assay while others were cryopreserved.

3.2.2. Cell Culture and Stimulation

My experimental set up was divided into three panels; one panel was considered for unstaining sample, two others for experimental samples (One for Tfh cells and another for Tfreg cells). A number of $2x10^5$, $2x10^6$ and $2.5x10^6$ PBMC were selected for unstaining, Tfh cells and Tfreg sample respectively. Cells were seeded into 12-well plate and each well was filled with 1ml complete media (RPMI-1640; Hyclone $^+$10% fetal bovine serum (FVS); Hyclone $^+$1% Penicillin-Streptomycin antibiotics; Hyclone). Cells were stimulated for intracellular cytokine staining (ICS) with T cell stimulator. Phorbol myristate acetate (PMA; 50ng/ml media; Sigma-Aldrich), ionomycin (500ng/ml media; Sigma-Aldrich) and golgistop (Monensin; 0.6µl/ ml media; BD Bioscience) were added to each well and mixed properly. Finally, cells were culture 5 hours.

3.2.3. Surface Staining, Fixation and Intracellular Staining

After 5 hours cells were washed 2 times with 1x PBS. During washing, the cells were spinned down by centrifugation at 6000 rpm for 2 minutes at 4^0C and resupended with 1x PBS. After washing, the cells were stained with fixable viability dye 700 and incubated 15 minutes in dark at 4^0C to discriminate live and dead cells. Then the cells were washed and spinned down at the same condition mentioned above with staining buffer 1% bovine serum albumin (BSA). The detailed information of buffer and solution are outlined in Table 3.2. The cells were then stained with antibodies against surface markers of different T cell phenotypes followed by 30 minutes incubation in dark at 4^0C. Anti-CD3, anti-CD4, anti-CXCR5 and anti-PD-1antibodies (Abs) were used to define Tfh cell population. Before surface staining the Abs were mixed (antibody-cocktail) with brilliant buffer staining reagent (BD Bioscience) by designing perfect antibody panel depending on their fluorochrome. The detailed information for antibodies and their fluorochromes are incorporated in Table 3.3. On the other hand, anti-FoxP3, anti-CD25 along with above mentioned Abs were stained for defining Tfreg cells. Additionally, anti-CXCR3, anti-CCR4 and anti-CCR6 Abs were used to stain for investigating other CD^+Th phenotypes as well as subtyping of Tfh cells. After incubating 30 minutes the cells were washed two times with staining buffer (1% BSA). The cells were fixed with cytofix solution containing 2% PFA and incubated for 30 minutes at the same condition as surface staining. Then, the cells were washed again two times with fixation- permeabilizaation buffer. In this

stage centrifugation was set at 8000 rpm for 5 minutes at 5^0C. Finally, intracellular cytokine staining was performed by staining PBMC with anti-IL-21, anti-IFN-γ, anti-IL-4 and IL-17 Abs. The antibody panel was designed and prepared same way for surface staining (Table 3.3). For Tfreg panel ICS was performed using anti-IL-10 Abs. FoxP3 stained was performed accordin to manufacturer protocol (BD Bioscience). After staining with ICS antibodies, the cells were incubated for 30 minutes at 4^0C in dark. Finally, after two times washing with 1X perm wash buffer the samples are taken to flow cytometry for acquiescing the data.

3.3. Data Acquisition and Analysis

3.3.1. Flow Cytometry

The study of immune cells and their subsets is very complex process because of their diversified phenotypes and classifications. Immune cells are widely studied through their surface markers, intracellular markers and transcription factors. But the combinations of huge markers generate lots of difficulties regarding identification and profiling immune subsets properly. However, multi-parameter analysis may allow us to identify and describe different immune cells under one panel. Recently, muticolour flow cytometry becomes a powerful tool to differentiate immune cells. Multicolour flow cytometry uses fluorochrome-conjugated antibody labeling of multiple cellular markers which paves the way of phenotypic assessment immune cells to identify exact cell population for a particular immune cell type.

For the acquisition of experimental data we used LSR Fortessa flow cytometer (BD Bioscience). Single-stained compensation beads were used as compensation controls. Unstained controls, internal control were processed alongside with experimental samples. During cell acquisition individual cells were compensated according to their fluorochrome to avoid spillover between cells. The flow cytomtric data were analyzed using FlowJo software. Initially the cells were arranged properly by excluding cell debris, doublets and dead cells to get fine singlet of lymphocytes (Figure 3.1). At least 20,000 lymphocytes were selected by gating with forward and side scattered (Figure 3.1).

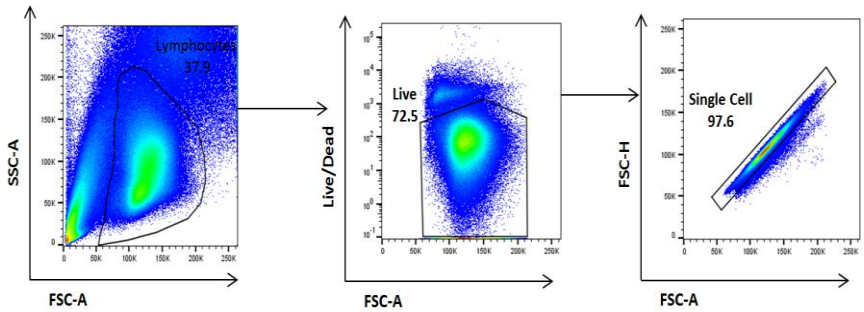


Figure 3.1 Early gating strategy for accessing fine singlets of lymphocytes. First the lymphocytes were gated. The live cells were selected by gating out the dead cell. Finally, single cell clumps were selected for further analysis by avoiding doublets, spill over problems.

Table 3.2 List of Buffers and Solutions Used for PBMC Isolation and Experimental Procedure

Procedure	Buffer & Solution	Details
PBMS isolation	PBS+EDTA	1xPBS (WEL-GENE) 2mm EDTA (WEL-GENE)
PBMC stimulation & culture	T cell stimulation	RPMI-1640 media PMA; 50ng/ml (Sigma-Aldrich) Ionomycin; 500ng/ml (Sigma-Aldrich) Golgistop; 0.6µl/ml (BD Bioscience)
PBMC staining	Staining buffer	1x PBS 1% BSA (Milipore)
PBMC fixation & permeabilization	Brilliant buffer	BD Bioscience
	Cytofix/ Cytoperm	BD Bioscience, ebioscience
	Permeabilization buffer	eBioscience
	BD Perm/Wash	BD Bioscience

Table 3.3 List of Fluorochrome-Conjugated Antibodies and Fluorescent Dyes

Antibody	Fluorochrome conjugation	Source
Tfh Panel		
CD3	PE-cy7	BD Biosceince
CD4	Buv496	BD Biosceince
CD8	FITC	BD Biosceince
CXCR5	Percp-cy5.5	BD Biosceince
PD-1	BV786	BD Biosceince
CXCR3	PE	BD Biosceince
CCR6	BV711	BD Biosceince
CCR4	BV510	BD Biosceince
IFN-γ	APC	BD Biosceince
IL-21	BV421	BD Biosceince
IL-17	BV650	BD Biosceince
IL-4	BV605	Bio Legend
Dead cell staining	Alexafluor-700	BD Biosceince
Tfreg Panel		
CD3	PE-cy7	BD Biosceince
CD4	Buv496	BD Biosceince
CD8	FITC	BD Biosceince
CXCR5	Percp-cy5.5	BD Biosceince
PD-1	BV786	BD Biosceince
CD25	APC-Cy7	BD Biosceince
FoxP3	PE	eBioscience
IL-10	PE-CF594	BD Biosceince

3.3.2 Statistical Analysis

Statistical significance was performed using prism software (Graphpad version5). Students t test was performed for two group analysis and one way ANOVA were used for continuous measurement in three groups. Two-tailed analysis was performed for statistically significant. A probability of less than 0.05 ($p<0.05$) was considered as statistical significance. All data were shown in Mean±SEM value.

4. Result

4.1. Prospective Analyzing Strategy for Immune Cells

This study aimed at defining the lineage of T cell subsets in both HC and MS. I chronologically analyzed $CD4^+$T cell, different subsets of Th cells, Tfh cells, Treg, Tfreg cells based on their surface markers. The substantial T cells were also analyzed and confirmed through the expression corresponding cytokines. The gating strategy for different T cells subtypes is shown in Table 4.1.

4.2. Circulating Tfh Cells were Significantly Increased in MS

Changes in the momentum of Tfh cells are known to play important role in humoral mediated autoimmune disease. 20 specimens of PBMC (10 healthy controls and 10 MS patients) were analyzed by flow cytometry to characterize Tfh cells. First I investigated whether any change observed in the frequency of $CD4^+$ T cells in MS patients or not. The average frequency of $CD4^+$ T cells did not show any significant changes between HC and MS **(**Fig 1a *(middle scatter plot)* & b). Then I evaluated the expression of cTfh ($CXCR5^+PD\text{-}1^+$) cells in MS comparing with HC. The frequency of cTfh cells were significantly elevated in MS patients compared to HC (Fig 1a *(right scatter plot)* & c), p=0.0002.

Table 4.1 Gating Strategy for Different Subsets of T Cell

Defined T cells population	Surface phenotyping	Signature Cytokines
Lymphocytes		
$CD4^+$ T cell	$CD3^+CD4^+$	
T follicular helper cells	$CD4^+CXCR5^+PD\text{-}1^+$	IL-21
CTfh1 Cells	$CD4^+CXCR5^+PD\text{-}1^+$ $CXCR3^+CCR6^-$	IL-21, IFN-γ
CTfh2 Cells	$CD4^+CXCR5^+PD\text{-}1^+$ $CXCR3^-CCR6^-$	IL-21, IL-4
CTfh17 Cells	$CD4^+CXCR5^+PD\text{-}1^+$ $CXCR3^-CCR6^-$	IL-21, IL-17
Non-follicular Th cells	$CD4^+CXCR5^-$ T cell	
Th1 Cells	$CD4^+CXCR3^+CCR4^-CCR6^-$	IFN-γ
Th2 Cells	$CD4^+CXCR3^-CCR4^+CCR6^-$	IL-4
TH17 Cells	$CD4^+CXCR3^-CCR4^-CCR6^+$	IL-17
Regulatory T follicular Cells	$CD4^+CXCR5^+PD\text{-}1^+$ $FoxP3^+CD25^+$	IL-10
nTfreg Cells	$FoxP3^+CD25^+$	IL-10
iTfreg Cells	$FoxP3^+CD25^-$	IL-10
$CD25^+$ Tfreg Cells	FoxP3-$CD25^+$	IL-10

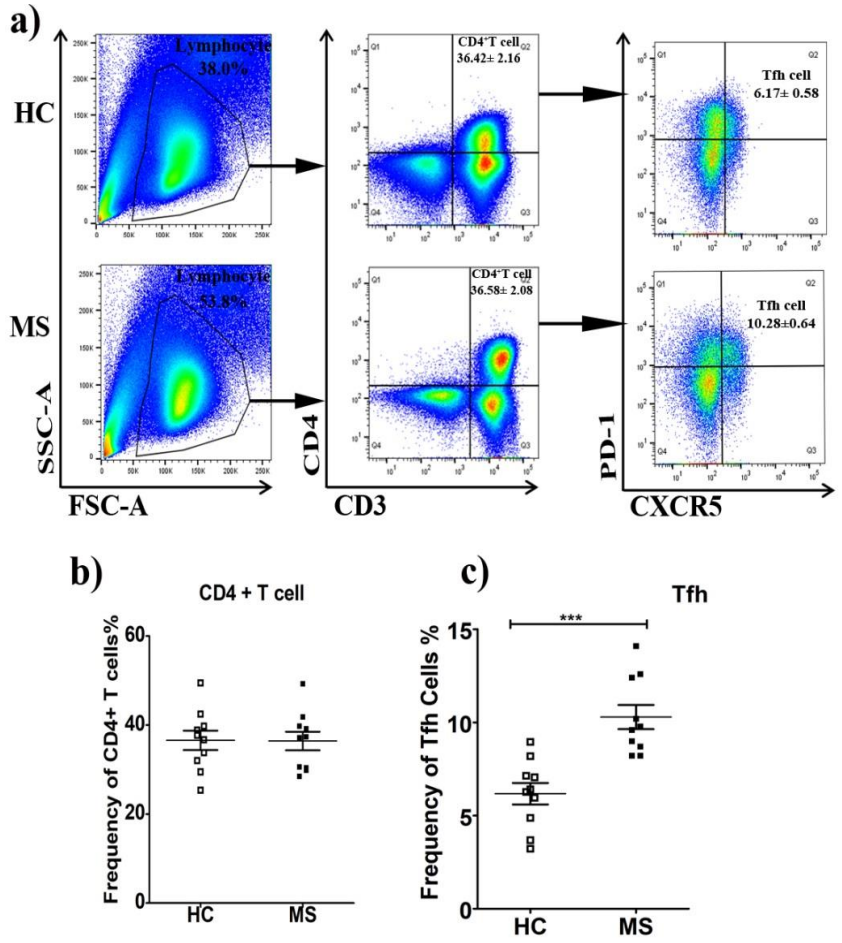


Figure 4.1 Frequency of circulating Tfh cells were significantly increased in MS. Fresh PBMC were stained with monoclonal antibodies against anti-CD3, anti-CD4, anti-CXCR5 and anti-PD-1 a) Gating strategy for cTfh cell population; $CXCR5^{+}PD\text{-}1^{+}$ *(right panel)* cells were selected by gating on $CD3^{+}CD4^{+}$ T cells *(mid panel)*, b) Percentage of $CD4^{+}$T cells were calculated and compared MS and HC, c) Percentage of cTfh cells in MS patients were calculated and compared with those of HC, ***p 0.0002. Each data point represent each individual subjects.

4.3. Tfh17 and $CXCR3^{+}CCR6^{+}$ Tfh Cells were the Major Subsets of Tfh cells that Significantly Increased in MS.

Tfh cells have been categorized into three different subsets (Table 1.1) based on Th1 and Th17 related surface markers (CXCR3, CCR6) where they play critical role in autoimmune disease (*121, 130*). $CXCR3^{+}$ Th1 and $CCR6^{+}$ Th17 cells were described earlier to play essential role in MS (*78*). I explored the persistence of CXCR3 and CCR6 markers in Tfh cells to classify Tfh subsets and evaluate their frequency in MS patients. This data represented that cTfh17 ($CXCR3^{+}CCR6^{+}$-Tfh) cells were significantly increased in MS patients compared to HC (Figure 4.2a & d), $p<0.0001$. Interestingly, It was found that $CXCR3^{+}CCR6^{+}$ cTfh were significantly increased in MS patients (Figure 4.2a & e), $p<0.0138$. Recently, co-expression of $CXCR3^{+}CCR6^{+}$ on Th cells has been recognized as new subtypes of $CD4^{+}$ T cells. These subtypes are commonly known as T-helper 1-like-Th-17 (Th17.1) cell (*140*). I also observed the similar co-expression of $CXCR3^{+}CCR6^{+}$ in follicular level suggesting us to recognize them as Tfh17.1 cell. On the counter wise, cTfh1 and cTfh2 cells were decreased in MS patients compared to HC (Figure 4.3. a-c), but no statistical difference was observed.

4.4. Non- follicular Th cells subsets showed no significant difference in MS.

As it was observed, the frequency of follicular T helper cells and their subsets were significantly changes in MS patients. Therefore, my investigations continued to decipher the non-follicular Th cells to observe their level of expression in MS compared to HC. Unfortunately, I didn't observe and noticeable difference between them. More precisely, the expression of Th1 cells were reduced in MS patients compared to HC (Figure 4.3a & b). However, Th2 cells were found to be expressed slightly higher in MS patients without any statistical difference (Figure 4.2a & c). Non- follicular Th17 cells were slightly decreased in MS **(**Figure 4.3a & d). Importantly, I also investigated non-follicular $CXCR3^{+}CCR6^{+}$ Th cell subsets termed as Thelper-1-like-Th7 (Th17.1) cells (*83*). But the result couldn't able to detect any significant alteration between HC and MS patients, although the percentages were bit lower in MS (Figure 4.3a & e).

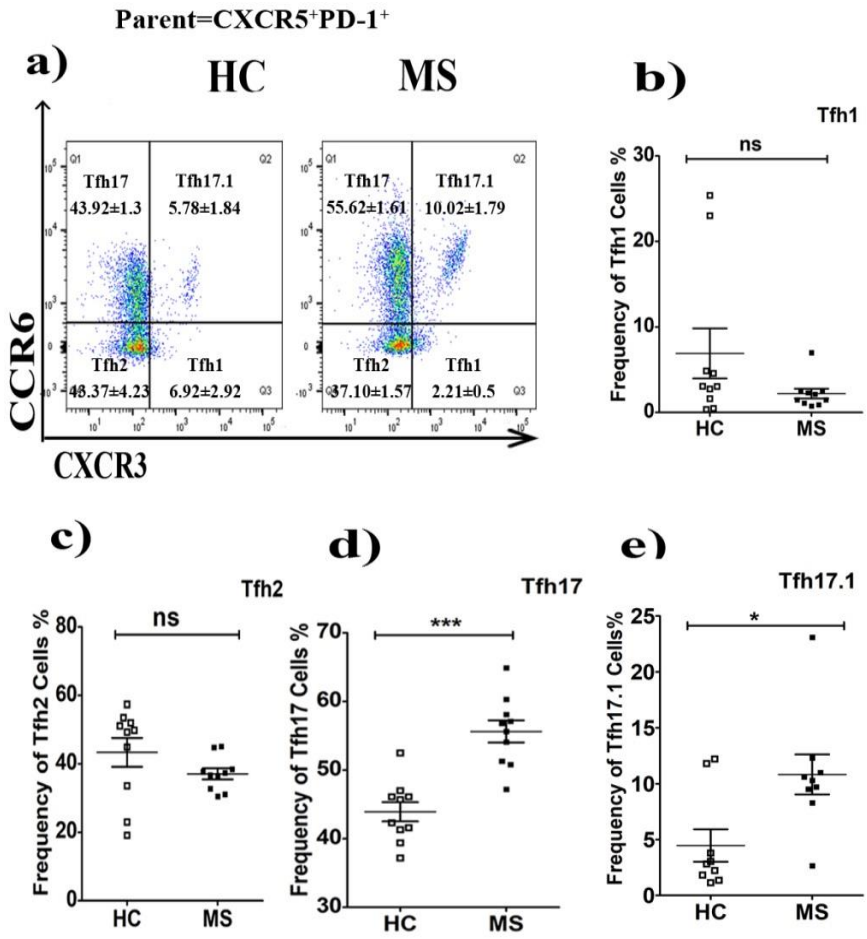


Figure 4.2 cTfh17 and CXCR3^{+}CCR6^{+} cTfh were the major subtypes that increased in MS. Fresh PBMC were surface stained with anti-CD3, anti-CD4, anti-CXCR5 anti-PD-1, anti-CXCR3 and anti-CCR6 antibodies a) Gating strategy; CXCR3 and CCR6 were gated on CD4^{+}CXCR5^{+}PD-1^{+} cells to delineate Tfh subtypes where CXCR3^{+}CCR6^{-} selected as Tfh1, CXCR3^{-}CCR6^{-} is for Tfh2 and CXCR3^{-}CCR6^{+} defined as a Tfh17 cells and CXCR3^{+}CCR6^{+} recognized as Tfh17.1 b-e) Proportion of different subtypes of Tfh were calculated in MS and compared with those HC, ***$p<0.0001$ and *$p<0.0138$. Each data point indicates each individual**.**

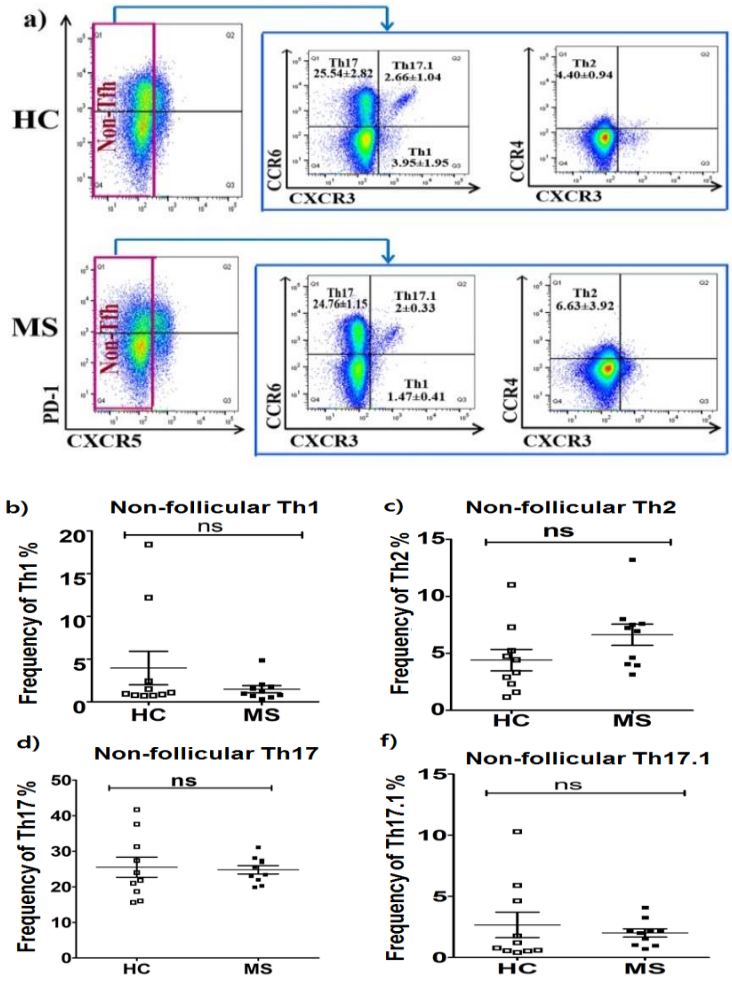


Figure 4.3 Frequency of non-follicular T cell subsets didn't show any difference between HC and MS. Fresh PBMC were surface stained with anti-CD3, anti-CD4, anti-CXCR5 anti-PD-1, anti-CXCR3, anti-CCR6 and anti-CCR4 antibodies a) Gating strategy; CXCR3 and CCR6 were gated on $CD4^{+}CXCR5^{-}$ cells to delineate Th subtypes where $CXCR3^{+}CCR4^{-}CCR6^{-}$ selected as Th1, $CXCR3^{-}$ $CCR4^{+}CCR6^{-}$ is for Tfh2 and $CXCR3^{-}$ $CCR4^{-}CCR6^{+}$ defined as a Th17 cells, and $CXCR3^{+}CCR6^{+}CCR4^{-}$ are defined as Th17.1 cells, b-e) Proportion of different non-follicular Th subpopulations were calculated in MS and compared with those HC. Each data point indicates each individual.

4.5. Frequency of IL-21 producing $CD4^+$T cells were significantly increased in MS

Characterization of pro-inflammatory cytokines has been accepted one of the key strategies to study autoimmune disease. This study evaluated some key inflammatory cytokines such as IFN-γ, IL-4, IL-17 and IL-21 in MS patients and compared with those of HC. I first investigated the secretion of these cytokines by lymphocytes followed by sequential analysis cytokine production by T cells and B cells. I found that the level of IL-21 secretion by lymphocytes was significantly enriched in MS patients compared to HC, p=0.0013 (Figure 4.4a & b and Table 4.2) whereas secretion of IFN-γ, IL-4 and IL-17 by lymphocytes didn't showed any significant difference between HC and MS (data not shown). Then I analyzed $CD3^+$T and $CD3^-$ (mostly B cell) cells to observe the secretion of IL-21 by those cell types. I observed that $CD3^+$T cells but not $CD3^-$ cells were engaged with increased production of IL-21 in MS compared to HC, p=0.0203 (Table 4.2). $CD4^+$T and $CD8^+$T cells are two main subtypes of $CD3^+$T cells which involve in great variety of cytokine production. It is needed to testify whether they contribute to elevated secretion of IL-21 in MS. This data revealed that among $CD3^+$T cells, $CD4^+$T cells were significantly involved with higher production of IL-21 in MS compared to HC whereas $CD8^+$T secreting IL-21 showed no significant alteration between HC and MS (Figure 4.4c & d and Table 4.2). Finally, I evaluated IFN-γ, IL-4 and IL-17 producing $CD4^+$Ts cell to investigate and compare their frequency between HC and MS, but I couldn't

observe any significant alteration those cytokines between HC and MS (Figure 4.4e-g).

4.6. cTfh cells revealed as the main producer of IL-21 in MS

Production of IL-21 is the distinctive feature of Tfh cells has been widely studied in different autoimmune disease because of their incentive action on autoreactive B cells (*143, 144*). As it is described earlier Tfh cells belong to $CD4^{+}$T cell which are significantly higher in MS patients (Figure 4.1a & c). Moreover, IL-21 $CD4^{+}$T producing cells also increased in MS patients (Figure 4.4c & d). Now, I further investigated whether Tfh were engaged with predominant IL-21 secretion. The result has shown that in accordance with increased proportion of Tfh, IL-21 secretion by Tfh were also significantly increased in MS patients compared to HC, p=0.0002 (Figure 4.5a & b). Additionally, I checked if there exist any difference of IL-21 secretion by Tfh and non-follicular Th cells. Comparative analysis IL-21 production by follicular versus non-follicular Th cells revealed that Tfh dominantly secreted IL-21 in both HC and MS, where the secretion level was remarkably higher in MS patients, p=0.0001(Figure 4.5a & b). However, in HC, there was no notable difference between follicular and non-follicular Th cells have observed in terms of IL-21 production. Taken together, it is clear from this data that Tfh cells and their signature cytokine (IL-21) abundantly express in MS where Tfh is the main source of IL-21 production.

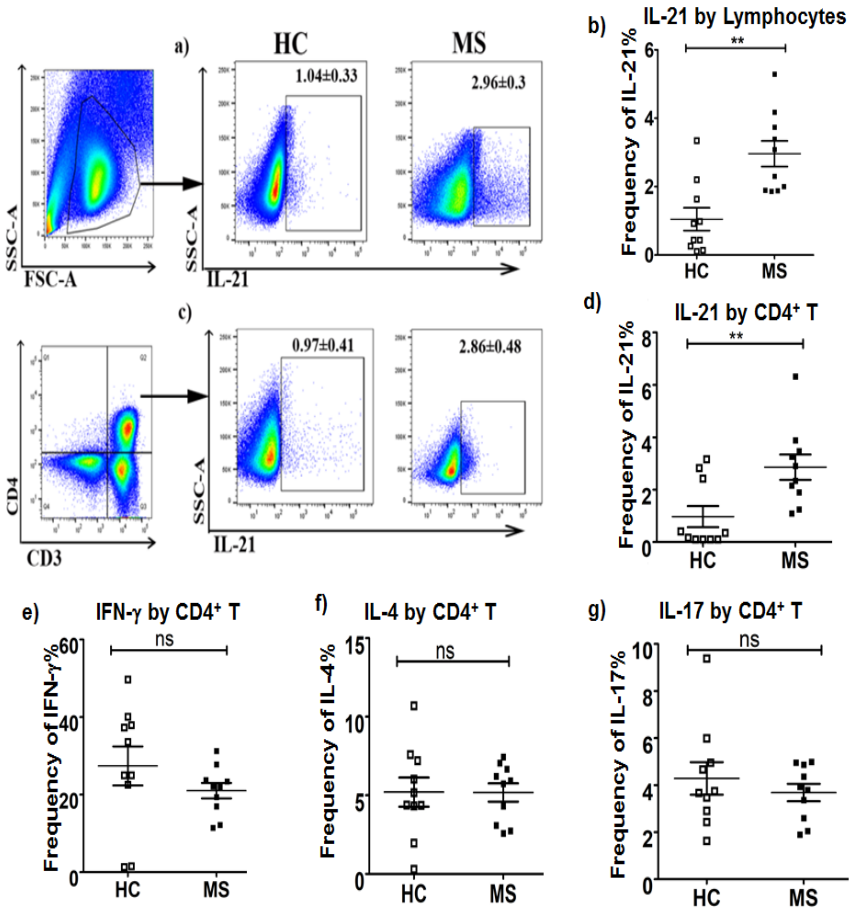


Figure 4.4 Frequency of IL-21 producing $CD4^+$T cell was significantly increased in MS. Fresh PBMC from both HC and MS were intracellular stained with anti-IFN-γ, anti-IL-4, anti-IL-17 and anti-IL21 antibodies after stimulating 5 hours with PMA, ionomycin and golgistop, a) Gating strategy; IL-21 population was selected by gating on lymphocyte, b) Proportion of IL-21 secreted by lymphocytes were calculated in MS patients and compared with those of HC, **p=0.0013, c) Gating strategy; IL-21 population was selected by gating on $CD3^+CD4^+$ T cells, d) Percentage of IL-21 production by $CD3^+CD4^+$T cells were calculated in MS patients and compared with HC, p=0.0078, e-g) Proportion of IFN-γ, IL-4 and IL-17 were examined in MS patients and compared with that of HC. Each data point indicates each individual**.**

Table 4.2 Secretion of IL-21 among Different Immune Cells

Cell types	IL-21		
	HC	MS	P value
Lymphocytes	1.04 ±0.33	2.96 ±0.3	0.0013
$CD3^+$ T cell	0.81 ±0.34	1.95 ±0.32	0.0203
$CD3^-$ cell	1.38 ±0.49	1.26 ±0.21	0.8200
$CD4^+$ T cell	0.97 ±0.40	2.86 ±0.48	0.0078
$CD8^+$ T cell	0.69 ±0.31	1.29 ±0.33	0.2085
Tfh cell	4.10 ±1.75	14.58 ±1.75	0.0002

4.7. Elevated Tfh17 and cTfh17.1 Cells were Associated with Increased Production of IL-21 in MS

From this experiment I already showed that cTfh17 and cTfh17.1 were the dominant subtypes for Tfh cells that expressed higher in MS (Figure 4.2a, d & e). Thereafter, I needed to determine whether these subtypes also contribute to the increased production of IL-21 in MS or not. For this purpose I performed surface staining as well as ICS using same stimulating condition mentioned above (Figure 4.2 & 4.4). Although, all the subtypes were engaged with the production of IL-21, only cTfh17 ($CXCR3^-CCR6^+$-Tfh) and cTfh17.1 ($CXCR3^+CCR6^+$-Tfh) showed statistically significant difference regarding IL-21 secretion between HC and MS, $p<0.0001$(Figure 4.6a & b). Interestingly, I observed that it was cTfh17.1 cell who were engaged with maximum production of IL-21 off all subtypes (Figure 4.6 b). However, as far it is concerned, Tfh17 can also produce IL-17, and I sequentially analyzed and compared IL-17 production by Tfh17 in

HC and MS followed by comprehensive analysis of IL-17 production by Tfh17 vs non- follicular Th17 cells in HC and MS patients. I didn't observe any significant difference between them (Figure 4.6c & d). On the other hand, significant increase of CXCR3 and CCR6 co-expression on cTfh17.1 cells in MS patients made us curious to elucidate them further based on different cytokines. I evaluated the secretion of IFN-γ, IL-17 and IL-21 by this subset of Tfh. It was shown that cTfh17.1 released all three cytokines with different significant level (Figure 4.6e). More precisely, secretion of IL-21 and IL-17 by this cellular subtypes significantly increased in MS than that of HC, $p<0.0001$ & $p=0.0415$ respectively. Importantly, IL-21 was the dominant cytokine secreted by cTfh17.1 over others. Collectively, the findings suggested that both elevated cTfh17 and cTfh17.1 cells were also responsible for overproduction of IL-21 in MS where cTfh17.1 cells were the main secretor of IL-21 in MS.

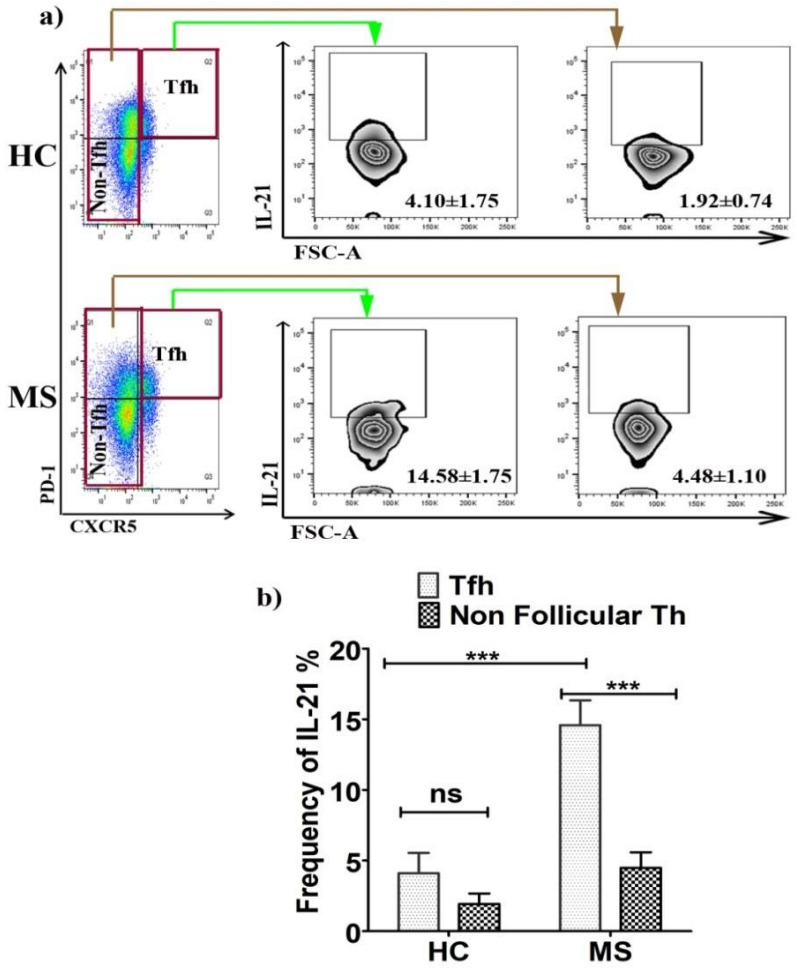


Figure 4.5 Frequency of IL-21 producing Tfh was significantly higher in MS. Fresh PBMC were surface stained as well as ICS as describe in (Figure 4.1& 4.4) a) Gating strategy: $CXCR5^{+}PD\text{-}1^{+}$ and $CD4^{+}CXCR5^{-}$ cells were selected as a source of IL-21 from $CD4^{+}$ T cells. Then, IL-21 population was derived from Tfh and non- follicular Th cells, b) Percentage of IL-21 production by Tfh and non-Th cells were calculated and compared between MS and HC. ***p=0.0002 denotes the comparative analysis of IL-21 secretion by Tfh cells between MS and HC. ***p=0.0001 represent the statistical analysis of IL-21 production by Tfh and non-Tfh cells in MS.

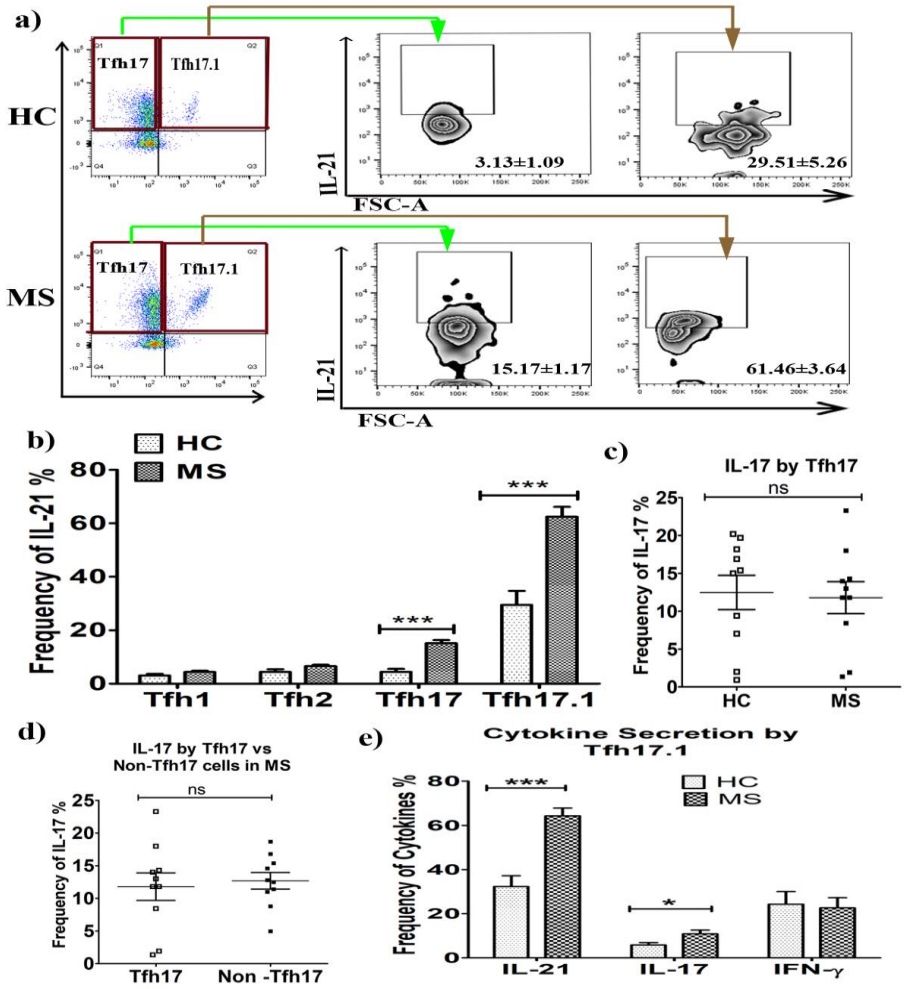


Figure 4.6 Increased frequency of Tfh17 and cTfh17.1 cells were involved in elevated secretion of IL-21 in MS. a) Gating strategy; IL-21 population was selected from $CXCR3^{-}CCR6^{+}$ and $CXCR3^{+}CCR6^{+}$ Tfh cells which were the derivative of $CXCR5^{+}PD\text{-}1^{+}$ Tfh cells (Figure 4.1), b) Percentage of IL-21 production by different subtypes of Tfh cells were calculated in MS and compared with those of HC, ***p <0.0001, c) The frequency IL-17 production by Tfh17 in MS and HC were calculated and compared. d) IL-17 production by Tfh17 and non-follicular Th17 cells in MS showed no significant difference, e) Percentage of IL-21, IL-17 and IFN-γ production by $CXCR3^{+}CCR6^{+}$ Tfh cells were calculated in MS and compared with those of HC, ***p<0.0001, *P=0.0415 respectively. Each data point represents each individual.

4.8. The Frequency of nTfreg were Significantly Decreased in MS

In contrast to Tfh cells, Tfreg are known to inhibit the activity of Tfh and prevent them helping antibody mediated immune response. Tfreg cells are classically defined by the expression of $CXCR5^{+}PD\text{-}1^{+}FoxP3^{+}CD25^{+}$ cells that can produce IL-10. In autoimmune disease, Tfreg cell population found to be decreased that helps Tfh cells to become hyperactive (*135*). As previously described that Treg cells can be divided into three subgroups naming nTreg, iTreg and $CD25^{+}$Treg (described in 1.1.6 section) (*141*). I also followed the same strategy to define Tfregs. It was shown from this data that nTfreg cells were significantly decreased in MS patients compared to HC, p=0.0008 **(**Figure 4.7a & b). However, I didn't find any remarkable changes in iTfreg frequency between HC and MS (Figure 4.7a & c). On the other hand I found that, $CD25^{+}$Tfreg cells were significantly increased in MS patients compared to HC, p=0.0431 (Figure 4.7a & d).

4.9. Non-follicular nTreg didn't show any significant difference between HC and MS

Several reports have been published that nTreg population are impaired in MS patients where they play critical role in disease activity and severity (*95, 142*). In Figure 4.7 I have already shown that nTfreg were expressed lower in MS compared to HC. To verify the result I needed evaluate and compared the

frequency of total and non-follicular nTreg cells. Therefore, I investigated the frequency total nTreg cells followed by non-follicular nTreg cells to check if there any difference exists between HC and MS. As expected, the frequency of nTreg cells were reduced significantly in MS patients compared to HC, p=0.0004 (Figure 4.8a & c). However, we couldn't obverse any remarkable difference regarding the expression of non-follicular Treg cells between HC and MS (Figure 4.8b & d).

4.10. Frequency of IL-10 producing Tfreg cells were higher than their counterpart non-follicular Treg cells

IL-10 is the most common anti-inflammatory cytokines studied in autoimmune disease. In this study, I observed that the secretion of IL-10 by lymphocytes was decreased significantly in MS than in HC, p=0.0016 (Figure 4.9a & b). Although IL-10 is secreted by most of the regulatory T cells, but role of Tfreg on IL-10 production is not documented fully. Therefore, I analyzed and compared the secretion of IL-10 by Tfreg and non-follicular Treg among HC and MS. The result showed that Tfreg cells were highly engaged with IL-10 production compared to non-follicular Treg in both HC and MS, p<0.0001 **(Figure 4.9 c-e)**.

4.11. Depleted nTfreg Cells were Associated with Reduction of IL-10 in MS

Although the frequency of nTfreg was impaired in MS patients, yet the effect of these impaired Tfreg cells on IL-10 secretion needed to explore. Therefore, I targeted nTreg, nTfreg, iTfreg, $CD25^{+}$Tfreg and non- follicular nTreg cells to investigate how their frequency change affects IL-10 production. It was shown that similar to the tendency of nTfreg cells decreased in MS patients, IL-10 secretion by Tfreg also remarkably reduced in MS patients compared to HC (Figure 4.10a & b), p=0.0384. But I didn't observe any significant change in IL-10 secretion by iTfreg and $CD25^{+}$ Tfreg cell between these two groups (Figure 4.10c & d) although the frequency of $CD25^{+}$Tfreg cells were enriched in MS. Finally, I investigated the production of IL-10 by total nTreg and non-follicuar nTreg cells. Similar to Tfreg, IL-10 secretion by total nTreg was significantly lower in MS (Figure 4.10 f). However, I couldn't observe any significant change in IL-10 secretion by non- follicular Treg between HC and MS (Figure 4.10 e**)**.

Collectively, these results suggest that Tfreg and IL-10 may connect each other to take part in the disease activity of MS.

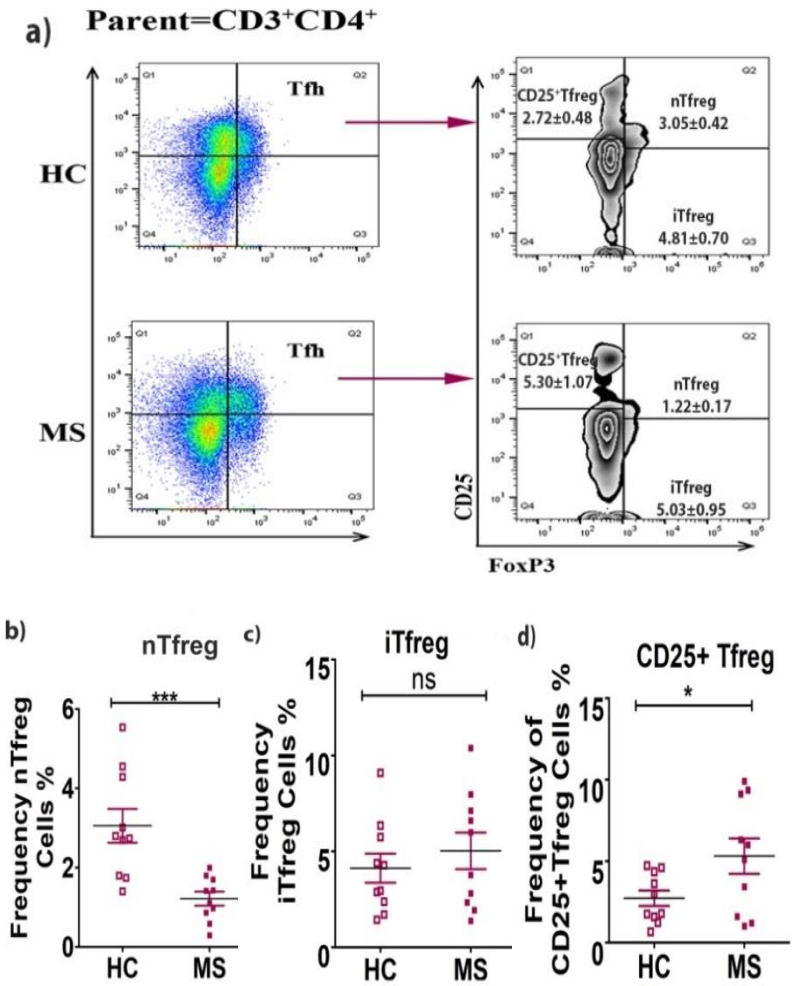


Figure 4.7 Tfreg cells were significantly reduced in MS patients. PBMC were surface stained with anti-CD3, anti-CD4, anti-CXCR5, anti-PD-1 antibodies. FoxP3 staining with anti-FoxP3 antibody was performed according to manufacturer protocol, a) Gating strategy: nTfreg, iTfreg and $CD25^{+}$Tfreg were defined based on $FoxP3^{+}CD25^{+}$, $FoxP3^{+}CD25^{-}$ and $FoxP3^{-}CD25^{+}$ respectively. The cells were gated on $CD4^{+}CXCR5^{+}PD\text{-}1^{+}$ cells (Figure 4.1), b) Percentage of nTfreg cells were calculated in MS and compared with those HC. ***p=0.0008. c) Percentage of iTfreg cell were calculated in MS and compared with those HC. d) Finally, the absolute proportion $CD25^{+}$Tfreg cells were compared between HC and MS, *p=0.0431. Each data point indicates each individual.

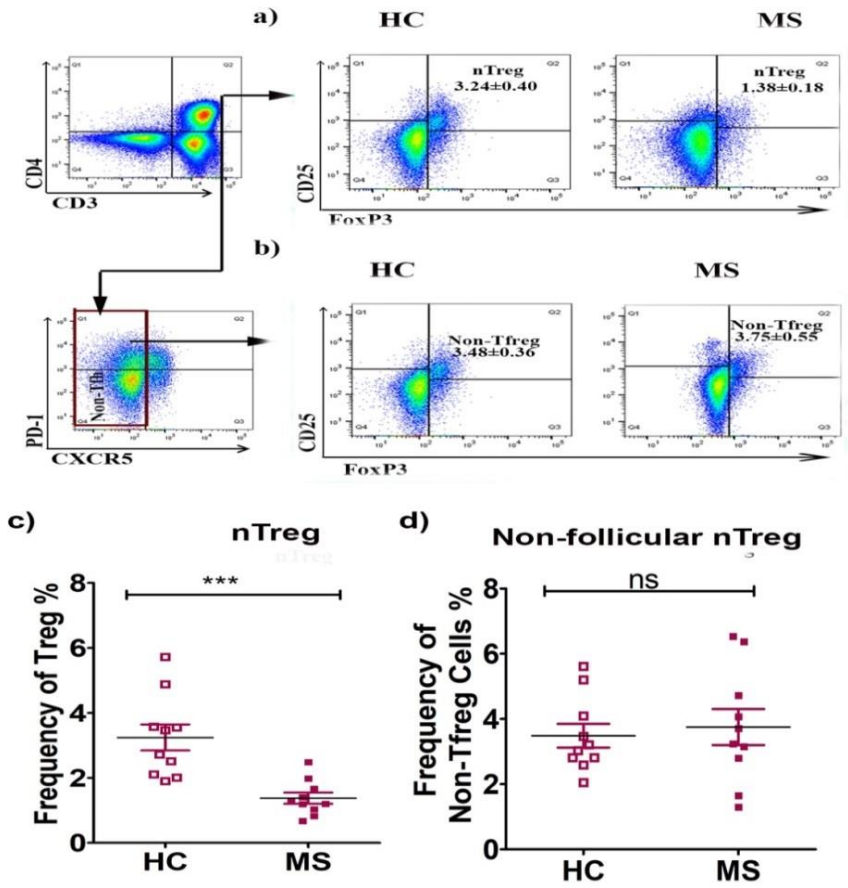


Figure 4.8 Non-follicular Tfreg showed no significant difference between HC and MS**.** Fresh PBMC from both HC and MS surface stained exactly same as described in Figure 4.7, a) Gating strategy: nTreg cells were determined by selecting $FoxP3^{+}CD25^{+}$ cells on $CD4^{+}$ T cell, b) Gating strategy: non- follicular nTreg cells were determind same as Treg but $CD4^{+}CXCR5^{-}$ Th cells were considered as a source of non-Tfreg cells. c) Absolute number of nTreg were significantly reduced in MS patients compared to HC, ***p=0.0004, d) Percentage of non-follicular didn't show any significant difference. Each data point indicates each individual**.**

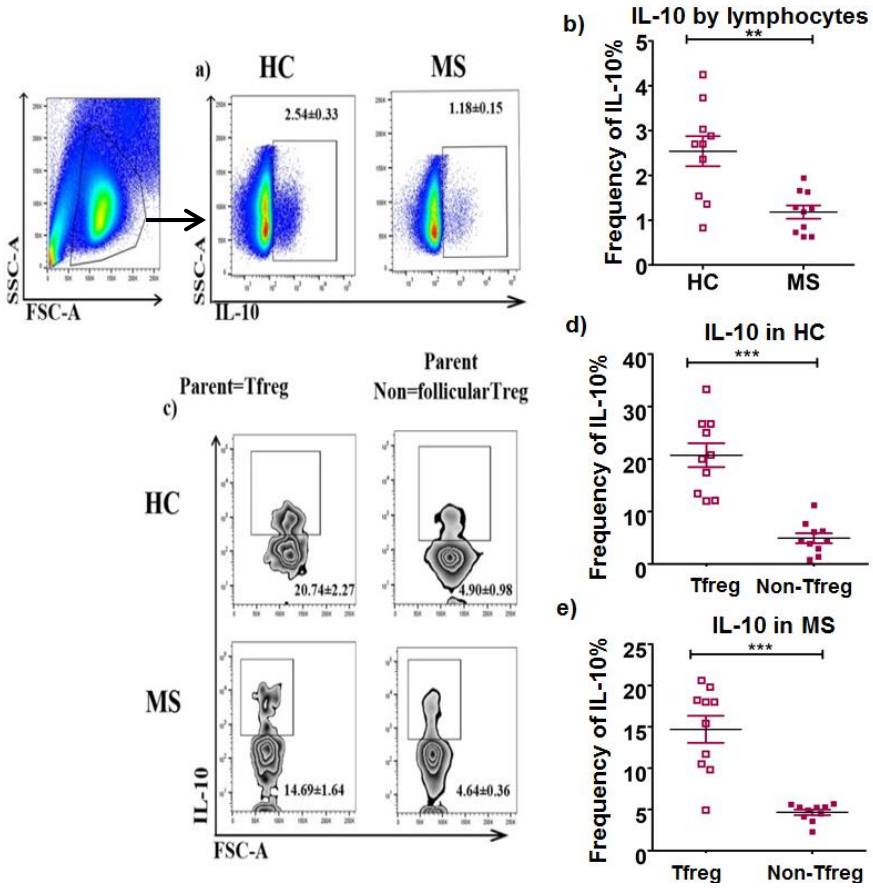


Figure 4.9 Frequency of IL-10 producing Tfreg cells were higher in both HC and MS. Fresh PBMC from both HC and MS were surface stained with monoclonal antibodies described in Figure 4.7. ICS was performed with anti-IL-10 antibody after 5 hours stimulation with PMA, ionomycin and Golgi stop. a) Gating strategy: IL-10 was gated directly on lymphocyte from HC and MS PBMC, b) Proportion of IL-10 secretion by lymphocytes were calculated in MS and compared with that of HC, **p=0.0016, c) Gating Strategy: IL-10 positive cells were selected by gating on $CD4^{+}CXCR5^{+}PD\text{-}1^{+}FoxP3^{+}CD25^{+}$ Tfreg and $CD4^{+}CXCR5^{-}FoxP3^{+}CD25^{+}$ non-follicular Tfreg cells, d-e) Percentage of IL-10 secreted by Tfreg and non-follicular Treg were calculated and compared in both HC and MS, ***p<0.0001 for both HC and MS. Each data point indicates each individual.

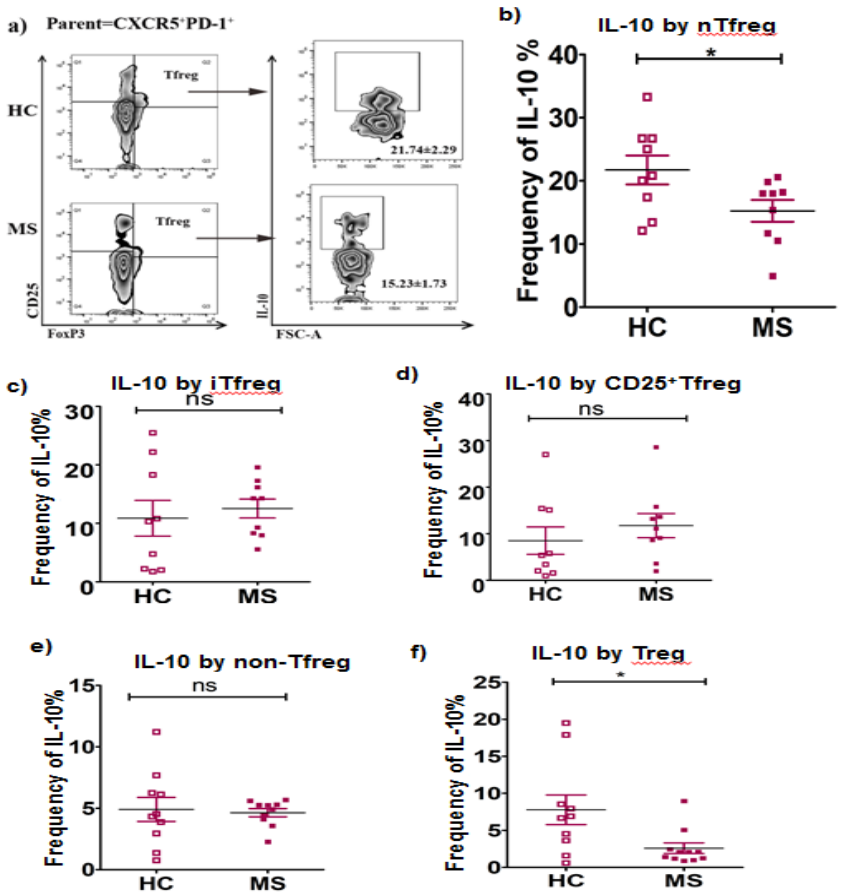


Figure 4.10 Level of IL-10 secretion was decreased proportionally to impaired frequency of Tfreg cells in MS. Fresh PBMC were surface and intracellular stained same as described Figure 4.7 & 4.9. a) Gating strategy: IL-10 positive population was selected by gating on $FoxP3^{+}CD25^{+}$ Tfreg cells which are derived from $CD4^{+}CXCR5^{+}PD\text{-}1^{+}$ (Figure 1), b) Percentage of IL-10 production by Tfreg in MS PBMC were calculated and compared with those of HC, *p=0.0384 c-d), Percentage of IL-10 secretion by iTreg, CD25+Treg were calculated in MS and compared with HC, e & f) Proportion of IL-10 secretion by non-follicular Treg and total nTreg cells were calculated and compared between HC and MS, *p=0.02. Each data point indicates each individual.

5. Discussion

Breakdown of immune tolerance is considered as the key immunopathogenic episodes in MS. Dysregulated effector and regulatory T cells alter the pro-inflammatory and anti-inflammatory cytokine milieu that result the autoreactive immune response in MS (*143*). Tfh and Tfreg cells are the newest member of $CD4^+$T cells that comprise a compact immune regulatory system. Any disturbance of this regulatory system causes autoimmune disease (*137, 144-146*). Despite ongoing research, very little is known about the role of Tfh and Tfreg in MS pathogenesis.

In this present study, I showed an increase frequency of circulating Tfh ($CXCR5^+PD\text{-}1^+$) cells expressed in MS patients compared to HC. Along with Tfh, IL-21 secretion also significantly elevated in MS patients. Subsequent analysis of IL-21 production by follicular and non-follicular Th cells revealed that Tfh cells were the main secretor of IL-21 where the frequencies were remarkably higher in MS patients. Very low secretion of IL-21 by non-follicular Th cells suggests that Tfh cell and IL-21 constitute a distinctive feature of $CD4^+$ T cells. On the other hand, different subsets of $CD4^+$T cells did not result any alteration in their frequency between HC and MS. Moreover, comparative analysis of IFN-γ, IL-4, IL-17 secretion by $CD4^+$T cells in showed no significant change between HC and MS. Initially, the role of Tfh in autoimmune disease was described in murine model (*126*). But recent reports with human data in RA, SLE, MG, autoimmune thyroid disease revealed that increased expression of Tfh cells exist among those

diseases where they associated with disease activity and severity through the amplification of autoreactive B cells and pathogenic autoantibody production (*128, 129, 144, 146*). Moreover, IL-21 is signaturely produced by Tfh cells, which has been reported to secret abundantly in autoimmune diseases that is responsible for autoantibody production and class switching Igs production in disease context (*144, 146*). My result admitted those results but more importantly for MS, I convincingly connect Tfh cells and IL-21 that were expressed high suggesting their potential role in the pathogenesis of MS. Interestingly, most of the patients used for this study were undergone continuous treatment with different immune modifiers. But still the frequency of Tfh and IL-21 was elevated in MS even though other $CD4^{+}$T cell subsets and their corresponding cytokines showed no alteration in their frequency between HC and MS. This finding suggests that those treatments have minimum effects on Tfh and IL-21 regarding their expression and secretion in MS which may target as a novel biomarker for studying MS more insights.

As described earlier, Tfh cells can be further categorized into Tfh1, Tfh2 and Tfh17 based on their surface phenotypes. Proportional bias among Tfh subtypes reported to be linked with autoantibody production in autoimmunes disease like SLE and MG (*144, 146*). This study has observed that cTfh17 and cTfh17.1 cells were the major subtypes of cTfh cells that expressed high in MS patients. In line with increased frequency, IL-21 production by these two subtypes was also significantly higher in MS. Although IL-17 producing Th17 was previously

reported to involve in MS, I couldn't observe any remarkable change in the frequency of non- follicular Th17 between HC and MS. Moreover, comparative analysis of IL-17 production by Tfh17 and non-follicular Th17 showed no significant difference between HC and MS. This discrepancy might be generated from the enrolled patients who were continuously undergone treatment with different immune modifiers except only one with treatment naïve. IL-17 producing Th17 was reported to increase in untreated patients with multiple sclerosis compared to HC. Moreover, expression of IL-17 mRNA was found to increase in MS-CSF rather than to MS peripheral blood mononuclear cells. Meanwhile, data from patients with treatment naïve showed the frequency of both Th17 and IL-17 were increased compared to HC (*147, 148*). The exact role of Tfh17 in MS is not clear. Morita et al. reported that cTfh17 cells proficiently help both naïve and memory B cell to produce Igs (IgG, A & M) which needs to be elucidated in MS (*121*).

On the other hand, this study identified cTfh17.1 cells were significantly increased in MS patients compared to HC. Recently, the frequency non-follicular Th17.1 cells have reported to be augmented in MS (*140*). I also observed similar result but importantly among Tfh cells. I also found that elevated cTfh17.1 cells were associated with over production of IL-21 in MS. Additionally, cTfh17.1 cells were also engaged with significantly higher secretion of IL-17 secretion in MS compared to HC. Importantly, cTfh17.1 cells were engaged with maximum production of IL-21 over other Tfh subtypes both in HC and MS where the

secretion level was even higher in MS. Collectively, the present study demonstrated that cTfh17.1 may play a crucial role in MS pathogenesis through their incentive actions on elevated secretion of IL-21, IL-17.

Alternatively, It is well known that pathogenesis of MS is highly linked to failure of regulatory mechanism by Treg cells (*95, 97, 143*). In this study, the main focus was to investigate whether the proportion of nTfreg disturbed in MS patients or not. I simultaneously evaluated the percentage of Treg, Tfreg & their subtypes and non-follicular Treg cells in MS. The data revealed that total circulating nTreg ($CD4^{+}FoxP3^{+}CD25^{+}$) population was markedly reduced in MS patients compared to HC which resemble with previous studies. I further showed that among nTreg cells, frequency of natural cTfreg ($CXCR5^{+}PD\text{-}1^{+}FoxP3^{+}CD25^{+}$) cells were significantly lower in MS patients compared to HC while non- follicular Treg ($CXCR5^{-}FoxP3^{+}CD25^{+}$) showed no significant alteration in MS. Even though unlike GC-Tfreg, cTfreg doesn't express BCL-6 and ICOS, they commonly share the functional property to control excessive humoral mediated immunity (*149*). Recently, in vitro co-culture assay with responder T cell and Tfreg cell described that both cTfreg and tonsilar Tfreg cells provide equal suppressive activity to the responder cells (*150*). Natural cTfreg cells are found to be defective in different autoimmune diseases (*137, 144, 150*). My finding supports those observations and suggests that cTfreg cells are impaired in MS that may result uncontrolled immunoregulation regarding the disease context. However, this study was not limited to evaluate only nTfreg cells.

I also simultaneously investigated the expression of Tfreg subsets like iTfreg ($CXCR5^{+}PD\text{-}1^{+}FoxP3^{+}CD25^{-}$) and $CD25^{+}$Tfreg ($CXCR5^{+}PD\text{-}1^{+}FoxP3^{-}CD25^{+}$) cells in MS and compared with that of HC. The data revealed mixed observations where no significant alteration found for iTftreg frequencies between HC and MS whereas the frequency of $CD25^{+}$Tfreg was remarkably increased in MS patients. It has been reported that iTreg were found to be lower in MS patients compared to HC and the $CD25^{+}$Treg cells were increased in MS-CSF but not in the peripheral blood (*151, 152*). I couldn't correlate with those observations in follicular level although $CD25^{+}$Tfregs were enriched in MS PBMC. The effect of treatments may influence on the alteration of these subtypes. But importantly, the treatments didn't change the reduced frequency of natural cTfreg cells.

The mechanism underlying the suppressive activity of Tfreg over other effector T cells is still elusive. It is not clear that whether Tfregs directly inhibit the Tfh cells by blocking their transcriptional activation or they use IL-10 signaling pathway to set up their inhibitory milieu. However, this study tried to explore the connection between IL-10 and Tfreg in MS. First I examined absolute number of IL-10 secretion by lymphocyte in MS followed by a substantial investigation of IL-10 production by nTfreg cells. It was shown that IL-10 production by nTfreg was higher than their counterpart non-follicular Treg cells in both HC and MS. This observation suggested that nTfreg cells were the mainly involved in IL-10 secretion off other Tregs. Following this observation I further compared the secretion of IL-10 by nTfreg cells between HC and MS as observed earlier that

the frequency of nTfreg was decreased in MS compared to HC. This result has shown that depleted nTfreg cells were also significantly engaged with parallel reduction of IL-10 in MS compared to HC. Comprehensive analysis for IL-10 production by iTfreg and $CD25^{+}$ Tfreg cells didn't result any detectable change between HC and MS. On the other hand, IL-10 secretion by non-follicular nTreg didn't show any notable reduction in MS, although IL-10 production by total nTreg was significantly reduced in MS patients compared to HC. The exact role of IL-10 in MS remains controversial. Meanwhile depleted level of IL-10 has been report to increase the severity of MS (*97*). Recently, IL-10 has been reported to stimulate Foxp3 to differentiate naïve CD4+ T cells into Treg (*153*). The current study revealed similar observations which collectively suggest nTfreg may play a critical role in IL-10 secretion, whose deregulated distribution may involve with autoimmune pathogenesis of MS.

7. Conclusion

Despite the fact that my study had some limitations including small sample size, treated patients with low immune modifiers, lack of confirmative studies through animal model, I was able to find a powerful interaction of Tfh cells in MS patients that may exacerbate via IL-21. More precisely, Tfh17 and Tfh17.1 cells were significantly higher in MS that were associated with overproduction of IL-21. Contrarily, Tfreg cells and its signature cytokine IL-10 were remarkably defective in MS. Taken together, Our study established that an imbalance ratio of Tfh:Tfreg persist in MS. However, extensive study with larger cohort may validate and clarify our observation regarding the role of Tfh cells and their subsets in MS. Functional studies through the co-culture of purified Tfh and B cells may open up new horizon to understand MS and other autoimmune disease better that eventually guided us to identify therapeutic interventions.

8. Bibliography

1. A. Nylander, D. A. Hafler, Multiple sclerosis. *The Journal of Clinical Investigation* **122**, 1180-1188 (2012).
2. A. Scalfari, A. Neuhaus, M. Daumer, G. C. Ebers, P. A. Muraro, Age and disability accumulation in multiple sclerosis. *Neurology* **77**, 1246-1252 (2011).
3. D. S. Reich, C. F. Lucchinetti, P. A. Calabresi, Multiple Sclerosis. *New England Journal of Medicine* **378**, 169-180 (2018).
4. L. Barin, A. Salmen, G. Disanto, H. Babacic, P. Calabrese, A. Chan *et al.*, The disease burden of Multiple Sclerosis from the individual and population perspective: Which symptoms matter most? *Multiple sclerosis and related disorders* **25**, 112-121 (2018).
5. Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet. Neurology* **18**, 269-285 (2019).
6. S.-E. Chung, H.-K. Cheong, J.-H. Park, H. J. Kim, Burden of disease of multiple sclerosis in Korea. *Epidemiology and health* **34**, (2012).
7. C. H. Polman, S. C. Reingold, B. Banwell, M. Clanet, J. A. Cohen, M. Filippi *et al.*, Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Annals of neurology* **69**, 292-302 (2011).
8. F. D. Lublin, S. C. Reingold, J. A. Cohen, G. R. Cutter, P. S. Sørensen, A. J. Thompson *et al.*, Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology* **83**, 278-286 (2014).
9. C. Hirst, G. Ingram, T. Pickersgill, R. Swingler, D. Compston, N. P. Robertson, Increasing prevalence and incidence of multiple sclerosis in South East Wales. *Journal of Neurology, Neurosurgery & Psychiatry* **80**, 386-391 (2009).
10. D. H. Mahad, B. D. Trapp, H. Lassmann, Pathological mechanisms in progressive multiple sclerosis. *The Lancet. Neurology* **14**, 183-193 (2015).
11. C. Stadelmann, C. Wegner, W. Bruck, Inflammation, demyelination, and degeneration - recent insights from MS pathology. *Biochimica et biophysica acta* **1812**, 275-282 (2011).
12. Lublin, S. C. Reingold, Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* **46**, 907-911 (1996).
13. C. Confavreux, S. Vukusic, Natural history of multiple sclerosis: a unifying concept. *Brain : a journal of neurology* **129**, 606-616 (2006).
14. M. J. Tullman, R. J. Oshinsky, F. D. Lublin, G. R. Cutter, Clinical characteristics of progressive relapsing multiple sclerosis. *Multiple sclerosis* **10**, 451-454 (2004).

15. J. M. Frischer, S. Bramow, A. Dal-Bianco, C. F. Lucchinetti, H. Rauschka, M. Schmidbauer *et al.*, The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain : a journal of neurology* **132**, 1175-1189 (2009).
16. F. D. Lublin, S. C. Reingold, J. A. Cohen, G. R. Cutter, P. S. Sorensen, A. J. Thompson *et al.*, Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology* **83**, 278-286 (2014).
17. F. D. Lublin, New Multiple Sclerosis Phenotypic Classification. *European Neurology* **72(suppl 1)**, 1-5 (2014).
18. S. E. Butterworth, G. Ingram, N. P. Robertson, Advances in biomarker research in multiple sclerosis. *J Neurol* **263**, 621-623 (2016).
19. A. J. Thompson, B. L. Banwell, F. Barkhof, W. M. Carroll, T. Coetzee, G. Comi *et al.*, Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *The Lancet. Neurology* **17**, 162-173 (2018).
20. D. Miller, F. Barkhof, X. Montalban, A. Thompson, M. Filippi, Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. *The Lancet. Neurology* **4**, 281-288 (2005).
21. W. J. Brownlee, T. A. Hardy, F. Fazekas, D. H. Miller, Diagnosis of multiple sclerosis: progress and challenges. *Lancet* **389**, 1336-1346 (2017).
22. A. B. Chu, J. L. Sever, D. L. Madden, M. Iivanainen, M. Leon, W. Wallen *et al.*, Oligoclonal IgG bands in cerebrospinal fluid in various neurological diseases. *Ann Neurol* **13**, 434-439 (1983).
23. J. R. Avasarala, A. H. Cross, J. L. Trotter, Oligoclonal Band Number as a Marker for Prognosis in Multiple Sclerosis. *Archives of Neurology* **58**, 2044-2045 (2001).
24. B. J. Kaskow, C. Baecher-Allan, Effector T Cells in Multiple Sclerosis. *Cold Spring Harbor perspectives in medicine* **8**, (2018).
25. J. M. Fletcher, S. J. Lalor, C. M. Sweeney, N. Tubridy, K. H. Mills, T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol* **162**, 1-11 (2010).
26. A. P. Jones, A. G. Kermode, R. M. Lucas, W. M. Carroll, D. Nolan, P. H. Hart, Circulating immune cells in multiple sclerosis. *Clin Exp Immunol* **187**, 193-203 (2017).
27. Y. Miyazaki, M. Niino, T. Fukazawa, E. Takahashi, T. Nonaka, I. Amino *et al.*, Suppressed Pro-Inflammatory Properties Of Circulating B Cells In Patients With Multiple Sclerosis Treated With Fingolimod, Based On Altered Proportions Of B-Cell Subpopulations (P1.198). *Neurology* **82**, P1.198 (2014).
28. M. E. Fransson, J. Liljenfeldt Ls Fau - Fagius, T. H. Fagius J Fau - Tötterman, A. S. I. Tötterman Th Fau - Loskog, A. S. Loskog, The T-cell pool is anergized in patients with multiple sclerosis in remission.

29. L. Börnsen, J. R. Christensen, R. Ratzer, A. B. Oturai, P. S. Sørensen, H. B. Søndergaard *et al.*, Effect of natalizumab on circulating CD4+ T-cells in multiple sclerosis. *PloS one* **7**, e47578 (2012).
30. L. Durelli, L. Conti, M. Clerico, D. Boselli, G. Contessa, P. Ripellino *et al.*, T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-β. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society* **65**, 499-509 (2009).
31. K. M. Danikowski, S. Jayaraman, B. S. Prabhakar, Regulatory T cells in multiple sclerosis and myasthenia gravis. *J Neuroinflammation* **14**, 117 (2017).
32. C. J. Willer, D. A. Dyment, N. J. Risch, A. D. Sadovnick, G. C. Ebers, Twin concordance and sibling recurrence rates in multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12877-12882 (2003).
33. T. Hansen, A. Skytthe, E. Stenager, H. C. Petersen, H. Bronnum-Hansen, K. O. Kyvik, Concordance for multiple sclerosis in Danish twins: an update of a nationwide study. *Multiple sclerosis* **11**, 504-510 (2005).
34. E. Canto, J. R. Oksenberg, Multiple sclerosis genetics. *Multiple sclerosis* **24**, 75-79 (2018).
35. S. Sawcer, G. Hellenthal, M. Pirinen, C. C. Spencer, N. A. Patsopoulos, L. Moutsianas *et al.*, Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **476**, 214-219 (2011).
36. B. Brynedal, K. Duvefelt, G. Jonasdottir, I. M. Roos, E. Åkesson, J. Palmgren *et al.*, HLA-A confers an HLA-DRB1 independent influence on the risk of multiple sclerosis. *PloS one* **2**, e664 (2007).
37. L. Moutsianas, L. Jostins, A. H. Beecham, A. T. Dilthey, D. K. Xifara, M. Ban *et al.*, Class II HLA interactions modulate genetic risk for multiple sclerosis. *Nature genetics* **47**, 1107-1113 (2015).
38. T. Olsson, L. F. Barcellos, L. Alfredsson, Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nature reviews. Neurology* **13**, 25-36 (2017).
39. S. Simpson, Jr., L. Blizzard, P. Otahal, I. Van der Mei, B. Taylor, Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *Journal of neurology, neurosurgery, and psychiatry* **82**, 1132-1141 (2011).
40. C. R. Gale, C. N. Martyn, Migrant studies in multiple sclerosis. *Progress in neurobiology* **47**, 425-448 (1995).
41. P. Berg-Hansen, S. M. Moen, L. Sandvik, H. F. Harbo, I. J. Bakken, C. Stoltenberg *et al.*, Prevalence of multiple sclerosis among immigrants in Norway. *Multiple sclerosis* **21**, 695-702 (2015).
42. C. Ahlgren, A. Oden, J. Lycke, A nationwide survey of the prevalence of multiple sclerosis in immigrant populations of Sweden. *Multiple sclerosis* **18**, 1099-1107 (2012).

43. A. Raghuwanshi, S. S. Joshi, S. Christakos, Vitamin D and multiple sclerosis. *Journal of cellular biochemistry* **105**, 338-343 (2008).
44. B. Kalman, E. Toldy, Genomic binding sites and biological effects of the vitamin D--VDR complex in multiple sclerosis [corrected]. *Neuromolecular medicine* **16**, 265-279 (2014).
45. J. Salzer, G. Hallmans, M. Nystrom, H. Stenlund, G. Wadell, P. Sundstrom, Smoking as a risk factor for multiple sclerosis. *Multiple sclerosis* **19**, 1022-1027 (2013).
46. A. E. Handel, A. J. Williamson, G. Disanto, R. Dobson, G. Giovannoni, S. V. Ramagopalan, Smoking and multiple sclerosis: an updated meta-analysis. *PloS one* **6**, e16149 (2011).
47. A. Manouchehrinia, C. R. Tench, J. Maxted, R. H. Bibani, J. Britton, C. S. Constantinescu, Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study. *Brain : a journal of neurology* **136**, 2298-2304 (2013).
48. A. Ascherio, K. L. Munger, Epstein-barr virus infection and multiple sclerosis: a review. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* **5**, 271-277 (2010).
49. L. I. Levin, K. L. Munger, E. J. O'Reilly, K. I. Falk, A. Ascherio, Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann Neurol* **67**, 824-830 (2010).
50. K. L. Munger, R. W. Peeling, M. A. Hernan, L. Chasan-Taber, M. J. Olek, S. E. Hankinson *et al.*, Infection with Chlamydia pneumoniae and risk of multiple sclerosis. *Epidemiology* **14**, 141-147 (2003).
51. M. R. Hammerschlag, Z. Ke, F. Lu, P. Roblin, J. Boman, B. Kalman, Is Chlamydia pneumoniae present in brain lesions of patients with multiple sclerosis? *J Clin Microbiol* **38**, 4274-4276 (2000).
52. K. Berer, L. A. Gerdes, E. Cekanaviciute, X. Jia, L. Xiao, Z. Xia *et al.*, Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 10719-10724 (2017).
53. K. Berer, M. Mues, M. Koutrolos, Z. A. Rasbi, M. Boziki, C. Johner *et al.*, Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* **479**, 538-541 (2011).
54. D. T. Chard, C. M. Griffin, G. J. Parker, R. Kapoor, A. J. Thompson, D. H. Miller, Brain atrophy in clinically early relapsing-remitting multiple sclerosis. *Brain* **125**, 327-337 (2002).
55. W. Bruck, P. Porada, S. Poser, P. Rieckmann, F. Hanefeld, H. A. Kretzschmar *et al.*, Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol* **38**, 788-796 (1995).
56. B. F. Popescu, I. Pirko, C. F. Lucchinetti, Pathology of multiple sclerosis: where do we stand? *Continuum* **19**, 901-921 (2013).
57. M. Sospedra, R. Martin, Immunology of multiple sclerosis. *Annu Rev Immunol* **23**, 683-747 (2005).

58. P. K. Stys, G. W. Zamponi, J. van Minnen, J. J. Geurts, Will the real multiple sclerosis please stand up? *Nat Rev Neurosci* **13**, 507-514 (2012).
59. M. Pette, K. Fujita, D. Wilkinson, D. M. Altmann, J. Trowsdale, G. Giegerich *et al.*, Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. *Proc Natl Acad Sci U S A* **87**, 7968-7972 (1990).
60. J. M. Greer, P. A. Csurhes, K. D. Cameron, P. A. McCombe, M. F. Good, M. P. Pender, Increased immunoreactivity to two overlapping peptides of myelin proteolipid protein in multiple sclerosis. *Brain* **120 (Pt 8)**, 1447-1460 (1997).
61. J. Zhang, S. Markovic-Plese, B. Lacet, J. Raus, H. L. Weiner, D. A. Hafler, Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* **179**, 973-984 (1994).
62. N. K. de Rosbo, J. F. Kaye, M. Eisenstein, I. Mendel, R. Hoeftberger, H. Lassmann *et al.*, The myelin-associated oligodendrocytic basic protein region MOBP15-36 encompasses the immunodominant major encephalitogenic epitope(s) for SJL/J mice and predicted epitope(s) for multiple sclerosis-associated HLA-DRB1*1501. *J Immunol* **173**, 1426-1435 (2004).
63. J. J. Bajramovic, H. Lassmann, J. M. van Noort, Expression of alphaB-crystallin in glia cells during lesional development in multiple sclerosis. *J Neuroimmunol* **78**, 143-151 (1997).
64. T. Derfuss, K. Parikh, S. Velhin, M. Braun, E. Mathey, M. Krumbholz *et al.*, Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals. *Proc Natl Acad Sci U S A* **106**, 8302-8307 (2009).
65. U. Traugott, E. L. Reinherz, C. S. Raine, Multiple sclerosis. Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. *J Neuroimmunol* **4**, 201-221 (1983).
66. B. Brynedal, K. Duvefelt, G. Jonasdottir, I. M. Roos, E. Åkesson, J. Palmgren *et al.*, HLA-A confers an HLA-DRB1 independent influence on the risk of multiple sclerosis. **2**, e664 (2007).
67. L. Legroux, N. Arbour, Multiple Sclerosis and T Lymphocytes: An Entangled Story. *J Neuroimmune Pharmacol* **10**, 528-546 (2015).
68. S. Sakaguchi, T. Yamaguchi, T. Nomura, M. Ono, Regulatory T cells and immune tolerance. *Cell* **133**, 775-787 (2008).
69. T. Chitnis, The role of CD4 T cells in the pathogenesis of multiple sclerosis. *Int Rev Neurobiol* **79**, 43-72 (2007).
70. S. W. Brostoff, D. W. Mason, Experimental allergic encephalomyelitis: successful treatment in vivo with a monoclonal antibody that recognizes T helper cells. *J Immunol* **133**, 1938-1942 (1984).

71. I. Raphael, S. Nalawade, T. N. Eagar, T. G. Forsthuber, T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* **74**, 5-17 (2015).
72. N. Gagliani, M. C. Amezcua Vesely, A. Iseppon, L. Brockmann, H. Xu, N. W. Palm *et al.*, Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* **523**, 221-225 (2015).
73. J. Geginat, M. Paroni, S. Maglie, J. S. Alfen, I. Kastirr, P. Gruarin *et al.*, Plasticity of human CD4 T cell subsets. *Front Immunol* **5**, 630 (2014).
74. T. R. Mosmann, H. Cherwinski, M. W. Bond, M. A. Giedlin, R. L. Coffman, Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-2357 (1986).
75. A. Rostami, B. Ciric, Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. *J Neurol Sci* **333**, 76-87 (2013).
76. T. Korn, E. Bettelli, M. Oukka, V. K. Kuchroo, IL-17 and Th17 Cells. *Annu Rev Immunol* **27**, 485-517 (2009).
77. A. Waisman, J. Hauptmann, T. Regen, The role of IL-17 in CNS diseases. *Acta Neuropathol* **129**, 625-637 (2015).
78. G. Arellano, E. Acuña, L. I. Reyes, P. A. Ottum, P. De Sarno, L. Villarroel *et al.*, Th1 and Th17 Cells and Associated Cytokines Discriminate among Clinically Isolated Syndrome and Multiple Sclerosis Phenotypes. *Front Immunol* **8**, 753 (2017).
79. J. Rasouli, B. Ciric, J. Imitola, P. Gonnella, D. Hwang, K. Mahajan *et al.*, Expression of GM-CSF in T Cells Is Increased in Multiple Sclerosis and Suppressed by IFN-beta Therapy. *J Immunol* **194**, 5085-5093 (2015).
80. A. Schottelius, The role of GM-CSF in multiple sclerosis. *Drug Res* **63 Suppl 1**, S8 (2013).
81. F. J. Hartmann, M. Khademi, J. Aram, S. Ammann, I. Kockum, C. Constantinescu *et al.*, Multiple sclerosis-associated IL2RA polymorphism controls GM-CSF production in human TH cells. *Nat Commun* **5**, 5056 (2014).
82. R. Noster, R. Riedel, M. F. Mashreghi, H. Radbruch, L. Harms, C. Haftmann *et al.*, IL-17 and GM-CSF expression are antagonistically regulated by human T helper cells. *Sci Transl Med* **6**, 241ra280 (2014).
83. A. F. Wierenga-Wolf, I. M. Spilt, J. van Langelaar, M. M. van Luijn, M. Janssen, R. Q. Hintzen *et al.*, T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain* **141**, 1334-1349 (2018).
84. D. A. Vignali, L. W. Collison, C. J. Workman, How regulatory T cells work. *Nat Rev Immunol* **8**, 523-532 (2008).
85. S. Sakaguchi, N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh *et al.*, Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* **182**, 18-32 (2001).

86. J. D. Fontenot, M. A. Gavin, A. Y. Rudensky, Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**, 330-336 (2003).
87. H. Zhang, H. Kong, X. Zeng, L. Guo, X. Sun, S. He, Subsets of regulatory T cells and their roles in allergy. *J Transl Med* **12**, 125 (2014).
88. A. M. Bilate, J. J. Lafaille, Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu Rev Immunol* **30**, 733-758 (2012).
89. M. G. Roncarolo, S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, M. K. Levings, Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* **212**, 28-50 (2006).
90. E. M. Shevach, Biological functions of regulatory T cells. *Adv Immunol* **112**, 137-176 (2011).
91. A. L. Zozulya, H. Wiendl, The role of regulatory T cells in multiple sclerosis. *Nat Clin Pract Neurol* **4**, 384-398 (2008).
92. S. Hori, M. Haury, A. Coutinho, J. Demengeot, Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc Natl Acad Sci U S A* **99**, 8213-8218 (2002).
93. M. J. McGeachy, L. A. Stephens, S. M. Anderton, Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol* **175**, 3025-3032 (2005).
94. G. Frisullo, V. Nociti, R. Iorio, A. K. Patanella, M. Caggiula, A. Marti *et al.*, Regulatory T cells fail to suppress CD4T+T-bet+ T cells in relapsing multiple sclerosis patients. *Immunology* **127**, 418-428 (2009).
95. V. Viglietta, C. Baecher-Allan, H. L. Weiner, D. A. Hafler, Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* **199**, 971-979 (2004).
96. J. Huan, N. Culbertson, L. Spencer, R. Bartholomew, G. G. Burrows, Y. K. Chou *et al.*, Decreased FOXP3 levels in multiple sclerosis patients. *J Neurosci Res* **81**, 45-52 (2005).
97. K. Venken, N. Hellings, M. Thewissen, V. Somers, K. Hensen, J. L. Rummens *et al.*, Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* **123**, 79-89 (2008).
98. P. Schaerli, K. Willimann, A. B. Lang, M. Lipp, P. Loetscher, B. Moser, CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* **192**, 1553-1562 (2000).
99. D. Breitfeld, L. Ohl, E. Kremmer, J. Ellwart, F. Sallusto, M. Lipp *et al.*, Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* **192**, 1545-1552 (2000).

100. C. D. Allen, T. Okada, J. G. Cyster, Germinal-center organization and cellular dynamics. *Immunity* **27**, 190-202 (2007).
101. N. M. Haynes, C. D. Allen, R. Lesley, K. M. Ansel, N. Killeen, J. G. Cyster, Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol* **179**, 5099-5108 (2007).
102. C. S. Ma, S. Suryani, D. T. Avery, A. Chan, R. Nanan, B. Santner-Nanan *et al.*, Early commitment of naive human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. *Immunol Cell Biol* **87**, 590-600 (2009).
103. L. Bossaller, J. Burger, R. Draeger, B. Grimbacher, R. Knoth, A. Plebani *et al.*, ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol* **177**, 4927-4932 (2006).
104. R. I. Nurieva, Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma *et al.*, Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* **29**, 138-149 (2008).
105. N. Simpson, P. A. Gatenby, A. Wilson, S. Malik, D. A. Fulcher, S. G. Tangye *et al.*, Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* **62**, 234-244 (2010).
106. G. Roncador, J. F. Garcia Verdes-Montenegro, S. Tedoldi, J. C. Paterson, W. Klapper, E. Ballabio *et al.*, Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma. *Haematologica* **92**, 1059-1066 (2007).
107. J. Shi, S. Hou, Q. Fang, X. Liu, X. Liu, H. Qi, PD-1 Controls Follicular T Helper Cell Positioning and Function. *Immunity* **49**, 264-274.e264 (2018).
108. S. R. Greisen, T. K. Rasmussen, K. Stengaard-Pedersen, M. L. Hetland, K. Horslev-Petersen, M. Hvid *et al.*, Increased soluble programmed death-1 (sPD-1) is associated with disease activity and radiographic progression in early rheumatoid arthritis. *Scand J Rheumatol* **43**, 101-108 (2014).
109. D. Baumjohann, S. Preite, A. Reboldi, F. Ronchi, K. M. Ansel, A. Lanzavecchia *et al.*, Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* **38**, 596-605 (2013).
110. C. G. Vinuesa, M. A. Linterman, D. Yu, I. C. M. MacLennan, Follicular Helper T Cells. *Annual Review of Immunology* **34**, 335-368 (2016).
111. T. Chtanova, S. G. Tangye, R. Newton, N. Frank, M. R. Hodge, M. S. Rolph *et al.*, T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* **173**, 68-78 (2004).

112. D. Yu, S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe *et al.*, The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* **31**, 457-468 (2009).
113. A. L. Dent, A. L. Shaffer, X. Yu, D. Allman, L. M. Staudt, Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* **276**, 589-592 (1997).
114. A. T. Bauquet, H. Jin, A. M. Paterson, M. Mitsdoerffer, I. C. Ho, A. H. Sharpe *et al.*, The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* **10**, 167-175 (2009).
115. V. L. Bryant, C. S. Ma, D. T. Avery, Y. Li, K. L. Good, L. M. Corcoran *et al.*, Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol* **179**, 8180-8190 (2007).
116. N. Chevalier, D. Jarrossay, E. Ho, D. T. Avery, C. S. Ma, D. Yu *et al.*, CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol* **186**, 5556-5568 (2011).
117. Q. Huang, J. Hu, J. Tang, L. Xu, L. Ye, Molecular Basis of the Differentiation and Function of Virus Specific Follicular Helper CD4+ T Cells. **10**, (2019).
118. J. S. Hale, R. Ahmed, Memory T follicular helper CD4 T cells. *Front Immunol* **6**, 16 (2015).
119. R. Forster, T. Emrich, E. Kremmer, M. Lipp, Expression of the G-protein--coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood* **84**, 830-840 (1994).
120. A. Asrir, M. Aloulou, M. Gador, C. Pérals, N. Fazilleau, Interconnected subsets of memory follicular helper T cells have different effector functions. *Nature communication* **8**, 847 (2017)
121. R. Morita, N. Schmitt, S. E. Bentebibel, R. Ranganathan, L. Bourdery, G. Zurawski *et al.*, Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* **34**, 108-121 (2011).
122. Y. Chung, S. Tanaka, F. Chu, R. I. Nurieva, G. J. Martinez, S. Rawal *et al.*, Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* **17**, 983-988 (2011).
123. M. A. Linterman, W. Pierson, S. K. Lee, A. Kallies, S. Kawamoto, T. F. Rayner *et al.*, Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* **17**, 975-982 (2011).
124. H. W. Lim, P. Hillsamer, C. H. Kim, Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest* **114**, 1640-1649 (2004).
125. M. Aloulou, E. J. Carr, M. Gador, A. Bignon, R. S. Liblau, N. Fazilleau *et al.*, Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun* **7**, 10579 (2016).

126. M. A. Linterman, R. J. Rigby, R. K. Wong, D. Yu, R. Brink, J. L. Cannons *et al.*, Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* **206**, 561-576 (2009).
127. K. Szabo, S. Papp G Fau - Barath, E. Barath S Fau - Gyimesi, A. Gyimesi E Fau - Szanto, M. Szanto A Fau - Zeher, M. Zeher, Follicular helper T cells may play an important role in the severity of primary Sjogren's syndrome. . *Clinical Immunology* **147**, 95-104 (2013).
128. R. Liu, Q. Wu, D. Su, N. Che, H. Chen, L. Geng *et al.*, A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. *Arthritis Res Ther* **14**, R255 (2012).
129. C. Zhu, J. Ma, Y. Liu, J. Tong, J. Tian, J. Chen *et al.*, Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab* **97**, 943-950 (2012).
130. C.-J. Zhang, Y. Gong, W. Zhu, Y. Qi, C.-S. Yang, Y. Fu *et al.*, Augmentation of Circulating Follicular Helper T Cells and Their Impact on Autoreactive B Cells in Myasthenia Gravis. *The Journal of Immunology* **197**, 2610 (2016).
131. M. Akiyama, K. Suzuki, K. Yamaoka, H. Yasuoka, M. Takeshita, Y. Kaneko *et al.*, Number of Circulating Follicular Helper 2 T Cells Correlates With IgG4 and Interleukin-4 Levels and Plasmablast Numbers in IgG4-Related Disease. *Artritis & Rheumatology* **67**, 2476-81 (2015).
132. I. Arroyo-Villa, M.-B. Bautista-Caro, A. Balsa, P. Aguado-Acín, M.-G. Bonilla-Hernán, C. Plasencia *et al.*, Constitutively altered frequencies of circulating follicullar helper T cell counterparts and their subsets in rheumatoid arthritis. *Arthritis Research & Therapy* **16**, 500 (2014).
133. C. Le Coz, J.-L. Joublin A Fau - Pasquali, A.-S. Pasquali Jl Fau - Korganow, H. Korganow As Fau - Dumortier, F. Dumortier H Fau - Monneaux, F. Monneaux, Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. . *Plos one* 8, e75319 (2013).
134. Y. Zhu, L. Zou, Y. C. Liu, T follicular helper cells, T follicular regulatory cells and autoimmunity. *Int Immunol* **28**, 173-179 (2016).
135. W. Fu, X. Liu, X. Lin, H. Feng, L. Sun, S. Li *et al.*, Deficiency in T follicular regulatory cells promotes autoimmunity. *The Journal of Experimental Medicine* **215**, 815 (2018).
136. Y. Wen, B. Yang, J. Lu, J. Zhang, H. Yang, J. Li, Imbalance of circulating CD4(+)CXCR5(+)FOXP3(+) Tfr-like cells and CD4(+)CXCR5(+)FOXP3(-) Tfh-like cells in myasthenia gravis. *Neurosci Lett* **630**, 176-182 (2016).
137. B. Xu, S. Wang, M. Zhou, Y. Huang, R. Fu, C. Guo *et al.*, The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. *Clin Immunol* **183**, 46-53 (2017).
138. Y. Cui, Y. Guan, W. Liu, Y. Li, H. Li, M. Guo, [The changes of circulating follicular regulatory T cells and follicular T helper cells in

children immune thrombocytopenia]. *Zhonghua Xue Ye Xue Za Zhi* **35**, 980-984 (2014).
139. J. L. Quinn, G. Kumar, A. Agasing, R. M. Ko, R. C. Axtell, Role of TFH Cells in Promoting T Helper 17-Induced Neuroinflammation. *Front Immunol* **9**, 382 (2018).
140. J. van Langelaar, R. M. van der Vuurst de Vries, M. Janssen, A. F. Wierenga-Wolf, I. M. Spilt, T. A. Siepman *et al.*, T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain* **141**, 1334-1349 (2018).
141. M. A. Gavin, J. P. Rasmussen, J. D. Fontenot, V. Vasta, V. C. Manganiello, J. A. Beavo *et al.*, Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771-775 (2007).
142. K. Venken, N. Hellings, M. Thewissen, V. Somers, K. Hensen, J.-L. Rummens *et al.*, Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* **123**, 79-89 (2008).
143. I. Mexhitaj, M. H. Nyirenda, R. Li, J. O'Mahony, A. Rezk, A. Rozenberg *et al.*, Abnormal effector and regulatory T cell subsets in paediatric-onset multiple sclerosis. *Brain* **142**, 617-632 (2019).
144. C. J. Zhang, Y. Gong, W. Zhu, Y. Qi, C. S. Yang, Y. Fu *et al.*, Augmentation of Circulating Follicular Helper T Cells and Their Impact on Autoreactive B Cells in Myasthenia Gravis. *J Immunol* **197**, 2610-2617 (2016).
145. I. Arroyo-Villa, M.-B. Bautista-Caro, A. Balsa, P. Aguado-Acín, M.-G. Bonilla-Hernán, C. Plasencia *et al.*, Constitutively altered frequencies of circulating follicullar helper T cell counterparts and their subsets in rheumatoid arthritis. **16**, 500 (2014).
146. C. Le Coz, A. Joublin, J. L. Pasquali, A. S. Korganow, H. Dumortier, F. Monneaux, Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS One* **8**, e75319 (2013).
147. X. Zhang, S. Markovic-Plese, Interferon beta inhibits the Th17 cell-mediated autoimmune response in patients with relapsing–remitting multiple sclerosis. *Clinical Neurology and Neurosurgery* **112**, 641-645 (2010).
148. D. Matusevicius, P. Kivisäkk, B. He, N. Kostulas, V. Özenci, S. Fredrikson *et al.*, Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Multiple Sclerosis Journal* **5**, 101-104 (1999).
149. P. T. Sage, D. Alvarez, J. Godec, U. H. von Andrian, A. H. Sharpe, Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* **124**, 5191-5204 (2014).

150. T. Dhaeze, E. Peelen, A. Hombrouck, L. Peeters, B. Van Wijmeersch, N. Lemkens *et al.*, Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis. *The Journal of Immunology* **195**, 832 (2015).
151. I. H. Mohiuddin, V. Pillai, E. J. Baughman, B. M. Greenberg, E. M. Frohman, M. P. Crawford *et al.*, Induction of regulatory T-cells from memory T-cells is perturbed during acute exacerbation of multiple sclerosis. *Clinical immunology (Orlando, Fla.)* **166-167**, 12-18 (2016).
152. U. Feger, C. Luther, S. Poeschel, A. Melms, E. Tolosa, H. Wiendl, Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. *Clinical and experimental immunology* **147**, 412-418 (2007).
153. M. Murai, O. Turovskaya, G. Kim, R. Madan, C. L. Karp, H. Cheroutre *et al.*, Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* **10**, 1178-1184 (2009).

ACKNOWLEDGEMENT

I want to express my greatest gratitude to Professor Ho Jin Kim who always supported and guided me throughout my entire journey in South Korea to fulfill my master degree. He was my advisor, whose expertise and invaluable advocacy helped me to fulfill this dissertation especially formulating the research topic, designing methodology. More importantly he continuously guided me to trace out the troubleshooting and overcome them while conducting the research. Besides, he always provoked and encouraged me to think creatively and scientifically regarding the needs for solving any biological research phenomena. He always showed me, by his example, what a good scientist should be and how to reach that level.

I am especially indebted to Professor Ho Lee who remained my co-advisor. He has been supportive to me since very beginning through his influential advices to complete my research as well as academic goals. He always provided me the best suggestion that make my journey smooth at NCC-GCSP.

I sincerely would like to thank professor Eun Jung Park, the chairman of thesis committee who encouraged me through her insightful positive comments on my thesis.

I am grateful to all of my lab members with whom I had the pleasure time to work with on this and other related projects. I want to convey my sincere acknowledgement to Yeseul Kim who helped me to adopt and adept with the laboratory environment as well as laboratory techniques. She has been very co-operative to teach me the techniques what need to complete this dissertation.

Finally, I want to convey my special thanks to Tae-Sik Kim (Flow cytomtry core facility) for his effortless help to acquire and compensate my data to make them figural, attractable.