Evaluating the *in vitro* effects of selective and potent non-selective fibroblast growth factor receptor inhibition in human BL2 cells as potential therapeutic targets for multiple sclerosis

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1 Introduction

1.1 Multiple Sclerosis (MS)

"a woman lost the sight of both eyes and on the next day she lost her speech . . . on the third day a candlewick was put around her head and she then recovered the sight of one eye and was able to open both . . . on [Sunday] she recovered her speech and on the feast of St Michael . . . the sight of the eye that has previously been blind" (Compston, 2005).

An early case description like this one in the Icelandic saga of St.Thorlakr, as documented by Margaret Cormack and supported by Poser in 1995, was of Saint Lidwina van Schiedman (born 1380, died 1433) (Compston, 2005). This historical narrative outlines a pattern of symptoms and partial recoveries strikingly consistent with modern descriptions of multiple sclerosis (MS).
In 1849, Friedrich Theodor von Frerichs first described its characteristic symptoms, while Robert Carswell and Jean Cruveilhier identified pathological features as “remarkable lesions of the spinal cord with atrophy” (Carswell, 1838). In 1868, Charcot consolidated clinical and pathological knowledge, establishing MS as a distinct disease through his lectures on “Histologie de la Sclérose en plaques” (Murray, 2018). This significant work shifted MS research from

an exploratory approach of single case reports to high-level clinical research, resulting in several decades of intensive research into the causes of the disease, MS pathogenesis, and possible therapeutic approaches.

MS is an inflammatory disorder of the brain and spinal cord characterized by episodes of neurological dysfunction leading to chronic neurodegeneration and often times to progressive disability (Compston and Coles, 2008; Dobson and Giovannoni, 2019). Today, MS is the most common chronic inflammatory and demyelinating disease of the central nervous system (CNS). In 2023, around 2.8 million people worldwide were affected, and the global prevalence increases (MSIF, 2020). The regional distribution varies across the world. Europe, North America, Australia, and New Zealand are the populations with the highest prevalence. Factors that might contribute to the global and regional distribution are population genetics, environment, and socioeconomic structures (Koch-Henriksen and Sørensen, 2010). What is striking from an epidemiological standpoint, MS mainly affects females (69%), and the age at onset is around 32 years. The gender ratio varies among regions from 2-4:1 (F:M). Possible reasons could be different female hormonal, genetic, lifestyle, and environmental exposures compared to males. However, not only adults are affected; around 30,000 children living with pediatric MS (under the age of 18) have been reported worldwide (MSIF, 2020).

1.1.1 Etiology of MS

Scientists consider that the multifactorial influence of genetic predisposition and environmental aspects contribute to the development of the disease. Various genetic, microbial, and environmental risk factors are identified as potential contributing factors, but no definite cause has been detected (Waubant et al., 2019).

Genome screening showed that many genes increase the risk of MS. Human leukocyte antigen (HLA) II or major histocompatibility complex (MHC) II genes were identified to be strongly linked to the disease. Further, family studies supported the genetic association and showed that identical twins have a higher risk (25%) than close relatives (3-5%). Nevertheless, the low incidence of both homozygous twins developing MS indicates that other factors, beyond genetics, contribute to the development of this disease (Koch-Henriksen and Sørensen, 2010; Parnell and Booth, 2017).

Furthermore, Epstein-Barr virus (EBV) infection has been identified as a significant risk factor (Ascherio and Munger, 2010; Høglund, 2014). Emerging evidence indicates a causal relationship in which EBV infection occurs before the onset of MS, significantly increasing the probability of developing the disease (Bjornevik et al., 2022). The virus is associated with immune dysregulation and the development of autoreactivity (Dobson and Giovannoni, 2019; Lanz et al., 2022). In particular, Lanz et al. (2022) highlighted how EBV can contribute to MS

through molecular mimicry between EBV antigens and CNS proteins. This molecular similarity could trigger the activation of autoreactive immune cells. B cells are especially central to this process, as EBV-infected B cells evade apoptosis and persist as carriers of chronic infection. These infected B cells accumulate in target organs, where they act as antigen-presenting cells (APCs) and provide survival signals to autoreactive T cells, leading to continued immune activation, inflammation, and tissue damage, ultimately contributing to the initiation and progression of MS (Lucas et al., 2011; Pender, 2003).

In addition, socioeconomic status, employment, smoking, sex hormones, late delivery, birth control, and obesity were associated as possible risk factors (Koch-Henriksen and Sørensen, 2010). In addition, Vitamin D deficiency in the northern hemisphere is discussed as a contributing environmental factor (Ascherio and Munger, 2010; Høglund, 2014; Thouvenot et al., 2025).

There is no simple contributive association between gene and environmental interactions, and although some risks have been identified, research gaps exist.

1.1.2 MS pathophysiology

MS is an autoimmune disorder characterized by inflammation, demyelination, and axonal degeneration within the CNS. The exact pathophysiology remains unclear, but the idea that peripheral immune

dysregulation and intrinsic CNS mechanisms contribute to MS development and progression is widely accepted (Filippi et al., 2018).

The experimental autoimmune encephalomyelitis (EAE) disease model has been instrumental in identifying numerous pathophysiological mechanisms relevant to MS. Findings from EAE helped shape our understanding of how activated autoreactive T and B lymphocytes contribute to neuroinflammation. The development of these aggressive effector cells is proposed to be influenced by molecular mimicry, the presentation of new autoantigens, genetic and environmental factors. Once activated in the periphery, these cells migrate through the blood-brain barrier (BBB), trigger inflammation and cause tissue damage within the CNS. Upon entering the CNS, they provoke an inflammatory cascade by releasing cytokines, recruiting other inflammatory cells such as monocytes, T cells and B cells, while activating microglia and astrocytes. This cascade contributes to demyelination through the loss of oligodendrocytes (OLs), driven by direct cellular mechanisms and the release of inflammatory and neurotoxic mediators. Axonal injury can occur early in the disease process as a direct consequence of inflammation or, due to the failure of neuroprotective and regenerative mechanisms. Over time, persistent inflammation leads to inadequate remyelination, gliosis, neuroaxonal degeneration, and disrupted neuronal signaling, all of which correlate with clinical symptoms and disability progression in MS patients (Dargahi et al., 2017; Filippi et al., 2018; Yamout and

Alroughani, 2018).

In addition to peripheral immune mechanisms, intrinsic CNS processes play a role in MS pathogenesis. Compartmentalized inflammation, characterized by the continuous activation of immune cells within the CNS, has been considered as a key contributing factor, particularly in the progressive stages of MS. This compartmentalized inflammation involves the formation of ectopic lymphoid-like structures, especially in the meninges. These structures contain B cells, T cells, and follicular dendritic cells and maintain chronic immune activation, contributing to cortical demyelination, axonal loss, neurodegeneration (Dendrou et al., 2015).

Furthermore, antigens originating from the CNS can be transported to peripheral lymphoid tissues, where they may stimulate the activation of autoreactive immune cells. Once activated, these immune cells have the potential to migrate into the CNS through a damaged BBB, thereby reinforcing a cycle of persistent inflammation and progressive neurodegeneration as described above. The disruption of the BBB in MS is mediated by pro-inflammatory cytokines such as TNFα, IL6, and IL17, which increase vascular permeability and promote the expression of adhesion molecules on endothelial cells, facilitating immune cell infiltration. This bidirectional interaction between peripheral immune activation and CNS infiltration plays an important role in maintaining chronic inflammation (Dendrou et al., 2015; Filippi et al., 2018; Lassmann, 2018).

Demyelinated lesions, a hallmark of MS, are found in the white and gray matter of the brain and spinal cord. These lesions can be classified as acute, chronic active, or inactive. Chronic lesions show significant loss of myelin, fibrous gliosis, and minimal immune cell infiltration. In comparison, active lesions show acute inflammation, active demyelination, and axonal injury, with less extensive myelin destruction. Although inflammation appears at all stages of lesion development, early acute lesions are dominated by macrophages with myelin debris and T, B, and plasma cell infiltrates. During this acute phase, peripheral immune cells invade the CNS through a damaged BBB, initiating inflammatory responses. Additionally, early MS lesions often contain oligodendrocyte progenitor cells (OPCs), which attempt to initiate remyelination through the formation of new myelin sheaths (Dendrou et al., 2015; Popescu et al., 2013). However, as the disease progresses, remyelination efforts frequently fail, and the exhaustion of regenerative capacity is accompanied by ongoing neuronal and axonal loss, myelin degradation, and brain atrophy. These pathological changes strongly correlate with clinical symptoms and disabilities observed in MS patients (Dendrou et al., 2015). Therefore, therapeutic approaches focus on reducing neuroinflammation, protecting neurons, and enhancing repair processes to slow down disease progression and maintain neurological function.

1.1.3 MS symptoms

The symptoms, as presented in Figure 1, that may occur in MS are heterogeneous, and the clinical course of the disease is individually different and unpredictable. Optic neuritis, paraesthesia, and non-specific symptoms like fatigue are usually the first manifestation of MS (Hacke, 2010; Tafti et al., 2024). MS can cause optic neuritis, color blindness up to temporary blindness, and internuclear ophthalmoplegia (INO) (L. Chen and Gordon, 2005). In addition, patients experience cranial nerve palsy, neuropathic pain, sensory and motor impairments, central and central-vestibular symptoms, autonomic dysfunction, fatigue, and cognitive and psychological changes (Hacke, 2010; Mattle et al., 2013).

The diverse clinical symptoms in MS result from the distribution of lesions throughout the CNS, affecting regions like the brainstem, spinal cord, cerebellum, juxtacortical areas, and periventricular white matter. Central to these diverse symptoms are shared pathological mechanisms, such as neuroinflammation, demyelination, and ongoing neurodegeneration as described in Section 1.1.2 (Filippi et al., 2018; Mahajan et al., 2025).

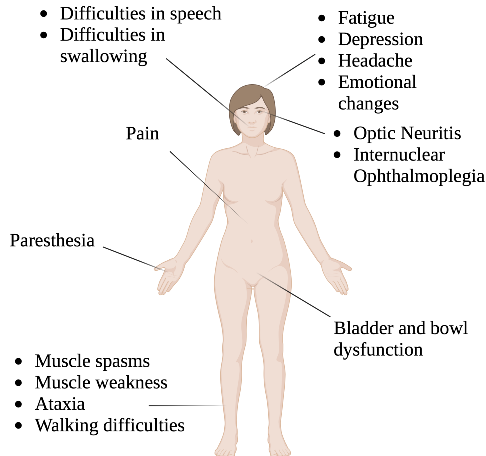

Figure 1: Simplified overview of symptoms associated with multiple sclerosis (MS). Illustrating the diverse most common neurological, physical, and psychological impairments experienced by patients with multiple sclerosis (Tafti et al., 2024). Image created with BioRender (biorender.com).

1.1.4 Phenotypes of MS

As discussed in Section 1.1.2, MS is driven by inflammatory and neurodegenerative mechanisms, leading to a heterogeneous clinical presentation. Inflammatory processes drive immune cell infiltration, lesion formation, neurodegeneration, and relapse activity. Some individuals experience successful remyelination, while others experience persistent myelin and axonal loss, resulting in progressive disability. The interplay between these pathological processes shape the disease phenotype (Dendrou et al., 2015; Filippi et al., 2018).

To better define these patterns, MS has been classified into differ-

ent subtypes, relapsing-remitting multiple sclerosis (RRMS), primary progressive multiple sclerosis (PPMS), and secondary progressive multiple sclerosis (SPMS) (Fig. 2). It is important to note, however, that clinically isolated syndrome (CIS), defined as a single episode of neurological symptoms caused by inflammation or demyelination in the CNS, is not classified as MS according to established guidelines. Nonetheless, CIS can represent an early manifestation and has the potential to progress to MS (Lublin et al., 2014).

The most common clinical course is RRMS, accounting for approximately 90% of all MS cases. It is characterized by exacerbations followed by periods of complete or partial remission of symptoms, as seen in the life of Saint Lidwina van Schiedman (cf. Section 1.1). A slow and steady increase in disability can appear as the disease progresses (Plantone et al., 2016; Tafti et al., 2024). A significant number of individuals with RRMS eventually transition to SPMS, characterized by a pattern of continuous progression of symptoms, with or without relapses (Plantone et al., 2016). About 10% of all MS patients have PPMS, which manifests a continuous worsening of symptoms from the onset of the disease, occasionally with periods of temporary stability or mild clinical improvement (Lublin and Reingold, 1996; Lublin et al., 2014).

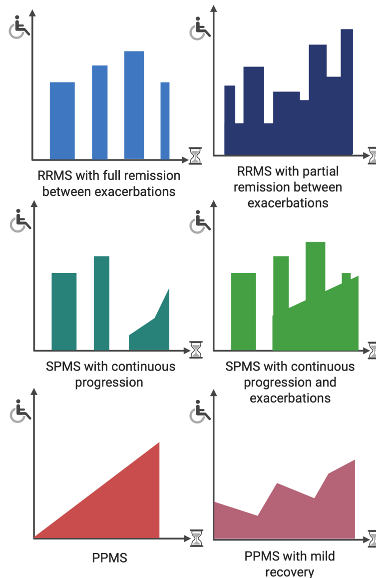

Figure 2: Systematic visualization of the clinical phenotypes of multiple sclerosis (MS). The figure illustrates the key characteristics and progression patterns of relapsing-remitting MS (RRMS), secondary-progressive MS (SPMS), and primary-progressive MS (PPMS) (Lublin et al., 2014). The x-axis represents time, while the y-axis represents the level of disability. Image created with BioRender (biorender.com).

1.1.5 MS diagnosis

MS diagnosis depends on clinical findings, imaging, and laboratory results. Table 1 outlines the 2017 McDonald criteria, adapted from Thompson et al. (2018), used to diagnose MS to provide a concise and practical reference. The dissemination in time (DIT) refers to a new appearance of CNS lesions across distinct time intervals, while

the criteria of dissemination in space (DIS) indicates the presence of different lesions in the CNS across various regions (Dobson and Giovannoni, 2019; McGinley et al., 2021).

Table 1: Key Elements of the McDonald criteria for MS diagnosis adapted from Thompson et al. (2018).

Clinical Manifestations	**Number of Lesions with Clinical Evidence**	**Additional Data for a Diagnosis of MS**
≥2 clinical attacks	≥2 clinical objective lesions	None
≥2 clinical attacks	1 clinical objective lesion	Dissemination in space (MRI or more clinical attacks implicating different sites)
1 clinical attack	≥2 clinical objective lesions	Dissemination in time (MRI or demonstration of CSF-specific oligoclonal bands or clinical attacks)
1 clinical attack	1 clinical objective lesion	Dissemination in space and time

Magnetic resonance imagings (MRIs) of MS patients typically reveal multiple sclerotic plaques, associated with demyelination and reactive gliosis. These lesions are primarily found in the periventricular white matter, juxtacortical and infratentorial regions of the brain, and spinal cord. In addition to MRI, the detection of oligoclonal bands (OCB) in the cerebrospinal fluid (CSF) is an important diagnostic tool for diagnosing MS. OCB reflect the increased production of immunoglobulin G due to intrathecal inflammation. Other diagnostic findings include lymphocytic pleocytosis in the CSF, as well as abnormalities in visually evoked potentials (Hacke, 2010; Thompson et al., 2018). In the case of recurrent typical clinical attacks with corresponding neurological

findings and MRI evidence of dissemination in space, no additional tests are necessary for a diagnosis (Fig. 3).

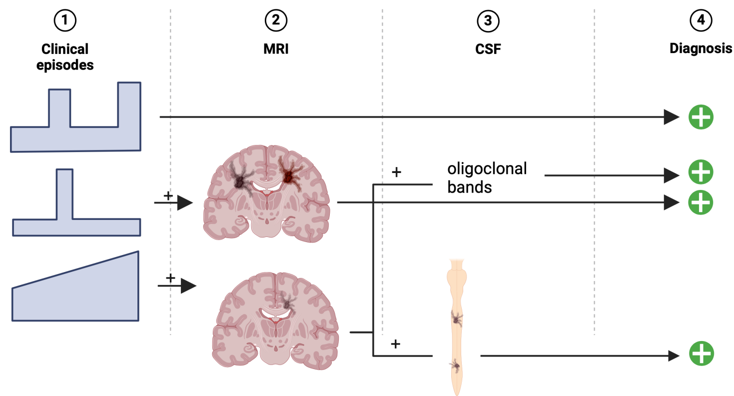

Figure 3: Simplified illustration of the McDonald criteria for diagnosing multiple sclerosis (MS).The figure outlines the key components of the diagnostic criteria, including the evidence of dissemination in space and time, based on clinical, radiological (MRI), and cerebrospinal fluid (CSF) findings. This simplified diagram provides an overview of how the McDonald criteria are clinically applied in the diagnosis of MS (Thompson et al., 2018). Image created with BioRender (biorender.com).

1.1.6 MS treatment

Since there is no definitive cure for MS, the main goal of treatment is to manage symptoms, prevent further attacks, and slow the progression of the disease. The treatment of patients can be divided into the man-

agement of acute exacerbations and long-term disease modification strategies. Acute exacerbations are ideally treated with high-dose glucocorticoids (Grauer et al., 2001). Alternative treatments for exacerbations are plasma exchange and immunoadsorption (Hemmer, 2023). Long-term management includes disease-modifying drugs (DMDs), lifestyle modification (e.g., exercise, mediterranean diet, stress prevention), vitamin D supplementation, and the management of comorbidities and symptoms (Thompson et al., 2018).

DMDs primarily prevent relapses, reduce the accumulation of MRI lesions, and slow the progression of disability by modulating the immune response (Gholamzad et al., 2018). DMDs are interferon beta (IFN-β), glatiramer acetate (GA), dimethyl fumarate (DMF), teriflunomide, cladribine, sphingosine 1-phosphate receptor modulators (e.g., fingolimod, siponimod, ozanimod, ponesimod), monoclonal antibodies (e.g., alemtuzumab, natalizumab, ocrelizumab, ofatumumab, ublituximab), and mitoxantrone.
IFN-β decreases exacerbations and the progression of RRMS by suppressing T cell activity and reducing CNS pro-inflammatory cytokines and lymphocyte invasion. Although its role has diminished, due to the availability of more effective therapies (Goldschmidt and Hua, 2020). GA remains an efficient therapy for reducing inflammation by shifting Th1 to anti-inflammatory Th2 lymphocytes. Although there is long-term clinical experience, these substances have been

largely replaced by newer therapies that are more tolerated and effective. DMF targets the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), important for balancing oxidative and antioxidative processes in cells, helping to prevent inflammation or cell damage. Fumaric acids reduce neuronal cytotoxicity, regulate the peripheral immune response, and inhibit leukocyte migration across the BBB. Teriflunomid inhibits B and T cell proliferation. Oral application of teriflunomide reduces exacerbations, decreases MRI lesion numbers, and slows down MS progression. Cladribine, an oral immunosuppressant, is used in patients with highly active relapsing MS. It selectively depletes lymphocytes, reducing the activity and progression of MS. Fingolimod, approved as the first oral DMD for patients with relapsing MS, is a sphingosine-1-phosphate (S1P) receptor antagonist. S1Ps inhibit lymphocyte egress from lymph nodes, thereby limiting the entry of auto-reactive lymphocytes into the CNS. Further S1Ps interact with astrocytes and oligodendrocytes, leading to anti-inflammatory and neuroprotective effects. Studies have demonstrated that these agents, particularly fingolimod, show a more significant beneficial impact in reducing exacerbations, slowing disability progression, and minimizing brain volume loss compared to first-line treatments such as GA and IFN-β (Amin and Hersh, 2021; Dargahi et al., 2017).

Four monoclonal antibodies (i.e., alemtuzumab, natalizumab, ocre-

lizumab, and ofatumumab) are approved for MS therapy. In detail, alemtuzumab, a CD52 antibody, depletes B and T lymphocytes through cytolysis. Natalizumab acts as a monoclonal antibody against α4-Integrin, efficiently inhibiting the invasion of lymphocytes to the CNS (Dargahi et al., 2017). Ocrelizumab and ofatumumab are antibodies against CD20 and deplete B cells, resulting in profound suppression of disease activity and slowing the development of disability (Sellebjerg et al., 2020). Mitoxantrones act as a nonselective potent immunosuppressant, used in exceptional situations in advanced forms of MS or when other DMDs fail.

While DMDs primarily target neuroinflammation, there remains a significant gap in therapies that effectively address the neurodegenerative aspects of the disease. Although DMDs reduce relapse frequency and delay disability progression by modulating the immune response, they have limited capacity to prevent or repair axonal damage and neuronal loss, which are key drivers of permanent disability in MS. Consequently, there is a great need for treatments that promote remyelination and neuronal repair. Despite ongoing research into neuroprotective and regenerative therapies, these approaches are still largely experimental and are not yet part of standard clinical practice. Addressing these therapeutic gaps is important to improve the long-term prognosis for MS patients (Dargahi et al., 2017; Reich et al., 2018).

There is a continuously growing field of DMD, with treatment choices tailored to MS subtype, patient characteristics, and emerging options. Therefore, treatment guidelines are frequently updated. As an example, Figure 4 presents an adapted overview of the current German recommendation for patients with RRMS (Bayas et al., 2021).

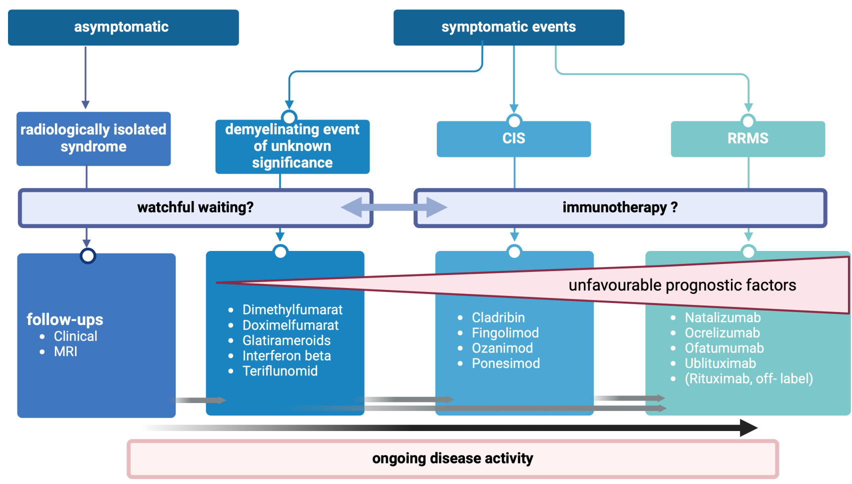

Figure 4: Overview of current treatment recommendations for relapsing-remitting multiple sclerosis (RRMS) according to German guidelines 2021 (Hemmer, 2023). The figure outlines the first-line, second-line, and escalation therapies, highlighting approved disease-modifying drugs (DMDs) and their use based on disease activity and patient characteristics (Bayas et al., 2021). Image created with BioRender (biorender.com).

The remarkable clinical success of B cell-depleting therapies, such as ocrelizumab and rituximab, fundamentally shifted the understanding of

the pathogenesis of MS - a disease historically considered primarily T cell-mediated. These therapies reduce antigen presentation and pro-inflammatory cytokine production by depleting B cells, thus suppressing MS activity and limiting the formation of new CNS lesions. These findings lead to a reevaluation of immunopathological mechanisms, emphasizing that B cells contribute via complex interactions to inflammation and tissue damage within the CNS (Hauser et al., 2008; R. Li and Bar-Or, 2019).

New treatments are being developed to selectively target pathogenic B cells that drive MS disease progression. A promising approach includes targeting alternative signaling pathways, such as FGFRs, which modulate key signaling pathways involved in cell survival, differentiation, and repair within the CNS (Gurski et al., 2025; Kamali et al., 2021; Klimaschewski and Claus, 2021; Rajendran et al., 2018). Additionally, attention was drawn to the inhibition of Bruton's tyrosine kinase (BTK), an enzyme central to BCR signaling pathways. BTK is a critical mediator in pathways such as PI3K, MAPK, and nuclear factor 'Kappa-Light-Chain-Enhancer' of activated B cells (NFkB), important for cell survival, activation, proliferation, and differentiation into antibody-producing plasma cells (Carnero Contentti and Correale, 2020). BTK inhibitors evaluated in phase II/III clinical trials suggest their ability to inhibit B cell activation, reduce cytokine production, and alter APCs functions (Torke and Weber, 2020). Moreover,

BTK inhibition demonstrates potential in modulating disease-driving interactions between B cells and T cells, effectively reducing the formation of demyelinating lesions within the CNS (Montalban et al., 2019). However, recent developments in evaluating BTK for MS have yielded mixed results. For instance, a phase III clinical trial evaluating the highly selective BTK inhibitor evobrutinib did not meet its primary objective of reducing annualized relapse rates in patients with RRMS compared to teriflunomide (Merck KGaA, 2023). However, in another phase III trial that focused on progressive MS, the BTK inhibitor tolebrutinib demonstrated a delay in disability progression, suggesting potential efficacy in this subgroup (Fox et al., 2025). Despite ongoing challenges, BTK inhibition shows potential, but its effectiveness may depend on identifying responsive patient subgroups.

In conclusion, although DMDs and B cell-depleting treatments have already advanced the management of MS, the need for more target and selective therapies remains. Emerging strategies, including BTK inhibitors and interventions targeting alternative signaling pathways like FGFRs, aim to modulate immune responses, prevent neurodegeneration, and promote remyelination, thereby addressing significant unmet needs in comprehensive MS management (Klimaschewski and Claus, 2021; Lindner et al., 2015; Rajendran, Böttiger, Dentzien, et al., 2021; Rajendran, Böttiger, Stadelmann, et al., 2021). These innovative approaches promise to advance treatment beyond con-

ventional anti-inflammatory strategies, offering the potential for more effective long-term outcomes. Further research is important to broaden the therapeutic landscape and ultimately find a cure for MS. Understanding the immune system's role in MS is key to advancing treatment strategies, with immune modulation that influences both disease progression and remission.

1.2 B lymphocytes

The immune system, comprising both innate and adaptive branches, relies on the balance between these components to maintain homeostasis, as dysregulation can lead to immunodeficiency, infections, allergies, or autoimmunity (Marshall et al., 2018). Inflammation represents the immune system's response to tissue damage or pathogens, driven by immune cell activation and cytokine release, which can lead to tissue injury when dysregulated (Medzhitov, 2008). Within this immunological framework, B cells have become a compelling therapeutic target due to their multifaceted functions in both immune defense and disease pathogenesis (Duddy et al., 2007). The following sections will explore their development, function, and specific involvement in MS pathology in greater detail.

1.2.1 B cell development

B cells originate and develop within the bone marrow, undergoing tightly regulated maturation processes to maintain immune tolerance. During this development, B cells encounter a critical mechanism known as negative selection. This negative selection process identifies and eliminates autoreactive B cell clones, which mistakenly recognize and target the body's own tissue. However, when this negative selection process is incomplete or defective, autoreactive B cells may escape into the peripheral circulation, contributing to the development of autoimmune disorders. In the context of MS, these autoreactive B cells can infiltrate and harm the CNS (Gururajan et al., 2014). This mechanism is further explained in Section 1.2.4, where I describe the specific processes underlying B cell-mediated neuroinflammation in MS. After passing this checkpoint, immature B cells leave the bone marrow to migrate to peripheral lymphoid organs, where they transition into mature follicular and marginal zone B cells. After these mature B cells interact with their specific antigens, they differentiate into antibody-secreting plasma or memory B cells. Mature B cells express CD19, CD20, CD21, CD40, MHCII, and B7 surface proteins, essential for B cell signaling, antigen presentation, and interactions with T cells. The B cell receptor (BCR), located on the surface of B cells, is crucial for antigen recognition, as well as B cell activation and maturation (Abbas et al., 2019; Rastogi et al.,

2022). Naive B cells, which are mature B cells, that have not been in contact with antigens, remain resting until stimulated. In peripheral lymphoid tissues, another negative selection process is performed to remove B cells with dysfunctional BCR rearrangements or high affinity for self-antigens. This second layer of immune tolerance is important to prevent the survival of autoreactive B cells, potential mediators of autoimmune diseases like MS (Abbas et al., 2019; Cencioni et al., 2021).

1.2.2 B cell activation

Naive B cells can be activated through Th cell interaction or independently through direct antigen binding. After activation, B cells differentiate into antibody-secreting plasma cells and memory B cells (Fig. 5).

In this process, the BCR initiates a signaling cascade (e.g., phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt), mitogen-activated protein kinase (MAPK) signaling pathway) upon binding the antigen. Additionally, the BCR internalizes the BCR/antigen complex through endocytosis to intracellular sites for antigen processing. The processed antigen fragments are then presented on the B cell surface via the MHCII receptor, priming Th cells specific to the same pathogen. Costimulation occurs through the interaction between the CD40 ligand on effector T cells and the CD40 receptor expressed on B cells,

activating the non-canonical NFkB signaling pathway. This interaction stimulates activated Th cells to secrete cytokines, including IL21, which enhances B cell division, proliferation, survival, and facilitates their maturation into antibody-producing plasma cells or memory B cells. This activation is mediated by the transcription factor STAT3. On the other hand, B cells can respond directly to non-protein antigens, including thymus-independent antigens or gram-negative lipopolysaccharides. These stimuli initiate T cell-independent activation of B cells, leading to a rapid but transient immune response characterized by the secretion of unspecific IgM antibodies (Murphy, 2017; Rawlings et al., 2017).

A critical feature of T cell interaction, particularly through the cos-timulation of the CD40 ligand, is immunoglobulin class switching. B cells transition from the initial IgM immune response to producing other immunglobuline isotypes (e.g., IgA, IgE, or IgG). This switch in immunglobulin isotype occurs within the germinal center of secondary lymphoid tissues, where somatic hypermutation and affinity maturation optimize antibody specificity. During this process, B cells undergo clonal selection, interacting with T cells to refine antigen recognition, and produce antibodies with higher affinity. Following class switching, some B cells differentiate into long-lived memory B cells, which are primed for rapid responses during subsequent exposures to the same antigen, and secrete isotype-specific antibodies (e.g., IgG) (Murphy, 2017; Rawlings et al., 2017).

The activated B cells then circulate between the blood and secondary lymphoid organs like lymph nodes, the spleen, and mucosa-associated lymphoid tissue (MALT). Combining class switching and affinity maturation enables B cells to produce antibodies with higher specificity and efficacy. However, altered B cell-intrinsic signaling through the BCR and extrinsic co-receptor signaling pathways, essential to B cell activation, can lead to the survival of autoreactive B cells. These B cells may secrete pathogenic antibodies or show autoreactive receptors that bind to self-antigens, contributing to autoimmune diseases, highlighting the critical role of tightly regulated B cell activation in maintaining immune tolerance and preventing autoimmunity (Murphy, 2017; Parkin and Cohen, 2001; Rawlings et al., 2017). In the context of neuroinflammation, these dysregulated B cells infiltrate the CNS and drive inflammation, leading to myelin degradation and axonal damage. Thus, highlighting the importance of targeting B cell responses as part of therapeutic strategies to prevent CNS autoimmunity in MS (Dendrou et al., 2015).

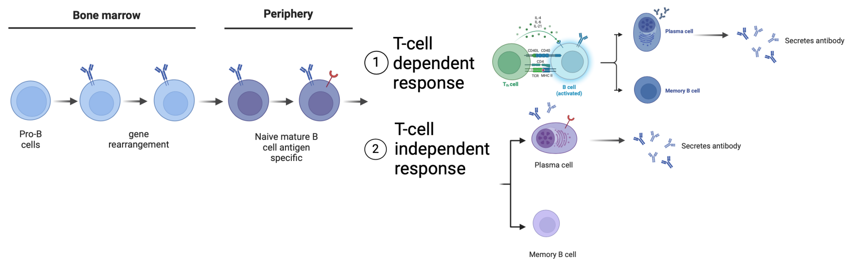

Figure 5: Simplified overview of B cell development and activation. B cell development begins in the bone marrow, where progenitor B cells undergo genetic rearrangement to create a receptor capable of recognizing a broad range of antigens. Once matured, naive B cells travel to secondary lymphoid organs. They are activated upon antigen binding. During T cell-dependent activation, B cells process and present antigens via MHC II to T-helper cells, receiving essential costimulatory signals through the CD40-CD40L interaction and cytokines such as IL-21. These signals trigger B cell proliferation, class switching, and the maturation into plasma cells that secrete antibodies, as well as memory B cells. T cell-independent activation occurs when B cells directly recognize antigens, enabling them to rapidly produce antibodies without the involvement of T cells, leading to the formation of resting memory cells and plasma cells that secrete antibodies (Abbas et al., 2019; Cencioni et al., 2021). Image created with BioRender (biorender.com).

1.2.3 B cell function

B cells play various roles in immunity. Most importantly, they mediate humoral immunity through the production of immunoglobulins and participate in the development of cellular immunity by serving as APCs for T cells and B cells, which release a range of different cytokines

(Cencioni et al., 2021).

These antibodies can either be presented as surface immunoglobulins on B cells or can be secreted. Further, they activate the classic pathway of the complement system via C1q binding, and engage fragment crystallizable receptor (FcR) on immune cells to activate and recruit macrophages and other immune cells. The immunoglobulin isotype and the binding affinities to the FcR on immune cells determine the exerted effector functions (Hoffman et al., 2016). B cells become activated and potent APCs by presenting specific antigens. This results in CD4 T cell activation and differentiation through MHCII and co-stimulatory molecules expressed by B cells, promoting immunity. As APCs, activated B cells can positively or negatively impact T cell function, resulting in T cell activation or T cell tolerance (X. Chen and Jensen, 2008).

In addition, activated B cells release pro- and anti-inflammatory cytokines (i.e., IL2, IL4, IL6, IL10, IL35, IFNγ, TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which modulate immune responses. Some cytokines have the potential to alter B cell development by affecting the growth, survival, and class switching of these cells. B cells can be classified into anti-inflammatory, cytokine-producing, “regulatory”, and pro-inflammatory cytokine-producing “effector” B cells. Activated B lymphocytes also release chemokines (i.e., CCL22 and CCL17), which are important for Th2 cell recruitment. Effector cytokines from B cells such as IL6, IFNγ and TNFα induce

inflammation (Hoffman et al., 2016; Murphy, 2017; Vazquez et al., 2015). Further, IFNγ, and TNFα can directly generate endothelial and epithelial cell injury. In contrast, the production of IL10 or IL35 by B cells exerts regulatory function (regulatory B cell (Breg)) through modulation of dentritic cells (DCs) (e.g., decrease of IL6 and IL12), macrophages, NK cells, and T cells (Hoffman et al., 2016).

In addition, described as an in situ immune response, B cells contribute to the formation of tertiary lymphoid organs within peripheral tissues. These tertiary lymphoid organs typically form in response to chronic inflammation or ongoing infection and can lead to tissue injury. This process can be observed in autoimmune diseases. In MS these lymphoid structures occur mainly within the meninges of the CNS and are associated with chronic inflammation (Mitsdoerffer and Peters, 2016). B cells play a dual role in immune regulation, contributing to pathological and protective processes in neuronal damage and repair. The review article *"Heterogeneity of B Cell Functions in Stroke-Related Risk, Prevention, Injury, and Repair"* by Selvaraj et al. (2016) highlights how B cells influence stroke risk factors, such as hypertension and atherosclerosis, through pro-inflammatory actions, while regulatory B cells secrete anti-inflammatory cytokines like IL10, promoting tissue repair and neuroprotection (Selvaraj et al., 2016). These findings underscore the ability of B cells to modulate neuroinflammatory environments, which is particularly relevant in MS. Figure 6 is an adapted illustration from Selvaraj et al. (2016) of several

key functions of B cells in both the immune system and neuroimmunological processes. It emphasizes the crucial involvement of B cells in maintaining immune homeostasis and their active participation in both protective and pathological mechanisms, including immune responses, neuroprotection, and inflammation. Targeting specific B cell functions may reduce inflammation and enhance neuronal repair and remyelination, presenting promising therapeutic strategies for MS treatment.

B cell function

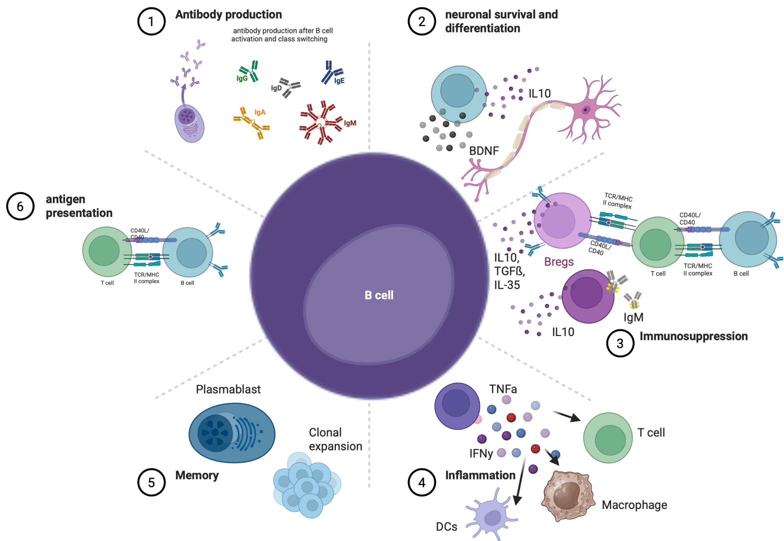

Figure 6: This image illustrates the multifaceted roles of B cells in both the immune system and neuroimmunological processes. 1. Antibody production: B cells, upon encountering specific antigens, differentiate into plasma cells and produce antibodies (immunoglobulins). 2. Neuronal survival and differentiation: B cells contribute to neuronal health by secreting cytokines and growth factors, which promote neuronal survival and differentiation, particularly in response to central nervous system injury or neuroinflammation. 3. Immunosuppression: Regulatory B cells (Bregs), produce anti-inflammatory cytokines, suppressing excessive immune responses, helping maintain immune tolerance and preventing autoimmune damage. 4. Inflammation: B cells produce proinflammatory cytokines and interact with other immune cells, contributing to inflammation and the development of tissue damage. 5. Memory: Memory B cells are formed after an initial immune response to an antigen and can rapidly produce antibodies upon re-exposure. 6. Antigen presentation: B cells function as antigen-presenting cells, capturing, processing, and presenting antigens to T cells. This interaction initiates and strengthens adaptive immune responses (Selvaraj et al., 2016). Image created with BioRender (biorender.com).

1.2.4 B cells in MS

The presence of intrathecal B cells and the resulting abnormal increase in immunoglobulin synthesis, along with the typical OCB found in the CSF of patients, imply the involvement of B cells in the pathophysiology of MS. The positive IgG OCB are diagnostic markers. However, specific CNS reactive antibodies consistently present within the CSF across MS patients have not been identified (Antel and Bar-Or, 2006; Dendrou et al., 2015). OCB may represent immune responses to neoantigens generated during demyelination rather than primary autoimmune reactivity. These neoantigens, arising from post-translational modifications or structural changes in myelin proteins, may amplify ongoing immune responses (Pryce and Baker, 2018). Furthermore, cross-reactive antibodies against EBV nuclear antigen EBNA1 and glial cell adhesion molecule have been found in the CSF of MS patients, with evidence of viral mimicry contributing to disease activity (Lanz et al., 2022). However, beyond antibody production, B cells drive disease pathogenesis by activating T cells, presenting antigens, and secreting pro-inflammatory cytokines such as IL6, underscoring their multifaceted role in driving disease pathogenesis. Memory B cells from MS patients have the ability to induce spontaneous proliferation of auto-reactive CD4+ T cells. Activated B cells act as potent APCs when binding the same antigen as the T cell, resulting in effector T cell activation and the production of regulatory T cell (Treg)

cells. This complex interplay of T and B cells could contribute to MS pathophysiology (Cencioni et al., 2021; Dendrou et al., 2015). This possibility is also supported by B cell-specific MHCII knockout EAE experiments, where mice were resistant to the MOG-induced disease, suggesting an MHCII dependent APC function of B cells in EAE (Dendrou et al., 2015; R. Li et al., 2018).

A dysregulation between pro- and anti-inflammatory cytokines is recognized in patients with MS. Activated B cells produce excessively cytokines, including TNF, lymphotoxin α, IL6, and GM-CSF. For example, IL6, known to provoke Th17 cell responses, is believed to drive the pathogenesis of EAE and MS. GM-CSF secreting B cells are also considered to be pro-inflammatory through effectively increasing myeloid cell pro-inflammatory responses and by co-expressing high levels of IL6 and TNF (Dendrou et al., 2015). On the other hand, B cells are able to suppress inflammation and the immune response by producing anti-inflammatory cytokines such as IL10, IL35, and TGFβ. The selective knockout of IL10 in EAE resulted in more severe EAE symptoms (Bettelli et al., 1998). Molecular mechanisms involved in human cytokine regulation by B cells are altered in MS patients, where more phosphorylation of STAT5 and STAT6 has been observed, resulting in increased proinflammatory GM-CSF and decreased production of the anti-inflammatory cytokine IL10. Also, microRNAs were described to underlie B cell cytokine dysregulation of MS patients, like the overexpressed miR-132 resulting in increased

TNF secretion (Dendrou et al., 2015).
In MS, aberrant activation of peripheral immune cells and their subsequent trafficking across the BBB into the CNS lead to chronic neuroinflammation. B cell-rich aggregates of infiltrating immune cells in the meninges of the CNS were observed in MS patients. Here, B cells drive compartmentalized inflammation and contribute to CNS injury by secreting inflammatory and cytotoxic factors into the CSF, fostering a inflammatory environment within the brain. These B cell clones are maintained over time within the CNS by factors like B cell activating factor (BAFF), secreted by astrocytes, supporting B cell survival and activation. Chronically activated B cell-rich infiltrates in the meninges are associated with extensive neuronal, astrocyte, and oligodendrocyte loss and active gray matter demyelination. Activated and demyelinated cortical lesions with perivascular immune cell infiltrates are common in early MS (Dendrou et al., 2015). Further B cell accumulation has been observed to correlate with a worse clinical disease course, indicating B cell contribution to CNS inflammation (Cencioni et al., 2021; Dendrou et al., 2015; R. Li et al., 2018).

1.3 Cytokines

Cytokines are signaling proteins, peptides, or glycoproteins that regulate immunity, inflammation, and hematopoiesis. They are produced

by immune and non-immune cells and act in autocrine, paracrine, and endocrine manners and can have overlapping, synergistic, or antagonistic effects. The cytokine superfamily includes interleukins, chemokines, colony-stimulating factors (CSF), interferons, transforming growth factors (TGF), and TNF. Cytokines can be classified by their pro-inflammatory (i.e., IL 1, 6, 8, 12, 18, interferons and TGF) and anti-inflammatory (i.e., IL4, 10, 11, 13 and TNFβ) functions. Pro-inflammatory cytokines induce fever, inflammation, and tissue damage by stimulating the synthesis of inflammatory mediators, the production of acute phase proteins, and the recruitment of immune cells (Sino Biological, 2023). However, their classification as strictly pro- or anti-inflammatory is oversimplified, as cytokines such as IL6 can exert both effects depending on the context (Scheller et al., 2011). Cytokines signal via receptor binding of JAKs or other tyrosine kinases, leading to intracellular signal cascade activation predominantly involving molecules like STATs, Src-kinases, protein phosphatases, and other signaling proteins such as Shc, Grb2, and PI3K (Sino Biological, 2023).

1.3.1 The role of cytokines in MS

In the CNS, cytokines modulate neurodevelopment, synaptic transmission, and neuronal signaling (Zipp et al., 2023). However, their dysregulation can lead to neuronal inflammation, neurodegeneration,

and demyelination. By producing pro-inflammatory and neurotoxic factors, cytokines cause neuronal and glial cell damage, promote immune cell infiltration across the BBB, thereby amplifying neuronal injury and inflammation (Ramesh et al., 2013). Preclinical studies, particularly EAE models, have provided valuable information on cytokine modulation. However, translating EAE findings into MS remains a significant challenge. The limitations of preclinical models in capturing the complexity of cytokine networks and the multifaceted pathogenesis of MS contribute to discrepancies between experimental results and clinical efficacy (Göbel et al., 2018).

IL6

IL6 is activated during systemic inflammation, regulating innate immunity by stimulating acute phase reactant synthesis and directing leukocyte activation and trafficking (Akdis et al., 2011). In MS, increased IL6 levels in both serum and CSF have been detected (Stampanoni Bassi et al., 2020). IL6 compromises the BBB, facilitates immune cell infiltration into the CNS, and exacerbates inflammation by recruiting inflammatory cells, activating autoreactive T cells, and promoting the production of pathogenic antibodies (Vazquez et al., 2015). IL6 enhances the inflammatory Th17 cell differentiation, thus stimulating the production of IL6, reactive oxygen species, and nitric oxide by astrocytes, causing neuronal damage (Serizawa et al., 2021). IL6 modulates B cell differentiation and antibody production through

the JAK-STAT3, PI3K-Akt, and RAS-MAPK pathway. In support of IL6 crucial role in MS pathogenesis, IL6-deficient mice are resistant to develop MS similar symptoms in EAE models (Rothaug et al., 2016).

IL12

IL12 is secreted by activated monocytes, macrophages, neutrophils, microglia, DCs, and B cells (Akdis et al., 2011) and promotes Th1 cell differentiation and NK cells activation. IL12 primarily signals through the JAK - (STAT)4 pathway and plays a key role in EAE pathogenesis (Comabella et al., 1998; Jee et al., 2001). Elevated serum levels of IL12 are observed in patients with MS and correlate with disease activity (Wang et al., 2018).

TNFα

TNFα is found in active MS lesions and is elevated in the serum and CSF, correlating with disease severity and progression (Göbel et al., 2018). TNFα mediates neurotoxicity by promoting glutamate production and inducing oligodendrocyte death in a calcium-dependent manner (Ramesh et al., 2013). However, it also supports remyelination. Preclinical studies showed that TNFR1 deficient mice were partially resistant to EAE, suggesting a potential therapeutic target. However, TNFα blockade in MS patients worsened the disease, highlighting its dual role in both neuroinflammation and remyelination (Göbel et al., 2018).

IFNγ

IFNγ is a cell-signaling glycoprotein critical for innate and adaptive immunity. It is a potent activator of macrophages, enhancing phagocytosis and NK cells to diminish infected target cells. INFγ downregulates Th2 cell response and stimulates MHCII expression and antigen presentation in cells. It also promotes class switching of B cells to IgG3 and exerts synergistic effects of TNF on macrophages. Often, IFNγ is associated with autoimmune diseases. IFNγ levels are elevated during active MS, and IFNγ was detected in MS lesions. In both EAE and MS, inflammation is strongly associated with the Th1 cell response, which produces IFNγ. IFNγ activates the JAK signaling pathway, which is mainly regulated through the STAT1 pathway (Wang

et al., 2018). Studies showed that treatment with IFNγ can aggravate the disease course of MS patients, on the other hand EAE studies have suggested a potential beneficial role. IFNγ supports OL survival and may reduce demyelination through ERK pathway modulation in EAE (Lees and Cross, 2007).

CCL2 and CX3CL1

Cytokines with chemotactic activities are considered chemokines. CCL2 is a chemotactic factor that draws monocytes and is associated with the pathogenesis of diseases with monocytic infiltrates. CX3CL1 is a chemokine ligand of the fractalkine gene family that regulates endothelium leukocyte adhesion and migration (Sino Biological, 2023). In MS, the specific chemokine CCL2 mediates the recruitment of inflammatory cells to CNS inflammation sites (Høglund, 2014). CCL2 is overexpressed in active and chronic MS lesions. Here, inflammatory cells, predominantly astrocytes, secrete CCL2, leading to additional microglial recruitment and activation. This, as well as inhibiting mature OL production, results in enhanced demyelination. Studies on CCL2 serum levels show inconsistent results, but mostly reduced CCL2 levels in the CSF. This reduction is linked to radiologically defined MS disease activity, suggesting that CCL2 plays a role in CNS demyelination and contributes to neurodegeneration in MS (Mahad and Ransohoff, 2003). Neuronal CX3CL1 modulates microglia-neuron interactions, promoting survival, synaptic transmission, plasticity, and

network maturation (Limatola and Ransohoff, 2014). It regulates cytokine release from microglia, reducing pro-inflammatory mediators like TNF, IL1β, IL6, and nitric oxides, offering neuroprotection during neuroinflammation. Additionally, CX3CL1 may influence neuroprotection or neurotoxicity by regulating microglial phagocytosis of neurons (Limatola and Ransohoff, 2014).

1.4 FGFs and FGFRs

The FGFs that signal through FGFRs are ubiquitous in various cell types (e.g., endothelial cells, fibroblasts, and immune cells like B, and T cells). They play key roles in maintaining essential physiological functions such as metabolic and tissue homeostasis, endocrine regulation, development, and injury response (Nobuyuki Itoh and David M. Ornitz, 2011; Ornitz and Itoh, 2015).

The FGF family consists of 23 members, which are structurally related signaling proteins with diverse biological roles. These members are classified into different subfamilies based on their function and expression patterns. The subfamilies include iFGFs (e.g., FGF11, FGF12, FGF13, and FGF14), hFGFs (e.g., FGF15, FGF21, and FGF23) and cFGFs. Notably, FGF15 is absent in humans and its functional human ortholog is FGF19 (Itoh and Ornitz, 2008).

FGFs function in intracrine, paracrine, and endocrine signaling. In the paracrine and endocrine pathways, FGFs interact with heparan

sulfate proteoglycans (HSPGs) on the cell surface, which facilitate their interaction with FGFRs. These interactions mediate their signaling effect on target cells. On the other hand, iFGFs exhibit a distinct mode of action, operating mostly independently of FGFRs. They function intracellularly and diffuse directly after being produced. This intracellular signaling pathway includes the binding of FGFs to intracellular domains of voltage-gated sodium channels, particularly in the nucleus, leading to distinct downstream effects compared to the activation of FGFR mediated signaling on the cell surface, which follows a typical tyrosine kinase mechanism (Beenken and Mohammadi, 2009). Upon FGF binding, fibroblast growth factor receptors (FGFRs) undergo dimerization, trans-/autophosphorylation, and subsequent phosphorylation of key downstream molecules, including MAPK/ERK, PI3K/Akt, PLCγ/PKC, and STAT. While all FGFRs activate these pathways, they do so with varying intensities (Brewer et al., 2016).

The four FGFRs (FGFR1 to FGFR4) mediate diverse functions through FGF ligand binding. Each FGFR consists of extracellular, transmembrane, and intracellular domains. The extracellular region includes three immunoglobulin-like domains (D1-D3), where D2 and D3 primarily mediate FGF binding. Alternative splicing in the IgIII domain generates two isoforms, IgIIIb and IgIIIc, which dictate epithelial or mesenchymal ligand specificity. This splicing pattern ensures that epithelial IgIIIb isoforms bind mesenchyme-derived FGFs (e.g., FGF7 and FGF10), while mesenchymal IgIIIc isoforms

interact with epithelium-derived FGFs (e.g., FGF2, FGF4 and FGF8). FGF1 is the exception, activating both isoforms, facilitating broader FGFR signaling. This selective binding mechanism is essential for epithelial-mesenchymal interactions. The IgI domain and the serine-rich acid box linking D1 and D2 play a key role in receptor autoinhibition. The transmembrane domain connects the extracellular region to the intracellular tyrosine kinase domains (TKI and TKII), which contain the catalytic activity (Beenken and Mohammadi, 2009). Dysregulated FGF signaling can contribute to pathological conditions through gain and loss of functions of FGF ligands or FGFRs. For example, pathological FGFR1 signaling through a gain-of-function mutation has been identified in glioblastoma (Rand et al., 2005). In craniosynostosis syndromes, kinase domain mutations of FGFR2 were found, and single nucleotide polymorphisms in FGFR2 are known to play a role in BRCA2 mutation breast cancer (Beenken and Mohammadi, 2009). Dysregulated FGF signaling is a pivotal factor in disease development. Advances in molecular biology and targeted therapy paved the way for innovative treatment strategies aimed to restore normal FGF function. Further research into the intricate mechanisms of FGF/FGFR dysregulation will be essential for developing precision medicine approaches, ultimately improving patient outcomes in conditions driven by aberrant FGF signaling.

1.4.1 FGF/FGFR signaling pathway

The binding of FGF to the FGFR induces receptor dimerization, leading to cross-phosphorylation of the kinase domains, thus recruiting downstream effector molecules and activating four key downstream pathways (i.e., RAS-RAF-MAPK-ERK, PI3K-Akt, JAK-STAT and phospholipase Cy (PLCy)). These FGF/FGFR1 signaling pathways primarily regulate cell proliferation, differentiation, and migration. A key component of this downstream signaling is the Akt pathway, which plays a crucial role in controlling cell survival, cell differentiation in size and growth, and modulating cell metabolism. Additionally, the downstream effects of Akt signaling contribute to essential cellular processes, including proliferation, tissue invasion, while also contributing to neovascularization (Altomare and Testa, 2005). The ERK signaling pathway also plays an important role in cell proliferation, differentiation, migration, metabolism, growth, and survival. It is also activated in response to cellular stress and DNA damage, particularly during inflammatory responses. In the CNS, ERK signaling has a fundamental impact on brain development, and alterations in this pathway could lead to neuronal disabilities (cf. Section 1.4.2) (Lavoie et al., 2020).

In FGF/FGFR signaling, several adapter proteins activate signal transduction in the downstream pathway. FGFR substrate 2 (FRS2) plays an essential function as a signaling transducer by binding the

juxtamembrane region of the receptor. Phosphorylation of FRS2 enables the recruitment of SOS and Grb2, which together activate the RAS-RAF-MAPK-ERK pathway. Simultaneously, the growth factor receptor-bound protein 1 (Grb1) - FRS2 complex initiates the activation of the PI3K-Akt signaling pathway. Another significant signaling mediator is PLCy, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG). This reaction triggers protein kinase C (PKC) activation and intracellular calcium release. PKC then contributes to the RAS-RAF-MAPK-ERK pathway activation through RAF phosphorylation. On the other hand, the JAK pathway activates STAT, which then undergoes dimerization and translocates to the nucleus, to modulate gene expression (Babina and Turner, 2017). In line with their involvement in cytokine-driven inflammation and immune responses, JAK-mediated activation of STAT3, involved in regulating cell survival and proliferation, can contribute to oncogenesis when dysregulated (Babina and Turner, 2017; Yu et al., 2009). Ligand binding is a crucial step in FGF signaling, and this process can be regulated by various proteins that influence receptor internalization and signaling activity. Some of these proteins act as negative regulators, modulating the signaling pathways triggered by FGF ligands. For example, fibroblast growth factor receptor-like 1 (FGFRL1) and similar expression to FGF (SEF) are known to reduce the signaling output. Additionally, proteins such as sprouty (SPRY), Casitas B-lineage lymphoma (CBL), and

MAPK phosphatases (e.g., MKP1 and MKP3) regulate the intracellular signaling cascades by deactivating key components, such as the MAPK pathways. These regulatory mechanisms ensure a balance in FGF-mediated signal transduction and prevent overactivation, which is critical for maintaining cellular homeostasis and preventing pathological conditions (Turner and Grose, 2010).

1.4.2 FGF/FGFR signaling in MS

FGFR signaling is a key regulator of CNS homeostasis, playing a complex role in neuroinflammation, myelination, and tissue repair (Klimaschewski and Claus, 2021). FGFRs are widely expressed in the brain and spinal cord, with FGFR1 predominantly found in neurons and astrocytes, while oligodendrocytes and microglia express higher levels of FGFR2 and FGFR3 (M. Lee et al., 2011). Growing evidence indicates that FGF/FGFR signaling contributes to MS pathogenesis and its disease model EAE by influencing inflammatory responses and myelination. Analyses of the brain tissue of MS patients revealed the involvement of FGFs and FGFRs in the destruction of myelin sheaths as well as in remyelination (Lindner et al., 2015). Upregulation of FGF2, primarily expressed in astrocytes and macrophages/microglia, correlates with cellular inflammation and demyelination. Enhanced FGF2 expression was found in active lesions, where demyelination occurs, and in the periphery of chronic lesions (Clemente et al.,

2011). FGF2 is also known to play a beneficial role in intrinsic repair processes by promoting the recruitment and proliferation of OLs, thereby facilitating early stages of regeneration. However, in later stages with prolonged activation, FGF2 interferes with their differentiation, preventing them from maturing, negatively affecting remyelination. Furthermore, higher levels of FGF2 were found in the CSF of MS patients compared to a healthy control population, and levels increased significantly during disease relapse (Clemente et al., 2011; Rajendran, Böttiger, Stadelmann, et al., 2021; Sarchielli et al., 2008). Therefore, a correlation between FGF2 expression and disease severity might exist (Rajendran, Böttiger, Stadelmann, et al., 2021). Conditional deletion of FGFR1 and FGFR2 specifically in oligodendrocytes has been shown to reduce myelination, a process possibly linked to diminished ERK1/2-MAPK signaling (Furusho et al., 2012; Ishii et al., 2014). Under normal physiological conditions, FGFR2 appears to play a more significant role in myelination than FGFR1. Key downstream signaling pathways, such as Akt, mechanistic target of rapamycin (mTOR), and ERK, contribute to the regulation of myelin-related gene expression (Furusho et al., 2017). In MOG_{35-55}-induced EAE, OL-specific deletion of FGFR1 and FGFR2 resulted in a less severe disease course, characterized by decreased demyelination and axonal damage (Kamali et al., 2021; Rajendran, Böttiger, Dentzien, et al., 2021; Rajendran et al., 2018). Furthermore, immune cell infiltration into the spinal cord, specifically monocytes, T

cells and B cells, was markedly reduced in these knockout models, a finding that was similarly observed when pharmacological inhibition of FGFR was applied in EAE (Gurski et al., 2025; Rajendran et al., 2023).

These findings underscore the context-dependent nature of FGF/FGFR signaling in neural repair and inflammation. The observed reduction in B cell infiltration further emphasizes the role of FGFR signaling in regulating B cell responses, suggesting a broader impact on immune modulation and disease pathology.

1.4.3 Role of FGFs and FGFRs in B cells

FGFs and FGFRs play an important role in B cell development, especially during early stages in the bone marrow (Moroni et al., 2002). By interacting with FGFRs, FGFs promote the proliferation and differentiation of B cell precursors, facilitating their progression into mature B cells. Additionally, FGFRs help maintain the survival of mature B cells and regulate their activity during immune responses (Beenken and Mohammadi, 2009). Aberrations in FGF/FGFR signaling have been found in numerous human diseases, including B cell associated malignancies (e.g., CLL and multiple myeloma) (Ghosh and Kay, 2013; Lahiry et al., 2010). Beyond hematologic cancers, abnormal B cell activity has been observed in numerous solid tumors, such as breast, colon, lung, prostate, ovarian cancer, and melanoma,

where tumors are frequently infiltrated by high numbers of B cells (Somasundaram et al., 2017). This connection suggests that FGFR signaling may influence tumor progression and immune regulation through effects on B cells. In neuroblastoma, high B cell infiltration has been associated with the recruitment of other immune cells and the activation of immune-related pathways, strongly correlated with tumor prognosis (Schaafsma et al., 2021). Given the widespread expression of FGFRs on B cells, dysregulated signaling may affect their proliferation, survival, and function, potentially contributing to oncogenesis by influencing B cell behavior within the tumor microenvironment or shaping a broader immune response.

FGF2 ligand mediated FGFR activation has been implicated in oncogenic processes. In melanoma, tumor cells themselves secrete FGF2, which in turn stimulates B cells to produce inflammatory cytokines, promoting tumor heterogeneity and resistance to kinase inhibitors FGF2 (Akl et al., 2016; Somasundaram et al., 2017). This suggests that tumor-derived FGF2 plays a direct role in shaping the immune microenvironment to support cancer progression. Given that FGFR signaling influences immune responses across different tumor types, similar mechanisms may be relevant in other malignancies where B cell infiltration and FGFs contribute to disease pathology.

In pediatric B cell precursor acute lymphoblastic leukemia (BCP-ALL) *in vitro* cell studies, FGF2 stimulation reduced the sensitivity of leukemic cells to prednisolone therapy, indicating that FGF/FGFR

signaling may contribute to treatment resistance. The resistance was reversed by the FGFR inhibitor AZD4547, indicating a direct role of the FGF2–FGFR3 in glucocorticoid resistance (Jerchel et al., 2019). Similarly, elevated vascular endothelial growth factor (VEGF) and FGF2 levels in acute lymphoblastic leukemia (ALL) have been associated with enhanced leukemic cell survival and treatment resistance (Faderl et al., 2005). In chronic lymphocytic leukemia (CLL), constituive activation of receptor tyrosine kinase (RTK) pathways, including FGFRs, have been observed alongside elevated plasma levels of FGF2. These signals appear to sustain leukemic B cell survival as well as resistance to apoptosis (Ghosh and Kay, 2013). Flow cytometric and western blot analyses of CLL B cells showed low FGFR1, FGFR2, and FGFR4 expression levels, but FGFR3 expression was markedly elevated, suggesting it as a key driver of downstream signaling cascades involving common intermediate signaling molecules such as Src-kinases, PI3K, RAS/RAF, and PLCy, which activate Akt, ERK, PKC, and STAT that promote cell viability and resistance to cell death (Ghosh and Kay, 2013; Sinha et al., 2016).

Although these mechanisms are primarily characterized in cancer, they point to the general potential of FGFR signaling to influence B cell behavior. In the context of autoimmune diseases such as MS, where B cells play a central immunomodulatory role, similar signaling pathways may contribute to altered B cell function or survival. However, the involvement of the FGF/FGFR axis in B cell-mediated

immune regulation in MS remains largely unexplored. Advancing our understanding of this pathway could uncover novel mechanisms in B cell-driven diseases and identify new opportunities for targeted therapies.

1.4.4 Therapeutic approaches of FGFR inhibition

Aberrant FGF/FGFR signaling has been implicated not only in various cancers, but also in nonmalignant conditions, including immunological, hematological, and vascular disorders. In this context, aberrant activation of the FGF/FGFR pathway can drive cell-autonomous behavior, whereby cells maintain proliferation and survival independently of external regulatory signals. This intrinsic, self-sustaining signaling contributes to pathological conditions by enabling continuous growth, resistance to apoptosis, and altered cellular functions (Dai et al., 2019; Lahiry et al., 2010). Recognizing the central role of FGF/FGFR signaling in disease has prompted the development of targeted therapeutic strategies. Among these, small-molecule tyrosine kinase inhibitor (TKI)s have shown particular promise.

These compounds target the catalytic activity of the cytplasmatic kinase domain by binding the ATP-binding cleft. TKI therapies achieve this by selectively targeting FGFR (selective TKI) or targeting several growth factor receptors (multitargeting TKI). Another group of FGFR inhibitors is known as antagonistic monoclonal antibodies (mAbs)

or peptide inhibitors. They bind to the extracellular region of the receptor, thereby inhibiting the interaction between FGF and FGFR as well as preventing receptor dimerization. Another way to suppress FGF/FGFR interaction is through FGF ligand traps that bind multiple FGF ligands and receptors, thereby inhibiting signaling (Babina and Turner, 2017; Chae et al., 2017).

Many novel treatments targeting FGFRs are currently being investigated in preclinical studies and several clinical trials for different cancer types. For instance, erdafitinib has received approval for the treatment of advanced urothelial carcinoma with susceptible FGFR mutations or fusions, while pemigatinib and infigratinib are approved for cholangiocarcinoma with FGFR2 fusions or rearrangements (American Society of Clinical Oncology, 2023). Infigratinib, AZD4547, and dovitinib are among the promising FGFR inhibitors, currently under active investigation. Infigratinib, approved for cholangiocarcinoma with FGFR2 fusions, is also being explored for its potential in treating recurrent glioblastoma and other solid tumors driven by FGFR alterations (Ivy Brain Tumor Center, 2024). AZD4547, is undergoing evaluation in phase II studies targeting tumors with FGFR aberrations, including breast, urothelial, and cervical cancer (Chae et al., 2020). Dovitinib, a multi-targeted kinase inhibitor, is being tested in clinical trials for its efficacy in castration-resistant prostate cancer and advanced urothelial carcinoma (Choi et al., 2018; Milowsky et al., 2014).

Knight et al. (2022) suggests that infigratinib and AZD4547 exhibit promising characteristics for BBB penetration, which may enhance their efficacy in treating brain tumors (Knight et al., 2022). Given the evidence of CNS penetration, particularly in regions with BBB disruption, it is reasonable to assume similar activity for all inhibitors examined in this study. The following sections will discuss these inhibitors in greater detail.

1.5 Selective and potent non-selective FGFR tyrosine kinase inhibitors

1.5.1 Selective FGFR inhibitor - infigratinib

Infigratinib is a highly selective FGFR inhibitor of FGFR1, FGFR2, FGFR3, and FGFR4 (Dai et al., 2019; Kang, 2021). Infigratinib binds the ATP-binding cleft of the receptor and inhibits autophosphorylation and downstream signaling, resulting in decreased MAPK activity. *In vitro* studies with hepatocellular carcinoma models showed the selective potency of infigratinib inducing apoptosis and vessel normalization in cells with increased FGFR2-3 expression (Botrus et al., 2021). Also, infigratinib induced dose-dependent (1.4 µM / L) apoptosis and revealed antiproliferative/inhibitory effects against a human leukemia cell line (KG-1 cells) by sufficiently downregulating FGFR1, pAkt and phosphorylated p70 S6 kinase (pS6K) protein

expression (Jiang et al., 2018). In addition to preclinical studies, infigratinib showed promising antitumor effects in clinical trials, mostly in solid tumors including FGFR2-fusion cholangiocarcinoma, and FGFR3-altered urothelial carcinoma (Javle et al., 2021; Pal et al., 2018). Further, ongoing research suggests that infigratinib has a therapeutic potential beyond solid tumors. A study by Rajendran et al. (2023) explored infigratinib's immunomodulatory effects in EAE and revealed that infigratinib treatment led to reduced clinical severity, lowered CNS inflammatory infiltration, and decreased the proportion of $CD19^{+}$ B cells in the spleen during the early phase of the disease, suggesting a potential modulatory effect on peripheral B cells (Rajendran et al., 2023).

1.5.2 FGFR inhibitor - AZD4547

AZD4547 is a selective, orally available small-molecule inhibitor targeting FGFR1–4 (Gavine et al., 2012). In recent literature, AZD4547 is also known as fexagratinib. For clarity and consistency, this thesis uses the original development name, AZD4547, which was current at the time the experiments were conducted and is retained to ensure coherence and traceability. *In vivo*, AZD4547 inhibits proliferation in different cell lines, and demonstrated strong antitumor effects in preclinical models of tumors with abnormal FGFR signaling. AZD4547 inhibits FGFR downstream signaling, including the MAPK cascade

(Gavine et al., 2012). It not only suppresses RAS–MAPK signaling, but also modulates the PI3K pathway, with some studies observing mild upregulation of PI3K-associated gene products (Delpuech et al., 2016). Further the inhibition of ERK, Akt, and S6 have been observed, with selective increased STAT3 activation in responsive cell lines (Phanhthilath et al., 2020).

In medulloblastoma cell lines, FGFR inhibition by AZD4547 decreased proliferation and viability in a dose-dependent manner (between 5 µM to 50 µM) (Lukoseviciute et al., 2020). Significant dose-dependent tumor growth inhibition, and survival were also observed in *in vitro* and *in vivo* studies of gastric cancer (Xie et al., 2013). Modest response rates through application of AZD4547 have been observed in phase IIa trials of endocrine resistant breast cancer (Coombes et al., 2022). Notebly, AZD4547 reduced the growth and proliferation of Erythroblastic Leukemia Viral Oncogene Homolog 2 (ErbB2)-overexpressing human breast cancer cells, and decreased the stem-like properties of these cells, which are often associated with treatment resistance (Zhao et al., 2017). Additionally Chae et al. (2017) demonstrated that AZD4547 had modest efficacy in advanced cancers with FGFR mutations or fusions. Most research on AZD4547 has focused on various cancer cell lines, particularly in the context of tumors with alterations in the FGFR pathway (Babina and Turner, 2017). However, there is a lack of extensive studies investigating the direct impact of AZD4547 on immune cells, including B cells, which could open

new fields for therapeutic applications beyond oncology (Gurski et al., 2025).

1.5.3 Multikinase inhibitor - dovitinib

Dovitinib is an oral multikinase inhibitor targeting several receptors (e.g., FGFRs, VEGFRs, PDGFR-β, CSF-1R, c-Kit, RET, TrkA, and FLT3). It has been extensively studied in various cancer cell lines (Liu et al., 2021). For example in breast cancer studies, dovitinib suppressed cell proliferation in FGFR1- and FGFR2-amplified cell lines and decreased tumor growth in xenograft models with FGFR1 amplification (André et al., 2013). In endometrial cancer, dovitinib exhibited significant growth-inhibitory effects, with the strongest impact observed in cells with FGFR2 mutations. The treatment induced apoptosis, associated with reduced phosphorylation of the ERK and Akt signaling pathway (Konecny et al., 2013). In addition, in cellular models of human colon and colorectal cancer, dovitinib further reduced the proliferation and expression of the downstream signaling molecules pERK and pAkt (Gaur et al., 2014; C. K. Lee et al., 2015). The interactive mechanisms of FGF/FGFR and VEGF/VEGFR and, therefore, inhibiting both pathways simultaneously, revealed its potential to improve treatment efficacy in preclinical cancer studies (Liu et al., 2021). The inhibition of cellular functions, including cellular proliferation and survival, by dovitinib previously observed

in *in vivo* studies, are now transferred and used in anti-cancer treatment. In preclinical and clinical phase I/II tumor studies, dovitinib demonstrated antitumor activity in renal cell carcinoma, gastric cancer, prostate cancer, hepatocellular carcinoma, squamosa cell lung cancer, endometrial cancer, and breast cancer (Babina and Turner, 2017; Fumarola et al., 2017). These studies highlight dovitinib as a promising therapeutic agent across various cancer types, yet further investigation into its mechanisms and clinical applications is needed (Porta et al., 2015).

2 Aims

Recent studies indicate that FGF signaling pathways contribute to the pathogenesis of MS and its disease models, such as EAE, as explained in Section 1.4.2. Using a conditional knockout technique, the Experimental Neuroimmunology Group led by Prof. Dr. med. Martin Berghoff at Justus-Liebig-University Giessen - where this thesis was later conducted - found that the deletion of FGFR in OLs resulted in a less severe disease course, reduced inflammation, myelin loss, and axon degeneration. Notably, within demyelinating lesions, the number of T and B cells was downregulated (Rajendran et al., 2018, Kamali et al., 2021). Complementary pharmacological studies further showed strong anti-inflammatory effects in infigratinib-treated animals. These findings suggest that immunomodulatory mechanisms may occur early during disease onset and treatment, likely within the peripheral immune system (Rajendran et al., 2023).

Building on these findings, I hypothesized in this *in vitro* study, that pharmacological inhibition of FGFR would modulate FGF signaling pathways in B cells, leading to alterations in their proliferation and functional responses. To investigate this, I characterized the effects of three different tyrosine kinase inhibitors - AZD4547, infigratinib, and dovitinib - on FGFR signaling pathways in human BL2 cells. I analyzed their impact on FGFR1/2 expression, key downstream

signaling molecules such as pERK and pAkt, and cytokine production, with the aim of elucidating how FGF/FGFR signaling modulates B cell function. Given the established role of FGF/FGFR signaling in both immune regulation and myelin pathology, investigating its inhibition in immune cells, particularly B cells, is highly relevant to advance the understanding of MS pathophysiology and to identify novel targets for future immunomodulatory therapies.

To address this, the *in vitro* study was designed with the following specific aims:

- Evaluate FGFR1 and FGFR2 expression and its downstream signals (i.e., pERK and pAkt) in BL2 cells after selective FGFR or multikinase inhibition.

- Assess *in vitro* BL2 cell proliferation and cytotoxicity under selective FGFR or multikinase inhibition.

- Investigate the modulation of cytokines by selective FGFR or multikinase inhibition in *in vitro* BL2 cells, to better understand how B cells might contribute to inflammatory processes.

3 Materials and Methods

3.1 Cell line

The human Burkitt lymphoma cell line used in this study was derived in 1979 from the bone marrow of a 7-year-old boy diagnosed with nonendemic Burkitt lymphoma (DSMZ, 2025). In particular, these BL2 cells are EBV negative. The cells are small and exhibit a round to irregular shape, growing in suspension with an approximate rate of cell growth doubling time of 24 hours. These BL2 cells exhibit a MYC-immunglobuline light chain (MYC-IGL) gene fusion (DSMZ, 2025). In BTK studies, which are of growing interest for modulating B cell activity in MS, human Burkitt lymphoma cell lines were successfully used (Chu et al., 2018; Jeong et al., 2023). Thus, highliting the value of BL2 cells for evaluating the effects of tyrosine kinase inhibitors.

Table 2: Cell line.

Cell line	Provider
BL-2 (human Burkitt lymphoma cells)	DSMZ-German Collection of Microorganisms and Cell Culture GmbH

3.2 Materials

The tables 3-13 listed below summarize all the resources utilized in this study. All primers (Tab. 8) were purchased from Eurofins Genomics, Ebersberg, Germany.

Table 3: Primary antibodies.

Name	Species	Reactivity	Mol. weight [kDa]	Method	Article Nr.	Manu-facturer
GAPDH (G-9)	Mouse	H, M, R	37	WB	Sc-365062	Santa Cruz Biotech-nology, Pasco Robles, CA, USA
FGFR1 (Flg M2F12)	Mouse	H, M, R	48-140	WB	Sc-57132	Santa Cruz Biotech-nology, Pasco Robles, CA, USA

Continued on next page

Table 3 – *continued from previous page*

Name	Species	Reactivity	Mol. weight [kDa]	Method	Article Nr.	Manu-facturer
FGFR1	Rabbit	H, M, R	118	IF	ab58516	Abcam, Cam-bridge, UK
FGFR2 (Bek C-8)	Mouse	H, M, R	110/120, 50	WB	Sc-6930	Santa Cruz Biotech-nology, Pasco Robles, CA, USA
FGFR2	Rabbit	H, M, R	145	IF	Ab109372	Abcam, Cam-bridge, UK
Phospho-p44/42 MAPK (Erk1/2)	Rabbit	H, M, R	44/42	WB, IF	#4370s	Cell Sig-naling Tech-nology, Danvers, MA, USA

Continued on next page

Table 3 – *continued from previous page*

Name	Species	Reactivity	Mol. weight [kDa]	Method	Article Nr.	Manu-facturer
Phospho-Akt (Ser473)	Rabbit	H, M, R	60	WB, IF	#4060s	Cell Signaling Technology, Danvers, MA, USA
TNF-α (D5G9)	Rabbit	H	18/25	WB	#6945	Cell Signaling Technology, Danvers, MA, USA
IFN-γ (XMG1.2)	Rat	H, M, R	16	WB	MM700	Invitrogen, Carlsbad, CA, USA
IL-6 (D3K2N)	Rabbit	H	21/28	WB	#12153	Cell Signaling Technology, Danvers, MA, USA

Continued on next page

Table 3 – *continued from previous page*

Name	Species	Reactivity	Mol. weight [kDa]	Method	Article Nr.	Manu-facturer
IL-12A	Rabbit	H, M, R	35	WB	ab133751	Abcam, Cambridge, UK
MCP-1 (CCL2)	Rabbit	H	13-15	WB	#2027	Cell Signaling Technology, Danvers, MA, USA
CX3CL1	Rabbit	H, M, R	95/100	WB	ab25088	Abcam, Cambridge, UK

Table 4: Secondary antibodies.

Name	**Method**	**Article Nr.**	**Manufacturer**
Anti-mouse IgG HRP-linked	WB	#7076	Cell Signaling Technology, Danvers, MA, USA
Anti-rabbit IgG HRP-linked	WB	#7074	Cell Signaling Technology, Danvers, MA, USA
Anti-rat IgG HRP-linked	WB	#7077s	Cell Signaling Technology, Danvers, MA, USA
Alexa Fluor 488 F(ab´)2 fragment of goat anti-rabbit IgG (H+L)	IF	#400884	Invitrogen, Carlsbad, CA, USA

Table 5: Ladders for western blot.

Marker	**Article Nr.**	**Manufacturer**
PageRuler^{TM} Prestained Protein Ladder	#26616	Thermo Fisher Scientific, IL, USA
PageRuler^{TM} Plus Prestained Protein Ladder	#26619	Thermo Scientific, IL, USA

Table 6: Kits.

Kit	Manufacturer	Article Nr.	Method
BCA Protein Assay Kit	Thermo Scientific, Rockford, USA	23227	Protein quantification
Cell Proliferation Reagent WST-1	Roche Applied Science, Mannheim, DE	11644807001	Proliferation assay
Cytotoxicity Detection Kit (LDH)	Roche Applied Science, Mannheim, DE	11644793001	Cytotoxicity assay
iTaq Universal SYBR Green qPCR Supermix	Bio-Rad, CA, USA	1725124	PCR
QuantiTect Reverse Transcription Kit	Qiagen GmbH, Hilden, DE	205313	Reverse transcription
RNeasy Mini Kit (50)	Qiagen GmbH, Hilden, DE	74104	RNA isolation
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Scientific, Rockford, USA	34580	Western blot

Table 7: Buffers.

Buffer	Components	Volume
1x SDS-PAGE Running Buffer	Rotiphorese 10x running buffer, H_2O	100ml, 900ml
10x PBS (1L) pH 7.4	137mM NaCl, 2mM KH_2PO_4, 2.7mM KCl, 10mM Na_2HPO_4, H_2O	80g, 2.4g, 2g, 14.4g, 1000ml
10x TBS (1L) pH 7.2 to 7.6	Tris, NaCl, H_2O	24.2g, 87.7g, 1000ml
1x TBS-Tween (TBST) (1L) washing buffer	1xTBS, 0.1% Tween20	1000ml, 1ml
Lysis buffer (250ml) pH 7.4	NaCl, Tris, EDTA, Glycerol, NP40, NaN_3	2.19g, 0.61g, 0.07g, 25ml, 2.5ml, 0.025g
6x SDS-PAGE loading buffer	60mM Tris-HCl (pH 6.8), 2% SDS, 0.01% Bromophenol blue, 10% Glycerol, ddH_2O, ß-Mercaptoethanol	36ml, 60ml, 60mg, 60ml, 144ml, 65µL/ml
SDS-PAGE Transfer buffer (1L)	Rotiphorese 10x running Buffer, Methanol, ddH_2O	100ml, 200ml, 700ml
Blocking buffer for WB (5% BSA), Bovine Serum Albumin	BSA fraction V, TBST	5g, 100ml

Continued on next page

Table 7 – *continued from previous page*

Buffer	**Components**	**Volume**
Blocking buffer for IF (5% BSA), Bovine Serum Albumin	BSA fraction V, PBS	5g, 100ml
10x Trypsin EDTA	10x Trypsin, ddH_2O	1g, 10ml
10% Ammonium Persulfate (APS)	APS, ddH_2O	1g, 10ml
10% Sodiumdodecylsulfate (SDS)	SDS, ddH_2O	1g, 10ml

Table 8: Primers sequences for the genes of interest.

Gene	**5' $\rightarrow$ 3' Sequence**
Hu GAPDH	Forward ACAACTTTGGTATCGTGGAAGG
	Reverse GCCATCACGCCACAGTTTC
Hu FGFR1	Forward CCAAAGACGGTCGTTTAGTGG
	Reverse ACAGCCAAAGTAAAGTCAAGGTT
Hu FGFR2	Forward ACAGTTTCGGCTGAGTCCAG
	Reverse GGTGTCTGCCGTTGAAGAGA
Hu IL1β	Forward ATGATGGCTTATTACAGTGGCAA
	Reverse GTCGGAGATTCGTAGCTGGA
Hu IL6	Forward ACTCACCTCTTCAGAACGAATTG
	Reverse CCATCTTTGGAAGGTTCAGGTTG
Hu IL12A	Forward CCTTGCACTTCTGAAGAGATTGA
	Reverse ACAGGGCCATCATAAAAGAGGT
Hu IL21	Forward GGCAAGACCAGTATGAAGAGC
	Reverse TGACACTGAAAATGTCGTCGG
Hu CX3CL1	Forward ACCACGGTGTGACGAAATG
	Reverse TGTTGATAGTGGATGAGCAAAGC
Hu CCL2	Forward GCAATCAATGCCCCAGTCAC
	Reverse GACACTTGCTGCTGGTGATTC
Hu IFNγ	Forward TCGGTAACTGACTTGAATGTCCA
	Reverse TCGCTTCCCTGTTTTAGCTGC
Hu TNFα	Forward GAGACAGATGTGGGGTGTGAG
	Reverse AGCTGTCATATTTCCCGCTC

Table 9: Treatments used for experiments. AZD4547 refers to the compound now known as fexagratinib.

Compounds	**Name**	**Company**	**Cat. No.**	**Treatment Concentration**
AZD4547	ABSK 091	Selleck Chemicals, TX, USA	S2801	1 µM
Infigratinib	NVP-BGJ398	Selleck Chemicals, TX, USA	S2183	1 µM
Dovitinib	TKI-258	Selleck Chemicals, TX, USA	S1018	1 µM
FGF2	FGF basic/bFGF	R&D Systems, MN, USA	233-FB	25 ng/mL
DMSO	Dimethylsulfoxide	Carl Roth, Karlsruhe, DE	A994.1	1 ng/mL

Table 10: Chemicals used in the experiments.

Compound	Manufacturer
10x PBS for cell culture (DPBS)	PAN Biotech, Aidenbach, DE (Art.-Nr. P04-53500)
2-Mercaptoethanol	Sigma-Aldrich, Taufkirchen, DE
2-Propanol	Sigma-Aldrich, Taufkirchen, DE
Albumin Fraktion V	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 8076.3)
Ammonium persulphate (APS)	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 95923)
Aqua Resist	VWR International, Radnor, USA (Cat.-No. 462-7000)
Blot Stripping Buffer (Restore PLUS Western)	Thermo Scientific, Rockford, USA (Art.-Nr. 46430)
Bovine Serum Albumin (BSA)	Capricorn Scientific, Ebsdorfergrund, DE (Cat.-No. BSA-1T)
DAPI staining solution	Carl Roth, Karlsruhe, DE
Dimethylsulfoxid (DMSO)	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. A994.1)
DNase free H_2O	Millipore, Burlington, USA
Ethanol (100%)	Otto Fischer GmbH, Saarbrücken, DE

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Table 10 – *continued from previous page*

Compound	Manufacturer
Ethanol absolute ($\geq$99.8%)	Merck KGaA, Darmstadt, DE (M 32205-1I-M)
Fetal Bovine Serum (FBS)	Gibco, Invitrogen, Carlsbad, USA (Art.-Nr. 10270-106)
Fluorescence Mounting Medium	DAKO Agilent, CA, USA
Glycerol	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 3783.1)
Glycin	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 3790.2)
Glycerin	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 3783.1)
Methanol	Merck, Darmstadt, DE
Nonfat Dry Milk	Cell Signaling Technology, Danvers, MA, USA (9999S)
Nail Polish	Flormar, Turkey
Paraformaldehyde (PFA)	Sigma Aldrich, Taufkirchen, DE
Penicillin Streptomycin as medium supplement	Gibco, Invitrogen, Carlsbad, USA (Art.-Nr. 15070-063)
Prolong Gold Antifade Reagent mounting medium for IF	Cell Signaling Technology, MA, USA
Protease/Phosphatase Inhibitor cocktail	Roche, Mannheim, DE

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Table 10 – *continued from previous page*

Compound	**Manufacturer**
RNAse free H_2O	Millipore, CA, USA
Rotiphorese Gel (30% acryl-bisacrylamide mix)	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 3029.1)
Rotiphorese 10x SDS-PAGE	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 3060.2)
Roti-Load 1	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. K929.2)
RPMI Medium 1640 (1x)	Gibco, Invitrogen, Carlsbad, USA (Art.-Nr. 21875-034)
Stacking Gel Buffer pH 6.3	Bio Rad, Munich, DE
Sodium chloride (NaCl)	Carl Roth GmbH, Karlsruhe, DE
Sodiumdodecylsulfate (SDS)	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 2326.4)
Tetramethylethylendiamin (TMEDA)	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 2367.3)
Tris HCl	Carl Roth GmbH, Karlsruhe, DE (Art.-Nr. 9090.3)
Tris HCl buffer, pH 8.8 (Resolving Gel Buffer)	Bio Rad, Munich, DE
Tris/Glycine/SDS Buffer 10x	Bio Rad, Munich, DE
Trishydroxymethylaminomethan (Tris)	Carl Roth GmbH, Karlsruhe, DE
Triton X-100 solution	Sigma-Aldrich, Steinheim, DE

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Table 10 – *continued from previous page*

Compound	**Manufacturer**
Trypan Blue	Carl Roth GmbH, Karlsruhe, DE
Trypsin-EDTA 0.5% (10x)	Gibco, Invitrogen, Carlsbad, USA
Tween 20	Merck KGaA, Darmstadt, DE
Non-fat dry milk	Cell Signaling Technology, Danvers, USA
Restore PLUS western blot Stripping Buffer	Thermo Scientific, Rockford, USA

Table 11: Laboratory consumables.

Consumables	**Manufacturer**
Cellstar 6-well, 12-well and 96-well Cell Culture Plate	GreinerBioOne, Frickenhausen, DE
Cellstar U-shape with Lid, TC-Plate, 96-wells, sterile	GreinerBioOne, Frickenhausen, DE
Cellstar Plastikpipettes (5 mL, 10 mL, 25 mL)	GreinerBioOne, Frickenhausen, DE
Cellstar cell culture flasks (25 cm^2, 75 cm^2)	GreinerBioOne, Frickenhausen, DE
Cellstar sterile glass pipettes (5 mL, 10 mL, 25 mL)	GreinerBioOne, Frickenhausen, DE
Combs for SDS gels	BioRad, Munich, DE
Micro Centrifuge tube	Nerbe plus GmbH, Winsen, DE

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Table 11 – *continued from previous page*

Consumables	**Manufacturer**
Micro Amp Fast Reaction Tubes	Applied Biosystems, Darmstadt, DE
Micro Amp 8-Cap	Applied Biosystems, Darmstadt, DE
Cryobox	Ratiolab GmbH, Dreieich, DE
Cryo Tube TM vials (1.8 mL, 4.5 mL)	Sarstedt AG & Co, Nümbrecht, DE
Coverslips (24x36mm, 0.13-0.16 thick)	R. Langenbrinck GmbH, Emmendingen, DE
Falcon tubes (15 mL, 50 mL)	GreinerBioOne, Frickenhausen, DE
Glass Pasteur pipettes (150 mm)	Brand, Wertheim, DE
Ministart Syringe Filter (0.2 µm)	Sartorius Stedim Biotech GmbH, Göttingen, DE
Micro tube (1.5 mL)	SARSTED AG & Co., Nürnbrecht, DE; Nerbe plus GmbH, Winsen, DE
Pipette tips sterile (10 µL, 20 µL, 100 µL, 200 µL)	Nerbe plus GmbH, Winsen, DE
Filter paper for transblotting	BioRad, Munich, DE
Pipette tips (nonsterile) (10 µL, 100 µL, 200 µL, 1000 µL)	SARSTED AG & Co., Nürnbrecht, DE
Eppendorf tubes (1.5 mL, 2 mL)	Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, DE
Glass plates for SDS gels	BioRad, Munich, DE
Glasswares (different sorts)	Schott AG, Mainz, DE; VITLAB, Grossostheim, DE

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Table 11 – *continued from previous page*

Consumables	**Manufacturer**
AmershamTM ProtranTM Nitrocellulose blotting membrane (0.2 µm)	GE Healthcare, Buckinghamshire, UK
Microscope Slides	R.Langenbrinck GmbH, Emmendingen, DE
MicroAmp®Fast Reaction Tubes	Applied Biosystems, Darmstadt, DE
MicroAmp®Optical Cap Strips	Applied Biosystems, Darmstadt, DE

Table 12: Chemicals used in the experiments.

Instrument	**Manufacturer**
Axioplan 2 Fluorescence Microscope	Carl Zeiss, Jena, DE
Multiscan EX (ELISA-Reader)	Thermo Electron Corporation, Langenselbold, DE
Casting frame SDS gels	BioRad, Munich, DE
Cell culture cabinet	NuAire, Plymouth, USA
Centrifuge Universal 32 R	Hettich GmbH, Tuttlingen, DE
Centrifuge cell culture	Sigma, Osterode am Harz, DE
Centrifuge Universal (cooling)	Hettich, Tuttlingen, DE
Consort EV231 Power Supply for Electrophoresis	Sigma-Aldrich, Steinheim, DE
Microscope for cell culture	A. KRÜSS Optic GmbH, Hamburg, DE

Continued on next page

Table 12 – *continued from previous page*

Instrument	**Manufacturer**
Mini-Protean Tetra Cell for SDS-PAGE	BioRad, Munich, DE
Magnetic stirrer	IKA Werke GmbH, Staufen, DE
Nanophotometer	Implen GmbH, Munich, DE
pH-Meter (HANNA)	Merck KGaA, Darmstadt, DE
Pipette boy	Integra Biosciences GmbH, Fernwald, DE
Pipette different types	BIOHIT Proline Plus, Merck KGaA, Darmstadt, DE; Eppendorf, Hamburg, DE
Refrigerators and freezers (+4℃, -20℃, -80℃)	Different companies
SANYO Incubator for cell culture	Ewald Innovationstechnik GmbH, Bad Nenndorf, DE
Scale (New Classic MS)	Mettler Toledo, Gießen, DE
StepOne Real-Time PCR System	Applied Biosystems, Darmstadt, DE
Vortexer Vortex-Genie2	Heidolph Instruments GmbH & Co. KG, Schwabach, DE
Water bath	Memmert GmbH & Co. KG, DE
Western blotting system	BioRad, Munich, DE
Thermomixer (1.5 mL)	Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, DE

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Table 12 – *continued from previous page*

Instrument	**Manufacturer**
Trans-Blot SD (Semi-Dry Transfer Gel)	BioRad, Munich, DE
ECL Chemocam Imager	Intas-Science-Imaging Instruments GmbH, Göttingen, DE
Rotating mixer RM5	Assistant, Sondheim vor der Rhön, DE
Feather Disposable Scalpel	Feather, Osaka, JPN
Neubauer Improved Chamber	Karl Hecht “Assistant”, Altnau TG, CH
Secure work bench	NUAIRE Integra Biosciences, Fernwald, DE
PCR & qPCR Thermocycler	Biometra Bioscience, Analytik Jena GmbH, Jena, DE
Micro Amp 48-Well Base	Applied Biosystems, Darmstadt, DE
Thermocycler MyCycler	BioRad, Munich, DE

Table 13: Software used for analysis and imaging.

Software	Manufacturer
ChemoStar Imager (0.4.18.0, 2016)	Intas-Science-Imaging Instruments GmbH, Göttingen, DE
MacOS	Apple Inc., USA
Microsoft Office 2018	Microsoft Corporation, USA
SigmaPlot 10 software	Systat, San Jose, CA, USA
Image J software (ImageJ 1.52a)	National Institute of Health, USA
StepOne RealTime PCR Software v2.1	Applied Biosystems, Darmstadt, DE
Graph Pad Prism Software Version 9	GraphPad Software, Inc., CA, USA
ZEN software for microscope (Zen 2.3)	ZEISS, Jena, DE

3.3 Methods

3.3.1 BL2 cell culture

The human Burkitt lymphoma cells (BL2), provided by the Leibniz Institute DSMZ (DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, DE), were cultured in suspension. The growth medium consisted of 80% Rosewell Park Memorial Institute (RPMI-1640) (Gibco, Invitrogen, Carlsbad, USA) medium supplemented with 20% Fetal Bovine Serum (FBS) (Gibco, Invitrogen, Carlsbad, USA) and 1% penicillin / streptomycin (Gibco, Invitrogen, Carlsbad, USA). Cells were seeded in 12-15 ml of growth medium

in T75 flasks at a concentration of 0.5×10^6 cells / mL. They were maintained at 0.5 cells to 2×10^6 cells / mL, growing at 37 ℃ in an incubator in a controlled atmosphere, with 95% humidity and 5% CO_2. At maximum confluency of 80%, cells were sub-cultured and resuspended in fresh prewarmed growth medium or used for experimental treatments.

Thawing and freezing BL2 cells

Cells were stored at -80℃ in 2 ml cryotubes and thawed at room temperature. When thawed, the freezing medium was diluted with growth medium and centrifuged (4 min at 12000 rpm). The supernatant was then removed, and the spare pallet was gently resuspended in 1 ml of prewarmed growth medium for cell count. Subsequently, the resuspended cells were added to a 15 ml prewarmed growth medium in appropriate cell culture flasks. Thawed cells were divided at least two times before they were used in experiments. For freezing, cells were collected from the flasks and centrifuged (4 min at 12000 rpm). The cell pellet was dissolved in a 1.5 ml freezing medium and added to cryotubes. This freezing medium contained 20% FBS RPMI and 10% DMSO to prevent cell damage while cells were stored at -80℃ or in liquid nitrogen.

Cell counting of BL2 cells

Cells were observed daily for quality control under the microscope and counted. Notably, the Neubauer improved chamber is suitable

for counting cultivated cells and was used regularly to determine the general growth parameters of the BL2 cells. The cell count was performed to ensure predetermined cell concentrations for all experiments. Cell counting with this method used 10 µL of cell suspension mixed with 10 µL Trypan Blue. This compound helps distinguish dead cells from living cells because, in contrast to viable cells, dead cells with damaged membranes absorb the dye. The four corner squares were used to count the viable cells, following the scheme in Figure 7. The mean value of the cell count in all four large squares was multiplied by 10^4 to determine the cell concentration per milliliter. The value of the cell concentration per milliliter was then multiplied by the dilution factor to estimate the total number of viable cells in the volume of suspension (Gstraunthaler and Lindl, 2021).

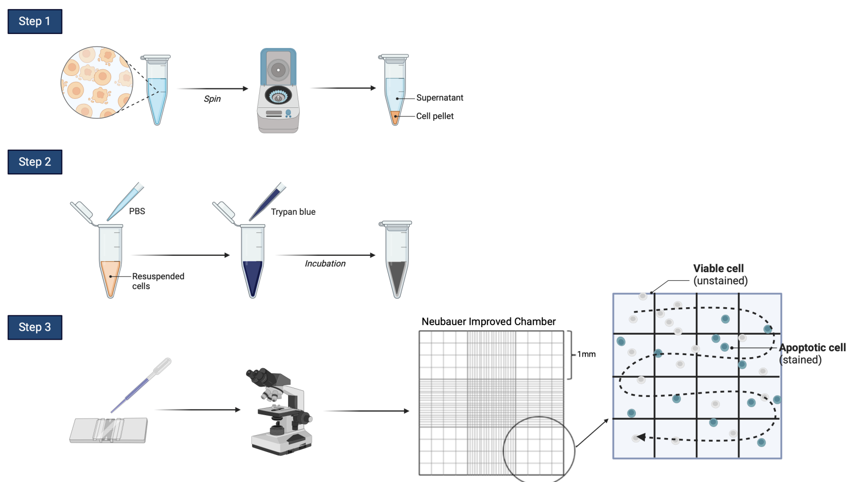

Figure 7: Cell viability assay conducted using the Trypan Blue exclusion method and cell counting with a Neubauer improved counting chamber. The Trypan Blue exclusion assay, steps 1 through 3 were followed for assessing cell viability. Cell counting was performed under a microscope, distinguishing between stained dead cells and unstained viable cells. Viable cells within the large square of the Neubauer improved counting chamber were counted according to the illustrated orientation diagram. Image created with BioRender (biorender.com).

3.3.2 Experimental treatment of BL2 cells

For each experiments described in the paragraphs below, groups of cells were subjected to five different treatments: addition of AZD4547 (1 µM now known as fexagratinib; Selleck Chemicals, TX, USA), addition of infigratinib (1 µM; Selleck Chemicals, Houston, TX, USA), addition of dovitinib (1 µM; Selleck Chemicals, TX, USA), addition of FGF2 (25 ng/mL; R&D Systems, Minneapolis, MN, USA), addition of DMSO (1ng/ml; Carl Roth, Karlsruhe, DE) or no added chemical compound to serve as a control (Tab. 14).

Table 14: The experimental treatment group setup.

Control	AZD4547	Infigratinib	Dovitinib	FGF2	DMSO
No treatment	1 µM	1 µM	1 µM	25 ng/mL	1 µM

All results and interpretations presented under the name AZD4547 refer to the compound now commanly known as fexagratinib, since the name AZD4547 was commonly used at the time this study was initiated and is consistently applied throughout the experimental setup and data analysis, the original designation is retained for clarity and consistency. Until use, AZD4547, infigratinib, dovitinib, and FGF2 were dissolved in 1% BSA in DPBS (Biotech, Aidenbach, DE). All compounds were added to RPMI growth medium at a concentration of 1 µM (AZD4547, Infigratinib, Dovitinib) and 25 ng / mL (FGF2) and incubated for 24 hours. Later, an appropriate number of cells was used for each experiment and for further analysis. All experiments were performed three times and the following treatment setup was used in triplicates (Tab. 14). Figure 8 illustrates the experimental treatment procedure for BL2 cells used in this study.

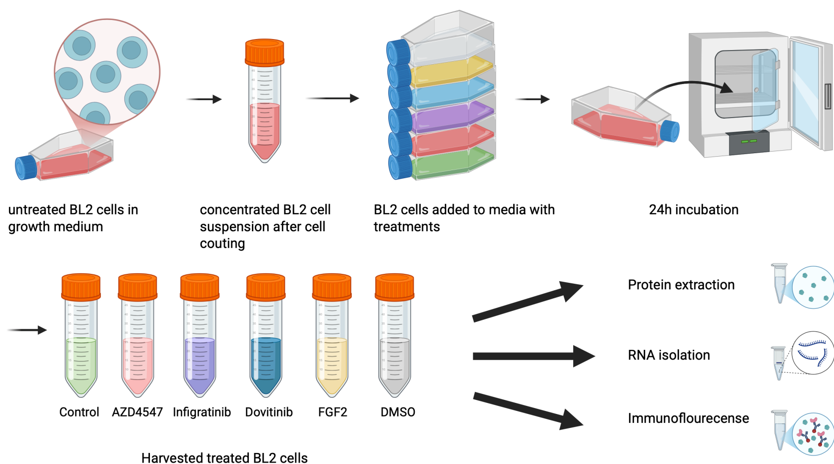

Figure 8: Simplified illustration of the experimental treatment procedure for BL2 cells. The schematic outlines the workflow used to treat BL2 cells with various compounds. Exponentially growing BL2 cells were collected by centrifugation, the supernatant was removed, and the pellet was resuspended to reach a defined cell concentration. This suspension was transferred into flasks pre-filled with growth medium containing treatment compounds at concentrations exceeding the final desired level. Mixing the cell suspension with this medium resulted in dilution of the compounds to the target concentration. Following 24 hours of incubation, cells were harvested for analyses of protein, RNA and immunoflouresence. Image created with BioRender (biorender.com).

3.3.3 Cell proliferation assay

In this study, a photometric evaluation was conducted to analyze the number of active metabolic cells in the cell culture under the different experimental treatments. A WST-1 (water-soluble tetrazolium salt-1) assay (Roche Applied Science, Mannheim, Germany) provides a method to detect induction and inhibition of cell proliferation *in*

vitro. The assay is based on the reduction of tetrazolium salt WST-1 to solvable formazan by mitochondrial conversion. Dependent on enzyme activity, different absorbance spectrums in ELISA reading are detected and correlated with the number of viable cells in each well (Roche, Cell Proliferation Reagent WST-1). The cells were seeded in a 96-well plate (CELLSTAR, Greiner Bio-One, Frickenhausen, Germany) at a density of 5×10^4 cells per well. For each experimental group, treatments were performed in technical triplicates (three wells per group) across all three independent experiments. The treatments were applied as described in Section 3.3.2. After 24 hours of incubation with the indicated substances, 10 µL of the WST-1 cell proliferation reagent was added to each well to obtain a final volume of 100 µL/well. Wells were incubated for 4 hours in a humidified atmosphere (37 ℃, 5% CO_2). Then the wellplate was shaken for 1 min, and the absorbance was measured against a blank as a background control, using a microplate ELISA-Reader (Multiscan EX, Thermo Fisher Scientific, Langenselbold, Germany) at 450 nm and the reference wavelength of 620 nm. The relative proliferation was calculated according to the protocol provided in the manufacturer's datasheet for the cell proliferation reagent WST-1 (Roche, content version: Cell Proliferation Reagent WST-1, content version: Feb. 2011).

3.3.4 Cytotoxicity assay

The “Cytotoxicity Detection Kit (LDH)” assay from Roche Diagnostics (Mannheim, Germany) was performed to examine the possible cytotoxic effects of the different treatment compounds. This method measures the lactate dehydrogenase (LDH) released into the supernatant by damaged cells after treatment. LDH is considered to be a stable cytoplasmic enzyme in all cell types. nicotinamide adenine dinucleotide (NADH) produced by the reversible reaction of Pyruvate to Lactate catalyzed by LDH can be used for the conversion of yellow-colored tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium) to the red colored water-soluble formazan salt. This can be measured by absorbance at 492 nm since only formazan shows significant absorption at this wavelength. The measured absorbance is directly proportional to the amount of LDH in the supernatant of the cell culture. Because LDH is rapidly released when the plasma membrane is damaged, the LDH level directly correlates with the number of damaged cells and the number of cells undergoing apoptosis and necrosis and can, therefore, indicate the cytotoxic effects on BL2 cells (Kumar et al., 2018). To perform this assay, the cells were seeded in a 96-well U-bottom plate at a concentration of 2×10^6 cells / mL and treated for 24 hours, as described in Section 3.3.2. Each well contains a final volume of 100 µL per well and a concentration of 1×10^4 cells per well. Cell suspension with Triton

X100, which results in high cell lysis, was used as a high control, while a cell-free suspension served as blank. Following incubation, the plate was centrifuged for 10 min. Ensuring strict conservation of cell debris, only the supernatant was carefully transferred to a flat-bottom tissue culture plate and 85 µL of LDH reagent was added to each well. The plate was then kept at room temperature for 20 min, protected from light. The absorbance was measured at 492 nm and 620 nm as a background control, using an ELISA-Reader (Multiscan EX) to calculate cytotoxicity according to the manufacturer's protocol (Roche Diagnostics, Cytotoxicity Detection Kit, version 7 March 2016).

3.3.5 Protein biochemistry

Protein extraction and quantification

Prior to protein extraction, all cells (>5 × 10^6 cells per group) were treated for 24 hours in T-75 flasks at 37 ℃ as described in Section 3.3.2. Medium removal and a washing step with PBS by centrifugation was (4 min at 1200 rpm) performed. Once PBS was removed, 500 µL of lysis buffer was added and mixed gently with the cell pellet. After 30 min of incubation on ice, cells were again centrifuged at 14000 rpm for 30 min at 4 ℃, and the supernatant was collected. The supernatant was used for protein quantification. The protein concentrations of the cell lysate were quantified using a nanophotometer (IMPLEN GmbH, Munich, Germany) according to the manufacturer's instructions. The

protein level for each sample was normalized (2 µg/µL) and stored at -20℃ until further use.

SDS-PAGE and western blot

Western blot is a laboratory technique for the special identification and characterization of proteins. Proteins are separated by their molecular weight through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Next, the separated proteins are electrophoretically transferred to a nitrocellulose membrane. This process is described as transblotting. The membrane is then incubated with a specific primary antibody, binding the protein of interest. After the application of the secondary antibody, which binds the primary antibody and is connected to an enzyme called horseradish peroxidase (HRP), which converts the substrate to a chemiluminescent signal. This signal allows the detection of the protein of interest using a camera (Mahmood and Yang, 2012). In this experiment, 10 µL loading dye protein buffer was added to 30 µL protein (60 µg protein) from each treatment group and denaturalized through 5 min heat application at 95℃. 35 µL (60 µg protein) of the normalized samples were loaded and separated by 10% SDS-PAGE gel electrophoresis. The first step for running the gel was a program for 30 min at 1: 90 V, 300 mA, 150 W, followed by 120 V, 1000 mA, and 150 W for 2 hours and 30 min. The separated proteins were then transblotted (25 V, 250 mA, 150 W for 1 hour and 10 min) to a nitrocellulose

membrane and blocked with 5% BSA for 1 hour. The primary antibody was then supplemented in the appropriate concentration, diluted in TBST with 5% BSA and incubated overnight at 4℃. The antibodies used in this study are listed in process can be found in Tabel 3 and Tabel 4. The next day the membrane was washed with 1xTBST three times for 5 min before the secondary antibody (concentration 1:1000), diluted in non-fat dry milk, was added and incubated for 1-2 hours at room temperature. Then again, washed three times for 5 min with 1xTBST buffer. 2ml per membrane of SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) was then added to the membrane for 3 min and used for protein visualization. The visualized protein bands were developed in an ECL Chemocam Imager (Intas-Science, Göttingen, Germany). As a loading control, GAPDH was used for all protein analyses. The protein bands were quantified using ImageJ software (NIH, Bethesda, Maryland, USA) with the untreated control samples and the GAPDH as control references.

3.3.6 Molecular biology

RNA extraction and quantification

For RNA extraction, 1×10^6 cells per group were treated with the FGFR inhibitors according to their assigned groups and incubated for 24 hours as explained in Section 3.3.2. Next, media was removed,

and cells were washed with PBS and centrifuged (4 min at 1200 rpm) in order to later remove the washing buffer. For RNA extraction, the RNeasy Mini Kit (QIAGEN, Hilden, Germany) protocol was followed. A small amount (1 µL) of RNA was quantified with the nanophotometer, according to manufacturer's instructions, with RNAse free H_2O as a blank value. The RNA was normalized (1 µg / 20 µL), diluted in RNAse-free H_2O and stored at -20℃ until further use. Reverse transcription and cDNA synthesis Reverse transcription was performed to obtain a complementary DNA strand (cDNA) from our extracted RNA. A small amount (1 µg) of RNA was used for subsequent cDNA synthesis with the QuantiTect Reverse Transcription Kit (Thermo Fisher Scientific, QIAGEN, Hilden, Germany). To 20 µL of diluted (1 µg/20 µL) RNA, 3 µL genomic DNA wipe out buffer was added and incubated for 2 min at 42℃. Then 6 µL cDNA synthesis master mix was prepared for all samples, containing 4 µL RT buffer, 1 µL RT primer, and 1 µL RT master mix. This master mix was added and incubated for 30 min at 42℃ and 3 min at 95℃. Next, 170 µL RNAse free H_2O was complemented. The total volume of 200 µL synthesized cDNA was stored at -20℃ for qRT-PCR.

Relative reverse transcription PCR quantification of BL2 cells was performed in 40 repetitive cycles, consisting of 15 sec of denaturation at 95℃ followed by 1 min of annealing at 60℃. The amplified DNA was heated up to 95℃, and the absorbance was measured. The StepOne Real-Time PCR system (Applied Biosystems, Darmstadt,

Germany) with StepOne TM Software v2.3 (Applied Biosystems, Darmstadt, DE) was utilized. cDNA (1000 µg) was added to a master mix prepared beforehand in Micro Amp Fast Reaction Tubes (Applied Biosystems, Darmstadt, Germany) for each sample. This master mix contained 10 µL SYBR-Green qPCR Supermix (Bio-Rad, CA, USA), 1 µL forward primer, 1 µL reverse primer, and 7 µL H_2O.

The primers used to quantify the target genes are listed in Tabel 8. GAPDH was used as an internal control gene. Only the data, with template cDNA in the technical duplicates, was included for the gene expression analysis of all three biological replicates.

The Delta CT method (Eq. 1) was implemented for the evaluation of the relative quantification of gene expression (Livak and Schmittgen, 2001).

$$\Delta C_T = 2^{-\Delta C_T(\textit{target gene} - \textit{control gene})} \tag{1}$$

3.3.7 Immunoflourecence

Immunofluorescence was performed to visualize specific proteins within cultured BL2 cells (Im et al., 2019). All treated and untreated cells were stained with a specific primary antibody (Tab. 3), which emits fluorescence under a fluorescence microscope when bound by a fluorophore-conjugated secondary antibody (Tab. 4). For this experiment, cells were seeded (density 5x105 cells/group) in 6-

well plates and treated according to the procedures outlined in Section 3.3.2 for their respective experimental groups. After 24 hours incubation, cells were collected and added to a 2 mL epitube. After centrifugation, the cells were washed with 200 µL PBS and fixed in 500 µL 4% PFA, incubating for 15 min at room temperature. Then the cells were centrifuged again, followed by another washing step with PBS. Afterward, for the process of permeabilization, the cells were incubated 15 min at room temperature in PBS with 0.1% Titron-X100, to facilitate antibody penetration. After incubation, the cells were again washed with PBS and centrifuged. To minimize non-specific binding, BL2 cells were blocked with 5% BSA (in 0.3% Titron X-100 in PBS) for 1 hour at room temperature before adding the primary antibody (Tab. 3). After another washing step with PBS and centrifugation, the primary antibody targeting the protein of interest (Tab. 3), diluted in 1% BSA in PBS was added and incubated overnight at 4℃. The next day, the secondary fluorescent antibody (Tab. 4) diluted in 1% BSA in PBS was added and incubated for 1 hour at room temperature after another step of washing with PBS and centrifugation. After 1 hour of incubation, the cells were washed with 0.1% Triton X in PBS and centrifuged. To the leftover pellet, 300 µL DAPI stain (1 µg / mL) was added, gently mixed, and incubated for 10 min at 4℃, and protected from light. The cells were then centrifuged, and the DAPI was removed. The pallet was gently vortexed, mounted onto microscope slides, and fixated with DAKO Fluorescence Mounting

Medium (Carpinteria, CA, USA). The slides were then sealed with nail polish and stored at 4℃ until use. Images were taken with an Axioplan 2 fluorescence microscope (Carl Zeiss, Jena, Germany) at a magnification of 50x and 400x using ZEN software (Zen 2.3, ZEISS, Jena, Germany) for image acquisition and processing. On each slide, fluorescence intensity and signal localization were assessed in 30 cells selected from 10 randomly chosen areas across the entire slide. Cells exhibiting high-intensity staining were identified and compared to the total cell population determined by DAPI counterstaining. These high-intensity cells were classified as positive or intensively stained cells (Fig. 9). Positive staining was quantified by calculating the proportion of fluorescence-positive cells relative to the total number of cells in the sample (Eq. 2).

$$\textit{\% of positive cells} = \frac{\textit{DAPI positive cells}}{\textit{total number of cells}} \times 100 \qquad (2)$$

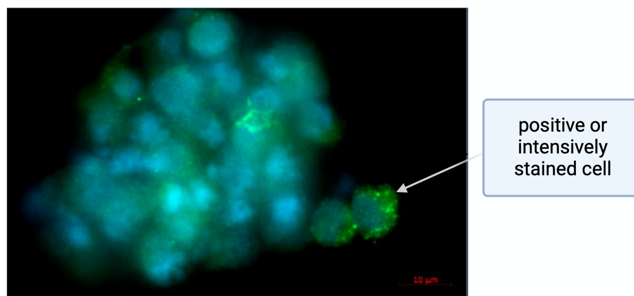

Figure 9: Example of positive, intensively stained cell. The image was captured at 10 µm magnification.

3.3.8 Statistical analysis

All experiments were conducted with a minimum of three independent replicates at least three times. Statistical analysis for proliferation assays, cytotoxicity, real-time PCR data, and protein quantification was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. The software GraphPad Prism (version 9.5.0; GraphPad Software Inc., Boston, MA, USA) for macOS was utilized for statistical analysis. Results are presented as mean ± standard error of the mean (SEM). The control group was set as the baseline at 100%. For protein and real-time PCR analyses, the SEM of the control group is not shown separately, as all relative values are normalized to this baseline. P values of $p < 0.05$ were considered statistically significant (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$).

4 Results

4.1 All substances do not affect the proliferation of BL2 cells

Considering the critical role of FGF/FGFR signaling pathways in regulating cell development and growth (Nobuyuki Itoh and David M. Ornitz, 2011), we investigated the effect of FGFR inhibition on BL2 cell proliferation. A WST-1 assay performed after treatment revealed no significant effect on BL2 cell proliferation compared to untreated cells, by any of the FGFR inhibitors (Control vs. AZD4547: $p = 0.9935$; Control vs. Infigratinib: $p > 0.9999$; Control vs. Dovitinib: $p = 0.8771$; Control vs. DMSO: $p = 0.4662$). Furthermore, the application of FGF2 did not alter BL2 cell proliferation (Control vs. FGF: $p = 0.9965$) (Fig. 10).

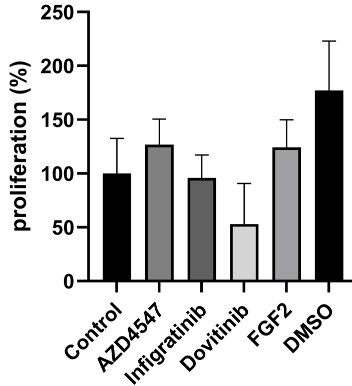

Figure 10: Cell proliferation of BL2 cells incubated with AZD4547, Infigratinib, Dovitinib, FGF2, and DMSO. These inhibitors did not affect BL2 cell proliferation compared to untreated cells. FGF2, and DMSO application did not alter BL2 cell proliferation. Data are presented as mean ± SEM. Values for control cells were normalized to 100%, and all other results were considered in relation to control values.

4.2 The FGFR inhibition has no cytotoxic impact on BL2 cells

An LDH assay was performed to detect possible cytotoxic effects of FGFR inhibition in BL2 cells and further study the role of FGFR inhibition on BL2 cell survival. FGFR inhibitors did not exert cytotoxic effects on BL2 cells compared to untreated cells (Control vs. AZD4547: $p = 0.6669$; Control vs. Infigratinib: $p = 0.2650$; Control vs. Dovitinib: $p = 0.9056$; Control vs. DMSO: $p = 0.5176$). Furthermore, the application of FGF2 had no cytotoxic impact on BL2 cells (Control vs. FGF: $p = 0.1897$) (Fig. 11).

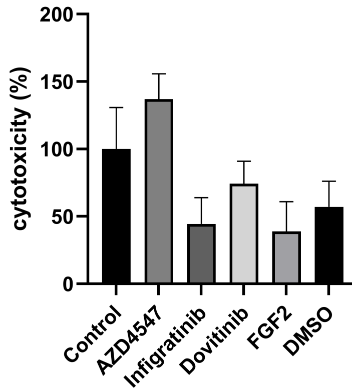

Figure 11: Cytotoxic effects of FGFR inhibition with AZD4547, Infigratinib, Dovitinib, and the application of FGF2 and DMSO on BL2 cells. FGFR inhibitors did not exert cytotoxic effects in BL2 cells. Data are presented as mean ± SEM. Values for control cells were normalized to 100%, and all other results were considered in relation to control values.

4.3 The multikinase inhibitor dovitinib decreases cellular FGFR1 expression

To investigate the expression of FGFR1 in BL2 cells after FGFR inhibition, western blot, IF and qRT-PCR were performed. The application of the multikinase inhibitor Dovitinib resulted in a decreased number of BL2 cells that exhibited a high staining intensity for FGFR1 (Control vs. Dovitinib: $p = 0.0110$) compared to untreated cells (Fig. 12A, 12B). IF staining intensity for FGFR1 was not altered by the selective inhibitors Infigratinib and AZD4547 compared to the control

(Control vs. AZD4547: $p = 0.0692$; Control vs. Infigratinib: $p = 0.0515$) (Fig. 12A, 12B). No changes in FGFR1 protein expression were observed in western blot analyses (Fig. 12C, 12D), and the application of Infigratinib, AZD4547, or Dovitinib did not change FGFR1 mRNA levels in BL2 cells (Fig. 12E).

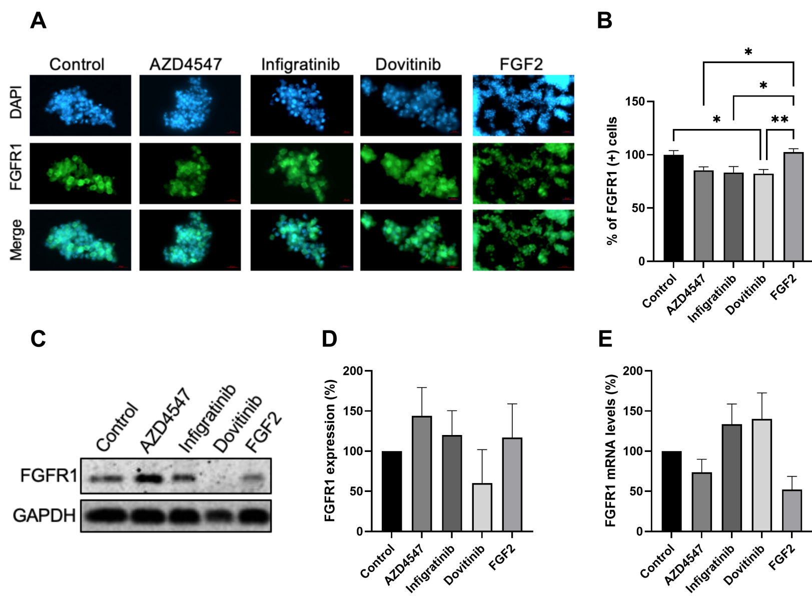

Figure 12: FGFR1 expression (A-E) of BL2 cells treated with AZD4547, Infigratinib, Dovitinib, and FGF2. Representative sections of immunofluorescence staining. The scale bar represents 20 µm for images of the Control, AZD4547, Infigratinib, and Dovitinib groups, while for the FGF2 group, it corresponds to 50 µm (A). Quantitative analysis of immunofluorescence staining revealed reduced numbers of FGFR1+ cells after Dovitinib treatment compared to the control (B). All treatment showed reduced FGFR1+ cells compared to FGF2 application (B). Representative western blot images of FGFR1 analysis (C). Quantification of western blot showed no significant regulation of the protein expression of FGFR1 by any compounds (D). qRT-PCR showed no regulation of FGFR1 mRNA levels (E). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$. Values for control cells were considered 100%, all other results were put in relation to control values. The SEM of the control group is not shown separately in figure D, E , as all relative values are based on this normalization.

4.4 Reduced FGFR1 cellular expression after FGFR inhibition compared to FGF2-treated cells

In the quantitative analysis of IF, the cellular FGFR1 expression in BL2 cells was significantly reduced after treatment with the three different tyrosine-kinase inhibitors compared to the cells with FGF2 application (Infigratinib vs. FGF2: $p = 0.0142$, AZD4547 vs. FGF2: $p = 0.0172$, Dovitinib vs. FGF2: $p = 0.0022$) (Fig. 12A, 12B). The application of FGF2 did not increase the intensity of the stain compared to untreated cells (Fig. 12A, 12B). Furthermore, no changes in FGFR1 protein expression in western blot or FGFR1 mRNA levels were observed in qRT-PCR after FGF2 application (Fig. 12C, 12D, 12E).

4.5 Selective and multikinase FGFR inhibition decreases FGFR2 mRNA levels

To analyze whether inhibition of FGFR by the three inhibitors Infigratinib, AZD4547, and Dovitinib alters the expression of the FGFR2 protein or genetic levels of FGFR2, IF, western blot, and qRT-PCR was carried out. FGFR2 mRNA levels after FGFR inhibition were decreased ($p < 0.0001$) compared to the control (Fig. 13E). Further, FGF2 application reduced FGFR2 mRNA levels ($p < 0.0001$) in BL2 cells compared to untreated cells. None of the compounds altered FGFR2 protein expression compared to untreated cells as determined

by western blot or IF (Fig. 13A, 13B, 13C, 13D). However, in IF, the application of the multikinase inhibitor Dovitinib led to increased cellular FGFR2 expression in BL2 cells compared to FGF2 application ($p < 0.001$) (Fig. 13A, 13B).

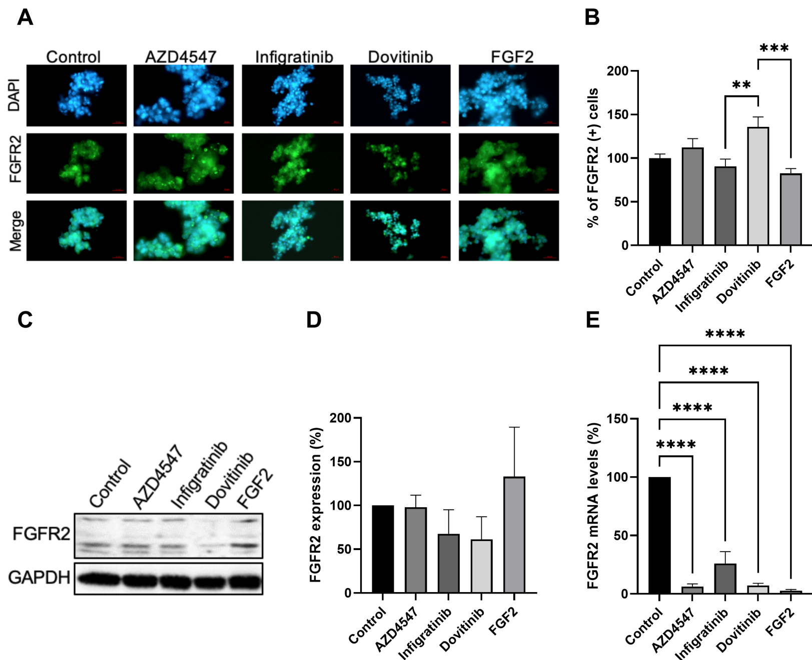

Figure 13: FGFR2 expression (A-E) of BL2 cells treated with AZD4547, Infigratinib, Dovitinib and FGF2. Representative sections of immunofluorescence staining. The scale bar of the images corresponds to 20 µm(A). Quantitative analysis of immunofluorescence staining revealed increased numbers of FGFR2+ cells after Dovitinib treatment compared to FGF2 application (B). Representative western blot images of FGFR2 analysis (C). Quantification of western blot showed no significant regulation of the protein expression of FGFR2 by any compounds (D). qRT-PCR showed strongly decreased FGFR2 mRNA levels of all compounds compared to untreated cells (E). Data are presented as mean ± SEM. ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$. Values for control cells were considered 100%, all other results were put in relation to control values. The SEM of the control group is not shown separately in figure D, E , as all relative values are based on this normalization.

4.6 FGFR inhibition modulates FGFR downstream molecule pERK

To evaluate the effect of FGFR inhibition on the FGFR downstream signaling in BL2 cells, the phosphorylation status of ERK, was analyzed. ERK phosphorylation (pERK) was quantified using IF and western blot. This study observed a significantly increased number of intensively stained pERK BL2 cells after treatment with the selective inhibitor Infigratinib compared to untreated BL2 cells (Control vs. infigratinib: $p = 0.0198$). Also, the staining intensity of pERK cells after Infigratinib treatment was increased compared to the cells after FGF2 application (Infigratinib va. FGF2: $p = 0.0198$) (Fig. 14A, 14B). Dovitinib treatment significantly reduced pERK expression compared to the Control (Control vs. Dovitinib: $p = 0.0050$) (Fig. 14C, 14D). Additionally, pERK phosphorylation was lower with Dovitinib than with AZD4547 treatment (AZD4547 vs. Dovitinib: $p = 0.0123$) (Fig. 14C, 14D). AZD4547 application did not significantly affect pERK phosphorylation in either western blot analyses or IF compared to untreated cells (Fig. 14A, 14B, 14C, 14D).

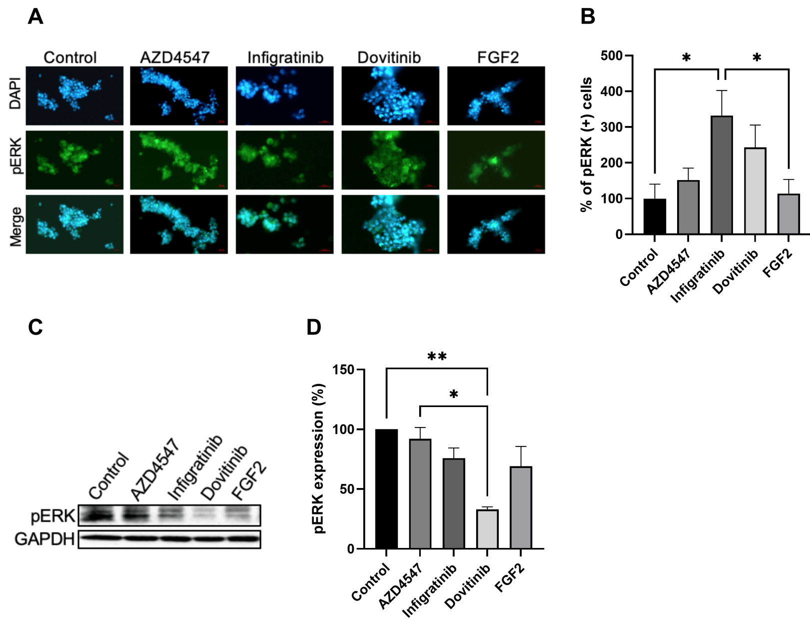

Figure 14: Cellular distribution (A, B) and protein expression (C, D) of pERK in BL2 cells incubated with FGFR inhibitors AZD4547, Infigratinib, Dovitinib, and FGF2. Representative sections of immunofluorescence staining. The scale bar of the images corresponds to 20 µm (A). Quantification of staining showed an increase in pERK+ cell count for Infigratinib treated cells compared to untreated cells and FGF2 application (B). Representative western blot image of pERK analysis (C). Quantification of western blots showed decreased pERK expression in Dovitinib treated cells compared to untreated cells. The multikinase inhibitor Dovitinib decreased pERK phosphorylation compared to the treatment with the selective inhibitor AZD4547 (D). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$. Values for control cells were normalized to 100%, and all other results were considered in relation to control values. The SEM of the control group is not shown separately in figure D, as all relative values are based on this normalization.

4.7 Selective FGFR and multikinase inhibitors increase cellular pAkt expression in BL2 cells

To investigate the effects of selective FGFR and multikinase inhibition on the FGF signaling pathway, western blot and IF of the downstream signaling molecule pAkt was performed. Akt is phosphorylated and therefore, activated upon ligand binding to the receptor. We observed an increased number of intensively stained pAkt cells (Control vs. AZD4547: $p = 0.0023$; Control vs. Infigratinib: $p < 0.0001$; Control vs. Dovitinib: $p = 0.0332$) (Fig. 15A, 15B). The highest number of intensively stained pAkt cells were seen after the application of infigratinib compared to untreated cells and compared to the cells after FGF2 application (Control vs. Infigratinib: $p < 0.0001$; Infigratinib vs. FGF2 $p = 0.0007$) (Fig. 15A, 15B). No regulation of the protein expression of pAkt was seen in the western blot by any components (Control vs. AZD4547: $p > 0.9999$; Control vs. Infigratinib: $p = 0.6638$; Control vs. Dovitinib: $p = 0.1837$; Control vs. FGF2: $p = 0.0782$) (Fig. 15C, 15D).

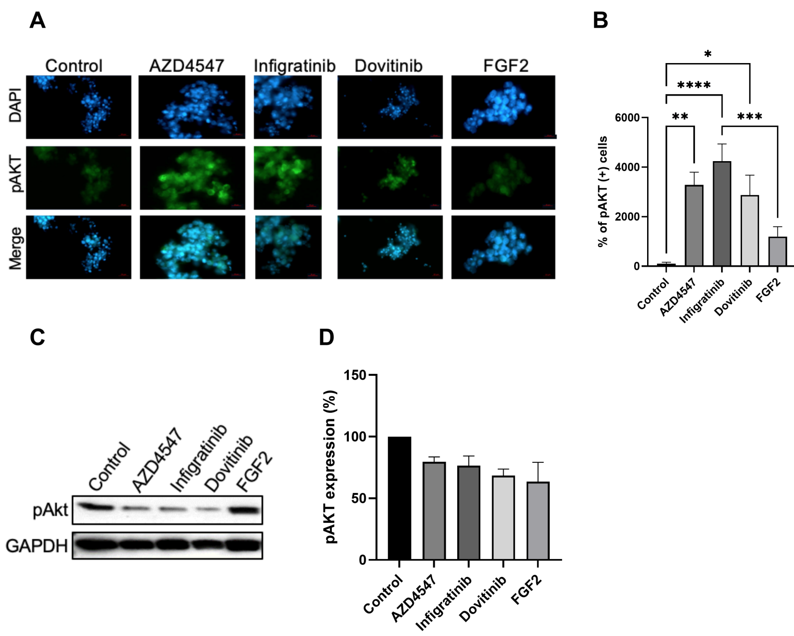

Figure 15: Cell distribution (A, B) and protein expression (C, D) of pAKT. Representative sections of immunofluorescence staining. The scale bar of the images of the Control, AZD4547, Infigratinib, and Dovitinib group corresponds to 20 µm , and FGF2 to 10 µm (A). Quantification of staining showed an increased number of pAKT+ cells compared to untreated cells (B). Also, Infigratinib treatment showed significantly more pAKT+ cells compared to FGF2 application (B). Representative western blot image of pAKT analysis (C). Quantification of western blots showed no regulation of the protein expression of pAKT by any compounds (D). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$. Values for control cells were normalized to 100%, and all other results were considered in relation to control values. The SEM of the control group is not shown separately in figure D, as all relative values are based on this normalization.

4.8 Decrease of proinflammatory cytokines IL6, IL12 by dovitinib

To investigate possible regulations of cytokines in BL2 cells by the three FGFR inhibitors, we studied the protein expression and mRNA levels of IL6, IL12, IFNγ, TNFα, as well as the chemokines CCL2, and CX3CL1. The application of Dovitinib resulted in reduced IL6 (Control vs. Dovitinib: $p = 0.0231$) and IL12 (Control vs. Dovitinib: $p = 0.0233$) protein expression in BL2 cells (Fig. 16A, 16B) compared to the untreated cells. When looking at IL12 regulation in BL2 cells, the multikinase inhibitor Dovitinib not only decreased the protein expression compared to untreated cells but also in comparison to the selective FGFR inhibitor AZD4547 (Control vs. Dovitinib: $p = 0.0233$; AZD4547 vs. Dovitinib: $p = 0.0096$) (Fig. 16A, 16B). No changes were found in mRNA levels in the treatment group compared to the control (Fig. 17C). The administration of FGF2 led to reduced mRNA IL12 levels compared to cells treated with the selective inhibitor AZD4547 (AZD4547 vs. FGF2: $p = 0.0379$) (Fig. 17C).

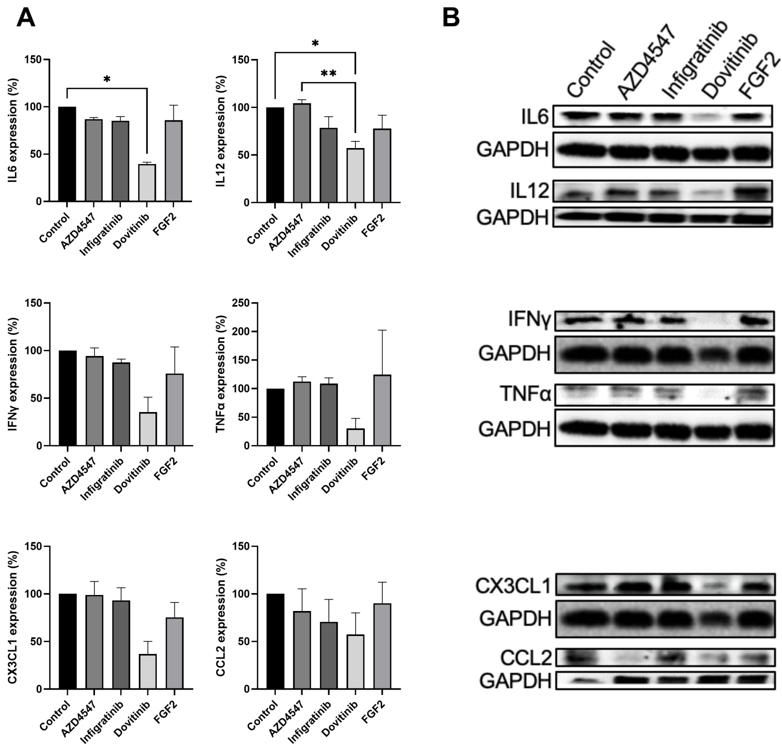

Figure 16: Protein expression (A, B) of IL6, IL12, IFNγ, TNFα, CX3CL1, and CCL2 in BL2 cells after the application of AZD4547, Infigratinib, Dovitinib and FGF2. Quantification of western blot showed decreased IL6 and IL12 protein expression after Dovitinib treatment compared to the control. The multikinase inhibitor Dovitinib decreased the protein expression compared to the selective FGFR inhibitor AZD4547. IFNγ, TNFα, CX3CL1, and CCL2 protein expression were not regulated by any compound (A). Representative western blot images of IL6, IL12, IFNγ, TNFα, CX3CL1, and CCL2 inanalysis (B). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.005$. Values for control cells were considered 100%, all other results were put in relation to control values. The SEM of the control group is not shown separately, as all relative values are based on this normalization.

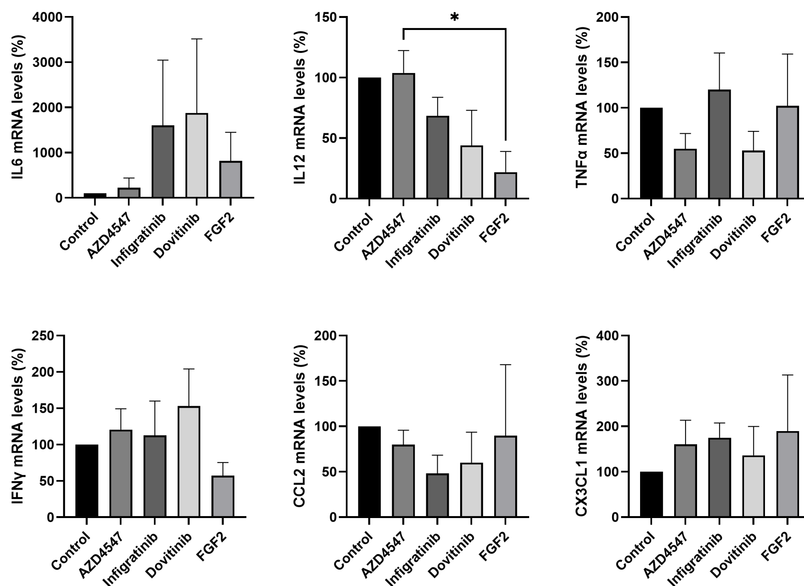

Figure 17: mRNA levels (C) of IL6, IL12, IFNγ, TNFα, CX3CL1, and CCL2 in BL2 cells after the application of AZD4547, Infigratinib, Dovitinib, and FGF2. qRT-PCR showed no regulation of IL6, IL12, IFNγ, TNFα, CX3CL1, and CCL2 mRNA levels in BL2 cells compared to the control (E). Data are presented as mean ± SEM. Values for control cells were considered 100%, and all other results were put in relation to control values. The SEM of the control group is not shown separately, as all relative values are based on this normalization.

5 Discussion

FGFs that signal through FGFRs are ubiquitously expressed across a variety of cell types. FGFR-mediated signaling plays a fundamental role in the regulation of key physiological processes, including tissue homeostasis, metabolism, development, endocrine function, and injury response (Nobuyuki Itoh and David M. Ornitz, 2011; Ornitz and Itoh, 2015). Therefore, it is not surprising that dysregulated FGF signaling can contribute to pathological conditions (Lahiry et al., 2010). Current studies revealed the importance of FGFR signaling in neurodegenerative disease models, indicating that FGF pathways are involved in the pathophysiology of MS, a chronic inflammatory and demyelinating disease of the CNS (Dendrou et al., 2015; Kamali et al., 2021; Rajendran, Böttiger, Stadelmann, et al., 2021). In the context of MS, FGF signaling pathways contribute to disease progression by modulating the immune response, promoting or inhibiting myelin repair, and affecting neuronal survival (Lindner et al., 2015; Rajendran, Böttiger, Stadelmann, et al., 2021). Specifically, these pathways may influence immune cell activation, CNS repair processes, and the balance between neuroprotection and neurodegeneration in MS (Rajendran, Rajendran, et al., 2021). However, the precise role of FGF/FGFR signaling pathways in MS pathophysiology remains mostly undefined, and further research is needed.

The Experimental Neuroimmunology Group led by Prof. Dr. med. Martin Berghoff - where this thesis was later conducted - demonstrated that deletion of FGFR in OLs in $MOG_{35–55}$-induced EAE resulted in a milder disease course, less lymphocyte and macrophage/microglia infiltrates, as well as decreased neurodegeneration (Kamali et al., 2021; Rajendran, Rajendran, et al., 2021; Rajendran et al., 2018). These findings led to a transition to pharmacological approaches to explore the clinical potential of FGFR inhibition. In animal models, treatment with FGFR inhibitors such as infigratinib and AZD4547 reduced inflammation and demyelination, further supporting the therapeutic relevance of targeting this pathway (Gurski et al., 2025; Rajendran et al., 2023). Building on these findings, and considering that the immunomodulatory effects observed in the infigratinib study emerged early in disease progression, likely involving the peripheral immune system, the attention shifted to investigate the role of immune cells, as the underlying mechanisms remained unclear (Rajendran et al., 2023). Notably, the deletion of FGFR in OLs reduced the number of B cells infiltrating demyelinated lesions (Rajendran, Rajendran, et al., 2021), suggesting a possible link between FGF/FGFR signaling and B cell behavior.

The findings of this study demonstrate that inhibition of FGFRs point towards a potential anti-inflammatory role in the context of neuroinflammation. First, FGFR inhibition did not alter proliferation

or induce cytotoxicity in BL2 cells, indicating that BL2 cell viability remains unchanged under *in vitro* conditions. Treatment with the multikinase inhibitor dovitinib led to a reduction in FGFR1 surface protein expression and downregulation of pERK, alongside a significant decrease in the secretion of pro-inflammatory cytokines such as IL6 and IL12. Moreover, surface expression of FGFR1 was reduced following inhibitor treatment when compared to FGF2 application, suggesting an alteration in receptor regulation at the membrane level. All FGFR inhibitors also reduced FGFR2 mRNA levels, reinforcing a transcriptional component to receptor modulation. Interestingly, infigratinib treatment led to an elevated expression of pERK compared to untreated cells and cells treated with FGF2, while all inhibitors increased pAkt expression, indicating a change in the dynamics of cell signaling. These results suggest that multikinase inhibition attenuates inflammatory responses of BL2 cells by modulating key signaling pathways, including pERK and pAkt and reducing cytokine production. The multifaceted and context-specific consequences of FGFR pathway inhibition, in BL2 cells, will be further explored and interpreted.

5.1 Effects of selective FGFR and multikinase inhibitors on BL2 cell proliferation and cytotoxicity

In the present *in vitro* study, the application of AZD4547, infigratinib, and dovitinib was not cytotoxic to BL2 cells and did not decrease BL2 cell proliferation, in contrary to what was initially hypothesized. Applying FGF2 as a growth factor did not significantly alter BL2 cell proliferation or exerted cytotoxic effects.

These results suggest, that FGFR signaling may not play a central role in regulating proliferation or survival of BL2 cells. Instead, alternative signaling pathways may be more critical in this context. Notably, Herzog et al. (2009) demonstrated that pre-B cell receptor engagement activates SYK and Src-kinases, which leads to the activation of PI3K, resulting in the production of PIP3 and phosphorylation of Akt, a key effector promoting cell survival and proliferation (Herzog et al., 2009). In the present study, the observed increase in pAkt expression in IF following FGFR inhibition with all three inhibitors may reflect compensatory activation of the SYK–PI3K–Akt axis. This compensatory mechanism could account for the absence of cytotoxic or antiproliferative effects in BL2 cells, as it may sustain essential survival signals.

The evidence supporting the antiproliferative potential of FGFR inhibition originates primarily from oncology research, where FGFR inhibitors have shown efficacy in multiple tumor models. The multi-

kinase inhibitor dovitinib, which targets FGFRs among other kinases, has been shown to suppress cell proliferation in renal cell carcinoma, endometrial carcinoma, and hepatocellular carcinoma (Porta et al., 2015). Dovitinib inhibited proliferation through downregulation of the MAPK and Akt signaling pathways in colon carcinoma cells (Gaur et al., 2014) and reduced STAT3 and STAT5 phosphorylation by modulating PI3K downstream signaling in breast cancer models (Chiu et al., 2019). Similarly, infigratinib, a selective FGFR1-3 at low nanomolar half maximal inhibitory concentration (IC50) values (Guagnano et al., 2011), has been investigated in phase I to III clinical trials of primarily solid tumors (Dai et al., 2019). In cancer cell lines, infigratinib decreased cell proliferation and exerted antitumor activity in tumors such as cholangiocarcinoma (Javle et al., 2021). Another selective compound, AZD4547, decreased proliferation and viability in medulloblastoma cell lines in a dose-dependent manner (between 5 µM to 50 µM) (Lukoseviciute et al., 2020).

Inhibition of cell growth is often accompanied by increased cytotoxicity, and significant cytotoxic effects were also observed in different tumor cell lines treated with the three compounds tested in this study (Babina and Turner, 2017). These cytotoxic effects exerted on cells could be explained since not only cell proliferation is mediated through FGF/FGFR signaling, but also cell survival (Turner and Grose, 2010). It must be considered that TKI efficacy and cytotoxicity are closely related (Thomson et al., 2022). Furthermore, possible cytotoxic side

effects need to be considered when applying FGFR inhibitors *in vivo*.

In MS, activated B cells cross the BBB, stimulate autoreactive T cell proliferation, release excessive amounts of pro-inflammatory cytokines and exert cytotoxic effects on OLs and neurons, driving CNS inflammation and damage (Cencioni et al., 2021; R. Li et al., 2018; Magliozzi et al., 2010). The efficacy of B cell depleting therapies such as ocrelizumab and ofatumumab highlight their central role in MS pathogenesis (Cencioni et al., 2021). Current research focuses on targeting BTKs to regulate B cell survival, activation, proliferation, and differentiation via PI3K, MAPK, and NFκB signaling pathways, without causing full B cell depletion (Carnero Contentti and Correale, 2020). Recent clinical trials have investigated several BTK inhibitors, such as evobrutinib, tolebrutinib, and fenebrutinib, demonstrated their ability to cross the BBB, reduce inflammatory activity, and potentially address compartmentalized CNS inflammation (Fox et al., 2025; Langlois et al., 2024; Merck KGaA, 2023; Montalban et al., 2019). These orally available, functionally selective compounds offer a promising approach for progressive disease forms, with the potential to suppress pathogenic B cell activity while preserving broader immune function, thus representing a potential alternative to B cell depletion. The suppression of autoreactive B cells invading the CNS and modulating their function remains a key therapeutic objective in MS. In this context, targeting pathways that regulate B cell function and prolif-

eration presents an attractive strategy. FGF/FGFR signaling is known to modulate cell proliferation, therefore targeting this pathway could have antiproliferative effects and suppress autoreactive B cells in both the periphery and the CNS, providing an additional or complementary mechanism to existing therapies (Ornitz and Itoh, 2015).

Further research is essential to fully elucidate how B cell proliferation might be effectively targeted through FGFR inhibition. A key aspect will be to investigate the complex interaction between FGFR signaling and alternative cell survival pathways. It is also important to acknowledge that the present *in vitro* findings may not directly translate to *in vivo* conditions, where various additional factors influence proliferation and cytotoxicity. Moreover, it remains to be determined whether targeting B cell proliferation is the most critical therapeutic goal or if addressing their proinflammatory activity is more important. Targeting specific intracellular signaling pathways to suppress their proinflammatory activity may ultimately provide a more refined therapeutic strategy, one that suppresses pathogenic B cell functions while preserving overall immune competence, and thus avoiding the broad immunosuppressive effects associated with complete B cell depletion experienced under current B cell depleting MS therapy.

5.2 FGFR1 and FGFR2 protein and gene expression in BL2 cells

Investigations of brain tissue from MS patients have highlighted the significance of FGF/FGFR signaling in the disease pathophysiology. FGF expression primarily located in astrocytes and microglia, was found to be upregulated in active lesions. This upregulation strongly correlated with cellular inflammation and demyelination and was further observed in the periphery of chronic plaques (Clemente et al., 2011). Beyond the CNS, elevated levels of FGF2 were noticed in CLL B cell plasma (Ghosh and Kay, 2013). Tumor studies further support the role of FGF2, showing that increased secretion from tumor cells can amplify cytokine production and inflammatory factor release by B cells (Somasundaram et al., 2017). In MOG_{35-55}-induced EAE animal models, OL-specific deletion of FGFR1 and FGFR2 reduced the numbers of infiltrating macrophages/microglia, and T and B cells into the CNS. This decreased immune cell infiltration, and led to diminished inflammatory activity, resulting in a less severe disease course with reduced myelin and axon degeneration (Kamali et al., 2021; Rajendran, Rajendran, et al., 2021).

In this *in vitro* study, BL2 cells showed a decreased FGFR1 expression at the cellular level following treatment with the multikinase inhibitor dovitinib. While this observation is consistent with previous findings in cancer biology, where FGFR expression was shown to decrease after TKI treatment, it is important to note that this BL2 cell *in vitro*

study lacks the tumor microenvironment and cell-cell interactions present in solid tumor systems. For instance, Fumarola et al. (2017) demonstrated that treatment with dovitinib led to a reduction in FGFR1 expression in squamous cell lung cancer cells (Fumarola et al., 2017). Similarly, Zhao et al. (2017) reported a downregulation of FGFR expression in breast cancer cell lines treated with the selective inhibitor AZD4547 (Zhao et al., 2017). Furthermore, Prawira et al. (2021) highlighted that infigratinib application inhibited FGFR activity by reducing the levels of FGFR1-4 in hepatocellular carcinoma models (Prawira et al., 2021).

The observed reduction in FGFR1 in BL2 cells may reflect a direct effect of dovitinib treatment, potentially through receptor internalization and intracellular sequestration rather than transcriptional downregulation or degradation, since total FGFR1 protein expression and mRNA levels were not affected by any inhibitor or FGF2 application.

The difference in protein expression may also arise from the inherent characteristics of each technique. In general, IF detects localized, cell-specific phosphorylation, while western blot measures average expression across all cells. Although less sensitive to subtle changes, western blot offers more standardized, objective quantification and is less prone to subjective bias compared to IF. IF results may be influenced by variability in staining quality, background fluorescence, or signal interpretation, which can lead to overestimation or misrepresentation of phosphorylation levels (Im et al., 2019; Mahmood

and Yang, 2012). Additionally, it must be acknowledged that the immunofluorescence images obtained in this study were of limited resolution and quality, which may further affect the reliability of quantitative or localization-based interpretations. Therefore, these technical limitations warrant cautious interpretation of the data when assessing the effects of FGFR inhibition in BL2 cells.

The pronounced effect of dovitinib on BL2 cells could be attributed to its broad inhibitory spectrum, as it targets multiple tyrosine kinase receptors, including c-Kit, PDGFR, VEGFR, and EGFR, FLT3 (Katoh, 2016; Porta et al., 2015). Among these, VEGFRs play a crucial role in processes such as cell development, cell survival, proliferation, permeability, and migration, all of which are effectively suppressed by dovitinib. Recent studies highlight the functional interplay between FGF and VEGF signaling pathways, showing how their interaction influences immune regulations within the tumor microenvironment and tumor cell apoptosis (Liu et al., 2021). Crosstalk between these pathways can lead to compensatory mechanisms, for example, inhibition of one pathway may result in the upregulation or increased reliance on the other (Boichuk et al., 2024; Liu et al., 2021). This underscores the importance of targeting both pathways simultaneously for therapeutic efficacy. In support of this, Ghosh and Kay (2013) suggest that dual inhibition of FGFR/VEGFR signaling is critical to impair CLL B cell survival (Ghosh and Kay, 2013). This finding may help explain

the stronger impact of dovitinib on BL2 cells compared to the more selective FGFR inhibitors. The enhanced effectiveness of dovitinib could be attributed to its multi-target activity, particularly its ability to inhibit both FGFR and VEGFR signaling pathways simultaneously. AZD4547 and infigratinib, which are designed to selectively inhibit FGFR1–4 (Gavine et al., 2012; Roskoski, 2019), were less effective at downregulating FGFR1 surface expression, possibly due to their limited activity beyond FGFR signaling. Dovitinib's multikinase profile may promote FGFR1 internalization through additional mechanisms (Porbska et al., 2018). No changes in FGFR1 gene levels were detected by RT-PCR, despite the observed downregulation of cellular protein expression in IF, suggesting that post-transcriptional mechanisms, such as increased degradation or reduced protein stability, might mediate FGFR1 downregulation without altering mRNA levels. Alternatively, compensatory transcriptional upregulation of FGFR1 may occur as a cellular response to its downregulation at the protein level. These potential mechanisms highlight the complexity of FGFR signaling in BL2 cells. Future studies should focus on confirming FGFR1 downregulation at the protein level and explore the unique regulatory mechanisms of FGFR1 expression in BL2 cells to better understand its posttranscriptional regulation and cellular autoregulation.

Compared to treatment with the growth factor FGF2, all TKI treatment

groups showed a significant decrease in cellular FGFR1 expression. This was unexpected, as FGF2 is known to induce FGFR1 internalization upon ligand binding in many cell types (Porbska et al., 2018). These findings suggest, that in BL2 cells, FGF2 application may not induce receptor internalization and degradation to the same extent as observed with TKI treatment, and therefore, does not lead to a comparable decrease in cellular FGFR1 expression. The reduction in FGFR1 expression following FGFR inhibition could result from impaired receptor recycling and reduced synthesis, ultimately limiting the reappearance of FGFR1 at the cell surface after internalization.

When looking at FGFR2 cellular staining intensity, dovitinib showed a significantly higher number of intensively stained BL2 cells compared to BL2 cells with FGF2 application. Dovitinib significantly blocks FGFR1 in BL2 cells, which may influence FGFR2 expression or stability through compensatory regulatory mechanisms between the receptors (Katoh, 2016; Turner and Grose, 2010). The apparent increase in signal may be due to receptor stabilization or impaired degradation following FGFR1 inhibition. However, specific studies detailing this compensatory relationship in BL2 cells are currently lacking. More research is needed to elucidate the interaction between FGFR1 inhibition and FGFR2 regulation in this context.

On the other hand, FGF2 binding to FGFR2 could trigger the above mentioned receptor internalization as part of its natural trafficking

cycle, temporarily reducing its surface availability for staining, before recycling back to the membrane (Porbska et al., 2018). However, FGFR2 internalization by FGF2 may temporarily reduce surface staining, contrasting with TKI-induced degradation, which might lead to a permanent receptor loss. These findings highlight the complexity of FGFR signaling pathways and the interplay between FGFR1 and FGFR2 in BL2 cells, as well as emphasizing the different effects of receptor trafficking after receptor inhibition and ligand binding. Future studies utilizing live-cell imaging and receptor-specific trafficking assays will be essential to clarify these mechanisms and their potential impact on therapeutic strategies.

Further, FGF2 application led to a reduction in FGFR2 mRNA levels in BL2 cells compared to untreated cells. Similarly, all tested FGFR inhibitors significantly decreased FGFR2 mRNA levels compared to the control. However, none of the treatments affected FGFR2 protein expression in western blot analyses.
This discrepancy, that the downregulated FGFR2 mRNA levels were not reflected by FGFR2 protein expression could be due to several post-transcriptional mechanisms that regulate gene expression (Becker et al., 2018). One possible explanation could be mRNA stabilization. Despite the downregulation of transcription, the stability of the mRNA could be maintained through RNA-binding proteins or microRNAs, which prevent mRNA degradation (Gebauer and Hentze,

2004; W. Li et al., 2022; Vos et al., 2019). This mechanism could allow sustained translation and constant protein expression in BL2 cells, although mRNA synthesis is reduced by TKIs. Additionally BL2 cells might activate compensatory bypass mechanisms, such as alternative RTKs, which could maintain similar signaling activity and therefore result in unchanged protein levels despite the reduced FGFR2 mRNA levels after TKI. Such compensatory receptor regulation has been observed in previous studies, where cells compensate FGFR signaling by upregulation of alternative receptors such as insulin-like growth factor 1 receptor (IGF1R) or erythroblastic leukemia viral oncogene homologue receptor (ERBB) (Babina2017, Gebauer2004). These posttranscriptional and compensatory mechanisms are crucial for cellular homeostasis and might explain the discrepancy between FGFR2 mRNA levels and protein expression in BL2 cells.

This study indicates that dovitinib more effectively reduces FGFR1 expression in BL2 cells than selective FGFR inhibitors, likely due to its broader kinase inhibition including VEGFR. The unchanged FGFR1 mRNA levels suggest post-transcriptional regulation. The increased FGFR2 signal after dovitinib treatment compared to FGF2 application may represent a compensatory response to FGFR1 inhibition, reflecting the functional interplay within the FGFR family. Stable FGFR2 protein expression despite reduced mRNA levels by all inhibitors may point to compensatory bypass mechanisms. To further validate these

hypotheses, future studies should include live-cell imaging of receptor trafficking, protein degradation assays, and analysis of alternative receptor tyrosine kinase activity to explore compensatory signaling pathways.

5.3 Effects of selective FGFR and multikinase inhibitors on FGFR signaling downstream molecule pERK

The phosphorylation of the downstream protein ERK, dominantly channeled by the RAS/RAF pathway, is crucial to many cellular processes, including cell metabolism, cell proliferation, cell differentiation, cell survival, and migration (Roskoski, 2012). Due to its central role in regulating key cellular processes and the fact that its dysregulation is implicated in numerous human diseases, ERK signaling has become a major focus of therapeutic research (Lavoie et al., 2020; Samatar and Poulikakos, 2014). Emerging evidence suggests that FGFR inhibitors can influence ERK phosphorylation, a critical pathway in cell signaling (Huynh et al., 2012; Rajendran, Böttiger, Stadelmann, et al., 2021; Rajendran, Rajendran, et al., 2021; Rajendran et al., 2018).

In the present study, a decrease in pERK levels was observed in BL2 cells upon treatment with dovitinib, supporting the hypothesis that ERK activity is modulated via FGFR inhibition and aligns with the findings of other *in vitro* studies, which demonstrate the inhibitory effects of dovitinib on ERK phosphorylation across various cancer models. For

instance, Gaur et al. (2014) demonstrated that dovitinib reduced ERK phosphorylation in colorectal cancer cells (Gaur et al., 2014). Furusho et al. (2012) showed that FGFR1 and FGFR2 signaling in oligodendrocytes regulates myelin sheath thickness through MAPK/ERK activation, with receptor deletion leading to hypomyelination and reduced ERK phosphorylation (Furusho et al., 2012). Similarly, Kamali et al. (2021) demonstrated that oligodendrocyte-specific FGFR2 deletion in an EAE model attenuates disease severity and led to a downregulation of ERK phosphorylation, suggesting that reduced ERK activity is a downstream effect of FGFR inhibition (Kamali et al., 2021). However, regional differences in the expression of FGFRs, ERK, or associated signaling components exist. In the cerebellum, oligodendrocyte-specific deletion of FGFR1 did not regulate ERK phosphorylation, indicating that ERK may not be the dominant downstream effector of FGFR1 in this brain region (Rajendran, Rajendran, et al., 2021).

These findings highlight the complexity of FGFR/ERK signaling across different CNS regions and cell types. ERK operates within broad signaling networks, responding to diverse inputs, with its effects shaped by the cellular context. The observed reduction in pERK following dovitinib treatment in BL2 cells may thus reflect a combination of direct kinase inhibition and pathway crosstalk.

In addition to its effects on the FGFR/ERK signaling cascade, dovitinib exerts inhibitory effects in other signaling pathways. One such pathway involves BDNF/TrkB, which has been implicated in

neuroprotection and remyelination (Kamali et al., 2021; Nociti and Romozzi, 2023; Stadelmann et al., 2002). Interestingly, B cells have been shown to express TrkB and to produce BDNF under certain conditions (Brigadski and Leßmann, 2020; Kerschensteiner et al., 1999). BDNF production by B cells is not simply a side effect of immune activation, it plays an active role in regulating both neuronal and immune system functions (Kerschensteiner et al., 1999; Torres et al., 2025). As demonstrated by Nociti and Romozzi (2023), BDNF contributes to neuronal repair and remyelination, suggesting a potential neuroprotective mechanism mediated by immune cells (Nociti and Romozzi, 2023). This aligns with findings by Stadelmann et al. (2002), who demonstrate that immune cells, are a significant source of BDNF within MS lesions, suggesting a potential neuroprotective role of immune-derived BDNF in the inflamed CNS (Stadelmann et al., 2002). Together, these studies underscore the dual role of immune cells in the CNS: while capable of promoting inflammation, they can also contribute to repair processes under certain conditions. Given dovitinib's broad target spectrum, including interactions beyond FGFR inhibition, it is plausible that its impact on cellular signaling extends to the BDNF/TrkB axis as well. This hypothesis is supported by the findings of Rajendran, Böttiger, Dentzien, et al. (2021), who proposed that dovitinib may reduce ERK phosphorylation but also increase BDNF/TrkB signaling in OL (Rajendran, Böttiger, Dentzien, et al., 2021). These insights collectively point toward a multifaceted

mechanism of action for dovitinib, involving both direct kinase inhibition and indirect modulation of neuroimmune crosstalk.

In contrast, no significant change in pERK expression was observed, following treatment with the selective FGFR inhibitor AZD4547. Interestingly, BL2 cells treated with infigratinib, another selective FGFR inhibitor, exhibited an increase in intensively stained pERK BL2 cells. *In vivo*, Rajendran, Rajendran, et al. (2021) also observed increased ERK phosphorylation via selective FGFR knockout in mice, resulting in enhanced myelination and a less severe disease course (Rajendran, Rajendran, et al., 2021). Although *in vivo* and *in vitro* systems differ, these findings support the observation that selective FGFR inhibition can increase ERK phosphorylation. However, these different effects of FGFR inhibition on modulating the downstream molecule pERK in BL2 cells, could result from different binding specificities. Dovitinib, acting as a multikinase inhibitor, could strongly influence ERK phosphorylation by targeting ERK through other signaling pathways, or exerting synergic effects through targeting multiple receptors, compared to selective inhibitors AZD4547 and infigratinib. While it is currently unclear whether this phenomenon is specific to BL2 cells, due to a lack of targeted studies in this cell line, the observed variation suggests that signaling network complexity may play a critical role.

These findings suggest that FGFR inhibition alone may not be the primary driver of the downstream effects observed in BL2 cells. This

challenges the view of FGFR as a key regulator of ERK phosphorylation and raises questions about the efficacy of selective FGFR inhibitors in the context of B cell modulation. It also underscores the importance to explore the broader effects of the multikinase inhibitor dovitinib in BL2 cells, as such broad inhibition is typically associated with increased off-target effects and a higher potential for side effects, as commonly observed with multikinase inhibitors (Fan et al., 2024). Future research should therefore focus on investigating the specific contributions and interplay of the various pathways targeted by dovitinib in BL2 cells to better understand its therapeutic relevance and limitations.

5.4 Effects of selective FGFR and multikinase inhibitors on FGFR signaling downstream molecule pAkt

This study investigated Akt, a key intermediate in signaling pathways, particularly those activated by receptor tyrosine kinases (RTKs) and PI3K. Rather than acting in isolation, Akt functions as a dynamic regulator of key cellular processes, including proliferation, survival, and metabolic activity (Manning and Toker, 2017). The Akt signaling cascade is known to contribute to different malignancies and is dysregulated in various types of cancers, making it a compelling focus for targeted therapeutic strategies (Altomare and Testa, 2005). In cancer cell studies, the application of FGFR inhibitors resulted

in suppressed Akt phosphorylation (Dey et al., 2010). However, Y. Hu et al. (2014) demonstrated that while pAkt inhibition by FGFR inhibitors is critical, it is not sufficient on its own to effectively suppress tumor growth, suggesting that compensatory signaling pathways can counteract the effects of FGFR inhibition (Y. Hu et al., 2014). This concept was previously discussed in Section 5.1 addressing the limited antiproliferative effects observed in BL2 cells. Y. Hu et al. (2014) findings highlight the importance of targeting not only FGFR, but also downstream effectors such as PI3K/Akt to supress Akt phosphorylation effectively and to achieve enhanced therapeutic efficacy. Supporting this, Terp et al. (2021) showed that a dual inhibition strategy was effective *in vivo*, pointing to dual inhibition as a promising strategy to overcome resistance mechanisms associated with monotherapy (Terp et al., 2021).

This study showed alterations of Akt phosphorylation in BL2 cells by treatment with AZD4547, infigratinib, and dovitinib. But in contrast to previous studies (Y. Hu et al., 2014; Terp et al., 2021), detecting suppressed Akt phosphorylation after FGFR inhibition, enhanced pAkt cellular surface protein expression of BL2 cells compared to untreated cells were observed. The most intensively stained pAkt cells in IF were seen after applying infigratinib. However, no regulation of Akt protein phosphorylation was observed in western blot analyzes.

A similar mechanism has been reported in a *in vivo* study by

Rajendran, Böttiger, Stadelmann, et al. (2021), where conditional knockout of FGFR1 in mice led to increased phosphorylation of Akt, along with increased expression of BDNF and its TrkB receptor (Rajendran, Böttiger, Stadelmann, et al., 2021). These findings support the idea that cells can engage alternative pathways to sustain key functions when FGFR activity is disrupted and highlights the inherent plasticity of cellular signaling networks, especially in systems under stress or therapeutic intervention.

In light of this, the enhanced staining intensity of pAkt observed in this study may reflect a compensatory activation of the PI3K-Akt signaling pathway via alternative growth factor receptors (Babina and Turner, 2017). Notably, the BDNF/TrkB axis is capable of engaging the PI3K/Akt cascade and has been implicated in both neuronal repair and immune cell signaling (Sajanti et al., 2020; Sochal et al., 2022; Torres et al., 2025).

These compensatory mechanisms could counteract the inhibition of FGFR to maintain essential cellular functions, including cell survival, metabolism, and differentiation mediated by pAkt. The observed increase in Akt phosphorylation in BL2 cells following FGFR inhibitor treatment thus suggests the ability of BL2 cells to adapt to FGFR inhibition by engaging backup signaling pathways (e.g. TrkB, EGFR, IGF1R) to sustain proliferative and survival signals despite targeted inhibition (Altomare and Testa, 2005; Manning and Toker, 2017).

The lack of detectable changes in Akt phosphorylation in western blot

analysis, despite the increased signal seen in immunofluorescence, may reflect methodological differences between the two techniques (Im et al., 2019; Mahmood and Yang, 2012), as outlined in a previous section, and should be considered a limitation of this study.

In summary, this chapter underscores the adaptability of BL2 cells in maintaining Akt signaling despite FGFR inhibition. The observed upregulation of pAkt cellular protein expression suggests the activation of alternative pathways that compensate for FGFR blockade, reflecting the intrinsic plasticity of the signaling network. These insights emphasize that FGFR inhibition alone may not be sufficient to block survival signaling in BL2 cells and support the use of combination therapies that target both FGFR and additional signaling pathways, including the PI3K/Akt axis, to improve therapeutic efficacy and potentially overcome resistance mechanisms.

5.5 Modulatory effects of selective FGFR and multikinase inhibitors on cytokines

The significant decrease in ERK phosphorylation was accompanied by a reduction in the protein expression of IL6 and IL12 after dovitinib treatment. This effect was not seen in IF and did not reach significance in the comparison between treatment groups. Therefore suggesting a potential link between the ERK signaling pathway and cytokine regulation in BL2 cells remains speculative.

Despite the speculative nature of a direct link between ERK signaling and cytokine regulation in BL2 cells, previous studies support a connection between kinase pathways, B cell development and cytokine expression in B cells (Carnero Contentti and Correale, 2020; Lucas et al., 2022; Yasuda et al., 2008). Carnero Contentti and Correale (2020) reported a decreased secretion of pro-inflammatory cytokines through BTK inhibiton, emphasizing the impact of kinase signaling on cytokine regulation in B cells (Carnero Contentti and Correale, 2020). In addition, elevated cytokine levels, including IL6 and IL12, in the plasma of CLL B cells have been associated with continuous activation of RTKs, leading to leukemic cell survival and apoptosis resistance (Ghosh and Kay, 2013). These findings raise the possibility, that ERK pathway, might play a role, mediating IL6 and IL12 expression. However, more studies are needed to confirm whether the observed cytokine regulation in this study is directly related to ERK signaling or occurs through alternative mechanisms, including activation of parallel signaling pathways, feedback loops, or crosstalk between kinases.

In MS, B cells are known to contribute to disease pathogenesis by secreting excessive amounts of distinct inflammatory factors like TNF, lymphotoxin α, IL6, and GM-CSF (Michel et al., 2015).
Among these, IL6 plays a pivotal role in the development of EAE, driving inflammation and disease progression. Experimental models have shown that selective deletion of IL6 in B cells reduces disease

severity in EAE, highlighting this pathway as a central mechanism linking B cells to neuroinflammation (Rothaug et al., 2016; Serada et al., 2008). IL6 supports the development of pathogenic Th17 cells, limits regulatory T cell responses, and promotes immune cell infiltration into the CNS (Barr et al., 2012; Serada et al., 2008). Through IL6 secretion, B cells enhance pro-inflammatory signaling and IL6 has been identified as a potent modulator of B cell proliferation, plasma cell survival, and immunoglobulin secretion (Rothaug et al., 2016; Vazquez et al., 2015). This cytokine plays a critical role in promoting the recruitment of inflammatory cells, enhancing their migration across the BBB, activating autoreactive T cells, and stimulating the production of pathogenic antibodies. Elevated IL6 levels play a significant role in driving inflammation and autoimmune responses (Vazquez et al., 2015).

The enhanced phosphorylation of STAT5 and STAT6, downstream molecules of JAK or other tyrosine kinase signaling pathways such as PI3K-Akt and RAS-MAPK, were described as a signaling pathways involved in increased production of proinflammatory cytokines (Yao et al., 2014, Sino Biological, 2023). Therefore, targeting FGFR signaling in B cells may help reduce the expression of pro-inflammatory cytokines such as IL6, potentially limiting neuroinflammation, demyelination, and neurodegeneration by disrupting key immune and inflammatory pathways.

In this *in vitro* study, FGFR inhibition in B cells resulted not only in less pro-inflammatory expression of the IL6 protein, but also reduced the expression of IL12 in BL2 cells. IL12 is primarily modulated through the STAT4 signaling pathway and regulates NK activation and T cell differentiation, enhancing inflammation (Jee et al., 2001). In MS, elevated serum levels of IL12 are associated with increased disease activity, as IL12 plays a crucial role in the promotion of Th1 cell expansion. This connection is further supported by findings from EAE studies (Comabella et al., 1998; Wang et al., 2018). Therefore, the reduced IL12 protein expression in BL2 cells after dovitinib treatment could impact MS pathogenesis, possibly by limiting Th1 mediated inflammatory responses. Among the kinase inhibitors evaluated, dovitinib demonstrated the strongest cytokine-suppressive effects in BL2 cells. Unlike the more selective FGFR inhibitors, dovitinib targets a broader range of kinases, including VEGFRs, PDGFRs, EGFRs, FLT and c-Kit, possibly accounting for its superior efficacy.

However, other key pro-inflammatory cytokines such as IFNγ, TNFα, and the cytokines CCL2 and CX3CL1 strongly involved in the pathogenesis of MS (Ramesh et al., 2013), were not significantly affected in this *in vitro* study. No alteration in protein expression or mRNA levels were observed.

One possible explanation could be that these cytokines are mainly regulated through the JAK and STAT pathway, which is known to be

essential for cytokine-induced gene expression and plays a key role in modulating immune cell functions (B. Hu et al., 2021; Wang et al., 2018). Another factor might be the limited observation period of 24 h, which could have been insufficient for B cells to fully differentiate or activate under the specific experimental conditions required for cytokine production (Vazquez et al., 2015). Moreover, this timeframe may not have allowed for the detection of delayed transcriptional or translational responses, particularly those affecting mRNA cytokine levels.

The absence of co-stimulatory signals or immune cell interactions, which are essential for full B cell activation and cytokine production, may also explain the limited cytokine modulation observed *in vitro*. In vivo, B cells operate within a highly dynamic and interactive immunological microenvironment, where continuous crosstalk with other immune and non-immune cells significantly influences their cytokine expression profiles (Dendrou et al., 2015; R. Li et al., 2018). This level of complexity is inherently absent in monoculture systems, such as this BL2 cell *in vitro* model. As a result, indirect or chronic inflammatory signals, such as those contributing to the formation of chronic lesions or slowly progressing inflammation in the CNS of MS patients, cannot be accurately replicated. This limitation is particularly relevant given the proposed feedback interactions between peripheral immune cells and CNS-resident cells, including microglia and astrocytes, which are thought to play a critical role in modulating cytokine responses during

neuroinflammation (Dendrou et al., 2015; Lassmann, 2018).

In general, *in vitro* models are limited in their ability to reflect the complex immune responses that occur in living organisms. They cannot reproduce systemic effects or the progression from acute to chronic disease stages, as seen in EAE models or in tissue from MS patients. Although this study provides initial insights, how FGFR inhibition affects cytokine expression in B cells, these results must be interpreted with caution due to the simplified nature of the experimental setup. Future studies should use more advanced models, such as co-cultures, tissue explants, or organotypic cultures, which better reflect the physiological environment. Co-culture systems involving B cells and CNS-resident cells (e.g., microglia, astrocytes, OLs) could enable the investigation of reciprocal cellular interactions. These include the influence of B cell-derived cytokines on glial activation, as well as the capacity of CNS-resident cells to modulate B cell phenotype and function through both contact-dependent mechanisms (e.g., CD40–CD40L interactions) and soluble mediators (e.g., IL-6, TNF). Furthermore, such systems may help determine whether FGFR inhibition impacts B cell communication with T cells, potentially altering T cell activation and downstream effector responses, key components of MS pathophysiology. These approaches would allow for a more accurate analysis of cell-to-cell interactions, signaling dynamics, and the potential therapeutic effects of kinase inhibitors under conditions

that more closely resemble the *in vivo* situation.

Another limitation of this study is the use of BL2 cells, a human Burkitt lymphoma B cell line, which differs from B cells found in patients with MS. As a result, the findings derived from this study are difficult to directly extrapolate to the behavior of B cells in MS, where B cells are involved in both pro-inflammatory and regulatory processes. To strengthen the translational relevance of the data, future studies should incorporate B cells isolated from MS patients. This would allow for a more accurate assessment of how FGFR-targeted therapies influence inflammatory signaling in disease-relevant immune cells and provide deeper insight into their potential therapeutic value in neuroinflammatory conditions.

This chapter explored how selective FGFR inhibitors and the multikinase inhibitor dovitinib modulate pro-inflammatory cytokine expression in BL2 cells, showing that dovitinib significantly reduced IL6 and IL12 expression. This suggests a possible role for FGFR and associated kinase signaling, such as ERK, in regulating inflammatory responses. In contrast, more selective FGFR inhibitors showed limited impact, highlighting the broader efficacy of multikinase inhibition. The study also emphasized the complexity of cytokine regulation, noting that certain key inflammatory mediators involved in MS, including IFNγ and TNFα, were not affected under the experimental conditions. Factors such as limited observation time, lack of co-stimulation, and

the simplified *in vitro* model likely contributed to these findings. To visualize these findings, the following Figure 18 illustrates the impact of dovitinib on key signaling pathways in BL2 cells, summarizing its effects on cytokine expression and intracellular signaling dynamics.

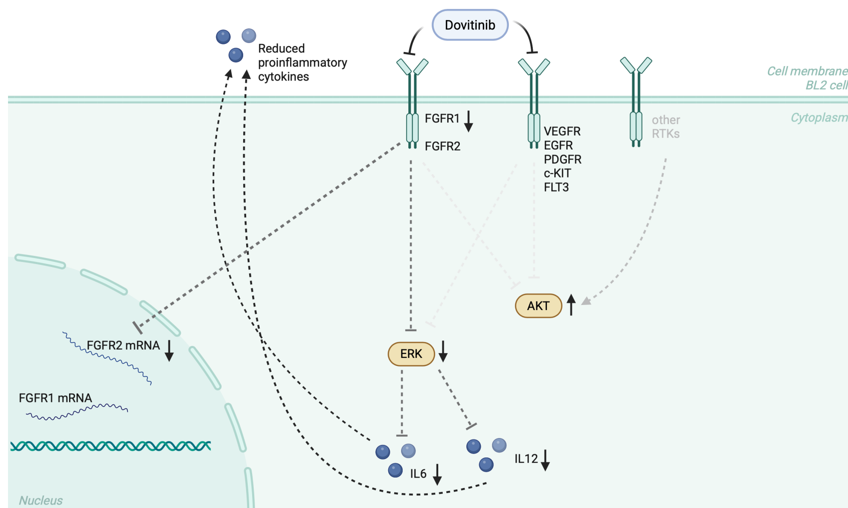

Figure 18: Impact of dovitinib on cell signaling in BL2 cells. This figure illustrates the key findings from this study on how the multikinase inhibitor dovitinib might alter cell signaling in BL2 cells. Dovitinib treatment leads to a decrease in FGFR1 expression, which results in downregulation of pERK activity and a reduction in the secretion of proinflammatory cytokines, indicating a suppression of key inflammatory pathways. On the other hand, dovitinib treatment induces the upregulation of pAKT, suggesting a compensatory activation of of other signaling pathways to counterbalance the effects of FGFR inhibition and maintain proliferation. Additionally, FGFR2 mRNA levels are downregulated. The black arrows in the figure represent the upregulation or downregulation of specific molecules, while the dashed arrows indicate potential downstream signaling, highlighting the complexity and interconnectedness of the signaling networks involved. Image created with BioRender (biorender.com).

This study highlights the potential of kinase inhibition, especially through multikinase inhibitors like dovitinib, to modulate pro-inflammatory cytokine expression in B cells. The reduction of IL6 and IL12 levels following treatment points to a possible role of FGFR signaling in driving inflammatory responses in BL2 cells, supporting further investigations into its potential therapeutic role in neuroinflammatory diseases like MS. Validation in more physiologically relevant models and patient-derived cells will be essential to assess clinical relevance.

6 Conclusion

This study examined the potential of FGFR signaling as a regulatory axis in B cell-mediated inflammation, with a particular focus on pharmacological FGFR inhibition in BL2 cells using two selective FGFR inhibitors (AZD4547, infigratinib) and the multikinase inhibitor dovitinib. This work explored FGFR1/2 receptor dynamics, the impact of FGFR pathway inhibition on intracellular signaling (pERK, pAkt), and the cytokine expression in BL2 cells.

The findings provide several novel insights. First, FGFR inhibition did not suppress BL2 cell proliferation or induce cytotoxic effects under the *in vitro* conditions tested. This contrasts with findings from oncology studies, where FGFR inhibition typically suppresses tumor cell proliferation (Dai et al., 2019; Lukoseviciute et al., 2020; Porta et al., 2015), suggesting that FGFR signaling may not be essential for B cell viability. Interestingly, an increase in pAkt following inhibitor treatment, particularly with infigratinib, suggests that compensatory mechanisms, such as alternative survival pathways (e.g., via PI3K-Akt), may be activated to maintain cell homeostasis despite FGFR inhibition.

Second, dovitinib demonstrated a more pronounced regulatory effect on both receptor expression and intracellular signaling compared to the selective inhibitors. Dovitinib reduced FGFR1 surface protein

expression, and downregulated total pERK protein, a key signaling molecule involved in cell proliferation, survival, and cytokine regulation (Lavoie et al., 2020). Simultaneously, dovitinib treatment increased pAkt surface expression, suggesting a shift in signaling likely due to broader kinase inhibition. Therefore, dovitinib effects may result from the cumulative impact of blocking multiple kinases and disrupting the broader receptor signaling network.

Third, dovitinib significantly decreased the expression of proinflammatory cytokines IL6 and IL12 in BL2 cells, key mediators implicated in MS pathogenesis. IL6 drives the differentiation of pathogenic Th17 cells and promotes immune cell CNS infiltration, while IL12 supports Th1 responses and amplifies inflammatory cascades (Akdis et al., 2011; Vazquez et al., 2015; Wang et al., 2018). The ability of dovitinib to suppress these cytokines suggests that broad kinase inhibition can effectively modulate the proinflammatory capacity of B cells, potentially altering their contribution to MS pathology. However, this effect appears to be independent or at least not specific to FGFR inhibition, raising the question of whether FGFRs are the primary mediators or simply one component within a larger signaling landscape. In contrast, selective FGFR inhibitors had a limited impact on cytokine expression, indicating that a more specific inhibition of FGFR1–4 alone may be insufficient to modulate B cell inflammatory activity. This differential effect supports the notion that multikinase inhibition may offer a promising approach to modulating immune cell

function in the context of neuroinflammation. Rather than eliminating B cells entirely, a strategy that can affect immune surveillance, targeting key signaling pathways that drive their inflammatory response, may have a better balance between efficacy and safety. This could allow for suppression of pathogenic B cell activity while preserving their regulatory or homeostatic functions. Furthermore, given the multifactorial nature of the pathogenesis of MS, therapies should aim to balance, rather than eradicate, dysregulated immune responses (Dendrou et al., 2015; Filippi et al., 2018; Lassmann, 2018). FGFR signaling, such as the disease itself, is multifaceted, with effects shaped by receptor isoform expression, cellular context, and interaction with other pathways (Beenken and Mohammadi, 2009; Itoh and Ornitz, 2008; Ornitz and Itoh, 2015). This complexity could actually present a therapeutic advantage, as modulating a key signaling axis, such as FGFR, may allow precise immune regulation without requiring the identification of specific antigens or cell types.

In summary, this work identifies FGFR signaling, and more broadly, RTK signaling, as a modifiable axis in B cells with relevance to neuroinflammation. Although selective inhibition of FGFR showed limited effects, inhibition by the multikinase inhibitor dovitinib altered signaling activity and reduced the expression of pro-inflammatory cytokines, supporting its potential as an immunomodulatory agent. These findings suggest that broad inhibition of interrelated pathways, rather than single-target interventions, may be better suited to address

the complex immunopathology of MS.

Each experimental insight contributes to a broader understanding, while simultaneously revealing new complexities. This doctoral thesis contributes to this goal by elucidating how FGFR pathways influence B cell function, signaling behavior, and inflammatory potential. By highlighting the distinct immunomodulatory effects of multikinase inhibition, particularly through dovitinib, this work underscores the importance of looking beyond single-target strategies but towards a broader network signaling modulation. In doing so, it offers a valuable framework for future investigations into B cell-directed therapies and supports the pursuit of precision-based interventions that move beyond symptom management toward true disease modification in MS.

Abstract

B lymphocytes play a key role in the pathogenesis of multiple sclerosis (MS). While B-cell-depleting therapies have demonstrated clinical efficacy, emerging therapeutic strategies focus on functional modulation of B cells to preserve overall immune homeostasis.
In addition to B cells, molecular signaling pathways such as the B cell receptor pathway, targeted by Bruton's tyrosine kinase inhibitor and fibroblast growth factor (FGF/FGFR) signaling, important for neurodegeneration and immune regulation in the central nervous system, have gained attention. Experimental autoimmune encephalomyelitis models showed that conditional deletion of FGFR1/2 in oligodendrocytes resulted in a milder disease course, reduced inflammation, and decreased myelin and axon degeneration. In demyelinating lesions, the number of immune cells was significantly reduced, indicating that FGF/FGFR signaling might play an important role in immune cell modulation.
This study investigated the effects of pharmacological FGFR inhibition on BL2 cells. Selective FGFR inhibitors (AZD4547, infigratinib) and the multikinase inhibitor dovitinib were investigated for their effects on BL2 cell proliferation, cytotoxicity, receptor expression (FGFR1/2), intracellular signaling (pERK, pAkt), and cytokine production using WST-1 and LDH assays, immunofluorescence, western blot, and RT-

PCR.

Under *in vitro* conditions, FGFR inhibition did not affect BL2 cell proliferation or exerted cytotoxic effects. Dovitinib significantly reduced the surface expression of FGFR1, pERK activation, and the secretion of the pro-inflammatory cytokines IL6 and IL12, both important in the pathophysiology of MS. In contrast, selective FGFR inhibitors had only minor effects. FGFR2 mRNA levels were reduced across all treatment groups, and protein levels remained unchanged, suggesting post-transcriptional regulation or the activation of compensatory feedback mechanisms. An increase in pAkt activity, particularly following infigratinib treatment, further indicates the activation of alternative signaling pathways in response to FGFR blockade. The enhanced efficacy of dovitinib might be due to its broader kinase inhibition profile, targeting not only FGFR but also additional tyrosine kinases.

In summary, this study shows that the multikinase FGFR inhibitor dovitinib can modulate pro-inflammatory B cell activity without compromising cell viability, offering a promising strategy as a targeted immunomodulatory approach for MS therapy.

Zusammenfassung

B-Lymphozyten spielen eine zentrale Rolle in der Pathogenese der Multiplen Sklerose (MS). Während B-Zell-depletierende Therapien wirksam sind, rücken modulierende Ansätze zur Erhaltung der Immunhomöostase zunehmend in den Fokus. Neben der direkten B-Zell-Modulation sind intrazelluläre Signalwege wie der B-Zell-Rezeptor- und der FGF/FGFR-Signalweg von Bedeutung, welcher relevant für neurodegenerative und immunologische Prozesse im zentralen Nervensystem ist. Experimentelle autoimmunen Enzephalomyelitis Studien zeigen, dass die Deletion von FGFR1/2 in Oligodendrozyten mit milderem Verlauf, geringerer Myelin- und Axondegeneration und reduzierter Immunzellinfiltration einhergeht. Dies weisst auf die immunmodulatorische Relevanz von FGF/FGFR in Immunzellen hin.

Diese Arbeit unstersucht die Effekte einer pharmakologischen FGFR-Inhibition auf die BL2 Zelllinie. Hierzu wurden sowohl selektive FGFR-Inhibitoren (AZD4547, Infigratinib) als auch der Multi-Tyrosinkinase-Inhibitor Dovitinib eingesetzt. Die Effekte auf Proliferation, Zytotoxizität, Rezeptorexpression (FGFR1/2), intrazelluläre Signalaktivierung (pERK, pAkt) sowie die Zytokinproduktion wurden mittels WST-1- und LDH-Assay, Immunfluoreszenzfärbung, Western Blot und RT-PCR evaluiert.

Die FGFR-Inhibition zeigte unter den gewählten *in-vitro*-Bedingungen

keine Veränderung der Proliferation und keine Zytotoxizität in BL2-Zellen. Dovitinib reduzierte signifikant die FGFR1-Oberflächenexpression, die pERK-Aktivierung und die Sekretion der MS- relevanten proinflammatorischen Zytokine IL6 und IL12. Selektive FGFR-Inhibitoren zeigten hingegen nur geringe Effekte. Trotz Abnahme der FGFR2-mRNA bei allen Inhibitor-Gruppen blieb die Proteinexpression unverändert, was auf posttranskriptionale Regulation oder kompensatorische Mechanismen hinweist. Eine verstärkte pAkt-Aktivität nach FGFR Inhibition, besonders unter Infigratinib, deutet auf eine kompensatorische Aktivierung alternative Signalwege als Antwort auf eine FGFR-Blockade hin. Die ausgeprägtere Wirkung von Dovitinib dürfte auf das erweiterte Zielprofil weiterer Tyrosinkinasen zurückzuführen sein.

Zusammenfassend zeigt sich, dass Dovitinib proinflammatorische B-Zell-Aktivität wirksam modulieren kann, ohne die Zellvitalität zu kompromittieren. Dies eröffnet eine vielversprechende Perspektive für eine differenzierte immunmodulatorische MS-Therapie.

List of Abbreviations

ALL	acute lymphoblastic leukemia
APCs	antigen-presenting cells
Akt	protein kinase B
ATP	adenosine triphosphate
BAFF	B cell activating factor
BBB	blood-brain barrier
BCP-ALL	B cell precursor acute lymphoblastic leukemia
BCR	B cell receptor
BTK	Bruton’s tyrosine kinase
Breg	regulatory B cell
CBL	Casitas B-lineage lymphoma
CCL2	CC-chemokine ligand 2
cFGFs	C-terminal fibroblast growth factors-23
CIS	clinically isolated syndrome
c-Kit	cellular kit
CLL	chronic lymphocytic leukemia
CNS	central nervous system
CSF	cerebrospinal fluid

CSF-1R	colony-stimulating factor 1 receptor
CX3CL1	C-X3-C motif chemokine ligand 1
DAG	diacylglycerol
DCs	dentritic cells
DIT	dissemination in time
DIS	dissemination in space
DMF	dimethyl fumarate
DMD	disease-modifying drug
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EBNA1	Epstein-Barr nuclear antigen 1
EGFR	epidermal growth factor receptor
ERBB	erythroblastic leukemia viral oncogene homologue receptor
ERK	extracellular signal-regulated kinases
FcR	fragment crystallizable receptor
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGFRL1	fibroblast growth factor receptor-like 1

FLT3 Fms-like tyrosine kinase 3

FRS2 FGFR substrate 2

GA glatiramer acetate

GM-CSF granulocyte-macrophage colony-stimulating factor

Grb1 growth factor receptor-bound protein 1

Grb2 growth factor receptor-bound protein 2

hFGFs human fibroblast growth factors

HRP horseradish peroxidase

HSPGs heparan sulfate proteoglycans

IC50 half maximal inhibitory concentration

IF immunofluorescence

iFGFs intrecellular fibroblast growth factors

IGF1R insulin-like growth factor 1 receptor

IL interleukin

INO internuclear ophthalmoplegia

IP3 inositol trisphosphate

JAK janus kinase

LDH lactate dehydrogenase

MAPK mitogen-activated protein kinase

MALT mucosa-associated lymphoid tissue

mAbs	monoclonal antibodies
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MRI	magnetic resonance imaging
MS	multiple sclerosis
mTOR	mechanistic target of rapamycin
MYC-IGL	MYC-immunglobuline light chain
NADH	nicotinamide adenine dinucleotide
NFkB	nuclear factor 'Kappa-Light-Chain-Enhancer' of activated B cells
NK	natural killer cell
NK cells	natural killer cells
NRF2	nuclear factor erythroid 2-related factor 2
OCB	oligoclonal bands
OL	oligodendrocyte
OPC	oligodendrocyte progenitor cell
pAkt	phosphorylated protein kinase B
PDGFR	platelet-derived growth factor receptor
pERK	phosphorylated extracellular signal-regulated kinase

PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	protein kinase C
PLCγ	phospholipase Cγ
PPMS	primary progressive multiple sclerosis
RAF	rapidly accelerated fibrosarcoma
RAS	rat sarcoma
RET	rearranged during transfection
RRMS	relapsing-remitting multiple sclerosis
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
S1P	sphingosine-1-phosphate
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEF	similar expression to FGF
SEM	standard error of the mean
SOS	son of sevenless
SPMS	secondary progressive multiple sclerosis
SPRY	sprouty

pS6K	phosphorylated p70 S6 kinase
Src-kinases	sarcoma family kinases
STAT	signal transducer and activator of transcription
SYK	spleen tyrosine kinase
Shc	Src Homology 2 domain-containing protein
TGF	transforming growth factors
Th cells	T-helper cells
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TKI	tyrosine kinase inhibitor
TrkA	tropomyosin receptor kinase A
TrkB	tropomyosin receptor kinase B
Treg	regulatory T cell
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Publications

Publication derived from this study:

Rajendran R, Rajendran V, Böttiger G, Stadelmann C, Shirvanchi K, von Au L, Bhushan S, Wallendszus N, Schunin D, Westbrock V, Liebisch G, Ergün S, Karnati S, Berghoff M. The small molecule fibroblast growth factor receptor inhibitor infigratinib exerts anti-inflammatory effects and remyelination in a model of multiple sclerosis. Br J Pharmacol. 2023 Dec; 180(23): 2989-3007.
doi: 10.1111/bph.16186. Epub 2023 Jul 31. PMID: 37400950.

Science-Day 2021 JLU Giessen:

Investigation of FGF/FGFR signaling pathways in B cells by pharmacological FGFR inhibition von Au, L., Böttiger, G., Rajendran, R., Berghoff, M.

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