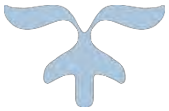
Differential effect of Fingolimod and Natalizumab treatments on B lymphocyte subpopulations and humoral responses in patients with relapsing remitting multiple sclerosis

Διαφορική επίδραση των Fingolimod και Natalizumab θεραπειών στους υποπληθυσμούς Β κυττάρων και στις χυμικές αποκρίσεις ασθενών με RRMS



Ioulia Boulogianni

Department of Molecular Medicine , Eberhard Karls University of Tübingen, Germany
Department of Biochemistry & Biotechnology, University of Thessaly, Greece
Spring 2014, Tübingen, Germany

Examiners-Thesis advisors

- **Dr. Felix Bischof** : private lecturer (PD) of neurosciences , department of molecular medicine , Karls Eberhard university of Tübingen, Germany

- **Anna Maria Psarra** : assistant professor of biochemistry, department of biochemistry and biotechnology, university of Thessaly

- **Dimitrios Leonidas** : associate professor of biochemistry, department of biochemistry and biotechnology, university of Thessaly

INDEX

Περίληψη

Η πολλαπλή σκλήρυνση (ή σκλήρυνση κατά πλάκας) είναι μια χρόνια αυτό-άνοση ασθένεια, της οποίας ο ακριβής μηχανισμός παθογένειας της δεν είναι πλήρως κατανοητός. Βιβλιογραφικά δεδομένα αποκαλύπτουν την εμπλοκή κυττάρων τόσο του ανοσοποιητικού όσο και του νευρικού συστήματος στην εκδήλωση της παθογένειας της νόσου. Όσο αφορά τα κύτταρα του ανοσοποιητικού, πρόσφατες μελέτες υποδηλώνουν ότι πέρα από την αρχικά διαπιστωμένη εμπλοκή των Τ κυττάρων στην εκδήλωση της παθογένειας της νόσου και τα Β κύτταρα φαίνονται να σχετίζονται με αυτήν. Στην συγκεκριμένη μελέτη, αξιολογήθηκε η επίδραση δύο φαρμακευτικών αγωγών, Natalizumab και Fingolimod, στα επίπεδα των υπό-πληθυσμών των Β λεμφοκυττάρων που εντοπίζονται στο αίμα ασθενών με σκλήρυνση κατά πλάκας σε σχέση με τα αντίστοιχα επίπεδα υγειών ατόμων. Σκοπός της μελέτης αυτής είναι να ενισχύσει την άποψη που θέλει τα Β λεμφοκύτταρα ως κρίσιμους ρυθμιστές της πολλαπλής σκλήρυνσης. Συγκεκριμένα, εξετάστηκαν τα επίπεδα των παρθένων Β κυττάρων (CD3- CD19+CD27-), των Β κυττάρων μνήμης (CD3- CD19+CD27+ CD20+) και των πλασματοκυττάρων (CD3- CD19+CD27+ CD20-) τόσο ασθενών με RRMS (Relapsing Remitting Multiple Sclerosis) όσο και υγειών ατόμων. Τα αποτελέσματα έδειξαν, ότι οι ασθενείς που τους χορηγήθηκε Fingolimod είχαν σημαντικά μειωμένα επίπεδα Β κυττάρων (CD3- CD19+) στο αίμα τους σε σύγκριση με αυτούς που λάμβαναν Natalizumab ($P < 0.001$). Επίσης , τόσο η χορήγηση του Natalizumab όσο και του Fingolimod προκαλεί μείωση στα επίπεδα των πλασματοκυττάρων σε σύγκριση με υγιείς ($P < 0.001$), παρόλο που η μείωση είναι πιο αξιοσημείωτη στο αίμα ασθενών που λαμβάνουν Natalizumab ($P=0.0098$). Επιπλέον η χορήγηση του Fingolimod οδηγεί σε δραματική μείωση των IgG+ πλασματοκυττάρων σε σύγκριση με τα άλλα δυο γκρουπ ατόμων. Τα αποτελέσματα αυτά, υποδεικνύουν διαφορές στην επίδραση των δυο θεραπειών στους υπό-πληθυσμούς των Β κυττάρων , με την μεγαλύτερη μείωση στα επίπεδα των Β λεμφοκυττάρων μετά την πρόσληψη του Fingolimod και την σημαντική μείωση στα επίπεδα των πλασματοκυττάρων στο αίμα ασθενών που λαμβάνουν Natalizumab. Σπουδαίο εύρημα αποτελεί ότι το Fingolimod προκαλεί δραματική μείωση στα IgG+ πλασματοκύτταρα ασθενών. Συμπερασματικά, οι θεραπείες αυτές επηρεάζουν διαφορετικά τα επίπεδα των υπό-πληθυσμών των Β κυττάρων. Η ένδειξη αυτήν μπορεί να αποτελέσει έναυσμα για την βελτίωση των υπάρχοντων θεραπειών και την εφαρμογή μιας εξιδανικευμένης θεραπείας ανάλογα με τον εκάστοτε ασθενή.

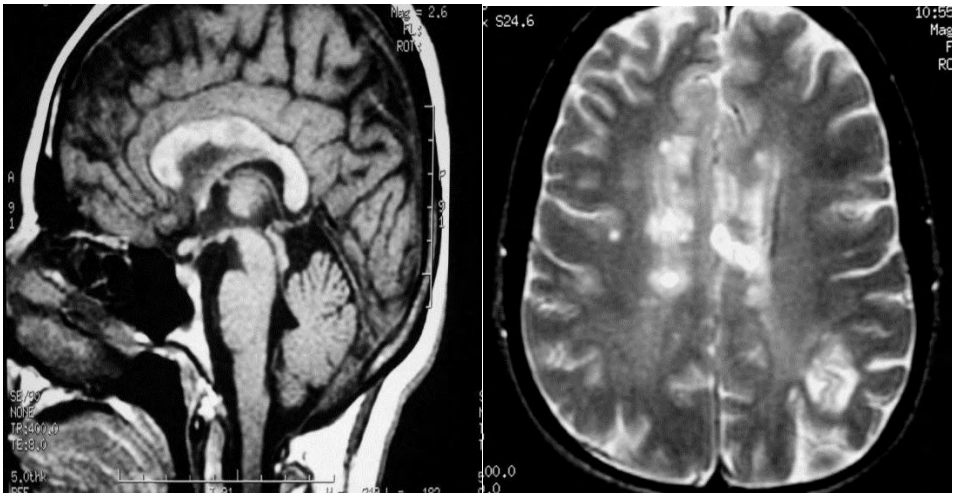
Abstract

Controversy has arisen about the etiology of Multiple sclerosis (MS) pathogenesis. Although it is originally described as being T cell-based autoimmune disease, some studies have recently reported that B cells have potential role in the pathogenesis of MS. In the present study, we evaluated the effects of Natalizumab (N= 9) and Fingolimod (N=9) treatments on the frequency of B lymphocyte subpopulations in the peripheral blood of patients with relapsing remitting multiple sclerosis (RRMS), providing insights of the important role of B cells in immune- pathogenesis of MS. Healthy people (N=9) were considered as control group. Particularly, differences in populations of naïve (CD3- CD19+CD27-)-, memory B cells (CD3- CD19+CD27+ CD20+) and plasmablasts (CD3- CD19+CD27+ CD20-) were evaluated in control group and groups of patients treated with Natalizumab or Fingolimod. The results of this survey showed that in contrast to Natalizumab therapy, Fingolimod treatment significantly reduced the frequency of CD3-CD19+ B cells in RRMS patients ($P < 0.001$). Both Fingolimod and Natalizumab treatments markedly reduced the rate of B cell subpopulation, plasma cells (CD20-CD27+ cells) in comparison with Healthy controls ($P < 0.001$), but the effect of Natalizumab was more dramatic than Fingolimod ($P=0.0098$). In addition, the RRMS patients with Fingolimod therapy showed essential reduction in the percentage of IgG+ cells inside of CD3-CD19+CD20-CD27+ terminally differentiated B lymphocytes. The results of present study demonstrate that both Fingolimod and Natalizumab therapies have considerable effects on the frequency of B Cells and B cell populations, with Fingolimod to be more effective in the reduction of B cells (CD3-CD19+) and Natalizumab to be much more conducive in the reduction of plasma cell (CD20-CD27+) subpopulation. The finding that Fingolimod has dramatically reduced the percentage of IgG+ B cells in the terminally differentiated B cells is the new finding that we present in this research. It seems that MS drugs differentially affect the distribution of B lymphocyte subsets. These findings can contribute to better understanding of MS pathology, bringing some insights for design of individual treatments of MS patients.

Introduction

Multiple sclerosis

Multiple sclerosis (MS), in essence, is a chronic inflammatory disease that affects over 1 million people worldwide (Heather L Wilson et al. 2012). Except of an inflammatory disorder, multiple sclerosis is one of the most serious neurological disorders, which damages the insulating covers of nerve cells in the brain and spinal cord.



1. Sagittal T1-weighted MRI depicts multiple hypointense lesions in the corpus callosum; this finding is characteristic of multiple sclerosis. 2. Axial T2-weighted MRI in a patient with multiple sclerosis demonstrates numerous white matter plaques in a callosal and pericallosal white matter distribution. (Brain imaging in Multiple sclerosis)

This demyelinating disease is presented most common in individuals aged 20–40 years and is associated with marked physical and cognitive disabilities, including mental and mood disorders and a shortened life span as well. (Heather L Wilson et al. 2012)

The spread in the clinical picture of MS has traditionally been seen as a strong evidence for heterogeneity of this disorder. MS is presented with diverse forms (relapsing forms, progressive forms). The classification of MS phenotypes based on **characterization of MS lesions (MS plaques)** (Gregory F. Wu et al. 2011) .The lesions mostly affect the white matter in the optic nerve, brain stem, basal ganglia, and spinal

cord, or areas of the white matter close to the lateral ventricles. The function of white matter cells is to transport signals between grey matter areas.

Especially as a pathologic hallmark of MS used to be plaques of inflammatory myelin breakdown within the CNS (Gregory F. Wu et al. 2011). These plaques are created due to the myelin destruction. The most common pattern of these plaques involves the presence of Abs, components of completment, T cells and macrophages. In this sense, MS plaque formation is a combination of function of innate and adaptive immune cells. Furthermore, MS is associated with the loss of oligodendrocytes, the cells responsible for creating and maintaining a fatty layer—known as the myelin sheath—which helps the neurons carry electrical signals (action potentials). This results in a thinning or complete loss of myelin and, as the disease advances, the breakdown of the axons of neurons.

Usually, MS plaque classification is based on progression (stages) of inflammatory destruction. Accordingly, **acute, chronic active, and chronic silent lesions** are thought to occur along a continuous timeline. The majority of MS patients initially **present with subacute attacks**, with symptoms and signs referable to the central nervous system (CNS) – defined as a clinically isolated syndrome **(CIS**). When the attack is followed by a complete or partial remission which is then followed by another attack(s), often focused in a different location in the CNS and possibly of higher intensity, **the disease course is defined as relapsing and remitting MS (RRMS)**. Patients who present with a gradually progressive course without a well-defined initial attack are presenting with **primary progressive MS (PPMS**). Secondary progressive **MS (SPMS**) is characterized by CIS or RRMS followed by progressive clinical worsening over time, generally 3 years or more after the onset of disease. (Heather L Wilson et al. 2012).

Neuro-immune pathogenesis of multiple sclerosis

This complicated autoimmune disorder has yet an unknown etiology. While the cause is not so clear, the underlying mechanism is thought to be either destruction by the immune system or failure of the myelin-producing cells (oligodendrocytes-

remyelination). Proposed causes for this include genetics and environmental factors such as infections. In any case, the main constituents of immune cells seems to be pathogenic T lymphocytes that are thought to underlie MS immune pathology.

It is well known that MS is caused by an autoimmune response to self-antigens in a genetically susceptible individual induced by gene-environment-infectious interactions. Principally, the demyelination and axonal lesion in MS patients mediated by T cells, especially by CD4+ T cells with a proinflammatory T helper (Th) 1 and Th17 subsets.

To begin with, the main function of the immune system is defense against external and internal antigens. Those of external origin usually belong to microorganisms and the internal antigens arise from malignant cells of organisms, after their destruction of replication mechanisms. Thus, it's clearly understandable that immune system except of its important role in attacking external ''enemies'', has a different but extremely important function that is prevention of autoreactive T and B cells activation. This activation, probably, will be a threat for the organism because in some cases could trigger autoimmune diseases, like MS. In order to be avoided this negative effect, there is a regulatory mechanism presented as recessive tolerance (RT). This mechanism based on deletion of autoreactive T and B cells in the thymus or in a bone marrow during the process of their maturation in these primary lymphoid organs. Sometimes the mechanisms of RT are not efficient to prevent all the autoreactive lymphocytes, a part of them escape their apoptosis, enter to periphery and finally reach the regions where are located the secondary lymphoid organs. Here, there is a high possibility of activation of autoreactive lymphocytes, if they come up against auto-antigens, cross reactive-antigens or in case of dysregulation of the immune system. This activation can cause a cascade of immune reactions that maybe induce autoimmunity.

Contribution of B cells and humoral responses in MS pathogenesis

During the past years, there is continuous attempt to clarify the role of immune cells in MS pathogenesis. T cells and mostly CD4+ helper cells have been considered as a ''key'' in MS pathogenesis. This hypothesis is further supported by the activated T lymphocytes in MS plaques. Despite the thought that MS being T cell driven disease, an emerging evidence comes from recent studies of EAE models and implicates the contribution of B cells and humoral responses in the pathology of MS. It is already known that an increased intrathecal production of immunoglobulins (Ig) in the CSF is remarked in more than 90% of Multiple Sclerosis patients (Kabat et al. 1948). The Igs which are principally produced, are involve IgG, IgA, IgD and IgM (Walsh et al. 1985, Sharief and Thompson 1991). Clonally expanded B cells exist in the central nervous system (CNS) and cerebrospinal fluid (CSF) and follicle like aggregates can be found to MS brain, suggesting to an antigen-driven B cells response. Evidence suggests that treatments with antibodies (like anti-CD20, acts on B cells compartment by depleting the B cells positive for the CD20) based on targeting on B cells subtypes are effectual in RRMS, confirming the pathogenic role of B cells in MS (G. Disanto et al. 2012). Likewise, it has been observed that increased concentrations of Abs in CSF of patients relate to episodes of MS worsening (Olsson and Link, 1973). Taken together, these findings indicate a B cell – driven pathology of MS, suggesting that the underlying mechanisms of MS are heterogeneous.

In spite of the great implication of antibodies in MS disorder, they cannot engender alone CNS inflammation. Remarkably, when the blood-brain-barrier (BBB) is broken by T cell mediated response, plasma cells and their antibodies may worsen the progress of disease. The implication of T cells in the onset of disorder is referred in the literature as a "first hit" while the implication of antibodies is called "second hit". (Markus Krumbholz et al. 2012)

For the determination of B cell participation in MS pathology, is useful to refer the basics of B cell development. First stage in the B cell maturation to pro- and pre- B cells take place in the bone marrow. The second lymphoid organs is the place where the immature naïve B cells develop into mature naïve ones. The B cell tolerance is achieved with different mechanisms analogous to the process while auto-reactive T cells are eliminated. The facts that naïve B cells encounter their antigens in the second lymphoid organs drive them to activation and proliferation. Part of these activated B cells migrate out of lymph follicles to become short lived plasma blasts, producing antibodies during a lifespan limited to 1-3 days. The rest antigen-activated naïve B cells follow an alternative path of development in germinal center reaction (GCs). There B cells (centroblasts) undergo processes like selection, somatic hypermutation , class switch and affinity maturation , which in a combination lead to production of memory B cells. Plasmablasts and plasma cells are included into antibody-secreting cells (ASC). Nonetheless, plasma cells are the terminally differentiated B-cells, which lost the ability of proliferation (in contrast to plasmablasts) and the recognition of present antigens. Plasma cells can be short or long lived. (E. Meint et al. 2006, M Duddy et al. 2007). Short term plasma cells develop in lymphatic organs and they can be detected in circulation.

It has been proven, that in around 90% -95% of MS patients present clonally expanded Ig secreting cells (Kabat et al. 1948). Ig production against autoantigens or foreign antigens (which cross-react with self-antigens) (Heather L Wilson et al. 2012) can preserved by different ways. This abnormality detected in CSF of MS patients and usually is based on B-lineage cells, precisely on antibody secreting cells, and are named oligoclonal bands (OCBs). This intrathecal Ig production create active ‘’spots’’ of inflammation in the CNS, which seem like lymphoid-like follicles (B cells follicles)(Markus Krumbholz et al. 2012, Heather Wilson et al.2012) . The prominent Ig in the OCBs typically is IgG isotype but IgM have also been tracked. (Heather L Wilson et al. 2012)

Regarding to the antibody dependent contribution of B cells in pathology of MS, first short-lived plasma cells (Ig producing cells) that are produced in secondary lymphoid organs pass the damaged BBB and they are gathered in the sites of inflammation in CNS (acute antigen response). Second, acute or chronic antigen activation cause the differentiation of memory B cells and the direct production of antibodies (antigen-driven or non-antigen driven-T cell mediation). Third, long lived plasma cells provide mainly Ig production in the sites of inflammation. These long term plasma cells occasion OCBs, a hallmark of MS. Short-lived plasma cells become long lived in case that they find a survival niche (milieu) providing them essential factors for their development and survival. This B cell- fostering milieu basically is located in bone marrow. However, some of them there are also located in the sites of inflammation. This survival environment can achieved by production of BAFF, which is one survival factor produced by astrocytes and by secretion of chemokines, which induce the migration and survival of plasma cells in sites of inflammation. The main survival mediators of plasma cells are the chemokines and especially the homeostatic or lymphoid chemokines. This type of chemokines are vital for the maintenance and development of lymphoid organs. (Markus Krumbholz et al. 2012, Meint et al. 2006) Sometimes when are upregulated , they prompt B cell attracting in the site of inflammation but not directly like inflammatory chemokines whose main role is to direct immune cells to the inflammatory sites. Except of attracking their irrefutable role is the maintenance of B cells in these B cell follicles. Some known homeostatic chemokines are the CXCL12 and CXCL13, (Meint et al. 2006) and are produced by monocytes, macrophages, and dendritic cells (Carlsen HS et al. 2002, Krumbholz M et al. 2012) and are remarkably correlated with Ig production, the number of B cells and plasmablasts. Taken together, is proposed that the inflammatory CNS provides a B cells fostering environment, indicating that some B lineage cells, (long-lived plasma cells) can survive for a long term, sometimes even for a lifetime. In this fostering environment is also included the intrathecal immunoglobulin (Ig)-producing cells.(Krumbholz M et al. 2012, Meint et al. 2006)

After the first implication of the OCBs and their production of Igs in the MS pathology (Kabat et al. 1948), the role of Ig secreting cells and their produced antibodies became incontrovertible due to the abiding attempt of many researchers to clarify how antibodies are involved in the pathology of MS. In this effort attempted to identify their targets antibodies. Some findings implicated specific Abs for recognizing components of myelin sheath, promoting demyelination (, Wang and Fujinami 1997 , Van der Goes et al. 1999). Although their dominant appearance in OCBs of the CNS, that is a hallmark of MS diagnosis, it is still unclear their specific distribution. Particularly, it isn't clear if they are implicated in the onset or in the progression of pathogenesis. (Klaus Lehmann Horn et al. 2013). Further evidence for the role of antibodies in pathology of MS indicates that the immune response, because of produced antibodies in the site of inflammation, may contribute to CNS demyelination (Breij et al. 2008, Merkler et al. 2006). According to some studies antibodies can't trigger alone the onset of EAE in otherwise healthy animals, but they can deteriorate the CNS damage, pushing the progression of disease. (Benkhoucha et al. 2012). Within plethora candidate myelin antigens, the most often implicated antibodies are the Abs to MOG (auto-antigen). While MOG is only a small "fraction" of myelin , its location (extracellular) makes prime and easily accessible target for the attack of specific antibodies. (Gardinier et al. 1992, Lalive et al. 2011). Except of MOG have also been investigated the role of Abs recognizing PLP and MBP in EAE animal models (Endoh et al. 1986 , van der Veen et al. 1986). An alternative but equally important role of Abs in an immune response is their capacity to recruit other cells in the sites of inflammation. Autoreactive T cells, monocytes and eosinophils are attracted to the sites of inflammation, resulting in the tissue damage.

Beyond their role as producers of antibodies, earlier maturation stages and as well as memory B cells can act as antigen-presenting cells (APCs) or as antigens to other APCs. In general, APCs can divided to resident (CNS) and non-residence (bone marrow derived) APCs (Klaus Lehmann-Horn et al. 2011). Astrocytes and parenchymal microglia are belong to resident APCs (Fontana et al. 1984). In contrast, B cells,

macrophages, and dendritic cells participate among the non-resident APCs. Accordingly, professional APCs express in their surface MHC class II molecules, which in this context are recognized from T cells (TH1, TH17). The crosstalk among the T – and B cells, lead to activation of CD4+ T cell subsets and may be also a ''key'' in the pathology of autoimmune disorders. Dendritic cells are the professional APCs that can sufficiently by their own induce CNS autoimmunity (Greter et al. 2005). Among the rest APCs, B cells are the main APCs in the initiation of T cells response to protein antigens (Constant et al. 1999) and also in the activation of T cells in case of limited amount of antigens (Tejada-Simon MV et al. 2001).

Besides, being a potent APC may contribute to the progress of inflammation by secreting pro- and anti- inflammatory Chemokines. Activated B cells produce substantial amounts of IL-6- proinflammatory chemokine, which is crucial for development of Th17 cells (Korn et al. 2008). A recent report supports that IL-6 production by B cells is a major pathogenicity factor for B cells and that, in essence, B cells in MS contain a higher frequency of activated IL-6-producing subsets (Barr et al. 2012). Except of B cells subsets, which contribute to the inflammation by secreting IL-6, there are also B cell subtypes with anti-inflammatory properties. These B cells subsets may promote development of induced regulatory T cells with suppressive capacity. These regulatory B cells may further exert regulatory properties through provision of anti-inflammatory IL-10.

All these activities of B cells summarize the complexity of B cell function in CNS autoimmune disease and highlight the possibility that unselective B cells directed therapeutic approaches may collaterally eradicate pre-existing B-cell regulation.

Treatments of MS

As described above, MS pathogenesis is likely more heterogeneous than previously thought. For that reason is very important to enrich the therapeutic armamentarium for the treatment of multiple sclerosis. Substantial interest has developed for testing B –cell depletion as a therapeutical strategy in MS because of the capacity of B cells to

act as potent APCs for activation of encephalitogenic T cells and to be developed into plasma cells secreting pathogenic antibodies. The target molecule is CD20, a surface molecule that well characterizes the B cell lineage. CD20 is expresses throughout B-cell maturation starting from pre B cells up to memory B cells and is only lost upon their terminal differentiation into plasma cells. Clinical trials of monoclonal antibodies against CD20 efficiently decrease circulating immature and mature B cells, but not the CD20-negative plasma cells. The positive effects of this drug associated with the reduction of B cells, which they serve as APCs and induce the inflammation. Contrary to the initial thought, the effectiveness of anti-CD20 is not derived from the reduction of Igs in many MS trials (antibody-independent activity) (Hauser et al. 2008).

Natalizumab was the first monoclonal antibody approved as a DMT for relapsing MS. This drug targets the alpha-4 subunit of the VLA-4 receptor that is expressed on activated T cells and other mononuclear white blood cells. Normally VLA-4 interacts with vascular cell adhesion molecule 1 on endothelium and fibronectin in tissue, allowing cell adherence to the vessel wall and trafficking within tissues. Blocking VLA-4 prevents inflammatory cell migration from the vasculature and within the CNS. (Bielekova B. et al. 2010)

Figolimod (FTY720) has a unique mechanism of action among the drugs that are currently used in treatment of MS. FTY720 also interferes with lymphocyte migration, but it isn't a monoclonal antibody like Natalizumab. This oral drug is an analog to sphingosine, binds to sphigosin-1-phosphate (S1P) receptors, being a superagonist that leads to internalization and following degradation of the receptors. This SP1 stimulation is blocked, which is very important for immune-cell-trafficking (S.Mandala et al. 2002). Taken together, the main action of Fingolimod in the immune system is to regulate the trafficking of lymphocytes between primary and secondary lymphoid organs and circulating fluids (blood or lymphs). Fingolimod causes reversible lymphopenia without affecting lymphocyte proliferation or survival or cytokine production. This lymphopenia results from the retention of lymphocytes in secondary

lymphoid organs. However, this effect differs among immune cell populations even among the subtypes of them.

Aim of the project

Accordingly, the multiple sclerosis is one of the worse autoimmune diseases, affecting also young individuals. Thus, it is crucial the therapeutic processes to be improved, declaring the function of given drugs or investigating new ones. In our project, we studied the effects of certain drugs on leukocyte populations, especially, on B lymphocyte subsets in the blood of patients with MS. These drugs consist of Natalizumab and Fingolimod, which both inhibit migration of lymphocytes into the CNS. It seems that MS drugs differentially affect T and B lymphocytes responsiveness and may further allow to differentiate RRMS patients into B cell-dominant and T cell-dominant patients. Moreover this analysis will take advantage of recent discoveries in lymphocyte differentiation and function and will include recently characterized terminally differentiated plasma cells and other B cell subsets which are central to the development of B cell-mediated autoimmunity, in contrast to the function of T cells subtypes TH17 (T helper 17 subtype) and TH9 (T helper 9 subtype), which are primarily involved in pathogenesis of auto-immune diseases. To determine whether each treatment induces alterations in distribution and function of peripheral B lymphocyte populations, identical analysis will be performed in patients with RRMS treated with Figolimod and Natalizumab. Finally this study could possibly help to identification and classification of B cell subpopulations of RRMS patients who influenced from Figolimod and Natalizumab treatment leading to improved condition of individuals.

Materials and methods

Study subjects

A total of 18 patients presenting with RRMS (9 women, 9 men) were recruited in the study at department of neuroimmunology and Hertie Institute for brain research in, Tübingen Germany. At the time of admission, all patients had undergone brain and

spinal cord MRI and CSF analysis to detect oligoclonal bands. Blood samples were collected from the RRMS patients in period of 4 months. These patients were classified in 2 groups. The first group consists of 9 well-characterized RRMS patients who were treated with Fingolimod (**MS-F**: 9 patients). Venous blood samples from these patients were collected. On the other hand, RRMS patients who were treated with Natalizumab belong to the second group (**MS-N**: 9 patients). For comparison there is a control group, which consists of healthy individuals (**HI**: 9 people). Patients with multiple sclerosis fulfilled the 2005 McDonald criteria for relapsing remitting or primary progressive multiple sclerosis. The written informed consent was obtained from each participant and the local ethics committee of Tübingen University approved the study.

	MS-F	**MS-N**	**HP**
Number of participants	**9**	**9**	**9**
female sex (%)	**67%**	**55%**	**33%**
	(6/9)	**(5/9)**	**(3/9)**

Table1: Patients cohorts

MS-F: Fingolimod-treated RRMS patients; MS-N: Natalizumab-treated RRMS patients; HP: healthy individuals

Phenotypic analysis (PBMCs isolation- Cell staining - FACS analysis)

A) PBMCS Isolation
B) Sample preparation-Cell staining
C) FACS analysis

A total of 20-40 ml EDTA blood were obtained from RRMS patients and healthy donors. Peripheral mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Biochrom) density gradient centrifugation and multicolor flow cytometric analyses were performed. Calculated amount (100-250µl) of fresh EDTA blood were added to a round-bottom 96-well plate (BD Biosciences) in order to study circulating B-cells subsets. Before the **cell staining** Fc-blocker (human IgG,) were used throughout all 9 wells to exclude unspecific antibody binding (25-50 µl depending on single or five color

staining). For all washing steps during the procedure of staining, 200µl phosphate-buffered saline (PBS, Sigma Aldrich) supplemented with 2% fetal calf serum (FCS) – (FACS buffer) was added to each well. The plate was centrifuged at 1400 X g for 4 minutes, and the supernatant was discarded. Specifically, for the cell staining the pellet was resuspended in 5 µl (single staining) and in 50 µl (Five color staining, 10 µl solution of each antibody) and incubated for 30 minutes at 4o C. After centrifugation and 2 washing steps in order to avoid unspecific binding, a secondary Ab for anti-IgM, anti-IgA, anti-IgG added and the cells incubated for another 30 minutes at 4° C. After the last washing steps the pellet was resuspended in 200µl FACS buffer for analysis with a flow cytometer (CyAnADP). The monoclonal antibodies, which were used for the cell staining : CD3 FITC, CD4 PE, CD4 APC, CD4 PB, CD4 APC-H7, CD27PE, CD20 APC-H7, IgM APC, IgG APC, IgA APC (all provided by BD Biosciences), CD19 Pacific Blue (Biolegend) and streptavidin (life technologies) which conjugates in the biotin of IgM, IgA and IgG. The secondary antidody is labeled with APC (Avidin Peroxidase Complex). Based on a SSC/CD19 gating (> 2500 CD19+ events), the above monoclonal anti-human Abs were used to analyze the different B cell-subsets, although a precise definition of the latter population would require additional markers. The B cell subsets were defined as follows: naïve: CD27- , Memory: CD27+, Plasma cells: CD20- CD27+ (Catalina Lee Chang, et al 2010). The variability between FACS results of patients was analyzed with Dako Summit 4.2. Software.

Flow cytometry is a powerful tool enabled by development of advanced flow cytometers. Robust assays can detect and monitor multiple analytes to produce a tremendous amount of data while conserving sample. Availability of different conjugates and putting the colors in the appropriate channels makes developing multicolor (>6) panels challenging. Data quality (results) depends greatly on proper panel design and optimization of instrument setup. In this study, was used a flow cytometer (CyAnADP) with three channels (488nm, 351nm, 635nm). Especially for the five color staining of human PBMCs, were used all the lasers. The term "compensation" as applied to flow cytometry typically refers to a mathematical

manipulation that subtracts or otherwise minimizes the effect of a spectral overlap across colors, thereby better resolving sub-populations, mainly using the same channel for two different colors. More accurately, compensation is the process by which the physical observation is mathematically manipulated to better observe biological significance. A common problem in resolving events that are positive in one color from events in another color is that the spectrum may be very close together. Worse yet, the spectrums may actually overlap. Thus, in assays that used clorochromes which are detected from the same channel, it is critical to undergo compensation between them. (e.g. FITC and PE, APC and APC-H7).

A. Uncompensated **B. Compensated**

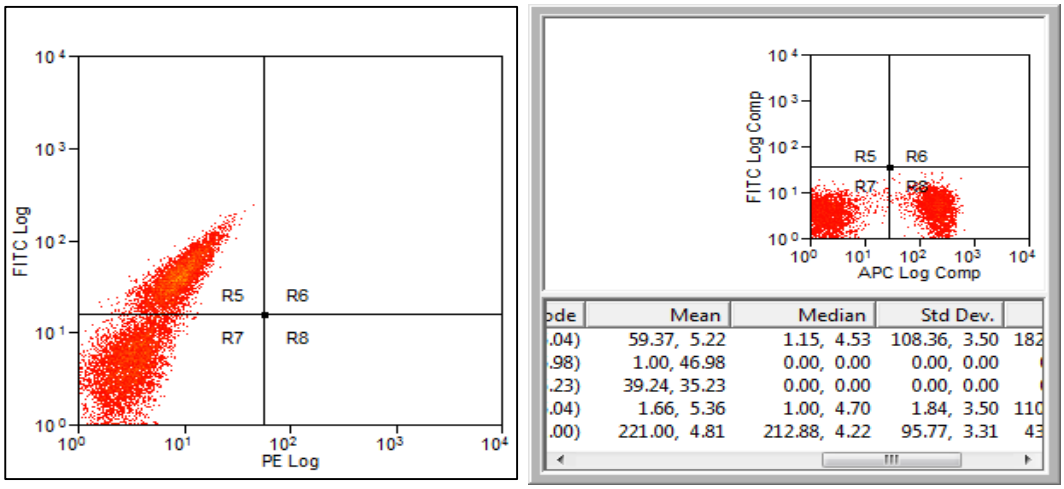


Figure 1: Compensation of clorochromes

A. single color staining: FITC against PE uncompensated. FITC and PE are two chlorochromes which are detected from the same laser (488 nm). B. FITC against APC compensated. In this case, parameter median should have difference less than 1. One of the reasons these markers are used together is that each is adequately excited by a 488nm laser. For example, many flow cytometers have one or two lasers, but two or three detectors may exist on each laser path. If many fluorescence markers can be sufficiently excited by a single laser, but emitted in different wavelengths, they can be properly resolved on relatively inexpensive cytometers (because only one laser is needed for multiple markers). Below is the relative excitation efficiencies for FITC and

PE, including a line identifying the commonly used 488nm laser line used to excite these markers.

Statistical analysis

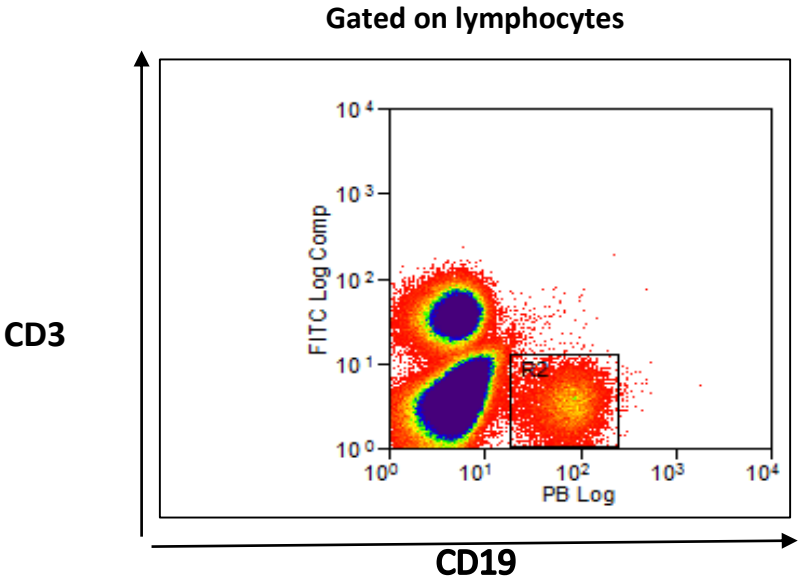
Statistical analysis was performed using GraphPad Prism software. Data are presented as means ± SE. One-way ANOVA was used to compare the different groups. Student`s t-test was used to compare data from two different groups. A value of $P<0.05$ was considered as significant.

Results

Reduction of circulating transitional B cells in Fingolimod-treated RRMS patients compared to Natalizumab -treated and healthy individuals.

Initially the population of circulating B cells in Fingolimod treated (MS-F) and Natalizumab treated patients (MS-N) was evaluated and compared with healthy controls. MS-F showed significantly lower amount of B cells in the circulation when they were compared to healthy individuals ($P < 0.001$). Natalizumab therapy could not change the frequency of B cell populations ($P= 0.1969$). The difference between the effect of Fingolimod and Natalizumab was also statistical significant ($P < 0.001$) (Fig. 2.B). In this study we were not able to analyze the proportions of B lymphocyte subsets from untreated MS patients, thus the comparison with the group of untreated patients is only discussed based on data from the literature. In this case, MS-F had also fewer B cells than patients without any disease modifying drugs (Yusei Miyazaki et al. 2014). The proportion of B cells was comparable between MS-N and untreated patients (Yusei Miyazaki et al. 2014, Kowarik et al. 2011).

A.



B.

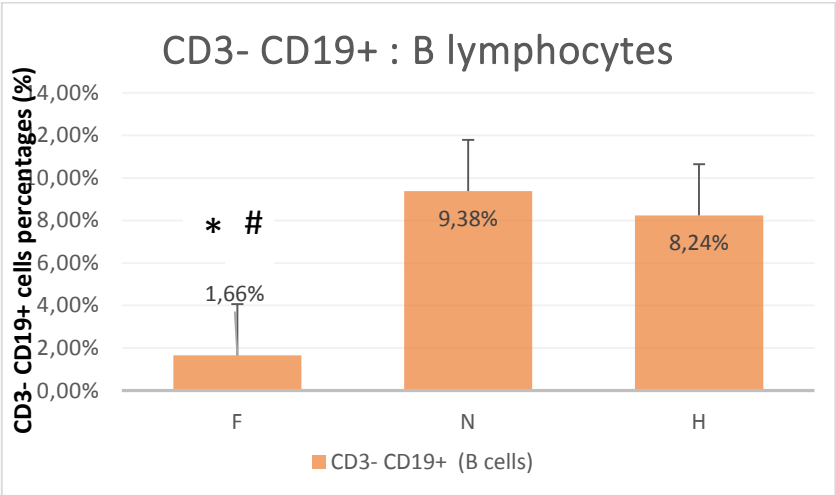


Figure 2: Fingolimod reduces and Natalizumab increases the number of B cells in the circulation (Differential effect of Fingolimod and Natalizumab on circulated B cells population)

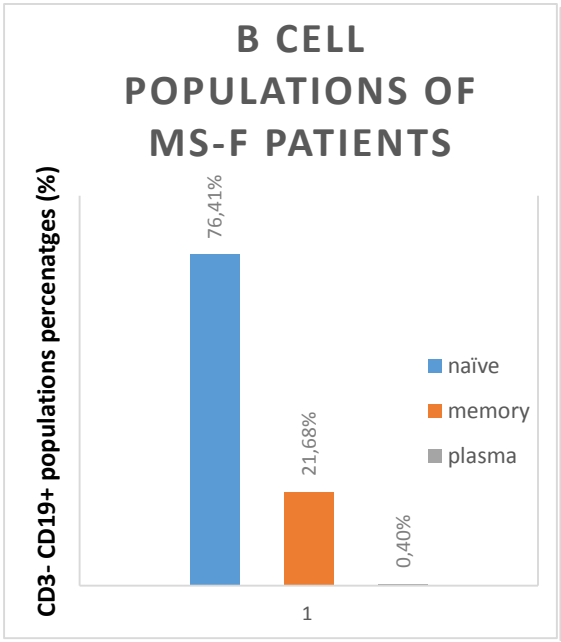
A. Gating strategy of B lymphocytes (CD19+). Representative dot blots from MS-F are shown. B. Proportions of B cells among total lymphocytes in MS-F, MS-N and healthy individuals (H). * MS-F significantly different compared to HP, $P < 0.001$. # Significantly different between MS-F and MS-N, $P < 0.001$. (MS-F significantly different compared to MS-N)

Distribution of B lymphocyte sub-populations (Naïve, Memory, Plasma) of MS-F and MS-N.

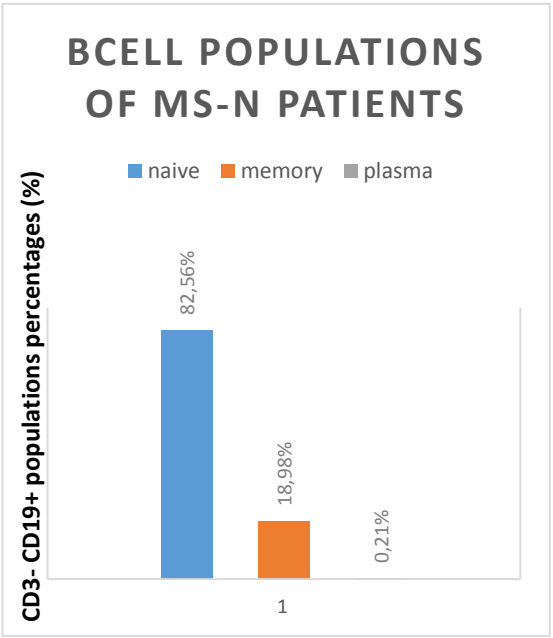
We next analyzed the distribution of B lymphocyte sub-populations in the inside of each group of patients (MS-F and MS-N) as well as in the control group (HP). Our results indicated that all three groups (MS-F, MS-N, HP) had higher proportion of naïve cells than memory cells.

The periphery of healthy individuals is more enriched in plasma cells than periphery of patients. However, the difference among the groups of patients and healthy individual is not important (Fig3.A).The fraction of naïve B cells was larger in MS-F and MS-N in compared to HP. (Fig 3.B). In addition, patients who received Natalizumab and Fingolimod drugs showed reduced percentage of memory cells (CD3- CD19+ CD27+ CD20+) when compared to healthy individual (Fig 3.C).

A.



B.



C.

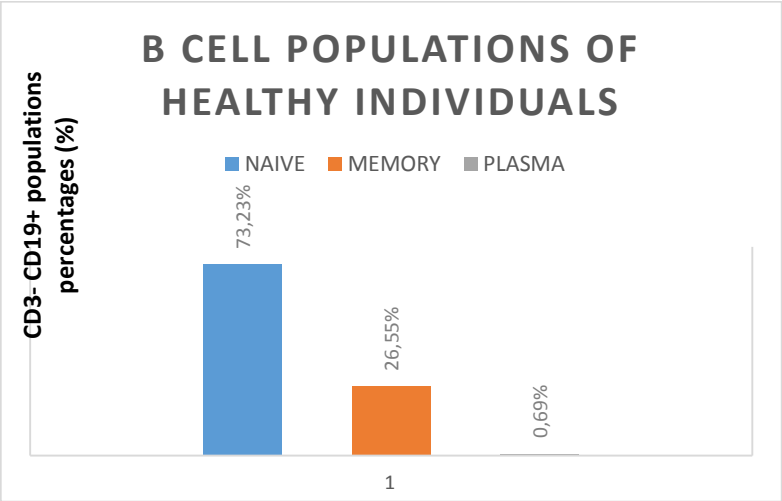


Figure 3: Increased proportion of naïve B cells and reduced proportion of memory B cells among the total B cells in the circulation of RRMS patients

A. Fractions of B cells subsets (naïve cells, memory cells, plasma cells) in fingolimod treated patients with MS (MS-F), B. natalizumab-treated patients with MS (MS-N) C. or healthy people (HP) in the peripheral blood.

	MS-F	MS-N	HP
NAÏVE CELLS	**76.41%**	**82.56%**	**73.23%**
MEMORY CELLS	**21.68%**	**18.98%**	**26.55%**
PLASMA CELLS	**0.40%**	**0.21%**	**0.69%**

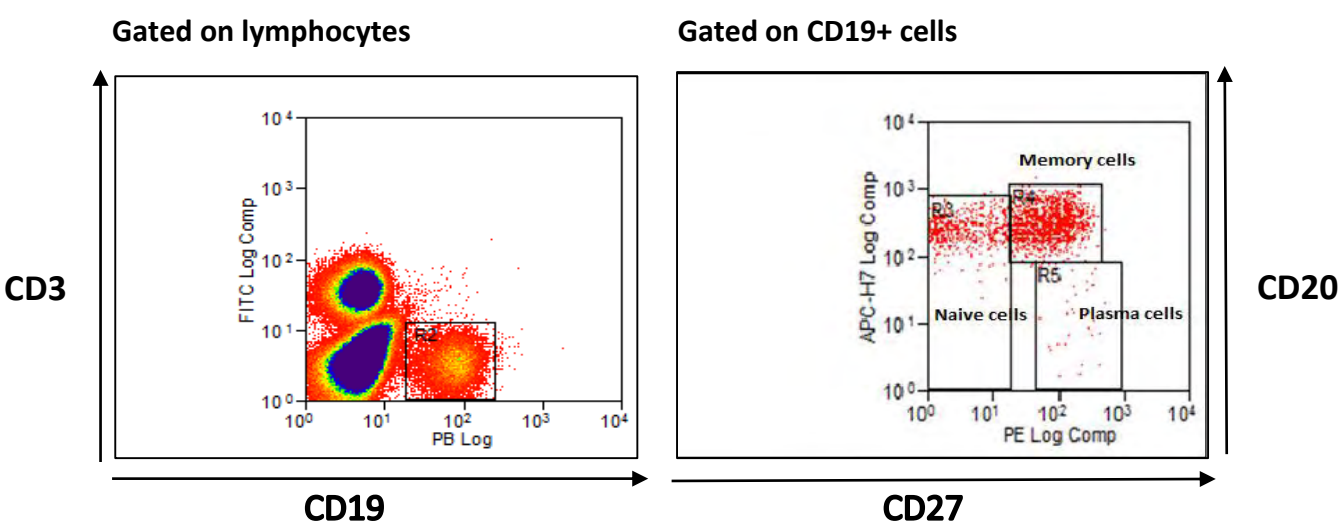
Table 2: B cell population percentages (%) in peripheral blood of patients with MS-F , MS-N and HP

MS-F: Fingolimod-treated RRMS patients; MS-N: Natalizumab-treated RRMS patients; HP: healthy individuals

Differential effects of Fingolimod and Natalizumab on B lymphocytes subtypes

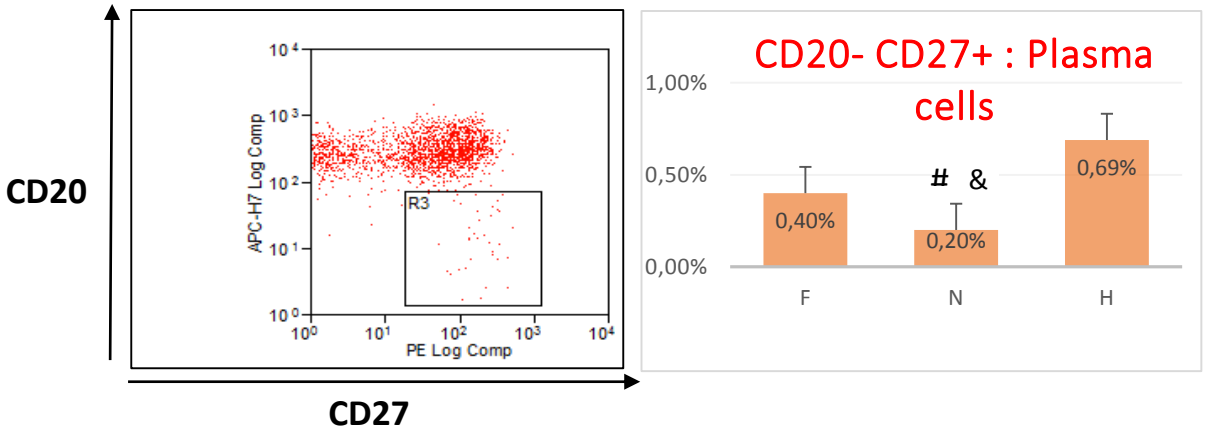
Thus based on total CD19+ cells (SSC/ CD19 gating), we analyzed the effect of different treatments on the proportions of circulating B cell subsets: memory cells (CD3- CD19+ CD27+ CD20+), naïve cells (CD3- CD19+ CD27-), plasma cells (CD3- CD19+ CD27+ CD20-) (Fig4.A). As it is depicted in Fig 4.B, both Fingolimod and Natalizumab treatments significantly reduced the frequency of CD20-CD27+ plasma cells in comparison to healthy people, but this was more pronounced for Natalizumab treated patients (P< 0.001). This difference between 2 therapies on the frequency of CD20-CD27+ plasma cells was statistically significant (P=0.0098). In contrast to CD20-CD27+ B cells, no major change was observed for the frequency of CD20+CD27+ B cells (Memory B cells) in the inside of CD3-CD19+ B lymphocytes after any therapy. There was a slight reduction in the percentage of memory B cells for Natalizumab treated patients, but this was not statistically significant (P> 0.05) (Fig 4.C). There was also no remarkable change in the rate of CD27- B cells (Naïve B cells) in response to therapies (P> 0.05). There was an induction in the percentage of CD27- B cells in the patients with Natalizumab therapy, but this increase was not statistically noteworthy (Fig 4.D)

A.



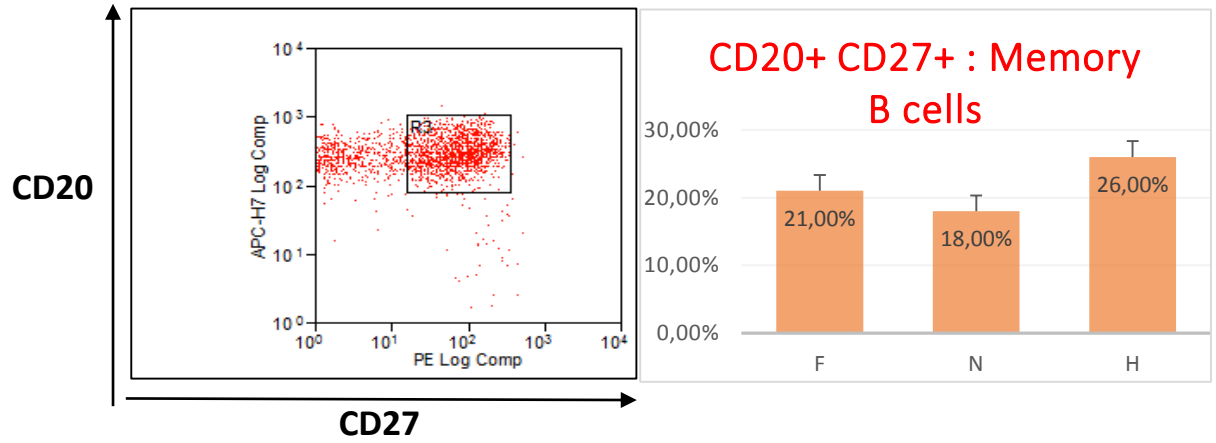
B.

Gated on CD19+ cells



C.

Gated on CD19+ cells



D.

Gated on CD19+ cells

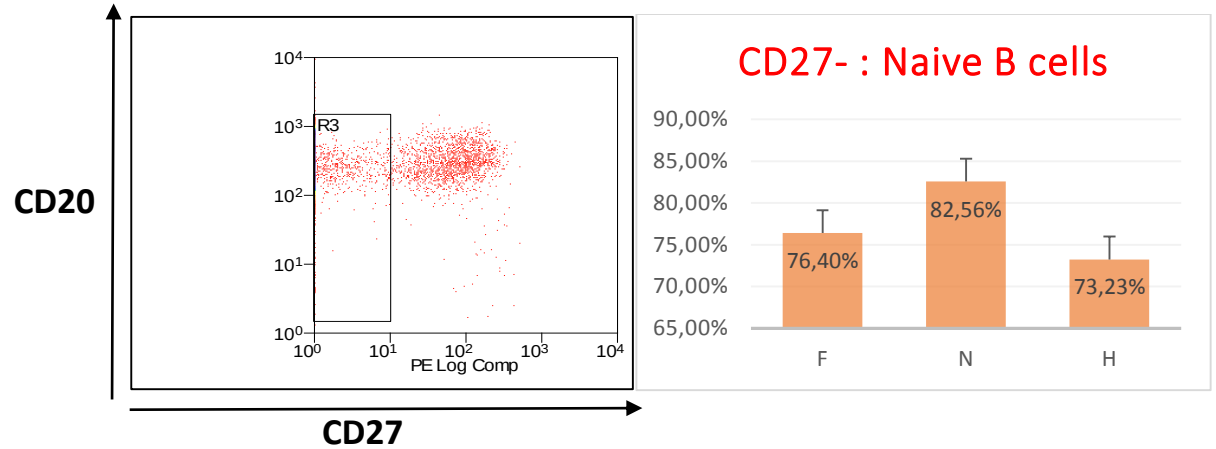


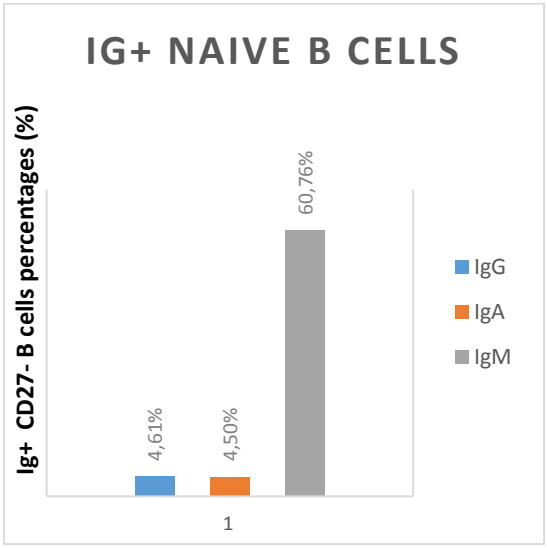
Figure 4: Differnent proportions of B cells subsets in the blood compartment of MS-F compared to MS-N

A. Gating strategy of B cells (CD19+), naïve (CD27-), memory B cells (CD27+, CD20+) and plasma cells (CD27+, CD20-) . Representative dot blots from MS-F are shown. B. Gating strategy of memory cells and proportions of plasma cells among total B cells in fingolimod-(MS-F) , natalizumab (MS-N)-treated patients and healthy people (HP). C. Gating strategy of naive cells and proportions of memory cells among total B cells in fingolimod-(MS-F) , natalizumab (MS-N)-treated patients and healthy people (HP). D. Gating strategy of naive cells and proportions of naïve cells among total B cells in fingolimod-(MS-F) , natalizumab (MS-N)-treated patients and healthy people (HP). * Statistically significant different F and H, $P < 0.001$. & significantly different between N and H, $P < 0.001$. # significantly different between F and N, $P < 0.001$.

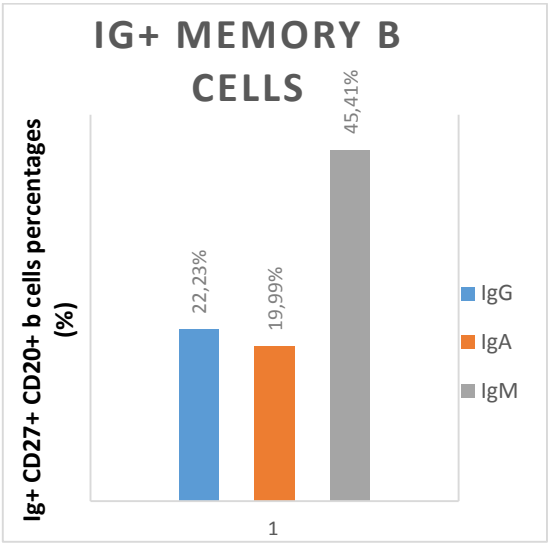
Spontaneous Ig expression in different subsets of B cells (naïve, memory and plasma cells)

We also evaluated the distribution of each membrane Ig in different subpopulation of B cells: Plasma cells (CD3- CD19+ CD27+CD20-), memory B cells (CD3- CD19+ CD27+ CD20+) and naïve B cells (CD3- CD19+ CD27-). We observed that the most prominent isotype of Ig in CD3- CD19+ CD27- B cells was the IgM (12 fold higher than IgG and IgA) (Fig5.A). Similar finding was also observed for CD3- CD19+ CD27+ CD20+ memory B cells, with IgM showing higher amount of induction compared to 2 other isotypes (Fig5.B). We must mention, however, the most abundant expressed isotype in the circulating plasma cells was IgA, which is mainly derived mucosal immune responses (Fig5.C)

A.



B.



C .

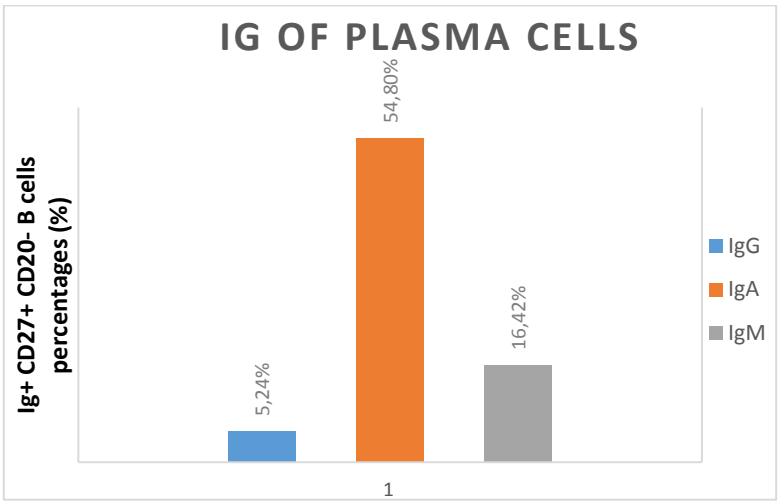


Figure 5: Spontaneous Ig expression in different subsets of B cells

A. Proportions of membrane IgG, IgA and IgM in naïve cells of HP B. Proportions of membrane IgG, IgA and IgM in memory cells of Healthy people (HP) C. Proportions of membrane IgG, IgA and IgM in Plasma cells of HP (Healthy People).

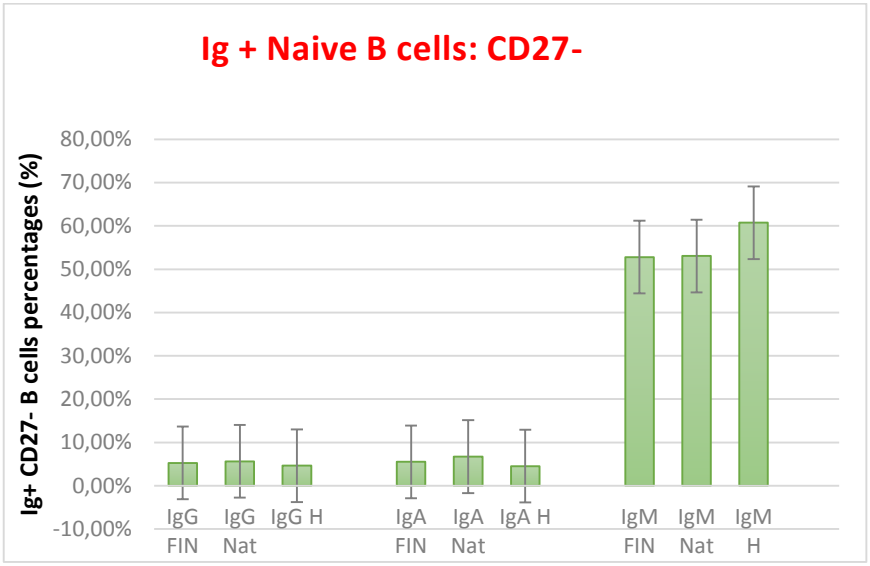
Effect of Fingolimod and Natalizumab treatments on the frequency of Ig positive B cell subtypes

Finally, we analyzed the effect of different therapies on the frequency of Ig positive B cell subtypes. As it is presented in figure 6 A-D, the was no significant effect of therapies (Fingolimod and Natalizumab) on the frequency of IgG+, IgM+, and IgA+ naïve B cells (P> 0.05).

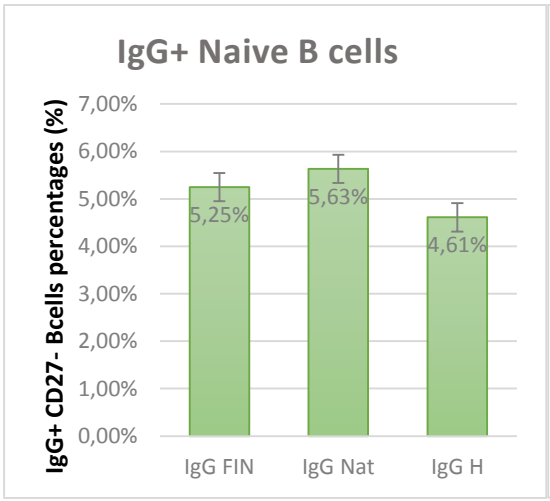
Similar to naïve B cells, both Fingolimod and Natalizumab therapies had no significant effect on the frequency of IgG+, IgA+ and IgM+ memory B cells (CD3- CD19+ CD27+ CD20+) compared to healthy people (Fig 7A-D). There was a slight induction in the percentage of IgG+ memory B cells in response to Fingolimod therapy but it was not remarkable (P> 0.05) (Fig 7.B). The slight reduction in the frequency of IgM+ CD3- CD19+ CD27+ CD20+ B cells in response to both Fingolimod and Natalizumab therapies was also not worthy (P> 0.05) (Fig 7D).

As regard to other B cell subsets, plasma cells (CD3- CD19+ CD27+ CD20-) showed some changes in the production of Igs in response to different therapies (Fig 8.A). To begin with, while Natalizumab therapy significantly induced the frequency of IgG+ CD3- CD19+ CD27+ CD20- B cells, the Fingolimod treatment significantly reduced their frequency compared to healthy control (Fig 8.B). On the other hand, in response to both treatments, only Natalizumab treated patient showed a significant reduction in the frequency of IgA+ CD3- CD19+ CD27+ CD20- B cells (P=0.0228). Fingolimod treated patients appeared also a reduction in IgA+ CD3- CD19+ CD27+ CD20- B cells but it was not significant (P=0.1359). Although there was an increase in the frequency of IgM+ CD3- CD19+ CD27+ CD20- B cells in MS-F group compared to MS-N and healthy groups, but this induction was not significant (P=0.0525). No remarkable difference was pointed out between MS-N and HP groups for the percentage of IgM+ CD3- CD19+CD20-CD27+ terminally differentiated B cells (Plasma Cells).

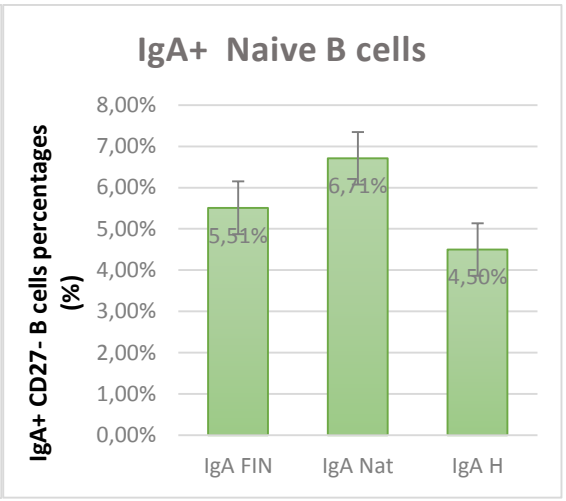
A.


Ig + Naive B cells: CD27-
Ig+ CD27- B cells percentages (%)
80,00%
70,00%
60,00%
50,00%
40,00%
30,00%
20,00%
10,00%
0,00%
-10,00%
IgG FIN
IgG Nat
IgG H
IgA FIN
IgA Nat
IgA H
IgM FIN
IgM Nat
IgM H

B.


IgG+ Naive B cells
IgG+ CD27- Bcells percentages (%)
7,00%
6,00%
5,00%
4,00%
3,00%
2,00%
1,00%
0,00%
5,25%
5,63%
4,61%
IgG FIN
IgG Nat
IgG H

C.


IgA+ Naive B cells
IgA+ CD27- B cells percentages (%)
8,00%
7,00%
6,00%
5,00%
4,00%
3,00%
2,00%
1,00%
0,00%
5,51%
6,71%
4,50%
IgA FIN
IgA Nat
IgA H

D.

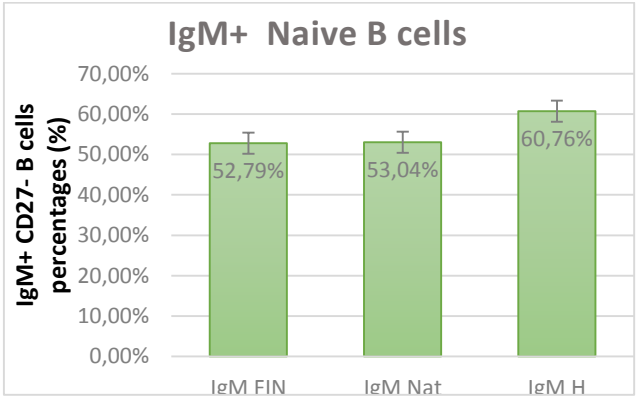
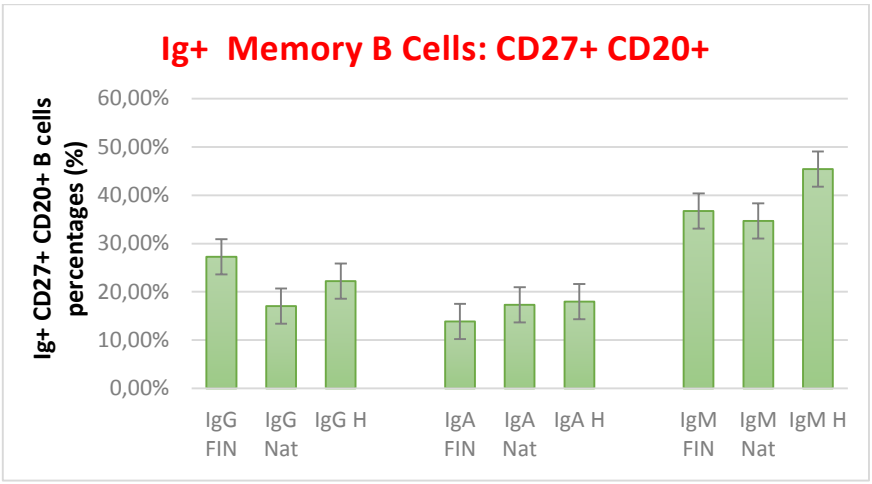
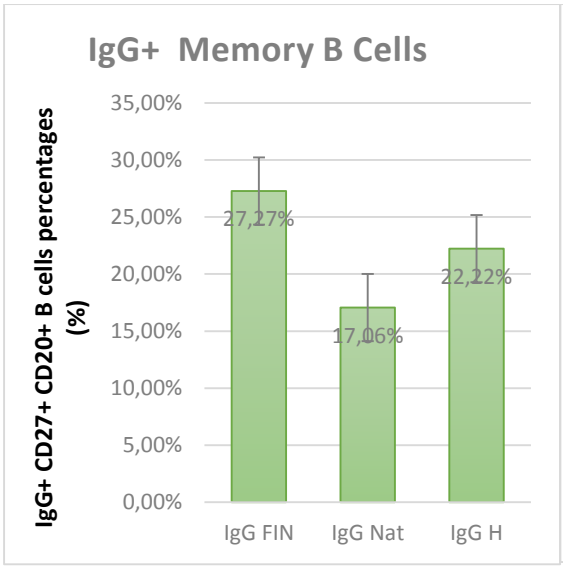

IgM+ Naive B cells
IgM+ CD27- B cells percentages (%)
70,00%
60,00%
50,00%
40,00%
30,00%
20,00%
10,00%
0,00%
52,79%
53,04%
60,76%
IgM FIN
IgM Nat
IgM H

Figure 6: Ig membrane forms in Naïve B cells in MS-F compared to MS-N and HP A. Proportions of Ig membrane forms in naïve B cells (CD3- CD19+ CD27-) of MS-F, MS-N and HP B. Proportion of IgG+ naïve cells among the naïve B cells (CD3- CD19+ CD27- IgG+) of MS-F, MS-N and HP C . Proportion of IgA+ naïve cells among the naïve B cells (CD3- CD19+ CD27- IgA+) of MS-F, MS-N and HP D. Proportion of IgM+ naïve cells among the naïve B cells (CD3- CD19+ CD27- IgM+) of MS-F, MS-N and HP. There was no significant difference in the percentage of IgG+, IgA+, IgM+ B naïve cells between 3 different groups. The P value was greater than 0.05 (P> 0.05).

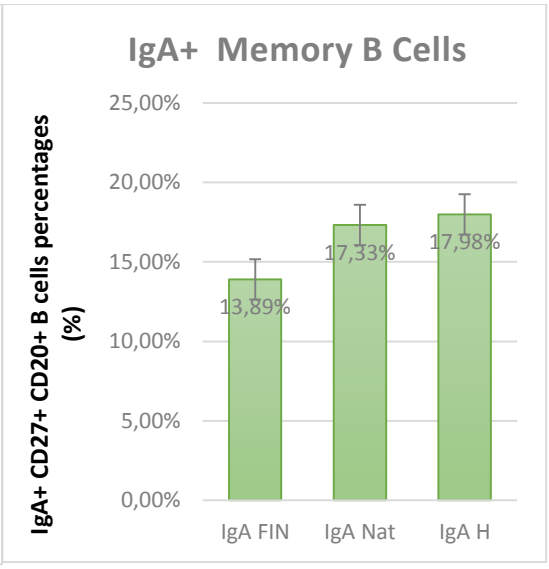
A.



B.



C.



D.

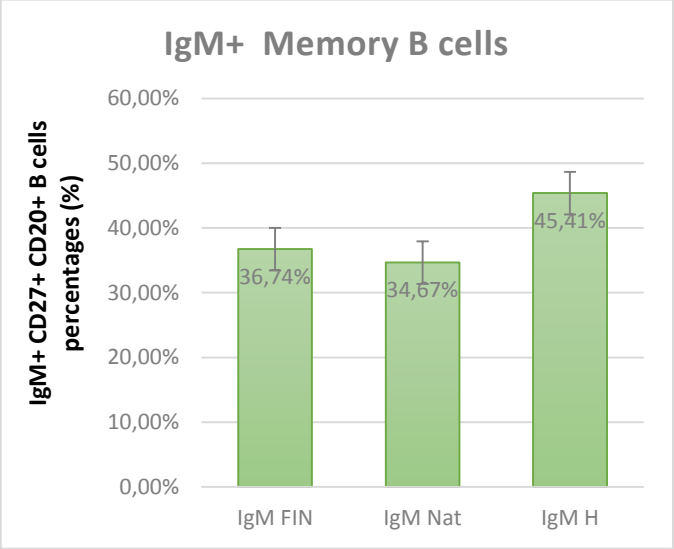
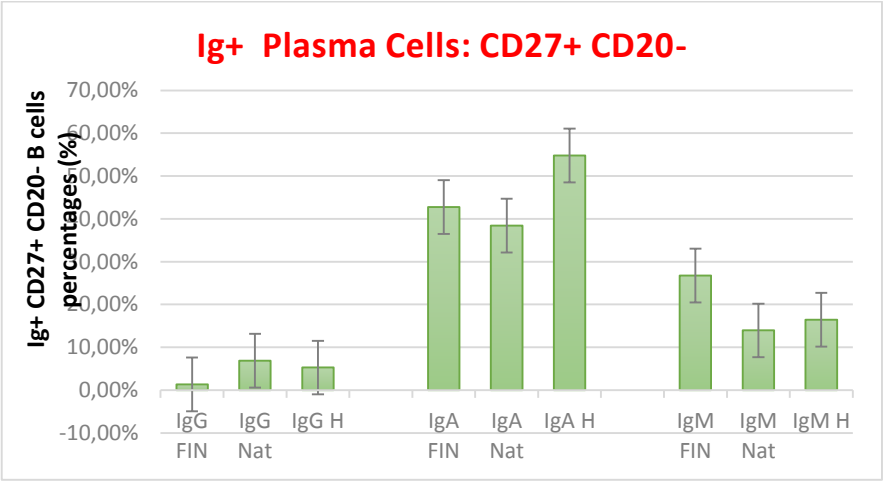
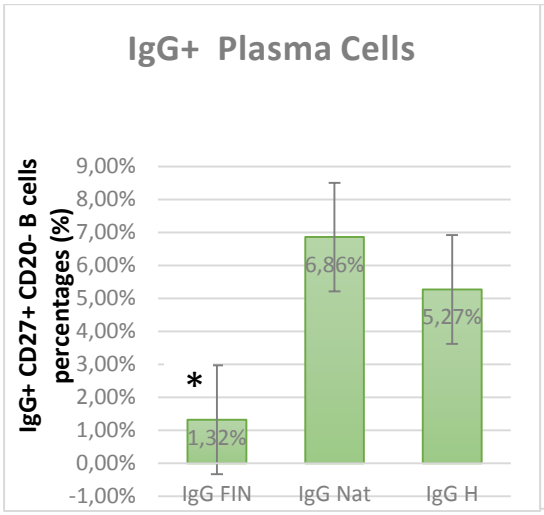


Figure 7: Ig membrane forms in Memory B cells in MS-F compared to MS-N and HP A. Proportions of Ig membrane forms in memory B cells (CD3- CD19+ CD27+ CD20+) B cells of MS-F, MS-N and HP B. Proportion of IgG+ memory cells among the memory B cells (CD3- CD19+ CD27+ CD20+ IgG+) of MS-F , MS-N and HP C . Proportion of IgA+ memory cells among the memory B cells (CD3- CD19+ CD27+ CD20+ IgA+) of MS-F, MS-N and HP D. Proportion of IgM+ memory cells among the memory B cells (CD3- CD19+ CD27+ CD20+ IgM+) of MS-F, MS-N and HP. There was no significant difference in the percentage of IgG+, IgA+, IgM+ memory B cells between 3 different groups. The P value was greater than 0.05 (P> 0.05).

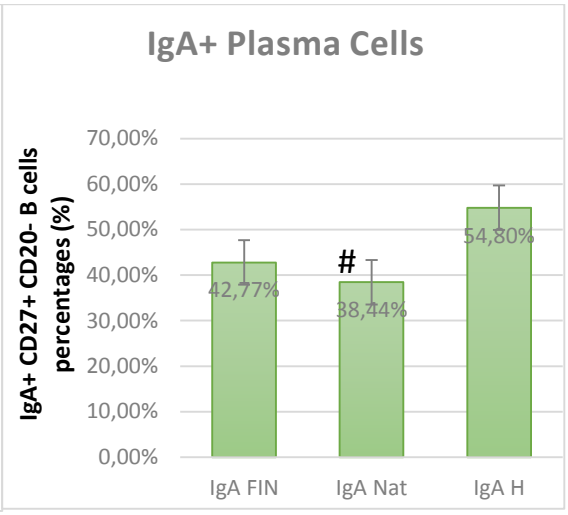
A.



B.



C.



D.

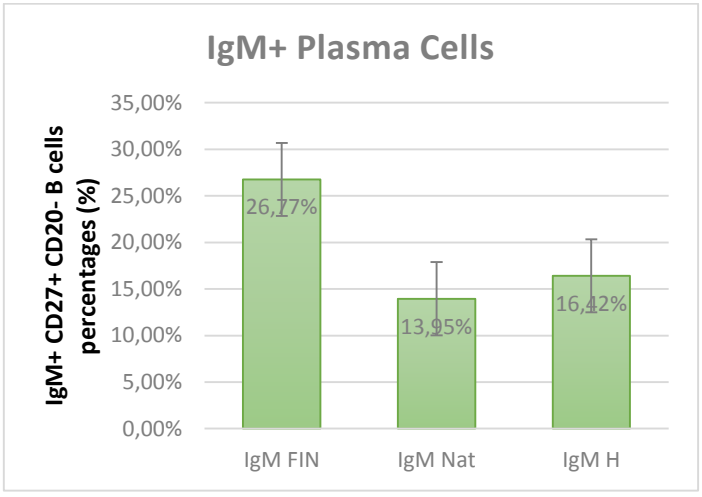


Figure 8: Ig membrane forms in Plasma B cells in MS-F compared to MS-N and HP

A. Proportions of Ig membrane forms in plasma B cells (CD3- CD19+ CD27+ CD20-) B cells of MS-F, MS-N and HP B . Proportion of IgG+ plasma cells among the plasma B cells (CD3- CD19+ CD27+ CD20- IgG+) of MS-F, MS-N and HP C . Proportion of IgA+ plasma cells among the plasma B cells (CD3- CD19+ CD27+ CD20- IgA+) of MS-F, MS-N and HP D. Proportion of IgM+ plasma cells among the plasma B cells (CD3- CD19+ CD27+ CD20- IgM+) of MS-F, MS-N and HP. *Significantly different between F and N, $P < 0.001$. #significantly different between N and H, $P < 0.001$. No significant difference was shown between the 3 groups for the percentage of IgM+ plasma cells

Discussion

The purpose of this study was to understand more about the impact of Fingolimod and Natalizumab treatments on the immune response within the blood compartment. In particular, we performed comparative studies on the effect of Fingolimod and Natalizumab on circulating B cells in patients with RRMS, with a focus on B cell subpopulations, antibody-dependent and antibody-independent activities.

Fingolimod treatment significantly reduces the lymphocytes (M.C. Kowarik et. al 2011) and as described in this study, significantly reduces the circulating B cell fraction in the periphery. The proportion of B cells (CD19+) in Fingolimod-treated patients was profoundly reduced even compared to healthy individuals (HP). These findings are in agreement with the lymphopenia in the peripheral compartment (T Bohler et. al 2007, M.C. Kowarik et. al 2011), which is induced by this drug via its involvement in immune cell trafficking. The beneficial function of this drug is based on the property of lymphopenia in the peripheral blood. However, the effect of reduction of circulating B cells was less pronounced following Natalizumab treatment. B cell levels in peripheral blood were unchanged compared to HP. Moreover, beneficial therapeutic effect of Natalizumab might be related much more to the lower fraction of these cells in the CSF (M.C. Kowarik et. al 2011).

It is proved that plasmablasts and memory cells are implicated for their crucial role in MS immune pathogenesis. Recent data indicates that also naïve B cells subsets might be involved in MS pathogenesis (M. Niino et. al 2009). In this survey, we focused on main circulating B-cell subsets: Memory B cells, Naïve B cells and Plasma cells. Mostly we investigated the impact of Fingolimod and Natalizumab treatments on peripheral blood with focus on the immune distribution in this compartment. Although MS treatments influence the proportions of B lymphocytes, the peripheral-B cell pool in patients groups is maintained. Furthermore a single B-cell subset appeared to be affected. The important finding in this study is the modified proportions of B cell

subtypes in the Fingolimod- and Natalizumab treated patients. Our findings highlighted that naïve B cells outnumber memory B cells in the blood of MS patients and healthy individuals in accordance with previous studies (C. Lee- Chang et. al 2010, M.C. Kowarik et. al 2011). Additional studies revealed that this effect is reversed in the CSF (C. Lee- Chang et. al 2010), while memory B cells outnumber naïve cells in this compartment. Last but not least memory B cells are reported to produce more pro-inflammatory cytokines (e.g. TNFa) and less anti-inflammatory cytokines compared with naïve B Cells (M.Duddy et. al 2007).

Concerning naive cells proportion, Fingolimod-treated patients showed no significant reduction in the periphery when compared with patients who were receiving Natalizumab treatment. The percentage of HP was also lower than MS patients. This implies that peripheral blood of patients consists of higher proportion of naïve cells, which have more anti-inflammatory properties, as a reflection to the inflammation due to the autoimmune disease. Furthermore, the results from the investigation of memory cells are in a line with the results from the naïve cells. Memory B cells were increased in the blood of HP compared to MS patients. In addition to, the CD27+ CD20+ B cells (memory) of Fingolimod- patients were more increased than Natalizumab-treated patients. Except of memory B cells, an important B cell subpopulation, which is profoundly implicated in MS due to its antibody-dependent participation, is the subset of plasmablasts. Patients who received Natalizumab (MS-N) had significantly reduced percentage of plasmablasts in the peripheral blood when were compared to HP. However, we should t mention, that the levels of plasmablasts of MS-N were not remarkably lower than Fingolimod-treated patients.

Our findings about the Fingolimod- and Natalizumab-induced alternation of B cell subsets and their contribution in immunity of MS, may be related with some recent studies. These studies suggest that B cells in general can be involved in immune regulation and particularly can contribute to MS pathogenesis through antibody-independent activities (H.C von Budingen et. al 2011, C.T Harp et. al 2010). Some of

them are the induction of T cell responses via pro-nflammatory cytokines, the presentation of the CNS antigens and the supporting of migration of activated lymphocytes in CNS (H.C von Budingen et. al 2011, C.T Harp et. al 2010). The B-cell mediated T cell activation, the presentation of antigens and the B cell clonal expansion are activities, which happen in the CNS and are mainly related with progress of inflammation. Except of the CNS and the site of inflammation, these activities can also take place in the peripheral lymphoid organs. The fate of activated lymphocytes in the periphery depend on production of chemokines by other immune cells.

Thus, the reduction in percentage of secreted B cell subsets led to enhanced proportions of pro-inflammatory chemokines (plasma cells, memory B cells) and the increased proportions of B cell subsets with anti-inflammatory features, probably contribute to inhibition of proinflammatory activated B cells entering the CNS. In this sense, it came from the fact that chemokines and their receptors play an important role in establishing the distribution of lymphocyte populations in primary and secondary lymphoid tissues and in the recruitment of leukocytes to sites of inflammation. (Sallusto F et. al 1998). Reduced percentage of plasma cells and memory ones and enhanced proportions of naïve cells are observed in MS-N compared to MS-F. This can lead to prevention of further neuronal damage and progress of disease and could imply a therapeutic benefit of the Natalizumab treatment.

Both chemokines, CXCL12 (SDF-1) and CXCL13 (BCA-1), regulate B cell migration in lymphoid tissues. In vitro human B cells migrate across the brain endothelium more rapidly than autologous T cells (Alter et al. 2003). These findings prove that there are differences in the migration of immune cells in the site of inflammation, based on different patterns of secreted chemokines. Different patterns of them can influence the migration of single B cell subset among the other B cell subsets, too. Although the levels of its B cell subset as well as the exact chemokine profile and their influence in the migration of individual B cell subsets in different MS patients are important.

Accordingly, the phenomenal benefit of Natalizumab treatment could overlapped from the Fingolimod treatment, in case that the most abundant naive B cells have an advantage in migration in the site of inflammation.

We also examined some anti-body dependent functions of these drugs in order to decide which treatment is properly designed for B-cell dominant MS patients. Previous studies have shown that long-lived plasma cells, originating from circulating plasmablasts or memory B cells, were able to survive in inflamed CNS and to induce the intrathecal synthesis of oligoclonal bands in MS (A. Corcione et. al 2004, S. Cepok et. al 2005). Plasma cells are the main B cell subtype, which implicated for their Ig production. Nevertheless, in this study we investigated the membrane bound form of Igs not only in circulating plasmablasts but also in other B cell subsets (Naïve and Memory B cells) in MS-N and MS-F patients. According to spontaneous expression of Igs of healthy individuals, we observed that the membrane Ig profile of naïve and memory B cells were not restricted to a specific isotype. Nonetheless, the most prominent Ig was the IgM in both subsets, which is typically associated with them. These IgM+ B cell subtypes induce the secretion of natural antibodies of the IgM isotype. Such antibodies do not require somatic mutation or gene rearrangement. By contrast, IgA was the most abundantly expressing isotype in the surface of plasmablasts (Mei HE et. al 2009). It has been discovered that natural autoantibodies among these also natural antibodies are detected in the serum of healthy individuals, which contains immunoglobulins directed against surface molecules on oligodendrocytes.

Naïve B cells are un-activated cells, which had not encountered antigen. These cells mostly present regulatory properties for the inflammation. Therefore, it is crucial to express a polyreactive Ig isotype in their surface and to produce natural Ig antibodies in the serum, in order to attack a plethora of foreign infectious or modified self - antigens. The polyreactivity is a property belongs to IgM isotype. Last but not least, IgM is expressed as a transmembrane monomeric form in all naïve B cells. This

contrasts with serum IgA and IgG, which are present at significantly lower levels under the same conditions (free of antigens). IgA and IgG are isotypes stems from somatic mutation and gene rearrangement of IgM isotype and present high affinity to specific antigens.

The proportions of IgM+, IgA+ and IgG+ naïve B cell subpopulations do not present major differences among the 3 groups. This implies, on the one hand that the proportions of IgM+, IgA+ and IgG+ naïve cells alter after the Natalizumab and Fingolimod treatment and reach the values of naïve B cells of healthy individuals. On the other hand that the proportions of those B cell subsets are not affected from these treatments but they are not involved in the pathogenesis of MS through their antibody-dependent activities. So no differences between MS patients and healthy individuals ensue. In both hypotheses the affection refers to the migration of IgM+, IgA+ and IgM+ naïve B cell subsets and not in their proliferation.

While naïve cells implicated in MS pathogenesis through indirect and regulatory responses, memory B cells and plasma cells are directly involved in the immune regulation of MS through antibody-dependent activities. Thus, the differences between the Ig+ B cell subsets among the 3 groups are more profound.

Our data from IgM+ memory B cells indicate that, this B cell subset of healthy individuals outnumber the same B cell subset of MS patients. These findings are supported by some studies in humans, which show a reduction in IgM in the blood of patients with systemic lupus erythematosus (Sle-autoimmune disease), which probably derived from the reduction in the IgM+ B cell subtypes. The decrease in sIgM may contribute to the increased number of apoptotic cells that are present in the peripheral blood of patients with Sle (Perniok A et al. 1998). Apoptotic cells express various autoantigens, and their persistence could generate harmful autoimmunity through the activation of self-reactive cells (Casciola Rosen et al. 1994). Natural IgM can bind to several autoantigens expressed by apoptotic cells, such as phospholipids,

thereby facilitating their clearance and preventing autoimmunity (Peng Y et al. 2005). In this case, we hypothesize that, the treatments (Fingolimod-Natalizumab) are not efficient to affect enough this B cell subset, providing clear therapeutical benefit. As a matter of fact, the examination of IgM+ plasma B cell subset indicated surprisingly unexpected differences among the groups. Despite the abundance of IgA+ plasma cells (typically associated with this subset), we observed that the IgM+ plasma cells of MS-F were unexpected higher than HP. This means, that this subpopulation is maybe affected by Fingolimod treatment, providing positive insights for this treatment's benefit in specific B cell subpopulations. However, it is essential to note that IgM isotype had also implicated for participating in pro-inflammatory activities, whereas it had detected in Oligoclonal bands (OCBs) of active lessions.

The IgA and IgG isostypes, as described above, are related with the high affinity recognition of foreign and self-antigens. Especially IgG is the most prominent Ig isotype in the OCBs of the CNS, so it is reasonable to be elevated in MS patients. Therefore the efficiency of each treatment derived from its capacity to ameliorate the proportion of IgG+ B cells. IgG+ memory B cells of Fingolimod-treated patients were increased compared to HP and Natalizumab treatment. In spite of reduction of B cells and consequently the reduction of IgG+ memory B cell subset in periphery, fingolimod-treated patients tend to have more increased proportion of this B cells subset. On the other hand, Natalizumab treatment induced the reduction of IgG+ memory B cell compared to HP. Although these differences are not significant and there aren't results from untreated patients to compare, these findings seems to be related with the therapeutical benefits of treatments, improving our knowledge about their influence on specific B cell subsets, which are involved in the onset and progress of MS. These results can be used as baseline for further studies in this research field as well. In the present work our outcomes showed that whereas IgG+ plasma cells are significantly reduced in MS-F . Natalizumab-treated patients pointed out comparable proportions of IgG+ plasma cells with HP. The IgG+ plasma cells of fingolimod patients

were 5-fold reduced even from HP. This might implies that Fingolimod treatment can inhibit the migration of this specific subpopulation in the periphery.

To sum it up, we examined the IgA+ B cells subset among the groups of patients. IgA as previous described is an antibody isotype stems from class switching and present higher affinity to specific antigens and in case of auto-immunity, higher affinity to auto-antigens as well. Thus, the increased proportions of IgA in the periphery maybe contribute the disease worsen. Comparing the IgA+ memory B cells among the groups we did not observe profound differences in their proportions. On the other hand remarkable decrease in IgA+ plasma cells was observed. Mainly, this reduction belongs to MS-N patients compared to healthy individuals and Fingolimod-treated. This modified proportion of IgA+plasma cells of MS-N open the way for other researchers so as to estimate the therapeutical efficiency of Natalizumab in B-cell based MS.

Although there are limitations in our study, due to the lack of data of untreated patients and lack of data of chemokine profiles of each group, the present study probably provides insights into the antibody-dependent and antibody-independent activities, which participate in the onset and progress of MS. In conclusion, such findings confirmed that these treatments alter the distribution of B cell subsets in the periphery, providing information for individual B cell-based MS treatments. However, in present work we can only hypothesize that there are differences in distribution of B cells subsets among the treatments, but the results are not significantly in order to decide which treatment is more efficient for B-cell based treatment. The aim of this study was to highlight the importance of B cells and especially of specific B cell subsets in the pathogenesis of MS. Many researchers can be based on such findings in order to investigate more treatments to find more suitable ones for B cell-based MS.

Perspectives

On the whole, our data provided some insights into the contribution of B cell subpopulations in the MS pathogenesis. Further studies may result in the classification of new B cell subsets, which participate in pathogenic immune responses of Multiple sclerosis. Furthermore, these studies will provide more details about the properties of these cell subtypes. This discrepancy suggests that there might be different B cell subsets with regulatory and pathogenic properties. Such findings will extend our knowledge about the mechanisms of this demyelinating disease. Particularly, through the understanding of which immune cell subsets are involved in processes of MS pathogenesis. In this sense, this knowledge will help in the establishing of new effective treatments or in the improving of the existent ones. Perspective studies of the effects of drugs in immune cell subpopulations should be in accordance with chemotaxis assays, in which it will be assessed the lymphocyte migration due to some chemokines such : CXCL13 (BCA) , CCL21 (SLC), CCL2 (MCP-1), CCL5 (RANTES) and CXCL12 and survival factors (BAFF). As a matter of fact, it may further be allowed to differentiate RRMS patients into B cell-dominant and T cell-dominant patients. This classification probably can ''give birth'' in one developed individual treatment of autoimmune diseases, in which the side effects of treatments will be avoided.

Acknowledgments

I greatly acknowledge Dr. Felix Bischof who gave me this opportunity to work in his Lab for my Bachelor thesis, and also thanks for his support and supervision during my work. I greatly thank my supervisor Asghar Abbasi for his important guidance and supervision during my lab rotation. Furthermore I would like to thank him, who have willingly shared his precious time during the period of 6 months of my lab rotation. Special thanks are also given to Evelyn Dubois for her technical assistance and my collegues Aleksandar, Kirsi, Ray, Fabian, Miriam, Benjamin and Claudius who supporting me thorough entire process. I would like also to express my deep gratitude

to my bachelor thesis advisor Anna Maria Psarra for spending time read this thesis and providing useful suggestions about it. I would like to thank my professor D. Leonidas from my home university. Last but not least important, my friends Antranik and Yashar who support me all these months. Finally, I own more than thanks to my family members for their encouragement throughout my life.

References

Corcione et al. CCL19 and CXCL12 trigger in vitro chemotaxis of human mantle cell lymphoma B cells. Clinical Cancer Research. 2004 Feb 1; 10(3):964-71

S. Cepok et al. Short-lived plasma blasts are the main B cell effector subset during the course of multiple sclerosis. Brain: a journal of Neurology. Mar 30 2005 Jul; 128(Pt 7):1667-76

H.C von Budingen et al. B cells in multiple sclerosis: connecting the dots. Current Opinnion in Immunology. 2011 Dec; 23(6):713-20

C.T Harp et al. Memory B cells from a subset of treatment-naïve relapsing-remitting multiple sclerosis patients elicit CD4(+) T-cell proliferation and IFN-γ production in response to myelin basic protein and myelin oligodendrocyte glycoprotein. European Journal of Immunology. 2010 Oct; 40(10):2942-56

M. Duddy et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. Journal of Immunology.2007 May; 178(10):6092-9

C. Lee- Chang et al. Susceptibility to experimental autoimmune encephalomyelitis is associated with altered B-cell subsets distribution and decreased serum BAFF levels. Immunology letters. 2011 Mar; 135(1-2):108-17

M.C Kowarik et al. CXCL13 is the major determinant for B cell recruitment to the CSF during neuroinflammation. Journal of Neuroinflammation. 2012 May; 16;9:93

M. Niino et al. Estrogens as potential therapeutic agents in multiple sclerosis. Central Nervous System Agents in Medical Chemistry. 2009 Jun; 9(2):87-94.

T. Bohler et al. Differential effects of single dose FTY720 on CD62L+ B-cells in stable renal allograft recipients. International Immunopharmacology. 2007 Jan; 7(1):88-95

B. **Bielekova** et al. Monoclonal antibodies in MS: mechanisms of action. Neurology. 2010 Jan; 5;74

S.L. Hauser et al. Multiple lessons for multiple sclerosis. The New England journal of medicine. 2008 Oct; 359(17):1838-41

MV Tejada-Simon et al .Reactivity pattern and cytokine profile of T cells primed by myelin peptides in multiple sclerosis and healthy individuals. European journal of immunology. 2001 Mar; 31(3):907-17

T. **Korn** et al. Pathophysiology of multiple sclerosis. Journal of Neurology. 2008 Dec; 10.1007/s00415-008-6001-2.

T.A. Barr et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. The Journal of Experimental Medicine. 2012 May 7; 209(5):1001-10

Endoh et al .Chronic experimental allergic encephalomyelitis and antibody responses in rabbits immunized with bovine proteolipid apoprotein. Journal of Neurology. 1986 Jun; 11(4):321-33

Klaus Lehmann-Horn et al. Anti-CD20 B-cell depletion enhances monocyte reactivity in neuroimmunological disorders. Journal of Neuroinflammation. 2011 Oct; 8:146

Fontana et al. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. Nature. 1984 Jan; 307(5948):273-6

Greter et al. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nature Medicine. 2005 Mar; 11(3):328-34

Kabat et al. Experimental studies on acute disseminated encephalomyelitis in the rhesus monkey. Annals of allergy. 1948 Mar-Apr; 6(2):109

Wang and Fujinami et al. Enhancement of EAE and induction of autoantibodies to T-cell epitopes in mice infected with a recombinant vaccinia virus encoding myelin proteolipid protein. Journal of Neuroimmunology. 1997 May; 75(1-2):75-83

Van der Goes et al. The role of anti-myelin (auto)-antibodies in the phagocytosis of myelin by macrophages. Journal of Neuroimmunology. 1999; 101(1):61-7

Klaus Lahmann Horn et al. Targeting B cells in the treatment of multiple sclerosis: recent advances and remaining challenges. Therapeutic advances in neurological disorders. 2013 May; 6(3):161-73

Breij et al. Homogeneity of active demyelinating lesions in established multiple sclerosis. Annals on neurology. 2008 Jan; 63(1):16-25

Merkler et al. Myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in the common marmoset reflects the immunopathology of pattern II multiple sclerosis lesions. Multiple Sclerosis (Houndmills, Basingstoke, England). 2006; 12(4):369-74

Benkhoucha et al. IgG glycan hydrolysis by EndoS inhibits experimental autoimmune encephalomyelitis. Journal of Neuroinflammation. 2012 Sep; 9:209.

Gardinier et al. Myelin/oligodendrocyte glycoprotein is a unique member of the immunoglobulin superfamily .Journal of neurosciences research. 1992 Sep; 33(1):177-87

Lalive et al. Highly reactive anti-myelin oligodendrocyte glycoprotein antibodies differentiate demyelinating diseases from viral encephalitis in children. Multiple Sclerosis (Houndmills, Basingstoke, England). 2011 Mar; 17(3):297-302

Meint et al. B lineage cells in the inflammatory central nervous system environment: migration, maintenance, loca l antibody production, and therapeutic modulation. Annals of Neurology 2006 May; 59(6):880-92

Carlsen HS et al. B cell attracting chemokine 1 (CXCL13) and its receptor CXCR5 are expressed in normal and aberrant gut associated lymphoid tissue. Gut immunology. 2002 Jan; 51(3):364-71

Krumbholz M. et al. B cells and antibodies in multiple sclerosis pathogenesis and therapy. Nature reviews. Neurology. 2012 Nov; 8(11):613-23

Sharief MK, Thompson EJ. Intrathecal immunoglobulin M synthesis in multiple sclerosis. Relationship with clinical and cerebrospinal fluid parameters. Brain: a journal of neurology. 1991 Feb; 114 (Pt 1A):181-95

G. Disanto et al. The evidence for a role of B cells in multiple sclerosis. Neurology. 2012 Mar; 78(11):823-32

Olsson JE and Link H. Immunoglobulin abnormalities in multiple sclerosis. Relation to clinical parameters: exacerbations and remissions. Archives of neurology. 1973 Jun; 28(6):392-9

S. Sinha . Immune regulation of multiple sclerosis by CD8+ T cells.Immunologic research. 2014 Aug; 59(1-3):254-65

M. Kleinewietfeld . Regulatory T cells in autoimmune neuroinflammation. Immunological reviews. 2014 May; 259(1):231-44

E. Schmitt. Th9 cells, new players in adaptive immunity. Trends in immunology. 2014 Feb; 35(2):61-8

K. Lehmann-Horn et al .Targeting B cells in the treatment of multiple sclerosis: recent advances and remaining challenges. Therapeutic advances in immunological disorders. 2013 May; 6(3):161-73

F. Wu Gregory et al. The immunopathophysiology of multiple sclerosis. Neurologic clinics. 2011 May; 29(2):257-78

Heather L Wilson et al. B cells contribute to MS pathogenesis through antibody-dependent and antibody-independent mechanisms. Biologics: targets & therapies. 2012 May; 6:117-23

Yusei Miyazaki 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. Clinical Immunology. 2014 Apr; 151(2):127-35

MC Kowarik et al. Differential effects of fingolimod (FTY720) on immune cells in the CSF and blood of patients with MS. Neurology .2011 Apr. 76(14):1214-21

F Sallusto et al. Activated murine B lymphocytes and dendritic cells produce a novel CC chemokine which acts selectively on activated T cells. The journal of experimental medicine. 1998 Aug; 188(3):451-63

A. Perniok et al. High levels of circulating early apoptic peripheral blood mononuclear cells in systemic lupus erythematosus. Lupus. 1998 Feb; 7(2):113-8

Casciola Rosen et al. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. The journal of experimental medicine. 1994 Apr; 179(4):1317-30

Y. Peng et al. The role of IgM antibodies in the recognition and clearance of apoptotic cells. Molecular immunology. 2005 May; 42(7):781-7

Constant SL. B lymphocytes as antigen-presenting cells for CD4+ T cell priming in vivo. Journal of Immunology. 1999 May; 162(10):5695-703

Walsh MJ et al. Immunoglobulin G, A, and M--clonal restriction in multiple sclerosis cerebrospinal fluid and serum--analysis by two-dimensional electrophoresis. Clinical immunology and immunopathology. 1985 Jun; 35(3):313-27

Van der Veen RC et al. Chronic experimental allergic encephalomyelitis and antibody responses in rabbits immunized with bovine proteolipid apoprotein. Journal on neuroimmunology. 1986 Jun; 11(4):321-33

Mandala S et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science. 2002 Mar; 12; 296(5566):346-9

Alter A. et al. Determinants of human B cell migration across brain endothelial cells. Journal of Immunology. 2003 May; 1; 170(9):4497-505

Mai HE et al. Blood-borne human plasma cells in steady state are derived from mucosal immune responses. Blood 2009 Mar; 113(11):2461-9