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DISSERTATION

Cytokine Patterns of T helper cells in Healthy Donors and Patients with Multiple Sclerosis

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“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”

Marie Curie

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IV. List of Abbreviations

AF	Alexa Fluor
AF750	Alexa Fluor 750 succinimidyl ester
AP1	Activator protein one
APC	Antigen-presenting cell
BFA	Brefeldin A
BP	Bandpass filter
Bregs	B-regulatory cells
BSA	Bovine serum albumin
BUV	Brilliant ultraviolet
BV	Brilliant violet
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
CI	Confidence interval
CIS	Clinically isolated syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCR	C-X-C chemokine receptor type
DAPI	4’,6-Diamidine-2-phenylindole, 4’,6-Diamidine-2-phenylindole
DMF	Dimethyl fumarate
DMSO	Dimethyl sulfoxide
EAE	Experimental Autoimmune Encephalitis
EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
FA	Formaldehyde
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FSC	Forward scatter
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
HLA	Human leukocyte antigen
IL	Interleukin

INF-γ	Interferon gamma
JAK	Janus kinase
LP	Longpass filter
mAB	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MBP	Myelin basic protein
MFI	Mean flourescence intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	N-hydroxysuccinimide
NK cells	Natural Killer cells
Nrf2	Nuclear-Factor-(Erythroid-derived-2)-like-2
NSCLC	Non-small-cell lung cancer
PacB	Pacific blue
PacO	Pacific orange
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PI3-kinase	Phosphatidylinositide-3-kinase
PKC	Protein-kinase-C
PMA	Phorbol-12-myristate-13-acetate
PPMS	Primary-progressive Multiple Sclerosis
PRI	Pattern Recognition of Immune Cells
RA	Rheumatoid arthritis
RPMI	Roswell Park Memorial Institute
RRMS	Relapsing-remitting Multiple Sclerosis
SLE	Systemic lupus erythematosus
SPMS	Secondary-progressive Multiple Sclerosis
SSC	Sideward Scatter

STAT	Signal Transducers and Activators of Transcription
Tcm	Central memory T cells
TCR	T cell receptor
Te	Effector T cells
Tem	Effector memory T cells
Th	T helper
Tmem	Memory T cells
Tn	Naïve T cells
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells

V. Abstract

The activation of autoreactive T helper cells (Th cells) plays an essential role in the pathogenesis of demyelinating chronic autoimmune diseases, such as Multiple Sclerosis (MS). Uncovering the molecular mechanisms of these cells, by analyzing cytokine patterns and surface activation markers, therefore, provides an important insight into the activated T cell repertoire and consequently helps to unveil auto-immunological mechanisms.

In this study, CD4+ T cells obtained from 16 healthy donors and 20 MS patients were analyzed according to their memory features, expression of immune checkpoint receptors and cytokine profiles in general and relating to disease-associated changes. Complex relationships in the modulation of cytokine production were displayed with the help of a novel bioinformatic approach for “pattern recognition of immune cells” (PRI) developed in our group. Cytokine production in different Th subsets was analyzed, as well as patterns in cytokine co-producing memory T cells.

We found that IFN-ɣ, IL-10 and IL-21 frequency in memory T cells were increased with PD-1 upregulation whereas IL-2, IL-22 IL-17, GM-CSF, TNF-α showed no direct correlation with PD-1 expression. In terms of cytokine production, flow cytometric data revealed elevated frequencies of IL-10 producing effector memory T cells (Tem) in MS patients compared to healthy donors. Furthermore, an increased frequency of the OX40+ Th cells was found in MS patients. A subset of CD20+ T cells was identified that showed increased cytokine production indicating polyfunctional capacities. However, frequencies of CD20+ T cells in the blood of MS patients were comparable to those in healthy donors. We demonstrated the occurrence of a variety of complex Th cell subsets with memory and activation features and proposed PRI as a viable tool to characterize these subpopulations.

With MS being an extremely heterogeneous disease, frequencies of circulating T cells and cytokine-producing subsets showed significant spreading and did not generally correlate with disease status. Still, the analysis of cytokine profiles as well as surface receptor analysis provides important insight into the complex pathogenic mechanism in MS.

VI. Zusammenfassung

Autoreaktive T-Helferzellen und deren Aktivierung spielen eine wesentliche Rolle bei der Pathogenese chronisch-demyelinisierender Autoimmunerkrankungen, wie z.B. der Multiplen Sklerose. Um die zugrunde liegenden molekularen Mechanismen besser zu erkennen und zu verstehen, wurden in dieser Arbeit kombinatorische Zytokinmuster und Oberflächenaktivierungsmarker analysiert.

Es wurden CD4+ T-Zellen von 16 gesunden Spendern und 20 MS-Patienten bezüglich der Expression von Gedächtnismarkern, Immun-Checkpoint-Rezeptoren und Zytokinprofilen von T-Helferzellen im Allgemeinen, sowie im Hinblick auf krankheitsassoziierte Veränderungen analysiert. Komplexe Zusammenhänge in der Modulation der Zytokinproduktion wurden mit Hilfe eines in unserer Gruppe entwickelten neuartigen bioinformatischen Ansatzes zur „Mustererkennung von Immunzellen" (PRI) dargestellt. Die Zytokinproduktion in verschiedenen T-Helfer-Zell-Subpopulationen, sowie Muster in Zytokin-koproduzierenden Gedächtnis-T-Zellen wurden analysiert.

Es konnte gezeigt werden, dass die Frequenzen von IFN-ɣ+, IL-10+ und IL-21+ T-Zellen mit der Expression von PD-1 positiv korrelierten. Weiterhin konnte eine Erhöhung der Frequenz von IL-10 produzierenden T-Zellen bei Patienten mit Multipler Sklerose im Vergleich zu gesunden Spendern beobachtet werden. Darüber hinaus wurde bei den untersuchten Patienten eine erhöhte Frequenz der OX40+ T-Helferzellen festgestellt. Die Analyse der CD20+ T-Zellen zeigte eine erhöhte Zytokinproduktion und Aktivierungsmerkmale und weist somit auf polyfunktionale Kapazitäten dieser Zellen hin. Die Häufigkeit von CD20+ T-Zellen bei MS-Patienten war jedoch mit der von gesunden Spendern vergleichbar.

Diese Arbeit zeigt das Auftreten einer Vielzahl von komplexen T-Helfer-Zell-Subsets mit ihren Zytokinproduktionen und Aktivierungsfunktionen auf und präsentiert PRI als neues und hilfreiches Werkzeug zur Charakterisierung multifunktionaler Zell-Subpopulationen. Die Arbeit liefert insgesamt wichtige Einblicke in die komplexen Aktivierungsmechanismen von T-Zellen, die unter anderem bei der Pathogenese der Multiplen Sklerose eine wichtige Rolle spielen.

1 Introduction

1.1 Immunological Background

The human immune system forms the basis for the defense against the variety of pathogens or common microorganisms potentially invading and attacking the host's integrity. The two main subsystems working together, as pillars for the immunological defense, are known as the innate- and the adaptive immune system.

The innate immune system is characterized by its rapid response capabilities. Common features of pathogens can be detected via invariant receptors and consequently an immediate response is initiated. Involved in this response is the secretion of cytokines and chemokines as well as a variety of innate leukocytes i.e., dendritic cells, phagocytic cells, natural killer cells (NK cells), and the complement cascade. A main important function of the adaptive immune system is the formation of immunological memory. Necessary to that end is the adaptive immune system, also known as the specific immune system. It has the ability to generate differentiated memory lymphocytes through clonal selection, leading to antigen-specific effector cells and thus an enhanced and more effective response upon antigen re-encounter. Both innate and adaptive immune systems comprise features of humoral as well as cellular defense mechanisms.

T cells play a crucial role in both of these branches of the adaptive immune system, especially in the context of cell-mediated immunity. The T cell receptor (TCR) recognizes antigens or peptide fragments presented by major histocompatibility complex (MHC) molecules (1). Cytotoxic T cells then directly eliminate intracellular pathogens whereas extracellular pathogens are combated via activation of B cells and macrophages by Th cells (2). The majority of T cells in humans are termed αβ-T cells for the αβ chain on the TCR; these will be the ones regularly mentioned in this work. Furthermore, there is a small subset, the ɣδ-T cells, with a more limited TCR repertoire. These cells are mainly located in the epithelia, e.g., of the skin and intestine, and differ significantly in their development and function from αβ-T cells. They are commonly more closely classified as part of the innate immune system (3, 4). T cells not only play a central role in mediating an appropriate immune response but can also be involved in the pathogenesis of inflammatory autoimmune diseases such as MS when the immune system is dysregulated and immunological self-tolerance cannot be maintained.

1.1.1 T cell Activation and Regulation

Antigen-Specific Activation

To activate a naïve T cell in the first place, an antigen-specific signal is required. This consists of the interaction of the respective TCR with the MHC complex of the antigen-presenting cell (APC) (1). After a successful TCR antigen recognition the activated T cell activates transcription factors like nuclear factor of activated T cells (NFAT), activator protein one (AP1) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (5), which are essential to IL-2 transcription and which in turn induce T cells proliferation by clonal expansion (6). Furthermore, the APC will initiate at least two more signals, essential to successful T cell activation and differentiation. For one thing, a costimulatory signal is needed to ensure survival and expansion of the T cell (costimulatory signals are further detailed under 1.2.1) and second, a signal to control differentiation into a specific effector T cell subtype. This applies in particular to CD4+ T cells. This differentiation is mainly, albeit not exclusively, driven and regulated by cytokines (7). Different transcription factors of Signal Transducers and Activators of Transcription (STATs) function as gene expression promoters towards specialized effector Th and regulatory T cells (Tregs) (8). Individual subsets are aimed at distinct response tasks like e.g., pathogen types, characterized by master transcription factors and signature cytokines (Figure 1).

1.1.2 Cytokines in T cell Activation and Differentiation

All effector T cells (Te) produce cytokines. Cytokines are defined as ‘proteins that are released by various cells in the body, [...] and they induce responses through binding to specific receptors (9). Their general function can be summarized as the activation and stimulation of other T cells, B cells, and macrophages, hematopoietic and other somatic cells. They are a structurally diverse group and specific effects depend and vary according to the respective target. In synergy with other effector molecules, combinatorial functions can be achieved. These effects can cause an impact either locally in an autocrine or paracrine manner, as well as in the various peripheral tissues, in an endocrine manner (7). The cytokines play a central role in regulating the differentiation of the Th cell spectrum, into the diverse subtypes, including Th1, Th2, Th3, Th9, Th17, Tregs or T follicular helper cells (Tfh). Interleukin-12 (IL-12), for example, induces Th1 cells. In contrast, the influence of Interleukin-4 (IL-4) causes the

induction of Th2 (Figure 1) (10). Subtypes show a certain degree of plasticity that allows differentiation from one type into another.

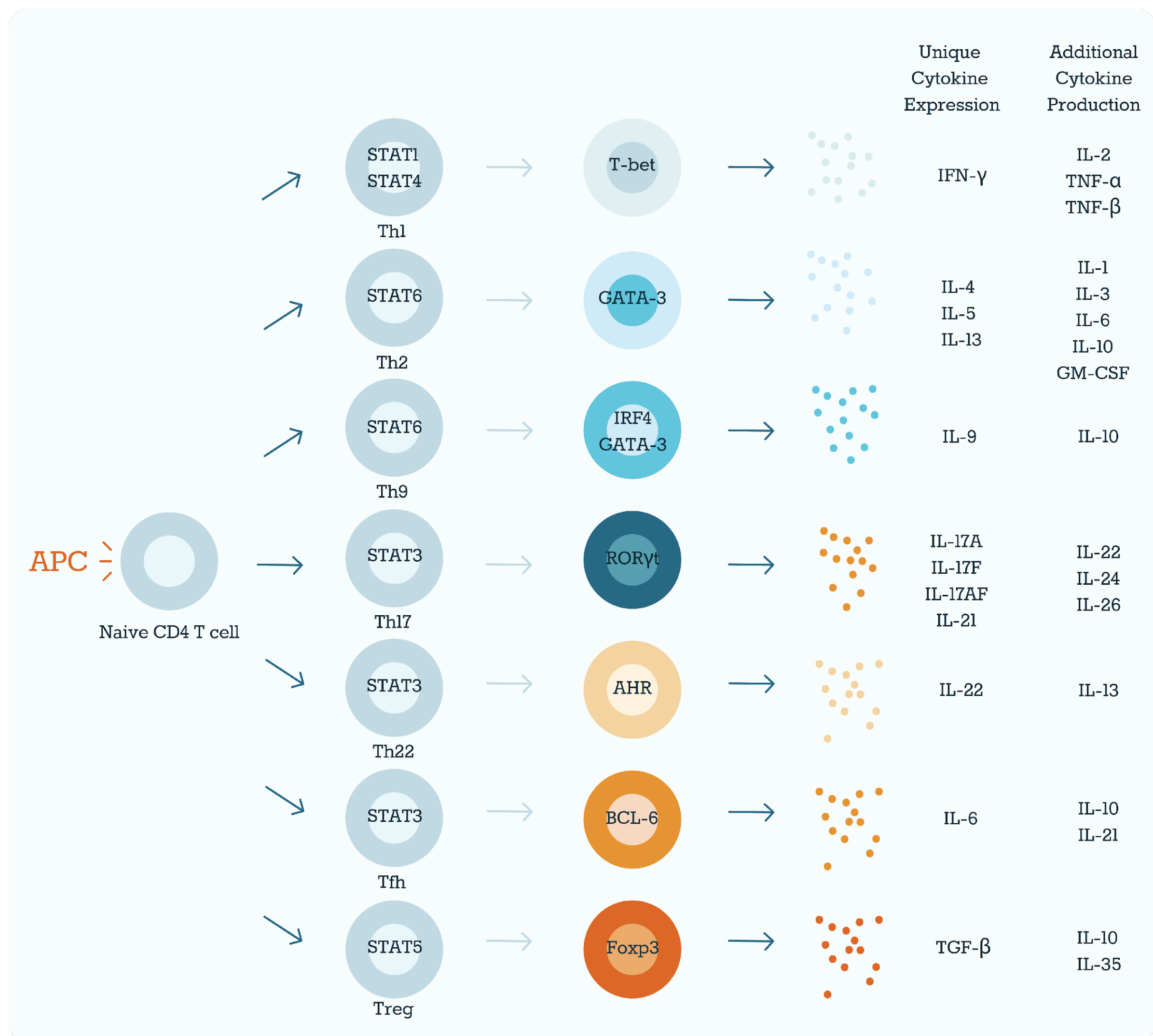

Figure 1: Th Plasticity

APCs transmit antigen-specific activation signals. Subsequently, naive CD4+ T cells are differentiated into different effector Th and Treg subtypes. These are characterized by lineage-specific properties. Figure 1 shows the differentiation of the different subgroups, driven by individual transcription regulators (STATs), as well as the key transcription factors and unique cytokines primarily secreted by the respective cell type. Apart from these, additional cytokines can be secreted by each subset as indicated in the column on the right.

Not only do cytokines drive the physiological T cell differentiation process but they also have important implications in pathological inflammatory mechanisms. INF-γ for example, which is produced by Th1 cells, has been known to play a major role in autoimmune diseases such as MS (11) or Diabetes mellitus (12).

Multi-cytokine-producing virus-specific CD4+ T cells are functionally superior to single-cytokine-producing T cells. Among the triple producers, more cells express the activation marker CD40L. They exhibit a higher degranulation potential as well as a higher level of cytokines per cell (13). All these features are indicators of higher functional capacity of multi-cytokine-producers within the CD4 repertoire. Therefore, they play a relevant role in the genesis of infectious and autoimmune diseases and make for an important target of diagnostic and therapeutic approaches.

Effector Function

IL-2 induced proliferation of T cells eventually leads to differentiation into effector cells after approximately 4 to 5 days of circulation. These now do not require a costimulatory signal for activation, however the activation threshold will be lowered by the presence of co-stimulatory molecules. This applies to the helper and cytotoxic T cells equally. An effector T cell also has a modified repertoire of surface molecules, such as cell adhesion molecules and receptors. In contrast to naïve cells, there are for example no receptors for L-selectin or CD45RO on activated effector cells. However, lately they do express receptors for integrin VLA-4 and CD45RA.

CD4+ T cells then go on to differentiate into a variety of functionally different effector cells. The first ones to be described were Th1, Th2 and were therefore named accordingly. Other subsets are named for their signature cytokine, like Th17 cells, that are known for secreting IL-17, or for their functions, like Tfh cells, that provide help to B cells within the lymphoid follicles and Tregs that downregulate T cell activation. In this, the priming APC and the local environment (cytokines, etc.) during activation have a determining influence on the fate of the individual progenies of a naïve T cell.

1.1.3 Immune Checkpoint Receptors

Even after primary selection within the thymus, a small number of autoreactive T cells may remain undetected, then go on to circulate peripherally and therefore potentially lead to autoimmune reactions. To modulate the complex immune pathways, checkpoints are needed to enhance or moderate the variety of signals in order to warrant a more differentiated host response. Figure 3 shows a selection of checkpoint markers with corresponding ligands and their effects that are necessary to ensure an appropriate response while maintaining host integrity.

1.1.3.1 Stimulatory Checkpoint Molecules

Earlier theories concerning immune checkpoint receptors assumed two steps toward successful activation of naïve T cells via two consecutive signals, typically from the same APC. The first interaction entails the interaction between the antigen-MHC-complex and the TCR. Afterward, the second step is a costimulatory signal mediated by specific receptors expressed respectively on APCs and T cells. A classic example of this would be the binding of CD80 or CD86, surface proteins from the B7 family to the costimulatory TCR CD28 on T cells (14). If no costimulatory signal follows the initial TCR activation, the consequence will be T cell anergy. T cell anergy is the mechanism by which the functional inactivation of lymphocytes after antigen contact occurs. In the presence of a costimulatory signal, however, phosphatidylinositide-3-kinase (PI3-kinase) is activated and amplifies synthesis or activation of IL-2 and high-affinity IL-2 receptors (6, 15). Since IL-2 drives proliferation, this cascade enhances cell growth and survival. Therefore, only an antigen-specific signal followed by the costimulatory cascade will lead to T cell activation. This ensures an appropriate immune response and acts as a safeguard against autoimmunity. This theory was expanded with the discovery of the co-inhibitory signals (1.1.3.2). **OX40 (CD134)** is one of the key costimulatory molecules involved in controlling T cells besides the-CD28 pathway. OX40 and its Ligand OX40L or CD252 are members of the TNFR/TNF superfamily. OX40 can be expressed on activated cytotoxic and Th cells but is not found on resting naïve T cells or most resting memory T cells. OX40L can be induced on professional APC such as B cells, dendritic cells, and macrophages. The primary function of an OX40 signal is the induction of cell division and survival in T cells, which in turn leads to clonal expansion of effector and memory populations (Figure 2). Furthermore, suppression of Tregs has been described as an effect of OX40 signaling. A secondary function of OX40 and OX40L is also the regulation of cytokine production from T cells, APCs, NK cells, and NKT cells. After the effector-phase response OX40 is downregulated to baseline levels. Subsequently, in the event of a re-encounter with the antigen, it can be rapidly re-expressed on memory T cells (16).

OX40/OX40L interactions have been shown to have critical involvement in the development of a variety of inflammatory and autoimmune diseases (17). In other words, in active white matter lesions of MS patients an upregulation could be observed

(18). Additionally, the treatment with OX40L monoclonal antibodies (mAB) or deleting OX40+ myelin-reactive T cells led to the regression of the disease (18, 19).

Therefore, blocking the OX40 ligand holds great potential in managing autoimmune disease while further investigation into the receptor is needed to evaluate potential risks and benefits of its medical use.

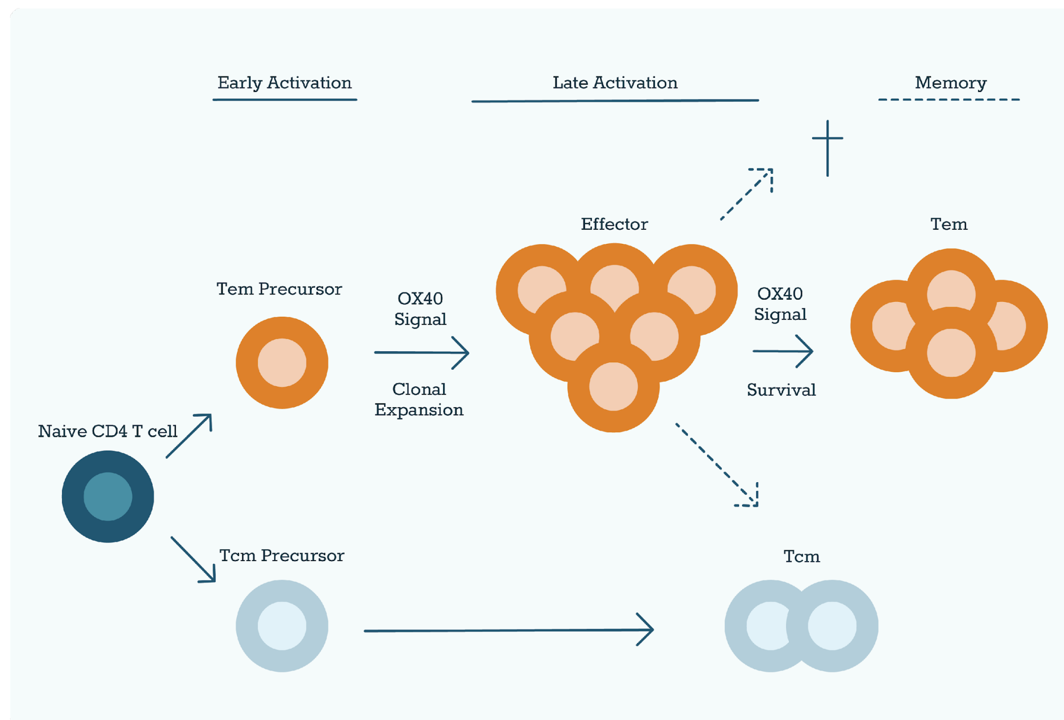

Figure 2: Role of OX40 Signaling in T cell Differentiation

OX40 affects the size of the Tem cell pool. OX40L binds to OX40 on recently activated naïve T cells. The OX40 signal induces clonal expansion and promotes the survival of Tem precursors. Tcm precursors develop independently of OX40. OX40 is therefore crucial for the CD4+ T cell memory, as Tem generally predominates.

1.1.3.2 Inhibitory Checkpoint Molecules

Inhibitory signals are mediated by co-inhibitory receptors that act as immune checkpoints and make a significant contribution to maintaining immune tolerance. One of the most important representatives is the B7-1/B7-2- CD28/CTLA-4 pathway, essential for detecting potentially autoreactive T cells and ensuring adequate activation (20). **CTLA-4** (Cytotoxic T-lymphocyte-associated protein 4) is generally expressed on Tregs and gets upregulated in conventional T cells only after activation. CTLA-4 has a higher binding affinity to B7 molecules CD80 and CD86 than CD28 (21) and since CD28 transmits a stimulatory signal but CTLA-4 an inhibitory one, both molecules compete for the same receptors, ultimately achieving a down-regulatory effect. Therefore, CTLA-4 can potentially inhibit T cell activation in the absence of CD28 (22).

PD-1 Programmed Cell Death Protein 1 (CD279) and its ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are members of the B7/CD28 family that function as negative immune regulators and are mainly expressed on the surface of activated T cells (23). Initially named for its involvement in programmed cell death (24), the complexity of PD-1 pathway functions is an ongoing subject of investigation. By initiating a co-inhibitory signal on T cells, they play a key role in mediating T cell response. PD-1 attenuates TCR signaling by influencing the cell cycle through PI3-kinase/Akt and Ras/MEK/Erk pathways (25). Apart from inhibition of self-reactive T cells, PD-1 is also associated with Treg induction, thereby playing a key role in maintaining immune homeostasis. Transient PD-1 upregulation is a sign of T cell activation, whereas sustained expression points to T cell exhaustion (26). During chronic viral infection expression of coinhibitory receptors is linked to disease severity (26). Ishida et al. contributed to understanding the PD-1 function by showing, that PD-1 deficient mice developed an autoimmune phenotype, (arthritis, splenomegaly, and glomerulonephritis), albeit with delayed onset (24, 27). PD-1 pathways have been shown to play a crucial role in transplantation, autoimmunity and tumor immunity.
Recently, the Nobel Prize for Medicine or Physiology award in 2018 drew widespread public attention to this topic and highlighted the importance of research into these structures. James P. Allison was honored for showing that the in vivo administrations of CTLA-4 antibodies induced an anti-tumor response leading to tumor regression (28) and Tasuku Honjo for isolating the PD-1 gene and demonstrating its involvement in the classic type of programmed cell death (24). Within the context of tumor research, groundbreaking insights have been gained in this regard, but in immunology, particularly in the complex interaction of cytokines, detailed data are lacking. Detailed new insights into the role of PD-1 in MS pathogenesis, therefore, offer promising clinical approaches, especially considering the successful therapeutic application of PD-1 modulation in tumor therapy.

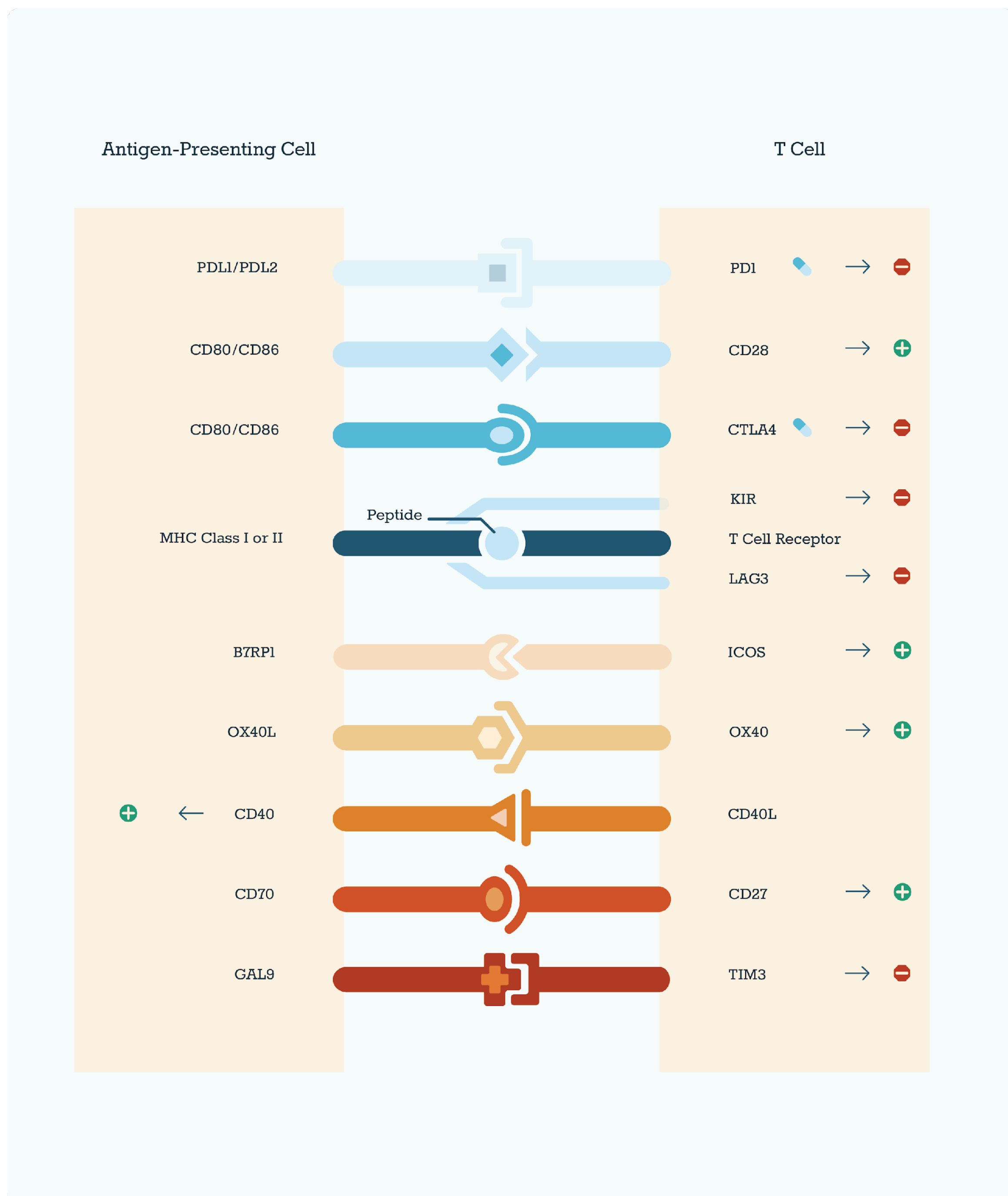

Figure 3: Checkpoint Molecules in T cell Immunology

The Figure shows various ligand-receptor interactions between T cells and APCs that regulate the T cell response to antigen. The MHC-peptide complex is shown at the center. TCR - MHC interaction is the prerequisite for any other signal to trigger a signal cascade. Examples of both co-stimulatory signals and inhibitory signals are shown. Ligands can also bind to opposing receptors. Examples are CD28 and cytotoxic T lymphocyte-associated antigen 4 (CTLA4). Typically, the co-stimulation receptor is expressed on naïve and resting T cells, but the inhibitory receptor is often upregulated after activation of the T cells. The communication between T cells and APCs is bidirectional, which means that the ligands themselves can also transmit a signal to the APC. This is the case, for example, with the upregulation of CD40L, which stifles receptors on APCs. Checkpoint pathways marked with pill symbols can currently be pharmacologically influenced by checkpoint inhibitors already on the market and have been approved for application.

1.1.4 Human CD3+ CD20+ T cells

Human T Lymphocytes have been known to partially express the CD20 Antigen for quite some time (29). Initially, these findings have been disregarded as artifacts or contamination and were controversially discussed. However, it was proven that they do not express CD19 but instead αβ or ɣδ TCRs (30). They have increasingly become a focus of interest, especially in the context of autoimmunity, while their function remains largely unclear.

Of all circulating human T cells, 3-5 % express CD20 on their surface and transcribe both CD3 and CD20. Functionally, increased frequencies of IL-4-, IL-17-, INF-γ, and TNF-α-producing cells could be detected, compared to T cells that do not express CD20. They have been found not only in peripheral blood, but also within the thymus, bone marrow, and secondary lymphatic organs. They could also be detected in cerebrospinal fluid (CSF) even in the absence of inflammation (31).

In conclusion, they can be described as a cytokine-producing T cell population with some type of memory features but of yet unclear significance.

Human CD3+CD20+ T cells in Autoimmunity

In Rheumatoid Arthritis (RA) patients a population of cytokine producing terminally differentiated CD20+ T cells has been described. Though not elevated in frequency compared to healthy individuals, it was shown that Rituximab also depleted them during therapy (32). In RA patients (32, 33) they tend to display more of a Th17 phenotype compared to healthy controls which in comparison had a rather Th1 like phenotype (33).

In psoriasis patients, a positive correlation was found between the levels of cytokine production (IL-17A, TNF-α, and IL-2) in CD3+CD20+ T cells and disease severity scores (34).

In MS patients their role has also not yet been clarified, although they could be detected in the blood of patients as well as in chronic white matter lesions, so that some type of pro-inflammatory role can be assumed (35).

1.1.5 Generation of Immunological Memory

Immunological memory forms the basis for an adaptive immune response. Secondary immune responses enable a significantly more effective and timely response upon prior engagement with an antigen. Memory B cells, primed by infection or vaccination for example have a higher antigen affinity and a higher level of MHC class II molecules and hence produce a quantitatively and qualitatively improved response thanks to their populating secondary lymphoid organs as well as the peripheral blood.

After a primary immune reaction, a distinct set of long-living T cells, with special characteristics in response to certain stimuli, regarding survival and also different surface proteins persist. These are termed memory T cells (Tmem). Since in contrast to B cells, the TCR does not undergo class-switching or somatic hypermutation for T cells the distinction naïve vs. memory was first made on the basis of the different CD45 isoforms (CD45 is needed for modulation of TCR signaling) expressed on the respective cell type, whereby RA is found on naïve and RO on memory T cells. A further subdivision can be made by the different expressions of the C-C chemokine receptor type 7 (CCR7) or CD197. CCR7 affects the lymph node homing potential and the T cells that express it are then called central memory T cells (Tcm) (CCR7+CD45RO+). After stimulation by an antigen, Tcm lose their CCR7 receptor and differentiate into effector memory cells (36, 37).

1.2 Multiple Sclerosis

MS is considered a chronic inflammatory, demyelinating autoimmune disease of the central nervous system (CNS) that over the course of the disease also shows neurodegenerative features. Characteristic is the relapsing course of the disorder with polymorphic, neurological symptoms although there are various different possible clinical courses. It is assumed that autoreactive T-helper cells are originally activated peripherally, which then migrate into the CNS, where autoantigens lead to reactivation and, in the course of time, to a local inflammatory reaction, which ultimately leads to demyelination. Pathomorphological correlates are demyelination foci in the CNS. Diagnosis is based on the revised MacDonald criteria that include clinical symptoms, typical lesions in MRI and detection of pleomorphic lymphocytosis and oligoclonal bands in the cerebrospinal fluid (38). The worldwide incidence is estimated to be around 2.5 million, of which 700 000 are affected in Europe (39). Often times MS

significantly impacts the quality of life, leading to considerable personal and societal burdens (40).

1.2.1 Etiology

While the distinct cause of the disease remains partly unresolved, different factors are believed to contribute. Epidemiological studies have shown, that susceptibility is influenced by a combination of genetical, as well as environmental factors (e.g. gender, early childhood infections, sun exposure). Increased disease risk has been linked to the human leukocyte antigen (HLA)-DRB1 gene (41) and the distinctly asymmetrical incidence with higher female affection, also points to some likely sex- or hormonally-linked causes. And as far as environmental factors, there are different viral candidates especially Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) under investigation (42). A proposed pathogenetic mechanism is the virus-induced molecular mimicry (43) which in turn enables demyelination (44).

1.2.2 Pathomechanisms

It is assumed that besides B-cells, activated microglial cells and macrophages, T cells in particular play a central role in the immunopathogenesis of MS. In this case, the cells are autoreactive Th cells. Initially circulating naïve T cells will interact with APCs presenting cognate antigens. Well described are i.e., myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) two myelin sheath proteins known to induced antibody production with a central role in demyelinating diseases. The activated Th1 and Th17 cross the blood-brain barrier into the CNS, where a reactivation occurs. Cytokines crucially contribute to the development of autoimmune neuroinflammation, by enabling the lymphocyte invasion and loss of integrity of the blood-brain barrier. Subsequently, cytokine-and chemokine mediated excitation of B cells leads to clonal expansion and consequently differentiation of microglial cells to immune effector cells occurs (11). The inflammatory cascade is initiated; macrophages and antibodies attack myelin sheaths. Preferred predilection sites for lesions are juxtacortical and periventricular mainly white but also grey matter, brainstem, cerebellum, and the spinal cord. Localized inflammatory damages (inflammation) result in loss of neuronal myelin sheaths (demyelination) and axonal and neuronal damage (neurodegeneration). This cascade of events leads to permanent neurodegenerative damage with disturbed neuronal conduction, which in turn will lead to the multitude of MS-related symptoms

1.2.3 Immunological Mechanisms in MS

Autoantibodies against myelin sheath antigens, like anti-MBP or anti-MOG circulate within the peripheral blood and the secondary lymphoid organs, like the lymph nodes and activate antigen-specific T cells. These recirculate and then, with the help of inflammatory mediators migrate across the blood-brain barrier. In the CNS a reactivation ensues, triggering an autoinflammatory cascade leading to inflammation, demyelination, and neurodegeneration.

1.2.3.1 The Role of Th1 and Th17 in MS

In the mouse model of Experimental Autoimmune Encephalitis (EAE), the disease could be transmitted from sick to healthy animals by transferring isolated CD4+ T cells (45). For a long time, it was assumed that these T cells primarily secrete Th1 cytokines such as INF-γ, TNF-α, and IL-2, thus attributing the IL-2-Th1-INF-γ axis the central role in central nervous autoimmune diseases (46). With the discovery of the Th17 cell series and its importance in autoimmune diseases, its role in neuroinflammation was also examined in more detail (47). This activation takes place within the secondary lymphatic organs before the Th17 cells involved enter the CNS. These Th17 cells migrate into the CNS, where they secrete their eponymous cytokine IL-17A. Interestingly, IL-17 suppression led to milder disease progressions in the EAE model, but complete healing was not achieved (48). Therefore, it can be assumed that the two cell lineages (Th1/TH17) interact in the development and maintenance of the disease (49).

1.2.3.2 T cell Cytokine Networks in MS

Cytokines play a central role in disease development and progression. However, many aspects of their exact functions and interplay still remain unclear. INF-γ administration i.e. has been shown to provoke antagonistic effects. Especially in the initial phase of the disease, the effector phase, INF-γ administration led to improvement. On the other hand, interferon-gamma injected into the CNS triggered inflammation, yet, the elimination of the genes coding for interferon-gamma and the administration of INF-γ-blocking antibodies leads to exacerbation of the disease (50, 51). This indicates that cytokine regulation must be complex and multiple factors or immune mediators might play into disease mechanisms.

In addition to the Th1 and Th17 associated cytokines mentioned above, there are other cytokines that have been reported to play a role in MS pathogenesis. GM-CSF, for

example, plays a key role in the neuroinflammatory effect of both Th1 and Th17 cells. In contrast to INF-γ or IL17A, GM-CSF seems to have an obligatory role in CNS inflammation considering that GM-CSF deficient T cells cannot trigger EAE. Yet, this is not the case for INF-γ or IL17A-deficiency (52).

Also, a positive feedback mechanism involving IL-23 has been demonstrated as promoting the migration of Th17 cells into the CNS. GM-CSF stimulated APCs secrete IL-23 which in turn propagates the encephalogenecity of Th17 cells. IL-21, a cytokine produced by Th17 as well as Tfh cells that helps amplify IL-17 production in autoimmunity, is an interesting subject for further investigation with regard to its role in MS (53).

The balance of pro- and anti-inflammatory properties thus seems to play an important role. The exact functions of the respective T cell lines and the cytokines secreted by them, therefore, ought to be characterized in more detail.

1.2.4 Clinical Presentation

Neurological symptoms are polymorphous and can range from minor motor weakness and sensorimotor disturbances, to a variety of other conditions, like the blurring of vision, cognitive deficits, incontinence or paralysis, which in worst cases can lead to permanent physical disablement.

Patients tend to have different types of disease progression and are categorized accordingly, whereby a majority of 80 % suffers from the relapsing type referred to as relapsing-remitting MS (RRMS). RRMS can develop into secondary-progressive MS (SPMS) characterized by a progressive increase in disability (with or without superimposed relapses) after the previous relapsing course. Approximately 15 % of patients suffer from primary-progressive MS (PPMS) showing a progressive increase in disability since onset. Furthermore, there is also a type of primary manifestation called clinically isolated syndrome (CIS) that can but does not necessarily develop into a proper MS. It is defined as an episode of neurologic symptoms, (which can be either mono- or multifocal) which lasts 24 hours or longer and is caused by CNS inflammation or demyelination. While typically with MS a monosymptomatic onset can be observed, a plethora of manifestations might follow as the disease progresses. With patients complaining of neurological symptoms such as sensory and locomotor disturbances, ataxia, gait impairment, and other cerebellar symptoms, but also general fatigue as well as cognitive and psychological changes or vegetative symptoms like loss of libido

or urinary incontinence. A quantification of the severity of the disease, based on the affected systems, is usually done using the Expanded Disability Status Scale. Among the prognostically favorable factors is good remission after the first clinical presentation, onset before the age of 35 and monosymptomatic sensitive symptoms. Considering the possible severity of the disease the overall life expectancy with this diagnosis is merely reduced by approx. 6-7 years (54). Nonetheless, the individual disease course varies considerably between patients and can hardly be predicted in advance.

1.2.5 Therapy

Therapy options in MS are divers and complex. So far, there is however no causal approach among the therapy options currently available. A distinction is made between relapse therapy and progression-modifying therapy/relapse prophylaxis. In addition, individually adapted supportive measures are used according to distinct necessities. Glucocorticoids are in the foreground in the therapy of acute attacks. The second option is plasmapheresis. The following table (Table 1) gives a simplified overview of the currently recommended therapy scheme by the German Society of Neurology with so-called disease-modifying drugs for exacerbation prophylaxis in simplified form (55-57).

Table 1: Stage Therapy in MS - Simplified Schematic

Indication	Clinically isolated syndrome	Relapsing-remitting MS (RR-MS)	Secondary progressive MS (SP-MS)	Primary progressive MS (PP-MS)
Basic therapy	Glatiramer acetate Interferon (IFN-β)	Glatiramer acetate Interferon (IFN-β) Dimethylfumarate	Interferon (IFN-β) Mitoxantrone	Ocrelizumab Ofatumumab
Escalation therapy		1. Line: Alemtuzumab Fingolimod Natalizumab 2.Line: Mitoxantrone		

modified after S2 guideline German Neurological Society

Table 2 and Table 3 show a selection of currently available drugs for patients suffering from a moderate (Table 2) or highly active (Table 3) disease form with recommended indications respectively. Though many agents' detailed mechanism of action is not very well elucidated, postulated or known mechanisms are described in the aforementioned tables. Agents are listed alphabetically.

Table 2: Disease-Modifying Therapy - Moderate Presentation

Active Substance	Brand Names	Disease Form	Mode of Action
Dimethyl Fumarat	Tecfidera®	RR-MS	Activation of the Nuclear-Factor-(Erythroid-derived-2)-like-2 (Nrf2)-transcription pathway
Glatiramer acetate	Copaxone®	CIS RR-MS	Induction of Tregs
IFN-β	Rebif® Betaferon®	CIS RR-MS SP-MS	General immunomodulatory effect Enhancement of suppressor activity of peripheral lymphocyte Downregulation of pro-inflammatory cytokines, reduction of trafficking towards BBB
Mitoxantrone	Mitoxantron® Novantron® Onkotrone® Ralenova®	SP-MS	Topoisomerase II inhibition Cytotoxic effect non-selective immunosuppressive effect Reduced production of inflammatory cytokines, reduced antibody production, reduced myelin degradation by macrophages Third line treatment and mechanism of action:
Teriflunomide	Aubagio®	RR-MS	De-novo pyrimidine synthesis inhibition

Table 3: Disease-Modifying Therapy – Highly Active Progression

Active Substance	Brand Names	Disease Form	Mode of Action
Alemtuzumab	Lemtrada®	RR-MS	Binds to CD52+ T and B cells Antibody dependent T cell cytolysis and complement-mediated lysis
Daclizumab - no longer on the market due to adverse effects	Zinbryta®	RR-MS	Depletion of CD25+ memory B cells, CD4+ T cells, and enhancement of NK cell activity
Fingolimod	Gilenya®	RR-MS	A modulator of the sphingosine 1-phosphate receptor (S1PR) on lymphocytes and on nerve cells Blocks the lymphocytes ability to leave the lymph nodes Redistribution reduces the infiltration of pathogenic lymphocytes, including pro-inflammatory Th17 cells into the CNS
Mitoxantrone	Mitoxantron® Novantron® Onkotrone® Ralenova®	RR-MS	Topoisomerase II inhibition Cytotoxic effect non-selective immunosuppressive effect Reduced production of inflammatory cytokines, reduced antibody production, reduced myelin degradation by macrophages Third line treatment and mechanism of action:
Natalizumab	Tysabri®	RR-MS	Binds specifically to α4-Integrin Prevents transendothelial migration of mononuclear leukocytes into inflamed parenchyma tissue of CNS
Ocrelizumab, Ofatumumab	Ocrevus® Arzerra®	PP-MS	Depletion of CD20+ B cells

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1.3 Objective

The prerequisite to appropriately understanding pathological dysregulations in autoimmunity is to first also gain a deeper understanding of the sensitive hubs regulating the balance between pro- and anti-inflammatory cytokines. By their unbalanced cytokine production, differentiation and regulation T cells enhance inflammatory processes and contribute to MS pathogenesis.

The aim of this study is, therefore, to comprehensively analyze the Th cell repertoire and its associated cytokines and surface markers and immune checkpoint receptors in MS patients and healthy donors. The focus was particularly on Th1- and Th17-associated cytokines and or cytokines which are attributed a role in MS pathogenesis in activated Th cell subsets (INF-γ, GM-CSF, IL-10, IL-21, IL-17, TNF-α, IL-2, IL-22).

To achieve these objectives, the following tasks are planned:

1. The analysis of T-helper cells, their cytokine production, co-production and patterns in cytokine production associated with PD-1 expression in healthy volunteers.
2. The analysis of Th cell subpopulations in MS patients including frequencies of circulating subsets, cytokine production and checkpoint receptor expression in MS patients.
3. The analysis of CD20+ T cells, regarding their memory features, features of activation and cytokine production capacities and comparison of those in healthy donors and MS patients.

Multiparametric flow cytometric data will be used to detect and visualize patterns of dysregulated cytokines and surface markers associated with inflammation in patients with autoimmune disease. Using a novel bioinformatic approach for Pattern Recognition of Immune Cells (PRI) developed in our group will help to visualize combinatorial protein expressions in an easily comprehensible way. Thereby, PRI facilitates the identification of complex relationships between cytokines by perceiving their expression patterns.

2 Material and Methods

2.1 Patient Cohort

Patients and healthy controls were recruited from the CIS- and VIMS- Cohort (neuro-ophthalmologic register) at the NeuroCure Clinical Research Center. All patients had been diagnosed with Relapsing-Remitting MS for at least three years according to the revised McDonald criteria at the time point of blood collection. The Ethics Committee of the Charité has approved both studies. All donors were over the age of 18 and signed informed consent to participation was obtained. Patients and healthy donors were age and sex matched. Patients included were medicated as follows: no medication (7) Interferon-beta (Betaferon®, Rebif®) (5) Teriflunomide (Aubagio®) (3) Glatiramer acetate (Copaxone®) (4) Daclizumab (Zinbryta®) (1).

Exclusion criteria:

- An eye disease that could interfere with OCT (e.g., glaucoma, diabetic retinopathy)
- Secondary Progressive MS
- Pregnancy
- Contraindications for MRI, e.g., pacemaker, metal implants, allergy against gadolinium
- Alcohol or drug abuse
- Ongoing treatment with Fingolimod (Gilenya®) or Dimethyl fumarate (DMF) (Tecfidera®)
- Treatment with systemic glucocorticoids within four weeks
- Acute symptoms within previous four weeks
- Acute lymphopenia

Table 4: Size and Demographic Characteristics of the Cohort

	Healthy Controls	Patients
N total	16	20
N female	11	12
N male	5	8
Median age	41,5	44
Average age	40,33	45,5
Average age male	47,04	47,37
Average age female	42,3	40,23
Min. age total	23	23
Max age total	60	65

2.2 Materials

2.2.1 Chemicals and Reagents

Table 5: Chemicals and Reagents

Reagent	Manufacturer
4’, 6-Diamidine-2-phenylindole (DAPI)	Sigma-Aldrich
Alexa Fluor 750 succinimidyl ester (AF750)	Molecular Probes
β-mercaptoethanol	Gibco Life Technologies
Bovine serum albumin (BSA)	New England Biolabs
Brefeldin A (BFA)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	New England Biolabs
Ethylenediaminetetraacetic acid (EDTA)	Merck
Fetal calf serum (RA)	Merck
Ficoll-PlaqueTM PLUS	GE Healthcare
Formaldehyde (FA)	Merck
Ionomycin	Merck
N-hydroxysuccinimide (NHS)	Molecular Probes
Pacific orange succinimidyl ester (PacO-NHS)	Molecular Probes
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich
Saponin	Sigma-Aldrich

2.2.2 Buffers and Media

Table 6: Buffers and Media

Buffer or Medium	Composition
Roswell Park Memorial Institute (RPMI) medium 1640 GlutaMaxTM	90 % RPMI1640
	10 % RA
	10 mM L-glutamate
	10 µg/ml β-mercaptoethanol
	100 U/ml penicillin
Phosphate buffered saline (PBS) (pH 7.4)	137 mM NaCl
	2.7 mM KCl
	1.4 mM KH_2PO_4
	4.3 mM $Na_2HPO_4 \cdot 2H_2O$
PBS/BSA (pH 7.4)	PBS + 0.5 % BSA (w/v)

2.2.3 Antibodies and Beads

Table 7: Antibodies

Antibody Specificity	Clone	Conjugate	Manufacturer
α-hu CCR7	G043H7	PerCP-Cy5.5	BioLegend
α-hu CCR7	G043H7	BV650	BioLegend
α-hu CD127	HLR-7R-M21	BUV395	BD Bioscience
α-hu CD134	AKT35	APC-Cy7	BioLegend
α-hu CD19	BU12	AF700	DRFZ
α-hu CD20	LT20	FITC	Miltenyi Biotec
α-hu CD279	EH12.2H7	PE	BioLegend
α-hu CD3	OKT3	AF647	DRFZ
α-hu CD4	TT1	PacB	DRFZ
α-hu CD45RA	T6D11	PE-Vio770	Miltenyi Biotec
α-hu CD45RA	HI100	BUV737	BD Bioscience
α-hu CD45RO	UCHL1	AF405	DRFZ
α-hu CD8	GN11/134D7	PE	DRFZ
α-hu CXCR5	J252D4	BV785	BioLegend
α-hu GM-CSF	BVD2-21C11	FITC	DRFZ
α-hu GM-CSF	BVD2-21C11	PerCP-Cy5.5	BioLegend
α-hu IFN-γ	4S.B3	BV570	BioLegend
α-hu IFN-γ	4SB3	AF405	DRFZ
α-hu IL-10	JES3-9D7	PE-Cy7	BioLegend
α-hu IL-17A	CZ8-23G1	PE-Vio770	Miltenyi Biotec
α-hu IL-2	N7.48	APC-Vio770	Miltenyi Biotec
α-hu IL-21	3A3-N2	APC	Miltenyi Biotec
α-hu IL-22	22URTI	PerCP-eF710	eBioscience
α-hu TNF-α	Mab11	BV785	BioLegend

Table 8: Beads

Beads	Manufacturer
α-hu CD4	Miltenyi Biotec

2.2.4 Consumables

Table 9: Consumables

Consumable	Manufacturer
23 G cannula	B. Braun Melsungen AG
BD Safety-Lok™ Blood collection set	BD
BD Vacutainer ® Heparin tubes	BD
BD Vacutainer ® One-Use Holder	BD
Cell culture plates	CellStar Greiner Bio-One GmbH
CoolCell®LX	Biocision
Eppendorf tubes	Sarstedt
FACS tubes 5 ml	Sarstedt
Falcon tubes	Sarstedt
Glass pipettes	Brand GmbH+Co KG
MACS LS columns	Miltenyi Biotec
Pipet tips	Sarstedt
Pre-separation filters (30 µm)	Sysmex

2.2.5 Technical Equipment

Table 10: Technical Equipment

Equipment	Manufacturer
autoMACS® Pro Separator	Miltenyi Biotec
BD FACS Canto™ II	BD Biosciences
BD FACSymphony™	BD Biosciences
BD LSR Fortessa™	BD Biosciences
Heraeus™ Fresco™ 21 centrifuge	Thermo Fisher Scientific
Heraeus™ Multifuge 1S-R	Thermo Fisher Scientific
Heraeus™ sterile bench HS18	Thermo Fisher Scientific
Incubators	Binder
Inverted microscope ID-03	Carl Zeiss
Neubauer counting chamber	Carl Roth

2.2.6 Optical Layouts

All experiments were performed on the Flow Cytometers mentioned above. Only lasers and laser-setups actually used in the work process are listed.

2.2.6.1 BD FACS Canto™ II

Table 11: Optical Layout of BD FACS Canto™II

Optical Layout of BD FACS Canto™II			
Laser (nm)	**Longpass Filter (LP)**	**Bandpass Filter (BP)**	**Fluorochrome**
405	None	450/50	PacB
405	502LP	510/50	PacO
488	None	488/10	
488	502LP	530/30	FITC
488	556LP	585/42	PE
488	655LP	670LP	PerCP-Cy5.5
488	735LP	780/60	PeCy7
633	None	660/20	AF647
633	735LP	780/60	AF700

2.2.6.2 BD LSR Fortessa™

Table 12: Optical Layout of BD LSR Fortessa™

Optical Layout of BD LSR Fortessa™			
Laser (nm)	**Longpass Filter (LP)**	**Bandpass Filter (BP)**	**Fluorochrome**
405	None	450/50	PacB
405	505LP	525/50	PacO
488	None	488/10	
488	505LP	525/50	FITC
488	685LP	710/50	PerCP-Cy5.5
561	750LP	780/60	PE-Cy7
561	None	582/15	PE
633	None	670/30	AF647
633	750LP	780/60	AF700

2.2.6.3 BD FACSymphony™

Table 13: Optical Layout of BD FACSymphony™

Optical Layout of BD FACSymphony™			
Laser (nm)	**Longpass Filter (LP)**	**Bandpass Filter (BP)**	**Fluorochrome**
355	379/28	None	BUV395
355	735/30	690	BUV737
405	None	450/50	PacBlue, AF405
405	LP505	525/50	PacO
405	LP550	586/15	BV570
405	LP635	677/20	BV650
405	LP750	780/60	BV786
488	None	488/10	
488	LP505	530/30	FITC
488	LP685	710/50	PerCP-Cy5.5
561	None	586/15	PE
561	LP750	780/60	PE-Cy7
637	None	670/30	APC, Cy5, Alexa647
637	LP750	780/60	APC-Cy7, APC-H7

Table 14: Software

Software	Manufacturer
FACSDiva V8.0.1	BD Biosciences
FlowJo 10.4.1, 10.4.2	FlowJo LLC
GraphPad Prism 7	GraphPad Software
PRIanalyzer	Working group
PRIbase	Working group
R 3.4.2	Foundation for Statistical Computing
Packages:	
RSQLite_2.1.1	
Tcltk2_1.2-11	
R.devices_2.16.0	
Rstudio 0.99.902	Integrated Development for R. RStudio, Inc.

2.3 Methods

2.3.1 PBMC Isolation

Blood samples were processed no longer than two hours after collection. PBMCs were isolated by density gradient separation using Ficoll-Paque plus.

The samples were diluted with PBS in a 1:1 ratio, before being carefully layered into tubes filled with Ficoll in a 1:3 ratio without intermixing. Next, the samples were centrifuged at 600 xg for 20 minutes at 20 °C without a brake, resulting in the demarcation of distinct layers due to the differential migration of the blood components during centrifugation. From bottom to top containing: aggregated and sedimented erythrocytes at the bottom, granulocytes, Ficoll-Paque, mononuclear cells and topmost the plasma (Figure 4). After harvesting the layer containing the mononuclear cells, the solution was washed to remove any remaining platelets, plasma or any possible contaminating Ficoll- media. Lymphocytes were then counted using the Neubauer counting chamber. Trypan blue (0.04 %) was used to determine viability. All washing steps were performed by centrifugation (8 min, 500 xg, 4 °C) unless otherwise stated.

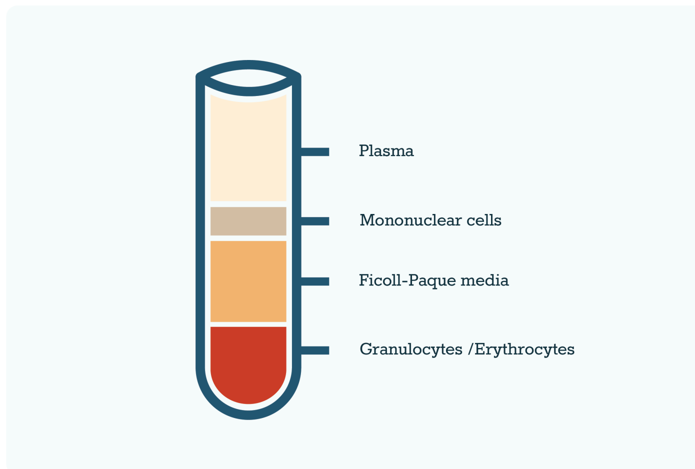


Figure 4: Distribution of Blood Components after Density Gradient Centrifugation

2.3.2 MACS Sort

The isolation of CD4+ T cells was achieved through Magnetic Activated Cell Sorting (MACS), using anti-CD4 MACS beads. Samples were suspended in 850 µl PBS/BSA per 10^8 total cells and mixed with 200 µl of CD4 MicroBeads per 10^8 total cells. During 15 minutes of incubation at 4 °C, samples were carefully remixed every 5 min. After washing, the cells were resuspended in PBS/BSA. The now magnetically labeled CD4 antibodies were automatically separated using the 'positive selection' program of the AutoMACS®. Essential to this process are two basic steps. First, a high magnetic gradient within the MACS column keeps the labeled antibodies bound so that the CD4-

fraction can be eluted. When deactivating the magnetic field, this bond is dissolved, allowing for the positive fraction to be separately collected. After separation, only the CD4+ fraction was processed further.
Small aliquots of each sample were obtained and stained for purity check with FACS after magnetic separation. Overall purity rates of over 95 % could be achieved. Therefore, a sufficient efficiency of the method can be assumed.

2.3.3 In-vitro Stimulation of Isolated CD4+ T cells

1×10^7 isolated CD4+ T cells per ml RPPMI medium were mixed with 10 % FCS/DMSO in a cryovial (58). The cryovials were then transferred to -80 °C freezer in the cooling box for cooldown at a rate of 1 °C per minute (59). After thawing samples were washed with PBS/BSA and kept at 37 °C for at least one hour before stimulation. In order to achieve a polyclonal activation of the cells and for them to obtain their maximal potential to secrete cytokines, PMA/Ionomycin, a strong and polyclonal stimulant was chosen. PMA acts as an activator of protein kinase C (PKC). Ionomycin additionally triggers calcium release. Therefore 1-2 $\times 10^6$ cells/ml CD4+ T cells per condition were in vitro stimulated with PMA (10 ng/ml) and Ionomycin (1 µg/ml). Cells were then incubated at 37 °C in a 5 % CO_2 atmosphere in RPMI1640 GlutaMAX medium supplemented with 10 % FCS, 100 U/ml penicillin, 10 µg/ml β-mercaptoethanol and 1 mM calcium for 5 h. Calcium at this concentration (1.5 mM) has been shown to be critical for a maximal response of Th cells to PMA/Ionomycin (60). For the last 4h BFA (5 µg/ml) was added. BFA inhibits the function of the Golgi apparatus and thus prevents the secretion of the produced cytokines so that they can later be detected using intracellular staining. Unstimulated controls were processed under equal conditions except that no PMA or Ionomycin but only BFA was added. Samples were thoroughly washed after stimulation.

2.3.4 Staining for Flow Cytometry

The cells were stained in 100 µl PBS/BSA for 20 minutes on ice using fluorochrome-labeled antibodies listed in Table 7. Fluorescence minus one (FMO) controls were included, consisting of stimulated cells stained with all but one fluorophore, providing a negative control for the missing fluorophore. The FMO control is used to determine spectrum overlaps of a fluorophore into one or more adjacent channels so that the results can be adjusted accordingly. Samples were washed with PBS/BSA to remove unbound antibodies. When processing live cells, like e.g., in the MACS purity check,

DAPI (2 µg/ml) was added immediately before the measurement to stain dead cells. DAPI links to dsDNA exposed in apoptotic or dead cells, dying them selectively. Before fixation, fixable dead cell staining was performed. Cells were stained with PacO-coupled NHS (0.5 µg/ml). The cells were resuspended in 500 µl PacO-NHS/PBS and stained for 20 minutes on ice. In order to remove any remaining dye, samples were washed with PBS. Before intracellular staining, fixation was performed to crosslink intracellular amino groups. 5×10^6 T cells were suspended in 500 µl formaldehyde (2 %) for 20 minutes at room temperature. Cross-linking of free amino groups leads to irreversible cell fixation. After fixation, the samples were washed with PBS/BSA to remove all excess formaldehyde. To stain intracellular antigens permeabilization of the cell membrane is necessary. Saponin is a detergent used to permeabilize cell membranes thus staining was performed in Saponin (0,5 %) in PBS/BSA for 30 minutes at room temperature. Fluorophore-coupled antibodies with respective clones used for intracellular staining are listed in Table 7 above. All antibodies were individually titrated for the respective use. The samples were then washed with PBS/BSA to remove all excess dye and permeabilizer.

2.3.5 Multicolour Flow Cytometry

Samples were measured on BD FACS Canto™ II, FACSymphony™ and BD LSR Fortessa™. Single stainings i.e., compensation controls were always used to calculate and compensate for spectral overlap within channels.

The technical variability, which is inevitably associated with FACS measurements, was largely minimized by the following aspects of the experimental set-up: Only the same clones of the respective antibodies were used to prevent the different binding behavior. The instrument fluctuations were kept as small as possible since all measurements for a specific question were performed on the same instrument and with a standardized optical layout. In addition, a calibration with CST beads was carried out before measurements on different measuring days.

The allocation of the channels and the panel design were carried out under consideration of the current guidelines (61): i.e., weak signal - bright color and v.v. Furthermore, no strongly correlating molecules were measured in adjacent channels or in channels with overlapping spectra.

2.4 Analysis

2.4.1 Data Processing

Data was acquired with FACSDiva Software on the cytometer and then exported in the .fcs format files into FlowJo software for processing. To identify only live single-cells, hierarchical gating was performed using Forward (FSC) and Sideward Scatter (SSC) and the dead cell staining as shown in Figure 5.

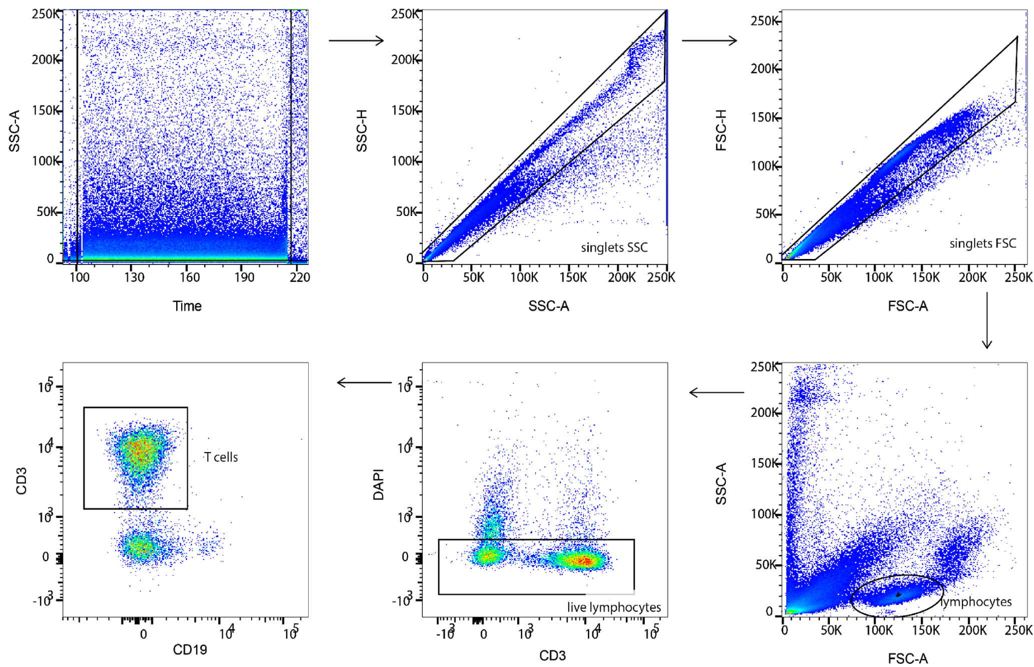


Figure 5: Exemplary Gating Strategy to Identify T cells

Cells were gated for consistent time flow (Time/SSC-A), then doublets were removed, lymphocytes were identified based on size and granularity (FSC-A/SSC-A), then live lymphocytes were identified using DAPI and an adjacent channel. T cells were gated based on CD3 positivity and CD19 negativity (CD19-CD3+).

Initially, cells were gated for consistent time flow (Time/SSC-Area), then doublets were removed based on Height/Area ratio of laser pulse first (SSC-Area/ SSC-Height) then (FSC-Area/FSC-Height), lymphocytes were identified based on size and granularity (FSC-Area/SSC-Area), then live lymphocytes were identified using DAPI and an adjacent channel. T cells were gated based on CD3 positivity and CD19 negativity (CD19-CD3+). Preprocessing and measurements were performed according to current guidelines (61).

2.4.2 Data Visualization

Conventional approaches in visualizing flow cytometry data including histograms, for the presentation of one parameter, or scatter plots and contour maps for two parameters were created in FlowJo. After compensating and gating to a specific population of interest for analysis in FlowJo, the fluorescence intensities of the measured channels for each event were exported as .fcs files. These files were then uploaded into our database management system (DBMS). Triplots were created with our in-house tool which was developed by group member Yen Hoang. The tool was coded in the programming language for statistical computing R. Columns with staining information were transformed with inverse hyperbolic arc-sine, which is a common transformation method for flow cytometry data. The area of the biaxial dot plot with parameter X and Y is partitioned into quadrant bins resulting in a grid of bins (Figure 6).

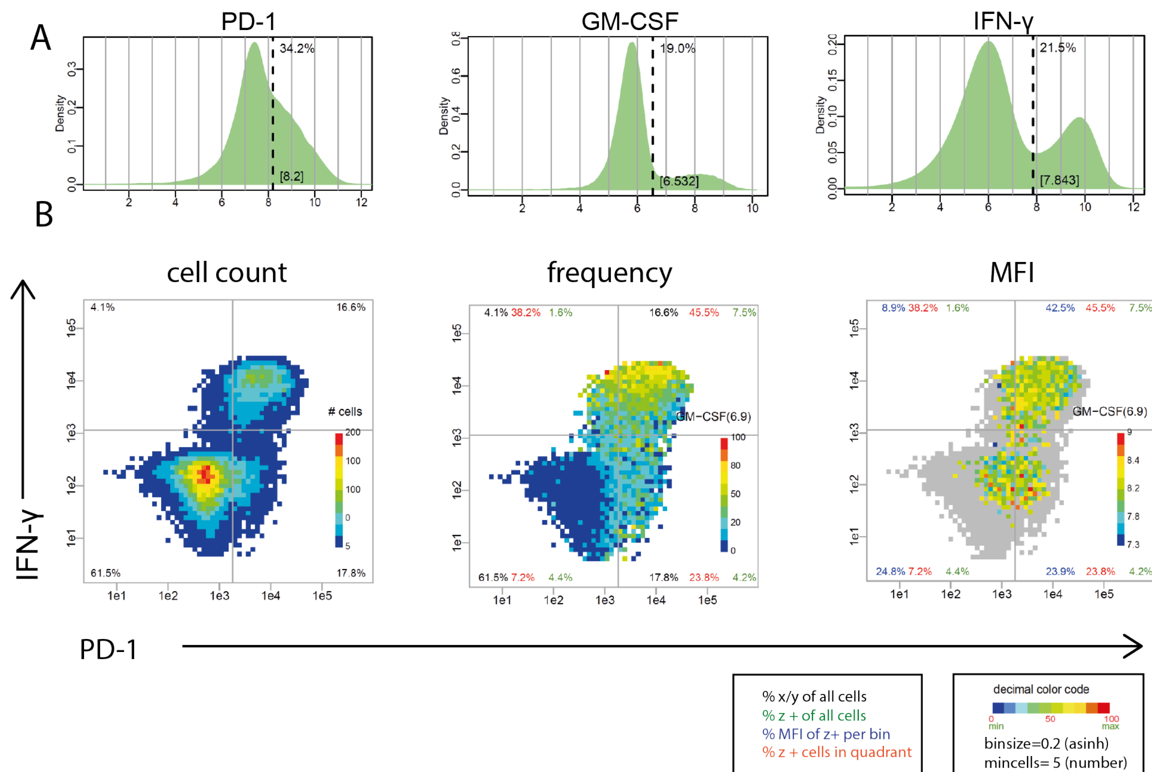


Figure 6: PRI-Pattern Recognition of Immune Cells

A: After exporting the population of interest and uploading to our DBMS, cut-offs were set according to negative controls and FMO controls (histograms with cut-offs shown here).

B: First Triplot shows cell count with % of all cells (black labeling) in each quadrant. Second Triplot shows frequency of third parameter per quadrant (red labeling) and % of the third parameter of all cells (green labeling) in each quadrant. The third Triplot includes MFI of third parameter Z+ per bin (blue labeling). Each plot was created with a minimum cell number of 5 per bin, binsize 0.2 (asinh). The decimal color code for third parameter representation is depicted on the right of each plot.

Each bin that contains at least 20 cells was displayed. This allows background signals and compensation artifacts to be corrected. These bin plots can be extended with statistical information about different parameters by calculating e.g., cell count, mean fluorescence intensity (MFI) or frequency of parameter Z production over the cells inside each bin. The intensity of the statistical measure is depicted in a color-coded manner. The frequency of parameter Z in each bin is plotted with frequencies of 0 % (blue) to 100 %.

2.4.3 Statistical Analysis

For statistical verification of the results obtained and graphics display, the programs GraphPad Prism 7 and Microsoft Office Excel were used. The data were first tested for normal distribution. If D'Agostino-Pearson Normality-test could prove normal distribution, then a t-test was performed in order to compare two groups. For comparison of more than 2 groups, one-way ANOVA was used. In the case of a non-Gaussian distribution or a sample size smaller than eight, the Mann-Whitney test for unmatched pairs was applied for comparisons of two groups and the Kruskal-Wallis test for more than two groups (Figure 7).

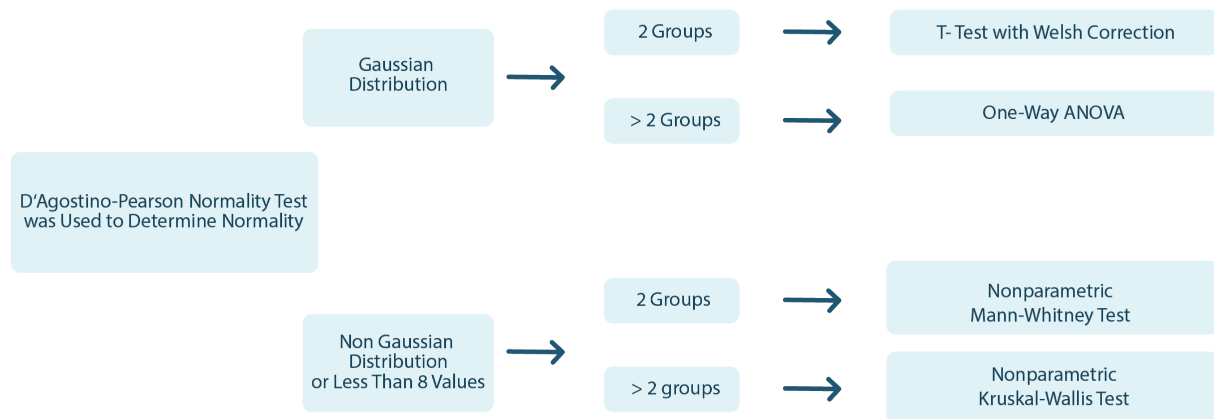


Figure 7: Statistical Tests - Decision Tree

If statistical testing showed an error probability (p) of > 0.05 the results were assumed as not significant. Comparisons, with a result of $p < 0.05$ were considered significant (*). Results were rounded to the fourth digit after the decimal point. P values less than 0.01 are depicted with two asterisks p values less than 0.001 are depicted with three asterisks and p values less than 0.0001 with four. Comparisons with a probability of error (p) of 0.05 - 0.1 can only be described as a trend. For variance inhomogeneity, a correction test (e.g., Welch correction) was used. If not otherwise stated the graphs display mean with 95 % confidence interval (CI).

3 Results

3.1 Analysis of Th Subsets

The CD4+ T cell compartment is extremely heterogeneous and functionally diverse. In this study, different subsets of Th cells were analyzed with respect to their activation features and inflammatory properties such as cytokine production. The aim was to find distinct patterns in cytokine-producing Tmem subpopulations and visualize them using a novel pattern recognition approach to characterize complex cell-subsets. Subsequently, an analysis of disease-related changes in MS patients was carried out (3.2 and 3.3).

3.1.1 Th cell Subsets

There is an inconsistency in the literature regarding the definition of the naive and memory Th cell subsets. For this study, they were defined based on the expression of CD45RA and CCR7, resulting in the division into four subsets, including CD45RA+CCR7+ naïve T cells (Tn), CD45RA-CCR7+ (Tcm), CD45RA-CCR7- (Tem), and CD45RA+CCR7- (Te) (62) (Figure 8).

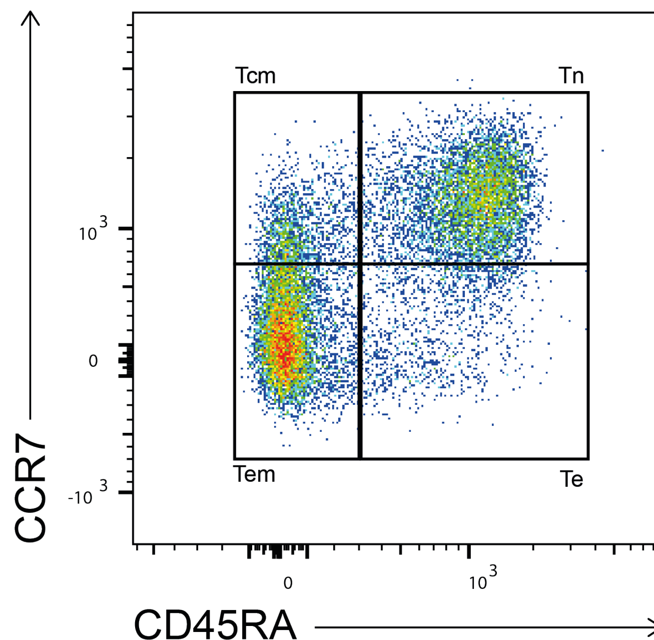


Figure 8: Identification of T cell Subsets by Expression of CCR7 and CD45RA

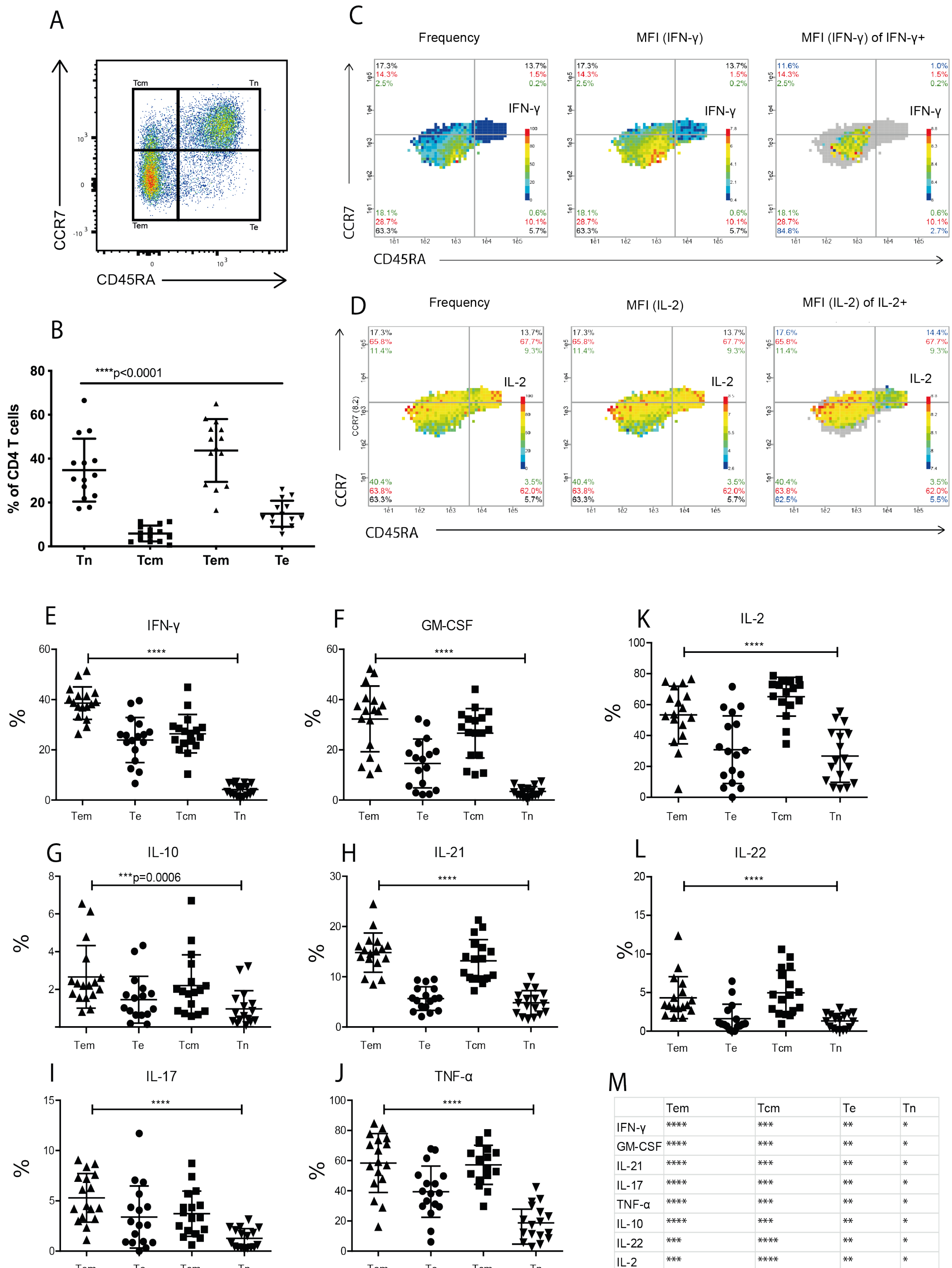
The figure shows the gating of CD4+ T cells, according to CD45RA and CCR7 receptor status.
Tn: CCR7+ CD45RA+, Tcm: CCR7+ CD45RA-, Tem: CCR7- CD45RA-, Te: CCR7- CD45RA+

3.1.2 Cytokine Production in T cell Subsets

Out of the four defined peripheral Th subsets (Figure 9A), Tem were found to circulate in the highest frequencies. The second largest subset was Tn cells, followed by Te cells. As central memory cells carry the lymph-node homing receptor CCR7, they circulate considerably less and lack immediate effector function. As expected, they were found to be reduced among overall CD4+ T cells isolated from PBMCs (Figure 9B).

A panel of eight cytokines (INF-γ, GM-CSF, IL-10, IL-21, IL-17, TNF-α, IL-2, IL-22), especially Th1- and Th17-associated cytokines and/or cytokines, which are attributed a role in MS pathogenesis were selected for measurement and analysis. In Figure 9C and Figure 9D, a pseudo-multiparametric view was chosen to help illustrate different characteristics of cytokine-producing subsets. As expected, IFN-ɣ+ cells accumulate within the memory populations (Figure 9C), whereas IL-2 is produced by memory and Tn cells alike (Figure 9D). It is however not only interesting to know where the cytokine producers are located in the diagram (representing the correlation with memory markers), but also to quantify their level of expression. We achieved this using mean fluorescence intensities (MFI) for a given parameter that were visualized using the color code (as demonstrated in 2.4.2, Figure 6). The MFIs can either be calculated from all cells (Figure 9 C, D in the middle) or only from a certain cell subpopulation (Figure 9 C, D right column). Figure 9 E-L show the frequencies (out of all CD4+ cells) of cytokine-producing Th cell subsets (Tn, Tcm, Tem, Te) for each cytokine measured.

After identifying the cytokine producing Th cells, we found that the Tem cells were proportionately present in the highest frequencies overall (Figure 9M). The only exceptions thereof were IL-2 and IL-22 where the higher expression levels could be found in Tcm, followed by Tem (Figure 9M). As we know, memory T cells circulate in the long term, contribute to cytokine production, in particular within the framework of their activation, and continue to be particularly relevant to diseases. Hence, the focus of further analysis was put on the memory subsets.



	Tem	Tcm	Te	Tn
IFN-γ	****	***	**	*
GM-CSF	****	***	**	*
IL-21	****	***	**	*
IL-17	****	***	**	*
TNF-α	****	***	**	*
IL-10	****	***	**	*
IL-22	***	****	**	*
IL-2	***	****	**	*

Figure 9: Subsets among CD4+ T cells

A: Naive and memory subsets among Th cells were defined based on the expression of CD45RA and CCR7 resulting in four populations: Tem, Tcm, Te, Tn. **B:** Out of all CD4+ cells, Tem were circulating in the highest frequencies (43,73 ± 14,29), followed by Tn (34,76 ± 14,31) and Te (14,89 ± 5,957), while Tcm were found in the lowest frequencies in peripheral blood of healthy donors (5,876 ± 3,605), (mean ± SD).

C, D: Representative bin plots are used to show distribution of cytokine producing Th cells in relation to T cell memory status. Displayed and statistically represented are respectively first the frequencies of the three parameters, then the MFIs and finally the MFIs exclusively of the cytokine positive cells. **C:** IFN-γ is preferentially produced by Tem. **D:** IL-2 production is largely unrelated to CCR7 and CD45RA status. **E-J:** INF-γ-, GM-CSF-, IL-10-, IL-21-, IL-17, and TNF-α+ Th cells were mostly Tem. **K-L:** IL-2 and IL-22+ Th cells were mostly Tcm. **M:** Schematic overview of cytokine production levels in different CD4 subsets after PMA/Ionomycin stimulation. Statistical analysis between subgroups was performed using 1way ANOVA.

3.1.3 Different Patterns of Cytokine Levels with T cell Exhaustion/Activation

To further describe the T cell memory compartment PD-1 was measured along with surface markers CD45RA and CCR7. Figure 10 shows how the highest frequencies, as well as MFIs for PD-1, are found in the quadrant of CD45RA- CCR7- cells, coinciding with Tem.

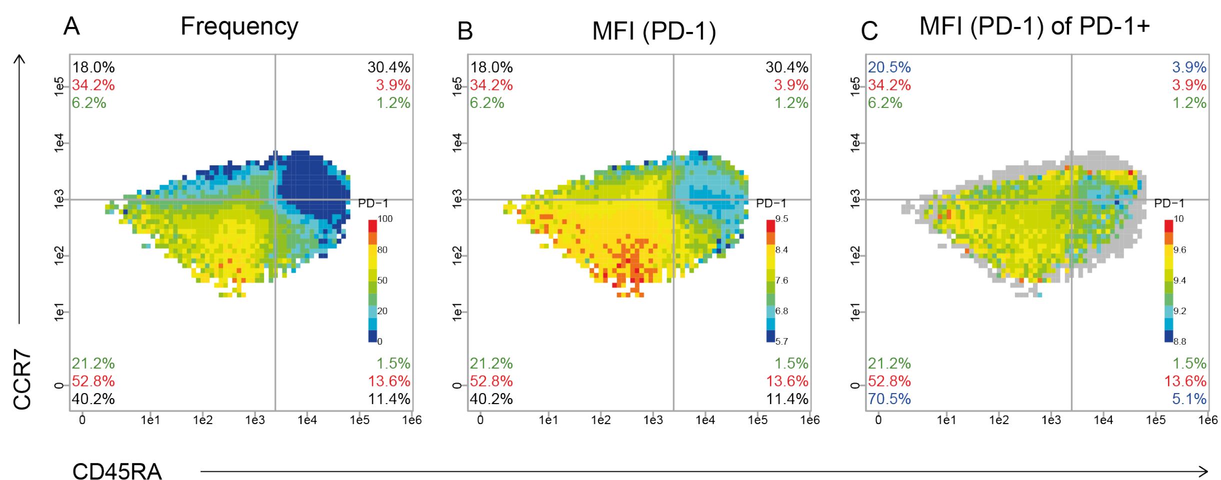


Figure 10: PD-1 Expression in Relation to CCR7 and CD45RA

PD-1 expression is plotted in relation to CCR7 and CD45RA expression. **A:** Highest frequencies as well as **B:** highest mean fluorescence intensities were found in the lower left quadrant, correlating with memory/activation status. **C:** Shows MFIs only of PD-1+ cells in bins.

PD-1 is typically upregulated with activation. Terminally upregulated and persistent PD-1 expression is linked to T cell exhaustion while transient PD-1 is a sign of activation. Therefore, to distinguish different levels of PD-1 expression, PD-1+ cells (PD-1+) were further divided into 20 % highest PD-1 expression (PD-1$^{20\%}$) and 10 % highest PD-1 expression PD-1$^{10\%}$. Next, the cytokine production in different PD-1 subsets was analyzed.

Two types of patterns could be observed in CD45RA- Th cells regarding cytokine expression with PD-1 upregulation: IFN-ɣ, IL-10, and IL-21 showed a positive correlation with PD-1 expression (Figure 11). Frequencies of cytokine producing T cell subsets significantly increased within each of the defined PD-1+ subsets (Table 15).

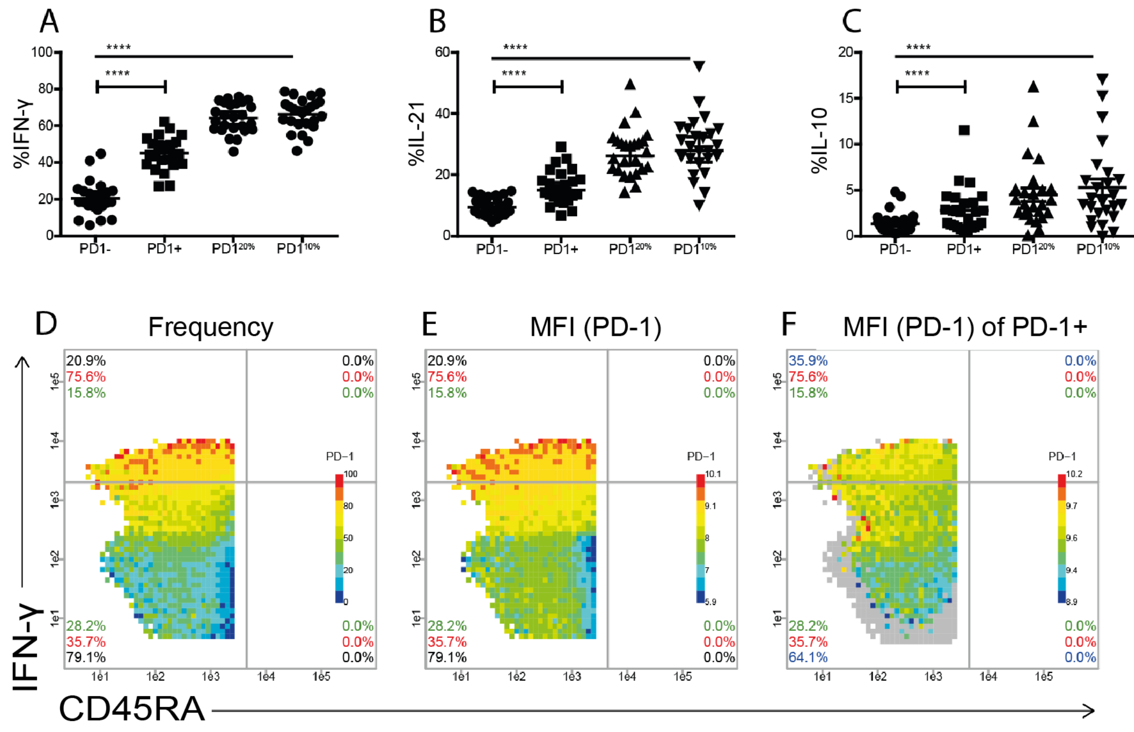


Figure 11: Correlation of IFN-ɣ, IL-10, and IL-21 with PD-1 Expression in Tmem

A-C: Show frequencies of cytokine-positive memory cells (gated for CD45RA-) in PD-1 subsets. **D-F:** Triplots illustrate the positive correlation of IFN-ɣ production with PD-1 expression in memory T cells (CD4+CD45RA-).

Table 15: Frequency of Cytokine-Positive CD45RA- cells in PD-1 Subsets

	PD-1- Mean (SD)	PD-1+ Mean (SD)	PD-1++ Mean (SD)	PD-1+++ Mean (SD)
IFN-ɣ	20,51 (9,106)	45,12 (8,792)	64,25 (8,167)	66,26 (8,476)
IL-21	9,870 (2,917)	15,99 (5,488)	27,16 (7,864)	29,54 (9,532)
IL-10	1,330 (1,212)	2,796 (2,420)	4,494 (3,649)	5,295 (4,451)

The second group of cytokines showed no direct correlation between PD-1 upregulation and cytokine production. Within this second group, a distinction of two subgroups can be made. With IL-17, GM-CSF, and TNF-α, an increase with PD-1 upregulation and a decrease again among the PD-1 highest population (PD-$1^{10\%}$) was measured (Figure 12 A-C and F-H). More precisely, a statistically significant difference was found in the comparison of the frequencies of PD-1+ vs. PD-1- cells regarding the level of cytokine production. However, the subgroup differences were only significant among IL-17 producers in the overall comparison (Table 16).

For IL-2 and IL-22 on the other hand, equal cytokine levels were measured in PD-1- and in PD-1+ Th cells (Figure 12 D, E, Table 16).

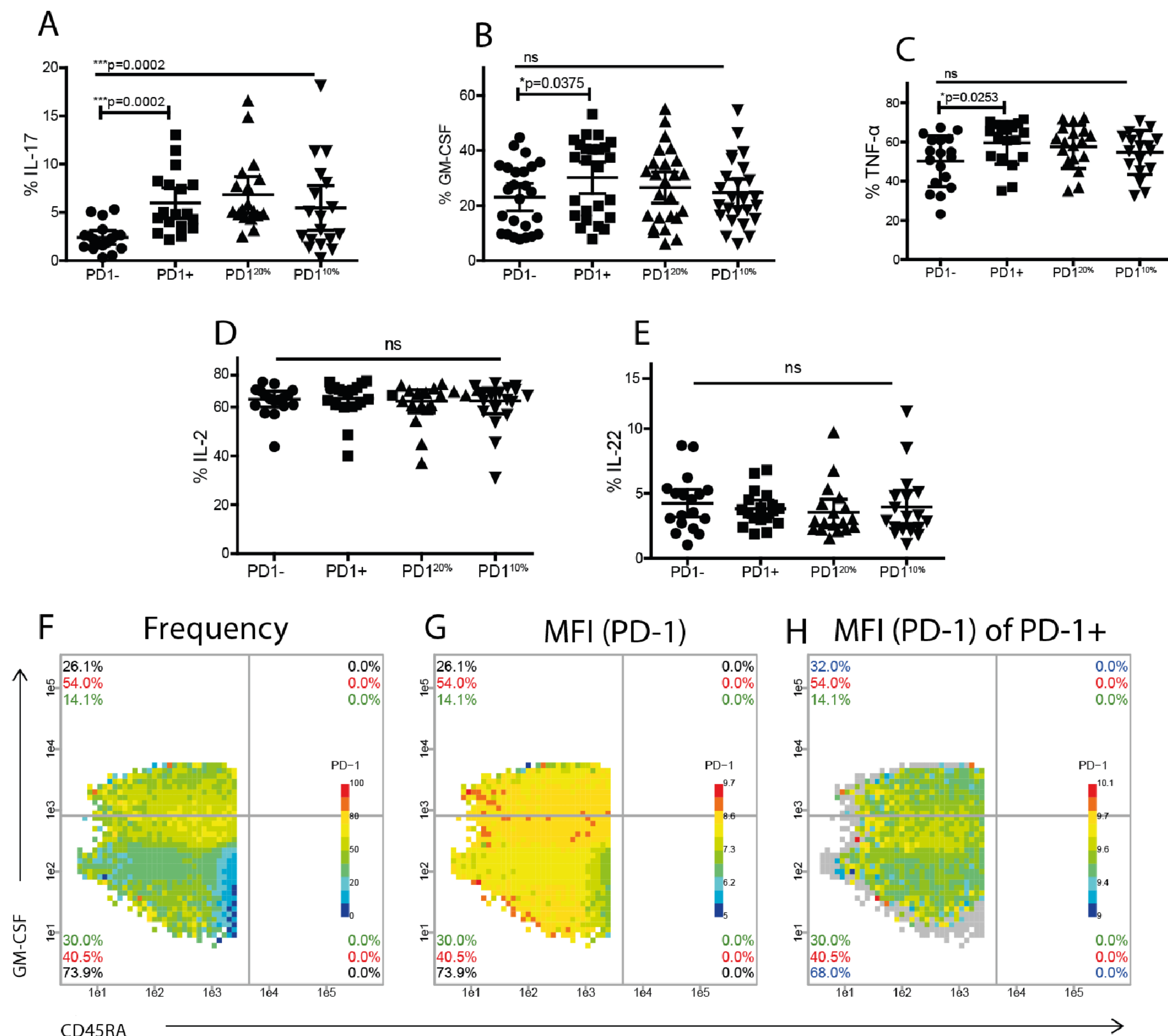


Figure 12: Cytokines with No Correlation with PD-1 Expression in Tmem

A-E: Show frequency of cytokine-positive cells in PD-1 subsets. **F-H:** Triplots illustrate the lack of correlation of cytokines with PD-1 expression in Tmem (CD4+CD45RA-).

Table 16: Frequency of Cytokine-Positive CD45RA- cells in PD-1 Subsets

	PD-1- Mean (SD)	PD-1+ Mean (SD)	PD-1++ Mean (SD)	PD-1+++ Mean (SD)
IL-2	63,19(6,517)	63,49 (8,024)	62,33 (9,450)	62,53 (10,64)
IL-22	4,244 (2,150)	3,827 (1,374)	3,545 (2,019)	3,961 (2,573)
IL-17	2,408 (1,425)	5,979 (3,161)	6,846 (3,784)	5,474 (5,474)
GM-CSF	23,10 (12,09)	30,11 (13,89)	26,53 (13,72)	24,64 (12,01)
TNF-α	50,17 (13,02)	59,61 (11,08)	57,54 (11,03)	54,65 (11,21)

Next, we looked at combinations of two cytokines to see if PD-1 could help to define distinct T cell subpopulations. Two markedly separate subpopulations of IFN-ɣ producing T cells can be observed well using PRI (Figure 13). In IFN-ɣ producing T cells, the co-production of GM-CSF is strictly concentrated in a separated bin area from IL-21 co-production. Also, with higher PD-1 expression the IL-21 production increases while GM-CSF is reduced in the PD-1 highest population, indicating a functional disparity between the two co-producing populations.

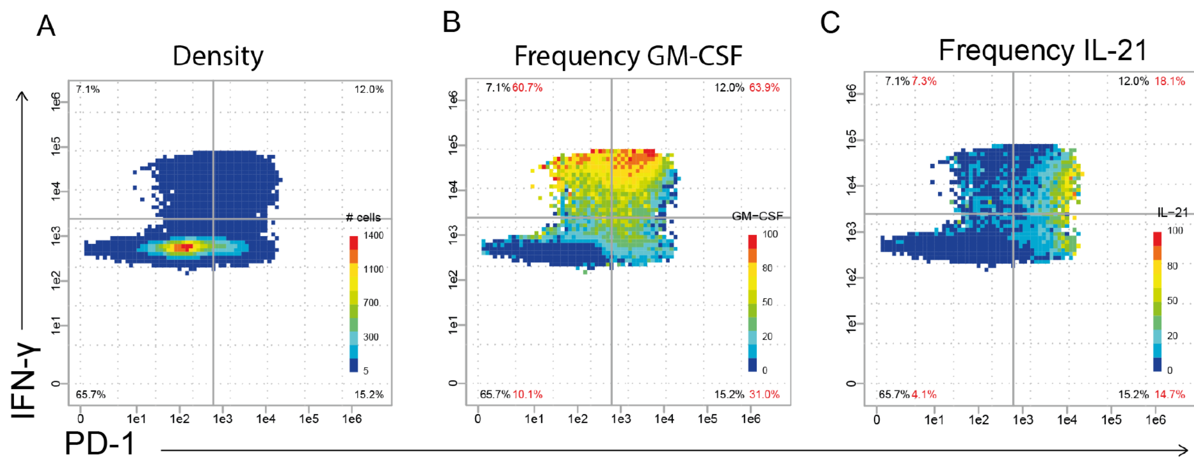


Figure 13: IFN-ɣ+GM-CSF+ and IFN-ɣ+IL-21+ Th cells

CD4 T cells are gated for IFN-ɣ and PD-1 status **A:** Shows frequencies of double negative, double positive and single positive cells as well as distribution via density plot. **B:** GM-CSF frequencies are color coded as the third parameter. **B:** IL-21 frequencies are color coded as third parameter; representative bin plots chosen.

3.1.4 Cytokine Co-Producers

Bin plotting was also used to depict the combinatorial properties of three cytokines within a Th cell subset. This goes beyond the usual representation of two parameters against each other and thus provides additional information about the combinatorics of the cytokines investigated. We mainly focused on potentially MS-associated cytokine combinations and investigated the combinatorial expression of Th1 and Th17 cytokines. Furthermore, IL-21 behavior in the interaction of other cytokine groups was of key interest. We observed that IL-21 is co-produced with both IFN-ɣ and GM-CSF. GM-CSF and IFN-ɣ, independently of IL-21, are also co-produced by the Th cells investigated here (Figure 14A). However, those GM-CSF/IFN-ɣ dual producers are not as likely to also be IL-21 producers (Figure 14B).

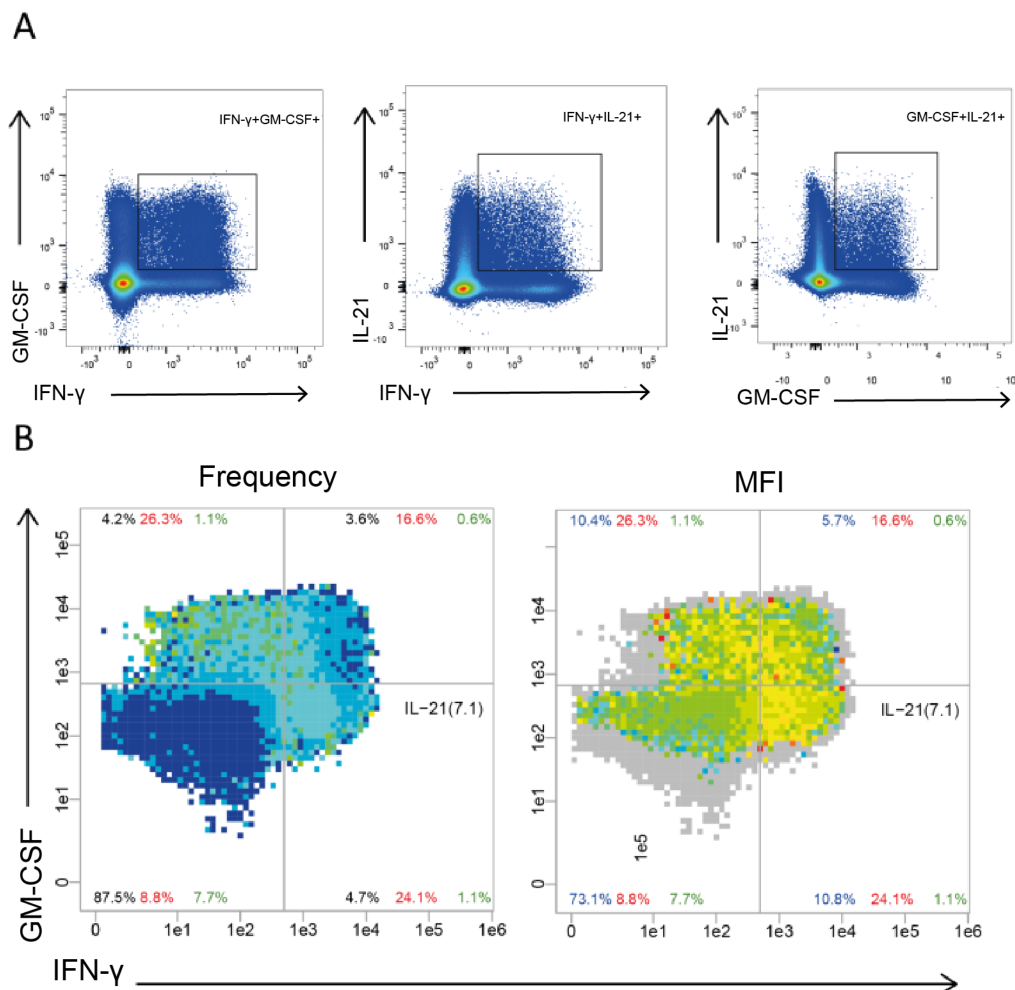


Figure 14: Co-Production of GM-CSF, IFN-ɣ, and IL-21

A: Depicts frequencies and distribution of GM-CSF, IFN-γ, and IL-21 in conventional dot plot. Color coded bin plot in **B** shows that only 0,6 % of GM-CSF+IFN-γ+ are IL-21+. The color code shows the likelihood of IL-21 decreases with increasing IFN-γ and GM-CSF expression intensities. Patterns were observed in the overall samples. Representative dot plot and bin plot examples are shown.

3.2 Th cell Analysis in MS Patients

For the analysis of disease-associated differences in Th cells, initially, frequencies of cytotoxic and helper T cells were compared between both groups. We found comparable frequencies in CD4+ T cells (Figure 15A). Differences between patients and controls were observed in the analysis of the PBMCs in the frequencies of circulating cytotoxic T cells (Figure 15B), Tcm cells and the CD4/CD8 ratio (Figure 15C).

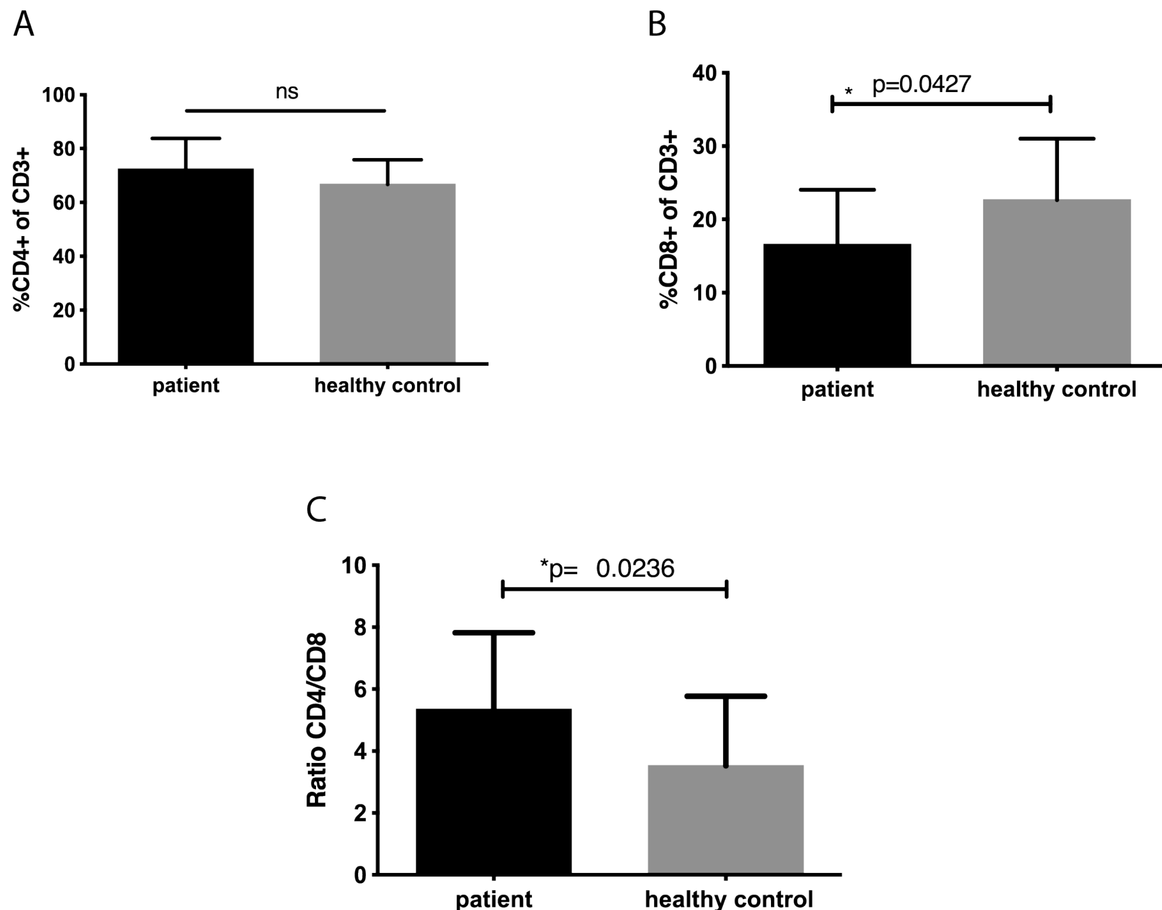


Figure 15: T cell Subsets - Comparison between Patients and Healthy Controls

A: Comparable frequencies of CD4+ T cells were found in MS patients (72,36 ± 11,44) and healthy controls (66,71 ± 9,16). **B:** Frequencies of CD8+ T cells in MS patients (16,57 ± 7,481) were reduced compared to healthy controls (2,65 ± 8,439, p = 0,0427). **C:** The Ratio CD4+/CD8+ was higher in MS patients (5,3 ± 2,465) compared to healthy controls (3,5 ± 2,242 p = 0,0236). (mean ± SD respectively)

Also, when comparing Tcm frequencies we observed a higher number of circulating cells within the healthy cohort whereas no differences were observed between frequencies of Te, Tn and Tem in patients and healthy controls (Figure 16).

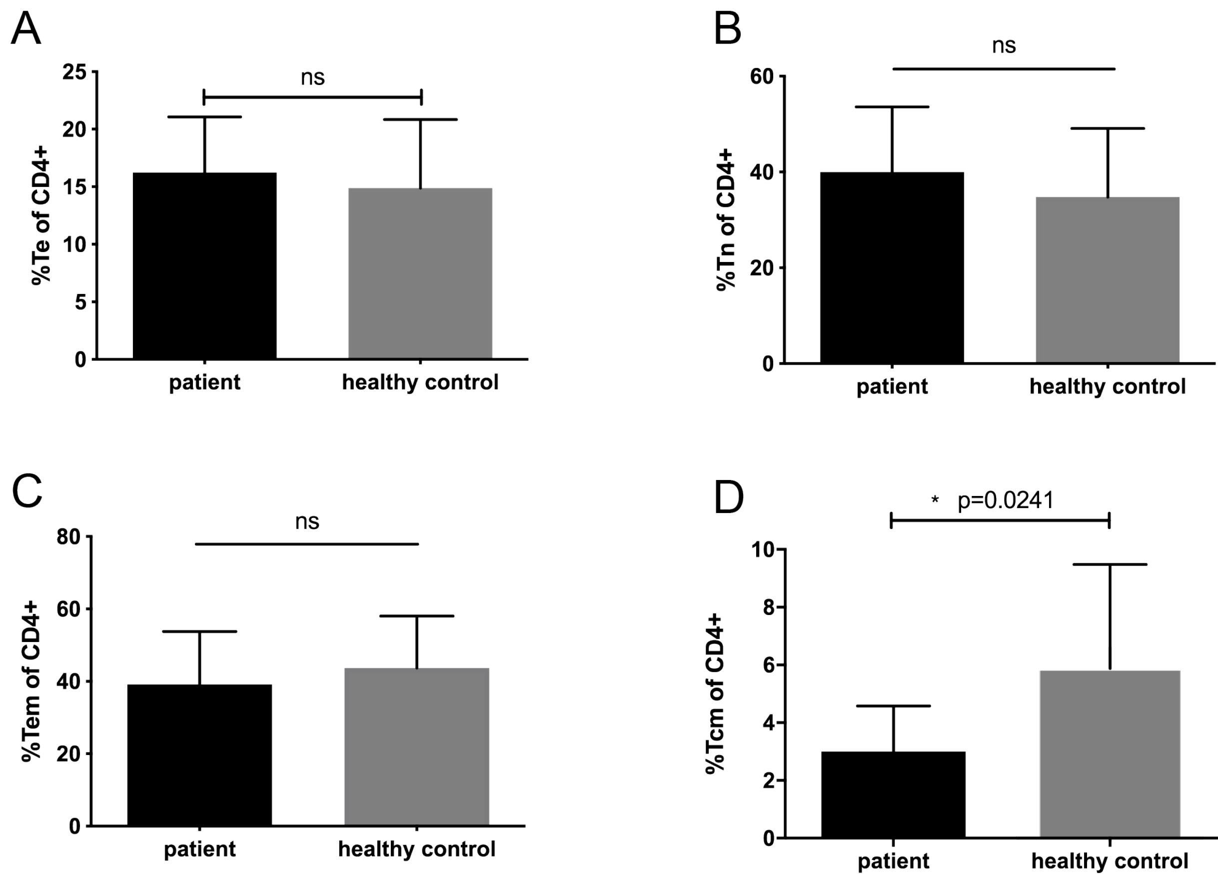


Figure 16: Frequencies of Th Subsets in Patients and Healthy Controls

No significant differences were found between circulating Te, Tn and Tem in patients compared to healthy controls. **A:** Te of all CD4+ MS patients: 16,24 ± 4,845 vs. healthy controls: 14,89 ± 5,957 **B:** Tn of all CD4+ MS patients: 39,99 ± 13,58 vs. healthy controls: 34,76 ± 14,31 and **C:** Te of all CD4+ MS: 39,15 ± 14,6 vs. healthy controls: 43,73 ± 14,29 **D:** Tcm in MS patients: 3 ± 1,578 vs. healthy controls: 5,876 ± 3,605 p = 0,0241 (mean ± SD respectively)

3.2.1 Cytokine-Producers in MS Patients

First, all of the cytokines selected for analysis were compared individually for the frequencies in Tem between patients and healthy individuals. IL-10 producers were found elevated in MS patients (patients 3,120 ± 1,591; healthy controls 2,145 ± 1,694, p = 0.0484) (Figure 17, Table 17). No differences were found in the frequencies of IFN-ɣ, GM-CSF, IL-21, TNF-α, IL-22, IL-2 and IL-17 between patients and healthy individuals (Figure 17, Table 17).

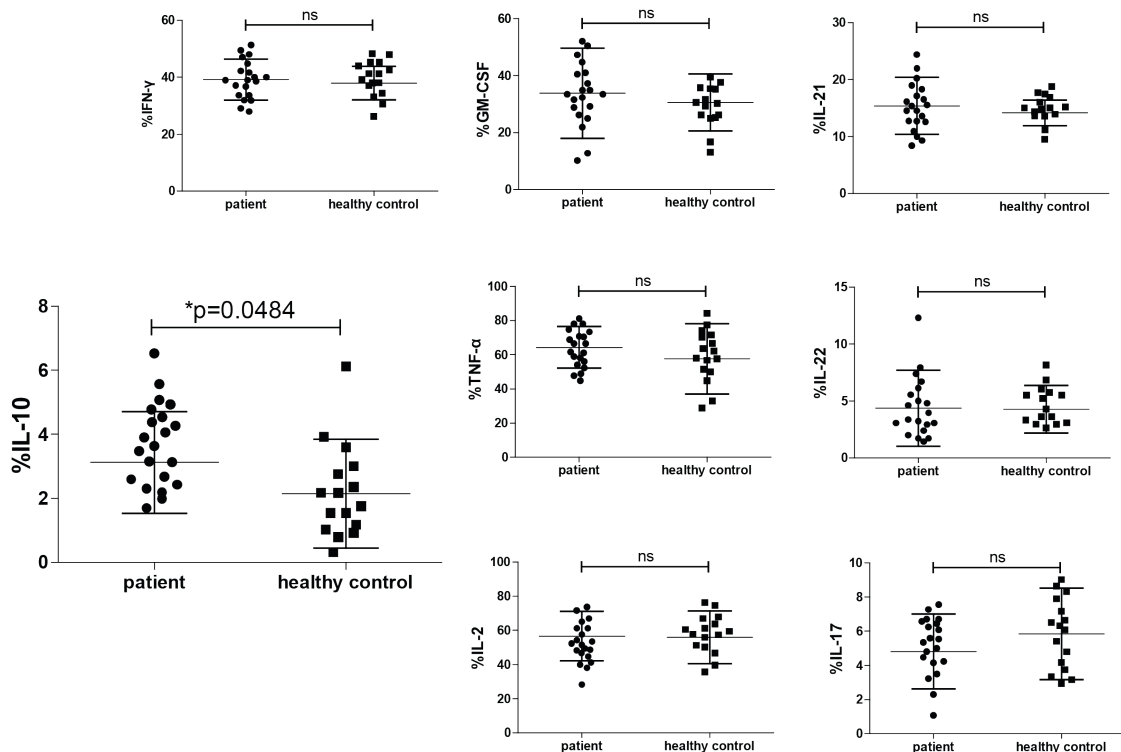


Figure 17: Cytokine Production in Tem in Patients and Healthy Controls

Frequencies of cytokine producers among Tem ($CD45RA^{-}CCR7^{-}$) in MS patients and healthy controls. IL-10 producers were found elevated in MS patients, frequencies of all other measured cytokines were comparable between both groups.

Table 17: Cytokine Production in Tem MS and Healthy Controls

	Patient **Mean ± SD**	**Healthy Controls** **Mean ± SD**
IFN-ɣ	39,21 ± 7,242	37,90 ± 5,860
GM-CSF	33,83 ± 15,79	30,58 ± 10,00
IL-10	3,120 ± 1,591	2,145 ± 1,694,
IL-21	15,35 ± 5,026	14,20 ± 2,248
TNF-α	64,33 ± 12,17	57,66 ± 20,54
IL-22	4,373 ± 3,329	4,276 ± 2,077
IL-2	56,63 ± 14,38	55,94 ± 15,35
IL-17	4,373 ± 3,329	4,276 ± 2,077

Multi-Cytokine Producers

Following the comparison of single cytokine producers, we also looked at double cytokine producers and compared frequencies circulating in patients with those of the control group. Finally, an analysis of multiple cytokine producers was performed, using Boolean analysis. We compared frequencies of a variety of potential cytokine combinations in healthy donors and MS patients. The analysis was structured into three groups focusing on IL-17, IL-21, and GM-CSF respectively.

Multi-Cytokine Producers: IL-17

Since Th17 cells are assumed to be primarily involved in the MS pathogenesis, an analysis of IL-17 co-producing Tem was performed. Similar frequencies were found in IL-17/GM-CSF as well as IL-17/TNF-α and IL-17/IFN-ɣ producers in MS and healthy controls (Figure 18). Slight increases within the patient cohort can at best be described as a trend. The Boolean analysis also revealed no differences in any of the IL-17 multi-cytokine-producing subpopulations. It was found that the co-expression patterns of TNF-α, GM-CSF and IFN-γ with IL-17 differed significantly. These patterns were consistent in all samples analyzed, independent of disease status. If IL-17 and GM-CSF are co-expressed, both are likely to have higher expression intensity, whereas the highest TNF-α expression did not correlate with IL-17.

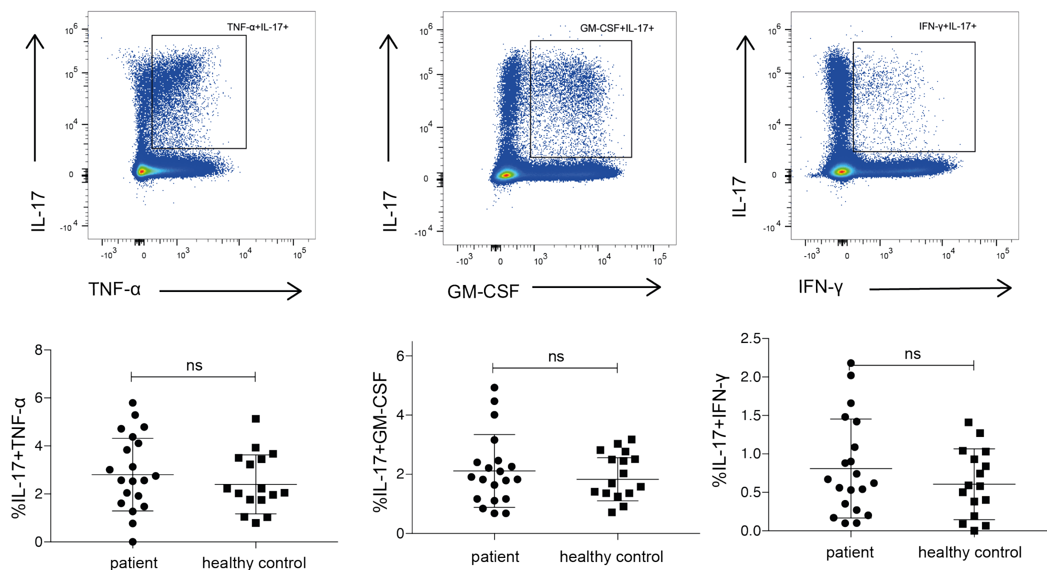


Figure 18: IL-17 Co-Producing Tem in Patients and Healthy Controls

We found comparable frequencies in IL-17+TNF-α Tem in MS patients (2,804 ± 1,511) and in healthy controls (2,399 ± 1,231). The same was true for IL-17+GM-CSF+ Tem in MS patients (2,115 ± 1,233) vs. healthy controls (1,829 ± 0,7282) and IL-17+IFN-ɣ+ in MS patients (0,532 ± 0,476) and healthy controls (0,442 ± 0,317). (mean ± SD respectively)

Cytokine Co-Producers: IL-21

IL-21/GM-CSF and IL-21/IFN-ɣ co-producing Tem show comparable levels in MS and healthy controls in the analyzed cohort (Figure 19). The Boolean analysis also revealed no differences in any of the IL-21 multi-cytokine-producing subpopulations. Both GM-CSF and IFN-γ were co-expressed regularly with IL-17 as expected. However, the GM-CSF producers with the lowest expression levels were least likely to also produce IL-21. IFN-γ/IL-21 co-expression appeared constant, independent of the respective cytokine intensities.

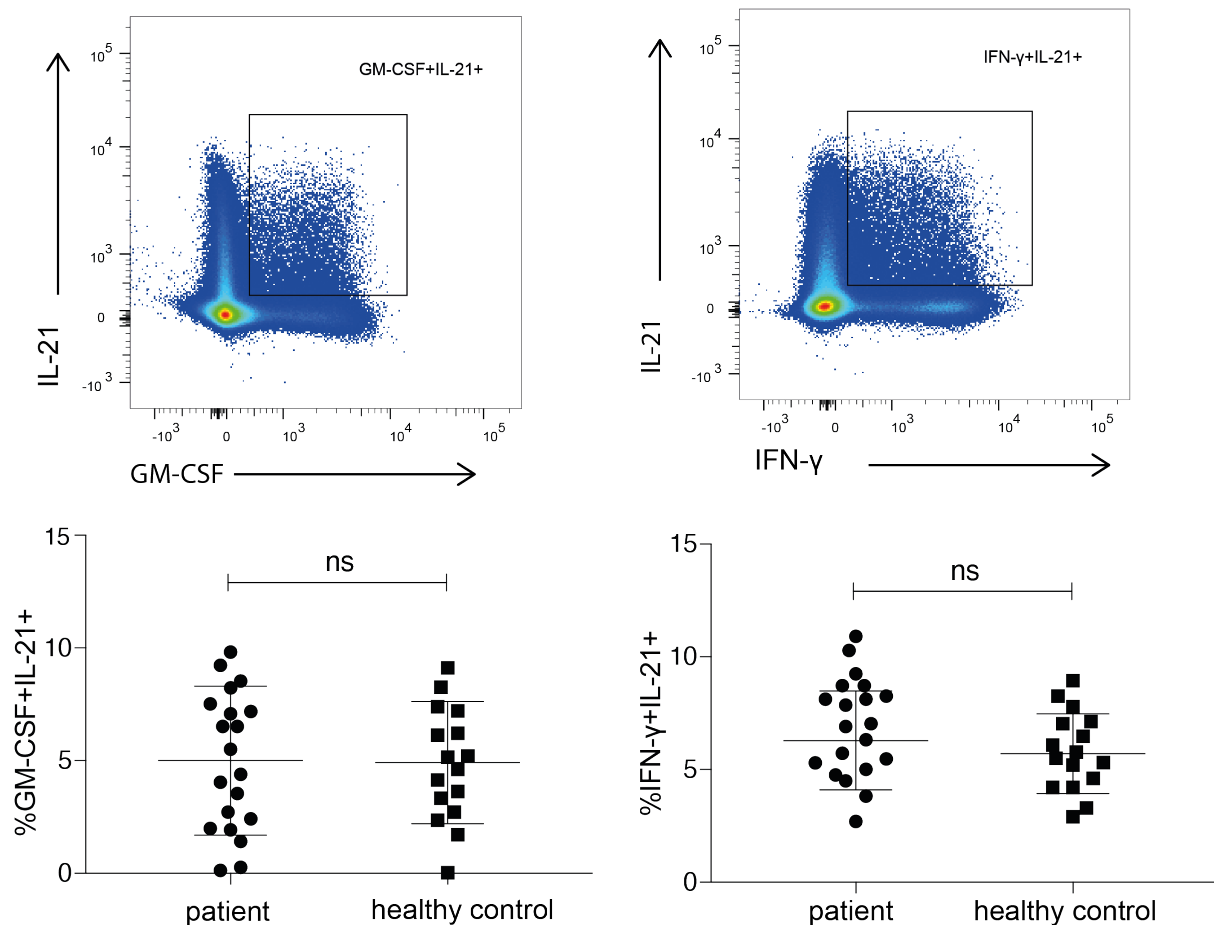


Figure 19: IL-21 Co-Producing Tem in Patients and Healthy Controls

No differences were found between frequencies in patients and controls. Frequency of IL-21+GM-CSF+ Tem in MS patients was 5,002 ± 3.003 and in healthy controls 4,916 ± 2,712. Frequency of IL-21+IFN-γ+ Tem in MS Patients was 6,287 ± 2,194 and in healthy controls 5,704 ± 1,770. (mean ± SD respectively)

Cytokine Co-Producers: GM-CSF

When looking at GM-CSF/IFN-γ and GM-CSF/TNF-α double producers, no differences could be found between patients and healthy persons either (Figure 20). The Boolean analysis also revealed no differences in any of the GM-CSF multi-cytokine-producing subpopulations. In general, IFN-γ lowest cells were less likely to co-produce GM-CSF, whereas GM-CSF lowest cells were unlikely to show a high TNF-α expression.

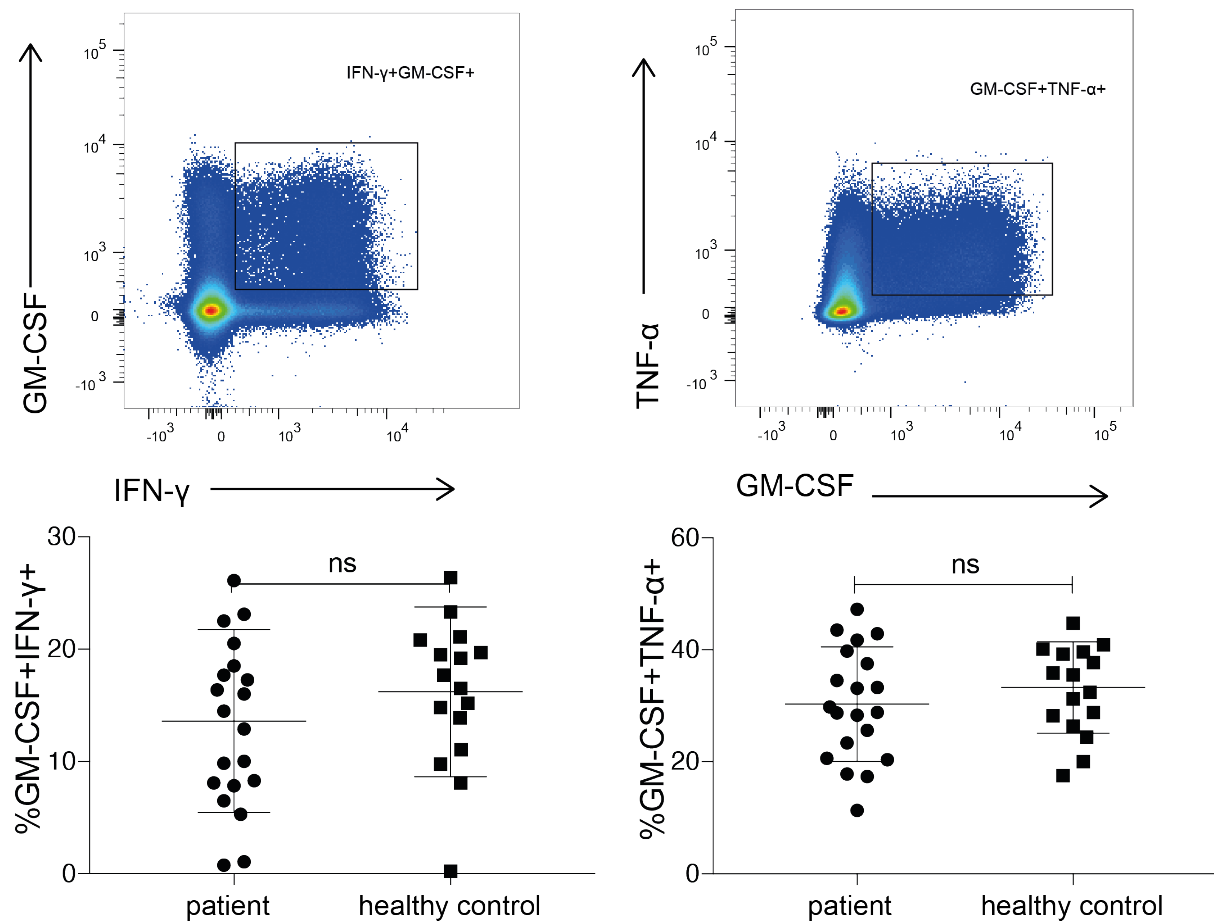


Figure 20: GM-CSF+IFN-γ+ and GM-CSF+TNF-α+ Tem in Patients and Healthy Controls

No differences were found between frequencies in GM-CSF+ double cytokine positive Tem in patients and controls. GM-CSF+IFN-γ+ in MS patients were 13,59 ± 8,137 and in healthy controls 16,21 ± 7,567. Frequencies of GM-CSF+TNF-α+ Tem in MS patients were 30,31 ± 10,22 and in healthy controls 33,26 ± 8,153. (mean ± SD respectively)

Cytokine Co-producers: IFN-ɣ/TNF-α and IFN-ɣ/IL-22

Neither IFN-ɣ/TNF-α nor IFN-ɣ/IL-22 co-producing Tem showed differences in frequencies of MS and healthy controls in the analyzed cohort either (Figure 21). Patterns of co-production were distinctly different in overall samples. While TNF-α co-production was more likely with increasing expression of IFN-γ, IL-22 co-production with IFN-γ was dependent on IL-22 expression intensity.

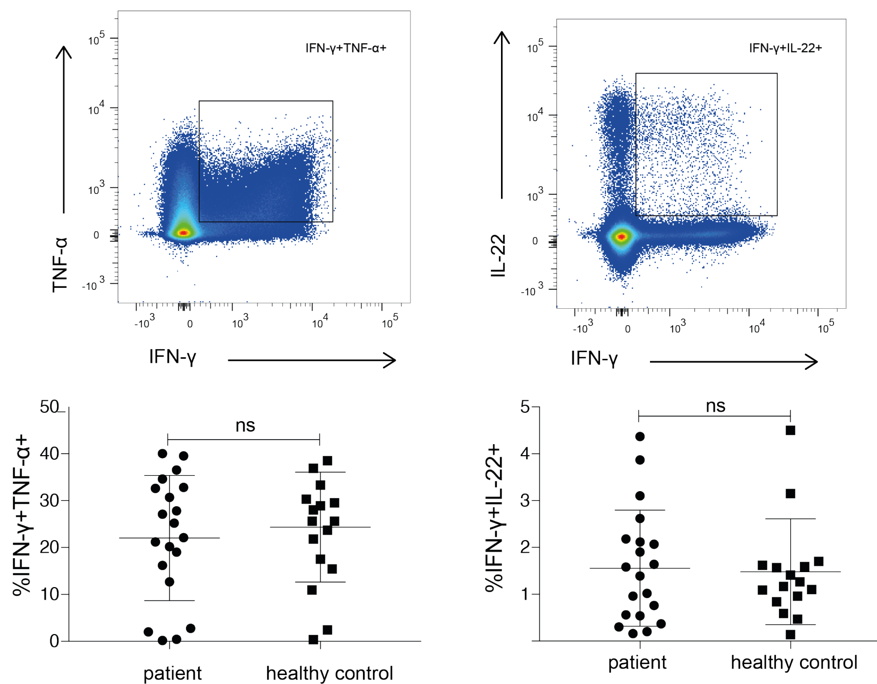


Figure 21: IFN-ɣ+TNF-α+ and IFN-ɣ+IL-22+ Tem in Patients and Healthy Controls

IFN-γ co-production with TNF-α was observed at comparable levels in MS Patients (27,53 ± 8,608) and healthy controls (28,55 ± 6,289) (ns). IFN-ɣ+IL-22+ double producers were found in equal frequencies in MS patients (1,557 ± 1,240) and controls (1,481 ± 1,130) as well. (mean ± SD respectively)

3.2.2 T cell Checkpoint Receptors in MS Patients

Next, we compared frequencies of the PD-1+ and OX40+ Tem cells. An increased frequency of the OX40+ Th cells was found, while PD-1 was expressed at comparable levels in the MS group (Figure 22).

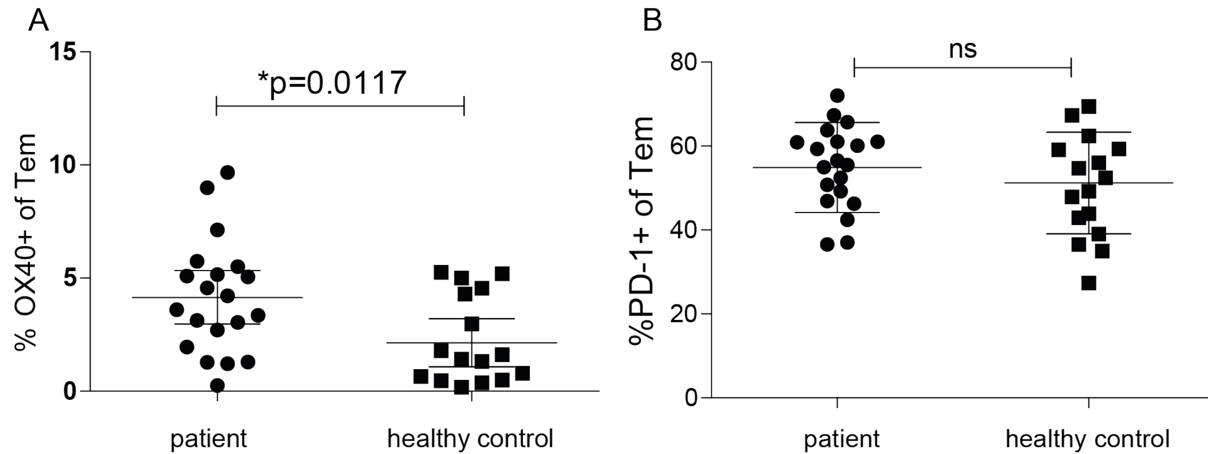


Figure 22: OX40+ and PD-1+ Tem in Patients and Healthy Controls

A: Frequency of OX40+ cells was elevated in Tem of MS patients (4,149 ± 2,515) compared to healthy controls (2,142 ± 1,925) (p = 0.0117). **B:** PD-1+ cells were found at comparable frequencies among Tem of patients (54.90 ± 10.75) and healthy controls (51.21 ± 12.09 (mean ± SD respectively).

3.2.3 Effect of Medication on T cell Subsets in MS

Since it was not possible to obtain a substantial cohort of unmedicated patients in this study, certain immunomodulatory substances are present in the cohort (Table 15). Naturally, it is of interest to know whether the medication affects the measured parameters. None of the substances presented here changed the frequencies of the circulating T cell subsets significantly so that subsequently the patients will be considered as an overall cohort with respect to the surface marker-based parameters. (Figure 23) shows an exemplary comparison for the influence of medication on the circulating frequencies of Tcm, Tn, Tem, and Te.

Table 15: Number of Patients Treated with the Respective Immunomodulatory Drug

	no medication	Interferon-beta (Betaferon®, Rebif®)	Teriflunomid (Aubagio®)	Glatirameracetat (Copaxone®)	Daclizumab
N	7	5	3	4	1

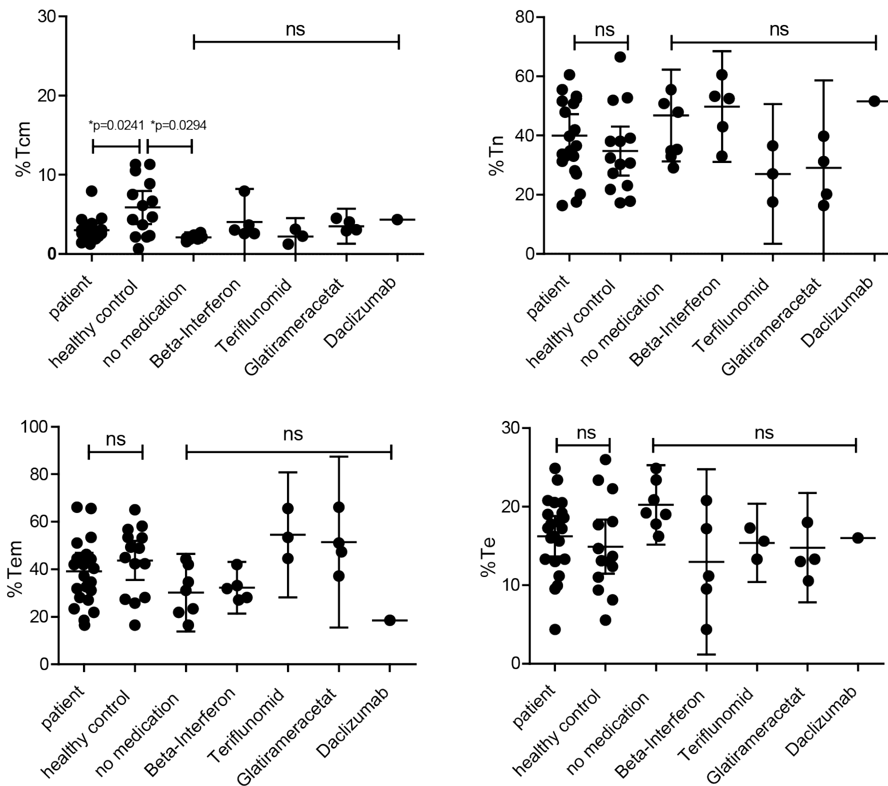


Figure 23: Frequencies of CD4+ Subsets - Effect of Medication

When comparing frequencies of different Th cell subsets with different effector and memory capacities, no differences were found using 1way ANOVA between subgroups medicated with Interferon, Teriflunomide, Glatiramer acetate, and Daclizumab and healthy controls of patients without immunomodulatory medication.

Effect of Medication on Cytokine-Producing Subsets

The next question was whether cytokine production is influenced by immunomodulatory medication. Table 18 summarizes trends observed in the comparison between unmedicated patients and the ones treated with the respective immunomodulatory drug. Overall, no statistically significant differences were observed. Figure 24 shows detailed results for frequencies of the measured cytokines.

Table 18: Influence of Medication on Cytokine Frequencies-Overview

	IFN-ɣ	GM-CSF	IL-10	IL-21	IL-17	IL-2	IL-22	TNF-α	Observed Trends
Beta-Interferon	+/-	↑	+	+/-	+/-	+/-	+/-	+/-	**+/-**
Teriflunomide	↑	↑	+	+/-	+/-	↑	+/-	+/-	↑
Glatiramer acetate	↑	↑	+/-	↑	+/-	↑	+/-	↑	↑
Daclizumab	↓	↓	+/-	↓	↓	+/-	+/-	↓	↓

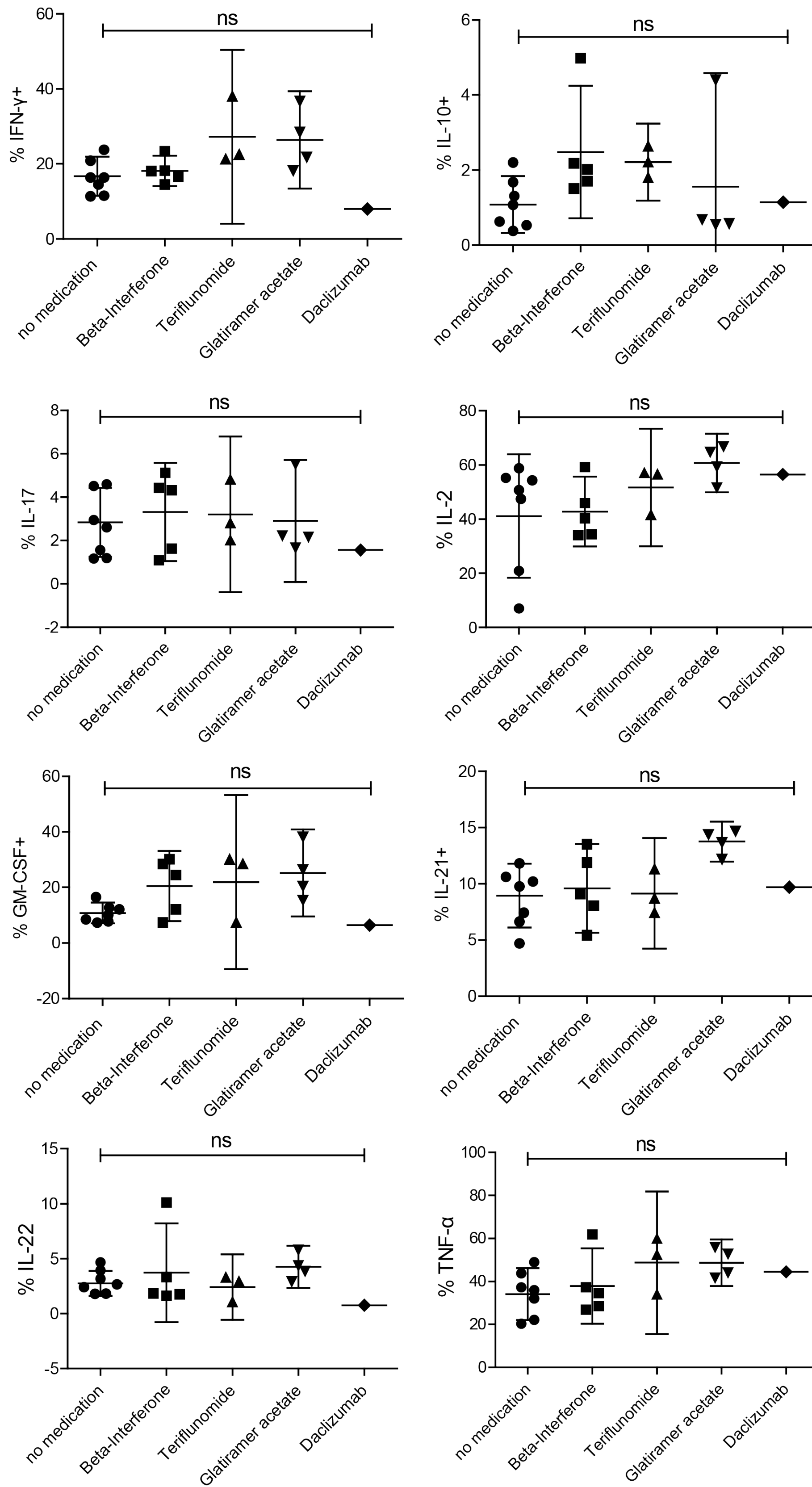


Figure 24: Cytokine-Producing CD4+ T cells - Effect of Medication

No significant differences were found in the production of any of the cytokines (INF-γ, IL-10, IL-17, IL-2, GM-CSF, IL-21, IL-22, TNF-α), using 1way ANOVA between subgroups of unmedicated patients and the ones treated with Beta-Interferon, Teriflunomide, Glatiramer acetate or Daclizumab.

3.3 CD3+ CD20+ T cells

Another objective was the analysis of CD20+ T cells. These cells are known to form a cytokine-producing T cell population with memory features. The aim was to gain a better understanding of their characteristics in healthy donors and MS patients. The analysis of the CD20+ T cells was conducted by first gating out CD19+ B cells. Next, the selection of CD20+CD3+ cells was performed and further analysis of CD4 and CD8 expression followed (Figure 25). Overall frequencies of 3,381 ± 0,4867 (mean ± SEM) of CD20+ out of all CD3+ T cells were observed among healthy donors. Results found in MS patients are detailed in 3.3.1 and Figure 27.

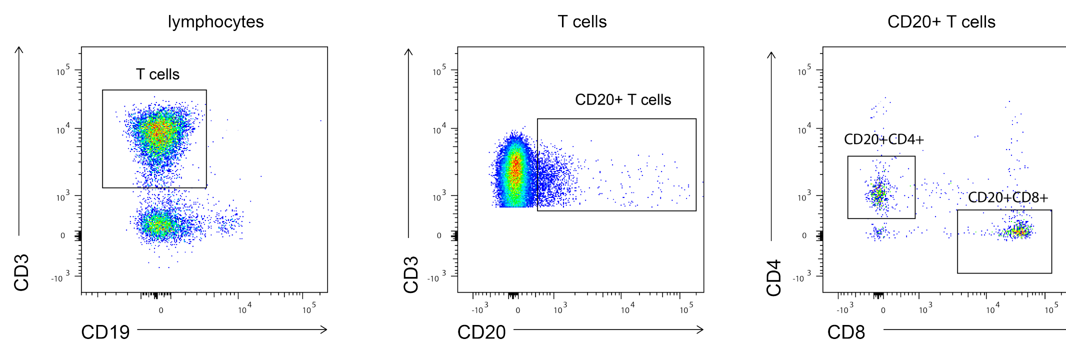


Figure 25: Gating Strategy to Identify CD20+ T cells

After initial gating for lymphocytes based on size and granularity, CD3+ and CD19- T cells were then gated for CD20 positivity and then further distinguished by CD4 and CD8 expression to identify cytotoxic as well as Th cells.

CD 20+ T cells - Distribution of CD4 and CD8

The frequency of cytotoxic T cells was found to be elevated among CD20+ T cells. Consequently, this leads to a higher CD4/CD8 ratio in CD20+ T cells (Figure 26). Still, overall, there were more CD4+ than CD8+ T cells within the CD20+CD3+ pool.

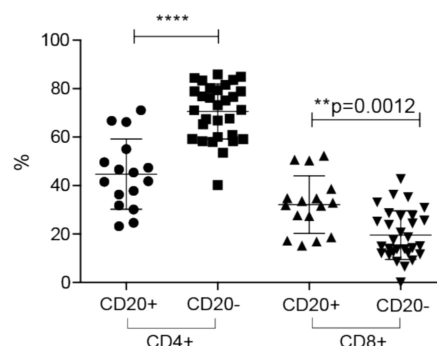


Figure 26: CD4/CD8 Ratio is Higher in CD20+ T cells compared to CD20- T cells

We found overall lower frequencies of CD20+CD4+ (44,69 ± 14,52) compared to CD20-CD4+ (70,52 ± 11,33, $p < 0.0001$). In contrast among cytotoxic T cells frequencies were higher in CD20+ CD8+ (32,14 ± 11,84) compared to CD20-CD8+ (19,57 ± 10,06, $p = 0.0012$). (mean ± SD respectively)

3.3.1 CD20+ T cells in MS

To determine disease-associated differences, the frequency of CD20+ T cells was compared between patients and healthy controls. First, all CD20+ T cells (CD19-CD3+CD20+) were analyzed and the two groups compared. No differences were found (Figure 27A). Since the ratio between helper and cytotoxic T cells was shifted among CD20+ T cells, the frequencies of CD4 and CD8 T cells of MS patients and healthy controls were compared to see if there were any variations. Again, no differences were found (Figure 27B, C).

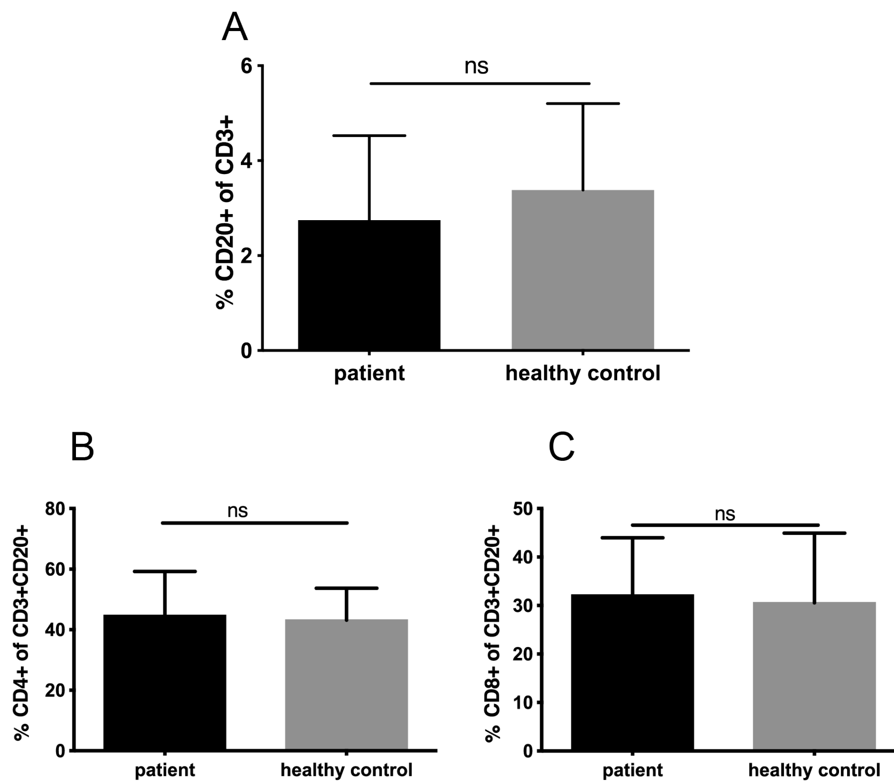


Figure 27: Frequencies of CD20+ T cells in Patients and Healthy Controls

A: CD20+ T cells occurred in MS patients (2,749 ± 1,779) and healthy donors (3,381 ± 1,821) in comparable frequencies (p = 0,2043). **B:** The same was true for helper T cells (CD4+ of CD3+CD20+) in patients (44,69 ± 3,63) vs. healthy controls (43,16 ± 2,812; p = 0,7415). **C:** Frequencies of cytotoxic T cells (CD8+ of CD3+CD20+) were also comparable in patients (32,14 ± 2,96) and in healthy controls (30,57 ± 3,834; p = 0,7482). (mean ± SD respectively)

Next, the Th cells of healthy controls and patients treated with different immunomodulatory drugs were compared regarding their frequencies of CD20+ cells. Patients treated with Teriflunomide were found to have elevated frequencies of circulating CD20+ T cells in PBMC compared to healthy and to unmedicated (Figure 28). None of the other inter-group comparisons revealed significant differences.

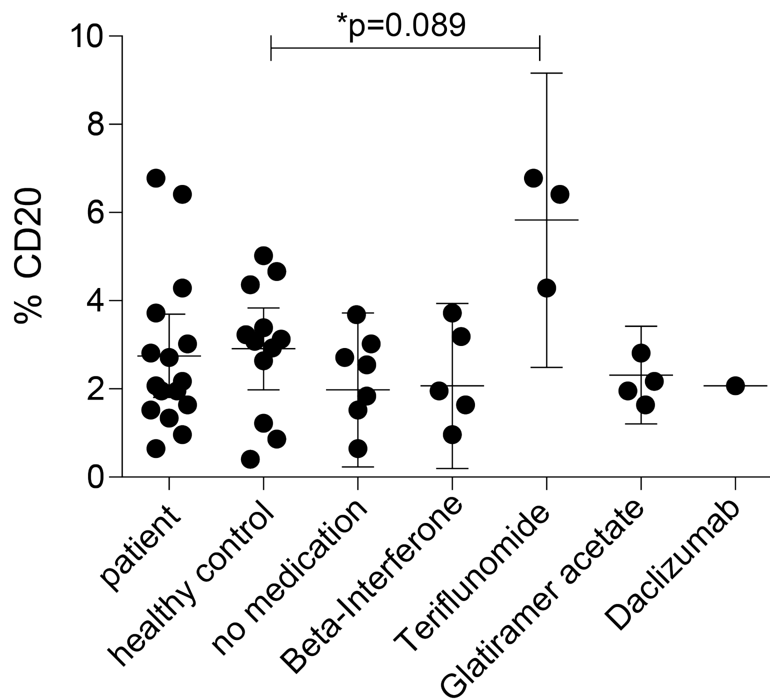


Figure 28: CD20+ T cells: Effect of Medication

Shown here are frequencies of CD20+ cells out of all CD3+ cells. Statistically relevant differences were found between the healthy control group (3,381 ± 1,821) and patients medicated with Teriflunomide (5,827 ± 1,344; p = 0,089). In the overall patient samples frequencies were 2,749 ± 1,779 and in unmedicated patients 1,975 ± 1,095. Among the other medicated patients (Beta-Interferon 2,068 ± 1,177 Glatiramer acetate 2,310 ± 0,4468, Daclizumab 2,070) differences in frequencies were not found statistically significant. (mean ± SD respectively)

3.3.2 Memory Phenotype of CD20+ Th cells

To determine memory and lymph node homing capacities CD20+ T cells were gated for CD45RA and CCR7 status, resulting in four subpopulations (Figure 9A). Figure 29 compares the frequencies of those subpopulations in CD20+ Th cells. Generally speaking, there were more CD45RA- and less CCR7+ among the CD20+CD4+ T cells. An elevated frequency of Tem cells (CD45RA-CCR7-) was observed (Figure 29C). Accordingly, Tn cells (CD45RA+CCR7+) were diminished among CD20+ compared to CD20-CD4+ T cells (Figure 29B). Therefore, it can be concluded that an effector memory phenotype predominates among CD20+ cells.

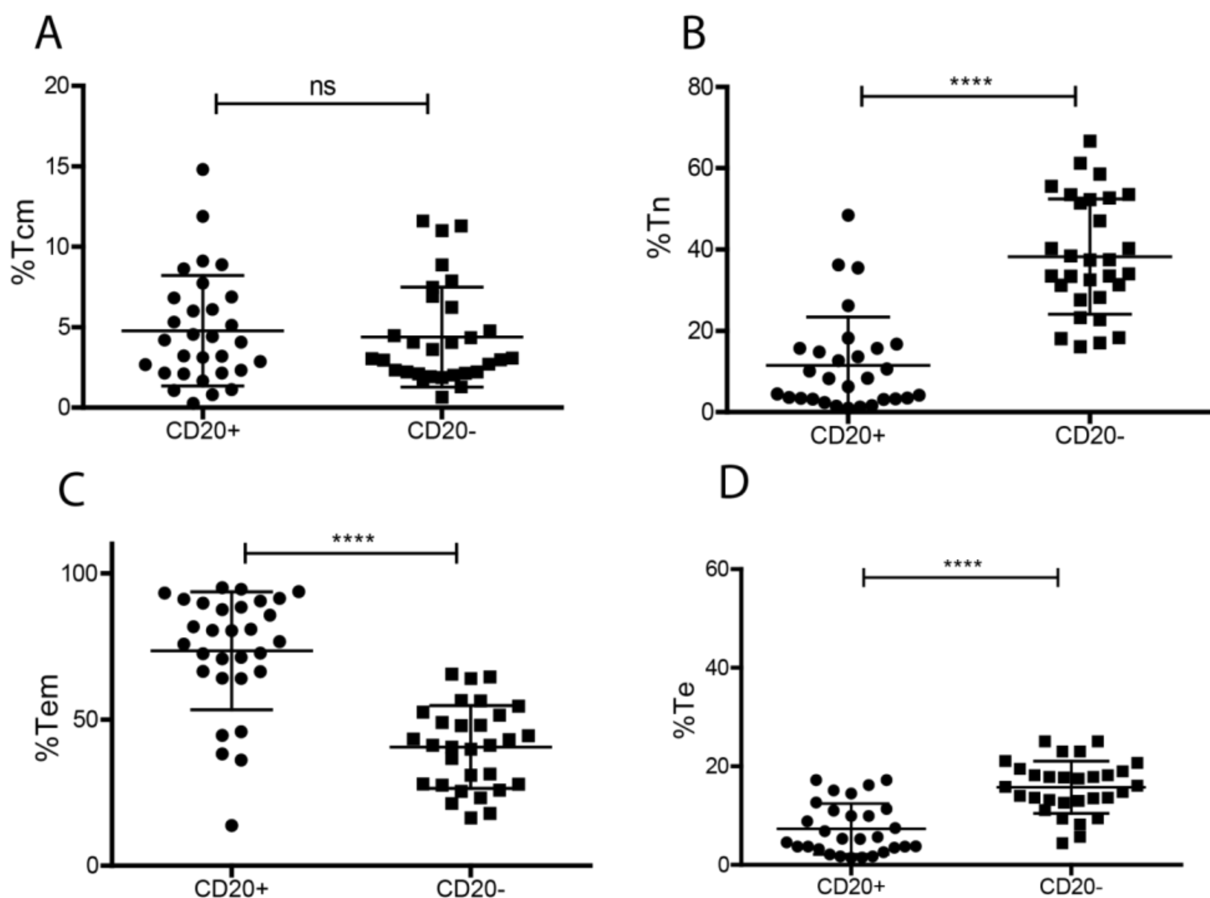


Figure 29: CD4+ CD20+ T cells Display Effector Memory Phenotype

A: Tcm cells were equally represented in both CD20+ (4,78 ± 3,422) and CD20- (4,399 ± 3,101) Th cells. **B:** In contrast CD20+ T cells were low in Tn cells (11,52 ± 11,85) compared to CD20- cells (38,25 ± 14,17; p < 0,0001). CD20+ Th cells were enriched in Tem cells. **C:** We found a predominant Tem population in CD20+ (73,48 ± 20,16) compared to CD20- T cells (40,58 ± 14,17; p < 0,0001). **D:** Te cells were significantly reduced in CD20+: (7,317 ± 5,149) compared to CD20- T cells (15,76 ± 5,297; p < 0,0001). (mean ± SD respectively)

3.3.3 Cytokine Production Capacities of CD20+ Th cells

To show functional capacities of CD20+ cells, cytokine production after PMA/Ionomycin stimulation was measured by intracellular FACS-Staining. Elevated production of a number of cytokines (INF-γ, TNF-α, GM-CSF, IL-2, IL-10) was observed in CD20+ Th cells (Figure 30).

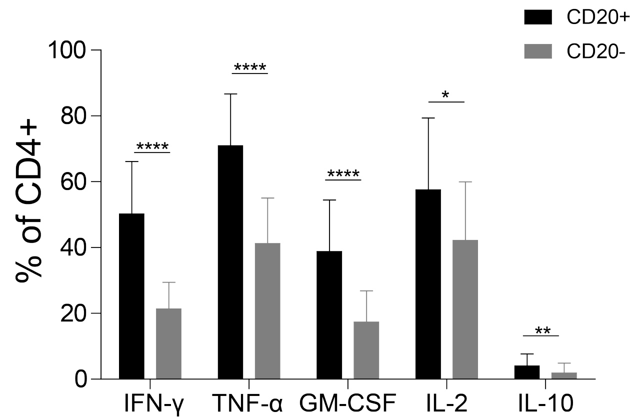


Figure 30: Cytokines Elevated in CD20+ Th cells

All cytokines presented here were significantly elevated in the CD20+ compared to CD20- Th cells. Frequency of INF-γ+ was 50,25 ± 15,90 in CD20+ and 21,35 ± 8,083 in CD20- with p < 0,0001. Frequency of TNF-α producers was 70,97 ± 15,77 in CD20+ and 41,25 ± 13,75 in CD20- with p < 0,0001. Frequency of GM-CSF producers in CD20+ was 38,79 ± 15,63 and 17,40 ± 9,448 in CD20- with p < 0,0001. Frequency of IL-2 producers was 57,53 ± 21,85 in CD20+ and 42,23 ± 17,72 in CD20- with p = 0,0450. Frequency of IL-10 producers was 3,991 ± 3,599 in CD20+ and 1,864 ± 2,973 in CD20- with p = 0,8351. (mean ± SD respectively)

With IL-17, IL-21, and IL-22 no significant differences could be found between cytokine production in CD20+ vs. CD20- Th cells (Figure 31).

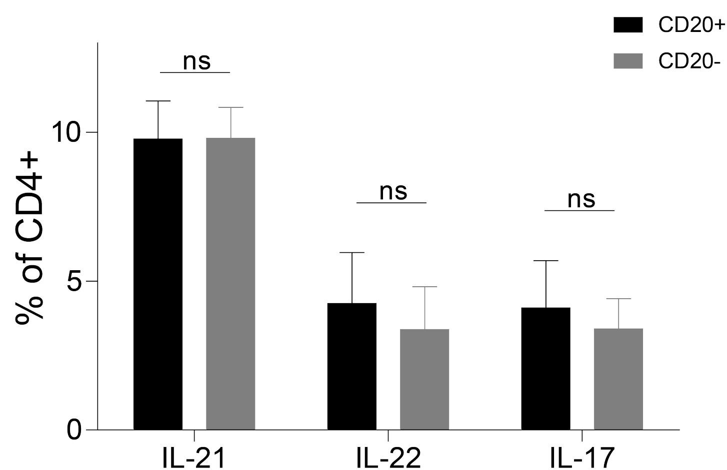


Figure 31: Cytokines with No Elevation in CD20+ Th cells

There was no significant difference in expression levels for the cytokines IL-21, IL-22 and IL-17 as shown above. Frequency of IL-21 producers in CD20+ was 9,701 ± 0,61 and in CD20- 9,803 ± 0,5099 with p = 0,8977. Frequency of IL-22 producers in CD20+ was 4,249 ± 0,7974 and in CD20- 3,379 ± 0,6753 with p = 0,3296. Frequency of IL-17 producers in CD20+ was 4,105 ± 0,7396 and in CD20- 3,395 ± 0,4727 with p = 0,4273. (mean ± SD respectively)

3.3.4 Features of Activation in CD20+ Th cells

In addition to cytokine production, PD-1 was also measured as a co-inhibitory receptor and surrogate for cell maturation/activation. We found that roughly two-thirds of CD20+ Th cells carried the PD-1 receptor, whereas only roughly one-third of CD20- ones did (Figure 32A). When gated for different expression levels of PD-1 (positive, 20 %highest, 10 % highest) the frequency of PD-1+ cells was more than doubled in CD20+ T cells compared to CD20-. The differences between CD20+ and CD20- T cells diminished with PD-1 upregulation, while still remaining significant (Figure 32B). The INF-γ expression was examined as the third parameter in this interaction. A clear concentration and positive correlation of the IFN-γ+ cells with PD-1 and CD20 upregulation was observed. This is reflected in the frequency (Figure 32C) as well as in the fluorescence intensity (Figure 32D, E). The cells with highest IFN-γ expression also expressed CD20 and PD-1 on their surface. A positive correlation could also be observed for the other cytokines investigated (TNF-α, GM-CSF, IL-2, IL-10), although not to an equally significant extent.

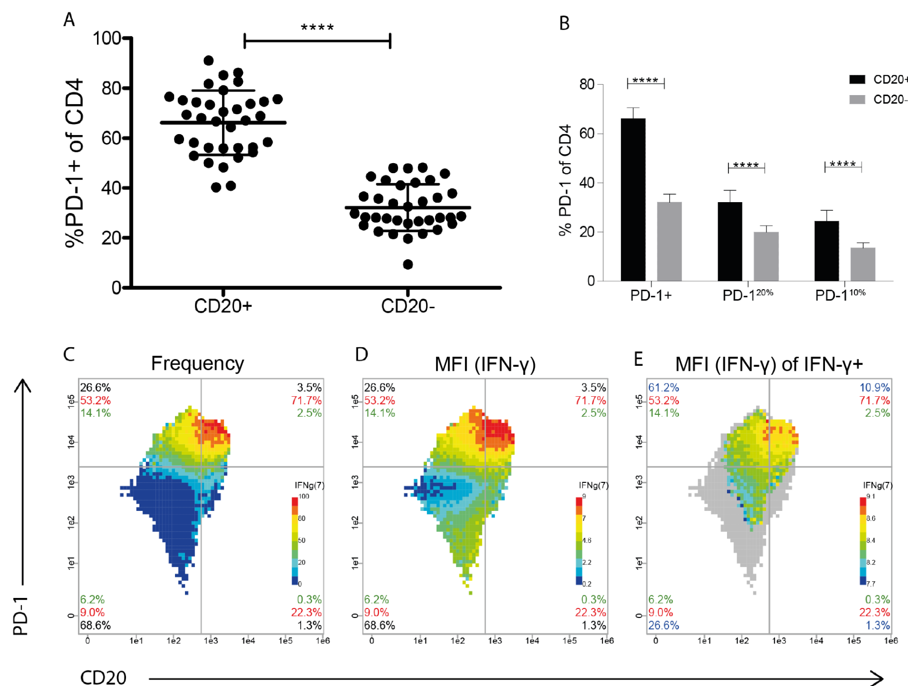


Figure 32: PD-1 is Upregulated in CD20+ Th cells

A: Shows enrichment of CD20+ T cells in PD-1. Frequency of PD-1+ Th cells in CD20+ was elevated (66,13 ± 12,87) compared to CD20- (32,15 ± 9,379, p < 0,0001). **B:** This enrichment persists with PD-1 upregulation and the differences between CD20+ and CD20- cells in PD-1 expression level remain significant also in the PD-1 highest population. **C:** Almost 72 % of PD-1+CD20+ cells also produce IFN-γ. **D:** The color code shows increasing expression intensity in IFN-γ with increasing PD-1 and CD20 expression. **E:** Even though only 10,9 % of all IFN-γ producers are PD-1+CD20+, those that are, have higher cytokine expression. (mean ± SD respectively)

4 Discussion

The aim of the study was the analysis of activated Th cell subpopulations. The characterization of these populations, including poly-functional cells, and the visualization of these populations using multicolor flow cytometry were complemented by multi-parametric data visualization with PRI (Pattern Recognition of Immune Cells), a tool developed in our group. Furthermore, we performed a comparative analysis of T cell populations in patients suffering from MS and healthy volunteers.

4.1 Th cell Subsets with Strong Activation Profile

Th subsets carry unique transcription factors but are also often identified by their signature cytokines or defined and categorized on the basis of these. However, some Th cells can produce more than one signature cytokine, which can lead to complex actions, causing a variety of effects. Strict adherence to the established Th cell model has therefore increasingly been questioned, based on the heterogeneity of the memory Th subset as well as the heterogeneity of pathogenic Th cells that play a role in different immune-mediated diseases (63).

Different approaches to defining pathogenic Th subsets should, therefore, be considered by analyzing functional capacities i.e. co-production of key transcription factors, receptor repertoire, cytokine production or regulatory capacities. Considering the multifacetedness of autoimmune disease this approach is likely to provide new insights into the complexity of interactions of T cells in health and disease.

4.1.1 IL-21-Producers Exhibit a Diverse Cytokine Profile

Here we found that IL-21 is co-produced with IFN-ɣ and GM-CSF but that GM-CSF-IFN-ɣ dual producers are not always IL-21 producers (Figure 14).

IL-21 is a Tcm cell-associated cytokine with pleiotropic properties (64). It stimulates Tfh differentiation via MAF and Bcl6 but can also inhibit the generation of pathogenic Th1/17 effector cells (65). IL-21 is also co-produced by memory Th1 cells with IFN-ɣ. It is discussed as a Tfh or Th17 cytokine. The IL-21 effect stimulates B cell differentiation and the germinal center formation and induces B-regulatory cells (Bregs) while inhibiting Tregs. It has distinct roles in gene regulation and cross-regulatory actions on dendritic cells. A pathological role of IL-21 in inflammatory bowel disease (66) was demonstrated in the context of Th17 cells. Also, in patients with dermatomyositis and Polymyositis upregulation of IL-21 was found (67).

Although only limited functional consequences can be drawn from this, it suggests a diversity of the IL-21-producing Th cell spectrum.

4.1.2 PD-1 Helps to Characterize Cytokine-Producing Subpopulations

My analysis of healthy donors showed that PD-1+ cells did preferentially express a majority of pro-inflammatory cytokines. The PD-1 expression level of a cell did not influence IL-2 or IL-22 cytokine levels (Figure 12 D, E). With other analyzed cytokines PD-1+ cells produced more cytokines than PD-1- cells but the patterns were more complex. In the case of IFN-ɣ, IL-10, and IL-21 a steady increase of cytokine-producing cells was measured with increasing PD-1 expression
(Figure 11). IL-17, GM-CSF, and TNF-α, on the other hand, showed an increase with PD-1 upregulation but then a decrease with maximum PD-1 expression (Figure 12 A, B, C). This may indicate that Th subsets exhibit different PD-1 expression levels on average. Tfh cells carry higher PD-1 and Th17 or Th17-like rather lower PD-1 receptor levels. The importance of PD-1 in autoimmunity is well established (68). However, the question remains if, or how PD-1 could help to characterize cytokine-producing subsets in human Th cells. PD-1+ memory CD4+ T cells that have been analyzed in HIV-Infected children had high inflammatory cytokine effector functions. They were shown to preferentially secrete the Th1 and Th17 cytokines INF-γ and IL-17A. These functional features persisted and were not reversed by the in vitro PD-1 blockade (69). CD8 exhaustion was observed in untreated patients with active disease. While it was linked to poor prognosis in viral infection, it was in contrast to this associated with a favorable outcome in autoimmunity (70). The individual patterns differ between cytokines with PD-1 upregulation. Therefore, a functional variety can be assumed according to PD-1 status.

4.1.3 CD20+ T cells likely Pro-Inflammatory Cells with Memory Potential

Our results demonstrate that CD20+ T cells are cytokine-producing (Figure 30), memory-profile-bearing (Figure 29), activated T cells (Figure 32), that constitute about 3 % of CD3+ cells. Therefore, showing a variety of features that could classify them as polyfunctional cells. In line with previously published data (30), we were able to demonstrate a clear shift towards the cytotoxic cell type among CD20+ cells (Figure 27). Further, consistent with findings of previous studies, (31) an effector memory phenotype was observed in CD20+ cells. CD20+ cells contain more CD45RA- and Tem cells (CD45RA-CCR7-). CD20+ T cells contain less CCR7+ Tn

(CD45RA+CCR7+) and Te cells (CD45RA+CCR7-) than CD20- T cells (Figure 29). The PD-1 expression on CD20 cells was analyzed as a surrogate for T cell activation. It was shown that the PD-1 expression in CD20+ Th cells was significantly higher than in CD20- cells (CD20+ 66.13 ± 12.87 CD20-: 32.15 ± 9.379) (Figure 32). The frequency of PD-1+++ (10 % highest PD-1 expression of total CD4mem), was evenly distributed in CD20+ and CD20-. Therefore, this suggests that the CD20+ Th cells should be classified as activated T cells.

Of all cytokines studied here, overall, CD20+ T cells in healthy individuals showed an increased production of IFN-ɣ in particular, but also of TNF-α, GM-CSF, IL-2, and IL-10 compared to CD20- T cells (Figure 30). Similar production of cytokines in CD20+ was observed of IL-21, IL-22 and IL-17 (Figure 31). This confirmed the results for TNF and IFN-ɣ shown by Schuh et al. also determined after PMA/Ionomycin stimulation and intracellular FACS measurement (31). GM-CSF, IL-2, and IL-10 production in CD20+ T cells had not yet been investigated. IL-21 production of CD20+ T cells had also not yet been investigated but was not increased compared to CD20+ T cells in this study. For IL-22 and IL-17 Schuh et al. showed a trend towards higher production in CD20+, whereas the frequencies in our sample were not significantly increased. Thus, according to the results presented here, CD20+ Th cells seem to be enriched in Th1 and Th2 types. In summary, all previous results indicate that the CD20+CD3+ population is an activated subset with memory properties and pro-inflammatory potential. It remains questionable whether these cells actually represent an independent population or rather a mixture of different lineages. From the data shown here, it is difficult to obtain a direct indication of the function of these cells, but the accumulation of these cells in the peripheral blood of MS patients was investigated hereafter.

4.2 Th cell Subsets in MS

Considering the role of immune cells in the pathophysiological processes of systemic autoimmune diseases, it stands to reason to analyze their distribution in peripheral blood in order to draw diagnostic or therapeutic conclusions. Changes in the frequencies and composition of lymphocyte subsets in peripheral blood were shown for RA, systemic lupus erythematosus (SLE) and other autoimmune diseases (71).

For MS an increase of Th17 cells in CSF and peripheral blood has been shown compared to healthy donors or patients with non-inflammatory neurological diseases.

Elevated frequencies were observed during a relapse or in patients with acute symptoms compared to remission phases (72).

The activation of memory CD4 and CD8 T cells has been shown in peripheral blood of MS patients and is likely associated with the exacerbation of MS and might be a reflection of systemic immunological dysregulation (73).

CNS lesions show large numbers of macrophages and CD8+ T cells (74) and, to a lesser extent, CD4 T cells, B cells and plasma cells (75). However, there is some discrepancy within the findings concerning frequencies of CD4 and CD8 T cells in peripheral blood (detailed references in 4.2.3).

4.2.1 Patient Cohort

Within the scope of the study carried out here, 36 subjects were recorded. Of these, there were 20 patients suffering from RR-MS in a relapse-free interval. Twelve of the examined samples were from women and 8 from men. This excess of the female sex reflects the gender distribution of the MS incidence where e.g. in Germany incidence of MS among women is almost twice as high as among men (76). The age range of the studied cohort of patients ranged from 23 to 65 years with a median of 41,5. The average age and the range examined here also correspond to the age at which the disease occurs in the German population as a whole where the prevalence reaches a peak between 45 and 54 years of age (76).

Overall, the patient cohort studied can be regarded as representative in relation to the population of stable RR-MS patients in general.

4.2.2 Analyzing T cells from Peripheral Blood

A fundamental challenge in the analysis of immunological mechanisms is the acquisition of relevant, affected material or tissue samples. If one considers the fact that T cell maturation and activation primarily takes place in lymphatic organs and the inflammation itself takes place typically in peripheral tissue or in the particular case of MS in the CNS, the question arises as to the value of the analyses of T cells from peripheral blood. However, it could be shown that activated T cells of the cytotoxic, as well as the helper type, can selectively cross the blood-brain barrier (77) and thus measurements in the peripheral blood can be used to represent pathological processes in the CNS. Since the immune cells migrate continuously via blood and lymphatic system during their migration, e.g. from their place of maturation to inflammation sites, etc., a large number of both naive and mature immune cells can

still be found in the blood. A clear advantage in the analysis of blood lies in the relatively easy accessibility (in contrast to e.g. CSF), which facilitates the clinical extraction and hence the potentially easier diagnostic applicability. A possible solution to this problem is the detection of certain migration or homing functions that can be detected by surface receptors such as chemokine receptors. CCR5, for example, could be used for this purpose (78). CXCR4 is also thought to have a pathogenic association with MS (79). By measuring the lymph node-homing receptor CCR7, for example, it was possible to classify the memory T cells into effector memory cells and thus classify them according to their potential effector function in this work. Access to and therefore analysis of CSF was not available for this study, but would likely provide interesting data complementary to the results presented here.

4.2.3 Frequencies of Circulating Lymphocyte Populations in MS

A large number of studies on frequencies of circulating T cells in MS patients compared to healthy donors showed heterogeneous results. There is evidence of elevated CD8+ cells in PBMCs and relative reduction in CSF (80). There are also studies describing increased frequencies of CD8 memory cells (CD8+CCR7+CD45RA-) (81) and CD8+CD45RA-CD27+ (82) in the blood of MS patients. Other studies found reduced frequencies of memory CD8+ T cells in CSF and blood of patients with MS (83), which corresponds to the data obtained in this research. In this study, the cytotoxic cells were reduced compared to the Th cells (Figure 15). In addition, CD4+ Tcm cells (CD45RA-CCR7+) were reduced in patients (Figure 16). Possible reasons for the diverging results include the heterogeneity of the disease, small cohort sizes, different genetic backgrounds, and different antibodies used to define T cell subpopulations. It is possible that there is a tissue redistribution of the populations and the CD8+ cells and CD4+ Tcm reduced here may have been shifted to the lymph nodes or the CNS in MS patients. This would be consistent with results obtained by TCR sequence analysis. It showed a very heterogeneous population of CD4 clones, as well as an increased number of CD8+ cells in perivascular CNS MS lesions. However, only two of the CD8 clones were also found in peripheral blood (74). After reviewing the existing literature and comparing it to my data, neither CD4 nor CD8 nor Tmem frequencies seem to be suitable to draw direct conclusions about the MS condition in the relapse-free interval.

CD4+/CD8+ Ratio in MS

The CD4+/CD8+ ratio (MS patients > healthy controls) was found to be elevated (Figure 15C). However, the overall ratio remained well above 5 in favor of CD4. This is consistent with other previously published data where the CD4/CD8 ratio was elevated in the blood (84) or in CSF of MS patients (85). In HIV patients the CD4/CD8 ratio was positively associated with activated CD4+HLA-DR+CD38+ T-cells (86). The ratio may serve as a surrogate marker for immune activation where a ratio of less than one (< 1) stands for immunosenescence. CD4+/CD8+ ratio inversion was associated with higher frequencies of senescent and exhausted CD4+ and CD8+ T cells (87). Natalizumab-associated shifts in the CSF T cell ratio have been implicated in facilitating progressive multifocal leukoencephalopathy (88), which is particularly dangerous when the ratio is shifted in favor of CD8. CD4/CD8 ratio can be used as a diagnostic differentiator between MS and Neurosarcoidosis (89) but the significance of minor changes in serum of remitting MS patients remains not entirely clear while it might very well hint to T cell activation in general.

Influence of Medication on Lymphocyte Subsets

Disease-modifying therapies cause changes in lymphocyte subpopulations, which can be detected in peripheral blood by flow cytometry. Treatment with monoclonal antibodies (Natalizumab and Alemtuzumab), Fingolimod and Dimethyl fumarate have a distinct effect on various peripheral blood lymphocyte subpopulations. In contrast, IFN-β, Glatiramer acetate, and Teriflunomide cause rather nonspecific changes (97). DMF for example has been shown to specifically reduce CD8+ T cells in MS (98, 99), while Natalizumab induced a decrease of CD3+, CD4+ T lymphocytes and the CD4/CD8 ratio (100). In the cohort studied in this study, no significant differences in circulating lymphocyte populations could be demonstrated between patients treated with Interferon-beta, Teriflunomide, Glatiramer acetate, and Daclizumab (Figure 23).

As a limitation, it must be pointed out that the individual drug patient groups were rather small. Furthermore, there were only measurements at one point in time, so that only a cross-section and no longitudinal section-analysis of the populations of the respective patients was achievable.

4.2.4 Frequencies of Cytokine-Producing T cells in MS

There have been a variety of studies implicating Th17 in MS pathogenesis. Analysis of associated cytokines, therefore, holds out the promise of some insightful findings. Muls

et.al. found IL-22 producing CD4+ CD45RA- cells elevated in relapsing RR-MS patients while in remission little/to no differences could be found (90).

INF-γ, GM-CSF, IL-10, IL-21, IL-17, TNF-α, IL-2, IL-22 were selected for analysis in this study due to their presumed role in the pathogenesis of MS. The frequency of cytokine-producing Th cells was measured on the basis of intracellular FACS-Analysis. First, all single-cytokine-producers were compared for their frequencies in CD4+, also in CD4+CD45RO+ and CD4+CD45RA- as well as CD45RA-CCR7- Tem neither subset showed differences in MS compared to healthy donors. IL-10 producers were slightly elevated in Tem (CD4+CD45RA-CCR7+) of MS patients (patients 3,120 ± 1,591 vs. healthy controls 2,145 ± 1,694, p = 0,0484) (Figure 17).

After this analysis, a comparison of the double producers was performed as well as a Boolean analysis of cytokine-producers in healthy donors and MS patients. Neither yielded any differences to be found between MS patients and healthy persons. Paradoxical effects of IL-10 have been described to date. While it can inhibit pro-inflammatory cytokine production, it has been shown to also induce growth and differentiation for T and B-cells (91), can promote autoantibody production and consequently has a pathogenic role in SLE (92) and RA (93). The elevation observed in this cohort of MS patients can therefore not necessarily be interpreted as an expression of a pro-inflammatory systemic status. However, possible applications of IL-10 targeting in immune-mediated diseases and cancer have been discussed and might be a promising therapeutic option in the future (94).

Reasons for the lack of detection of elevated cytokine production within my sample might be that the cohort consisted of patients in a symptom- free state (stable RR-MS) and only general T cell analysis was performed. In myelin-antigen specific cells, clear memory enrichment and cytokine enrichment was previously shown (IFN-ɣ, IL-17, GM-CSF)(95). In this cohort, peripheral blood was used as the primary source for analysis, where few differences were detectable. This could possibly be different in brain tissue or CSF. It would, therefore, be interesting for future studies to carry out analyses in these sources.

Stimulation

The stimulation protocol using PMA/Ionomycin as a strong universal stimulator leads to the retrieval of the maximum potential of the cell to release cytokines. The advantage is that the greatest possible release of cytokines can be obtained and measured.

However, this has the disadvantage that one does not know whether it corresponds to the realistic activation, as it would take place in vivo. Considering that surface receptors can be internalized or downregulated due to PMA/Ionomycin stimulations, those effects were tested in preliminary experiments and if the influence was significant, the antibody was removed from the panel. Overall, these are effects that one should be aware of when working with effects triggered by PMA/Ionomycin, while nonetheless, this method is an established way of inducing maximum cell activation for its potent results.

Disease-Modifying Therapy and Cytokines in MS

In the analyzed cohort Beta-Interferon tended to induce no changes, Teriflunomide tended to increase, Glatiramer acetate tended to elevate and Daclizumab tended to lower overall cytokine levels. None of these differences showed robust statistical significance. However, these trends indicate the probability of immunomodulatory-treatment induced changes in cytokine levels in the peripheral blood of MS patients. Cytokine levels showed promise as a biomarker for treatment response in MS therapy. For example, IL-17A serum baseline levels could predict treatment response to Interferon-β (96). Similar to the arguments pointed out in 4.2.3. "Influence of Medication on Lymphocyte Subsets" limitations to these results include the size of sample cohorts, cross-sectional measurements and the possibility of changes in antigen-specific subpopulations. Overall, immunomonitoring of general cytokine elevations in MS remains a limited source of information about general disease status in the remitting phase of the disease and grouping of the cohort is justifiable despite different immunomodulatory drugs present.

4.2.5 CD20+ T cells in MS

In the data presented at the top, it was demonstrated that CD20+ T cells have a pro-inflammatory potential and consequently their occurrence in MS patients was investigated.Frequencies of circulating CD20+ T cells were found to be similar in MS patients and healthy controls in this study (Figure 27). These findings coincide with results found in MS (35) and also in psoriasis patients where the frequencies of circulating CD3+CD20+ were comparable (34). There are however studies showing a modest elevation (101). A possible explanation could be that CNS tissue residents among this population or a proportionate increase of antigen-specific cells within the subset would not be detected by the experimental setup of this study.

Susceptibility to Antibody-Therapy and Immunomodulatory Drugs

The data presented here showed a statistically significant difference in circulating frequencies of CD20+ Th cells, treated with Teriflunomide (healthy control 3,381 ± 1,821 Teriflunomide 5,827 ± 1,344, p = 0,089). Frequencies in all other therapy groups (Beta-Interferon, Glatiramer acetate, Daclizumab) merely showed trends but no significant differences compared to healthy donors or MS patients without medication (Figure 28).

Interestingly, Rituximab in addition to the intended and expected B-cell depletion also shows an effect on pathogenic CD3+ CD20+ T cells. After depletion, they show earlier and higher repopulation than CD20+ B cells (30). This is particularly striking, considering the recent approval of new CD20 antibodies, such as Ocrelizumab, offering the first therapeutic option for secondary progressive MS patients. Moreover, CD20+ T cells have been shown to respond to MS disease-modifying drugs. After therapy with Fingolimod, Alemtuzumab, and Dimethyl fumarate, reduced frequencies of CD20+ T cells were found circulating in peripheral blood of MS patients. Natalizumab, however, had opposite effects and disproportionally decreased CD20+ T cells in the blood of MS patients (31). A recent study also showed the effectiveness of CD20 antibody Ocrelizumab in depleting CD20+ T cells (102). These cells, therefore, offer interesting evidence concerning the effect of CD20 antibodies not only on B cells but also directly on T cells. If a pathological role should be attributed to them, there are a variety of promising therapeutic options ranging from CD20 antibodies to Teriflunomide.

4.2.6 Immune Checkpoint Receptors in MS

Immune checkpoints, which were initially investigated as T cell regulators, have gained immense clinical importance in the treatment of malignant diseases.

The inhibitory immune checkpoint molecules CTLA-4, PD-1, and their ligands have successfully been established as targets in tumor therapy with promising results regarding tumor regression. The first approaches in therapeutic use were based on the amplification of co-activator signals in order to intensify or support tumor elimination via T cell activation. When the potency of the CTLA-4 receptors was discovered, this approach was expanded. Tumor regression is pursued by eliminating co-inhibitory signals that block the T cell antitumor response. Extremely successful results with these approaches, transferred to the PD-1 pathway, could be shown for various tumor

types, i.e. multiple melanoma, non-small-cell lung cancer (NSCLC) and renal cancer (103). Hence, it seems obvious to take the next steps and investigate their role in autoimmunity or rather to develop a possible strategy to engage these molecules in managing inflammation (104).

Significance of OX40+CD4+ T cell Elevation in MS patients

In our sample cohort an elevation of OX40+ Th cells was observed (patient 4,149 ± 2,515 vs. healthy control 2,142 ± 1,925 Mann Whitney test $p = 0.0117$) (Figure 22). In EAE models numerous co-stimulatory pathways have been studied regarding their role in promoting MS pathogenesis (104). The relevance of OX40 and the OX40 axis has been proven essential to T cell priming, migration and function in EAE (19, 105). OX40 specifically was found upregulated on autoreactive T cells within active lesions in MS patients and EAE (18). In CD28 (-/-) mice OX40L blocking ameliorated EAE (106). In addition, CD28:B7 or CD40:CD40L pathways seem to play a gatekeeping role because OX40L overexpressing mice did not develop EAE in the absence of CD28 or CD40. One other study in clinically active MS could not demonstrate an OX40+CD4+ increase in PBMCs of MS patients, however, in the publication referred to, very widely scattered measurement results are described as a possible source of error (82). Considering the results of this research in the context of previous findings, an enhanced memory generation induced by an OX40 upregulation seems likely to be a contributor within MS pathogenesis, while contradictory findings have been reported considering frequencies of circulating OX40+ Th cells.

4.2.7 PD-1+ Th cells in MS

Various mechanisms triggered by PD-1 contribute to the downregulation of the T cell activity. PD-1 has been analyzed mainly in the context of promoting CD8 exhaustion. Although PD-1 is not an absolute requirement for CD8 exhaustion the absence of PD-1 was shown to promote CD8 exhaustion in mice (107).

PD-1 has been shown to upregulate the transcription factor BATF. BATF, in turn, inhibits TCR-mediated T cell proliferation and cytokine secretion (108). These mechanisms are being investigated mainly within the context of HIV immunity to therapeutically reinforce T cell function (109). Furthermore, PD-1 inhibits the motility of virus-specific CD4+ and CD8+ cells and a blockade of PD-1 PD-L1 interaction could effectively reverse this motility inhibition (110). These mechanisms serve as interesting starting points for therapeutic immunomodulation.

In RR-MS patients quantitative real-time PCR analyses of CTLA-4, PD-1 and TIM-3 expression in PBMCs showed a reduction of inhibitory receptors. Especially PD-1 gene expression was significantly decreased in MS patients and thus indicated its importance for the maintenance of immunological homeostasis in the neuroimmunological context (111). However, my measurements of the frequency of PD-1+ Th cells showed no differences between the patients and the healthy cohort (Figure 22). However, as explained above, the co-stimulatory receptor OX40 was elevated, which supports the hypothesis on the importance of checkpoint regulators in the context of MS. It may be necessary to analyze tissue-resident or antigen-specific T cells to detect PD-1 changes in the disease context. Measurements of lymphocytes circulating in the CSF would also be informative in this respect.

4.3 Challenges in Analyzing High-dimensional FACS-Data

As described above, autoimmune processes in general and T cells in particular, play a central role in the pathogenesis of MS; therefore, a better understanding of the disease requires meaningful methods of analysis of these cells. Flow cytometry is particularly suitable for this purpose. Using this method, it is possible to characterize a large number of cells at a single-cell level in a very short time and thus to identify and analyze even small cell populations. Furthermore, it is possible to investigate both intra- and extracellular characteristics.

The conventional methods of representation in the two-dimensional plane, such as dot plots and Boolean gates quickly reach their limits when working with high-dimensional multicolor flow cytometry data (112). Rapid improvements in conventional flow cytometry (more lasers, colors, channels) were made, further pushing the previous visualization methods to their limits. Manual gating is not only impracticable for high dimensional data sets, but it is also known to not always be the most accurate approach. To keep up with this phenomenon, new visualization methods and computational approaches have been established. Our approach is a new three-dimensional visualization method named PRI “pattern recognition of immune cells”. In reaction to these developments, FlowJo has also integrated new functions with which expression distributions can be displayed using color maps. However, in contrast to PRI, no statistics based on a threshold for positivity can be generated there. PRI allows for a simple visual differentiation of correlative protein expression, using an R-based algorithm, which makes it possible to compare several parameters by means of triplots

and color-coding (Figure 6). Other methods for visualization and analysis include i.e. viSNE and SPADE. In viSNE cell populations are represented as islands on artificial axes. And with SPADE, with the help of k means clustering, gendograms are generated which are represented as "Minimum Spanning Trees". Both methods (in contrast to PRI) do not allow the specification of thresholds for the separation of positive versus negative values, which makes the identification of rare populations more difficult. The interpretation of both methods is very complex considering that similar cells are not necessarily found in neighboring areas.

Optimizing new bioinformatic analysis approaches like these, will facilitate gaining insights into the many lymphocyte subsets, allow for a better understanding of their role in health and disease, which can subsequently lead to new possibilities in terms of future clinical application.

4.4 Conclusion and Outlook

Overall, this work can be divided into two parts: The Th cell analysis carried out in healthy volunteers and the subsequent investigations performed in MS patients.

In healthy donors, several CD4+ T cell subpopulations with a highly active profile regarding cytokine production have been found: PD-1+ and CD20+ T cells. Those were enriched in IFN-γ, TNF-α, GM-CSF, IL-2, and IL-10. A shift towards a cytotoxic T cell type among all CD20+ cells was demonstrated and Tem (CD45RA-CCR7-) predominated among the CD4+CD20+ T cells. Overall, the CD20+ T cells were characterized as activated, cytokine-producing and memory-profile-bearing. The analysis of PD-1+ T cell subpopulations revealed different patterns in cytokine production contingent upon PD-1 upregulation: IL-21 showed a steady increase in cytokine-producing cells with increasing PD-1 expression. IL-17, GM-CSF, and TNF-α showed an increase with PD-1 upregulation, but a decrease with maximum PD-1 expression. PD-1 status had no effect on IL-2 or IL-22 cytokine levels.

As CD20+ and PD-1+ T cells show a differentiated and active T cell phenotype, their implication in MS was investigated. However, no enrichment of PD-1+ or CD20+ T cells was detected in RR-MS patients. Yet, noteworthy differences were observed in MS patients compared to healthy donors: reduced frequencies of cytotoxic T cells and CD4+ Tcm (CD45RA-CCR7+) in MS patients compared to healthy donors. IL-10 producers in Tem (CD4+CD45RA-CCR7+) and OX40+ Th cells were slightly elevated in MS patients compared to healthy donors.

This points to an altered immune regulation, even in RR-MS in a symptom-free interval. Within the cohort, patients taking different immunomodulatory drugs were included. However, cytokine and lymphocyte frequencies were comparable within the different subgroups, allowing a grouped analysis.

To clarify the results, the cohort size should be enlarged. Antigen-specific stimulation may provide further insights into the effector profile of auto-reactive T cells. Also, analysis of tissue-resident T cells or measurements of lymphocytes circulating in the CSF will solidify or enhance the information gained from peripheral blood. The data presented here shows once again the heterogeneity of MS. Diagnostic or therapeutic immunomonitoring may promise success as an individualized therapeutic approach but is probably not feasible as a 'one shoe to fit all' solution.

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„Ich, Meron Ariana de Oliveira Mekonnen versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Cytokine Patterns of Th cells in Healthy Donors and Patients with Multiple Sclerosis selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

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