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Characterization of patients with autoinflammation-related genetic variants and effects of long-term natalizumab treatment on peripheral and CNS immunoglobulin production

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TABLE OF CONTENTS

1. ABSTRACT

A closely regulated interplay between innate and adaptive immunity is necessary to ensure sufficient and appropriate function of the immune system; dysregulation can lead to autoinflammatory diseases on one- and autoimmunity on the other side of a spectrum.

This work deals with two aspects of dysfunctional immunity and consists of two projects. Project one aims to better characterize patients with genetic variants in autoinflammation-related genes, and project two evaluates the effect of long-term treatment with natalizumab on intrathecal and peripheral immunoglobulin production in patients with the autoimmune-inflammatory CNS disease multiple sclerosis.

Autoinflammatory disorders are genetic diseases caused by constitutive overactivation of the inflammasomes, cytosolic protein complexes that promote secretion of the proinflammatory cytokines IL-1β and IL-18 and pyroptosis, a form of inflammatory cell death. The most common forms of these overall rare diseases are linked to genetic variants in the genes *NLRP3*, *TNFRSF1A* and *MEFV*. Correct and timely diagnosis of these disorders is often difficult due to their rareness, a large symptomatic overlap with other diseases and the presence of low-penetrance mutations. Hence, there is a need for biomarkers and specific tests to facilitate the diagnosis of these diseases.

The aim of the first part of this work was to characterize patients carrying genetic variants in the aforementioned genes by immunophenotyping and by measuring the response of patients with MEFV variants to stimulation of the associated pyrin inflammasome.

In patients with *NLRP3* variants, we found a slight expansion of monocytes, especially of the $CD16^{high}$ monocytes, which are the strongest secretors of IL-1β among this cell class. In patients with *TNFRSF1A* variants, the proportion of T cells was slightly expanded. Patients with *MEFV* variants displayed an increased secretion of the pro-inflammatory cytokines TNFα and IL-4 by $CD4^{+}$ T cells.

Upon pyrin inflammasome stimulation, we observed an increased secretion of IL-1β in fresh PBMC of patients with *MEFV* variants and, even more pronounced, after stimulation of whole blood, while no difference compared to healthy controls could be seen in either IL-1β secretion or cell death rate of frozen PBMC.

These findings show the heightened inflammatory activity even in patients with low-penetrance or heterozygous variants and point to the possibility of developing a sensitive diagnostic assay for *MEFV*-associated autoinflammation utilizing IL-1β secretion after inflammasome stimulation.

In project two, we assessed the effects of long-term treatment of multiple sclerosis with the anti-VLA-4 antibody natalizumab on peripheral and intrathecal Ig production, since little is known about the development of these aspects of B cell mediated immunity in multiple sclerosis after long periods of natalizumab treatment.

To this end, we analyzed immunoglobulin production in a cross-sectional way in comparison to untreated patients with multiple sclerosis and in a longitudinal approach before and during natalizumab treatment. In addition, we assessed the expression of VLA-4 subunit α4 integrin on different B cell subsets.

Natalizumab strongly reduced intrathecal IgG production in a time-dependent manner, while important IgG mediated hallmarks of multiple sclerosis- MRZ reaction and oligoclonal band status- remained unchanged. In serum, it lead to an isotype-specific decrease in IgM levels, causing IgM hypogammaglobulinaemia in some patients.

These findings point to a reducing effect of natalizumab on short-lived plasmablasts, but not on long-lived plasma cells who are thought to be responsible for oligoclonal bands and MRZ reaction, supported by our finding of a high expression of α4 integrin on plasmablasts.

2. ZUSAMMENFASSUNG

Für den Erhalt einer funktionalen und angemessenen Funktion des Immunsystems ist ein enges Zusammenspiel seiner angeborenen und erworbenen Komponenten notwendig. Eine Fehlregulation führt zu autoinflammatorischen Erkrankungen am einen- und Autoimmunerkrankungen am anderen Ende eines Spektrums.

Diese Arbeit beschäftigt sich mit zwei Aspekten eines fehlregulierten Immunsystems und ist in zwei Teilprojekte untergliedert. In Projekt eins sollen Patienten mit genetischen Varianten in mit Autoinflammation assoziierten Genen besser charakterisiert werden; Projekt zwei befasst sich mit den Auswirkungen einer Langzeittherapie der autoimmun-entzündlichen ZNS-Erkrankung Multiple Sklerose mit Natalizumab auf die intrathekale und periphere Synthese von Immunglobulinen.

Autoinflammatorische Syndrome sind genetische Erkrankungen, welche von einer Überaktivierung der Inflammasome verursacht werden. Inflammasome sind zytosolische Proteinkomplexe, die zur Ausschüttung der entzündungsfördernden Zytokine IL-1β und IL-18 sowie zu Pyroptose, einer entzündlichen Form des Zelltodes, führen. Die häufigsten dieser insgesamt seltenen Erkrankungen sind mit Mutationen in den Genen *NLRP3*, *TNFRSF1A* und *MEFV* assoziiert. Die Seltenheit dieser Erkrankungen, die vor allem mit rheumatischen und psychosomatischen Erkrankungen überlappende Symptomatik und das Vorkommen von Niedrigpenetranzmutationen können eine korrekte und frühzeitige Diagnosestellung erschweren. Es werden daher spezifische Biomarker und funktionelle Tests zur Diagnose dieser Erkrankung benötigt.

Das Ziel des ersten Teils dieser Arbeit war, Patienten mit genetischen Varianten in den genannten Genen mittels Immunphänotypisierung besser zu charakterisieren und die Zellantwort von Patienten mit Varianten im *MEFV*-Gen auf eine Stimulation des Pyrin-Inflammasoms zu untersuchen.

Wir fanden einen leicht erhöhten prozentualen Anteil von Monozyten bei Patienten mit Varianten im *NLRP3*-Gen, insbesondere von CD16-hochexprimierenden Monozyten. Diese sind für einen Großteil der IL-1β-Ausschüttung dieser Zellklasse verantwortlich. Bei Patienten mit

TNFRSF1A-Varianten ergab sich ein erhöhter Anteil an T-Zellen und die $CD4^+$ T-Zellen von Patienten zeigte sich eine verstärkte Ausschüttung der entzündungsfördernden Zytokine TNFα und IL-4.

Nach Stimulation des Pyrin-Inflammasoms zeigte sich eine erhöhte IL-1β-Ausschüttung in frischen PBMC und Vollblut von Patienten mit *MEFV*-Varianten, während nach Inflammasomstimulation in gefrorenen PBMC keine Unterschiede in der Zytokinausschüttung und der Zelltodrate zwischen Patienten mit *MEFV*-Varianten und gesunden Kontrollen zu erkennen waren.

Insgesamt zeigen unsere Ergebnisse eine erhöhte Entzündungsbereitschaft auch von Patienten mit Niedrigpenetranz- oder heterozygoten Varianten. Aufgrund unserer Ergebnisse scheint die Entwicklung eines sensitiven funktionellen Tests zur Detektion MEFV-assoziierter Autoinflammation auf Basis der IL-1β-Ausschüttung nach Inflammasomstimulation denkbar.

Im zweiten Teil dieser Arbeit untersuchten wir die Auswirkungen einer Langzeittherapie der Multiplen Sklerose mit dem anti-VLA-4-Antikörper Natalizumab auf die periphere und intrathekale Immunglobulinsynthese, da bisher wenig über die Entwicklung dieser Aspekte B-Zell-vermittelter Immunität nach langer Applikation dieses Medikamentes bekannt ist.

Hierfür untersuchten wir die Immunglobulinproduktion in einer longitudinalen Untersuchung vor- und nach Natalizumabtherapie sowie in einer Querschnittsstudie im Vergleich zu unbehandelten Patienten mit Multipler Sklerose. Zusätzlich untersuchten wir das Expressionslevel von Integrin α4, einer Untereinheit von VLA-4, auf verschiedenen Gruppen von B-Zellen.

Natalizumab führte zu einer starken und behandlungszeitabhängigen Reduktion der intrathekalen IgG-Produktion, wohingegen wichtige IgG-vermittelte Anzeichen der Multiplen Sklerose- oligoklonale Banden und MRZ-Reaktion- weitgehend unbeeinflusst blieben. Im peripheren Blut führte Natalizumab zu einer Abnahme von IgM, welche bei manchen Patienten zu einer IgM-Hypogammaglobulinämie führte.

Diese Ergebnisse weisen auf einen möglichen Effekt von Natalizumab auf kurzlebige Plasmablasten, aber nicht auf langlebige Plasmazellen, welche für die Entstehung oligoklonaler Banden und der MRZ-Reaktion verantwortlich sind, hin. Diese Hypothese wird durch eine hohe Expression von Integrin α4 auf Plasmablasten in unserer Analyse gestützt.

3. List of Abbreviations

Adenosine triphosphate (ATP)

Cryopyrin-associated periodic syndrome (CAPS)

Clostridium difficile toxin B (TcdB)

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

C-reactive protein (CRP)

Danger-associated molecular pattern (DAMP)

Disease-modifying therapies (DMT)

Expanded Disability Status Scale (EDSS)

European Medicines Agency (EMA)

Epstein-Barr-Virus (EBV)

Familial Mediterranean fever (FMF)

Fetal Calf Serum (FCS)

Fluorescence-activated cell scanning (FACS)

Gasdermin D (GSDMD)

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Human leukocyte antigen (HLA)

Healthy control (HC)

Interferon γ (IFN γ)

Interleukin (IL)

Immunoglobulin (Ig)

Immunoglobulin A (IgA)

Immunoglobulin G (IgG)

Immunoglobulin M (IgM)

John Cunningham virus (JCV)

Lactate dehydrogenase (LDH)

Lipopolysaccharide (LPS)

Ludwig-Maximilians-Universität München (LMU)

Lumbar puncture (LP)

Measles, rubella, varicella zoster (MRZ)

Mediterranean fever (MEFV)

Magnetic Resonance Imaging (MRI)

Münster University Hospital (UKM)

Myeloid dendritic cell (mDC)

Myeloid-derived suppressor cells (MDSC)

Natalizumab (NTZ)

Natural killer cell (NK)

Natural killer T cell (NKT)

Non-steroidal anti-inflammatory drugs (NSAID)

Nucleotide binding and oligomerization domain and leucine-rich-repeat-containing (NLR)

Oligoclonal bands (OCB)

Pathogen-associated molecular pattern (PAMP)

Peripheral blood mononuclear cells (PBMC)

Protein kinase 1/2 (PKN 1/2)

Primary progressive multiple sclerosis (PPMS)

Pyrin and HIN domain-containing (PYHIN)

Relapsing-remitting multiple sclerosis (RRMS)

Serum-amyloid A (SAA)

Tumor necrosis factor (TNF)

TNF receptor subfamily member 1A (TNFRSF1A)

TNF-receptor-1A-associated periodic syndrome (TRAPS)

Universitätsklinikum Münster (UKM)

Vascular cell adhesion molecule-1 (VCAM-1)

Very late antigen-4 (VLA-4)

4. INTRODUCTION

The innate and adaptive immune system are closely intertwined to ensure optimal protection from harmful environmental influences; however, chronic or unsolicited activation of either entity can lead to disease. Immune-mediated diseases form a spectrum: from autoinflammatory disorders on the innate- to autoimmunity on the adaptive end (Doria, Zen et al. 2012) (Figure 1). Between the two, intense interaction has been described (Figure 1): The inflammasomes, part of innate immunity and involved in autoinflammation, play a role in the development of autoimmune diseases like multiple sclerosis (MS) and others (Gris, Ye et al. 2010, Kahlenberg and Kaplan 2014), and mutations in autoinflammation-related genes are more common in MS patients (Blaschek, R et al. 2018, Soares, Oliveira et al. 2019).

This work comprises two parts: Project one aims to better characterize patients with autoinflammatory syndromes, and project two evaluates effects of long-term therapy of MS patients with the monoclonal antibody natalizumab.

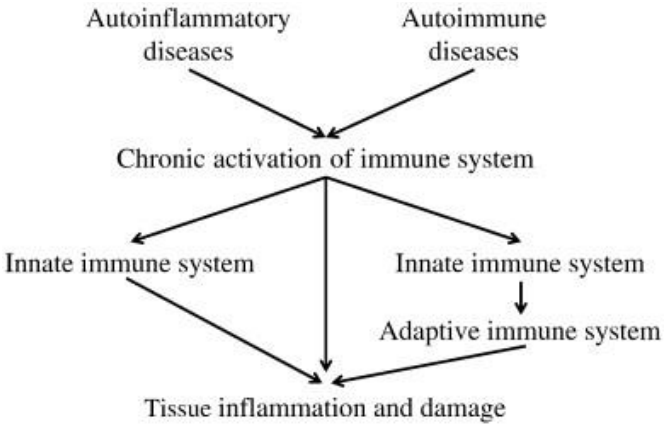

Figure 1. Interplay of autoinflammation and autoimmunity. From: (Doria, Zen et al. 2012)

4.1. The inflammasomes in the context of autoinflammatory diseases

The inflammasomes are multiprotein complexes which form in the cytosol upon recognition of certain molecular patterns related to pathogens or cell damage (Martinon, Burns et al. 2002).

Inflammasomes are part of the innate immune system (Dostert, Pétrilli et al. 2008). In principle, they are comprised of a cytosolic sensor protein, the proteolytic enzyme caspase 1, and in many cases an adaptor protein which enables interaction between the two (de Alba 2009). Receptors are mostly of either the nucleotide-binding-and-oligomerization domain and leucine-rich-repeat-containing (NLR) receptor family or the pyrin and HIN domain-containing (PYHIN) domain type (Rathinam, Vanaja et al. 2012); however, an inflammasome using pyrin as its receptor protein (pyrin inflammasome) has recently been described (Heilig and Broz 2018). Activation of the inflammasomes initiates a cascade which eventually leads to secretion of proinflammatory cytokines interleukin 1β (IL-1β) and interleukin 18 (IL-18) as well as to pyroptosis, an inflammatory form of cell death involving the formation of pores in the cell membrane by Gasdermin D (GSDMD, Figure 2) (Rathinam, Vanaja et al. 2012, Shi, Zhao et al. 2015). Upon ligand recognition, inflammasome sensor proteins oligomerize and recruit the adaptor protein ASC, which in turn interacts with procaspase 1 (Fernandes-Alnemri, Yu et al. 2009). Procaspase-1 is autoproteolitically activated into caspase 1, which cleaves the pro-forms of IL-1 and IL-18 into their mature forms which are then released from the cell via a not entirely clear mode of secretion (Broz, von Moltke et al. 2010). In recent years, different inflammasomes have been described, the most prominent one being the NLRP3 (Nucleotide binding and oligomerization domain and leucine-rich-repeat-containing) inflammasome which is seen as the prototype of an inflammasome. Genetic variants in genes coding for inflammasome proteins are linked to various autoinflammatory diseases, the most common being Cryopyrin-associated periodic syndrome (CAPS) and familial Mediterranean fever (FMF). The NLRP3 inflammasome, linked to CAPS, and the FMF-associated pyrin inflammasome are described below in more detail. Another more common autoinflammatory disease described below is TNF-receptor-1A-associated periodic syndrome (TRAPS), whose associated gene does not directly code for a known inflammasome and whose pathomechanistic details are less well understood.

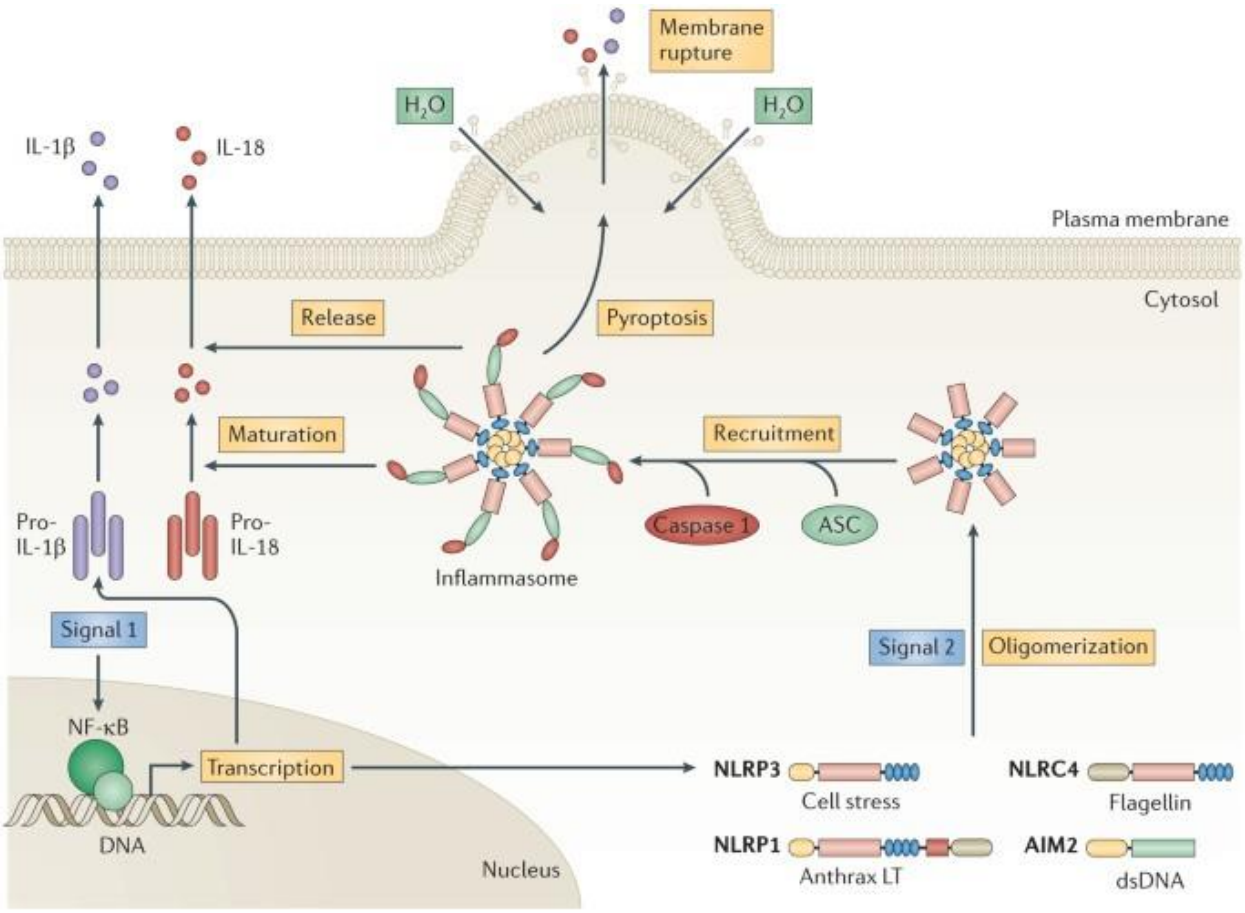

Figure 2. Activation of NLR- and PYHIN inflammasomes (Walsh, Muruve et al. 2014)

4.1.1. NLRP3 inflammasome

The NLRP3 inflammasome is activated by a broad variety of stimuli, including adenosine triphosphate (ATP), bacterial and viral nucleic acids, or urate crystals (Kanneganti, Ozoren et al. 2006, Mariathasan, Weiss et al. 2006, Martinon, Petrilli et al. 2006, Kanneganti 2010). Overall, NLRP3 is thought to recognize agents associated with cellular stress (Swanson, Deng et al. 2019). NLRP3 inflammasome activation is considered a two-step process requiring a first or "priming" signal, for example bacterial lipopolysaccharide (LPS), which triggers transcription of inflammasome components and the pro-forms of inflammatory cytokines IL-1β and IL-18 via transcription factor NF-κB (Bauernfeind, Horvath et al. 2009). If a second signal, consisting of a danger- or pathogen-associated pattern (DAMP or PAMP), is recognized, the inflammasome

components polymerize and recruit pro-inflammatory caspases, particularly caspase 1, via the adaptor protein ASC (Lu, Magupalli et al. 2014). Caspase 1 is activated by self-cleavage, and in turn cleaves zymogens pro-IL-1β and pro-IL-18 as well as GSDMD (Shi, Zhao et al. 2015, Boucher, Monteleone et al. 2018). GSDMD forms pores in the cell membrane which lead to cell death by pyroptosis (Ding, Wang et al. 2016). IL-1β and IL-18 are released by non-conventional secretion, partially with the aid of GSDMD pores (Monteleone, Stanley et al. 2018).

4.1.2. Pyrin inflammasome

Pyrin, sensor protein of the pyrin inflammasome, is encoded by the *MEFV* gene (Schnappauf, Chae et al. 2019). Pyrin indirectly senses inhibition of RhoA GTPases: In resting state, pyrin is dynamically phosphorylated by RhoA GTPases via their effectors PKN1 and PKN 2. In its phosphorylated form, pyrin can interact with chaperone proteins of the 14-3-3 family, which keep pyrin in an inactive state (Jéru, Papin et al. 2005, Xu, Yang et al. 2014, Gao, Yang et al. 2016). A number of bacterial toxins inhibit the function of RhoA GTPases; a typical RhoA inhibiting substance is Clostridium difficile toxin B (TcdB) (Dumas, Amiable et al. 2014, Xu, Yang et al. 2014, Van Gorp, Saavedra et al. 2016). Thus, pyrin is no longer being phosphorylated, ceases to interact with 14-3-3, and likely undergoes an activating conformational change (Gao, Yang et al. 2016, Masters, Lagou et al. 2016). Active pyrin recruits Caspase-1 via the adaptor protein ASC, initiating a downstream pathway analogous to that in the NLRP3 inflammasome (Yu, Wu et al. 2006). A priming signal does not appear to be necessary for this inflammasome, but LPS pretreatment leads to increased transcription of pyrin and increased IL-1β- and IL-18 secretion in an in vitro setup (Van Gorp, Saavedra et al. 2016). Pyrin inflammasome activation seems to be partially regulated by cytoskeleton structures (Figure 3). Mansfield et al. could show that pyrin colocalizes with microtubules (Mansfield, Chae et al. 2001); more recently, a model has been proposed in which wildtype pyrin depends on microtubules for activation, while pyrin mutations associated with FMF could lead to inflammasome activation without the necessity of microtubule interaction (Van Gorp, Saavedra et al. 2016).

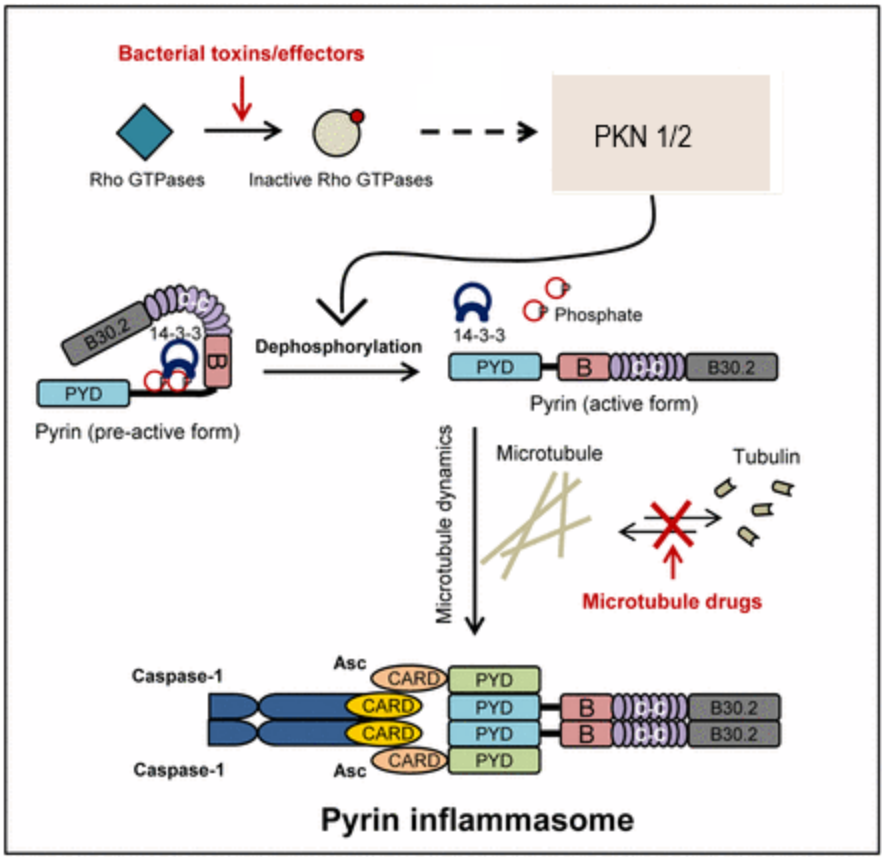

Figure 3. Activation of the Pyrin inflammasome. Adapted from(Gao, Yang et al. 2016)

4.2. Autoinflammatory syndromes

Autoinflammatory diseases are characterized by recurring systemic inflammation caused by different genetic variants. They are clinical diagnoses that can often be supported by a finding of genetic variants in corresponding genes. Fever and other symptoms can usually be explained by an excessive secretion of proinflammatory cytokines, mainly IL-1β, caused by inflammasome dysregulation (Ozen and Bilginer 2014). A major long-term complication of the diseases is AA amyloidosis caused by persisting inflammation with elevated levels of serum-amyloid A (SAA), potentially leading to organ- and especially kidney damage (Lachmann 2017, Papa and Lachmann 2018). Three of the more common autoinflammatory syndromes (CAPS, TRAPS, and FMF) will be described in more detail.

4.2.1. Genetics and epidemiology

A multitude of autoinflammatory syndromes have been described in recent years. CAPS, TRAPS and FMF all have a monogenetic cause, CAPS being associated with mutations in the *NLRP3*-gene, TRAPS with *TNFRSF1A* (tumor necrosis factor receptor superfamily 1A)-variants, and FMF with *MEFV*-gene alterations (Consortium 1997, McDermott, Aksentijevich et al. 1999, Hoffman, Mueller et al. 2001, Aksentijevich, Nowak et al. 2002, Gasperi, Salmen et al. 2019). Autoinflammatory syndromes are rare diseases: the prevalence of CAPS is approximately one to three per million without a clear impact of sex or ethnicity (Cuisset, Jeru et al. 2011). TRAPS occurs in a similar frequency of about one to two per million (Lachmann 2017). FMF is by far the most common monogenic autoinflammatory syndrome, especially in the eastern Mediterranean region, where the carrier frequency is as high as one in five (Lachmann 2017). This has led to the hypothesis that certain MEFV-mutations may in fact have an evolutionary benefit: Recently, they have been linked to improved resistance against Yersinia pestis, the bacterium causing plague (Park, Remmers et al. 2020). Importantly, low- and high-penetrance mutations have been described for all three disease entities. While a clear clinical diagnosis can often be found in patients with high-penetrance mutations, symptom spectra in patients with low-penetrance mutations vary severely, often impeding diagnosis (Federici, Calcagno et al. 2012, Cantarini, Rigante et al. 2014, Kuemmerle-Deschner, Verma et al. 2017, Mulazzani, Wagner et al. 2020). Especially for *MEFV*, it is not entirely clear whether these low-penetrance mutations lead to increased secretion of pro-inflammatory cytokines (Van Gorp, Huang et al. 2020). Hence, there is an urgent need for additional diagnostic assays to accurately identify patients with autoinflammatory syndromes.

4.2.2. Features and treatment of the most common autoinflammatory diseases

Autoinflammatory diseases are often termed periodic fever syndromes since the unifying symptom of classical, severe forms of these disorders is recurring fever. Other common

symptoms include arthralgia, myalgia, abdominal pain and skin rashes. Length of these flares of inflammation and intensity of symptom groups varies between the different diseases (Sag, Bilginer et al. 2017). Some patients with autoinflammatory syndromes suffer from additional CNS manifestations, mostly headaches, aseptic meningitis, cranial nerve impairment and MRI abnormalities (Schuh, Lohse et al. 2015, Canpolat, Gumus et al. 2017, Mamoudjy, Maurey et al. 2017). Importantly, some autoinflammation- associated genetic variants have been linked to a greater susceptibility for Multiple sclerosis (De Jager, Jia et al. 2009, Yahalom, Kivity et al. 2011a, Kümpfel, Gerdes et al. 2012, Soares, Oliveira et al. 2019), potentially suggesting a role of inflammasome activity in the pathogenesis of the neurological disease (Barclay and Shinohara 2017).

4.2.2.1. CAPS

CAPS is associated with dominant mutations in the *NLRP3*-gene, encoding cryopyrin. Today, CAPS is seen as a spectrum of syndromes with varying severity; the earlier distinction of three disease subtypes familial cold cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS) and chronic infantile neurologic cutaneous articular (CINCA, also known as neonatal-onset multisystem inflammatory disease [NOMID]) is not applied as strictly as it used to be (Kuemmerle-Deschner 2015). NLRP3 inflammasome overactivation leads to excess IL-1β secretion, causing systemic inflammation: Patients typically present with fever, malaise, urticarial rash, conjunctivitis and myalgia. Episodes typically last for less than two days, however, some patients experience continuous symptoms. In more severe cases, patients can suffer from sensorineural hearing loss, arthritis and skeletal abnormalities. Neurological manifestations range from headaches to aseptic meningitis, elevated intracranial pressure, and cranial nerve impairment; in very severe cases, varying degrees of neurocognitive impairment can be observed (Kuemmerle-Deschner 2015, Levy, Gérard et al. 2015, Lachmann 2017, Sag, Bilginer et al. 2017). According to the diagnostic criteria proposed by Kümmerle-Deschner et al. in 2017, diagnosis can be made in a patient with elevated serum inflammation markers (either C-reactive protein [CRP] or SAA) and two out of six of the major symptoms urticarial rash, cold- or stress-triggered

episodes, sensorineural hearing loss, musculoskeletal symptoms, chronic aseptic meningitis, and skeletal abnormalities (Kuemmerle-Deschner, Ozen et al. 2017). CAPS is classically a childhood-onset disease; however, symptoms can also begin later in life (Williams, Hawkins et al. 2019). As with most autoinflammatory diseases, the goals of treatment are a) alleviation of clinical symptoms and b) reduction of systemic inflammation to prevent secondary AA amyloidosis. The standard therapy for CAPS is anti-IL-1-treatment, since excessive IL-1 secretion is the main pathomechanism underlying CAPS. Currently, the IL-1-receptor antagonist Anakinra and the anti-IL-1-antibody Canakinumab are approved by the European Medicines Agency (EMA) for the treatment of CAPS (ter Haar, Oswald et al. 2015).

4.2.2.2. TRAPS

TRAPS has first been described as “Hibernian fever”, due to its description in an Irish/Scottish family, in 1982. It is associated with mutations in the *TNFRSF1A* gene, its mode of inheritance is autosomal-dominant (Williamson, Hull et al. 1982). To this day, the pathomechanisms underlying the disease are not entirely clear. Multiple mechanisms have been proposed; initially, the observation that patients with TRAPS have lower levels of soluble TNF-receptor 1 led to the hypothesis of defective receptor cleavage (McDermott, Aksentijevich et al. 1999). More recently, however, it has been suggested that *TNFRSF1A* mutations may lead to altered protein folding and aggregate formation of the receptor (Lobito, Kimberley et al. 2006). This, in turn, could lead to retention in the endoplasmatic reticulum, impaired receptor function and signaling independent of TNF (Ozen and Bilginer 2014). Moreover, overburdening of autophagy mechanisms responsible for the clearance of TNF receptor aggregates could serve as an inducer of NF-κB and IL-1β secretion (Bachetti, Chiesa et al. 2013). Clinically, TRAPS patients tend to present with long inflammatory episodes of more than six days, usually one to four weeks (Ozen and Bilginer 2014, Federici, Sormani et al. 2015). Apart from fever, the predominant symptoms are myalgia and abdominal pain. Skin manifestations can occur in the shapes of migrating erythematous- or urticarial rashes. Periorbital edema and conjunctivitis are the typical ocular manifestations of TRAPS. A cohort study of 158 TRAPS patients revealed an AA amyloidosis rate of 10%. (Lachmann,

Papa et al. 2014). Therapeutic decisions depend largely on disease severity: In mild cases, treatment of acute attacks with non-steroidal anti-inflammatory drugs (NSAID) and/or corticosteroids can be sufficient. However, loss of efficacy of these options, especially corticosteroids, has been reported (McDermott, Smillie et al. 1997). In more severe cases of TRAPS or after treatment failure with corticosteroids, biologicals can be applied. Etanercept, a decoy receptor for TNF, or anti-IL-1-treatment in analogy to that in CAPS is effective. The anti-IL-1-antibody Canakinumab has been approved for the treatment of TRAPS following a placebo-controlled trial in 2018 (De Benedetti, Gattorno et al. 2018). Prospective studies comparing the efficacy of different therapeutic options are, however, lacking (ter Haar, Oswald et al. 2015). Interestingly, anti-TNF-antibodies had a paradoxical pro-inflammatory effect in a small case series (Drewe, Powell et al. 2007).

4.2.2.3. FMF

FMF, the most common autoinflammatory disease, is endemic to the eastern Mediterranean region with a carrier frequency as high as one in five in Armenians (Touitou 2001). It is associated with mutations in the *MEFV* gene and was formerly considered a recessive disease. However, cases of FMF patients with heterozygous mutations are increasingly being recognized (Stoffels, Szperl et al. 2014, Boursier, Hentgen et al. 2019, Park, Remmers et al. 2020). A very broad variety of mutations have been described, however, five variants (V726A, M694V, M694I, M680I and E148Q) make up three quarters of FMF cases (Touitou 2001). Relatively short bouts of inflammation are characteristic for FMF with attacks lasting several hours to three days. In addition to fever, patients often have severe serositis, especially peritonitis and pleurisy, but also (mono-) arthritis. If the skin is involved, rashes are often erysipelas-like. Independently of attacks, some patients report exertional leg pain (Tunca, Akar et al. 2005, Ozen and Bilginer 2014). In a large study with 2838 Turkish FMF patients, 12.9% of patients had AA amyloidosis (Tunca, Akar et al. 2005). The most widely used diagnostic criteria for FMF in adults are the Tel Hashomer criteria, according to which either two major criteria (recurrent fever with serositis, AA amyloidosis without predisposing disease, favorable response to colchicine treatment) or one

major plus two minor criteria (recurrent febrile episodes, erysipelas-like rash, FMF in a first-degree relative) have to be fulfilled for diagnosis of FMF (Pras 1998). Standard treatment for FMF consists of colchicine as long-term medication to prevent inflammatory attacks as well as subclinical inflammation and subsequent amyloidosis. In colchicine-resistant or –intolerant patients, IL-1-blockade is usually effective (Ozen, Demirkaya et al. 2016, De Benedetti, Gattorno et al. 2018).

4.3. Autoinflammation and multiple sclerosis

Autoinflammation and genetic variants in autoinflammation-related genes have repeatedly been linked to the development of autoimmune diseases such as Crohn's disease, Systemic lupus erythematosus, and multiple sclerosis (Villani, Lemire et al. 2009, Zhang, Fu et al. 2016, Barclay and Shinohara 2017)

The NLRP3 inflammasome is known to play a crucial role in an animal model of MS, experimental autoimmune encephalomyelitis (EAE) (Gris, Ye et al. 2010, Shaw, Lukens et al. 2010). Moreover, caspase-1 and IL-1β are present in MS lesions (Merrill and Benveniste 1996, Ming, Li et al. 2002), and increased levels of caspase-1 and IL-18 have been measured in human peripheral blood mononuclear cells (PBMC) of therapy-naïve MS patients (Huang, Huang et al. 2004), pointing to a role of inflammasome activation also in MS.

This is reflected by the increased carrier frequency for variants in autoinflammation-related genes in MS patients (Blaschek, R et al. 2018;Soares, Oliveira et al. 2019) and the heightened rate of MS among FMF patients (Yahalom, Kivity et al. 2011b).

4.4. Aims of project one

Due to the rareness of these diseases, accurate diagnosis of autoinflammatory symptoms is often delayed or not made at all. Moreover, there is a large overlap of symptoms especially with rheumatic-, but also psychosomatic and sometimes neurological disorders. This problem

especially arises in patients with low-penetrance or heterozygous mutations, who may present with atypical or incomplete symptoms (Van Gijn, Ceccherini et al. 2018, Chuamanochan, Weller et al. 2019, Gattorno, Hofer et al. 2019).

We therefore strove to better characterize expression of cell surface markers and T cell cytokine secretion of patients with variants in autoinflammation-related genes compared to controls in order to find distinguishing characteristics of these patients.

Another approach to the differentiation of patients with a clinically insignificant carrier status for genetic variants in autoinflammation-related genes and patients with a real need for sufficient anti-inflammatory treatment is the development of functional assays that can be performed on easily accessible biomaterial. Therefore, we aimed to assess the response to inflammasome stimulation in different blood-derived materials in patients with genetic variants compared to HC. Since FMF is the most common autoinflammatory disorder and timely diagnosis is especially important because of FMF patients' relatively high risk of amyloidosis as a long-term complication, we focused on this patient cohorts' response to pyrin inflammasome stimulation.

4.5. Multiple Sclerosis

MS is an inflammatory demyelinating autoimmune disease of the central nervous system. A hallmark of the disease is the formation of inflammatory lesions in the brain and spinal cord (Dobson and Giovannoni 2019). Lesions contain T lymphocytes, especially CD8+ T cells, B- and plasma cells (Lassmann 2013). Demyelination occurs in the white and gray matter (Kutzelnigg, Lucchinetti et al. 2005). CD4+- and CD8+ T cells, B cells, and antibodies have been shown to play a role in the pathogenesis of MS (Meinl, Krumbholz et al. 2006, Krumbholz, Derfuss et al. 2012, Hohlfeld, Dornmair et al. 2016a, Hohlfeld, Dornmair et al. 2016b). Insufficiently treated, MS can cause irreversible disability (Confavreux and Vukusic 2006).

4.5.1. Epidemiology, genetics and risk factors

MS is one of the most common neurological diseases. In 2016, approximately 2.2 million people were estimated to be affected by the disease (Wallin, Culpepper et al. 2019). Patients' mean age at disease onset is around 30 years (Confavreux and Vukusic 2006), but pediatric MS has been described (Renoux, Vukusic et al. 2007). Female-to-male ratio is around 3:1, with an increasing proportion of affected women in some geographical regions since the 1950s (Orton, Herrera et al. 2006).

The cause of MS is not entirely clear but is most likely multifactorial with a genetic and an environmental component. Genetically, more than 100 susceptibility loci have been identified; the strongest association being with the class II human leukocyte antigen (HLA) gene cluster, especially the HLA type HLA-DRB1*15:01 (Hollenbach and Oksenberg 2015). Numerous contributing environmental factors have been suggested. Among others, Epstein-Barr-Virus (EBV)-infection, low levels of Vitamin D, and smoking seem to increase the risk of MS (Handel, Williamson et al. 2010, Handel, Williamson et al. 2011, Sintzel, Rametta et al. 2018).

4.5.2. Disease course

After a recently characterized prodromal phase (Wijnands, Zhu et al. 2019), patients typically present with first new, subacutely developed neurological symptoms. The most common initial findings are optic neuritis, brainstem- or spinal syndromes, yet clinical symptoms can vary greatly depending on the site of inflammation (Compston and Coles 2008, Dobson and Giovannoni 2019). Depending on additional Magnetic Resonance Imaging (MRI) - and CSF (cerebrospinal fluid) findings, these first symptoms can be classified as either clinically isolated syndrome (CIS) or MS, according to the 2017 McDonald criteria for the diagnosis of MS (Thompson, Banwell et al. 2018). Different disease courses of MS have classically been divided into relapsing-remitting (RRMS), primary progressive (PPMS), and secondary progressive MS (SPMS). Since 2014, the disease course of MS is classified as relapsing or progressive disease with or without disease

activity- defined as either clinical relapses or MRI activity- and with or without progression, defined by clinical worsening detected by at least annual clinical assessment (Lublin 2014).

The majority of MS patients first presents with a RRMS disease course, where relapses with new neurological symptoms develop over several hours to several days, persist for at least 24 hours, and may or may not resolve completely (Confavreux and Vukusic 2006). A PPMS disease course, where patients' neurological symptoms gradually worsen over time with or without additional relapses, is much less common. The proportion of patients with PPMS among MS patients is thought to be 10-15% (Miller and Leary 2007), even though this number seems to be decreasing (Westerlind, Stawiarz et al. 2016). Up to 80% of untreated RRMS patients enter a secondary progressive disease course after 10-20 years (Weinshenker, Bass et al. 1989, Tremlett, Zhao et al. 2008, Tedeholm, Skoog et al. 2015). New and more aggressive treatment options seem to reduce the risk of secondary progression substantially. "Hit hard and early" is becoming an increasingly popular therapeutic motto in MS (Brown, Coles et al. 2019).

Clinically, patients are often assessed using the Expanded Disability Status Scale (EDSS), first published in 1983. EDSS is a clinical score where points are given for patients' abilities in different functional systems (visual-, brainstem-, pyramidal-, cerebellar-, sensory-, bowel/bladder-, cerebral function, ambulation). Overall scores from zero (no findings in neurological examination) to ten (death due to MS) can be assigned (Kurtzke 1983).

4.5.3. Typical CSF findings

An important diagnostic tool for the diagnosis of MS is the analysis of cerebrospinal fluid (CSF), especially since the inclusion of CSF-specific oligoclonal bands (OCB) into the 2017 McDonald criteria for the diagnosis of MS (Thompson, Banwell et al. 2018). OCB are oligoclonal immunoglobulin G (IgG) bands mainly of the IgG1 subtype found upon isoelectric focusing and immunoblotting of CSF and corresponding serum samples (Freedman, Thompson et al. 2005, Stangel, Fredrikson et al. 2013). Bands that are only detectable in CSF, not in serum, are

considered CSF-specific; hence, they are a marker of intrathecal IgG production. They are thought to be produced by clonally expanded plasma cells in the brain (von Büdingen, Gulati et al. 2010).

Although not specific for MS, OCB have been described to occur in up to 95% of MS patients (Link and Huang 2006), which makes them an important hallmark of the disease. In CIS patients, positive OCB are an independent risk factor for conversion to MS (Arrambide, Tintore et al. 2018). Apart from this qualitative marker of intrathecal antibody production, IgG- and to a lesser extent, immunoglobulin M and –A (IgM, IgA)-synthesis is typically also elevated in MS patients when measured quantitatively (Link 1967, Reiber and Felgenhauer 1987). Elevated intrathecal immunoglobulin (Ig) production usually persists over the course of the disease (Walsh and Tourtellotte 1986).

Another antibody phenomenon in MS is MRZ (measles, rubella, varicella zoster)-reaction. Intrathecal Ig is polyspecific in MS patients; in up to 90% of MS patients, a reaction against virus antigens such as those of measles-, rubella-, and varicella zoster virus is observed (Reiber and Lange 1991, Reiber, Ungefehr et al. 1998). MRZ reaction has a higher specificity for MS than OCB (Jarius, Eichhorn et al. 2009, Hottenrott, Dersch et al. 2015)

Moreover, around 50% of patients initially show a lympho-monocytic pleocytosis of up to 50 cells/µl, although higher numbers can occur in a small proportion of patients (Polman, de Groot et al. 1987, Andersson, Alvarez-Cermeño et al. 1994)

Global blood-brain-barrier dysfunction, measured by CSF-to-serum albumin ratio, is rare and usually mild in MS. Blood-brain-barrier dysfunction might hint at an active lesion in close proximity to the lumbar CSF space (Liebsch, Kornhuber et al. 1996).

CSF-to-serum glucose ratio is typically normal in MS (Deisenhammer, Zetterberg et al. 2019).

4.5.4. Treatment options: overview

Disease-modifying therapies (DMT) for RRMS can be divided into three categories according to their efficacy: DMT with moderate, high and very high efficacy (Dobson and Giovannoni 2019).

In addition, Siponimod, a sphingosine-1-receptor modulator, has recently been approved for the treatment of SPMS with disease activity, i.e., with clinical relapses or MRI activity (Kappos, Bar-Or et al. 2018). Treatment decisions are made according to disease activity and –severity, comorbidities, and patients' wishes.

The DMT currently approved by EMA for the treatment of RRMS and SPMS/ PPMS with disease activity are listed in Table 1. To date, no specific treatment for PPMS or SPMS without disease activity is available.

Table 1: Current treatment options for MS. Adapted from (Dobson and Giovannoni 2019)

Efficacy	**DMT**	**Mechanism of action**	**Route of administration**
Moderate	Interferon-beta	Immunomodulatory, pleiotropic immune effects	Subcutaneous injection
	Glatiramer acetate	Immunomodulatory, pleiotropic immune effects	Subcutaneous injection
	Dimethyl fumarate	Pleotropic, NRF2 activation, downregulation of NFκB	Oral
	Teriflunomide	Dihydro-orotate dehydrogenase inhibitor (reduced *de novo* pyrimidine synthesis), anti-proliferative	Oral
High	Fingolimod Ozanimod	Selective sphingosine 1-phosphate modulator, prevents egress of	Oral

		lymphocytes from lymph nodes	
	Cladribine	Deoxyadenosine (purine) analogue, adenosine deaminase inhibitor, selective T- and B-cell depletion	Oral
Very high	Ocrelizumab	Anti-CD20, B-cell depleter	Intravenous
	Natalizumab	Anti-VLA4, selective adhesion molecule inhibitor	Intravenous
	Alemtuzumab	Anti-CD52, non-selective immune depleter	Intravenous
SPMS with disease activity	Siponimod	Selective sphingosine 1-phosphate modulator, prevents egress of lymphocytes from lymph nodes	Oral
PPMS with disease activity	Ocrelizumab	Anti-CD20, B-cell depleter	Intravenous

4.5.4.1. Natalizumab

As part of this work concerns the effects of natalizumab (NTZ) on peripheral and intrathecal Ig production, NTZ therapy is described in more detail.

NTZ is a highly effective DMT (Polman, O'Connor et al. 2006). It is a monoclonal antibody directed against the α_4 subunit of very late antigen-4 (VLA-4, $\alpha_4\beta_1$-integrin) which inhibits the interaction of VLA-4 with its binding partner vascular cell adhesion molecule-1 (VCAM-1), expressed on endothelial cells. Thus, it hinders transmigration of leukocytes from the periphery into the CNS

(Niino, Bodner et al. 2006, Hutchinson 2007). Part of the efficacy of NTZ may also stem from the inhibition of interaction of α_4 integrin with osteopontin and fibronectin, mediators of lymphocyte adhesion and migration, and T cell co-stimulants (Davis, Oppenheimer-Marks et al. 1990, Chan and Aruffo 1993, Bayless, Meininger et al. 1998, Chabas, Baranzini et al. 2001). B cells have long been known to play an important role in MS development and –disease course (Meinl, Krumbholz et al. 2006, Hohlfeld, Dornmair et al. 2016b), and B cell mediated antibody phenomena (as described in 4.5.3) are important diagnostic tools for MS. The influence of NTZ on these phenomena have been evaluated with mixed results (Mancuso, Franciotta et al. 2014, Warnke, Stettner et al. 2014, Largey, Jelcic et al. 2019). Generally, the role of adhesion molecules such as VLA-4 in the maintenance of CNS plasma cells and plasmablasts is poorly understood.

The most dire complication of NTZ therapy is progressive multifocal leukoencephalopathy (PML), a potentially fatal demyelinating disease of the brain, caused by an infection with John Cunningham virus (JCV) in immunosuppressed individuals (Padgett, Walker et al. 1971). The incidence of PML in NTZ patients worldwide is approximately four in 1,000 (Schwab, Schneider-Hohendorf et al. 2017). Important established risk factors are JCV serostatus, T cell lymphopenia, NTZ treatment duration and prior immunotherapy (Carson, Focosi et al. 2009, Schwab, Schneider-Hohendorf et al. 2017). To date, there are no effective treatment options for PML. The outcome depends on immune reconstitution after cessation of NTZ therapy. 77% of NTZ-associated PML patients survive the disease, however, 40% of survivors suffer severe- and 47% moderate disability (Pavlovic, Patera et al. 2015).

4.5.5. Aims of project two

While NTZ is a very efficient MS drug, it can lead to very severe infectious complications such as PML, especially after long periods of NTZ treatment (Carson, Focosi et al. 2009;Schwab, Schneider-Hohendorf et al. 2017). While an effort has been made to study the effects of NTZ on B cell mediated features of immunity, this yielded mixed results and only included patients after short-term treatment with NTZ (Mancuso, Franciotta et al. 2014;Warnke, Stettner et al. 2014;Largey, Jelcic et al. 2019).

In the second part of this work, we aimed to evaluate the effects of long-term treatment with NTZ on intrathecal and peripheral immunoglobulin (Ig) levels and on characteristic features of MS such as intrathecal Ig production, OCB and MRZ reaction in a cross-sectional and longitudinal manner.

5. MATERIALS AND METHODS

5.1. Materials for project one

5.1.1. Patient and healthy control materials

PBMC and whole blood of patients with genetic variants in autoinflammation-related genes and of healthy controls were obtained at the Institute of Clinical Neuroimmunology at LMU Klinikum (Munich, Germany). PBMC were isolated from EDTA blood by ficoll density gradient as described in 5.2.1and stored in liquid nitrogen at the biobank of the Institute of Clinical Neuroimmunology at LMU Klinikum (Munich, Germany), if necessary. Whole blood samples were taken in heparinized tubes and used immediately after blood withdrawal.

The local ethics committee (Ethik-Kommission der Medizinischen Fakultät der LMU, protocol number 159-03 and 163-16) approved the study. All patients and healthy controls gave written informed consent.

5.1.2. Generally used buffers and media

RPMI-1640 media with and without phenol red were bought from Sigma. PBS pH 7.4 was purchased from PAN biotech. FCS was bought from Biochrom; Supplements and antibiotics for cell culture medium were from either gibco or Sigma. DMSO was purchased from Sigma. Other reagents not mentioned here are specified in the corresponding methods section.

Table 2: Media prepared in-house

Complete RPMI	RPMI-1640 media with phenol red 10 % FCS 1 % Pen Strep 1% non-essential amino acid solution 1 % sodium pyruvate 1 % L-glutamine
LDH assay media	RPMI-1640 media without phenol red 5 % FCS 1 % Pen Strep 1% non-essential amino acid solution 1 % sodium pyruvate 1 % L-glutamine
Freezing media	FCS+10%DMSO

5.2. Methods of project one

5.2.1. PBMC isolation, storage and thawing

Blood was withdrawn under aseptic condition in EDTA tubes. Blood samples were kept at room temperature until further use to avoid coagulation. Blood samples were diluted 1:2 with PBS in 50ml Falcon tubes and subsequently layered on 15ml of Pancoll (Pancoll human, density 1.077 g/ml, PAN-Biotech), avoiding mixing of the layers. Tubes containing Pancoll and blood samples were centrifuged at 400g for 30 minutes without a brake on the centrifuge. This allows separation of a top layer with a density lower than that of Pancoll, containing a well-separated layer of PBMC, and a fraction with higher density, containing mainly erythrocytes and granulocytes, at the bottom of the tube. The PBMC layer was collected and washed twice by dilution with PBS and subsequent centrifugation at 300g for ten minutes. Washed PBMC were counted using an automated cell counter (Countess II, ThermoFisher Scientific) and trypan blue stain (ThermoFisher Scientific) as a discriminator for dead cells according to manufactorer's instructions. PBMC were either transferred to complete RPMI media and used directly or frozen at a density of 10^7 cells in FCS with 10% DMSO (Sigma) at -80°C in a freezing container for three days before transfer to liquid nitrogen for long-term storage. For thawing, PBMC were rapidly

transferred to 15ml of warm complete RPMI and washed twice with complete RPMI and centrifugation at 300g for 10 minutes.

5.2.2. FACS immunophenotyping of PBMC

PBMC of patients with autoinflammation-related genetic variants and age- and sex-matched HC were isolated as described in 5.2.1 and shipped to UKM in liquid nitrogen.

FACS staining and analysis were performed by the laboratory of Prof. Dr. Luisa Klotz at UKM according to their protocols. In short, PBMC were thawed and counted using a Countess II FL and trypan blue (both ThermoFisher Scientific). Three different staining panels, “basic”, “monocytes”, and “T cyto” were used. “Basic” and “monocytes” consist of cell surface staining only, while “T cyto” involved intracellular staining for cytokines. The antibodies used in this work are listed in Table 3.

For surface staining, the appropriate cell number (see Table 3) was seeded into 96-well plates. Plates were centrifuged at 300g for 4 minutes and supernatants discarded. 100µl staining solution per well was prepared by mixing FACS buffer (PBS containing 2% FBS and 2mM EDTA) with the appropriate antibodies. 100µl stainig mix were added to each well and plates incubated for 30 minutes at 4°C for the “basic” or 37°C for the monocyte panel. Plates were washed by adding 100µl FACS buffer and centrifuging at 300g for 4 minutes. Cells were resuspended in 80µl FACS buffer for analysis.

For “T cyto” staining, appropriate cell numbers were seeded into 96-well plates on day one. Plates were centrifuged (300g, 4 minutes) and supernatants discarded. 200µl X-VIVO 15 Serum-free Hematopoietic Cell Medium (Lonza Bioscience) were added and cells incubated overnight at 37°C, 5% CO_2. On day two, plates were centrifuged as before, supernatants discarded, and 100µl/well X-VIVO media containing 1µl Leukocyte Activation Cocktail (BD Biosciences), a cell activation mixture containing Phorbol 12-Myristate 13-Acetate (PMA), Ionomycin, and the protein transport inhibitor BD GolgiPlug (Brefeldin A, BD Biosciences) to elicit a cytokine response of the T cells. After four hours of incubation at 37°C, 5% CO_2, cells were centrifuged, supernatants

removed, and 100µl per well of surface antibodies (CD3, CD4, CD8, CD56) diluted in FACS buffer were added. Cells were incubated at 4°C for 30 minutes and washed as described for the surface staining panels. 50µl Fixation / Permeabilization Concentrate (ThermoFisher Scientific) was added and plates incubated at 4°C to achieve fixation and permeabilization of cell membranes. An additional 50µl per well of Permeabilization Buffer (ThermoFisher Scientific) diluted 1:10 in deionized H_20 was added before another centrifugation/discarding of supernatant step. 100µl/well of antibodies for intracellular cytokine staining were added in Permeabilization Buffer (ThermoFisher Scientific) diluted 1:10 in deionized H_20. After 30 minutes of incubation at 4°C, plates were washed by adding 100µl FACS buffer and centrifuging at 300g for 4 minutes. Cells were resuspended in 80µl FACS buffer for analysis.

FACS analysis was performed in a highly standardized way by staff of the laboratory of Prof. Luisa Klotz, UKM. FACS data was analyzed by M. Schlüter using Kaluza Software (Beckman Coulter) according to UKM standards.

Table 3: Antibodies used for immunophenotyping

Panel Cell no. per well	Marker	Fluorochrome	Manufacturer
Basic $0.25x10^6$	CD3	Brilliant Violet 650	Biolegend
	CD14	Brilliant Violet 510	Biolegend
	CD19	APC-A700	Beckman Coulter
	CD56	PE/Cy7	Beckman Coulter
	CD1c	FITC	Miltenyi
Monocytes $0.5x10^6$	CD14	Alexa Fluor 700	Biolegend
	HLA-DR	Brilliant Violet 510	Biolegend
	CD16	Brilliant Violet 785	Biolegend
	CD192	Brilliant Violet 605	Biolegend
	CD195	PE	BioLegend
	CD206	FITC	BioLegend

T Cyto $0.5x10^6$	CD3	APC-A750	Beckman Coulter
	CD4	ECD	Beckman Coulter
	CD8	APC-A700	Beckman Coulter
	CD56	APC	Miltenyi
	IFNγ	PE/Cy7	Beckman Coulter
	TNFα	PerCP/Cy 5.5	Biolegend
	GM-CSF	Brilliant Violet 421	BD Biosciences
	IL-4	Brilliant Violet 786	BD Biosciences
	IL-22	PE	eBioscience
	IL-17A	FITC	BioLegend

5.2.3. Inflammasome stimulation and IL-1β secretion assay

PBMC were either freshly isolated from EDTA blood or thawed as described before. Cells were counted and $2x10^6$ cells per well were seeded in 200µl complete RPMI per well. For use of whole blood, blood was withdrawn using lithium heparin coated tubes, blood samples were diluted 1:5 in complete RPMI media and 200µl per well of this dilution was transferred to a 96-well plate. Technical replicates of three wells were done for each condition and donor. Cells were allowed to rest overnight at 37°C. On day two, plates were centrifuged at 400g for five minutes and supernatants carefully removed. For conditions with LPS priming, media was replaced with 150µl complete RPMI containing 100ng/ml LPS (InvivoGen). For unstimulated wells, 150µl complete RPMI per well was added. Cells were incubated at 37°C for four hours. After incubation, 50µl of RPMI complete were added for unstimulated and LPS-only wells. For activation of the NLRP3 inflammasome, 50µl of complete RPMI containing either ATP (InvivoGen) or Nigericin (InvivoGen) to reach the desired final concentration were added. For pyrin inflammasome activation, 50µl of media containing Clostridium difficile Toxin B (TcdB, R&D Systems) to reach the desired concentration was added. Final concentrations of stimulants are given in each figure for the corresponding assay. Plates were incubated at 37°C for one hour before centrifugation (400g, five minutes) and collection of supernatants. Supernatants were immediately analyzed by ELISA.

ELISA was performed using the Human IL-1 beta ELISA Ready-SET-Go! Kit, 2nd Generation (ThermoFisher Scientific), according to manufacturers instructions except for the halving of all reagents to match the use of only 50µl per well of supernatant diluted 1:10 in ELISA Diluent supplied by the kit.

ELISA plates were measured at 405nm on a Victor^2 1420 Multilabel Counter (PerkinElmer life sciences) and a background value measured at 540nm was subtracted. For analysis, the mean of three technical replicates per condition and donor was used.

5.2.4. LDH cytotoxicity assay after inflammasome stimulation

The principle behind the LDH assay is the release of lactate dehydrogenase (LDH), a cytosolic enzyme, into the culture media upon cell death. With a coupled enzymatic reaction, an added salt is transformed to a red formazan product which can be measured at a wavelength of 490nm.

For LDH assay, PBMC were thawed as described under 5.2.1. For all further steps, LDH assay media containing no phenol red and only 5%FCS was used since these two ingredients increased the background signal of the assay. 50.000 cells per well were seeded in triplicates in 100µl of LDH assay media in a 96-well plate and incubated overnight at 37°C. On the second day, plates were centrifuged at 400g for five minutes and supernatants discarded. Inflammasome priming was performed analogous to that described under 5.2.3, but using 75µl of media per well containing 10ng/ml LPS. After four hours of incubation at 37°C, inflammasome stimuli were added in 75µl of media at the following final concentrations: ATP 2.5mM, Nigericin 2.5mg/ml, TcdB 0.5 µg/ml and 0.125 µg/ml. For determination of spontaneous LDH activity, a sample treated with media only and for maximum LDH activity, a sample treated with a lysis buffer supplied in the LDH assay kit were included. After one hour of incubation, 50µl of supernatant was transferred to a new plate and LDH assay (Pierce LDH Cytotoxicity Assay Kit, ThermoFisher Scientific) performed according to manufacturer's instructions.

Plates were measured at 490nm on a Victor^2 1420 Multilabel Counter (PerkinElmer life sciences) and a background value measured at 680nm was subtracted.

%Cytotoxicity was calculated as follows:

$$\frac{LDH\ activity\ after\ inflammasome\ stimulation - spontaneous\ LDH\ activity}{Maximum\ LDH\ activity - spontaneous\ LDH\ activity} \times 100$$

5.2.5. Statistical analysis of project one

Data were analyzed using Graphpad Prism 7 software (GraphPad Software Inc.). Differences in PBMC and lymphocyte composition were analyzed using two-way ANOVA with Dunnett's multiple comparisons test, correcting for multiple testing in the pairwise comparisons between HC and patients but not for simultaneous analysis of multiple outcomes ore subgroup analysis due to the expected loss of testing power with the small sample sizes. Hence, p values in this analysis are merely to be seen as hints at the actual situation and not to be overinterpreted. The same caveat applies to the analysis of monocyte subsets and T cell cytokine secretion by one-way ANOVA with Dunnett's multiple comparisons test.

IL-1β secretion of frozen PBMC and whole blood and LDH assay data after inflammasome stimulation was analyzed using multiple t tests with additional two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, false discovery rate 1%.

Differences were considered statistically significant for p values of *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 and marked accordingly in the figures.

5.3. Materials of project two

Parts of this project have been submitted for publication in Neurology® Neuroimmunology & Neuroinflammation.

5.3.1. Patients and samples

For the evaluation of long-term effects of NTZ, we investigated two groups of patients in a monocentric study: group 1 consisted of 49 RRMS patients treated with NTZ; group 2 was

comprised of 47 consecutive, age-matched, untreated patients with RRMS. Clinical features of all patients including disease duration, disease severity (EDSS), NTZ therapy duration and DMT before NTZ are summarized in Table 4.

All patients underwent lumbar puncture (LP) for clinical and diagnostic purposes. OCB status was available for 33 patients before NTZ treatment. Immunoglobulin data from a LP before NTZ therapy were available from 27 MS Patients in the NTZ treated group. All 49 NTZ treated patients switched therapy due to risk of PML and underwent another LP to exclude PML before timely start of subsequent cell depleting therapies. CSF and blood sampling at second LP was performed around the time point of patients' last NTZ application (mean time from last NTZ infusion to lumbar puncture +11 days; range -30 to 113 days). Untreated MS patients had a diagnostic LP usually at first diagnosis. CSF and serum were analyzed by routine methods for cell count, total protein and albumin levels, OCB, IgG, IgM, and IgA at the Institute of Laboratory Medicine, LMU Klinikum, Ludwig-Maximilians-Universität München, Munich, Germany, as described in 5.1.2. None of the patients, NTZ treated or untreated, underwent plasma exchange therapy in the 30 days prior to LP.

For additional analysis of α4 integrin expression on different B cell subsets, we analyzed PBMC of eight healthy donors (HC, mean age 30.5 years, male: female 5:3).

The local ethics committee (Ethik-Kommission der Medizinischen Fakultät der LMU, protocol number 159-03 and 163-16) approved the study. All patients and controls gave written informed consent.

Table 4: Characteristics of patients included in the NTZ study

	group 1 NTZ treated cohort	group 2 untreated cohort
Patient number	49	47
Diagnosis	49 RRMS	47 RRMS
Mean age at sampling (years)	41 (range 21–65)	39 (range 19–62)
Sex	30 female, 19 male (ratio 1.6 : 1)	28 female, 19 male (ratio 1.5 : 1)
Mean time disease onset - first lumbar puncture (years)	2 (range 0-10)	3 (range 0–22)
Mean time disease onset - first NTZ infusion (years)	8 (range 0-21)	n/a
Mean time first lumbar puncture – first NTZ infusion (years)	5 (range 0-14)	n/a
Mean time disease onset - lumbar puncture during NTZ (years)	14 (range 2–30)	n/a
Median EDSS	3.0 (range 0–7)	2.0 (range 1-5.5)
Mean NTZ treatment duration (years)	5.1 (range 1.2–11.8)	n/a
Mean number of DMT before NTZ	2 (range 0-7)	n/a
Mean time from last NTZ application to lumbar puncture (weeks)	+1.5 (-4.3–16.1)	n/a

5.1. Methods of project two

5.1.1. Analysis of intrathecal and peripheral Ig production

Cross-sectional study

We compared serum- and CSF IgG, IgA and IgM levels, Ig index, OCB status, and MRZ reaction of the NTZ treated patient cohort (49 patients) with corresponding data of 47 untreated RRMS patients.

Longitudinal study

Within the NTZ treated cohort, we performed paired analyses of serum and CSF IgG-, IgA-, and IgM levels and Ig index. In addition, percentage of OCB positive MS patients before and during NTZ therapy was determined and compared. Complete datasets before NTZ were not available for all patients of group 1; hence, patient number varied for the individual analyses (for details see “Results” section and figure legends). Of note, eight out of 27 patients with CSF data before NTZ therapy had their samples taken and analyzed in external clinics or practices.

5.1.2. Determination of serum- and CSF parameters

The following analyses were performed at the Institute of Laboratory Medicine, LMU Klinikum, Ludwig-Maximilians-Universität München, Munich, Germany, according to their standards. Protocols as follows have kindly been provided by Prof. Eichhorn, Institute of Laboratory Medicine, LMU Klinikum.

Determination of IgG, IgA, IgM and albumin levels

The quantitative determination of Immunoglobulins IgG, IgA, IgM and albumin in serum as well as in CSF were performed by means of immunonephelometry on an Atellica NEPH 630 analyzer using the diagnostic reagents supplied from Siemens Healthcare Diagnostics Products GmbH,

Marburg, Germany, in accordance with manufacturer`s instructions. For CSF IgM values below the quantification limit of the assay reaching from 0.12 mg/l to 0.15 mg/l, these values were used for further calculations. Ig indices were calculated as ((Ig ratio CSF/serum)/(Albumin ratio CSF/serum)) and used as measures of intrathecal Ig synthesis.

MRZ reaction

MRZ reaction was detected by calculation of the respective antibody indices (AI) as described earlier [20]. ELISAs (Euroimmun, Lübeck, Germany) were measured on an automated ELISA processing system (Analyzer I, Euroimmun). Virus-specific AI ≥1.5 was considered indicative of intrathecal antibody production against the respective virus. We analyzed MRZ reaction status of untreated and NTZ treated patients as one-fold positive (MRZ-1: reactivity against one of the three viruses) and two-fold positive (MRZ-2: reactivity against two viruses) MRZ reaction.

Determination of oligoclonal bands

Detection of OCB was performed as part of routine CSF analysis using a high-sensitive isoelectric focusing technique on agarose gel followed by immunofixation (Hydrasis Focusing instrument employing the Hydragel Isofocusing 9 CSF Isofocusing kit, both Sebia, France). Presence of OCB was assumed if there was >1 OCB exclusively in CSF. A sample stored at -80° in the biobank of the Institute of Clinical Neuroimmunology (LMU Klinikum, Munich) was used to analyze Ig band patterns of a patient in direct comparison before and during NTZ treatment.

5.1.3. FACS analysis of a4 integrin expression on B cell subsets

PBMC of eight HC were isolated using Pancoll density gradient, stored in liquid nitrogen and thawed as described in 5.2.1. After thawing, cells were resuspended in 80µl FACS buffer (consisting of PBS with 1% FCS) and incubated with 20µl FcR blocking reagent (Miltenyi Biotec) per 107 cells for 10 minutes at 4°C. Afterwards, cells were distributed to FACS tubes, stained

with the antibodies listed in Table 5 and incubated on ice for 30 minutes. Afterwards, cells were washed by adding 4ml of FACS buffer per tube, centrifuging at 300g for 10 minutes and decanting supernatants. For exclusion of dead cells, cells were resuspended in FACS buffer containing TO-PRO 3 stain (ThermoFisher Scientific) at a dilution of 1:4000. Samples were analysed on a FACSAriaFusion flow cytometer (BD Biosciences) at the Core Facility Flow Cytometry at the Biomedical Center, Ludwig-Maxmilians-Universität München. FACS data were analyzed using FlowJo 10.6.1 software.

Table 5: Antibodies for analysis of a4 integrin expression on B cell subsets

Antibody and fluorochrome	**Volume [µL] per sample**	**Clone**	**Manufacturer**
CD49d_FITC	2.5 µL	9F10	BioLegend
CD19_APC*Fire 750	2.5 µL	HIB19	BioLegend
CD27_BV605	2.5 µL	O323	BioLegend
CD38_eF450	2.5 µL	HB7	Invitrogen, ebioscience
CD3_AF700	2.0 µL	OKT3	Invitrogen, ebioscience
CD14_AF700	2.5 µL	M5E2	BioLegend
CD20_PerCP	2.5 µL	2H7	BioLegend
IgD_PerCP	2.5 µL	IA6-2	BioLegend
FITC Isotyp	2.5µL	MOPC-21	BioLegend

5.1.4. Statistical analysis of project two

Data were analyzed using Graphpad Prism 7 software (GraphPad Software Inc.). Differences in Ig levels were evaluated using Mann-Whitney test for cross-sectional- and Wilcoxon matched-pairs signed rank tests for longitudinal comparisons. OCB and MRZ data were analyzed using Fisher's exact test. Changes in antibody levels and indices with NTZ therapy duration were evaluated using Spearman nonparametric correlation. Potential differences in Integrin α-4 expression levels

were analyzed using Friedman test with Dunn's multiple comparisons test. Differences were considered statistically significant for p values of *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 and marked accordingly in the figures.

6. Results of Project one: Characterization of patients with genetic variants in genes related to autoinflammatory diseases

6.1. Immunophenotyping of PBMC of patients with MS or genetic variants in the *NLRP3*, *TNFRSF1A*, and *MEFV* gene

In collaboration with Universitätsklinikum Münster (UKM), we evaluated the composition of peripheral blood cells and cytokine secretion by T cells in patients with genetic variants in the autoinflammation-related genes NLRP3, TNFRSF1A and MEFV by flow cytometry and compared them to HC.

6.2. Patient cohort

We included samples of patients with genetic variants in the NLRP3-, TNFRSF1A- and MEFV gene and a control group of HC. Patient numbers and clinical characteristics are listed in Table 6.

Table 6: Clinical characteristics of patients for FACS immunophenotyping. Red lettering signifies high-penetrance mutations. F=female, m=male. Het.=heterozygous, hom.=homozygous. n/a=not applicable.

	HC	*NLRP3* variants	*TNFRSF1A* variants	*MEFV* variants
n	15	14	7	9
Sex	12 f 3 m	11 f 3 m	6 f 1 m	7 f 2 m
Mean age (range)	38 (20-56)	44 (20-63)	44 (28-58)	37 (20-51)
Genotype	n/a	11 het. Q703K 1 hom. Q703K 1 het. S726G 1 het. c.2124C>T	5 het. R92Q 1 hom. R92Q 1 het. N116S	2 het. E148Q 2 het. I591T 2 het. M694V 2 het. M680I 1 het. M680I & het. V726A 2 het. M694V & het. V726A
Diagnostic criteria fulfilled	n/a	2 yes (2 het. Q703K) 12 no	3 yes 2 no	3 yes (one patient het. M680I, two patients het. M694V & het. V726A) 6 no
Treated for suspected autoinflammatory syndrome	n/a	3	1	7
Periodic/continuous disease	n/a	6 periodic 6 continuous 2 mixed	5 periodic 2 continuous	6 periodic 3 continuous

6.3. PBMC and lymphocyte subset composition

First, we evaluated potential differences in the basic composition of PBMC or lymphocytes between patients with genetic variants in autoinflammation-related genes (*NLRP3* n=14, *TNFRSF1A* n=7, *MEFV* n=9*)* in comparison to healthy controls (HC, n=15). Statistical analysis of the basic percentages of the three cell types lymphocytes ($CD14^-$), monocytes ($CD14^+$), and myeloid dendritic cells (mDC, $CD14^-$ $CD19^-$ $CD1c^+$) showed a higher proportion of monocytes and a correspondingly lower level of lymphocytes in patients with variants in the *NLRP3* gene (adjusted p value=0.0015 for both comparisons; two-way ANOVA with Dunnett's multiple comparisons test; Figure 4A). An additional analysis of lymphocyte subtypes- B cells ($CD14^-$ $CD3^-$ $CD19^+$), T cells ($CD14^-$ $CD3^+$ $CD56^-$), natural killer (NK, $CD14^-$ $CD3^-$ $CD56^+$)- and natural killer T cells (NKT, $CD14^-$ $CD3^+$ $CD56^+$) showed a slight increase in the percentage of T cells in patients with *TNFRSF1A* variants (adjusted p value=0,0371, two-way ANOVA with Dunnett's multiple comparisons test; Figure 4B). A caveat is that analyses were only corrected for multiple testing in the pairwise comparisons between HC and patients; but the risk for an α error – falsely detecting a statistical significance – is also inflated by the fact that multiple outcomes are analysed simultaneously and, in case of the data shown in Figure 4B, subgroups are analysed. Hence, p values should not be overinterpreted and rather be seen as hints at the real situation.

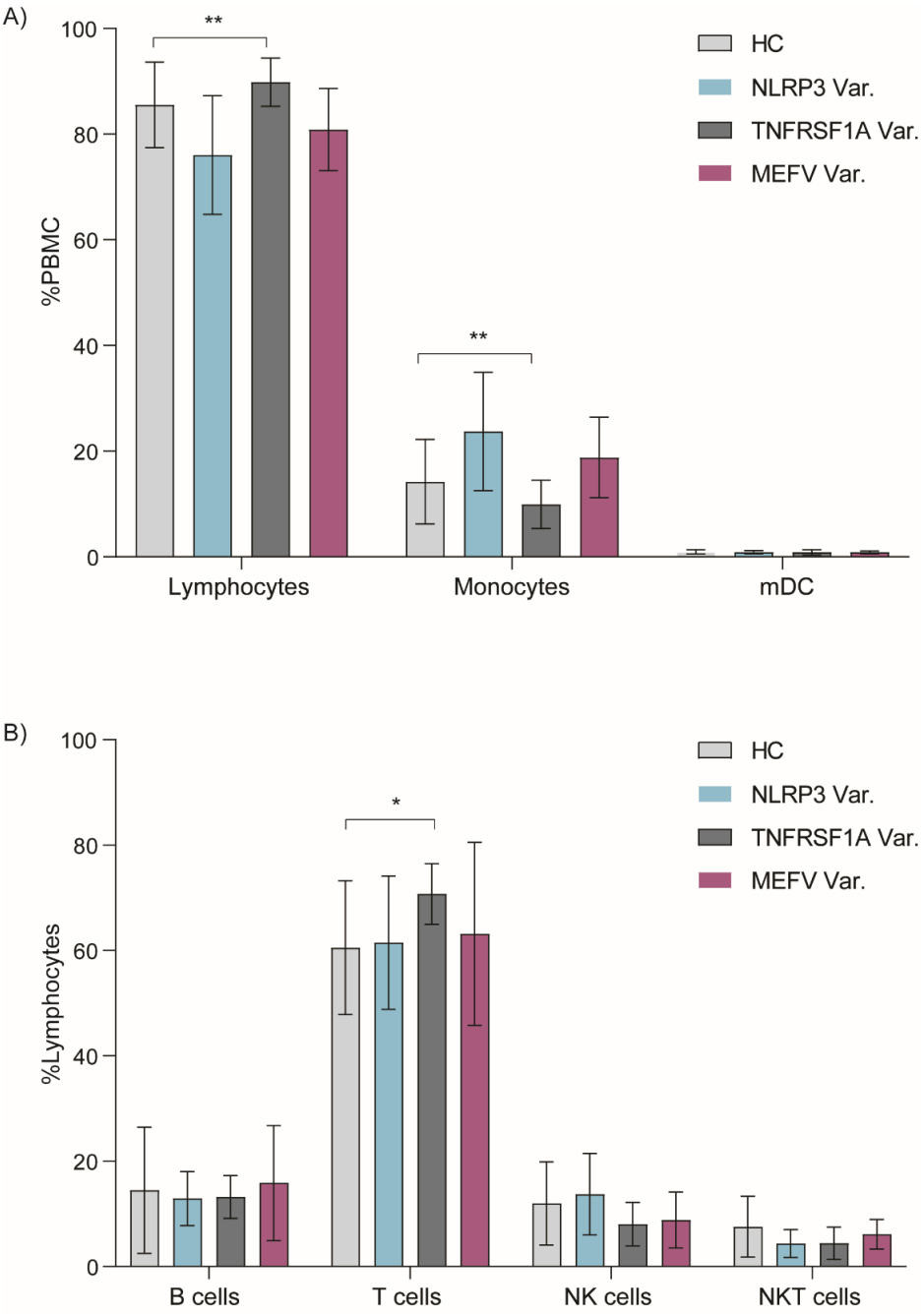

Figure 4. PBMC and lymphocyte subsets of patients with genetic variants in the *NLRP3*, *TNFRSF1A*, and *MEFV* gene and HC. FACS analysis of cell surface markers. Analysis of patients with variants in the *NLRP3*(n=14), *TNFRSF1A* (n=7), or *MEFV* (n=9) gene with those of healthy controls (n=15). Data is shown as mean +/-SD. two-way ANOVA with Dunnett's multiple comparisons test A) Comparison of percentages of lymphocytes ($CD14^-$), monocytes ($CD14^+$), and myeloid dendritic cells (mDC, $CD14^-$ $CD19^-$ $CD1c^+$). Significantly higher percentage of monocytes and lower proportion of lymphocytes in patients with *NLRP3* variants. B) Comparison of percentages of B cells ($CD14^-$ $CD3^-$ $CD19^+$), T cells ($CD14^-$ $CD3^+$ $CD56^-$), NK cells ($CD14^-$ $CD3^-$ $CD56^+$) and NKT cells ($CD14^-$ $CD3^+$ $CD56^+$). Higher percentage of T cells in

patients with *TNFRSF1A* variants. Var.= variant, mDC= myeloid dendritic cell, NK cells= natural killer cell, NKT cell= natural killer T cell.

6.4. Monocyte subset analysis

Since Monocytes are thought to be among the main cells involved in inflammasome activation, we analyzed monocyte subsets of patients with autoinflammation-related genetic variants (*NLRP3* n=14, *TNFRSF1A* n=7, *MEFV* n=9*)* in more detail and compared them to values measured in HC (n=15).

We observed no differences in the proportions of CD 16 negative monocytes, sometimes termed "classical monocytes" ($CD14^+$ HLA-DR^+ $CD16^-$ $CD192^{high}$), and CD16 intermediate ($CD14^+$ HLA-DR^+ $CD16^+$ $CD192^+$) monocytes, in some cases referred to as "intermediate monocytes". However, patients carrying *NLRP3* variants had a lower percentage of CD 16 high, so-called "non-classical" monocytes ($CD14^+$ HLA-DR^+ $CD16^{high}$ $CD192^-$; p= 0.0255; one-way ANOVA with Dunnett's multiple comparisons test; Figure 5A). CD 192 was included in the analysis to improve subset differentiation since its expression intensity is strictly opposite to that of CD16.

We further analysed percentages of M1- ($CD14^+$ HLA-DR^+ $CD16^-$ $CD192^{high}$ $CD195^+$) and M2 ($CD14^+$ HLA-DR^+ $CD206^+$) macrophages. This revealed similar percentages of M1- macrophages between HC and patients with variants in autoinflammation-related genes (one-way ANOVA with Dunnett's multiple comparisons test; Figure 5B).

The same caveat concerning testing of multiple outcomes and subgroup analyses as described in 6.3 applies.

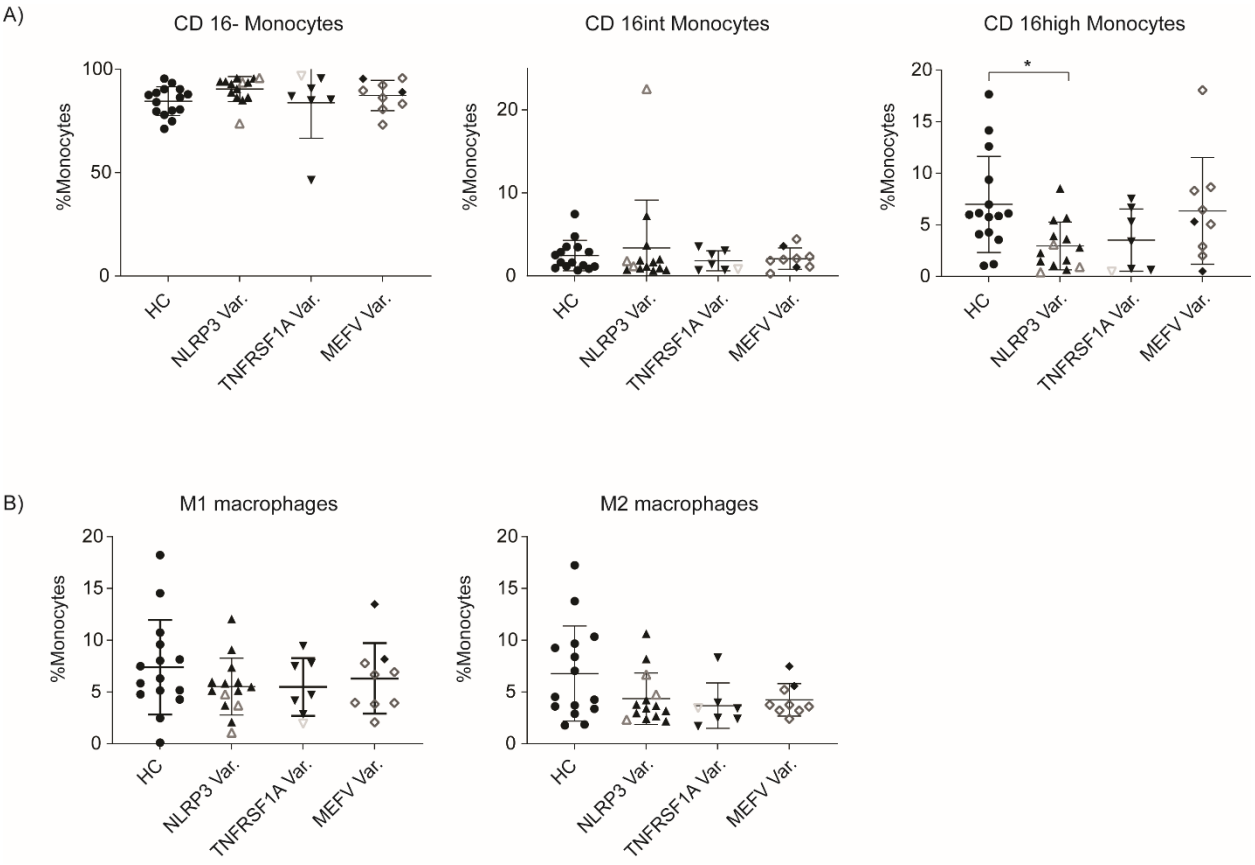

Figure 5. Monocyte subset analysis. FACS analysis of cell surface markers. Each point represents the percentage of a cell type of an individual patient. Open symbols signify patients under therapy for a suspected autoinflammatory disorder. HC: n=15; *NLRP3* Var.: n=14; *TNFRSF1A* Var.: n=7; MEFV Var. n=9. One-way ANOVA with Dunnett's multiple comparisons test A) Comparison of CD16 negative "classical", CD16 intermediate "intermediate" and CD16 high "non-classical" monocytes in patients with genetic variants in inflammation-related genes and HC. Lower levels of CD16 high monocytes in patients with *NLRP3* variants. B) Analysis of M1- and M2 macrophage subtypes in patients with genetic variants in inflammation-related genes and HC. No significant differences between patients and HC. Var.= variant, int.= intermediate.

6.5. Analysis of cytokine secretion by T cells

Because of the close interplay of innate and adaptive immunity, changes in T cell functionality in patients with variants in autoinflammation-related genes are conceivable. We therefore evaluated secretion of the cytokines interferon γ (IFN γ), TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-22 and IL17A by T cells of patients with mutations in the

genes *NLRP3* (n=14), *TNFRSF1A* (n=6, data not available for the seventh *TNFRSF1A* patient) or *MEFV* (n=9) in comparison to HC (n=15).

We found a significantly increased secretion of the pro-inflammatory mediator TNFα in CD4 T cells ($CD3^+$ $CD56^-$ $CD4^+$) of patients with variants in the *MEFV* gene (p=0.0054, one-way ANOVA with Dunnett's multiple comparisons test). Moreover, we observed a trend towards heightened IL-17A- and IFNγ-secretion in the same patient cohort (p=0.0603 and p=00639, respectively; one-way ANOVA with Dunnett's multiple comparisons test). We further observed increased secretion of the anti-inflammatory IL-4 in patients with *MEFV* variants (p= 0.001; one-way ANOVA with Dunnett's multiple comparisons test, Figure 6).

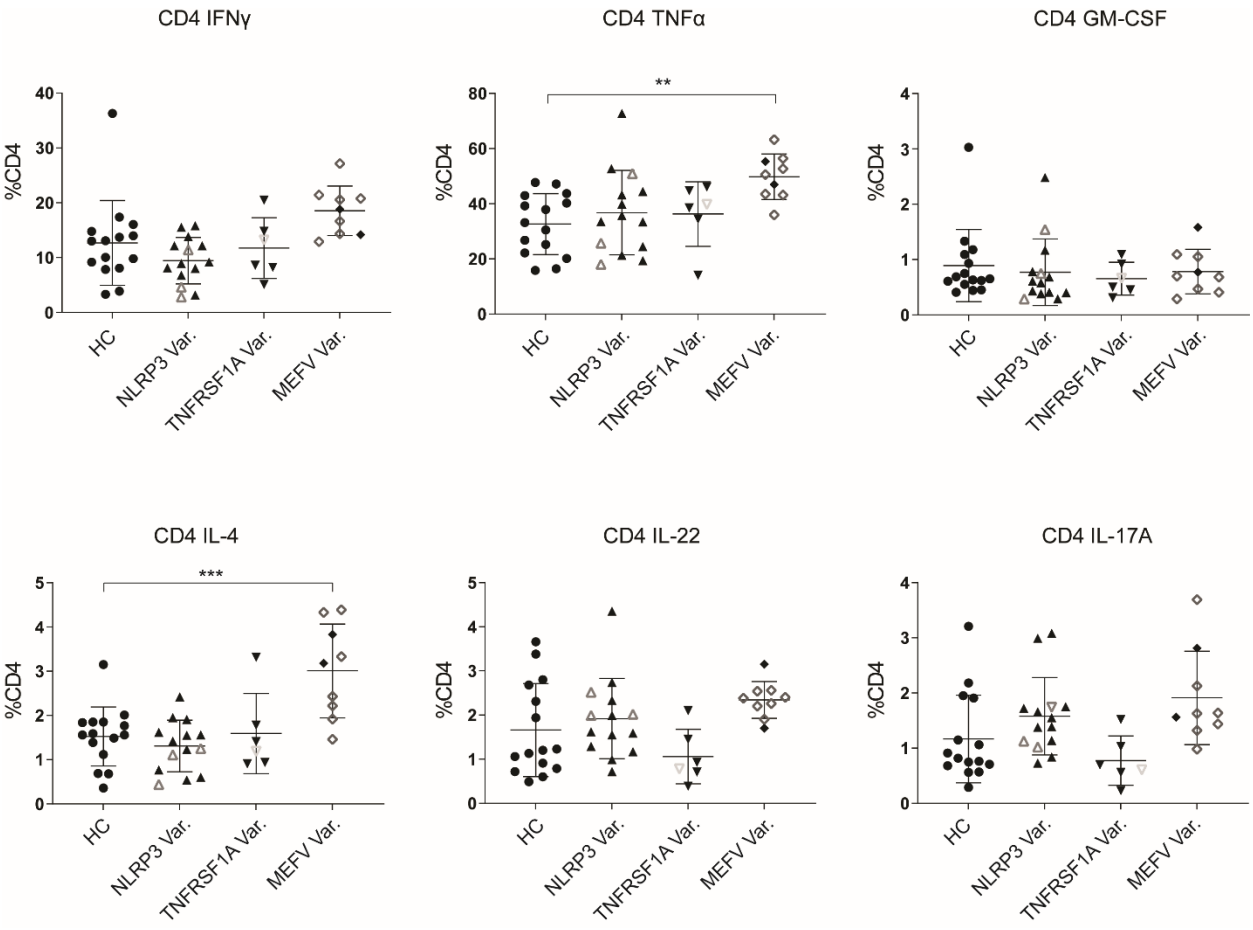

Figure 6. Cytokine secretion by CD4 T cells. FACS analysis of CD4 T cells after intracellular staining for different cytokines. Each dot represents the percentage of positively stained $CD4^+$ T cells of one patient. Open symbols signify patients under treatment. HC: n=15; *NLRP3* Var.: n=14; *TNFRSF1A* Var.: n=6; *MEFV*

Var. n=9. One-way ANOVA with Dunnett's multiple comparisons test. Significantly elevated secretion of TNFα and IL-4by $CD4^+$ T cells of patients with *MEFV* variants.

Analogous analysis of cytokine secretion of CD8 T cells ($CD3^+$ $CD56^-$ $CD8^+$) revealed similar cytokine secretion by CD8 T cells in patients and HC (Figure 7).

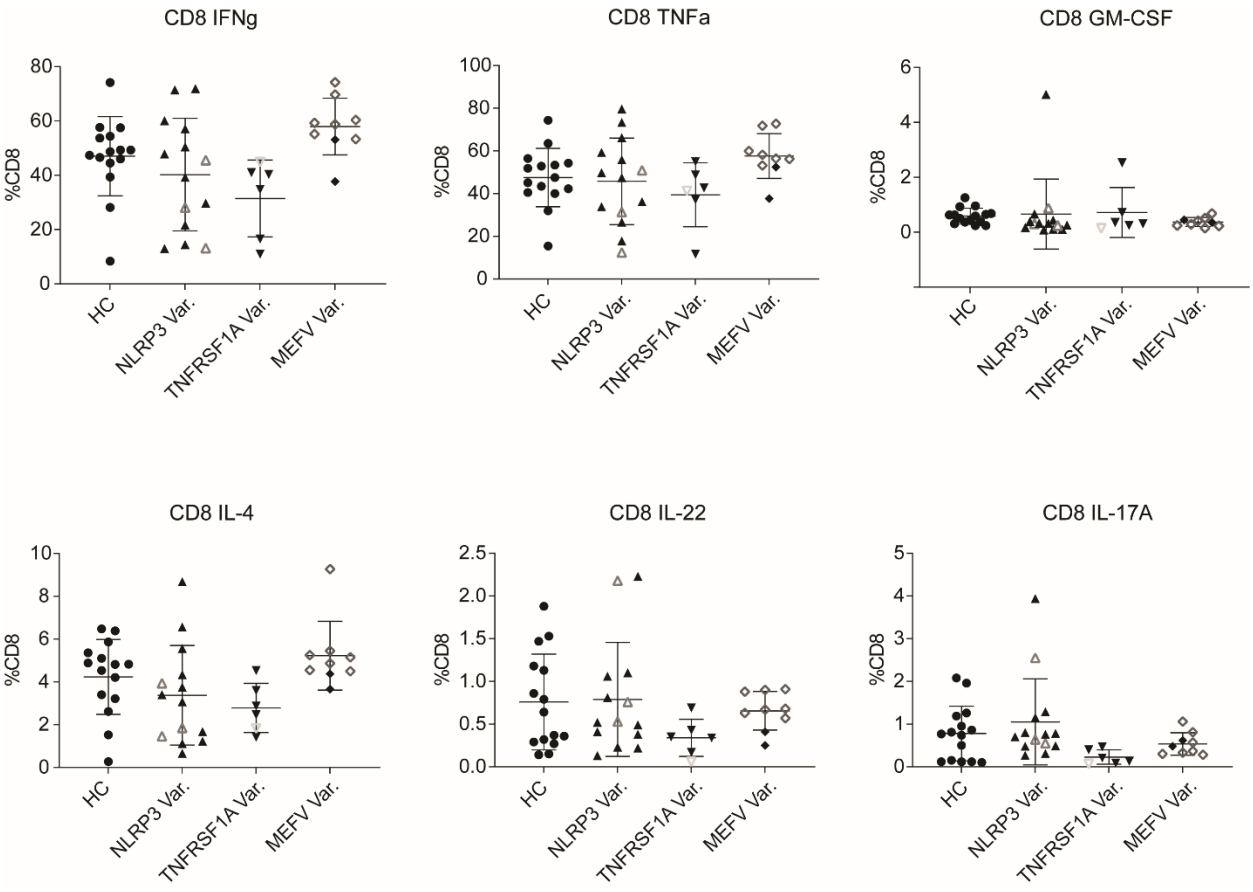

Figure 7. Cytokine secretion by CD8 T cells. FACS analysis of CD8 T cells after intracellular staining for different cytokines. Each dot represents percentage of positively stained CD8 T cells of one patient. Open symbols signify patients under treatment. HC: n=15; *NLRP3* Var.: n=14; *TNFRSF1A* Var.: n=6; *MEFV* Var. n=9. One-way ANOVA with Dunnett's multiple comparisons test. Similar levels of cytokine secretion by CD8 T cells in patients with variants in autoinflammation-related genes and HC.

6.6. Functional analysis of IL-1β secretion after inflammasome stimulation

6.7. PBMC

PBMC are an easily obtained, well storable and commonly used patient material for functional assays. Hence, we first evaluated IL-1β secretion of PBMC of patients with *MEFV* variants after pyrin inflammasome stimulation in comparison to HC.

A) frozen PBMC

Characteristics of patients and controls for this analysis are listed in Table 7.

Table 7. Characteristics of patients for IL-1β secretion analysis in frozen PBMC. Red lettering signifies high-penetrance variants. Het.=heterozygous, hom.=homozygous. n/a=not applicable.

	HC	MEFV variants
n	9	12
Sex	6 f 3 m	6 f 6 m
Mean age (range)	33 (20-47)	32 (18-47)
Genotype	n/a	2 het. E148Q 2 het I591T 1 het M680I 2 het. M694V & het. M680I 2 het. M694V & het. V726A 1 het. M680I & het. V726A 1 het. M680I & het. E148Q 1 het. M694V & het. R202Q

Diagnostic criteria fulfilled	n/a	4 yes (1 M694V & het. M680I; 1 het. M694V & het. V726A; 1 M680I & het. V726A; 1 M680I & het. E148Q)
Treated for suspected autoinflammatory syndrome	n/a	12 yes
Periodic/ continuous disease	n/a	9 periodic 2 continuous 1 mixed

To determine potential differences in IL-1β secretion upon inflammasome stimulation, we first analyzed PBMC stored in liquid nitrogen in the biobank of the Institute of clinical neuroimmunology (LMU, Munich). We tested samples of twelve patients with high- or low-penetrance mutations in the MEFV gene (six male, six female; mean age 32 years) and of nine healthy controls (HC; three male, six female; mean age 33 years). Four patients fulfilled the Tel Hashomer diagnostic criteria for FMF. After priming with LPS, cells were treated with either NLRP3 inflammasome activators ATP or Nigericin or with the Pyrin inflammasome activator Clostridium difficile toxin B (TcdB). We found no significant differences in the resulting IL-1β secretion between HC and patients with *MEFV* variants (multiple t tests with additional two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, false discovery rate 1%, Figure 8).

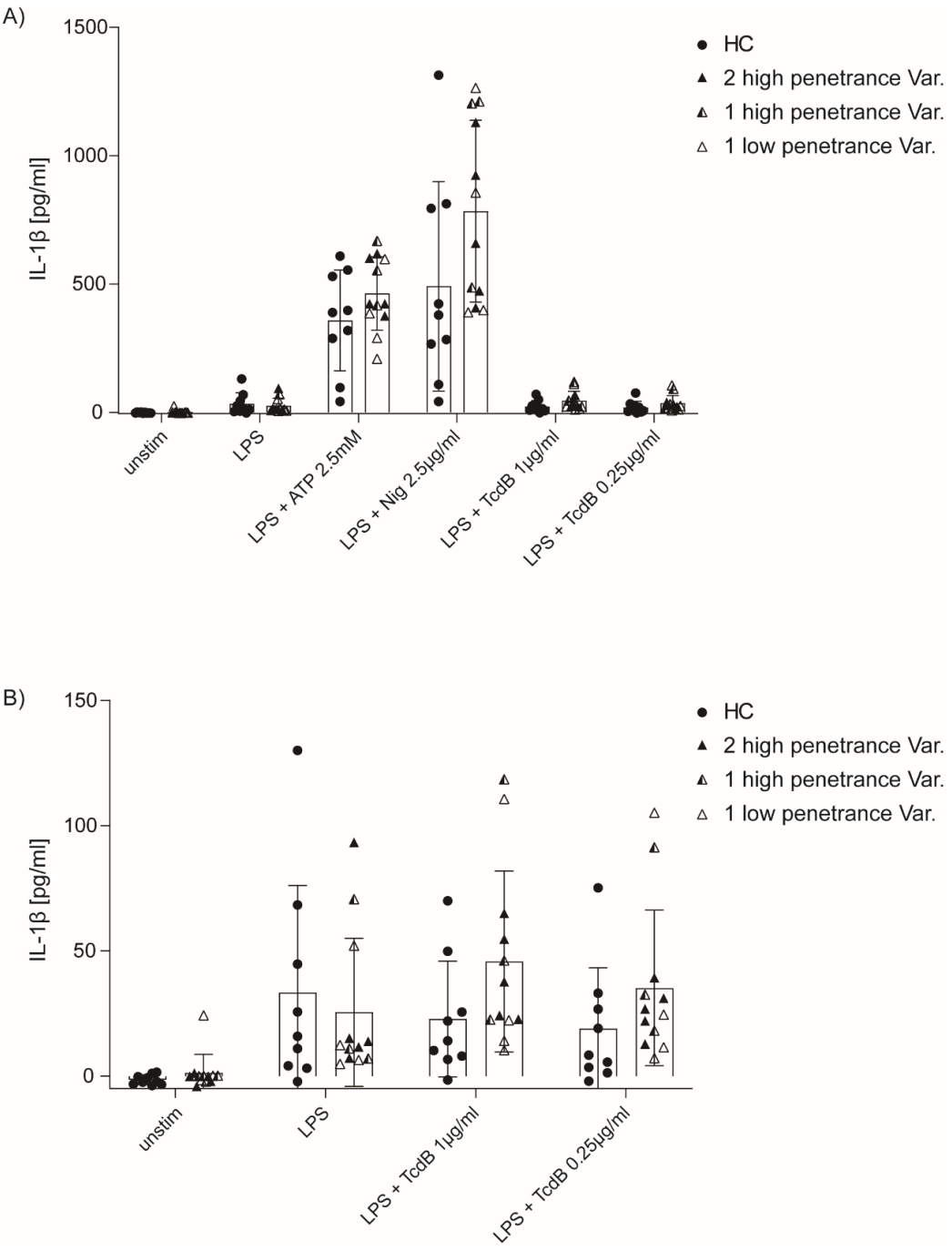

Figure 8. IL-1β secretion upon inflammasome stimulation in frozen PBMC of healthy controls and patients with *MEFV* variants. Filled triangles represent patients carrying two high-penetrance variants, half-filled triangles show values of patients with one high-penetrance- and open triangles those of patients with one low-penetrance variant in the *MEFV* gene. Multiple t tests with additional two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, false discovery rate 1%. A) IL-1β secretion after stimulation of the NLRP3-inflammasome with ATP or Nigericin (Nig) and of the Pyrin inflammasome with TcdB. No significant differences between healthy controls and patients with MEFV

variants. B) Shows an enlargement of the Pyrin inflammasome stimulation data and controls (unstim, LPS) from the same dataset as in A).

B) fresh PBMC

Characteristics of patients and controls for this analysis are listed in Table 8.

Table 8. Characteristics of patients for IL-1β secretion analysis in fresh PBMC. Red lettering signifies high-penetrance variants. Het.=heterozygous, hom.=homozygous. n/a=not applicable.

	HC	MEFV variants
n	5	1
Sex	2 f 3 m	f
Mean age (range)	25 (23-29)	55 (n/a)
Genotype	n/a	1 het M680I
Diagnostic criteria fulfilled	n/a	no
Treated for suspected autoinflammatory syndrome	n/a	yes
Periodic/ continuous disease	n/a	continuous

To exclude effects of the freezing process or of PBMC storage duration, we performed an inflammasome stimulation assay in analogy to the one described for frozen PBMC with freshly prepared PBMC. We analyzed samples of five healthy controls (male:female 3:2, mean age 25 years) and of a female patient aged 54 years with a heterozygous variant M680I in exon 10 of the *MEFV* gene, but not fulfilling Tel Hashomer diagnostic criteria for FMF. The patient was currently treated with colchicine, clinically, she had intermittent abdominal pain, headaches, and multifocal joint pain. The inflammation marker SAA was mildly elevated at 12.9 mg/l (reference value <6.5 mg/l) at the time of blood sampling. When stimulated with the NLRP3 inflammasome inducers ATP and Nigericin, responses of healthy controls and the patient were similar, with one

healthy control secreting even higher levels of IL-1β (Figure 9 A). Upon stimulation of the Pyrin inflammasome with TcdB, however, the patient secreted considerably higher amounts of IL-1 β with all concentrations of TcdB (Figure 9 B).

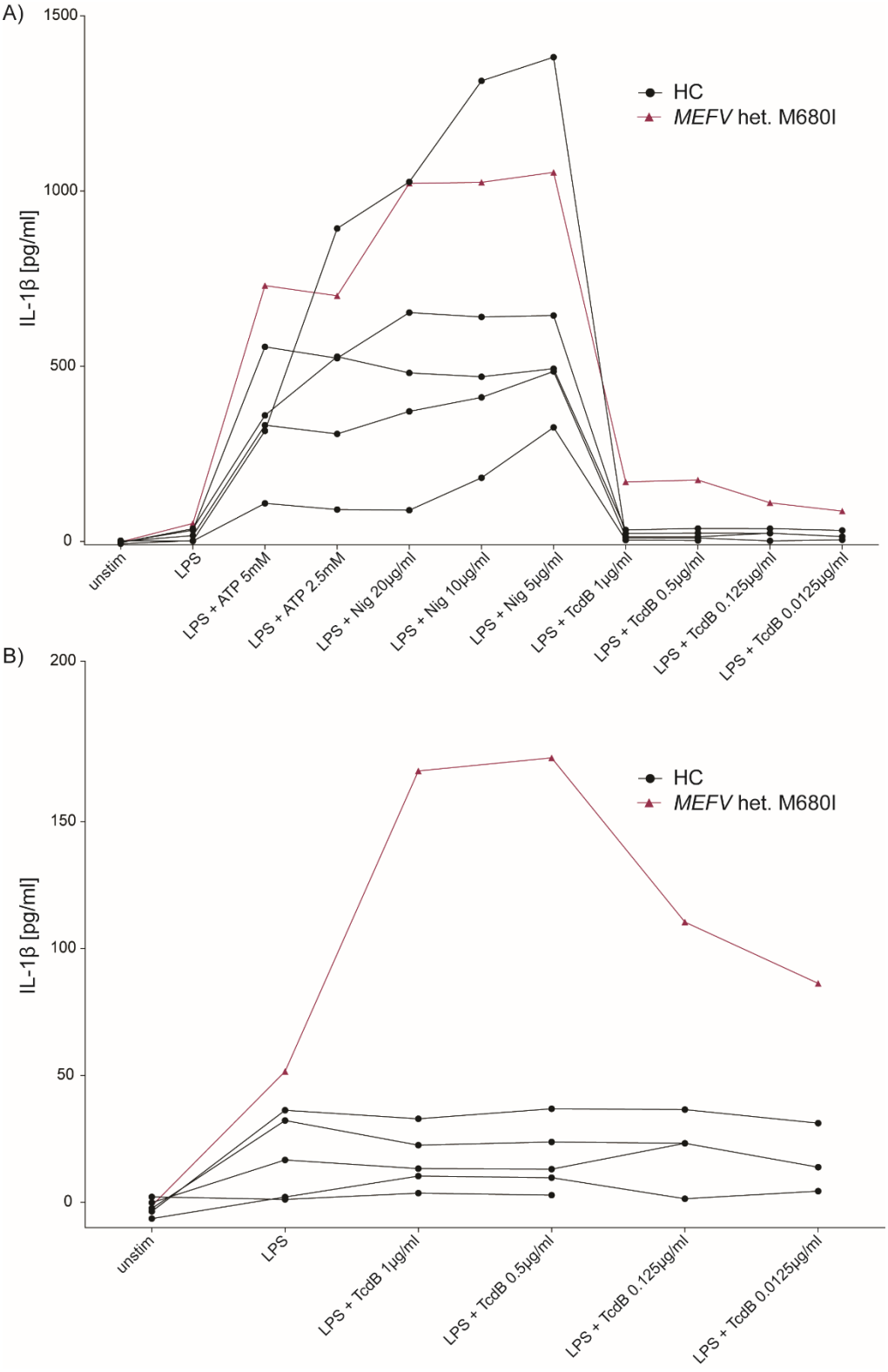

Figure 9. IL-1β secretion upon inflammasome stimulation in fresh PBMC of healthy controls and a patient with a heterozygous high penetrance variant M680I in the *MEFV* gene. Each symbol represents the value of an independent stimulation of one donor. Lines connect data of one donor. A) IL-1β

secretion after priming with LPS and stimulation with the NLRP3 inflammasome inducers Nigericin and ATP and the pyrin inflammasome inducer TcdB. Stronger IL-1β secretion by the patient upon pyrin inflammasome stimulation. B) Shows an enlargement of the Pyrin inflammasome stimulation data and controls (unstim, LPS) from the same dataset as in A). Considerably stronger IL-1β secretion upon pyrin inflammasome stimulation in the patient with the MEFV variant.

6.8. Whole blood

Characteristics of patients and controls for this analysis are listed in Table 9.

Table 9. Characteristics of patients for IL-1β secretion analysis in whole blood. Het.=heterozygous, hom.=homozygous. n/a=not applicable.

	HC	MEFV variants
n	6	4
Sex	2 f 4 m	1 f 3 m
Mean age (range)	27 (23-31)	32 (12-47)
Genotype	n/a	1 het. E148Q 3 hom. E148Q
Diagnostic criteria fulfilled	n/a	2 yes 2 no
Treated for suspected autoinflammatory syndrome	n/a	3 yes 1 no
Periodic/ continuous disease	n/a	4 periodic

Determining the clinical relevance of low penetrance variants in the *MEFV* gene in individual patients is often difficult. There is a broad overlap of symptoms especially with pain syndromes and psychosomatic disorders. Hence, we aimed to evaluate differences in IL-1β secretion upon inflammasome stimulation in diluted whole blood between patients with low-penetrance *MEFV* variants and HC. We tested blood samples of four healthy donors (three males, one female, mean

age 25 years) and of a family of four with the genetic variant E148Q in exon 2 of the MEFV gene (three male, one female, mean age 31). The mother and two sons of the family were homozygous for the genetic variant, the father heterozygous. The mother and one son fulfilled diagnostic criteria for FMF. Both parents and one son were treated with colchicine at the time of blood sampling, the mother was additionally treated with fingolimod for Multiple sclerosis.

Comparison of the E148Q family's IL-1β secretion with that of healthy controls revealed significantly higher levels of IL-1β secretion upon Pyrin inflammasome stimulation (p=0.0001 for TcdB 1µg/ml; p=0.0019 for TcdB 0.5µg/ml; multiple t tests with additional two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, false discovery rate 1%; Figure 10)

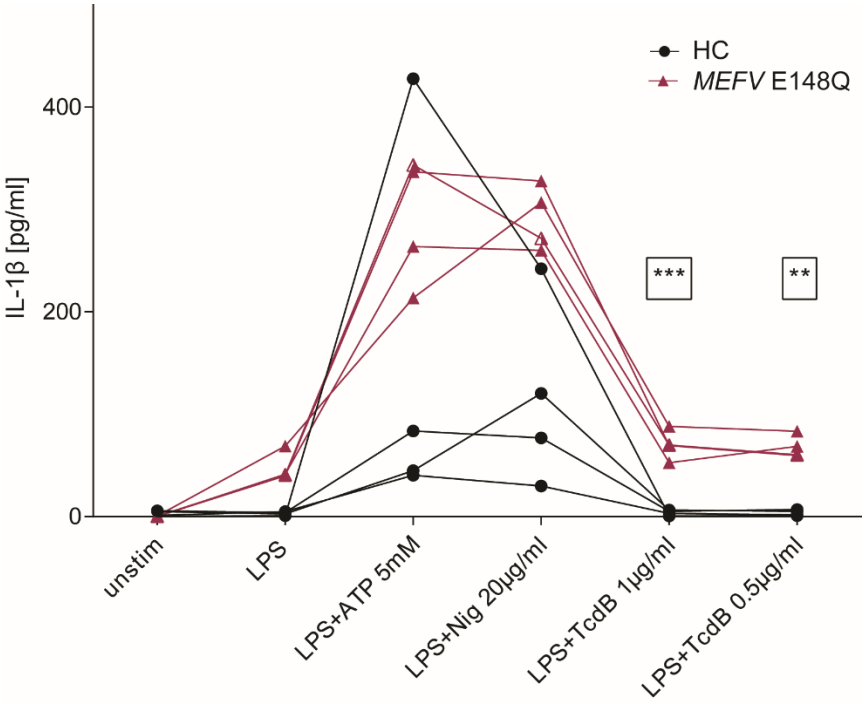

Figure 10. IL-1β secretion upon inflammasome stimulation in whole blood. Whole blood inflammasome stimulation assay of four healthy donors and a family of four with the genetic variant E148Q in exon 2 of the *MEFV* gene. Each symbol represents the value of an independent stimulation of one donor. Lines connect data of one donor. Four patients homozygous for the variant (full triangles), one heterozygous (half-filled triangles). Multiple t tests with additional two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, false discovery rate 1%. Significantly higher IL-1β secretion in patients with MEFV variants upon pyrin inflammasome stimulation with TcdB. Nig=Nigericin.

6.9. Cell death rate analysis after inflammasome stimulation

Characteristics of patients and controls for this analysis are listed in Table 10.

Table 10. Characteristics of patients for LDH cell death assay. Red lettering signifies high penetrance variants. Het.=heterozygous, hom.=homozygous. n/a=not applicable.

	HC	MEFV variants
n	8	10
Sex	5 f 3 m	5 f 5 m
Mean age (range)	33 (20-56)	33 (18-53)
Genotype	n/a	2 het. E148Q 2 het. M680I 2 het. M694V & het. M680I 2 het. M680I & het. V726A 1 het. M680I & het. E148Q 1 het. M694V & het. R202Q
Diagnostic criteria fulfilled	n/a	4 yes (1 het. M694V & het. M680I; 2 het. M680I & het. V726A; 1 het. M680I & het. E148Q 6 no
Treated for suspected autoinflammatory syndrome	n/a	9 yes 1 no
Periodic/ continuous disease	n/a	7 periodic 3 continuous

Since inflammasome activation leads to increased cell death by pyroptosis, we performed an LDH assay with PBMC thawed after storage in liquid nitrogen and inflammasome stimulation. We compared samples of eight healthy controls (3 male, 5 female, mean age 33 years) and ten

patients with different variants in the MEFV gene (5 male, 5 female, mean age 33). Four patients carried two, three patients one high-penetrance variant. Two patients had one low-penetrance variant and one patient a high- and a low-penetrance variant. All but one patient were treated with colchicine. As previous experiments showed an extremely high cell death rate for both patients and controls when primed with the otherwise used LPS dose of 100ng/ml and the previously used higher concentrations of ATP and Nigericin, we used an LPS concentration of 10ng/ml, ATP concentration of 2.5mM and Nigericin concentration of 2.5µg/ml for this assay. No significant differences in cell death rates as measured by percentage of cytotoxicity compared to unstimulated cells was observed (multiple t tests with additional two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, false discovery rate 1%). Interestingly, a TcdB concentration of 0.5µg/ml appeared to be slightly protective for both healthy controls and patients (Figure 11).

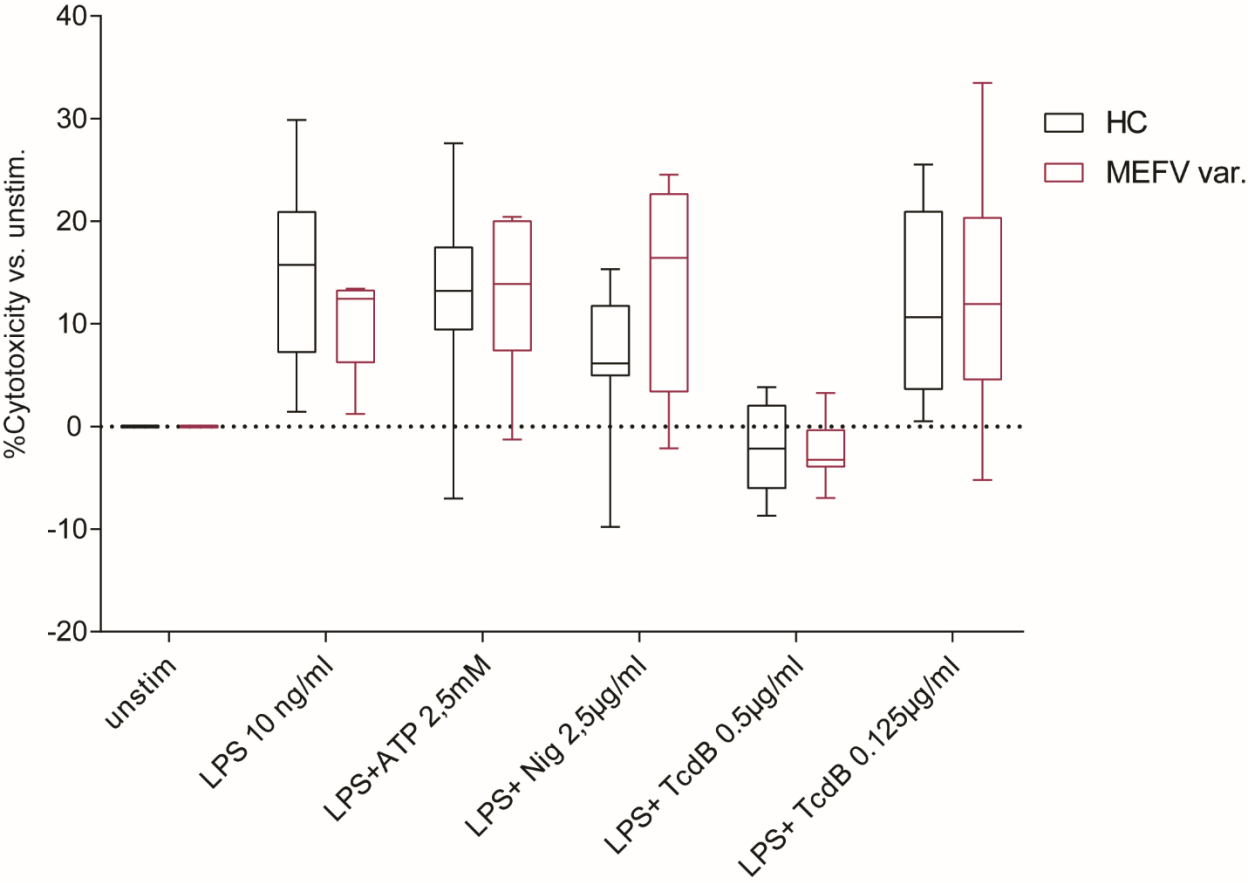

Figure 11. Cell death rate analysis after inflammasome stimulation. LDH assay of PBMC of eight healthy controls and ten patients with different MEFV variants after inflammasome stimulation. Results are displayed as %cytotoxicity of each inflammasome stimulus compared to unstimulated cells. Multiple t tests with additional two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, false discovery rate 1%. No significant differences between healthy controls and patients with MEFV variants.

7. Results of Project Two: Differential effects of natalizumab on features of peripheral and intrathecal immunoglobulin production

Parts of this project have been submitted for publication in Neurology® Neuroimmunology & Neuroinflammation.

The aim of this project was to better characterize the effects of long-term NTZ on different aspects of Ig production in MS patients and to evaluate the levels of VLA-4 expression on different B cell subsets.

7.1. NTZ reduces serum IgM- and IgG-, but not IgA levels

First, we analyzed serum Ig levels in a longitudinal and cross-sectional study approach.

In serum, we found a marked reduction of IgM levels in both the longitudinal comparison of patients before and during NTZ treatment ($p=0.0005$, $n=13$, Wilcoxon matched-pairs signed rank test) and in the cross-sectional study of NTZ treated versus untreated MS patients ($p<0.0001$, $n_{untreated}=41$, $n_{NTZ}=47$, Mann-Whitney U test). Quantitatively, the reduction in serum IgM was substantial, from a mean of 1307 mg/L to 653mg/L (50% reduction) after a mean of 3.7 years NTZ treatment. In three patients, serum IgM level decreased below reference value during NTZ treatment. In the cross-sectional part of the study, 13 patients (28%) in the NTZ group showed serum IgM levels below reference value compared to only 2 patients (5 %) in the untreated group ($p=0.0048$, Fisher's exact test). Serum IgG was slightly reduced in the longitudinal comparison ($p=0.0159$, $n=27$), but not in the cross-sectional part of the study. IgA levels were comparable both before and during NTZ treatment and between untreated and NTZ treated patients (Figure 12).

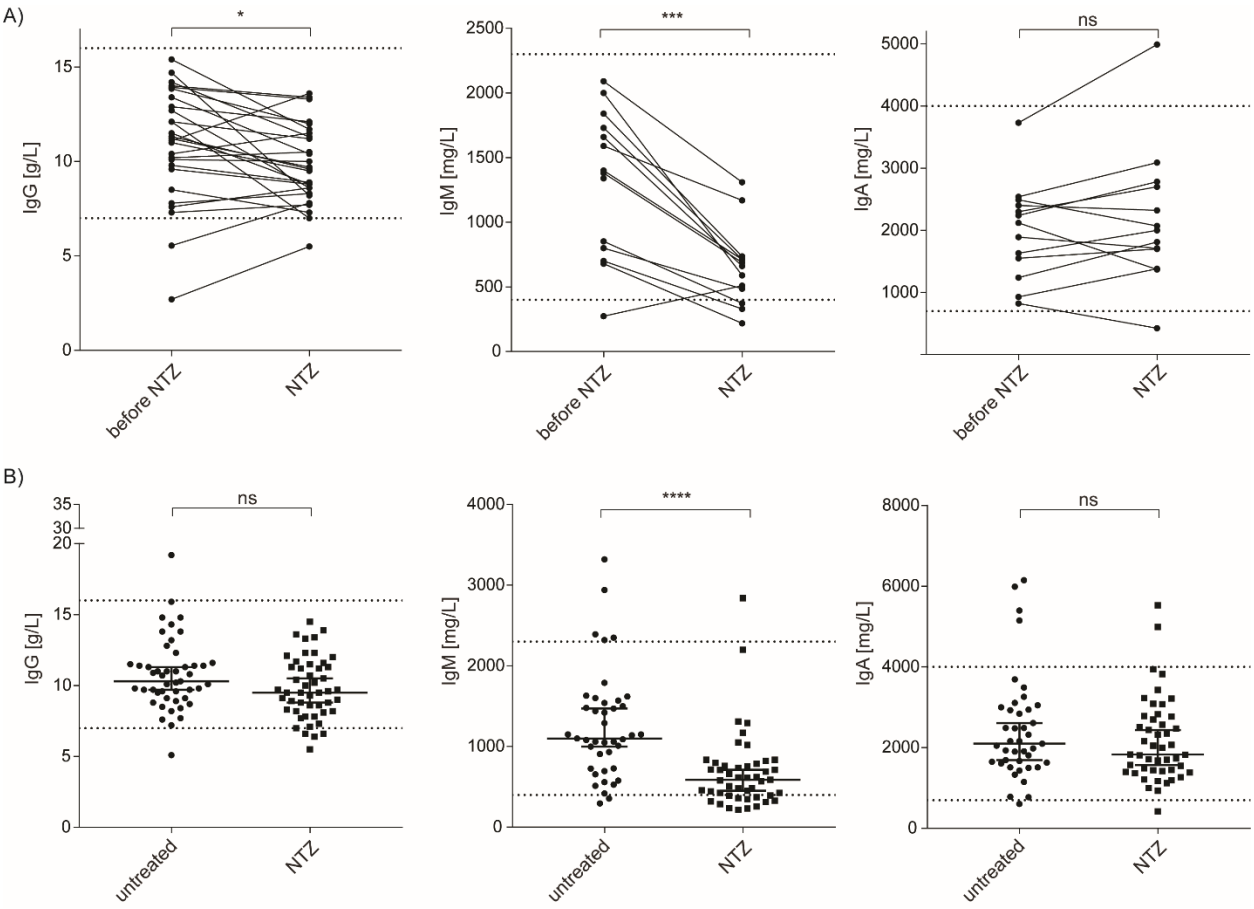

Figure 12: Serum Ig levels. Dotted lines represent reference values (IgG: 7-16 g/l, IgM: 400-2300 mg/l, IgA: 700-4000 mg/l). Serum IgM levels were reduced during NTZ therapy both in A) a longitudinal and B) a cross-sectional study. IgG- and IgA- levels remained stable. A) Reduction of serum IgG during NTZ treatment (n=27) and IgM levels (n=13). IgA levels remained stable (n=13). Wilcoxon matched-pairs signed rank test. B) Lower levels of serum IgM in NTZ treated (n=47) than in untreated MS patients (n=41). IgG- and IgA levels were comparable between the groups. IgG: $n_{untreated}$=47, n_{NTZ}=49; IgA: $n_{untreated}$=41, n_{NTZ}=47. Mann-Whitney U test.

7.2. NTZ treatment reduces overall CSF Ig levels

Next, we evaluated the amount of Ig in patients' CSF. Longitudinally, IgG- and IgM levels were reduced during NTZ treatment ($p<0.0001$, n=27 and $p=0.0012$, n=13, respectively; Wilcoxon matched-pairs signed rank test). Comparing untreated and NTZ treated patients, we found a global CSF Ig reduction in NTZ patients with the strongest effect on IgG ($p<0.0001$, $n_{untreated}$=47,

n_{NTZ}=49; Mann-Whitney U test) and IgM (p=0.0003, $n_{untreated}$=41, n_{NTZ}=47; Mann-Whitney U test), followed by IgA (p=0.0154, $n_{untreated}$=41, n_{NTZ}=47; Mann-Whitney U test, Figure 13).

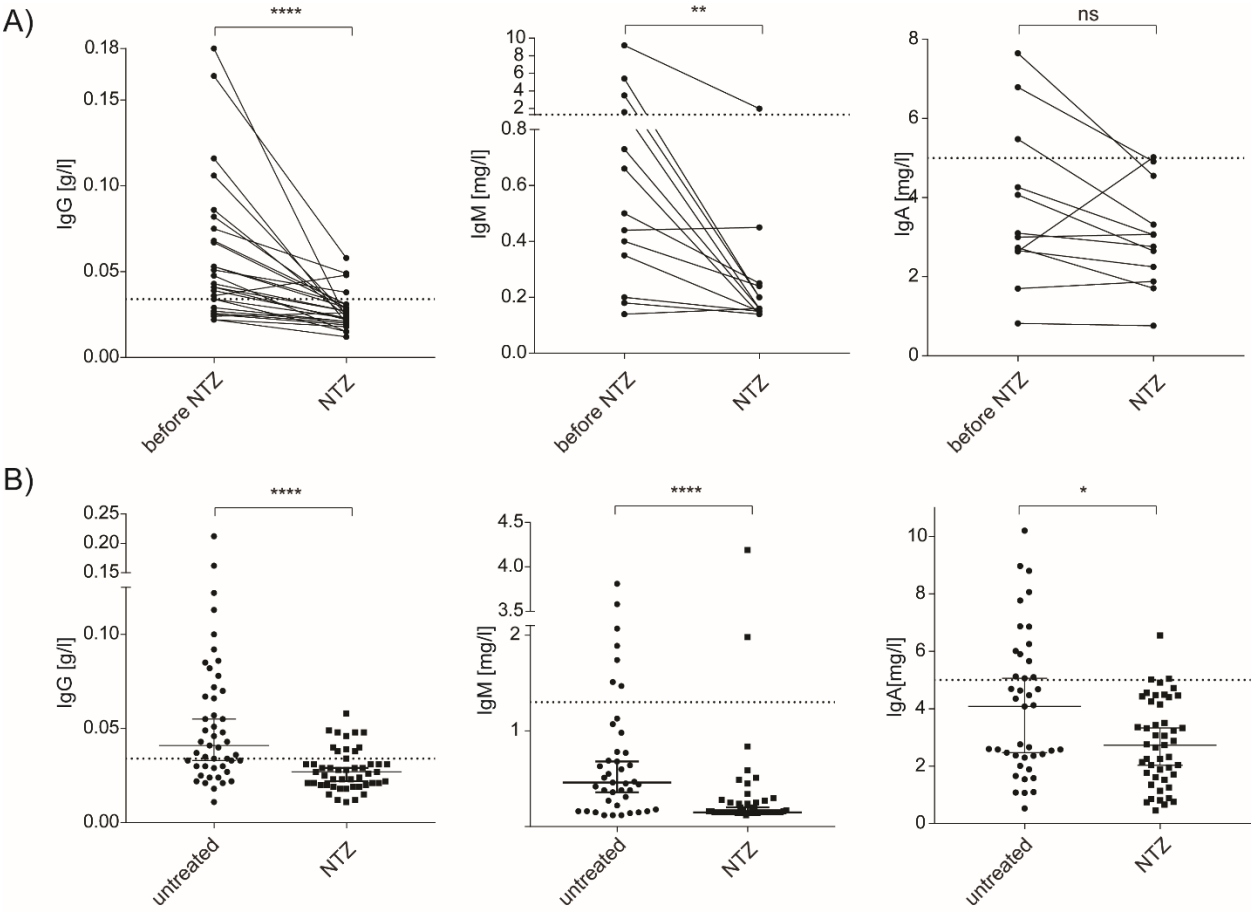

Figure 13: CSF Ig levels. Dotted lines represent reference values (IgG: 0.34 g/l, IgM: 1.3 mg/l, IgA: 5 mg/l). A) Longitudinal comparison. CSF IgG (n=27) and IgM (n=13) values were reduced during NTZ treatment. IgA: n=13. Wilcoxon matched-pairs signed rank test. B) Cross-sectional comparison. Considerably lower IgG- ($n_{untreated}$=47, n_{NTZ}=49) and IgM levels, slightly lower IgA levels in NTZ patients ($n_{untreated}$=41, n_{NTZ}=47). Mann-Whitney U test.

7.3. Isotype-specific reduction of intrathecal Ig production by NTZ

Using Ig index [(Ig in CSF/ Ig in serum)÷(albumin in CSF/albumin in serum)] as a surrogate parameter for intrathecal Ig production, we saw a strong reduction of intrathecal IgG production in patients during- compared to before NTZ treatment (p<0.0001, n=26; Wilcoxon matched-pairs

signed rank test). IgM- and IgA index remained unchanged in the longitudinal comparison. This finding was partially reflected in the cross-sectional study: IgG indices were significantly lower in NTZ treated patients (p=0.0007, $n_{untreated}$=47, n_{NTZ}=49; Mann-Whitney U test). However, we also found significantly lower IgA indices in the NTZ- compared to the untreated group (p=0.0076, $n_{untreated}$=41, n_{NTZ}=47; Mann-Whitney U test, Figure 14.

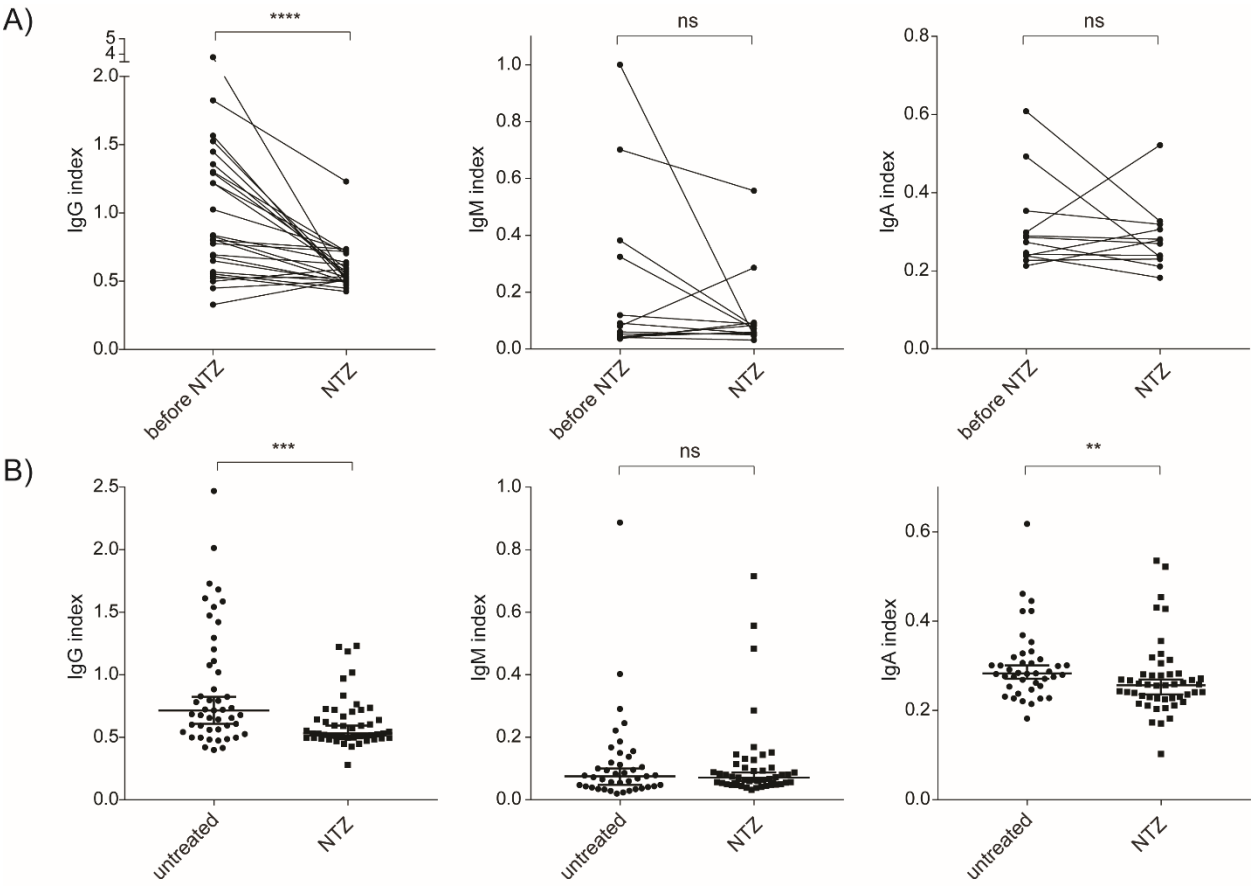

Figure 14. Intrathecally produced Ig as measured by Ig index. A) Longitudinal approach. IgG index (n=26) decreased in patients during NTZ treatment while IgM-(n=13) and IgA (n=12= index remained stable. Wilcoxon matched-pairs signed rank test. B) Cross-sectional approach. IgG- ($n_{untreated}$=47, n_{NTZ}=49) and IgA index ($n_{untreated}$=41, n_{NTZ}=47) were lower in NTZ treated patients than in untreated controls. IgM index ($n_{untreated}$=41, n_{NTZ}=47) was comparable between the groups. Mann-Whitney U test.

7.4. Differential effects of NTZ treatment duration on peripheral and intrathecal Ig levels.

Further, we analyzed the effect of patients' NTZ treatment duration on peripheral and intrathecal Ig production. While there was an inverse correlation of therapy duration with serum IgM- (p=0.0031, n=47, Spearman r= -0.4227; nonparametric Spearman correlation) and IgA levels (p=0.0354, n=47, Spearman r= -0.3077; nonparametric Spearman correlation), intrathecal IgG production declined over time (p= 0.0198, n=49, Spearman r= -0.3318, Figure 15; nonparametric Spearman correlation).

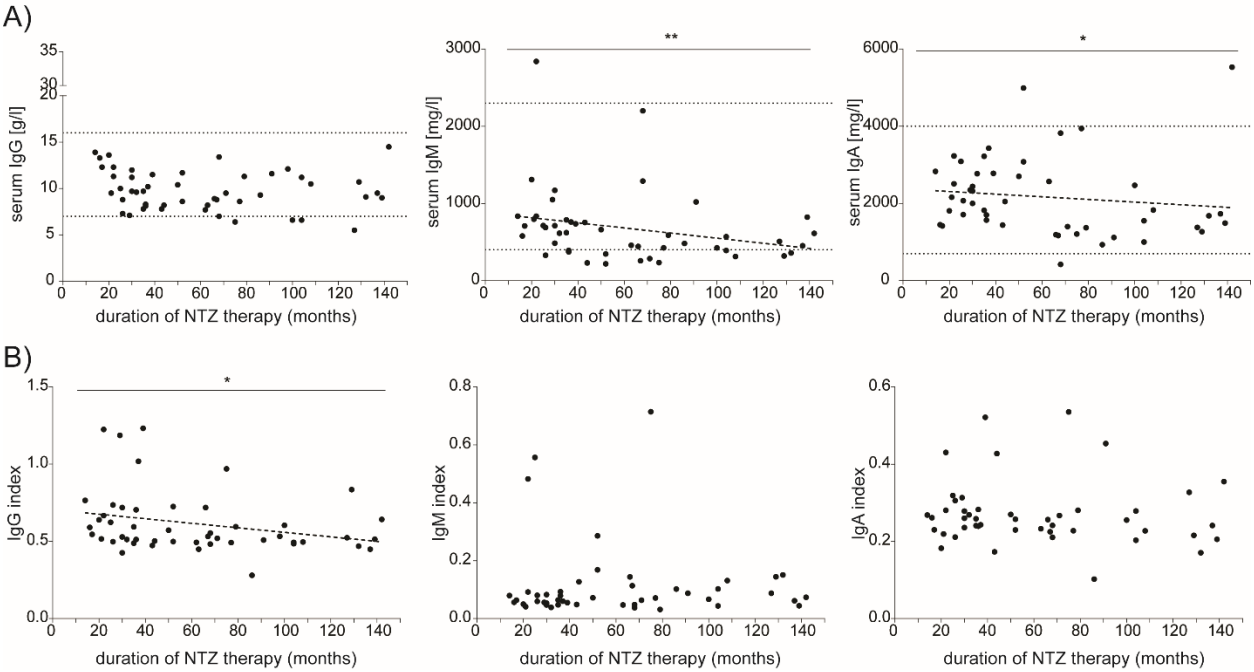

Figure 15. Differential effects of NTZ treatment duration on peripheral and intrathecal Ig levels. A) Levels of serum Ig and B) Ig indices for IgG (left), IgM (middle) and IgA (right) are shown along with NTZ therapy duration (months) until CSF and serum samples were obtained. Dotted horizontal lines represent upper and lower reference values for serum Ig (IgG: 7-16 g/l, IgM: 400-2300 mg/l, IgA: 700-4000 mg/l). Linear regression analysis and Spearman nonparametric correlation analysis were performed. Regression lines (dashed) are shown for significant correlations. A) Serum IgM- (n=47) and IgA levels (n=47) were lower in patients with a longer duration of NTZ treatment. IgG levels (n=49) were relatively stable over time. B) Intrathecal IgG production (n=49) correlated inversely with NTZ therapy duration, while treatment duration had no effect on IgM- (n=47) and IgA (n=47) indices.

7.5. OCB and MRZ reaction largely persist during NTZ treatment

Having noted a reduction in intrathecal IgG production in NTZ treated patients, we next evaluated two IgG-mediated hallmarks of MS: OCB and MRZ reaction. No differences in OCB status were detected in the cross-sectional comparison between NTZ and untreated patients ($n_{untreated}$ =47, n_{NTZ} =49; OCB positive: 87% [n=41] of untreated patients vs. 76% of NTZ patients [n=36], Figure 16 A). OCB status was largely stable over the course of NTZ treatment (n_{before} NTZ=33, nNTZ=49; OCB positive: 85% of patients before [n=28] vs. 76% of patients during NTZ [n=36], Figure 16 B). Of note, five patients out of 27 initially OCB positive patients (19%) lost their formerly positive OCB during NTZ treatment; these patients are analyzed further in section 7.5.1.

We had the opportunity to reanalyze OCB of a single patient in samples stored before and during NTZ treatment in parallel on the same gel. Banding patterns before and after 4.3 years of NTZ treatment were very similar with the majority of bands persisting. However, some individual bands became weaker or disappeared entirely (Figure 16 C).

Additionally, we evaluated the IgG mediated MRZ reaction, an unspecific intrathecal antibody reaction against measles-, rubella-, and varicella zoster virus. Positive MRZ reaction was defined as an intrathecal antibody reaction against one (MRZ-1) or two (MRZ-2) of the viruses No significant differences in the cross-sectional analysis of untreated and NTZ treated patients could be seen for MRZ-1 (p=0.1513, MRZ-1 positive: untreated 62% and NTZ treated 46%, Fisher's exact test.) or MRZ-2 (p= 0.1069, MRZ-2 positive: untreated 34% and NTZ treated 19%; Fisher's exact test; Figure 16 D).

Longitudinal data for MRZ reaction was not available.

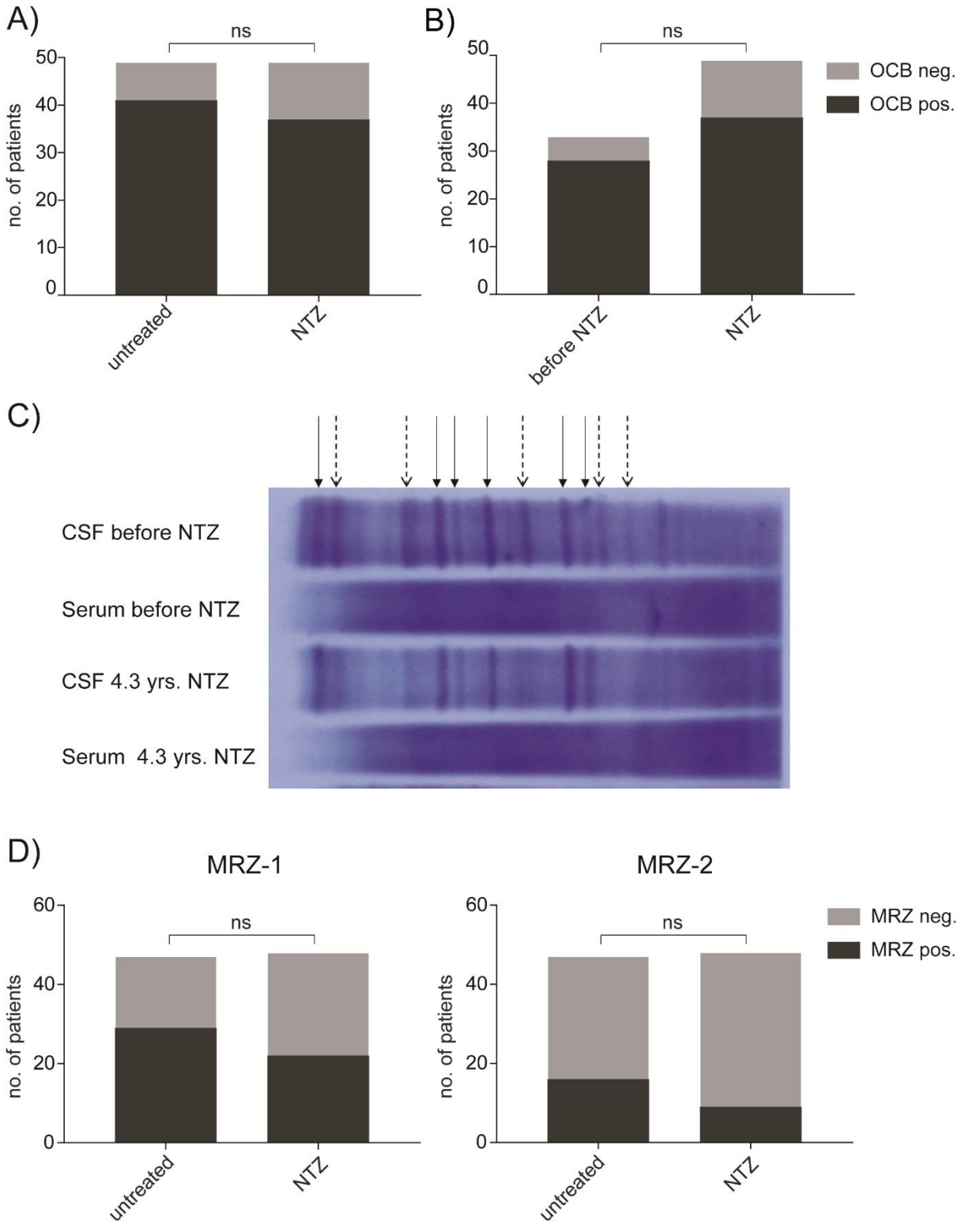

Figure 16. OCB and MRZ reaction largely persist during NTZ treatment. No significant differences (Fisher's exact test) in A) a cross-sectional and B) a longitudinal analysis of OCB status. C) OCB of a patient before and after 4.3 years of NTZ treatment measured in parallel on the same gel. Most bands

persist (solid arrows) while some bands disappear or fade over time (dashed arrows). D) Cross-sectional analysis of MRZ-reaction. No significant differences in MRZ-1 (reactivity to one virus) or MRZ-2 (reactivity against two viruses). Fisher's exact test.

7.6. OCB loss is not due to pronounced IgG reduction during NTZ

Next, we analyzed peripheral and intrathecal IgG production of the five patients who lost their OCB during NTZ treatment in comparison to subjects with persisting positive OCB. Paired IgG data was available for five patients with loss of OCB and 16 patients with persisting OCB. Loss of OCB does not seem to be due to a more pronounced reduction of peripheral or intrathecal IgG production (Figure 17). In patients with loss of OCB, mean serum IgG level was 9.5 g/l before and 8.5 g/l after NTZ therapy; in patients with persisting OCB, the corresponding values were 11.2 g/l and 10.0 g/l, respectively. OCB loss patients had a mean IgG index of 1.6 before and 0.5 after NTZ; patients with persisting OCB had mean IgG indices of 1.0 before and 0.6 during treatment. NTZ treatment duration was longer in patients with OCB loss (mean 7.0 years) than in patients with persistingly positive OCB (mean 4.4 years), on formal testing, this was not significant (Mann-Whitney U test).

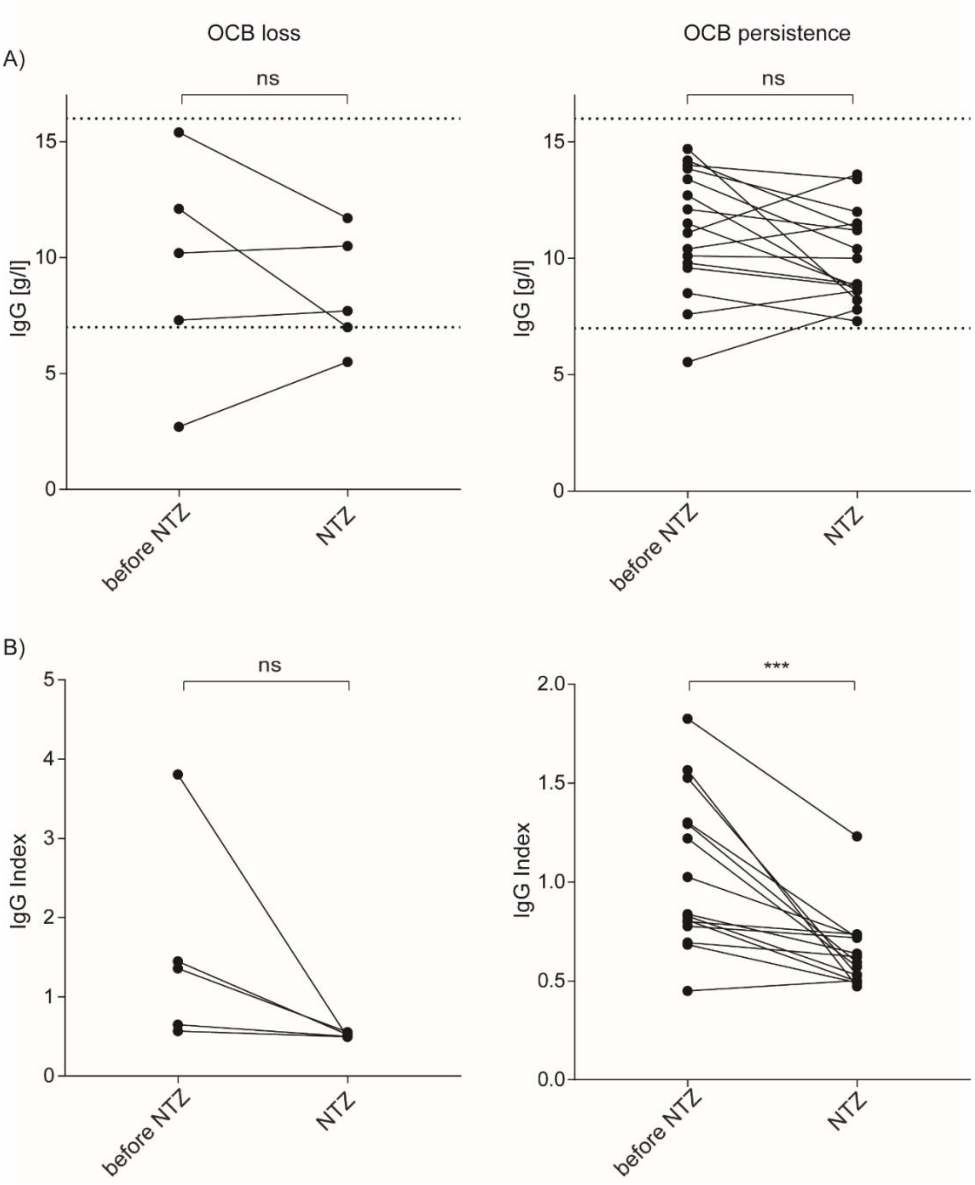

Figure 17. OCB loss is not due to pronounced IgG reduction during NTZ. Dotted lines represent reference values for serum IgG (7-16 g/l). A) Longitudinal comparison of serum IgG in patients with loss of OCB during NTZ therapy (n=5, left) and patients with persisting positive OCB (n=22) during NTZ treatment. No significant changes in serum IgG levels before and after NTZ in either group. Wilcoxon matched-pairs signed rank test .B) IgG index of patients with (left, n=5) and without (right, n=22) OCB loss. No significant difference of IgG indices before and during NTZ treatment in patients with loss of OCB but significant IgG index reduction in patients with persisting positive OCB (p=0.0001). Wilcoxon matched-pairs signed rank test.

7.7. α4Integrin expression on B cell subsets

To evaluate the intensity of α4 integrin expression on different B cell subsets, PBMC of 8 healthy donors (mean age 30.5 years, male: female 5:3) were stained with anti- α4 integrin and markers for the differentiation of B cell subtypes followed by flow cytometric analysis. This revealed different levels of α4 integrin expression on different B cell subtypes. We found the highest expression level of α4 integrin on plasmablasts ($CD19^+$ $CD38^+$ $CD27^+$), followed by memory B cells ($CD19^+$ $CD38^-$ $CD27^+$) and naïve B cells ($CD19^+$ $CD38^-$ $CD27^-$;Figure 18). Integrin α4 expression levels of memory cells and plasmablasts were significantly higher than of naïve B cells (p=0.0373 and p=0.0014, respectively). Friedman test with Dunn's multiple comparisons test.

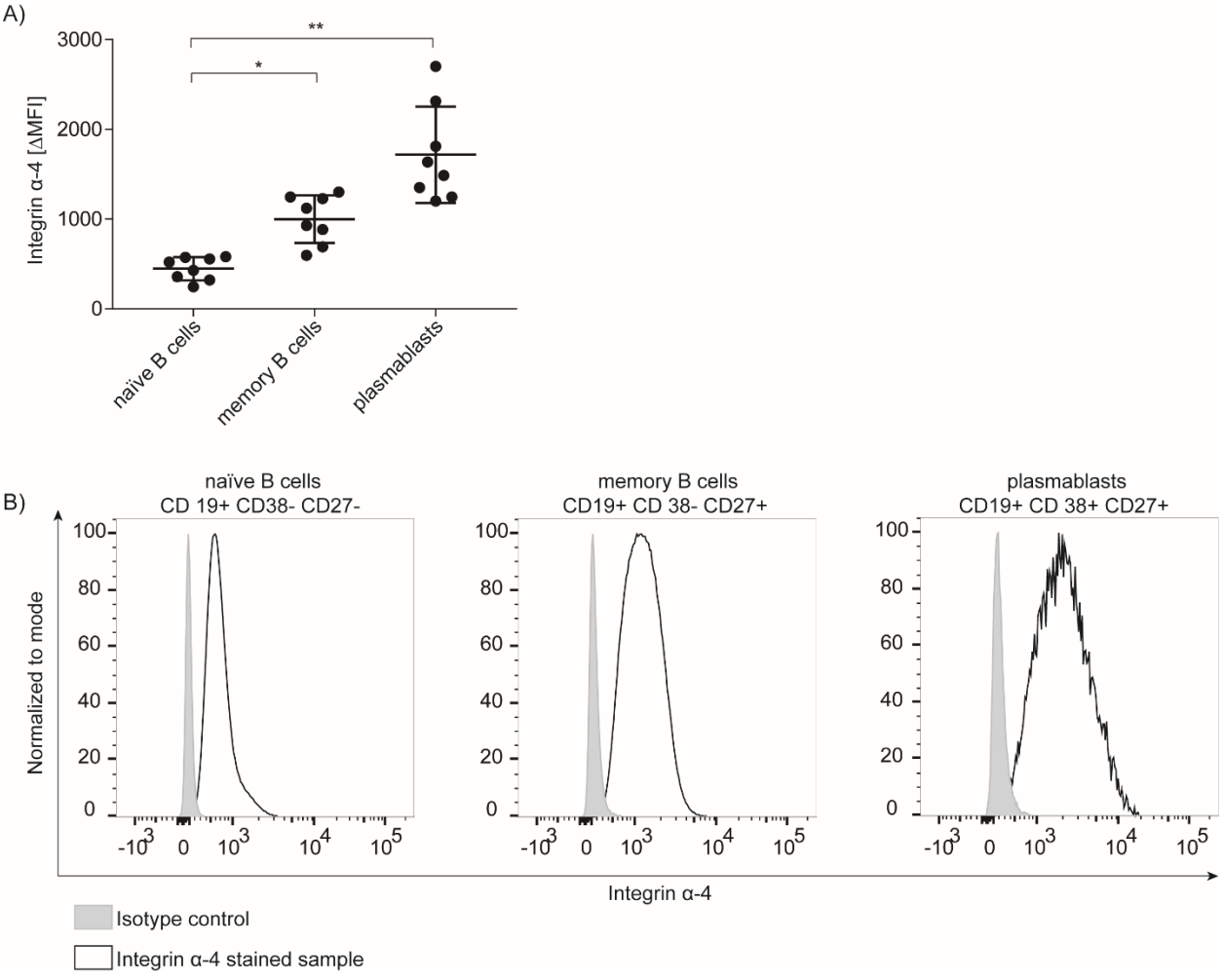

Figure 18. Integrin α-4 expression on B cell subsets. PBMC of healthy donors (n=8) were stained for Integrin α-4 (CD49d) and markers for differentiation of lymphocyte subsets. (A) Each dot represents ΔMFI (MFI[stained sample]-MFI[FMO control]) of one donor. Integrin α4 expression levels of memory

cells and plasmablasts were significantly higher than of naïve B cells (p=0.0373 and p=0.0014, respectively). Friedman test with Dunn's multiple comparisons test. (B) Representative Integrin α-4-staining of one donor. Light grey curves represent Isotype controls, black curves represent Integrin α-4 staining on naïve B cells ($CD3^-$ $CD19^+$ $CD38^-$ $CD27^-$), memory B cells ($CD3^-$ $CD19^+$ $CD38^-$ $CD27^+$) and plasmablasts ($CD3^-$ $CD19^+$ $CD38^+$ $CD27^+$).

8. DISCUSSION

8.1. Discussion of project one: Immunological characterization of patients with genetic variants in genes related to autoinflammatory diseases

Apart from the often delayed diagnosis of autoinflammatory syndromes due to their sheer rareness, patients with atypical symptoms, adult onset of disease, or only partially fulfilled diagnostic criteria pose a challenge to practitioners. There is a great symptomatic overlap chiefly with rheumatic diseases and psychosomatic disorders. Especially in patients with low-penetrance mutations or patients with heterozygous genetic variants in genes associated with diseases classically thought to have a recessive mode of inheritance like FMF, delayed diagnosis and over- or undertreatment is a potential danger to patients. Feasible diagnostic tests for these patients have not yet been established in the clinical routine.

In search of distinguishing features of patients with genetic variants in autoinflammation-related genes, we performed a) immunophenotyping of PBMC of patients with genetic variants in the genes *NLRP3*, *TNFRSF1A*, or *MEFV* and HC by FACS, and b) functional assays involving stimulation of the pyrin inflammasome in patients with variants in the *MEFV* gene and HC.

8.1.1. Immunophenotyping

A basic differentiation of PBMC subsets showed a heightened percentage of monocytes in patients with *NLRP3* low-penetrance variants compared to HC. Monocytes are the classical inflammasome-competent cells and represent the main producers of IL-1β among human PBMC (Netea, Nold-Petry et al. 2009). Constant inflammasome activation, the resulting chronic inflammation and high turnover rates among monocytes in patients with *NLRP3* variants could lead to an increase in circulating monocytes. The results of our analysis point in this direction,

especially since patients with minimal autoinflammation-associated symptoms (oral aphtae combined with cephalgia and or myalgia) with a potential “diluting” effect were included in the *NLRP3* variant group. An additional analysis in a larger cohort of patients with *NLRP3* low-penetrance variants and an additional analysis of patients with *NLRP3* high-penetrance mutations is needed to assess the clinical relevance of this finding.

In patients with low penetrance variants in the *TNFRSF1A* gene, we saw a slightly increased proportion of T cells among lymphocytes compared to HC. An expansion of T cells has been described in patients with high penetrance *TNFRSF1A* mutations, but not in carriers of low penetrance mutations (Pucino, Lucherini et al. 2016). The impact of mutations in the *TNFRSF1A* gene on TNFα signaling are not entirely clear, but evidence points towards a decrease in signaling via mutant TNFRSF1A receptors (Todd, Radford et al. 2004). Moreover, the ratio of TNF-receptor 1 to TNF-receptor 2 in serum has recently been shown to be lowered in TRAPS patients in inactive phases (Yasumura, Shimizu et al. 2020). Classical TNFRSF1A signaling leads to apoptosis, while TNF receptor 2 provides an activating and survival signal for T cells. One could speculate that a reduction of functional TNFRSF1A molecules could lead to a) reduced apoptosis in T cells and b) to alternate TNF signaling via e.g. TNF receptor 2, contributing to the expansion of T cells, potentially leading to an expansion of T cells.

We went on to study the proportions of different monocyte subsets, distinguished by their expression level of CD 16, and found a significant decrease in the proportion of CD 16^{high} monocytes in patients with *NLRP3* variants.

Mukherjee et al. could show that so-called “non-classical” monocytes - by their gating strategy, monocytes dimly expressing CD14 and highly expressing CD16 - were the strongest producers of IL-1β after stimulation with LPS among the basic monocyte subsets. Moreover, they suggested a strong antigen-presenting capability of “non-classical” monocytes. Overall, they attribute a distinctly pro-inflammatory role to this subtype of monocytes (Mukherjee, Kanti Barman et al. 2015). Even though our gating strategy differed in that we did not take different expression levels of CD14 into consideration, the lower percentage of CD16high monocytes in patients with NLRP3

low penetrance variants may be a counter-regulatory process as an attempt to prevent further inflammation caused by inflammasome over-activation.

In the analysis of T cell cytokine secretion data, patients with *NLRP3* variants did not show an increase in proinflammatory cytokines produced by $CD4^+$ or $CD8^+$ cells, even though increased secretion of TNFα and IFNγ have been described in patients with high penetrance *NLRP3* mutations (Haverkamp, van de Vosse et al. 2014). This might also be explained by a counter-regulatory process to prevent harmful inflammation. An interesting counter-regulatory pathway one could speculate on in *NLRP3* variant patients is the expansion of myeloid-derived suppressor cells (MDSC) observed in patients with CAPS (Ballbach, Hall et al. 2016). These cells' ability to inhibit T cell proliferation, Th1 and Th17 responses might be partially responsible for the normal levels of cytokine secretion in *NLRP3* variant patients' cytokine secretion by CD4+ cells. Important limitations to be considered in the interpretation of our FACS analysis of patients with NLRP3- and TNFRSF1A variants are first, the low patient numbers (NLRP3: n=14, TNFRSF1A: n=7) and second, the inclusion of asymptomatic carriers as wells patients with clinical symptoms, but lack of elevated serum inflammation markers in our FACS cohort. These patients with possibly no or very low inflammatory activity may well have a diluting effect on the results. Moreover, in these two groups, we did not have access to samples from patients with clearly pathogenic mutations as an additional reference group.

This situation is somewhat better in the *MEFV* variant group, as five out of nine patients carried at least one high-penetrance mutation. In *MEFV* variant patients, we observed an increased secretion of the pro-inflammatory cytokines TNFα and IL-17A, as well as a trend towards increased secretion of INFγ. Especially the increase in TNFα secretion may be of clinical relevance since this cytokine leads to high level expression of MEFV in cells (Papin, Cazeneuve et al. 2003). Interestingly, some FMF patients respond to anti-TNF treatment; MEFV upregulation by TNF α may be part of the mechanistic explanation (Ozgocmen and Akgul 2011, Erten, Erten et al. 2012). TNFα produced by CD4+ T cells has been reported to induce IL-1β secretion independently of inflammasome activation in mononuclear phagocytes, leading to systemic inflammation and T-cell driven autoimmunity (Jain, Irizarry-Caro et al. 2020). The increased level of TNFα secretion by CD4+ T cells in patients with MEFV variants could be part of the reason for the increased rate

of MS among FMF patients (Yahalom, Kivity et al. 2011b). Interestingly, high- and low penetrance *MEFV* mutations could not be separated by either cytokine secretion or proportion of cell subsets.

8.1.2. Functional analysis of IL-1β secretion after inflammasome stimulation

A feasible functional assay that can help determine the clinical relevance of genetic variants in autoinflammation-related genes would be of great use. Such an assay needs to be performable on easily accessible biomaterial, doable in a reasonable amount of time and, ideally, should use technical equipment and know-how that is already available in most clinical laboratories. Hence, an inflammasome stimulation assay with an ELISA readout on either whole blood or easily obtained blood cells like PBMC would be a good option.

In this work, we focused on patients with genetic variants in the *MEFV* gene and their response to stimulation of the pyrin inflammasome. Different approaches and materials were assessed. For PBMC, we found that frozen PBMC do not seem to be ideal for the determination of IL-1β levels after stimulation of the Pyrin inflammsome. As described previously, caspase-1 is pre-activated in PBMC, so that a priming stimulus like LPS is sufficient to induce a considerable amount of IL-1β secretion. (van de Veerdonk, Joosten et al. 2009). Moreover, when using frozen PBMC, the cell stress and -damage caused by the freezing- and thawing process might lead to endogenous DAMPs that may well suffice to activate the inflammasome in these cells. This may contribute to the large scatter of IL-1β values and the comparatively high levels of IL-1β secretion upon Pyrin inflammasome stimulation in healthy donors. This effect is less pronounced in fresh PBMC, but still, there is obvious IL-1β secretion upon LPS priming alone which reaches a level comparable to that under additional Pyrin inflammasome stimulation in healthy donors. Still, the analysis of fresh PBMC allowed discrimination of the tested patient with a heterozygous high penetrance *MEFV* mutation. The fact that the mutation was only heterozygous makes it more likely that this assay might also detect relatively low secretors of IL-1β, since heterozygous patients have been shown to secrete less IL-1β than homozygous ones in response to stimulation (Omenetti, Carta et al. 2014, Jamilloux, Lefeuvre et al. 2017) It may therefore be worthwhile to test whether patients with low penetrance mutations can also be detected by this assay. This

result will have to be confirmed in a larger patient cohort including patients with high- and low penetrance, heterozygous and homozygous mutations.

To resolve the issue of PBMC pre-activation, we applied the same inflammasome stimulation protocol to diluted whole blood and observed no increased IL-1β secretion with LPS only. We found a good discrimination between HC and four patients from a family carrying the homozygous (n=3) or heterozygous (n=1) low penetrance variant E148Q in the *MEFV* gene. The use of whole blood without any further preparation steps a) better represents the situation in patients' bodies because no components are willfully altered or removed and b) saves time in the clinical routine because no further preparation steps are necessary. This makes a whole blood assay a good option for future clinical use. Van Gorp and colleagues recently proposed a diagnostic assay for FMF in whole blood or PBMC based on their finding that colchicine and other microtubule-disrupting drugs do not suppress Pyrin inflammasome activation in FMF patients. This assay worked well for patients carrying at least one high penetrance variant, but could not separate patients with low penetrance mutations from healthy donors (Van Gorp, Saavedra et al. 2016, Van Gorp, Huang et al. 2020). Hence, further work on our approach might potentially add this additional use since we were able to discern patients carrying the low penetrance variant E148Q from healthy controls. Another recently published diagnostic test is based on the observation that Pyrin dephosphorylation alone without a second step involving microtubule dynamics activates the pyrin inflammasome in FMF patients, but not in HC (Magnotti, Lefeuvre et al. 2019). Therefore, an assay utilizing pyrin dephosphorylation due to the use of a kinase inhibitor could detect patients with high penetrance mutations, but it also missed a patient with a homozygous low penetrance mutation (Magnotti, Malsot et al. 2020).

8.1.3. Cell death analysis after inflammasome stimulation

While Magnotti et al. could recently demonstrate that cell death kinetics of freshly isolated monocytes after inflammasome stimulation by a BTK inhibitor perform well as a tool do detect FMF patients (Magnotti, Malsot et al. 2020), we saw no differences between HC and FMF patients in our LDH cell death assay. This may also be due to our use of frozen PBMC and their supposed pre-activated state and endogenous DAMPs. Another reason for the equal cell death rates

between HC and patients and also for the high IL-1β secretion rate in PBMC-with LPS priming alone is the potential of human monocytes to engage an alternative inflammasome pathway, leading to IL-1β secretion, but not to pyroptosis, without a second inflammasome signal (Gaidt, Ebert et al. 2016). For our experiments, this means a) high "background" IL-1β secretion because of LPS priming and b) an inability to specifically stimulate the pyrin inflammasome in these stimulate monocytes with already activated alternative inflammasome pathways, meaning a lack of differences in cell death rates between patients and HC. This might play a smaller role in the more realistic setting of whole blood, where other inflammasome-competent cells, for example, granulocytes, could still be stimulated. Overall, the use of whole blood without any further preparation steps a) better represents the situation in patients' bodies because no components are willfully altered or removed and b) saves time in the clinical routine because no further preparation steps are necessary.

8.1.4. Limitations

In the first part of this project, the analysis of cell subsets and T cell cytokine secretion by FACS, our main limitations were the lack of patients with clearly pathogenic mutations in the *NLRP3*- and *TNFRSF1A* groups and the overall small sizes of the groups. An important caveat for our analysis of this FACS data is that data were only corrected for multiple testing in the pairwise comparisons to HC but not for other parameters that had the potential to increase the risk of an α error like subgroup analysis or testing of multiple outcomes. The reason for this choice was that correction for all factors would likely have decreased testing power to a level on which statistical significance becomes next to impossible even for strong effects with this small sample size (Streiner 2015). Therefore, these data should be interpreted with caution and statistical significance should, in this case, be seen as a mere hint at a possible finding. Moreover, some patients especially in the NLRP3- and TNFRSF1A group presented with only very subtle autoinflammation-related symptoms. It is not clear how many of the patients in the group actually represent clinically healthy mutation carriers.

In the second part concerning the analysis of IL-1β secretion and cell death rates of patients with *MEFV* variants, it has to be considered that we found frozen PBMC to be no suitable material for

the functional analysis of the response to inflammasome stimulation; yet, we only had the chance to perform an analogous experiment on fresh PBMC of one single patient with a MEFV variant due to the rather time-intensive setup. It is unclear whether the result is representative until more patients have been tested. Moreover, in a system as sensitive to disruption of homeostasis as the inflammasome, PBMC may be too artificial a material to use. In the more "natural" whole blood assay, we did not adjust dilution of the blood samples to donors' cell numbers, which may have led to inequal stimulation and should be avoided in the future. Due to lack of appropriate patient material, we were not yet able to assess IL-1β secretion in whole blood of patients with high-penetrance variants.

8.1.5. Outlook

To take this research further, the FACS immunophentotyping- and T cell cytokine analyses could be repeated with the inclusion of larger patient numbers, more defined patient phenotypes and additional patients with clearly pathogenic mutations in the *NLRP3*- and *TNFRSF1A* genes, potentially in collaboration with other practitioners.

As a priority, however, the whole blood assay should be optimized since this is the most promising approach to a better and faster diagnosis of patients with variants in autoinflammation-related genes. Other readouts such as IL-18-, IL-1α- or protein S100 measurements could be added to potentially gain even more insights into the response of patients with *MEFV* variants to pyrin inflammasome stimulation. In the future an optimized version of this assay could be used in the clinical routine.

In addition, more patients' fresh PBMC should be analyzed to potentially confirm the discriminatory power of an inflammasome stimulation assay in fresh PBMC for MEFV variant carriers.

Moreover, some data points to a potential role of other cell types than the monocyte line, especially granulocytes (Gohar, Orak et al. 2016), in the development of autoinflammation in MEFV variant carriers. Since granulocytes are removed in the PBMC isolation process and are

hence often neglected in inflammasome research, the role of these cells should be studied in more detail.

8.2. Discussion of project two: Differential effects of natalizumab on features of peripheral and intrathecal immunoglobulin production

Intrathecal Ig production can be a prognostic marker for MS in an isotype-dependent manner. Intrathecal production of IgG is associated with clinical worsening (Gasperi, Salmen et al. 2019), while intrathecal IgM production is a risk factor for conversion from CIS to MS (Pfuhl, Grittner et al. 2019). In this analysis, intrathecal production of Ig was calculated using a linear index method, which is a commonly used tool to determine intrathecal Ig production (Warnke, Stettner et al. 2014, Largey, Jelcic et al. 2019). An important consideration is that the Ig index can be influenced by blood-brain-barrier dysfunction, measured by a high albumin ratio. Since albumin ratios were very similar in untreated patients (mean 5.8), patients before (mean 5.8) and during NTZ treatment (mean 4.9) and only few patients had a blood-brain-barrier dysfunction, this linear formula for intrathecal Ig production should yield valid results, especially since we did not interpret absolute levels of Ig production but rather the values in relation to each other.

8.2.1. Intrathecal Ig production, OCB and MRZ reaction

Intrathecal Ig are produced by short-lived plasmablasts (Cepok, Rosche et al. 2005) and long-lived plasma cells (Pollok, Mothes et al. 2017). Ig producing cells are located in the brain parenchyma and in perivascular and meningeal areas (Magliozzi, Howell et al. 2007, Pollok, Mothes et al. 2017). While we observed a reduction in absolute levels of CSF IgG and IgM in the longitudinal comparison, only the intrathecal production of IgG was significantly reduced in NTZ patients in the longitudinal study. This was, in most part, reflected in the cross-sectional study, however, we also saw lower absolute levels of IgA and lower intrathecal IgA production in the cross-sectional study. In contrast to this, intrathecal OCB and the MRZ reaction, prominent IgG phenomena in MS, persisted in most patients. We had the chance to reanalyze one patient's CSF samples before and during NTZ treatment for OCB in parallel on the same gel. This revealed persistence of a

number of bands despite the observed reduction in intrathecal IgG production during NTZ treatment. This persistence of OCB is in accordance with longitudinal assessments of OCB in MS patients, in which individual OCB remained unchanged for many years (Walsh and Tourtellotte 1986). This may be due to the potential of long-lived plasma cells to migrate to and persist in survival niches in the brain (Meinl, Krumbholz et al. 2006). Persistence of OCB after shorter durations of NTZ therapy has been described (Warnke, Stettner et al. 2014, Largey, Jelcic et al. 2019). Here, we could show that OCB persist even after long-term treatment with NTZ. We hypothesize that NTZ does not substantially affect long-lived plasma cells as producers of OCB in their survival niche. Loss or weakening of some bands could either be a partial NTZ effect on these cells or a natural event in the disease course since changes in banding patterns have been described in MS patients (Thompson, Kaufmann et al. 1983, Axelsson, Mattsson et al. 2013).

This is supported by the comparable MRZ reaction between NTZ treated and untreated patients. MRZ reaction typically persists at least in untreated MS patients (Meinl, Krumbholz et al. 2006). This, again, could be due to plasma cells finding a permanent survival niche which does not seem to be affected by NTZ. Largey et al. recently reported persistence of MRZ reaction after one year of NTZ, but mixed results for other antiviral antibodies (Largey, Jelcic et al. 2019). One may speculate that this is due to a differential formation of plasma cells in reaction to different viruses.

Since both OCB and MRZ reaction largely persist during NTZ therapy, it is unlikely that α4 integrin plays an important role in the formation or maintenance of survival niches. As a follow-up study, a potential role of laminin β1, an important adhesion molecule for IgG secreting cells in the bone marrow (Männe, Takaya et al. 2019), could be evaluated.

A smaller proportion of previously OCB positive patients (19%) did lose their OCB during treatment, a similar number to that described by Harrer et al.(Harrer, Tumani et al. 2013). This was not associated with a more pronounced reduction of intrathecally produced IgG. Treatment duration was longer in the OCB loss group; it could be argued that after very long treatment with NTZ, the cells supporting plasma cells in their niches cannot be replenished sufficiently anymore in some patients. Of note, one of the five patients with OCB loss developed PML.

The amount of intrathecally produced IgG has been found to correlate with the number of plasmablasts in the CSF; these cells are thought to be the main source of IgG produced in the CNS (Cepok, Rosche et al. 2005). Since the interaction of VLA-4 and VCAM1 enhances B cell activation (Carrasco and Batista 2006), its inhibition by NTZ may be a reason for the decrease in intrathecally produced IgG during NTZ treatment. Moreover, since plasmablasts in general are circulating cells (Odendahl, Mei et al. 2005) and B cells can migrate between the CNS and periphery (von Budingen, Kuo et al. 2012), NTZ might reduce homing of plasmablasts to the CNS, supported by our finding that plasmablasts express especially high levels of α4 intergrin. B cell migration to the CNS depends on VLA-4/ VCAM1 interaction (Alter, Duddy et al. 2003). Studies on an animal model of MS (experimental autoimmune encephalomyelitis, EAE) showed the importance of this process (Lehmann-Horn, Sagan et al. 2015, Lehmann-Horn, Sagan et al. 2016). Due to their very low numbers in peripheral blood, we could not analyze α4 integrin levels on plasma cells. This should be added in a follow-up study.

8.2.2. Serum Ig levels

In serum, NTZ treatment predominantly led to a reduction in IgM levels while having little or no effect on IgG- and IgA levels. Once more, this isotype-specific effect can be explained by effects of NTZ mainly on plasmablasts, but less on plasma cells. Serum IgG is mainly produced by long-lived plasma cells, while a large proportion of serum IgM stems from plasmablasts (Manz, Hauser et al. 2005), even though IgM plasma cells have also been described (Bohannon, Powers et al. 2016).

The reduction in serum IgM during NTZ treatment led to an increased proportion of patients with IgM hypogammaglobulinaemia. It has yet to be evaluated whether these patients will have a higher rate of infections during their subsequent B cell depleting treatment, and whether they more often develop persisting hypogammaglobulinaemia during B cell depletion. While this finding has to be confirmed in larger patient cohorts, measuring serum Ig and especially IgM in patients with infections during NTZ treatment and before switching to another therapy might increase patients' safety.

In summary, our findings in CSF and serum point to an effect of NTZ on short-lived plasmablasts, but not on plasma cells.

8.2.3. Limitations

An important limitation of this work is that there was a relatively long time span between the time of first LP and the beginning of NTZ therapy; moreover, many patients had other DMT before starting NTZ. Therefore, effects of other DMT or the natural disease course cannot be excluded. Moreover, eight out of 27 patients in the NTZ group had their first LP in external clinics, potentially limiting comparability of the assessed values.

In addition, we only had the chance to analyze OCB before and during NTZ in parallel in one single patient. Hence, we do not know if our finding of persistence of most bands holds true for other NTZ treated patients.

8.2.4. Outlook

In the future, more information should be gained on the composition of survival niches for plasma cells in the CNS. In a first step, a potential role of the adhesion molecule laminin β1 should be assessed.

Clinically, our finding of IgM hypogammaglobuliaemia during NTZ treatment might be of relevance. Patients often switch to B cell depleting therapies after NTZ. An important side effect of these therapies is hypogammaglobulinaemia and subsequent severe infections. We plan to assess whether patients with IgM hypogammaglobulinaemia during NTZ a) had more infectious complications during NTZ and b) whether they have a higher risk for persisting hypogammaglobulinaemia and infection during B cell depleting treatment. If so, a recommendation should be made to test for IgM hypogammaglobulinaemia both during NTZ treatment and before a change of therapy.

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