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**The effect of glatiramer acetate on
functional properties of B lymphocytes
in patients with MS**

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Abbreviations

APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell activation factor
BBB	Blood brain barrier
BCR	B cell receptor
BSA	Bovine serum albumin
CIS	Clinically isolated syndrome
CNS	Central nervous system
CpG	Cytosine-phosphate-Guanine
CSF	Cerebrospinal fluid
DC	Dendritic cell
dil. fac.	Dilution factor
DIS	Dissemination in space
DIT	Dissemination in time
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethyl fumarate
DMT	Disease-modifying therapy
DMSO	Dimethyl Sulfoxide
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ED	Encephalitis disseminate
EDSS	Expanded disability status scale
EDTA	Ethylene diamine tetraacetic acid disodium salt dihydrate
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	US Food and Drug Administration

FSC	Forward scatter
GA	Glatiramer acetate
GM-CSF	Granulocyte-macrophage colony stimulating factor
HHV-6	Human herpesvirus 6
HLA-DR	Human leukocyte antigen - antigen D related
i.m.	Intramuscular
IFNβ	Interferon beta
IgG	Immunoglobulin G
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-35	Interleukin 35
INFγ	Interferon gamma
int	Intermediate
ITP	Idiopathic thrombocytopenic purpura
JC virus	John Cunningham virus
LPS	Lipopolysaccharide
LT-α	Lymphotoxin-alpha
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MHC-II	Major histocompatibility complex-class II
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
NAWM	Normal appearing white matter
NEDA	No evidence of disease activity
NK cell	Natural killer cell

NMSS	National multiple sclerosis society (US)
N.s.	Not significant
NT3	Neurotrophin-3
PBMC	Peripheral blood mononuclear cells
p.o.	<i>Per os</i>
PBS	Phosphate-buffered salt solution
PFA	Para-formaldehyde
PKC	Protein kinase C
PLP	Proteolipid protein
PMA	Phorbol 12-myristate 13-acetate
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive relapsing multiple sclerosis
R	Pearsons correlation coefficient
R²	Coefficient of determination
RIS	Radiologically isolated syndrome
Rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute-1640
RRMS	Relapsing-remitting multiple sclerosis
s.c.	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
SIP	Sphingosine-1-phosphate
SLAM	Signaling lymphocytic activation molecule
SPMS	Secondary progressive multiple sclerosis
SSC	Side scatter
TERI	Teriflunomid
Th cell	T helper cell

TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is the most common immune-mediated disorder, which affects the central nervous system and leads to damage of myelin and axons (Compston und Coles 2008). It is also known that a loss of brain volume occurs in most patients throughout the MS course (De Stefano et al. 2015). The patients develop cognitive, neurological and psychological disabilities (Ghasemi 2017). The exact etiology of MS is still unknown. Multiple studies suggest that a combination of environmental, infectious and genetic factors may play a role in MS (Compston and Coles 2002).

1.1.1 Forms and symptoms

MS may cause any neurological symptom. The most common symptoms are visual, motor and sensory signs. Tornes and colleagues (Tornes et al. 2014) described such symptoms as nystagmus, tremor, dysmetria, truncal ataxia and dysdiadochokinesia in MS patients with cerebellar lesions. Rare manifestations such as cranial nerve involvement, pain syndromes, psychiatric or cognitive symptoms are also possible (Evlice et al. 2016).

There are different phenotypes of progression in MS, which were first described by Lublin and Reingold (Lublin and Reingold 1996). In 1996, the US National Multiple Sclerosis society (NMSS) distinguished four forms of MS clinical courses: relapsing-remitting (RR), secondary progressive (SP), primary progressive (PP) and progressive-relapsing (PR).

Depending on the phenotype of progression in MS, the prognosis and treatment options may change. Each phenotype is characterized by progression or relapses. Relapses in MS are defined as transient neurological dysfunctions, which cannot be explained through other clinical conditions than MS activity and last for at least 24 h (Kamm et al. 2014). The disease activity or progression is measured by expanded disability status score (EDSS score) (Kurtzke 2015). The RRMS is characterized by relapses, which are followed by periods with no disease activity (Tsang and Macdonell 2011). There is an initial form of RRMS, called clinically isolated syndrome (CIS). Patients with CIS display MS symptoms, but do not fulfill the diagnostic criteria of multiple sclerosis (Compston and Coles 2002). Approximately 30-70 % of those patients afterwards develop a RRMS disease course (Miller et al. 2005). Clinical trials have shown that around 65 % of the initial RRMS phenotype turns into SPMS disease course. SPMS is a phenotype characterized by progressive clinical disability between relapses and acute attacks, without defined periods of remission (Compston and Coles 2008; Lublin and Reingold 1996). Approximately 10-15 % of MS patients show progressive disability after disease onset. This group of patients has PPMS. PPMS is characterized by late onset and progressive clinical worsening, which takes months to years (Miller and Leary 2007).

Devic's neuromyelitis optica, Marburg's acute MS, Balo's concentric sclerosis, and Schilder's diffuse sclerosis are atypical forms of MS (Stadelmann and Brück 2004).

1.1.2 Diagnosis

A combination of clinical evaluation, radiological and laboratory data makes it possible to diagnose MS. Many criteria have been used in the past. The latest ones are the McDonald criteria. The first description of the McDonald criteria was in 2001. In the following years, they were reviewed three times (McDonald et al. 2001; Polman et al. 2005; Polman et al. 2011; Thompson et al. 2018) and are all based on clinical, radiological and laboratory findings at varying locations and times. The latest review of the McDonald criteria from 2017 (Thompson et al. 2018) suggests that the presence of CSF-specific oligoclonal bands demonstrates dissemination in time and allows, together with MRI findings, to diagnose MS at very early stages of the disease.

Table 1: McDonald criteria (Thompson et al. 2018)

Clinical presentation	Additional data needed
≥ 2 clinical attacks (relapses) and objective clinical evidence of ≥ 2 lesions	None
≥ 2 clinical attacks (relapses) and objective clinical evidence of 1 lesion	Dissemination in space , demonstrated by: an additional clinical attack, implicating a different CNS side or MRI
1 clinical attack (relapse) and objective clinical evidence of 2 lesions	Dissemination in time , demonstrated by: an additional clinical attack or MRI <i>or CSF specific oligoclonal bands</i>
1 clinical relapse and objective clinical evidence of 1 lesion	Dissemination in space , demonstrated by: an additional clinical attack implicating a different CNS side or MRI Dissemination in time , demonstrated by: an additional clinical attack or MRI <i>or CSF specific oligoclonal bands</i>

1.1.3 Pathogenesis

It is evident that the immune system plays a central role in MS. Studies suggest that microglia and macrophages are the immune cells that play a dominant role in the pathogenesis of MS.

Due to histopathological investigations, the mononuclear phagocytes, including microglia and macrophages, are the dominant immune cells in MS lesions (Hemmer et al. 2015). Investigations on animal models of experimental autoimmune encephalomyelitis (EAE) suggest that phagocytes contribute to both demyelination and remyelination (Shechter et al. 2013).

Several lines of evidence indicate that the adaptive immune system plays an important role in the immunology of MS. There are two major hypotheses in MS pathology during the early inflammatory phase (Hemmer et al. 2015).

The first one suggests that the initiating event occurs first in the central nervous system (CNS), causing the activation of microglia, which subsequently results in secondary recruitment of adaptive and innate immune cells. In 2009, Henderson and colleagues showed that there were no T or B cells in areas of initial oligodendrocyte loss, but rather phagocytes with incorporated myelin proteins. In contrast, the areas of complete demyelination were filled with B and T cells as well as IgG-positive plasma cells (Henderson et al. 2009).

The second hypothesis suggests that the antigen-specific immune activation occurs first in the periphery. Then, the activated immune cells cross the blood brain barrier (BBB) and transmigrate into the CNS. The experiments in EAE showed that transferred encephalitogenic T cells were first found in cervical lymph nodes followed by infiltration and reactivation within the CNS (Lassmann 2013; Sospedra and Martin 2005).

It has been considered that MS is a T cell-mediated autoimmune disease. Investigations showing the possibility to induce EAE by transferring encephalitogenic T cells in naïve animals support this consideration (Constantinescu and Gran 2014). CD4⁺ T cells were mostly found in acute lesions, while CD8⁺ T cells were predominantly present in chronic lesions. “Molecular mimicry” is the most popular theory of the pathogenesis of MS. According to this theory, the CD4⁺ T cells are activated by foreign antigen and cross-react with myelin antigens. Zhang and Raus (Zhang and Raus 1994) showed that activated myelin-reactive CD4⁺ T cells were found in both blood and CSF of MS patients; in contrast, nonactive CD4⁺ T cells were found only in blood of controls. The most accepted theory suggests that myelin-specific CD4⁺ T cells could be activated by certain antigens that show a molecular mimicry to CNS components. There are several examples showing the cross-reactivity of CD4⁺ T cells with a myelin basic protein (MBP) such as Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), coronavirus (Chitnis 2007).

Several lines of evidence demonstrate also an important role for B- and plasma cells in the pathogenesis of MS, which will be discussed in more detail in chapter 1.1.5.

Immunological mechanisms during the progressive phase of MS are also unclear. Researchers discuss actually two possible mechanisms called “Inside-Out” and “Outside-In”.

Investigators believing in the Outside-In model, suggest that the primary target in MS is myelin (outside) and that the axonal (inside) lesions occur secondary (Henderson et al. 2009). Myelin/Oligodendrocytes may be damaged by virus infections, macrophages, cytotoxic T cells and autoantibodies etc.

The Inside-Out theory suggests that the initial damage occurs in axons, triggering a secondary demyelination. Investigations on spinal cord sections showed that axonal damage may lead to oligodendrocyte apoptosis, causing a demyelination (Tsunoda and Fujinami 2002).

1.1.4 Pathology of MS lesions

There are three major effects, which can be seen in MS lesions: myelin loss or demyelination, axonal damage or neurodegeneration, and proliferation of astrocytes, which leads to gliosis. Additionally, in the early stages of the disease, a process of remyelination takes place (Lassmann 2013). MS has always been considered to be a white matter disease, but recent investigations also show demyelinated lesions in the cortical grey matter and normal-appearing white matter (NAWM) (Frischer et al. 2009).

White matter lesions are frequently observed in MS. There are different types of white matter lesions: slowly expanding lesions, classical active lesions, inactivated lesions and remyelinated shadow plaques. Active lesions contain lymphocytic inflammation and massive macrophage infiltration. The progressive stage of MS is characterized by slowly expanded lesions with an inactive core surrounded by infiltrations of macrophages and activated glia. Lesions without microglia activation and macrophage activity are called inactive plaques. Their number increases with the age of patients and disease duration (Lassmann 2013). It was also described that sometimes oligodendrocytes may reappear within the active lesions in the early stages of MS. As a result completely remyelinated plaques, the so-called shadow plaques may appear (Patrikios et al. 2006).

Cortical lesions were described in all stages of MS; however, they are present more frequently in the progressive stage. There are three types of cortical lesions: cortico-subcortical, intra-cortical and subpial lesions (Lassmann 2008). All types of cortical lesions were associated with active inflammation by T-cell infiltrates on the early stages of the disease. However, meningeal inflammation was described in cortical lesions in the progressive stage of MS (Fischer et al. 2013). In 2008, Moll and colleagues compared cortical lesions in progressive multifocal leukoencephalopathy (PML) and MS and concluded that subpial cortical lesions were highly specific for multiple sclerosis pathology (Moll et al. 2008).

In the progressive stage of MS new inflammatory lesions in the white matter are rare, but several changes in NAWM were described. It has been considered that these changes occur due to diffuse axonal injury, which leads to Wallerian degeneration (Kutzelnigg et al. 2005).

Lucchinetti and colleagues suggested that the mechanisms of demyelination may differ between MS patients and classified the MS lesions into four patterns (Lucchinetti et al. 2000; Popescu et al. 2013):

The Pattern I was found in approximately 15 % of biopsied MS patients and characterized by T cell and macrophage activation as well as infiltration with active demyelination. However, a high incidence of remyelination could also be noticed.

Pattern II is the most common pattern of MS lesions. This type of lesions is similar to the pattern I with the additional presence of plasma cells and complement deposits.

Pattern III is characterized by the apoptosis of oligodendrocytes and weak T cell infiltrations. There is no evidence of the presence of complement deposits or plasma cells in this type of lesions.

Pattern IV is the rarest type of MS lesions and considered to occur due to metabolic oligodendrocyte injury. In these lesions, primary degeneration of oligodendrocytes plays an important role.

1.1.5 B cells in multiple sclerosis

Although MS has historically been considered to be a T cell-associated autoimmune disease, investigations of recent years suggest that there is an important role of B cells in the pathogenesis of MS. There are three major roles of B cells: antibody production, antigen presentation and cytokine production (Dalakas 2008). Ectopic follicles were found in the meninges of patients with secondary-progressive multiple sclerosis. It could be shown that these ectopic follicles were enriched with B cells and plasma cells (Serafini et al. 2004). Mathey and colleagues found that patients with progressive MS had an increased concentration of neurofascin, which is a neuronal protein found at the nodes of Ranvier. Injecting anti-neurofascin antibodies into mice with EAE caused a complement activation, which led to axonal conduction (Franciotta et al. 2008). Furthermore, oligoclonal bands, which are immunoglobulins, were found in the CSF of 90 % MS patients throughout disease stages (Michel et al. 2015). Antibodies derived from the CSF of MS patients were able to cause a complement-mediated CNS injury through demyelination and astrocyte dysfunction in spinal cord samples of both mouse and human models (Blauth et al. 2015). Pattern II lesions, which are the most frequent lesion subtype found in MS patients, were first described by Lucchinetti and colleagues. This subtype of lesions shows immunoglobulin and complement depositions (Popescu et al. 2013). All these findings, mentioned above, confirm the important role of antibody production by plasma cells in the pathogenesis of MS. However, studies show that anti-CD20 therapy reduces the activity of relapsing MS, while the level of CSF antibodies seems to persist (Piccio et al. 2010). This therapeutic effect leads to the consideration that B cell depletion reduces new MS relapses through antibody-independent roles of B cells (Michel et al. 2015). These findings lead to more intensive investigations on the other roles of B cells, such as antigen presentation and cytokine

production. Some investigators consider that plasma cells and autoantibodies may play a more important role at the early stages of MS, as compared to later stages of the disease (Michel et al. 2015).

B cells may act as antigen-presenting cells (APC) via the activation of T cells. They initially recognize antigens with their surface B cells receptor (BCR) and present them to T cells (Pöllinger et al. 2009). There is a theory that B cells may drain from the CNS into the lymph nodes, then presenting CNS antigens to T cells, which leads to T cell activation and mediates relapses (Michel et al. 2015). B cell activation requires two major signals. The first one produced by antigen through BCR cross-linking and the second one from CD4⁺ T cells through the interaction of CD154 with the B cell surface receptor CD40. This interaction leads to B cell growth, proliferation, and production of costimulatory factors such as CD80 and CD86. Rodriguez-Pinto et al. showed in vivo that B cells can prime naïve CD4⁺ T cells in a CD154-CD40 dependent manner in the absence of other APC (Rodríguez-Pinto and Moreno 2005).

The third major function of B cells is the production of cytokines. Activated B cells can regulate inflammation through the secretion of pro-inflammatory and anti-inflammatory cytokines. Some B cells can secrete proinflammatory cytokines such as TNF, IL-6, GM-CSF, and Lymphotoxin-alpha, while other B cells play an anti-inflammatory role through secretion of IL-10 and IL-35. Investigations of recent years show that B cells from MS patients produce more pro-inflammatory- rather than anti-inflammatory cytokines (Bar-Or et al. 2010; Michel et al. 2015).

All these facts mentioned above, indicate an important role of B cells in the pathophysiology of MS. However, the strongest evidence of a crucial role of B cells in MS is the astonishing effect of anti-CD20 therapy. Investigators believe that the efficiency of this therapy occurs due to the loss of B cell cytokine-producing and antigen-presenting functions (Hauser et al. 2008).

1.1.6 Therapy of multiple sclerosis

The MS therapies can be divided into the following groups: treatment of acute attacks/relapses, treatment to prevent new relapses (disease-modifying therapy DMT), and symptomatic therapy.

New or worsening neurological symptoms lasting 24 hours or more in the absence of infection or fever are defined as MS relapses (Berkovich 2016). The therapy of acute relapses is an intravenous application of high dosed Methylprednisolone (common dosage is 1000 mg/day) for 3-5 days (Compston and Coles 2008). In case of unresponsiveness to the glucocorticoid therapy, plasma exchange should be considered (Ehler et al. 2017).

Disease-modifying therapies, such as glatiramer acetate or beta-interferons, were first introduced in the 1990s. They established safety and efficacy, although they had to be

administered via injections (Cross and Naismith 2014). The following drugs can be used as basic therapy in patients with low disease activity: interferon-beta (IFN- β), glatiramer acetate (GA), teriflunomide (TERI), dimethyl fumarate (DMF).

In contrast to GA or IFN- β , DMF and TERI can be administered orally. These drugs show a high efficacy and safety profile and are generally well tolerated by patients (Cross and Naismith 2014). Since IFN- β and GA require an administration via injections, they can cause skin reactions and flu-like symptoms (O'Connor et al. 2009). DMF is known to lead to adverse events such as gastrointestinal problems and flush. Teriflunomide should not be used within two years before pregnancy; because it is teratogenic. It is evident that all of these drugs reduce rates of MS relapses and decrease the rate of MS lesions (O'Connor et al. 2009; Fox et al. 2012; Kieseier et al. 2015; Ontaneda et al. 2018).

If there are MS relapses, new MRI lesions or a disability progress under the basic therapy, a so-called escalation therapy should be chosen. The drugs, shown below, are appropriate for the treatment of patients with disease activity: fingolimod, natalizumab, alemtuzumab, siponimod, cladribine.

These therapeutics are very effective but are not as safe as basic therapies. Fingolimod may cause bradyarrhythmia, herpes infection or liver injury (Akbulak et al. 2018). Therapy with Alemtuzumab can lead to thyroid-disorders, idiopathic thrombocytopenic purpura (ITP), and Good pasture syndrome. It has been considered that Alemtuzumab may cause a secondary auto-immunity in MS (Biernacki et al. 2017). Siponimod belongs to the sphingosine-1-phosphate (SIP) modulatory family such as Fingolimod. However, there is evidence that Siponimod-treated patients had a lower risk of adverse events such as bradycardia or vasoconstriction (Gajofatto 2017). Natalizumab is a selective adhesion-molecule inhibitor. It may lead to serious opportunistic infections, such as progressive multifocal leukoencephalopathy (PML) (Pardo and Jones 2017). It is known that patients having antibodies against the John Cunningham virus (JC virus) are associated with a higher PML risk (Zingaropoli et al. 2018). To estimate the individual risk of PML, antibodies against the JC-Virus should be measured before and during the therapy with Natalizumab (Ho et al. 2017). Cladribine selectively depletes lymphocytes. The major cladribine-associated adverse event is a herpes infection. Several phase III studies showed the efficacy of these drugs on reducing the disability progression, relapse rate, and MRI lesions (Grand'Maison et al. 2018; Pardo and Jones 2017).

Alternative escalation therapies include the following drugs: ocrelizumab, mitoxantrone.

There are only a few therapy options for the progressive forms of MS, SPMS and PPMS. Ocrelizumab is a monoclonal antibody, which selectively depletes CD20+ B cells. It was first approved by the US Food and Drug Administration (FDA) in 2017 (McGinley et al. 2017). Infections and infusion-related reactions are the most common adverse events associated with Ocrelizumab. Several studies demonstrated high efficacy of this therapy in reducing MS

relapses, disability progression and MRI lesions (Montalban et al. 2017; Gelfand et al. 2017). Mitoxantrone is approved for its use in aggressive forms of MS without superimposed relapses. This drug may cause AML (Acute myeloid leukemia) or cardiac problems (Edan 2004; Buttmann 2018).

Neurorehabilitation is very important in the treatment of MS-associated symptoms. Such MS-related symptoms as fatigue, depression, and spasticity can be improved by cognitive behavioral therapy and various psychotherapeutic measures, such as individualized physiotherapy (Kesselring and Beer 2005; Veauthier and Paul 2016). Although there are several oral antispasticity drugs used in Germany, such as baclofen, tizanidine or sirdalud, the effectiveness of these drugs is low and they do not affect the disease progression (Otero-Romero et al. 2016). Cannabidiol oromucosal spray was approved for the management of severe MS-related spasticity (Vermersch and Trojano 2016).

1.2 Glatiramer acetate

Glatiramer acetate or Copolymer 1 is a synthetic mixture composed of L-lysine, L-glutamic acid, L-alanine, and L-tyrosine, which are found in myelin basic protein (Sela and Teitelbaum 2001). It has been considered that myelin basic protein is the antigen causing an autoimmune reaction in MS and glatiramer acetate may play a role as a decoy for the immune cells. In 1996, Arnon et al. first showed that therapy with glatiramer acetate suppressed EAE in primates (Arnon 1996). Some successful clinical trials demonstrated that GA reduces relapse rate and progression of neurological disability so that in 1996 it was approved by the Food and Drug Administration under the brand name Copaxone for treatment of relapsing-remitting MS (Johnson et al. 1995). Copaxone is usually administered by subcutaneous injections. Side effects of GA therapy are usually mild. Approximately 30 % of patients develop injection-site reactions and about 10 % of users may have a fever and flu-like symptoms (O'Connor et al. 2009; Stewart and Tran 2012). Subcutaneous administration of 20 mg/mL once a day is approved in many countries to treat MS patients with a relapsing-remitting course. However, investigations of recent years showed that a high-concentration of glatiramer acetate 40 mg/mL administered three times a week reduced about 50 % of injection-site reactions compared to daily GA 20 mg/mL administration. It is important to mention that the efficacy of the three-times-weekly regimen is similar to that of the 20 mg/mL once-daily regimen (McKeage 2015). Several placebo-controlled studies showed that GA therapy delayed the conversion of clinically isolated syndrome (CIS) to clinically definite MS (Comi et al. 2013; Qizilbash et al. 2012).

1.2.1 Mechanism of action of GA

The precise mechanism of action of GA is still unknown. The therapeutic effect of glatiramer acetate is thought to be via neuroprotection and immunomodulation, for review see (McKeage 2015). Investigations suggest that GA treatment may affect T cells and B cells.

Glatiramer acetate has historically been thought to be a T-cell targeting therapy. It was thought that the beneficial effect of this therapy occurred due to a shift of the T-cell balance from a pro-inflammatory phenotype (Th1/Th17) to an anti-inflammatory one (Th2/T regulatory cells) (Bakshi et al. 2013; Basile et al. 2006). Some investigators believe that the mechanism of glatiramer acetate is its cross-reaction with myelin basic protein and bystander suppression of T cells (Yong 2002; Haas et al. 2009). Bystander suppression means that GA-specific T cells cross the blood-brain-barrier and secrete anti-inflammatory (Th2-specific) cytokines, inhibiting Th1 T cells (Aharoni et al. 1998). *In vitro* studies have shown that under GA therapy T cell lines secreted anti-inflammatory cytokines such as IL-10, IL-4, and TGF- β . However, the level of pro-inflammatory cytokines secreted by T cells such as TNF α and IFN- γ was decreased (Basile et al. 2006). In addition, it was demonstrated that GA-specific T cells can secrete brain-derived neurotrophic factor (BDNF) (Ziemssen et al. 2017). Similar findings could be demonstrated in the brains of GA-treated mice for neurotrophic factors such as neurotrophin NT3 and NT4, see review (Aharoni 2013). Thus, BDNF, NT3, and NT4 are important for neuronal survival (Lessmann et al. 2003), these findings show that GA may have some neuroprotective effects in patients with MS (Basile et al. 2006; Ziemssen et al. 2017). Regulatory T cells (anti-inflammatory phenotype of T cells) are functionally impaired in MS patients. Several studies showed that GA therapy restores the number and functionality of regulatory T cells, which seems to have an additional anti-inflammatory effect (Putheti 2003; Haas et al. 2009).

Monocytes and macrophages play an important role in MS pathogenesis. They are believed to be responsible for tissue damage. They dominate in active MS lesions and myelin degradation products can be found inside macrophages (Lassmann et al. 2001; Sospedra and Martin 2005).

Several lines of evidence show that GA may affect the T-cell-stimulating properties of antigen-presenting cells (APC). Thus, using *in vitro* cultures have shown that dendritic cells (DCs), which are professional antigen-presenting cells, had an impaired capacity to secrete Th1-polarizing factors after the treatment with glatiramer acetate and therefore induced more T cells of the Th2 phenotype (Hussien et al. 2001; Vieira et al. 2003). Monocytes are a major subset of APCs, which can easily be obtained from peripheral blood. In 2004, Weber et al. demonstrated for the first time both *in vitro* and *in vivo* that GA inhibits monocyte reactivity (Weber et al. 2004). It was also shown that GA had an inhibitory effect on TNF- α production by monocytes (Li et al. 1998). All these findings demonstrate that GA treatment leads to an additional anti-inflammatory effect through impairment of monocytes.

Once B cells cross the blood-brain barrier (BBB), factors such as BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand), which are produced by astrocytes, play an essential role in their survival (Dooley et al. 2016). Thus, the presence of these two factors in MS lesions was associated with increased B cell survival rates (Avery et al. 2003; Krumbholz et al. 2005), leading to EAE and worsening of MS course (Krumbholz et al.

2005). Begum-Haque et al. demonstrated that GA treatment downregulates the expression of BAFF and APRIL, as well as the BAFF receptor in mice with EAE (Begum-Haque et al. 2011). Furthermore, it could be demonstrated that GA reduces the concentration of cell-bound adhesion molecules on immune cells, including B cells, such as molecule-3 on B cells (Sellner et al. 2013). This effect may reduce the extravasation of immune cells into the CNS.

Kala et al. showed reduced expression of CD80 and CD86 on B cells isolated from GA-treated EAE-diseased mice. CD80 and CD86 are known to stimulate the proliferation of autoreactive T cells (Kala et al. 2010).

Investigations show that B cells from MS patients lost their ability to produce the anti-inflammatory cytokine IL-10, but produce more pro-inflammatory cytokines such as IL-6 and lymphotoxin-alpha (LT- α) (Bar-Or et al. 2010). Several investigations on GA-treated EAE mice models could demonstrate that secretion of IL-10 by B cells was increased. Inflammatory cytokines such as IL-6 were reduced (Kala et al. 2010; Begum-Haque et al. 2011). Ireland et al. however, demonstrated that GA therapy may diminish the IL-6 and TNF- α production by B cells only transiently (Ireland et al. 2012). IL-10 inhibits the differentiation of naïve T cells into Th1 T cells and antigen-specific T cell proliferation (Kala et al. 2010). Another anti-inflammatory cytokine, which is produced both by B cells and regulatory T cells, is IL-13. Begum-Haque and colleagues demonstrated that B cells of GA-treated EAE mice also tended to produce more IL-13 (Begum-Haque et al. 2011).

In vitro studies showed that GA binds MHC class II molecules, displacing other peptides from the MHC binding groove, which may prevent or reduce antigen presentation to T-cells and T cells activation (Fridkis-Hareli et al. 1994).

Several investigations demonstrated that almost all patients treated with glatiramer acetate developed anti-GA (IgG) antibodies over time (Brenner et al. 2001; Teitelbaum et al. 2003; Begum-Haque et al. 2011). These antibodies did not correlate with adverse events or efficacy of GA treatment and had no neutralizing effect (Brenner et al. 2001). Teitelbaum et al. showed that anti-GA antibodies could even be detected after 2 years of GA treatment (Teitelbaum et al. 2003). The antibodies peaked at 3 months after the therapy was initiated, decreased at 6 months and stayed low (Karussis et al. 2010). Isotype and IgG subclass analysis showed that the significant GA-reactive antibodies are of the IgG4 subclass (Farina et al. 2002; Basile et al. 2006). This finding is important because IgG4 antibodies are associated with a shift to Th2 responses, as anti-inflammatory cytokines regulate isotype switching to IgG4 in plasma cells (Bomprezzi et al. 2011).

1.3 Aims of the study

As mentioned above, the effects of GA are still not fully understood. A 15-year follow-up study compared patients who continued with GA therapy to patients, who dropped out of the trial. It could be demonstrated that patients who continued the GA therapy had reduced

relapse rates and decreased disability progression as compared to patients, who did not continue GA therapy (Ford et al. 2010).

It is unclear how GA affects antigen-presenting functions and cytokine production of both B cells and monocytes. Additionally, the effect on B cell activation is poorly investigated (Kala et al. 2010).

1.3.1 Blood cell distribution

It is known that GA reduces the total frequency of B cells and monocytes (Weber et al. 2004; Ireland et al. 2012). The current study aimed to re-evaluate the changes mentioned above and to analyze relative changes in the remaining lymphocyte pool. The peripheral blood mononuclear cells (PBMC's) of GA-treated and untreated patients were categorized by their surface molecules as follows:

Table 2: Blood cell distribution

B cells	CD19 ⁺
T-helper cells	CD4 ⁺
Cytotoxic T cells	CD8 ⁺
Monocytes	CD14 ⁺

1.3.2 Cell survival

In order to determine the fraction of necrotic and apoptotic cells, the cell status was defined as follows:

Table 3: Cell survival

Necrotic	Zombie ⁺
Apoptotic	size exclusion

1.3.3 B cell subsets

Several investigations show that GA therapy reduces the total frequency of B cells, plasmablasts and memory B cells (Ireland et al. 2014). The aim of this study was to determine B cell subsets and changes in their frequency in GA patients compared to untreated controls. Characterization of the B cell subsets can be performed through analysis of their surface molecules as follows:

Table 4: B cell subsets

Trans-B-cells	CD24 ^{high} , CD27 ^{neg} , CD38 ^{high}
Mature B cells	CD24 ^{high} , CD38 ^{low}
Activated B cells	CD27 ^{pos}
Memory B cells	CD27 ^{var} , CD38 ^{neg}
Plasmablasts	CD20 ^{neg} , CD27 ^{high} , CD38 ^{high}

1.3.4 Activation markers on B cells

It is very important to differentiate B cells through their activation markers. Thus, in this context it is easier to understand the pathogenic antigen-presenting, antibody-secreting or cytokine-producing role of B cells. In this study following surface markers were used: CD25 (alpha chain of IL-2 receptor), expressed on activated mononuclear immune cells, which may stimulate through IL-2 T cell growth and effector functions (Malek 2008); CD40 (CD40L receptor), which is a costimulatory molecule, found on APCs and is required for their activation (Teitelbaum et al. 2003); CD69 (Early activation marker), a transmembrane protein, which is involved in lymphocyte migration and-activation (Vazquez et al. 2009); CD80 (Lymphocyte activating antigen B7-1), a protein found on monocytes, activated B cells and dendritic cells, which plays an important role in T cell activation and survival (Aung und Balashov 2015); CD86 (Lymphocyte activating antigen B7-2), a protein expressed on APCs, which plays a crucial role in T cell activation and survival (Aung und Balashov 2015); MHC II, usually found on APCs,-plays an important role in the initiation of immune responses; CD95 (Apoptosis antigen 1; Fas receptor), a protein, which plays an important role immune cells apoptosis.

As mentioned above, the antigen-presenting functions of B cells and monocytes/macrophages seem to play an important role in the pathogenesis of MS (Teitelbaum et al. 2003; Aharoni 2013). To quantify the antigen-presenting function, the expression of MHC II and co-stimulatory molecules (CD80, CD86, CD40) was measured on B cells and monocytes in peripheral blood.

1.3.5 Cytokine production

As mentioned above, cytokine production by immune cells plays an important role in MS pathogenesis. Glatiramer acetate has been considered to stimulate the production of anti-inflammatory cytokines and reduce the secretion of pro-inflammatory ones (Begum-Haque

et al. 2010). In order to quantify the cytokines production of B cells and monocytes, three major cytokines as IL-6, IL-10 and TNF- α were measured in this study: Interleukin 6 (IL-6) plays a crucial role in the pathogenesis of neuroinflammatory diseases. Investigations in a murine model of MS, (EAE), showed complete resistance to disease induction in mice lacking IL-6, see for review (Rothaug et al. 2016). Furthermore, IL-6-deficiency impaired Th17 differentiation in a defective manner (Hernandez et al. 2015). These findings show that IL-6 is a pro-inflammatory cytokine. Interleukin 10 (IL-10) is an anti-inflammatory cytokine, predominantly produced by myeloid cells and lymphocytes (Moore et al. 2001). IL-10 suppresses the expression of pro-inflammatory cytokines, costimulatory factors on APCs and T effector functions (Ireland et al. 2015). Tumor necrosis factor alpha (TNF- α) is a pro-inflammatory cytokine, produced by macrophages, but also by lymphocytes, neurons, and other cells. TNF- α has similar effects on immune cells as IL-6. TNF- α is involved in inflammation and apoptosis and stimulates phagocytosis of macrophages (Locksley et al. 2001).

2 Material and Methods

2.1 Patients

The blood samples were drawn from patients of the Göttingen University Medical Center in Germany between 2015 and 2018 with confirmed RRMS. The diagnosis of RRMS was based on the McDonald criteria. The 22 MS patients treated with GA were compared to 18 untreated MS patients. Treated patients were taking GA for at least three months when the blood samples were drawn. Six patients treated with GA were analyzed longitudinally, meaning that blood samples were taken at two different time points with an interval of at least three months. The patients did not receive any kind of immunotherapy before GA. Additionally, a sample from a healthy person was used as an internal control.

Table 5: The disease and demographic characteristics of the patients

	Untreated	GA-treated
Number of patients	18	22
Age (y) (mean \pm SD)	32.4 \pm 9.7	42 \pm 9.4
Female sex (%)	77.7 %	54.5 %
EDSS score (mean \pm SD)	2.1 \pm 1.7	2 \pm 1.4
Disease duration (y) (mean \pm SD)	4.7 \pm 6.2	7.4 \pm 6.1
GA since (m) (mean \pm SD)	-	5.9 \pm 4.1

2.2 Materials

Table 6: Human monoclonal antibodies for flow cytometry

Antigen	Fluorochrome	Clone	Dilution	Manufacturing company
CD4	PE-Cy7	RPA-T4	1:100	BD Biosciences
CD8	PE	HIT-8a	1:100	eBioscience
CD14	BV421	M5E2	1:100	BD Biosciences
CD14	PE-CF594	M ϕ P9	1:100	BD Biosciences
CD19	APC	HIB19	1:100	BD Biosciences
CD19	FITC	HIB19	1:100	BD Biosciences
CD19	PerCp-Cy5.5	HIB19	1:100	BioLegend
CD19	PE-Cy5	HIB19	1:100	BD Biosciences
CD20	APC-Cy7	L27	1:100	BD Biosciences
CD24	PerCp-Cy5.5	ML5	1:100	BioLegend
CD25	BV605	BC96	1:100	BioLegend
CD27	PacificBlue	O323	1:100	BioLegend
CD38	FITC	HIT2	1:100	BioLegend
CD40	PE/Dazzle	5C3	1:100	BioLegend
CD69	FITC	FN50	1:100	BioLegend
CD80	PE-Cy7	L307.4	1:100	BD Biosciences
CD86	BV421	FUN-1	1:100	BD Biosciences
CD95	PE	DX2	1:100	BioLegend
CD150	BV421	A12	1:100	BD Biosciences
MHC II	APC	Tü36	1:100	BioLegend
IL-6	FITC	MQ2-13A5	1:50	BD Biosciences
IL-10	PE-CF594	JES3-19F1	1:50	BD Biosciences
TNF	A700	MAb11	1:50	BD Biosciences
Dead/Live	ZombieAqua™		1:100	BioLegend
Dead/Live	ZombieNIR™		1:100	BioLegend

Table 7: Reagents, protein and inhibitors

Product	Manufacturing company
BD FACS Clean™	BD Biosciences
BD FACS Flow™	BD Biosciences
BD FACS Rinse™	BD Biosciences
BioColl separation solution	Biochrom
CpG oligodeoxynucleotides	Sigma Aldrich
Cytofix/Cytoperm™	BD Biosciences
DMEM (Dulbecco's Modified Eagle's medium)	Sigma Aldrich
DMSO (Dimethyl-sulfoxide)	Sigma Aldrich
EDTA (Ethylene diamine tetraacetic acid disodium salt dihydrate)	Carl Roth
Ethanol 100 %	Merck Millipore
Fc-block™	BioLegend
FCS (fetal calf serum)	Sigma Aldrich
GolgiPlug™	BD Biosciences
Ionomycin	Sigma Aldrich
LPS (lipopolysaccharide)	Sigma Aldrich
PBS (phosphate buffered salt solution)	Sigma Aldrich
Perm/Wash™ buffer, 10x	BD Biosciences
PFA (para-formaldehyde)	Merck Millipore
PMA (Phorbol 12-Myristate 13 Acetate)	Sigma Aldrich
RPMI-1640 (Roswell park memorial institute-1640)	Sigma Aldrich
Sodium pyruvate 100mM	Sigma Aldrich
TMB (tetramethylbenzidine)	eBioscience
TrypanBlue	SigmaAldrich
β-mercaptoethanol	Sigma Aldrich

Table 8: Solutions, buffers and cell culture media

Solution	Composition
Cryo-medium	60 % RPMIcomplete, 20 % FCS, 20 % DMSO
ELISA blocking buffer	1.8 l distilled water, 200 ml PBS 10x, 20 g BSA
ELISA coating buffer	1 l distilled water, 8.4 g NaHCO ₃ , 3.5 g NaCO ₃
ELISA stop solution	1 M H ₂ SO ₄ solution
ELISA wash buffer	1.8 l distilled water, 200 ml PBS 10x, 1 ml Tween
FACS buffer	2 % FCS in PBS
RPMIcomplete	0.5 l RPMI-1640, 50 ml FCS, 5 ml sodium pyruvate, 5 ml L-glutamine, 0.5 ml β-mercaptoethanol

Table 9: Consumables

Product	Manufacturing company
96 well plates, flat bottom	Sarstedt
96 well plates, round bottom	Sarstedt
FACS tubes, 5ml	Sarstedt
Micro tubes (0.1ml, 0.5ml, 1ml, 1.5ml)	Eppendorf
Multistep pipettes, 1ml	Eppendorf
Nunc™ 96 well ELISA plates	ThermoScientific
Pipettes (10μl, 200μl, 1000μl)	Sarstedt
Pipettes (5ml, 10ml, 25ml)	Sarstedt

Table 10: Technical devices

Device	Manufacturing company
BBD 6220 cell incubator	ThermoScientific
Centrifuge 5415R	Eppendorf
Centrifuge 5810R	Eppendorf
CKX41 light microscope	Olympus
FACS LSRII Fortessa	BD Biosciences
IMARK™ microplate reader	Bio-Rad
Neubauer chamber	Superior Marienfeld
SAFE 2020 clean bench	ThermoScientific

Table 11: Software

Software	Company
Microplate Manager 6™	Bio-Rad
FACSdiva™ 6.1.2	BD biosciences
FlowJo™ 10.2	Tree Star Inc.
GraphPad Prism™ 6.01	GraphPad software Inc.
Excel	Microsoft Office 2016

2.3 Methods

All steps of PBMC isolation and cryopreservation were done according to the protocol, which was established in this laboratory.

2.3.1 Preparation of human peripheral blood mononuclear cells (PBMC)

The blood samples were drawn from the patients into EDTA tubes. Then, the blood was filled into a 50 ml tube and diluted with PBS. In the next step, the blood suspension was layered very carefully and slowly onto 20 ml of BioColl™ in another 50 ml tube. After a 35-minute centrifugation at 1500 revolutions per minute (rpm) and 21 °C with low acceleration and brake, the „ring” (the white layer), representing the peripheral blood mononuclear cells, was withdrawn, suspended in a new tube, and diluted with DMEM. After a ten-minute

centrifugation at 1250 rpm and 4 °C, the cell pellet was washed a second time with DMEM. The next step was to count the cells, solved in 5 ml DMEM. For this purpose, the cell suspension was diluted with DMEM to a concentration of $4 \times 10^6 \text{ ml}^{-1}$. Each cryo tube was filled with 500 μl of the cell suspension and 500 μl cryo-medium. A total cell count of 2 million cells per tube was achieved this way. The cryo tubes were stored at -80 °C in the ethanol cooling boxes until further steps of the experiments.

2.3.2 FACS (fluorescence-activated cell sorting) panels and stimulation regime

Different FACS panels and stimulation regimes were established to examine the aims of this study. The developed panels and stimulation regimes were tested and optimized before the patients' blood samples were used. The established FACS panels are shown below:

Table 12: FACS panels (1)

Cell distribution (b)	B cell subsets (a)	Cytokine production (d)
CD4 – PE-Cy7	CD19 – APC	CD14 – BV421
CD8 – PE	CD20 – APC-Cy7	CD19 – PE-Cy5
CD14 – PE-CF594	CD24 – PerCp-Cy5.5	IL-6 – FITC
CD19 – FITC	CD27 – PacificBlue	IL-10 – PE-CF594
CD150 – BV421	CD38 – FITC	TNF – A700
MHC II – APC	CD95 – PE	ZombieNIR
ZombieAqua	ZombieAqua	

Table 13: FACS panels (2)

Activation markers (c)	Antigen-presenting function (e)
CD19 – PerCp-Cy5.5	CD14 – FITC
CD25 – BV605	CD19 – PerCp-Cy5.5
CD40 – PE/Dazzle	CD40 – PE/Dazzle
CD69 – FITC	CD80 – PE-Cy7
CD80 – PE-Cy7	CD86 – BV421
CD86 – BV421	MHC II – APC
MHC II – APC	CD95 – PE
CD95 – PE	ZombieNIR
ZombieNIR	

In some panels, the cells were stimulated and incubated, because some activation markers and cytokines are only expressed or synthesized upon stimulation. For this purpose, the following substances were used: Lipopolysaccharide (LPS), a large molecule consisting of a lipid and a polysaccharide, found in the outer membrane of gram-negative bacteria. It is known that immune cells produce pro-inflammatory cytokines upon LPS binding to the TLR4 receptor; CPG (cytosine-phosphate-guanine), which is a dinucleotide and has a high abundance in microbial genomes. Due to this fact, CPG is recognized by immune cells as a pathogen-associated molecule. It promotes cytokine and antibody production. Ionomycin enhances the intracellular calcium level and stimulates the intracellular production of cytokines. It is an ionophore produced by the bacterium *Streptomyces globatus*; PMA or phorbol 12-myristate 13-acetate is commonly used together with Ionomycin to stimulate the intracellular cytokine production via a protein kinase C (PKC) pathway.

Table 14: Stimulation regimes

FACS Panel	Stimulation regime
Cell distribution (b)	No stimulation
B cell subsets (a)	No stimulation
Antigen-presenting function (e)	No stimulation
Cell distribution (b)	100 pg/ml LPS for 20 h
Activation markers (c)	2 µg/ml CpG for 20 h
Cytokine production (d)	1 µg/ml CpG for 20 h, then +GolgiPlug, after 2 h +500 ng/ml Ionomycin and 20 ng/ml PMA for 2 h

2.3.3 Unfreezing, cell counting and seeding

To unfreeze cells, the cell containing cryo-tubes had to be put in a 37 °C water quench for 30 seconds. Each of the frozen cryo-tubes contained two million cells and was stored at -80 °C. After thawing, the cell suspension was put into 40 ml RPMI. Each patient's sample was thawed in a different tube. The next step was centrifugation (1250 rpm, 4 °C, 10 minutes). After that the supernatant was discarded, and the sedimented cells were re-suspended in 1 ml RPMI for counting. For this purpose, a Neubauer chamber was used. In order to exclude dead cells, 10 µl cell suspension was diluted in 10 µl or 90 µl Trypan Blue (prediluted 1:10 in PBS), resulting in a dilution factor of 2 and 10, respectively. 10 µl of the mixture was given onto the counting chamber to count the cells. Hereby the following formula was used:

$$\text{Total cell count} = \frac{\text{Counted cells}}{4} * 10000 * \text{dil. fac.} * \text{volume of suspension [ml]}$$

The cell suspension was then diluted with RPMI to a concentration of $2 \cdot 10^6$ cells/ml. A total of 200'000 cells (100 µl of the prepared cell suspension) were plated per well. All patient samples were measured in duplicates. Additional cells were plated for single stainings. U-bottom 96-well plates were used for unstimulated panels. The staining of those samples was done on the same day, as shown below.

For stimulated panels flat bottom 96-well plates were used. 100 µl of CpG and LPS were added to the cell suspension, according to the stimulation regimes. The stimulated plates were incubated at 37 °C and 5 % CO₂ for 20 h. Supernatants were frozen at -18 °C until further examination.

2.3.4 Live/Dead staining

In order to exclude dead- from living cells, a Zombie™ dye was used. This dye reacts with primary amine groups on proteins. In dead cells, where the cell membrane is not intact, the dye enters and binds all kinds of proteins within the cytoplasm. These cells appear brighter in Zombie™ fluorescence than living cells. In living cells, in which the membrane is intact, the dye can only bind to surface proteins.

After standard centrifugation (1250 rpm, 4 °C, 7 min) and re-suspension in 200 µl PBS, the second centrifugation took place. After the supernatant was discarded, the cells were resuspended in 30 µl of the pre-diluted Zombie dye (1:500 in PBS). This excluded non-Zombie single stainings, which were resuspended in PBS. Thereafter the plates were incubated for 10 min at room temperature in the dark. Then the cells were washed in 200 µl FACS buffer.

2.3.5 Surface staining

In order to avoid the non-specific binding of antibodies to Fc receptors on the cell surface, 30 µl Fc-block™ (pre-diluted 1:100 in FACS buffer) was added to the cells in each well. After an incubation of 10 minutes at 4 °C in the dark, the cells had to be washed with 170 µl FACS buffer. Thereafter standard centrifugation (1250 rpm, 4 °C, 7 min) took place. After draining the supernatant, a surface staining was performed. For this purpose, a mastermix of fluorophore-labeled antibodies diluted 1:100 in FACS buffer was assembled for each panel. The 30 µl of the resulting mastermix, containing all fluorophores for one panel, was added to each patient sample. Single antibodies, which were used for single stainings, were also diluted 1:100 in FACS buffer. After an incubation of 15 minutes at 4 °C in the dark, the cells were washed twice with FACS buffer (140 µl and 150 µl) and then resuspended in 100 µl FACS buffer. The cell suspension was given into 5 ml FACS tubes for analysis.

2.3.6 Intracellular staining

After 20 hours of CpG stimulation, 10 µl GolgiPlug™ (pre-diluted 1:10 in RPMI) per well was added to the cells leading to a blockage of the Golgi apparatus and the accumulation of synthesized proteins inside the cells. After two hours, 50 µl Ionomycin and 50 µl PMA were added to each well. This step resulted in an even higher production of cytokines. Because of the Golgi apparatus inhibition, these cytokines accumulated inside the cells. After 2 hours of incubation, the cells were centrifuged. 230 µl supernatant of each well was stored at -18 °C in 96-well plates until further examination. The cells were stained for cell death and surface molecules, as shown in 2.3.4 and 2.3.5.

After the surface staining, 100 μ l Fix/PermTM was added to fix the cells and increase cell permeability. After an incubation of 30 minutes at 4 °C in the dark, the cells were centrifuged. Thereafter the cells were washed with 200 μ l Perm/WashTM per well. Then 50 μ l of prepared intracellular antibody mix (pre-diluted 1:50 in Perm/WashTM) was put onto the cells. The cells were then incubated for 18 hours at 4 °C in the dark. Thereafter the cells were washed twice in 100 μ l Perm/WashTM and 200 μ l FACS buffer. Then the cell suspension was resuspended in 100 μ l FACS buffer and put into 5 ml FACS tubes.

2.3.7 Flow cytometry

The fluorescence-activated cell sorting (FACS) was used to analyze the fluorophore-labeled cells. For this purpose, the LSRII Fortessa FACS machine by BD Biosciences was used. The cell suspension was acquired to record a maximum of 100 000 events for each tube. The same procedure was repeated for each tube. The same compensation was applied for all runs to make the comparison between the different runs more valid. The obtained data was analyzed with the FlowJo 10.2 software.

2.3.8 Gating

The next step was to determine the percentage of B cells, according to their characteristic cell surface molecules (2.3.2). In addition, B cells were divided into subgroups according to the gating strategy, as shown below:

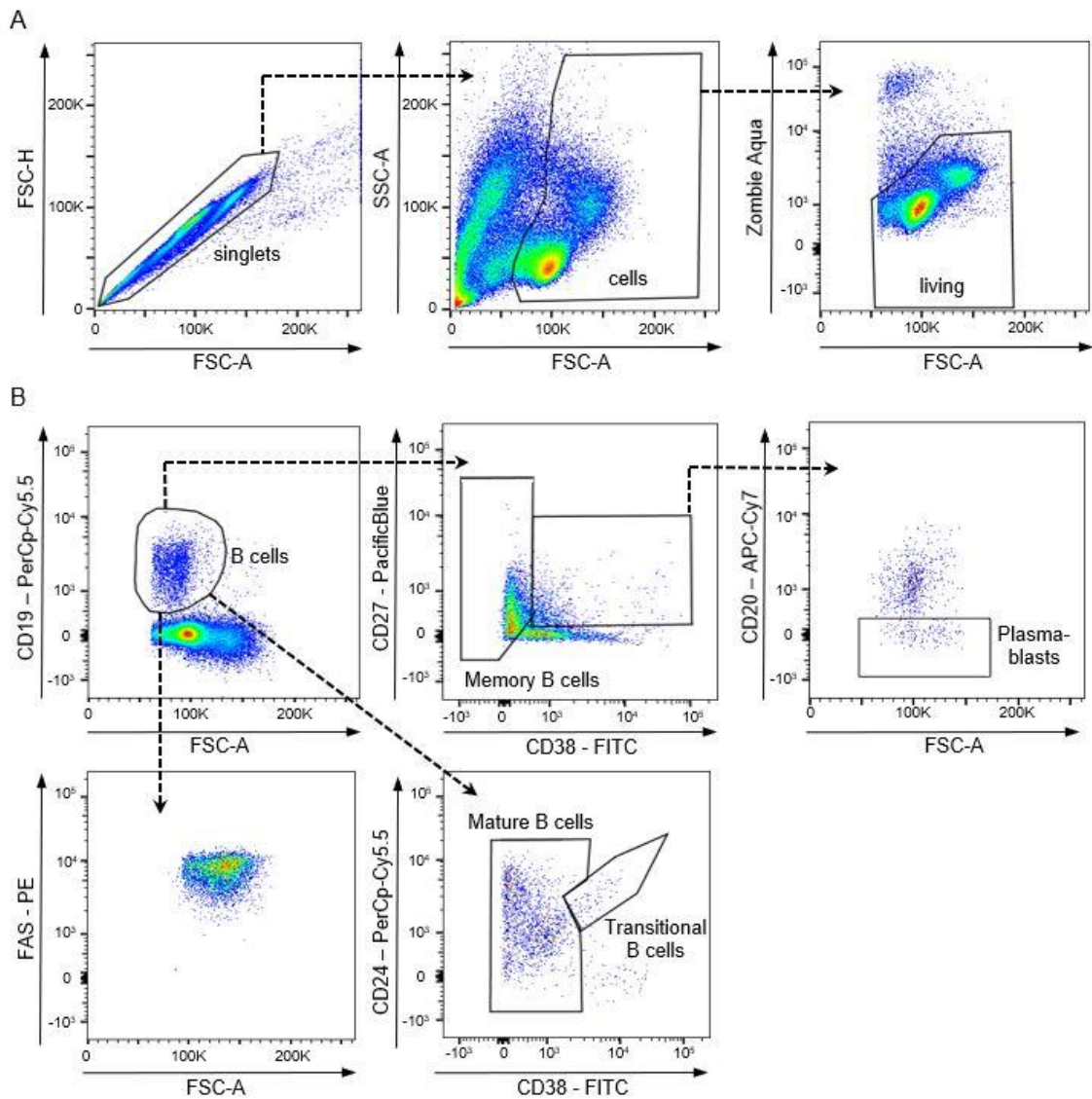


Figure 1: Pre-gating and gating strategy for B cell subsets and surface molecule expression.

(A) Singlets were gated to exclude doublets. Non-apoptotic cells were determined by size exclusion in FSC-A and SSC-A. Non-necrotic cells, meaning Zombie-negative cells were gated and defined as living cells. **(B)** The B cells gate was defined as CD19⁺, Memory B cells (CD27^{var}CD38^{neg}), Plasmablasts (CD27^{high}CD38^{high}CD20^{neg}), Mature B cells (CD24^{high}CD38^{low}), transitional B cells (CD24^{high}CD27^{neg} CD38^{high}).

2.3.9 Statistical analysis

The data of the patient groups were tested for Gauss distribution using the D'Agostino & Pearson omnibus normality test, the Shapiro-Wilk normality test, and the Kolmogorov-Smirnov normality test. For the comparison of two cross-sectional groups with Gauss distribution, an unpaired and for longitudinal samples, a paired t-test was used, respectively. If the data was not Gauss distributed, a Mann Whitney test was applied in the cross-sectional

analysis and the Wilcoxon matched-pairs signed-rank test was used for the longitudinal data. We also determined whether the cell distribution, B cell subsets, expression of surface markers, and cytokine production (variable 1) were correlated with GA-therapy duration (variable 2). For these purposes a Pearson's correlation coefficient, which is denoted by letter r , was determined. Pearson's correlation coefficient is the statistical association between two variables. R varies between -1 and 1. Zero means there is no correlation, 1 means a complete or perfect correlation, and a negative r means that the variables are negatively inversely related. When the correlation r is negative the regression slope, which is also negative.

R^2 which is the coefficient of determination shows the proportion of the variability of the dependent variable that can be attributed to its linear relation with the independent variable.

GraphPad Prism™ 6 was utilized for all statistical analyses. A p-value < 0.05 was considered statistically significant.

3 Results

Each sample of a GA-treated patient was measured together with a sample of an untreated patient in one run. In order to see if all experiments were performed under similar conditions, a sample of a healthy person was used (internal control) in all runs.

3.1 Cell distribution

3.1.1 GA has no effect on monocyte frequencies

There was no significant difference in the total amount of monocytes (being defined as CD14 positive cells) within the PBMC pool in the cross-sectional and longitudinal analyses of blood samples upon or without GA treatment. Stimulation with LPS did not cause any significant changes in the total amount of CD14⁺ cells within the PBMC pool of GA-treated patients compared to untreated controls.

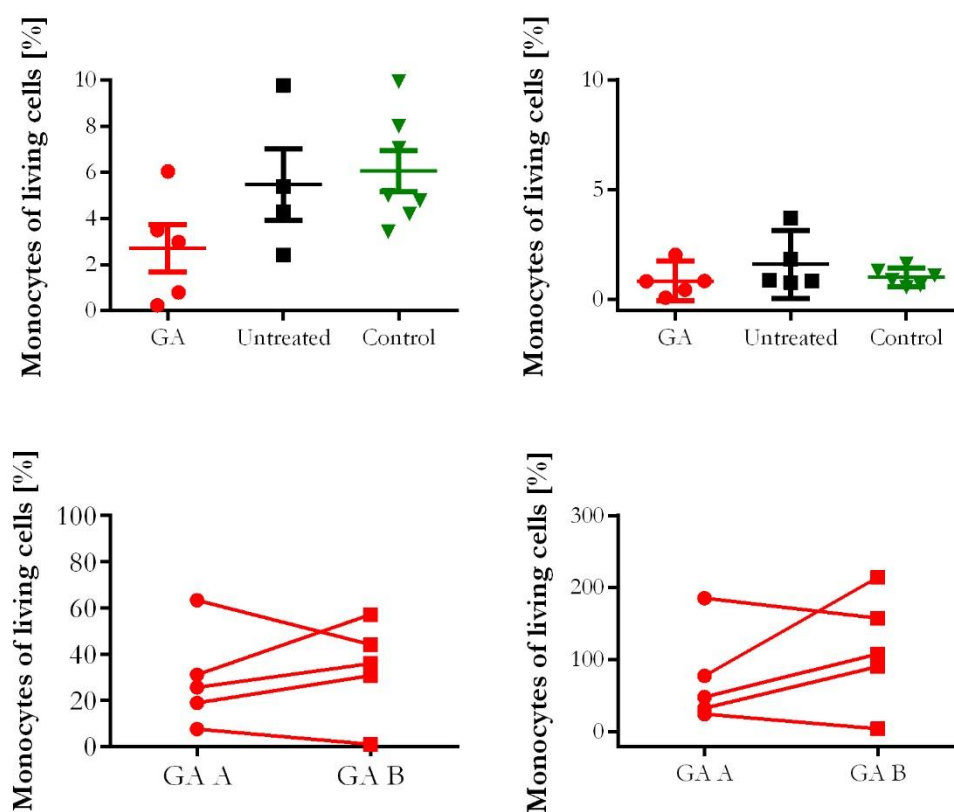


Figure 2: Monocyte frequency [% of Monocytes].

The upper graphs show individual patient data of the unstimulated $n=5$ (GA); $n=4$ (untreated); (upper left), and LPS stimulated $n=5$ (GA); $n=5$ (untreated), (upper right) cross-sectional experiment. Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the frequency of monocytes in the longitudinal analyses of blood samples of GA-treated RRMS patients in unstimulated $n=5$ (GA sample A and GA sample B), (lower left) and LPS stimulated measurements $n=5$ (GA sample A and GA sample B), (lower right). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.1.2 GA has no effect on T cell frequencies

The total frequency of T cells was determined by analyzing the frequency of $CD4^+$ and $CD8^+$ cells. GA had no effect on $CD8^+$ cells, also known as cytotoxic T cells. There was either no effect on $CD4^+$ T cells upon GA therapy.

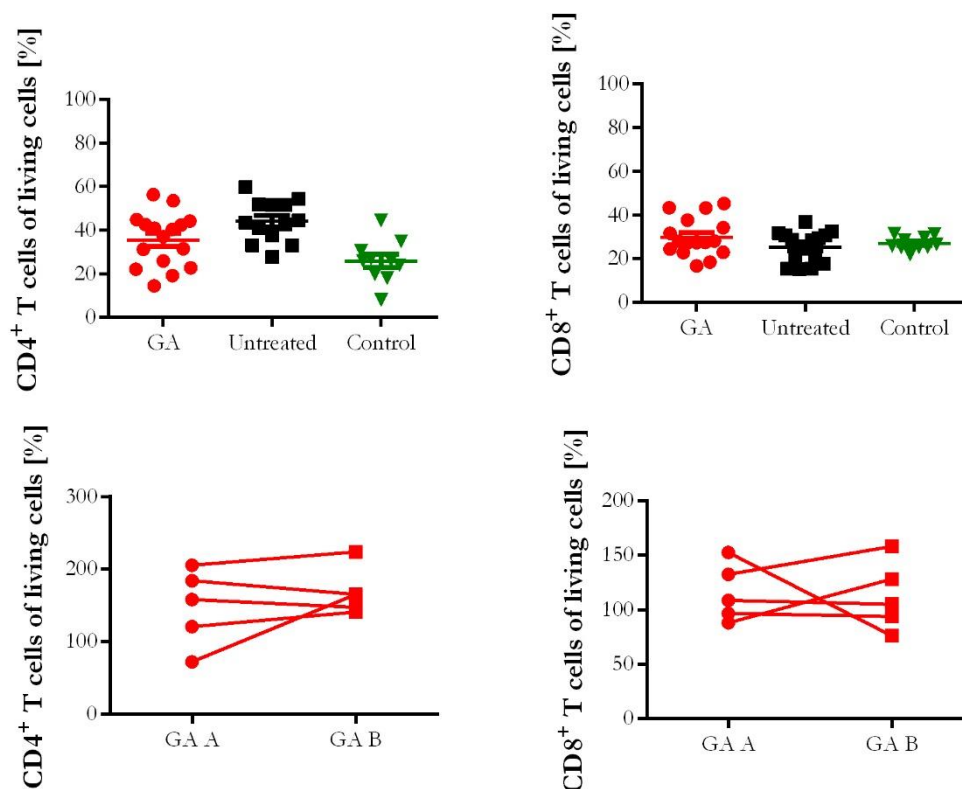


Figure 3: T cell frequency [% of T cells].

Changes in T cell subsets were analyzed in the unstimulated regimen. The upper graphs give an overview of CD4⁺ n= 16 (GA); n= 13 (untreated) and CD8⁺ T cells n= 13 (GA); n= 16 (untreated) in cross-sectional analyses of blood samples. Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the frequency of T cells in longitudinal analyses of blood samples of GA-treated RRMS patients n= 5 (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.1.3 GA reduces B cell frequencies

There was no significant change in the total amount of B cells in GA-treated patients when compared to untreated patients both in unstimulated and CPG stimulated experiments. However, it could be demonstrated that the B cell frequency was significantly reduced upon GA treatment in the longitudinal blood samples when stimulated with 2 μ g/ml CPG.

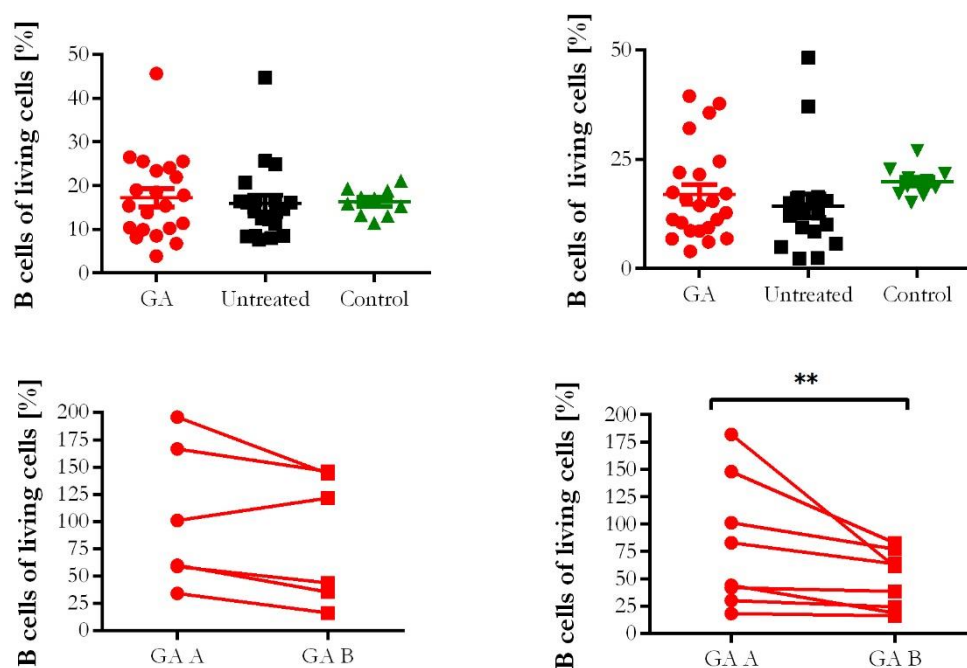


Figure 4: B cell frequency [% of B cells].

The left upper graphs show individual patient data of the unstimulated $n=21$ (GA); $n=20$ (untreated), (upper left) and CPG stimulated $n=23$ (GA); $n=18$ (untreated), (upper right) cross-sectional experiment. Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the significant reduction of B cells in longitudinal analyses of blood samples of GA-treated RRMS patients in unstimulated $n=6$ (GA sample A and GA sample B; lower left) and CPG stimulated measurement $n=8$ (GA sample A and GA sample B; lower right), (**= $p < 0.01$; unpaired t-test). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.2 The effect of GA on monocytes

The expression of activation markers such as CD150 and MHC II was examined on unstimulated monocytes ($CD14^+$ cells) and after LPS stimulation with 100 pg/ml LPS.

3.2.1 GA has no effect on the expression of CD150 on monocytes

There were no significant changes in CD150 expression on monocytes in both the cross-sectional and longitudinal experiments.

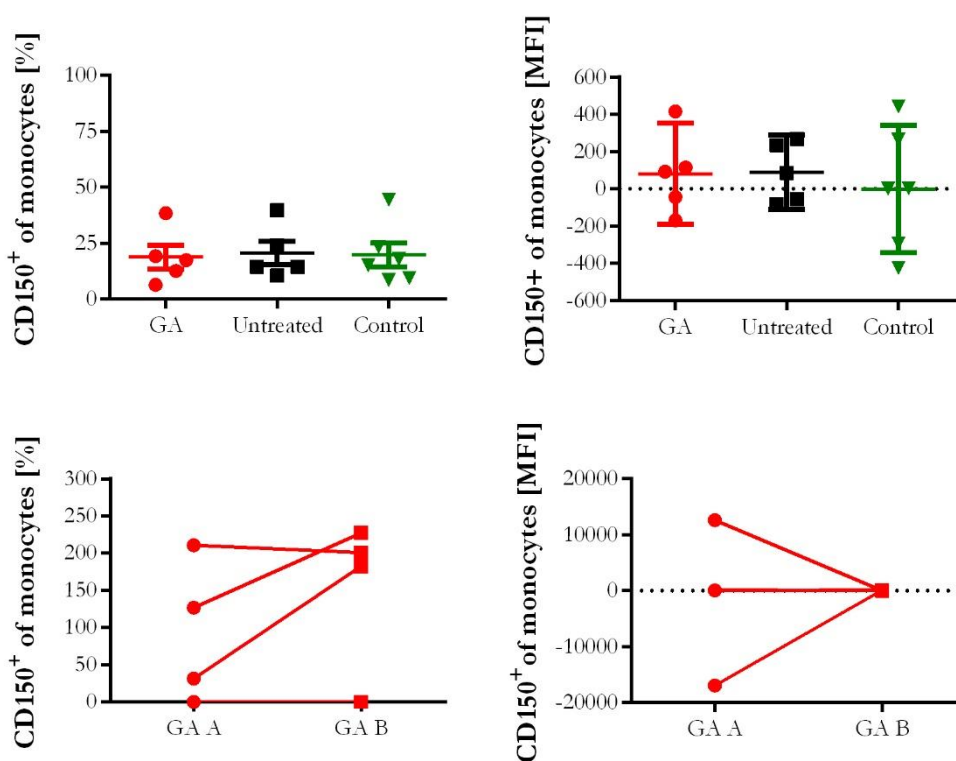


Figure 5: CD150⁺ Monocytes [% of Monocytes].

The left upper graph shows individual patient data in percentage of monocytes $n=5$ (GA); $n=5$ (untreated), the upper right one shows CD150⁺ monocytes in MFI $n=5$ (GA); $n=5$ (untreated). Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the expression of CD150 on monocytes in the longitudinal analyses of blood samples of GA-treated RRMS patients in percentage of monocytes $n=4$ (GA sample A and GA sample B), (lower left) and MFI $n=3$ (GA sample A and GA sample B), (lower right). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.2.2 GA has no effect on the MHC II expression on monocytes

As the antigen-presentation of monocytes plays an important role in the pathogenesis of MS, MHC II expression was examined on monocytes. There were no significant changes in the expression of MHC II on monocytes in both our cross-sectional and longitudinal groups.

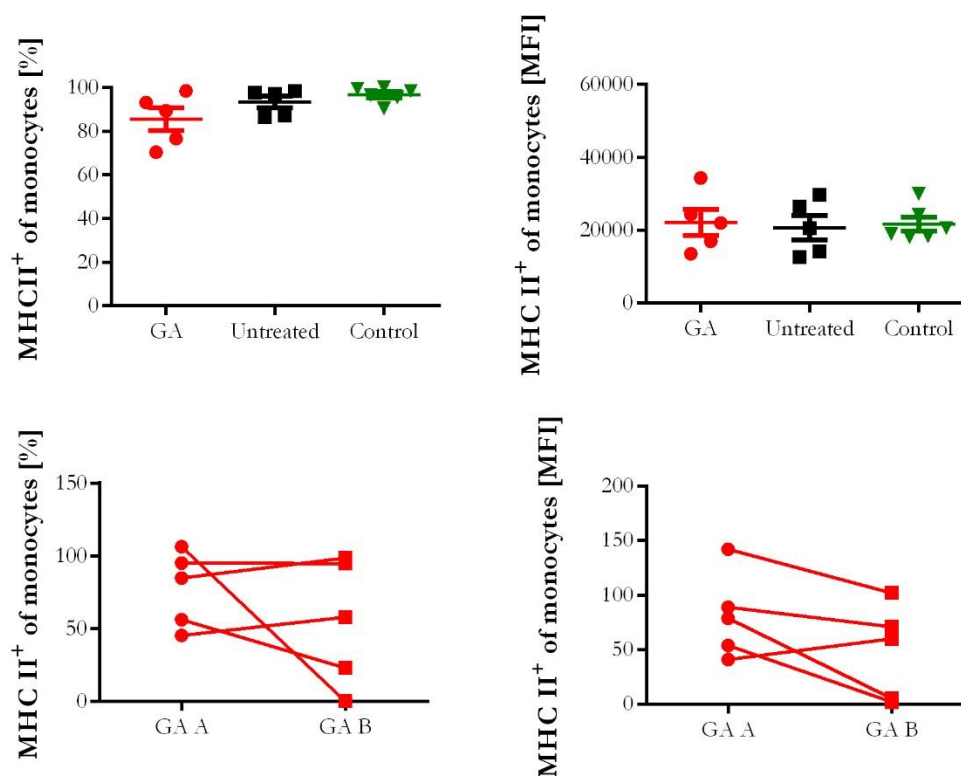


Figure 6: MHC II⁺ Monocytes [% of Monocytes].

The left upper graphs show individual patient data in percentage of monocytes $n=5$ (GA); $n=5$ (untreated), the upper right one shows MHC II⁺ monocytes in MFI $n=5$ (GA); $n=5$ (untreated), in cross-sectional experiment. Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the expression of MHC II on monocytes in the longitudinal analyses of blood samples of GA-treated RRMS patients in percentage of monocytes (lower left) and MFI (lower right), $n=5$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.3 B cell subsets

The different B cell subsets were determined, as presented in the table 4.

3.3.1 GA downregulates the frequency of transitional B cells

The intermediate stage of immature and mature B cells are transitional cells, which were determined as CD24^{high}, CD27^{neg}, CD38^{high}. There was no difference in the total frequency of transitional B cells upon GA treatment compared to their untreated controls. However, a significant reduction of transitional B cells could be observed in longitudinal samples, which also negatively correlated with GA treatment duration.

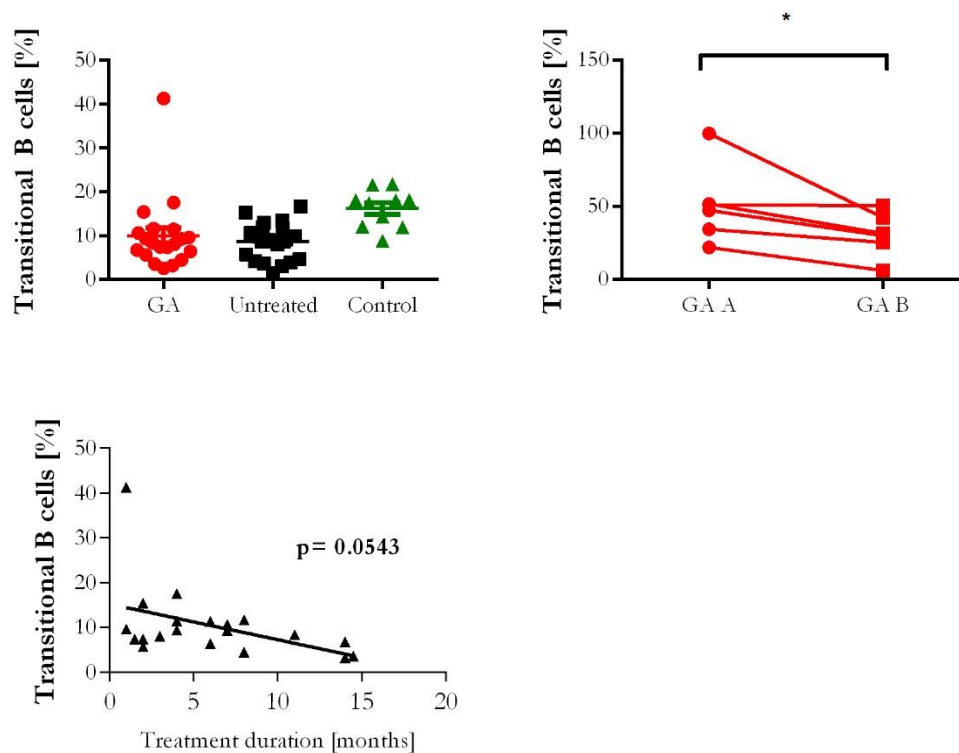


Figure 7: Transitional B cell frequency [% of B cells].

The proportion of transitional B cells (CD24^{high}, CD27^{neg}, CD38^{high}) stayed unaffected during GA therapy in the cross-sectional analysis. The left upper graph shows individual patient data of the unstimulated cross-sectional experiment, $n = 20$ (GA); $n = 19$ (untreated). Mean \pm SEM SD is represented by whiskers. There was a significant reduction in the total amount of transitional B cells in the longitudinal samples. The right graph demonstrates the significant downregulation of transitional B cells in longitudinal analyses of blood samples of GA-treated RRMS patients in unstimulated measurement, $n = 6$ (GA sample A and GA sample B), (*= $p < 0.05$; Mann Whitney test). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control. The left lower graph shows the amount of transitional B cells in relation to GA treatment duration. The trend line is based on linear progression.

3.3.2 GA has no effect on mature B cells

Mature B cells were defined as CD24^{high}, CD38^{low} cells. There was no impact of GA on mature B cells in both cross-sectional and longitudinal analyses of blood samples. However, a trend in the relation between the frequency of mature B cells and GA treatment duration could be observed.

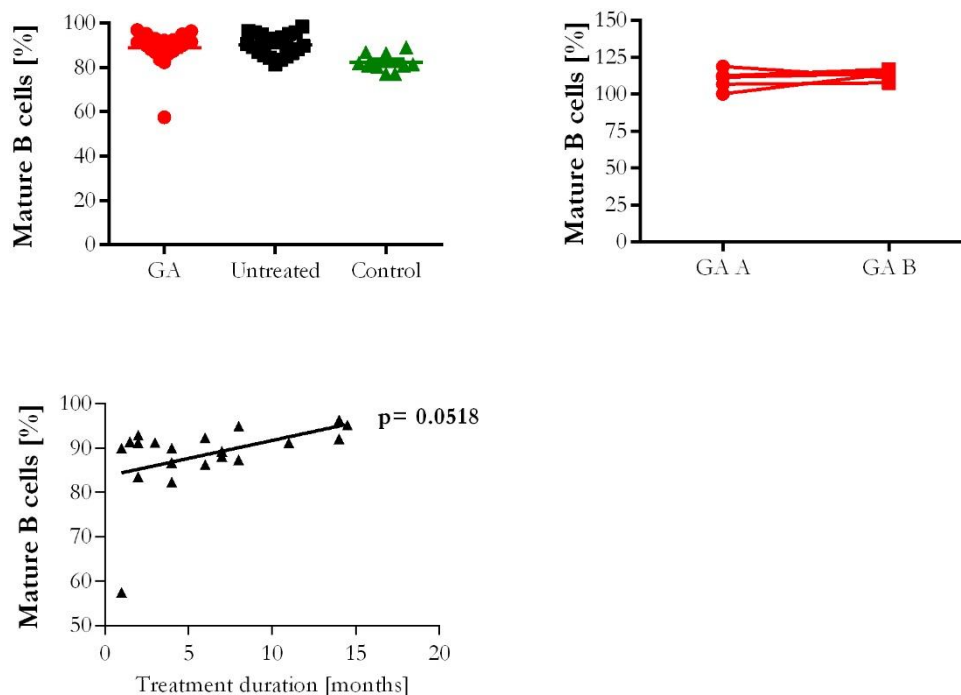


Figure 8: Mature B cell frequency [% of B cells].

The left upper graph shows individual patient data of the unstimulated cross-sectional experiment, $n=21$ (GA); $n=20$ (untreated). Mean \pm SEM is represented by whiskers. The right graph demonstrates the frequency of mature B cells in longitudinal analyses of blood samples of GA-treated RRMS patients in unstimulated measurements, $n=6$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativated to the internal control. The left lower graph shows the amount of mature B cells in relation to GA treatment duration. The trend line is based on linear progression.

3.3.3 GA has no effect on activated B cells

Activated B cells are known to have a high expression of CD27. GA had no impact on the total frequency of activated B cells compared to untreated patients. There were also no significant changes in the total amount of CD27⁺ B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients.

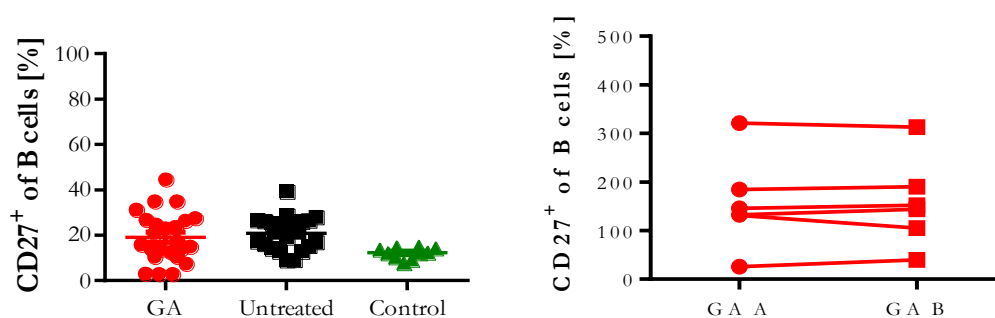


Figure 9: Activated B cell frequency [% of B cells].

The left graph shows individual patient data of the unstimulated cross-sectional experiment $n=21$ (GA); $n=20$ (untreated). Mean \pm SEM is represented by whiskers. The right graph demonstrates the frequency of activated B cells in longitudinal analyses of blood samples of GA-treated RRMS patients in unstimulated measurements, $n=6$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.3.4 GA treatment has no effect on memory B cell frequency

Memory B cells play an important part in the secondary immune response. These cells are defined as CD27^{var}, CD38^{neg}. There was no difference in the frequencies of memory B cells in GA-treated patients compared to untreated controls. The total amount of memory B cells also stayed unchanged in the longitudinal experiments.

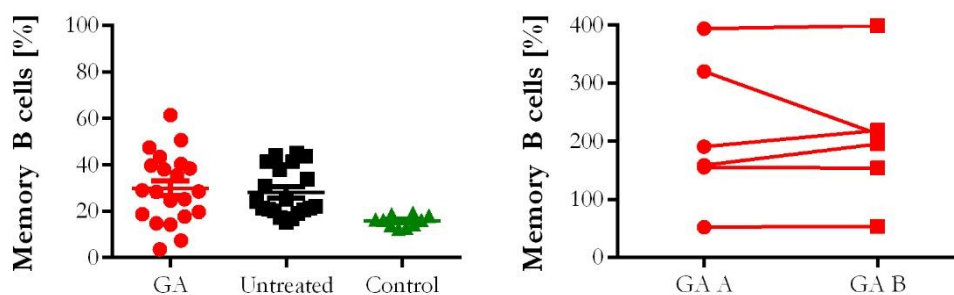


Figure 10: Memory B cell frequency [% of B cells].

The left graph shows individual patient data of the unstimulated cross-sectional experiment, $n=21$ (GA); $n=20$ (untreated). Mean \pm SEM is represented by whiskers. The right graph demonstrates the frequency of memory B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in unstimulated measurements, $n=6$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.3.5 GA decreases the total amount of plasmablasts

Plasmablasts were defined as CD20^{neg}, CD27^{high}, CD38^{high} B cells. Interestingly, GA decreased the frequency of plasmablasts (2.78 ± 0.43 %) when compared to untreated controls (4.68 ± 0.66 %). This effect could also be demonstrated in the longitudinal experiments.

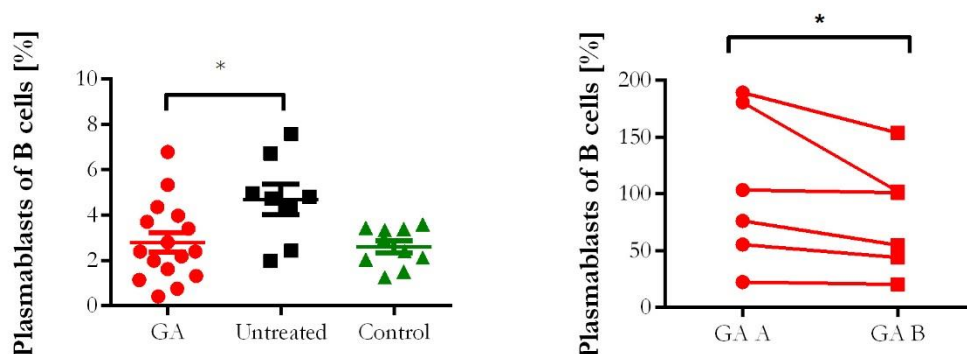


Figure 11: Plasmablast frequency [% of B cells].

The left graph shows individual patient data of the unstimulated cross-sectional experiment, $n=16$ (GA); $n=8$ (untreated). Mean \pm SEM is represented by whiskers. The right graph demonstrates the frequency of plasmablasts in longitudinal analyses of blood samples of GA-treated RRMS patients in unstimulated measurements, $n=6$ (GA sample A and GA sample B), ($*=p < 0.05$; Mann Whitney test). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was related to the internal control.

3.3.6 Changes in B cell subsets after GA therapy

In summary, there was a significant reduction in the total amount of plasmablasts in both the cross-sectional and longitudinal analyses of blood samples of GA-treated RRMS patients. In addition, a significant reduction of transitional B cells was observed in the longitudinal samples. For a detailed correlation between the B cell subsets and GA therapy duration, see table 16.

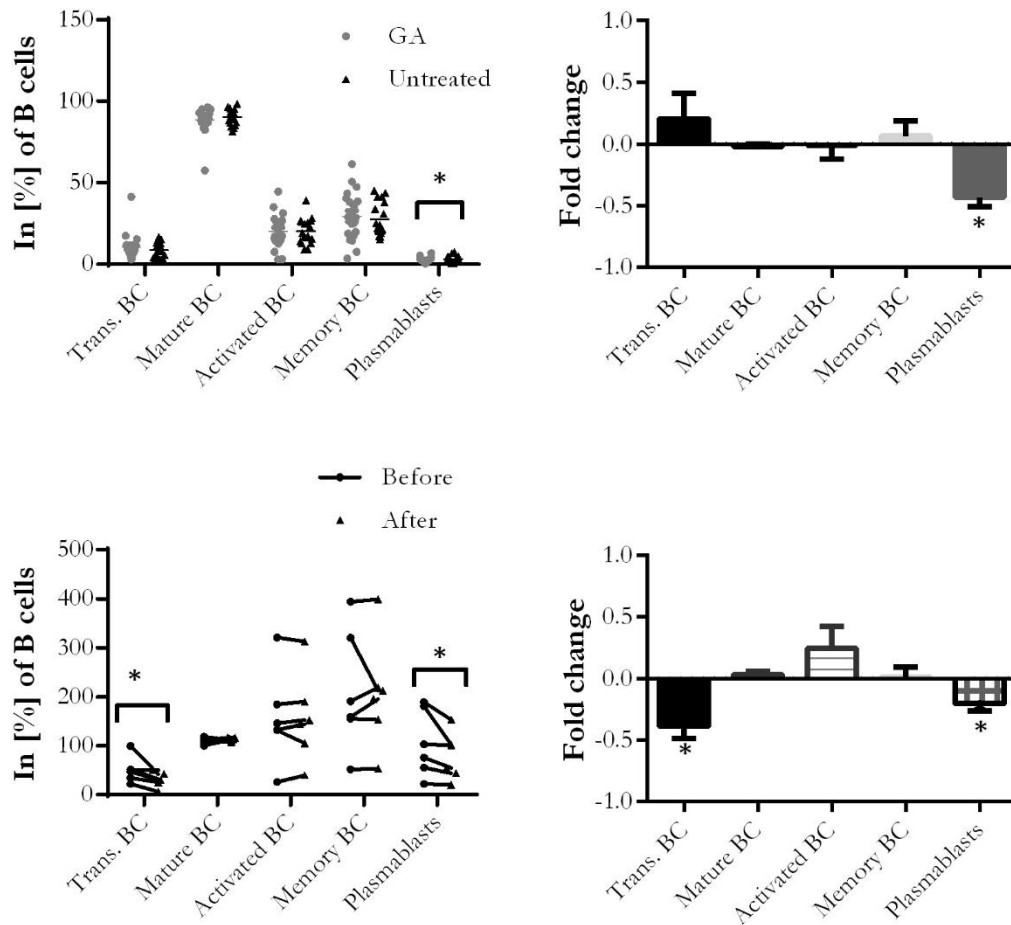


Figure 12: Changes in the B cell subsets after GA therapy.

The left upper graph provides a summary of the B cell subset frequencies in the cross-sectional samples. The right upper graph demonstrates the relative changes of the B cell subtypes in the cross-sectional samples. The left lower graph provides a summary of the different B cell subset changes in the longitudinal samples. The right lower graph demonstrates the relative changes of the B cell subtypes in the longitudinal samples. There was a significant reduction in the total amount of plasmablasts in both the cross-sectional (*= $p < 0.05$; unpaired t-test) and longitudinal samples (**= $p < 0.01$; Mann Whitney test). Additionally, there was a significant reduction in the frequency of transitional B cells in the longitudinal samples. In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.4 B cell activation

The expression of activation markers on B cells was measured using both the frequency of positive cells and the mean fluorescence intensity (MFI). In order to upregulate the expression of activation markers, B cells were stimulated with CpG.

3.4.1 GA reduces the expression of CD25 on stimulated B cells

There was a significant reduction of CD25 expression on B cells, when the blood samples of GA-treated RRMS patients were analyzed in a longitudinal manner. However, no differences in CD25 expression could be observed when the blood samples of GA-treated patients were compared to their untreated controls.

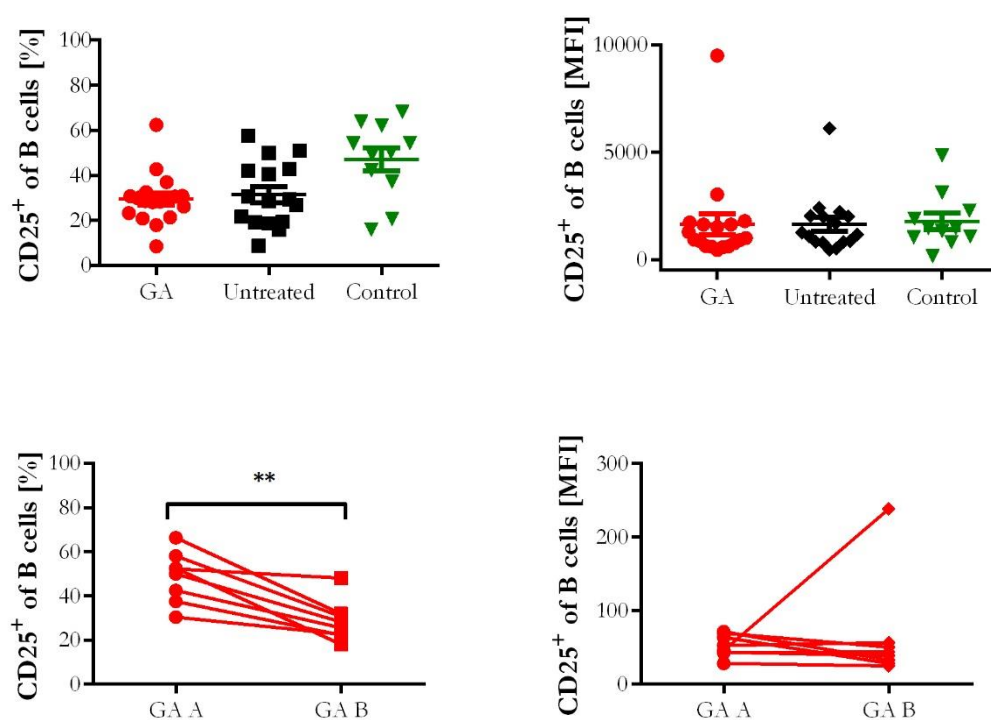


Figure 13: CD25⁺ B cells [% of B cells].

There were no significant changes in the expression of CD25 on the surface of B cells in the cross-sectional samples. The upper graphs show individual patient data in MFI (upper right) and percentage of B cells (upper left) in cross-sectional experiments, n= 22 (GA); n= 18 (untreated). Mean ± SEM is represented by whiskers. Lower graphs demonstrate the expression of CD25 on B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI (lower right) and percentage of B cells (lower left), n= 8 (GA sample A and GA sample B). GA downregulated the CD25 expression on B cells (**= p < 0.01; Mann Whitney test). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.4.2 GA has no effect on the CD40 expression on B cells

The expression of CD40 on B cells was unaffected during GA therapy in both the cross-sectional and longitudinal experiments.

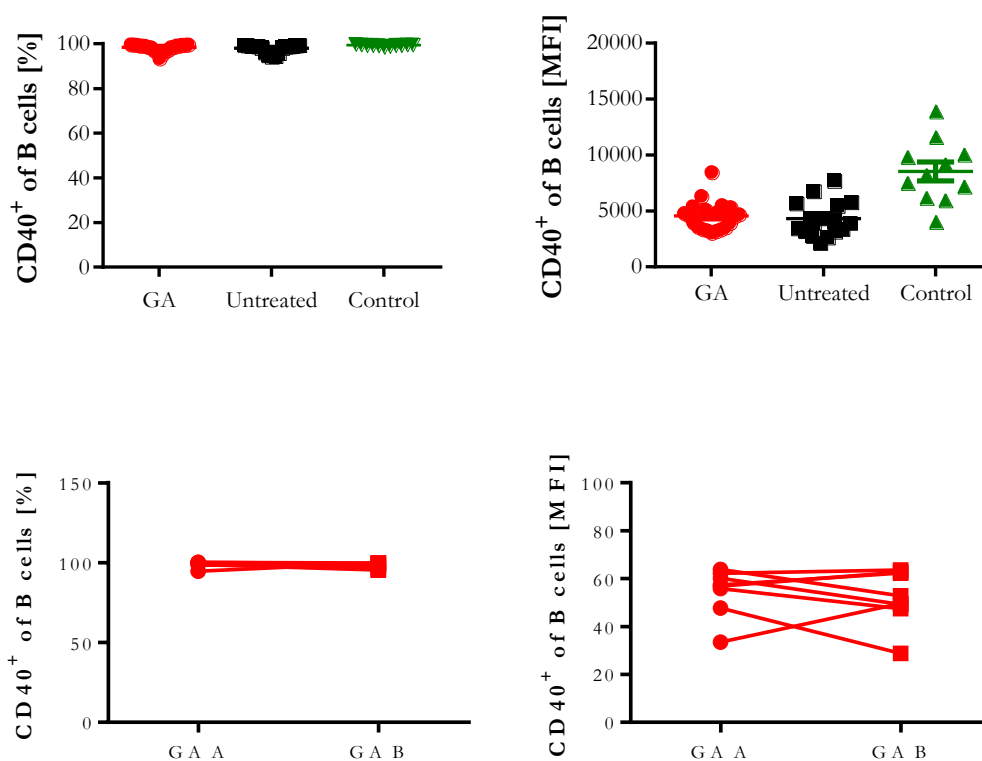


Figure 14: CD40⁺ B cells [% of B cells].

The upper graphs show individual patient data in MFI (upper right) and percentage of B cells (upper left) in cross-sectional experiments, $n = 22$ (GA); $n = 18$ (untreated). Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the expression of CD40 on B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI (lower right) and percentage of B cells (lower left), $n = 8$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.4.3 GA reduces the expression of CD69 on stimulated B cells

There was a significant reduction of CD69 expression on B cells when the blood samples of GA-treated RRMS patients were analyzed in a longitudinal manner. However, no differences were observed when the blood samples of GA-treated patients were compared to their untreated controls.

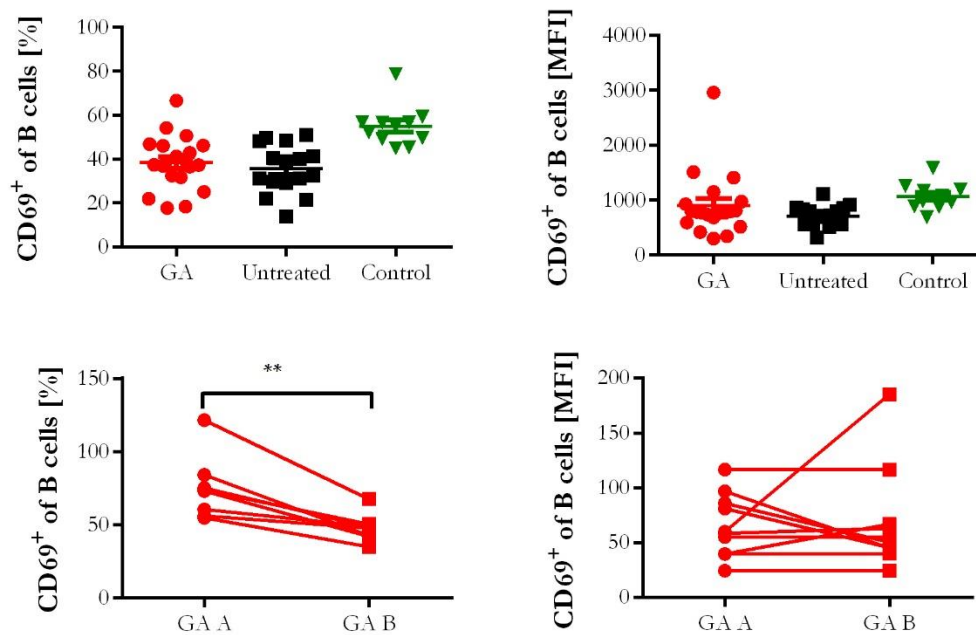


Figure 15: CD69⁺ B cells [% of B cells].

There were no significant changes in expression of CD69 on the surface of B cells in the cross-sectional samples. The upper graphs show individual patient data in MFI (upper right) and percentage of B cells (upper left) in cross-sectional experiments, $n=22$ (GA); $n=18$ (untreated). Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the downregulation of CD69 expression on B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI (lower right) and percentage of B cells (lower left), $n=8$ (GA sample A and GA sample B), (**= $p < 0.01$; Mann Whitney test). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.4.4 GA has no effect on the CD80 expression on B cells

The expression of CD80 on B cells was unaffected during GA therapy in both the cross-sectional and longitudinal experiments.

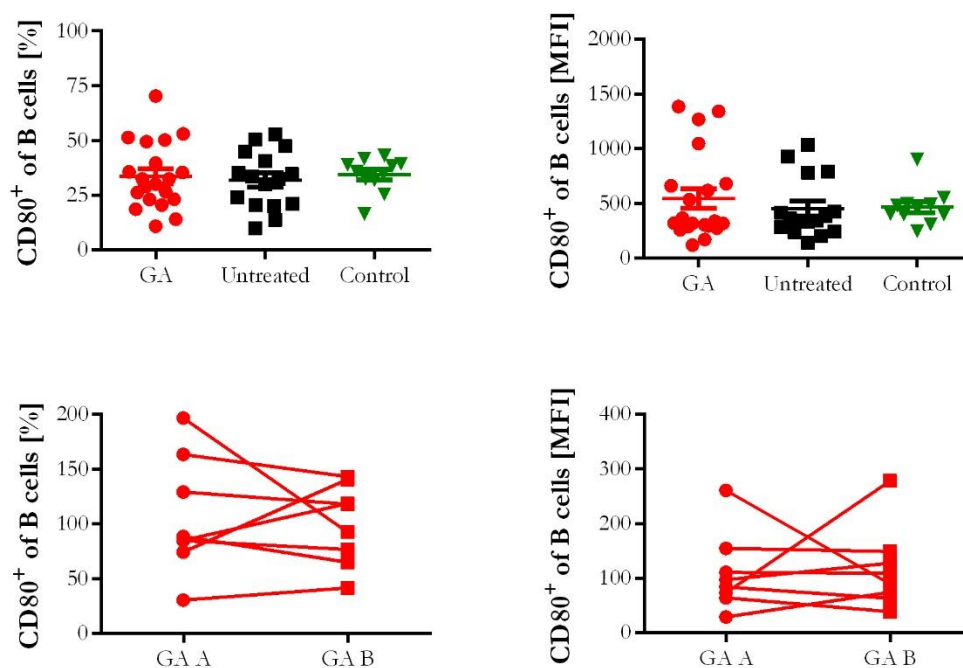


Figure 16: CD80⁺ B cells [% of B cells].

The upper graphs show individual patient data in MFI (upper right) and percentage of B cells (upper left) in cross-sectional experiments, $n=22$ (GA); $n=18$ (untreated). Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the expression of CD80 on B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI (lower right) and percentage of B cells (lower left), $n=8$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.4.5 GA has no effect on the CD86 expression on B cells

There was no significant difference in the expression of CD86 on B cells in GA-treated patients compared to their untreated controls. Additionally, no significant differences were observed in the longitudinal experiments.

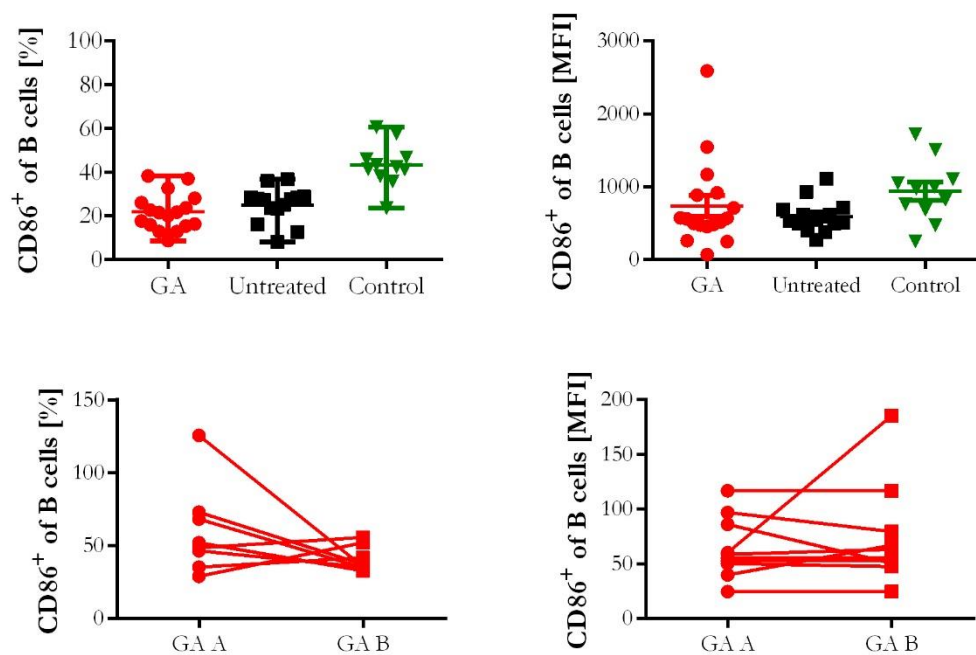


Figure 17: CD86⁺ B cells [% of B cells].

The upper graphs show individual patient data in MFI (upper right) and percentage of B cells (upper left) in cross-sectional experiments, $n=22$ (GA); $n=18$ (untreated). Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the expression of CD86 on B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI (lower right) and percentage of B cells (lower left), $n=8$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.4.6 The expression of CD95 on B cells positively correlates with GA treatment duration

The expression of the FAS receptor CD95 on B cells was not affected by GA therapy in both the cross-sectional and longitudinal analyses. However, a positive correlation between the frequency of CD95⁺ B cells and GA treatment duration could be observed.

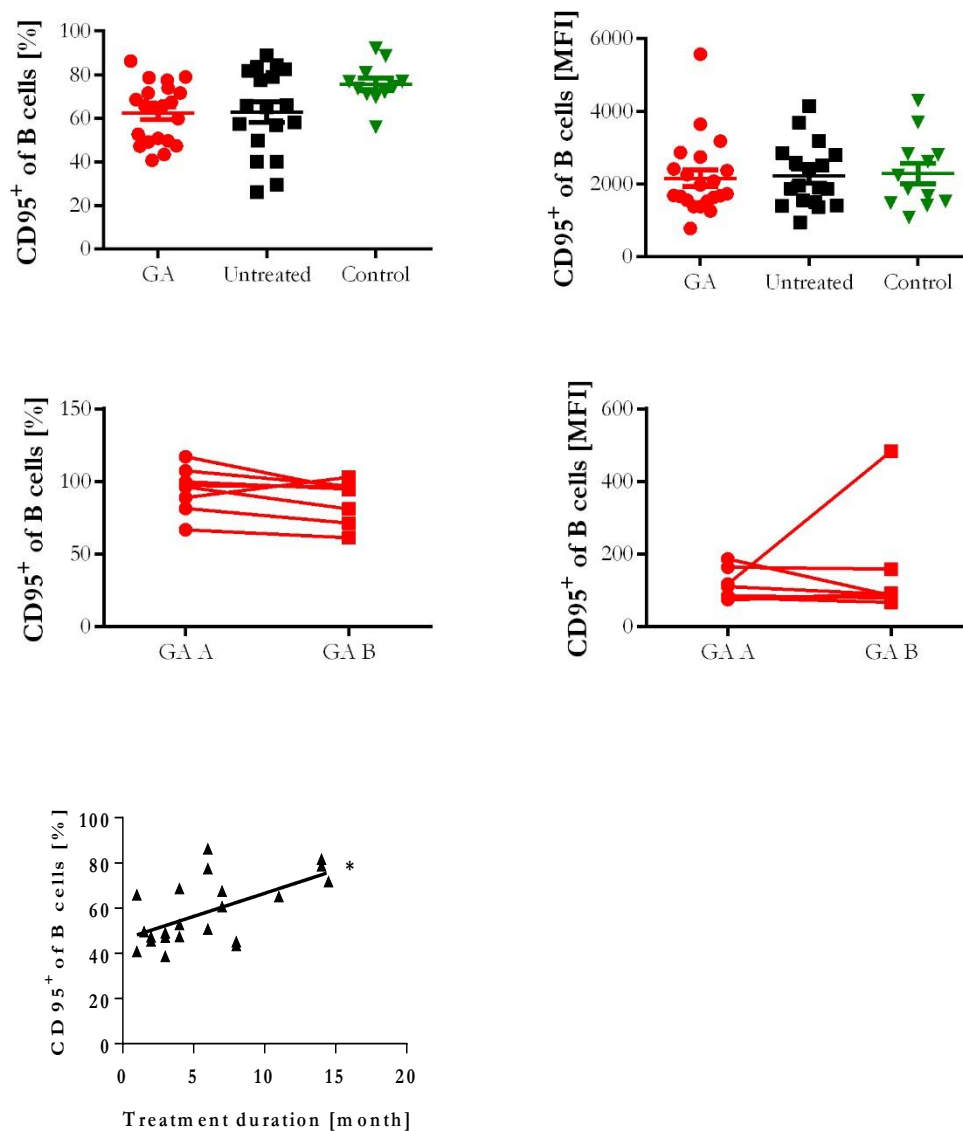


Figure 18: CD95⁺ B cells [% of B cells].

The upper graphs show individual patient data in MFI (upper right) and percentage of B cells (upper left) in cross-sectional experiments, $n=22$ (GA); $n=18$ (untreated). Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the expression of CD95 on B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI (lower right) and percentage of B cells (lower left), $n=8$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control. The left lowest graph shows the amount of CD95⁺ B cells in relation to GA treatment duration. The trend line is based on linear progression.

3.4.7 GA upregulates the expression of MHC II on B cells

The expression of MHC II on B cells was significantly higher in GA-treated patients (16705 ± 976.5 MFI) as compared to their untreated controls (13505 ± 1010 MFI). There were no significant changes in the expression of MHC II on B cells in the longitudinal experiments.

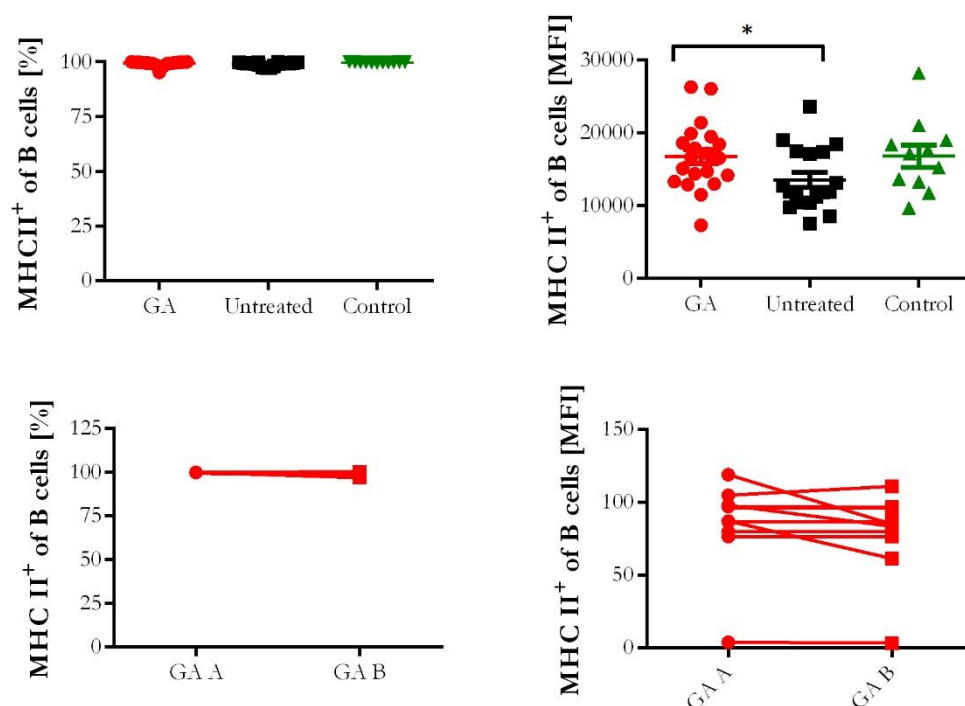


Figure 19: MHC II⁺ B cells [% of B cells].

There were no significant changes in the expression of MHC II on B cells in the longitudinal experiments. The upper graphs show individual patient data in MFI (upper right) and percentage of B cells (upper left) in cross-sectional experiment, $n=22$ (GA); $n=18$ (untreated). Mean \pm SEM is represented by whiskers. Lower graphs demonstrate the expression of MHC II on B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI (lower right) and percentage of B cells (lower left), $n=8$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.4.8 Summary of the GA-induced activation marker changes on B cells

In summary, there was a significant reduction in the expression of CD25 and CD69 in the longitudinal experiments. Additionally, the expression of MHC II on B cells in GA-treated patients was significantly higher compared to their untreated controls. Furthermore, a positive correlation between the frequency of CD95⁺ B cells and GA treatment duration

could be observed. For a detailed correlation between the cell surface markers and GA therapy duration, see table 17.

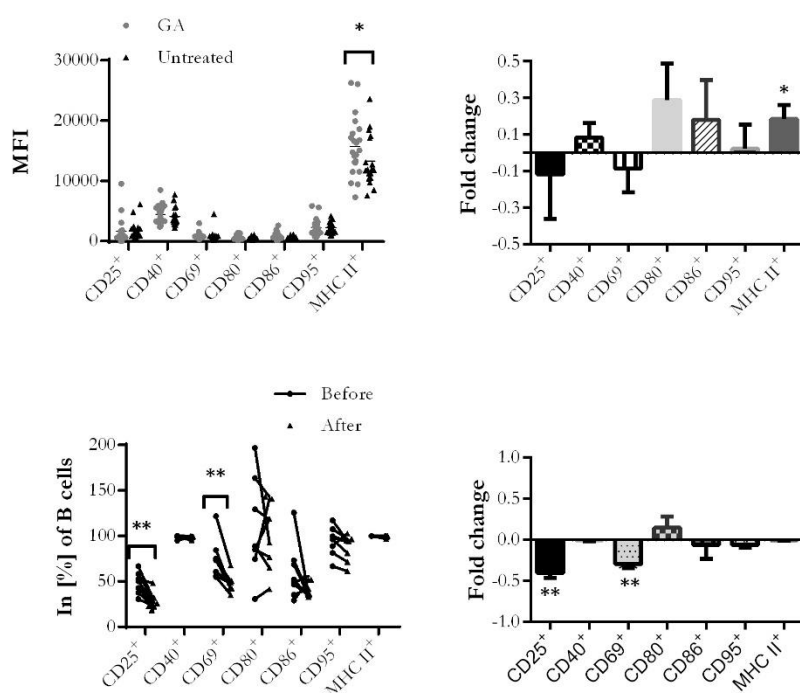


Figure 20: Changes in the expression of activation markers on B cells after GA therapy.

The left upper graph provides a summary of the expression of activation markers on B cells in the cross-sectional samples (MFI). The right upper graph demonstrates relative changes in the expression of activation markers on the surface of B cells in the cross-sectional samples. The left lower graph provides a summary of the expression of activation markers on B cells in the longitudinal samples (%). The right lower graph demonstrates relative changes in the expression of activation markers on B cells in the longitudinal samples. There was a significant reduction in the expression of CD25 and CD69 in the longitudinal experiments (**= $p < 0.01$; Mann Whitney test). However, the expression of MHC II on B cells was higher in GA-treated patients compared to untreated controls in the cross-sectional experiments (*= $p < 0.05$; unpaired t-test).

3.5 Cytokine production

In order to measure the effect of GA on cytokine production, intracellular staining of pro-inflammatory (IL-6 and TNF- α) and anti-inflammatory (IL-10) cytokines was performed. The cytokine production of B cells (MFI) was quantified by flow cytometry analyses after CpG stimulation.

3.5.1 GA has no effect on the IL-6 production in B cells

The production of IL-6 in B cells was unaffected by GA therapy in both the cross-sectional and longitudinal experiments.

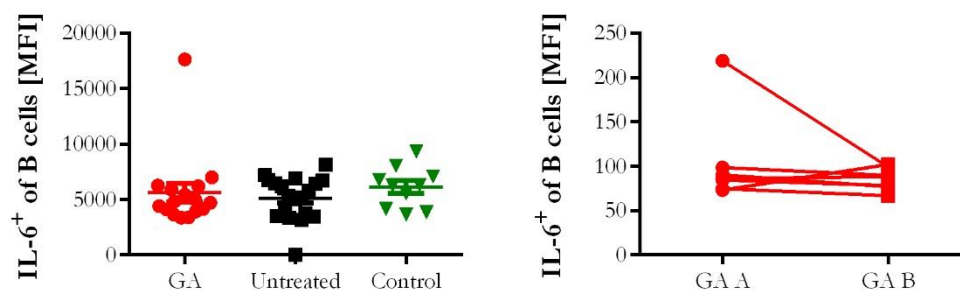


Figure 21: IL-6 production of B cells.

The left graph shows individual patient data in MFI in cross-sectional experiments, $n= 16$ (GA); $n= 18$ (untreated). Mean \pm SEM is represented by whiskers. The right graph demonstrates the production of IL-6 in B cells in longitudinal analyses of blood samples of GA-treated RRMS patients in MFI, $n= 7$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.5.2 GA has no effect on the IL-10 production in B cells

Both, the cross-sectional and longitudinal analyses of IL-10 secretion by B cells revealed no difference after GA therapy.

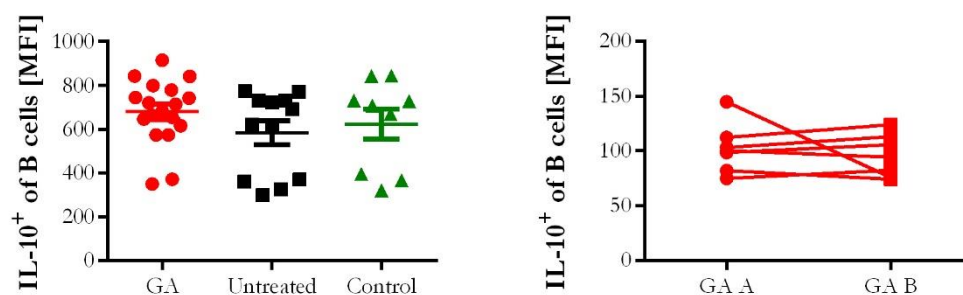


Figure 22: IL-10 production of B cells.

The left graph shows individual patient data in MFI in cross-sectional experiments, $n= 18$ (GA); $n= 12$ (untreated). Every dot in these plots represents an individual patient, whiskers indicate \pm SEM. The right graph demonstrates the production of IL-10 of B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI, $n= 7$ (GA sample A and GA sample B). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.5.3 GA downregulates the TNF- α production in B cells

TNF- α , a second major pro-inflammatory cytokine produced by B cells was quantified by flow cytometry. There was a significant reduction in the secretion of TNF- α by B cells under GA therapy in the longitudinal but not in the cross-sectional experiments.

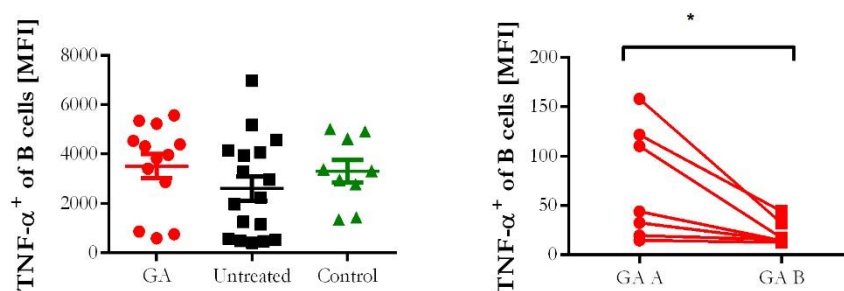


Figure 23: TNF- α production of B cells.

There were no significant changes in the production of TNF- α by B cells when GA-treated patients were compared to untreated RRMS patients in the cross-sectional experiments. The left graph shows individual patient data in MFI in cross-sectional experiments, $n= 13$ (GA); $n= 16$ (untreated). Mean \pm SEM is represented by whiskers. The right graph demonstrates the reduced production of TNF- α of B cells in the longitudinal analyses of blood samples of GA-treated RRMS patients in MFI, $n= 7$

(GA sample A and GA sample B), (*= $p < 0.05$; Mann Whitney test). In order to demonstrate that all longitudinal experiments were performed under the same conditions, each sample was relativized to the internal control.

3.5.4 Summary of the cytokine production upon GA therapy

In summary, no significant changes in the IL-6 and IL-10 production of B cells could be observed, in both the cross-sectional and longitudinal analyses. However, the TNF- α production was significantly reduced in the longitudinal experiments. For a detailed correlation between the cytokine production and GA therapy duration, see table 18.

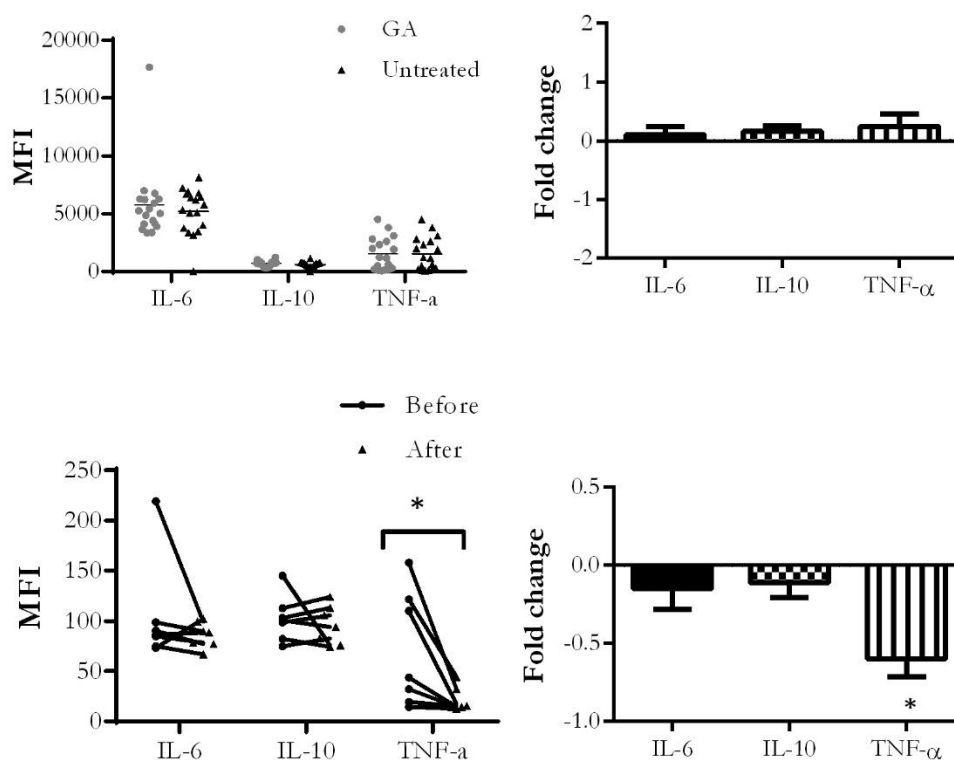


Figure 24: Changes in the B cell cytokine production after GA therapy.

The left graph provides a summary of the cytokine production by B cells in the cross-sectional samples (MFI). The right graph demonstrates the relative changes in cytokine secretion by B cells in the longitudinal data. There was a significant reduction in the production of the important pro-inflammatory cytokine TNF- α in the longitudinal experiments (*= $p < 0.05$; Mann Whitney test).

Table 15: Correlation between cell distribution and therapy duration

	Unit	slope (units/month)	R ²	R	p value	
B cells	%	-3,576	0,02955	0,171901134	0,5568	Ns
Monocytes	%	-23,3	0,01284	0,113313724	0,6997	Ns
CD4 ⁺ T cells	%	5,781	0,004213	0,064907627	0,8255	Ns
CD8 ⁺ T cells	%	4,161	0,01754	0,132438665	0,6518	Ns

Table 16: Correlation between B cell subsets and GA therapy duration

	unit	slope (units/month)	R²	R	p value	
Transitional B cells	%	-1,256	0,1906	0,4365776	0,0543	Ns
Mature B cells	%	1,236	0,1942	0,4406813	0,0518	Ns
Activated B cells	%	10,95	0,001492	0,0386264	0,8716	Ns
Memory B cells	%	1,46	0,04099	0,2024599	0,392	Ns
Plasmablasts	%	-20,75	0,01888	0,1374045	0,5635	Ns

Table 17: Correlation between cell surface markers and GA therapy duration

		unit	Slope (units/month)	R ²	R	p value	
CD25+ B cells	2 µg/ml CpG	%	-5,08	0,0040033	0,06327164	0,7789	Ns
CD25 on B cells	2 µg/ml CpG	MFI	-0,02358	0,007446	0,08629021	0,4278	Ns
CD40+ B cells	2 µg/ml CpG	%	10,01	0,07054	0,26559367	0,2322	Ns
CD40 on B cells	2 µg/ml CpG	MFI	0,03122	0,008358	0,0914221	0,6858	Ns
CD69+ B cells	2 µg/ml CpG	%	2,482	0,01929	0,13888844	0,5376	Ns
CD69 on B cells	2 µg/ml CpG	MFI	0,0299	0,06536	0,25565602	0,2508	Ns
CD80+ B cells	2 µg/ml CpG	%	1,29	0,04126	0,20312558	0,3646	Ns
CD80 on B cells	2 µg/ml CpG	MFI	0,1464	0,0057	0,07549834	0,7384	Ns
CD86+ B cells	2 µg/ml CpG	%	6,25	0,003956	0,06289674	0,781	Ns
CD86 on B cells	2 µg/ml CpG	MFI	0,04723	0,0214	0,14628739	0,5159	Ns
CD95+ B cells	2 µg/ml CpG	%	0,4892	0,3535	0,59455866	0,0035	*
CD95 on B cells	2 µg/ml CpG	MFI	0,0323	0,00923	0,09607289	0,6706	Ns
MHC-II+ B cells	2 µg/ml CpG	%	539,3	4,576	2,13915871	0,9762	Ns
MHC-II on B cells	2 µg/ml CpG	MFI	-0,004156	0,04418	0,21019039	0,3478	Ns

Table 18: Correlation between cytokine production and GA therapy duration

	Unit	slope (units/month)	R ²	R	p value	
IL-6 in B cells	MFI	-0,01163	0,01305	0,11423659658	0,06517	Ns
IL-10 in B cells	MFI	-0,1085	0,09817	0,313320922	0,2055	Ns
TNF in B cells	MFI	-0,005599	0,09801	0,313065488	0,2059	Ns

4 Discussion

4.1 Cell distribution

MS is an autoimmune disease of the central nervous system (CNS), and the immune system plays a central role in the disease pathogenesis. Therefore, it is very important to know the cell distribution of the MS patient's PBMC pool. The first part of the present study focuses on the determination of frequency changes of the distinct lymphocyte population.

4.1.1 B cells

Historically, MS is thought to be a T cell-mediated disease of the CNS. However, several lines of evidence showed that B cells are also key players in the pathogenesis of MS (Bar-Or et al. 2010; Rawlings et al. 2017; Baker et al. 2017). Major functions of the B cells are cytokine and antibody production and antigen presentation. The most convincing evidence of an important role of B cells in the pathogenesis of MS is the efficacy of anti-CD20 antibody therapy in reducing MS relapses and formation of new lesions in MRI (Moreno Torres and García-Merino 2017). The first aim of the study was to determine if glatiramer acetate changes the total amount of B cells in the PBMC pool. Several investigations showed that the absolute B cell counts diminished in patients taking GA (Teitelbaum et al. 2003; Sean Selva 2014; Ireland et al. 2012). The present study revealed that the B cell frequencies were unchanged when GA-treated patients were compared to untreated MS controls. However, there was a significant reduction of the B cell frequencies with longer GA therapy duration in the longitudinal samples. These findings suggest that the effect of GA therapy on B cells may only occur over time. Additionally to Rituximab, Ocrelizumab, and Ofatumumab, which are B cell-depleting therapeutics, there is evidence that Fingolimod and Mitoxantrone also decrease the total B cell numbers (Longbrake und Cross 2016; Gasperi et al. 2016). Taken together, all these findings demonstrate that the reduction of B cells is one of the most successful ways to treat RRMS.

4.1.2 CD4⁺ T cells

The most popular theory of the pathogenesis of MS is “molecular mimicry”. It means that CD4⁺ T cells activated by a foreign antigen cross-react with myelin antigen (Chitnis 2007). CD4⁺ T cells were shown to predominate in acute MS lesions (Raine 1994). In the EAE model, it could be shown that the injection of myelin components leads to a CD4⁺T cell-mediated MS-like disease. An adoptive transfer of encephalitogenic CD4⁺T cells into naïve animals caused EAE (Sospedra and Martin 2005). Some investigations showed that the total amount of CD4⁺ T cells is reduced in GA-treated patients (Rieks et al. 2003; Kantengwa et

al. 2007). This study revealed no changes in the CD4⁺ T cell frequencies in both the cross-sectional and longitudinal samples. As the present study focused on B lymphocytes, no further sub-analysis of CD4⁺ T cells was performed.

4.1.3 CD8⁺ T cells

There is evidence that CD4⁺ T cells recognize HLA class II molecules, expressed on macrophages, B cells, dendritic cells, and microglia cells. However, HLA class I molecules can be recognized by CD8⁺ T cells, which are expressed on all nucleated cells, including oligodendrocytes, astrocytes, and neurons (Hemmer et al. 2015). These findings suggest that CD8⁺ T cells may directly target these CNS resident cells. It could be demonstrated that in primary culture systems, activated CD8⁺ T cells injured neuronal axons and oligodendrocytes in an MHC class I-associated manner (Jurewicz et al. 1998; Sauer et al. 2013). Huseby and colleagues demonstrated in a mouse model that CD8⁺ T cells could cause a severe autoimmune disease of the CNS similar to MS (Huseby et al. 2001). These findings suggest that CD8⁺ T cells could play an important role as effector cells in the pathogenesis of MS.

There is evidence that untreated MS patients have an increased level of CD8⁺ T cells (Dooley et al. 2016). It was shown that GA is not able to suppress EAE in CD8⁺ T cell-deficient mice, which brought the authors to the conclusion that CD8⁺ T cells are very important for the therapeutic effect of glatiramer acetate (Tyler et al. 2013). In the present study, there were no significant changes in the CD8⁺ T cell frequencies both after GA therapy as compared to MS controls and in the longitudinal analyses of GA samples. In 2013, Ayers et al. showed an increased level of CD8⁺ T cells in the peripheral blood of MS patients after 12 hours of GA administration. However, these changes in CD8⁺ T cell numbers and suppressive abilities at 24 and 72 h were not statistically different from baseline (Ayers et al. 2013). It can be concluded that the suppressive effect of GA on CD8⁺ T cells seems to peak at the 12 h time point. As the present study primarily focused on B cells, no further analysis of T cell subsets was performed.

4.1.4 Monocytes

CD14 is a myeloid differentiation marker, which is found on the surface of monocytes and macrophages. A low concentration of CD14 can also be found on neutrophils (Shive et al. 2015). It is known that the CD14⁺ monocytes circulate in the blood and differentiate into macrophages upon tissue entry. CD14⁺ monocytes are mainly stored in red pulp of the spleen. Swirski and colleagues demonstrated for the first time that these monocytes may play a direct role in inflammatory processes even before their transformation into macrophages (Swirski et al. 2009). Several investigations demonstrated that CD14-deficient mice showed increased disease severity and infiltration of inflammatory immune cells in EAE (Halmer et

al. 2015; Walter et al. 2006). There is evidence that monocyte-derived macrophages can produce neuroprotective and anti-inflammatory cytokines (Miron et al. 2013; Shechter et al. 2013). However, they can also worsen neuroinflammation, leading to secondary neurodegeneration (Polfliet et al. 2002). Taken together, these findings suggest that monocytes and macrophages play a dual role in the pathogenesis of multiple sclerosis: pro-inflammatory and anti-inflammatory. The main roles of monocytes in the pathogenesis of multiple sclerosis are antigen-presentation, cytokine-production; and phagocytosis (Walter et al. 2006). In the present study monocyte frequencies and monocyte functions were analyzed. No significant changes were observed in the total frequency of monocytes within the PBMC pool after GA therapy as compared to MS controls. There was also no significant modulation of the CD14⁺ monocyte function during GA treatment. The present findings are in line with results published by Ayers and colleagues, who showed that the total amount of monocytes in peripheral blood stayed unchanged in GA-treated patients compared to untreated- or healthy controls (Ayers et al. 2013).

4.1.5 Conclusion: cell distribution

Not all cell subtypes within the PBMC pool seem to be equally affected after glatiramer acetate therapy. The present study showed that the B cell frequencies were significantly reduced under GA therapy over time. Other cell types such as CD8⁺ or CD4⁺ T cells and monocytes seem to be unaffected by glatiramer acetate. These findings are in line with the findings of other studies. There is only one investigation that measured the changes in all these cell types at the same time (Carrieri et al. 2015). As B-cell-depleting agents show growing efficacy in MS treatment, the fact that GA causes a significant reduction in the total amount of B cells over time is very important. Thus, it demonstrates the therapeutic efficacy of GA.

4.2 Cell survival

There is evidence that GA suppresses the expression of B cell surviving factors such as BAFF and a proliferation-inducing ligand (APRIL), potentially leading to reduced B cell survival and increased apoptosis and necrosis (Begum-Haque et al. 2010; Dooley et al. 2016). Apoptosis is a programmed cell death, which is regulated and triggered by several cascades. There is a consideration that a dysregulation between apoptotic (Bcl-2, FLIP) and anti-apoptotic (CD95) molecules may contribute to disease activity in multiple sclerosis (Seidi and Sharief 2002). In the present study, size exclusion was used to determine apoptotic cells while a Zombie dye stained for necrotic cells. The obtained results revealed a significant reduction of B cells in the longitudinal samples, as described above. The expression of FAS-receptor

(CD95), which causes apoptosis upon ligand binding, was unaffected when GA-treated MS patients were compared to untreated controls. However, a positive correlation between the frequency of CD95⁺ B cells and GA treatment duration could be observed. The results indicate that glatiramer acetate affects the apoptosis of cells over time. These findings could explain a significant reduction of B cells in the longitudinal samples, as described above. The exact mechanism of action of GA on the apoptosis of cells remains unclear. There are no similar investigations, demonstrating the role of FAS-receptor (CD95) in the pathogenesis of MS after GA therapy.

4.3 B cell subsets

As discussed above, B cells play an important role in the pathogenesis of multiple sclerosis. Therefore, it is important to detect possible effects of GA therapy on B cells. The aim of this study was to evaluate the impact of GA therapy on B cells. In the second part of the study, the impact of GA therapy on B cell differentiation and B cell function was analyzed.

4.3.1 Transitional B cells

The initial development of B cells takes place in the bone marrow. First, the immature B cells have to generate a functional B-cell receptor (BCR) and then overcome a negative selection for the reactivity with autoantigens. B cells, which survive these processes in the bone marrow, transit to the spleen to finish their maturation. These immature B cells found in the peripheral blood are called “transitional B cells”. Thus, transitional B cells represent a bridge in the development of B cells in the spleen and bone marrow, see for review (Sims 2005). According to B cell studies in literature, transitional B cells are defined as CD24^{high}CD27^{neg}CD38^{high} (Sims et al. 2005; Hamilton et al. 2017). Unfortunately, there are no studies showing how the total amount of transitional B cells changes under GA therapy. However, a significant reduction in transitional B cell numbers could be demonstrated in Fingolimod and Natalizumab treated MS patients (Haas et al. 2011; Chiarini et al. 2015). The current study showed a significant decrease in transitional B cells with longer ongoing therapy duration. There is evidence that the serum concentration of the B-cell activating factor of the tumor necrosis factor family (BAFF) positively correlates with the proportions and absolute count of transitional B cells (Miyazaki et al. 2018). Several studies demonstrated that GA therapy down-regulates the expression of BAFF (Dooley et al. 2016; Begum-Haque et al. 2010). Taken together, these findings could explain the significant reduction of transitional B cells under GA therapy through the down-regulation of BAFF expression. The current results point toward a decreased output of B cells from the bone marrow.

4.3.2 Mature B cells (naïve B cells)

As mature B cells express the maturation markers on their surface, they are defined as CD24^{high}CD38^{low} B cells. Mature or naïve B cells is the next stage of maturation of transitional B cells but not yet plasmablasts. Several studies described an elevation of naïve B cell numbers in MS patients treated with GA (Haas et al. 2011; Ireland et al. 2014; Rovituso et al. 2014). Additionally, there are studies, which demonstrate similar effects under the therapy with Fingolimod and Natalizumab (Skarica et al. 2011; Claes et al. 2014). In the current study, no significant changes in mature B cell frequencies under GA therapy could be found in both the cross-sectional and longitudinal analyses. As the changes observed in longitudinal samples tended to positively correlate with longer ongoing treatment duration, the results indicate that glatiramer acetate affects the mature B cells over time, in correlation with therapy duration. Taken together, these findings suggest that GA therapy shifts the B cell population toward less disease-promoting B cell subtypes.

4.3.3 Activated B cells

In the germinal center, naïve B cells interact with CD4⁺T cells via MHC II on their surface, presenting their specific antigen. This interaction leads to the activation and proliferation of B cells. This process results in an upregulation of several activation markers on the surface of B lymphocytes. CD27⁺ B cells were defined as activated B cells in this study. Activated B cells become short-living plasma cells or memory cells, after their clonal expansion in the germinal center (Hamel et al. 2012; Zhang et al. 2016). In the present study, there were no significant changes in the total amount of activated B cells in GA-treated patients as compared to untreated MS controls as well as in the percentage of activated B cells under GA therapy over time.

4.3.4 Memory B cells

There is growing evidence that memory B cells play a crucial role in the pathogenesis of MS. These cells are formed in the germinal center after naïve B cells interact with an antigen and undergo activation, proliferation and clonal expansion. In case of re-infection, memory B cells generate a more effective and accelerated antibody-mediated response (Airoidi et al. 2004). In the current study, memory B cells were defined as CD27^{var}CD38^{neg} cells. This gating strategy was also used in most other studies, investigating the role of memory B cells (Li et al. 2017). Recent studies suggest that memory B cells may be a major trigger for relapses in multiple sclerosis. It was found that current MS-treatments such a beta-interferons, rituximab/ocrelizumab, dimethyl fumarate, fingolimod or cladribine deplete memory B cells. Glatiramer acetate was shown to reduce the total amount of memory B cells (Bose 2017; Baker et al. 2017). In the current study, there were no significant changes in the percentage

of memory B cells in GA-treated patients neither compared to untreated MS patients nor over time under GA therapy.

4.3.5 Plasmablasts

Plasmablasts are precursors of antibody-producing plasma cells. Their development usually occurs in the blood. After 2-3 weeks, they either undergo apoptosis or differentiate into plasma cells. Although they can also produce antibodies, it is not as much as plasma cells do. Increased plasmablast numbers in blood are an indication for an active B cell response (Rivas et al. 2017). In MS patients the presence of plasmablasts is associated with an antibody-mediated pathology (Nakamura et al. 2015). It could be demonstrated that the frequency of plasmablasts correlated with MS activity according to MRI findings. In the blood of patients having their first clinical attack, plasmablast frequency was also elevated (Ligocki et al. 2013). Additionally, Rivas and colleagues could demonstrate for the first time that plasmablasts in the blood of untreated MS patients show high reactivity for proteins and cellular targets in the brain (Rivas et al. 2017).

GA was shown to reduce the total amount of plasmablasts within the PBMC pool (Ireland et al. 2014). The current findings are in line with these results. The frequency of plasmablasts was significantly reduced in GA-treated RRMS patients as compared to untreated MS controls as well as in the longitudinal GA therapy analyses. As it is considered that the amount of plasmablasts correlates with continuous antibody-mediated destruction and inflammation of the CNS (Avery et al. 2003; Kuerten et al. 2018), the present findings show that GA may prevent the antibody-associated destruction of myelin.

4.4 B cell activation

After the interaction of B cells with CD4⁺ T cells, B cells become activated and there is an upregulation of specific surface molecules. These surface molecules are called activation markers. Although activated B cells play an important role in the pathophysiology of multiple sclerosis, the effect of GA on the surface proteins of activated B cells is poorly investigated. Kala and colleagues observed reduced expression of CD80 and CD86 in GA-treated EAE mice (Kala et al. 2010). Another study demonstrated the reduced expression of CD40 molecules on the surface of B cells under GA therapy in humans (Chen et al. 2016).

In the current study, eight activation markers were included and directly analyzed ex vivo or after restimulation with CPG or LPS. CD40, CD80 and CD86 are representing specific costimulatory activation, whereas CD25, CD27, CD69, CD95 and CD150 are demonstrating an unspecific activation. No significant changes in expression of all parameters were observed in GA-treated patients as compared to untreated RRMS controls. However, the

expression of CD25 and CD69 was significantly downregulated in the longitudinal analyses of GA-treated patients.

CD25 is an α -chain of the IL-2 receptor. CD25⁺ B cells were shown to trigger CD4⁺ T cell proliferation in mixed cultures. Furthermore, CD25⁺ B cells represent a more activated and mature stage compared to CD25⁻ B cells (Amu et al. 2007). Brisslert and colleagues suggested that CD25⁺ B cells could contribute to antigen-presenting functions and could play a role in bridging innate and acquired immune responses. As a blockade of CD25 expression on B cells leads to disturbances in T cell activation, it was considered that CD25⁺ B cells play a major role in T cell activation (Brisslert et al. 2006; Amu et al. 2006). Furthermore, it could be demonstrated that in patients with rheumatoid arthritis the CD25⁺ B cells were enriched with EBV and might be an important source of this virus (Brisslert et al. 2013). As EBV may be involved in the pathogenesis of MS (Rizzo et al. 2016; Fernández-Menéndez et al. 2016; Guan et al. 2019), a downregulation of CD25⁺ B cells could be an advantageous effect of GA therapy.

Dendritic cells are antigen-presenting cells, which play a crucial role in the innate immune system. Several studies demonstrated that dendritic cells play an important role in the pathogenesis of multiple sclerosis (Weber et al. 2004; Swirski et al. 2009; Kala et al. 2010). There is evidence that B-cell induced activation of dendritic cells depends on the expression of CD69 on the surface of B cells (Maddur et al. 2014).

Taken together, these findings reveal an anti-inflammatory effect of GA-therapy, including the downregulation of CD25 and CD69, which was not discussed in the literature before.

4.5 Antigen-presenting functions of B cells

B cells are potent antigen-presenting cells (APCs). In particular, B cells are able to specifically bind antigens through their B cell receptor (BCR) and induce antigen-specific activation of T cells through the presentation of antigens. Several studies point out that B cells may play their crucial role in the pathogenesis of MS through their antigen-presenting role (Ireland et al. 2012; Bakshi et al. 2013; Sellner et al. 2013; Rovituso et al. 2015). B cells are able to perform their antigen-presenting function only in a complex with the major histocompatibility complex MHC II on their surface. Jackson and colleagues could demonstrate direct binding of GA to the B cells receptor (BCR) of murine and human B cells (Sean Selva 2014). There is evidence that the interaction of GA with the BCR may be a central mechanism of action of this drug. It could be demonstrated that the efficacy of GA therapy was abolished in mice with transgenic BCRs (Sean Selva 2014).

In the present study, there was a significant upregulation of MHC II expression on the surface of B cells in GA-treated patients compared with untreated RRMS controls after CpG restimulation. The present finding is in line with other studies showing that GA leads to the

clustering of MHC II molecules on the surface of B cells (Fridkis-Hareli et al. 1997). However, no significant changes were observed in MHC II expression on B cells in the longitudinal samples. Furthermore, there was a trend after stimulation with LPS, as 5 of 4 GA-treated patients showed a reduced expression of MHC II on the surface of monocytes compared to untreated controls. These findings are in line with the results of Li and colleagues, demonstrating a down-regulation of the expression of MHC II molecules on monocytes upon GA therapy (Li et al. 1998).

Taken together, these results suggest that the major targets of glatiramer acetate are the antigen-presenting function of B cells and the regulation of the activation of other APCs through B cells. In fact, these findings are in line with other investigations demonstrating that GA inhibits the activation of antigen-presenting cells (Weber et al. 2004; Sellner et al. 2013; Carrieri et al. 2015).

4.6 Cytokine production

There is evidence that B cells may play their crucial role in the pathogenesis of multiple sclerosis through their cytokine production (Bar-Or et al. 2010; Sean Selva 2014; Claes et al. 2015). Some studies show that therapy with glatiramer acetate causes a shift toward more anti-inflammatory cytokine production in B cells (Begum-Haque et al. 2010; Begum-Haque et al. 2011; Ireland et al. 2012; Sean Selva 2014).

Interleukin 6 is a pro-inflammatory cytokine and plays an important role in the pathogenesis of multiple sclerosis, as it leads to an increased differentiation of inflammatory Th17 cells (Li et al. 2017). Furthermore, it was shown that the production of IL-6 contributes to functional hyperactivity in APCs (Ireland et al. 2015). Several studies showed that GA reduced the total amount of IL-6 positive B cells (Sean Selva 2014; Ireland et al. 2014). In the present study, there were no significant changes in the frequencies of IL-6 positive B cells within the PBMC pool upon GA therapy, in both the cross-sectional and longitudinal analyses. It has been considered that suppressed secretion of IL-6 in GA-treated patients is a result of increased IL-10 secretion (Ireland et al. 2015). Since in the current study no significant changes in the IL-10 secretion levels (see below) could be demonstrated, it seems to be expectable that there were also no changes in the IL-6 production. Furthermore, there is evidence that GA therapy causes only a transient reduction in the IL-6 production of B cells during the first 32 months of therapy following a level indistinguishable to untreated controls at later time points (Ireland et al. 2014). In the present study the mean GA therapy duration was 5.9 ± 4.1 months covering early stages of GA modulation, which was possibly before the transient reduction of IL-6 secretion.

Interleukin 10 is an anti-inflammatory cytokine. It plays an important role in the suppression of inflammatory processes in multiple sclerosis. IL-10 inhibits the production of pro-

inflammatory cytokines such as IL-6, IL-12, IL-18, etc. In-EAE, a selective knockout of IL-10 in B cells was associated with severe disease progression (Bar-Or et al. 2010). Kala and colleagues demonstrated that IL-10 produced by B cells downregulated EAE severity. (Kala et al. 2010). In humans several studies showed an increase in IL-10-production in B cells after IFN- β , glatiramer acetate, fingolimod, and rituximab therapy (Aharoni 2013; Longbrake und Cross 2016). In the current study, no significant changes could be observed in the frequency of IL-10 positive B cells of GA-treated MS patients as compared to MS controls, which is in line with recent findings by Ireland and colleagues (Ireland et al. 2014).

TNF- α is also a pro-inflammatory cytokine and seems to play an important role in the pathogenesis of multiple sclerosis. TNF- α may induce apoptosis, cell proliferation, cell differentiation or production of other cytokines in the immune system. Several studies demonstrated that the frequency of TNF- α -positive B cells was diminished in GA-treated patients in comparison to untreated RRMS controls (Begum-Haque et al. 2010; Ayers et al. 2013; Sean Selva 2014). Jackson et al. demonstrated reduced concentrations of TNF- α in 50 % of GA-treated MS patients compared to untreated controls (Dooley et al. 2016). The findings in humans could be confirmed in EAE mouse models. B cells of GA-treated EAE mice showed reduced production of TNF- α (Begum-Haque et al. 2010; Ireland et al. 2014). The present study revealed decreased frequencies of TNF- α positive B cells in the longitudinal GA therapy analyses. However, no significant changes were observed when GA-treated MS patients were compared to untreated MS controls. Other approved drugs for the therapy of MS, such as fingolimod, dimethyl fumarate, and mitoxantrone were also shown to decrease TNF- α production in B cells (Longbrake and Cross 2016; Li et al. 2017). Taken together, the current findings suggest that GA may diminish inflammatory effects in MS through the reduction of pro-inflammatory cytokine production by B cells.

5 Summary and Outlook

Glatiramer acetate was approved for the treatment of RRMS in 1996. It is known to reduce relapse rates and disability progression. In some rare cases, it can cause injection site reaction and in some cases flu-like symptoms. Despite of approval since 1996, the exact mechanism of action of GA is still not clearly understood. Historically, GA has been considered to be a T-cell-targeting therapy. Several investigations of recent years demonstrated that GA affects B cells. The aim of the current study was to gain a deeper understanding of the effect of GA on functional properties of B cells. To evaluate the effects of GA, flow cytometry (FACS) analysis of peripheral blood mononuclear cells (PBMC) was performed. The samples were obtained from 40 RRMS patients, 22 of which had been treated with GA, and 18 were untreated controls. Both cross-sectional and longitudinal experiments were performed. Four acquisition panels for cell distribution, B cell subsets, activation markers, cytokine production, and antigen presentation were used.

The current study could confirm the huge effect of GA on B cells. The major target of glatiramer acetate seems to be the transitional B cells and plasmablasts. This effect of GA on B cell subsets had been reported before.

Moreover, in the current study, it could be demonstrated that activation markers, such as CD25 and CD69, were significantly reduced in longitudinal experiments under GA therapy. Since there are no studies describing similar effects of GA therapy, the results of the current study might be very important for future therapy strategies of RRMS. Results of Phase II and III clinical trials demonstrated that therapy with Daclizumab, a monoclonal antibody blocking CD25, was very efficient in reducing the relapse rate and T2 hyperintense MRI lesions in RRMS patients compared with placebo and IFN- β -1a (Papadopoulou et al. 2017). However, in March 2018, Daclizumab was withdrawn from the market after reports of encephalitis. Since then there has actually been no anti-CD25 directed therapy in clinical use. As GA significantly downregulated the expression of CD25 on B cells in the current study, this drug could be used as a follow-up therapy after stabilization of MS patients initially treated with very potent MS drugs with serious side effect profiles.

CD69 is an early activation marker and is involved in lymphocyte migration. Several investigations in mice demonstrated that the anti-CD69 monoclonal antibody treatment had antitumor effects and could potentially be used in the treatment of carcinomas (Esplugues et al. 2003; Wei et al. 2017). There is also evidence that anti-CD69 monoclonal antibodies could have a positive effect on rheumatologic diseases (Sancho et al. 2006). As the current study demonstrated a significant downregulation of CD69 expression on B cells upon GA therapy, it could be considered to choose GA primarily for the treatment of MS patients additionally suffering from cancer or rheumatologic diseases.

In the current study, the expression of MHC II, the most important surface molecule for antigen presentation, on B cells in GA-treated patients was significantly higher in comparison

to untreated RRMS controls. These findings are not surprising, since it has been suggested that after interaction with the B cell receptor (BCR), GA is internalized and then presented by MHC II to regulatory CD4⁺ T cells.

The second striking point was the shift to an anti-inflammatory cytokine production in B cells. No changes could be demonstrated in both IL-6 and IL-10 production. As it is known that changes in the cytokine production under therapy may be transient, it is possible that in the current study these transient changes could not yet be determined. However, a significant decrease in TNF- α secretion by B cells in GA-treated patients compared to untreated controls could be demonstrated.

In conclusion, the current study provokes questions that have to be answered in a larger cohort of longitudinal samples. The following aspects may be relevant for future MS treatment: more detailed understanding of the GA effect on antigen presentation, GA as a follow-up therapy after stabilization of MS patients initially treated with very potent MS drugs with serious side effects, use of GA in the treatment of MS patients additionally suffering from cancer or rheumatological diseases, determination of transient changes in cytokine production under GA therapy, and understanding the role of B cell activation markers in the pathogenesis of MS.

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Curriculum Vitae

My name is Zivar Hajiyeva. I was born on September 20, 1991, in Baku, Azerbaijan, as the first of two children. My father is an engineer and my mother is a teacher.

In 2008, I graduated from the School N:175 in Baku. At the age of 16, I entered the Azerbaijan Medical University (University entrance examination score 614/700) and studied there until 2010. In 2010, I was awarded an Azerbaijan National Scholarship and was transferred to the Medical Faculty of Istanbul University in Turkey. In 2013, I obtained an Erasmus Mundus Scholarship and was given the opportunity to do my internship at the Humboldt University in Berlin. In 2014, I graduated from Istanbul University and gained my degree of Doctor of Medicine (MD). From December 2014 to May 2016, I worked as a neurology resident at the Martin-Luther University, Halle-Wittenberg. In November 2015, I received a license to practise medicine in Germany. Since July 2017, I have been working at the Department of Neurology at the Georg-August-University of Göttingen as a resident neurologist. I speak Russian, Turkish, Azerbaijani and German fluently and have an excellent command of English.

