Fibroblast Growth Factor Receptor Inhibition in a Model of Multiple Sclerosis: Immunological Effects *in vivo* and *in vitro*

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Tag der Disputation: 6.11.2024

IF WE KNEW WHAT WE WERE DOING, IT WOULD NOT BE CALLED RESEARCH, WOULD IT?

WENN WIR WÜSSTEN, WAS WIR TUN, WÜRDE MAN ES NICHT FORSCHUNG NENNEN.

ALBERT EINSTEIN

I DEDICATE THIS WORK TO MY FAMILY, WHOM I LOVE DEARLY.

INDEX

I. INTRODUCTION

The iridescent, multifaceted complexity of neuroscience can be as overwhelming as it is captivating to the young researcher. The dissection of a single subject, even one as tightly circumscribed as the subject of this work, can lead to seemingly endless, though highly rewarding, inquiries. However, in the interest of a concise and accessible overview, many of these insights will not be part of this work.

Recent developments in neuroscience have had a redefining influence on the field. They have opened up fascinating avenues of research, contributing to an ever-growing understanding of the intricacies of the brain. One striking example is that of neural circuits that make predictions based on reafferent vs. exafferent input.

Another remarkable discovery is that of the *gut-brain* and the gut-brain-axis. It has revealed that the microbiome not only influences the permeability of our blood-brain barrier (BBB), but also shapes central nervous system (CNS) immunity and even our conscience.

As a further example, the vastly improved availability and resolution of neuroimaging allows us to dissect brain regions with near-microscopic precision using pulse waves, or to study the activity of very specific brain regions *in vivo* in humans. These samples offer just a glimpse of the astonishing recent advances in neuroscience that continue to amaze researchers in the field.

Yet a great deal of research and effort is still urgently called for since, despite all these breakthroughs, in 2021, 3.4 billion people – more than 3/7 of the world's population – either lived with a neurological disease or died of one. The total amount of morbidity and mortality increased by approximately 18% from 1990 to 2021 (GBD 2021 NSDC, 2024). Thus, there is still a great need for further advances in neuroscience, as attempted in this work. However, even a focused approach to the core topic of this thesis – *understanding a cell signaling pathway and its pharmacological manipulation* – is cloaked by numerous layers of complexity.

Because this work has utilized both an animal model and monocultured immortalized cell lines, the inherent oversimplifications of these models must be considered when deriving conclusions from these experiments. The effects of a drug can easily vary when interactions at the organ level come into play, just as the inferences drawn from cell culture versus tissue culture data will differ. Variations at the microenvironmental level depending on disease state and model add further complexity, as do cell-cell interactions *in vivo* vs. *in vitro*.

Despite the considerable methodological advances outlined above, the insights gained into this nascent field of study frequently prompt more questions than answers. One might be forgiven for feeling like a speleologist who becomes lost in a cave system but finds magnificent caverns along the way.

I. 1. MULTIPLE SCLEROSIS

Review of the disease multiple sclerosis (MS) can appear just as multilayered. It is a debilitating neuroinflammatory disease that leads to demyelination - loss of insulation from nerve processes in the CNS - and subsequent neuronal degeneration, in addition to direct neuronal cell death.

From a patients' perspective, often young, aspiring individuals are faced with a devastating diagnosis and the overwhelming fear of progressive disability that comes with it. Given the exemplary prevalence of approximately 300 per 100,000 in Germany, a country with one of the highest numbers of people with MS and its consequences for disablement, unemployment and health care costs, the societal burden of this illness is exceptionally high (Kobelt et al., 2017; Paz-Zulueta et al., 2020).

From a medical perspective, much progress has been made in the treatment and suppression of exacerbations, but little has been achieved in the treatment of disability progression. Furthermore, the pathophysiology of MS and its cause is still a matter of uncertainty (Attfield et al., 2022), although there are some intriguing approaches.

Considering the aforementioned socioeconomic challenges, especially the negative impact on patients' quality of life, the insufficiency of treatment options underscores the imperative to advance our comprehension of the pathophysiology of MS. Understanding it would be of paramount importance in developing tailored new pharmacological approaches or even preventive measures to alleviate the disease's burden.

I. 1. 1. Epidemiology and etiology

I. 1. 1. 1. Epidemiology

Prior to any further investigation, it is essential to ascertain the prevalence of the disease. This can enable a more comprehensive understanding of its distribution and potentially reveal underlying causes. Furthermore, the establishment of an increasingly robust epidemiological database for MS by the Multiple Sclerosis International Federation (MSIF) provides an even more powerful foundation upon which to base such propositions and can reveal an impressively high prevalence of 2.8 million individuals worldwide affected by the disease. Although this figure is most likely an underestimate, it is steadily

increasing, not only due to more sensitive diagnostic procedures and adapted criteria, but also likely due to an increase in incidence (MSIF, 2020; Koch-Henriksen and Magyari, 2021).

FIGURE 1: THE EPIDEMIOLOGY OF MS

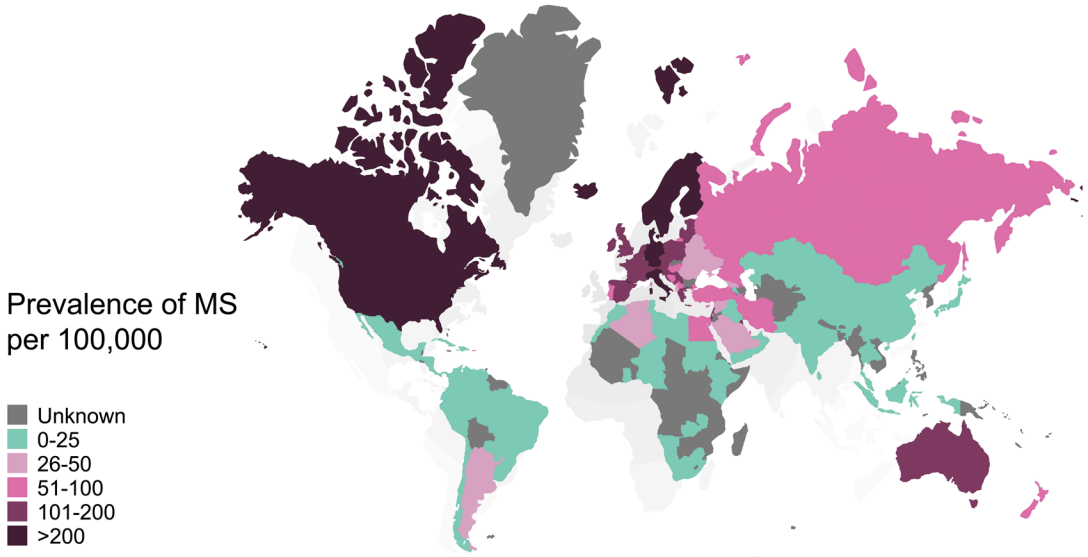


Figure 1 shows the most comprehensive epidemiological data currently available.
Source: MSIF – Multiple Sclerosis International Federation. Atlas of MS – 3rd Edition (September 2020). Modified and reproduced with the kind permission of MSIF.

There are marked differences in spatial and temporal distribution: The prevalence in Europe and the United States is considerably higher than in the rest of the world, although it should be noted that data from the Global South are often less comprehensive and, in some cases, not collected at all. In addition, there is a correlation between latitude and prevalence of MS (Simpson et al., 2011; Ghareghani et al., 2018; Simpson et al., 2019). There is some variation within subpopulations - particularly in terms of gender - but overall the majority of those affected are female (69%) and young adults (mean onset at 32 years; MSIF, 2020). Of all people with MS worldwide, at least 30,000 are under the age of 18; it is the most common neurological illness in young adults and the most common non-traumatic cause of disability (Dobson and Giovannoni, 2019; Wallin et al., 2019; MSIF, 2020). As a result, the individual and societal health burden is substantial, and the high prevalence of progression to disability provides a strong incentive for further research from both a socioeconomic and medical viewpoint (Kobelt et al., 2017).

I. 1. 1. 2. Etiology

In reviewing the extensive epidemiologic data, the question quickly arises as to the cause of this condition that is so costly in so many ways. This question is often answered as unsolved (Attfield et al., 2022), yet the following is known about the etiology of the disease.

I. 1. 1. 2. a. Epstein-Barr virus

Recently, a causal association between Epstein-Barr virus (EBV) and MS has been established:
i) EBV infection increases risk of MS 32-fold in patients who were neurofilament light chain-negative – a highly sensitive, albeit nonspecific marker of axonal damage (Gaetani et al., 2019; Bjornevik et al., 2022). In addition, almost all MS patients are EBV positive (Pakpoor et al., 2013; Dobson et al., 2017; Bjornevik et al., 2022).
ii) In some patients, cross-reactive Epstein-Barr nuclear antigen 1 (EBNA1) antibodies that also target hepatocyte cell adhesion molecule (hepaCAM, also referred to as glialCAM) were detected. This established a potential causative antigen and the corresponding molecular mimicry, given that antiEBNA/antihepaCAM antibodies have been shown to exacerbate CNS inflammation in experimental autoimmune encephalomyelitis (EAE) – a mouse model of MS (see I. 2). Interestingly, cluster of differentiation (CD) 8^+ T cells from MS patients are also activated by hepaCAM in culture (Lanz et al., 2022). In addition, post-translational modification of hepaCAM facilitates antibody affinity (Lanz et al., 2022).
Thus, post-translational modifications are thought to be a link between environmental and genetic factors in MS, which may explain the coexistence of these intrinsic and extrinsic susceptibilities (Hunter, 2012; Zavala-Cerna et al., 2014; Ricigliano et al., 2015; Dobson and Giovannoni, 2019). Epigenetics can potentially explain such antibodies against endogenous proteins modified by environmental factors and may explain tissue-specific differences as well as lack of central tolerance. Post-translational modifications do not occur in the thymus, so such modified proteins presented by professional antigen-presenting cells (APCs) are therefore recognized as exogenous (Zavala-Cerna et al., 2014).

There are many additional links between EBV and MS (Lünemann et al., 2008; Handel et al., 2010; Lünemann et al., 2010; Wergeland et al., 2016; Bar-Or et al., 2022), including antigen-independent ones (EBV persistence in self-reactive memory B cells: Tracy et al., 2012; class switching in B cells: van Langelaar et al., 2021). There are further

associations between known MS risk factors and EBV infection. *E.g.*, EBV induces genetic modifications of vitamin D binding sites (Ricigliano et al., 2015) and smoking may affect post-translational modifications implicated in the antigen presentation in the lung (see I. 1. 1. 2. c; Dobson and Giovannoni, 2019).

Such post-translational modifications, in addition to epigenetic alterations, may underlie the efficacy of cortisone, a crucial anti-inflammatory drug and hormone. Cortisone is a mainstay of acute relapse treatment and may mediate its therapeutic effects through changes in the epigenome (Hunter, 2012).

In any case, a nearly holoendemic infection such as EBV, demands an interplay of predisposing conditions to trigger a disease that is comparatively rare. As indicated above, this includes environmental and genetic factors - possibly at a vulnerable point in life (see Figure 2; Attfield et al., 2022). In summary, the (epi)genetic influences of obesity, sun exposure, vitamin D, and smoking on a genetically vulnerable immune system may set the stage from which EBV reactivation or (re)infection ultimately triggers the causal MS pathway (see also Figure 2). This may explain why only a miniscule subset of EBV-seropositive individuals develop MS (Sintzel et al., 2018; Dobson and Giovannoni, 2019).

I. 1. 1. 2. b. Microbiome

In light of the aforementioned (epi)genetic risk, the microbiome, as a major modulator of the epigenome, is intriguing to consider as a link to environmental factors: The gastro-intestinal tract (GIT) represents the largest exchange platform for immune stimuli due to its size, it being a direct contact site with exogenous substances, and its ubiquitous immune cells. The GIT is a mandatory requirement for our immune functions. This is evidenced by studies demonstrating that germ-free mice do not develop an intact immune system and are consequently protected from developing EAE (Berer et al., 2011; Berer et al., 2017). However, when these germ-free mice receive a microbiome transplant obtained from a diseased human, their susceptibility to developing MS (in a genetic mouse model) is restored. Furthermore, and very interestingly, when these mice are transplanted with a microbiome from a monozygotic human twin who is discordantly healthy (but whose monozygotic twin has developed MS), the incidence of autoimmunity is significantly lower (Berer et al., 2011; Berer et al., 2017).

The microbiota of the GIT serve as the foundation of the gut-brain axis, which – as the term implies – is implicated in numerous neurological diseases (Cryan et al., 2019) and is also regarded as pathological in MS (Parodi and Kerlero de Rosbo, 2021). Generally, in the Western world, the microbiome is depleted in terms of diversity (Segata, 2015), and end products such as short-chain fatty acids are often scarce. These have been allocated a beneficial role in EAE by modulating the immune system (Duscha et al., 2020). This may also be true for MS and is particularly pronounced in women, but further studies are needed to clarify causality and the mechanisms by which the microbiome interacts with an immune system predisposed to MS (Melbye et al., 2019; Becker et al., 2021).

Ultimately, however, short-chain fatty acids such as propionic acid may only be the final section of a healthy microbiome and may not necessarily execute beneficial functions due to their biochemical properties (Parks et al., 2020). Figure 2 summarizes important factors in the development of MS:

FIGURE 2: ETIOLOGICAL FACTORS OF MS

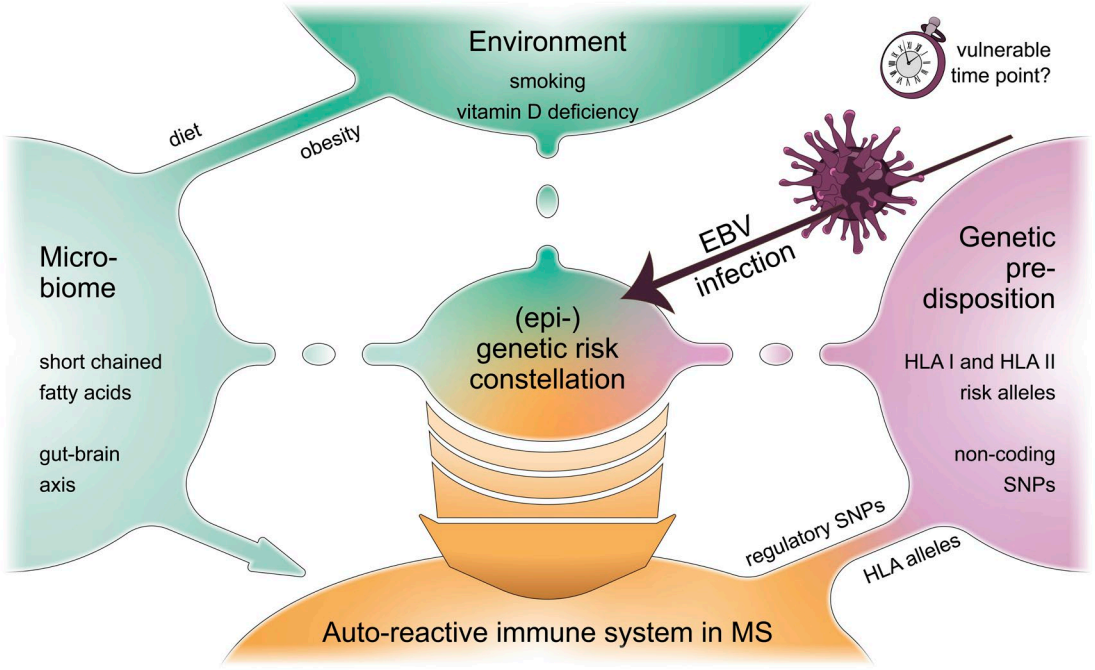


Figure 2 shows possible interplay between risk factors for developing MS.
EBV infection in the predisposed, possibly infecting or re-activating at a vulnerable time point, can lead to the inflammatory disease.

I. 1. 1. 2. c. Latitude and vitamin D

Not only are there associations between the microbiome, gut endotoxins and gut permeability, but several meta-analyses have also confirmed a correlation of latitude on a global scale as an important risk factor for MS (Simpson et al., 2011; Ghareghani et

al., 2018; Simpson et al., 2019). Ultraviolet (UV) radiation, especially UVB-light, modulates the immune system (Kripke, 1994; Slominski et al., 2018). Childhood sun exposure provides some protection against MS (Islam et al., 2007). However, the role of vitamin D is most likely independent of UV radiation – although the main source for vitamin D for most people is sunlight exposure (Ascherio et al., 2010). Vitamin D affects brain functions, cell cycle, hormonal homeostasis and modulates immune responses (Deluca and Cantorna, 2001; Niino et al., 2008; Shirazi et al., 2015; Danner et al., 2016). Despite mendelian randomization studies having attempted to establish a causality in MS (Mokry et al., 2015), there is mainly epidemiological evidence suggesting its protectivity (Munger et al., 2006; Ascherio et al., 2010; Dobson et al., 2013; Rodríguez Cruz et al., 2016; Bäcker-Koduah et al., 2020). Taken together, after decades of research there is still no compelling argument for a causal relationship between either vitamin D deficiency or UVB exposure and the etiopathogenesis of MS (as reviewed in Breuer et al., 2019).

I. 1. 1. 2. d. Risk alleles and genome wide associations

In light of these common environmental risk factors, genetic analyses have been employed to specify the genetic predispositions necessary for susceptibility to MS. In the 1970s, it was established that there are alleles in the human leukocyte antigen (HLA) class II that represent genetic risk factors for MS. The most important are HLA-DQB1*03:02, HLA-DRB1*03:01, HLA-DRB1*08:01, HLA-DRB1*13:03 and HLA-DRB1*15:01. Given the relevance of HLA class II for antigen presentation, this association led to the dominant paradigm that T cells are critical in the pathogenesis of the disease. However, this paradigm has since been seriously challenged by several subsequent findings. The sequencing capabilities of the 2000s enabled the identification of gene correlates for MS. However, the hope of thereby revealing the cause of MS did not materialize – even though more than 200 single-nucleotide polymorphisms (SNPs) associations were discovered (Gresle et al., 2020). Most of these SNPs are located near promoters of immune genes, but are also present in 5% of healthy individuals and cannot explain the incidence of MS. Nevertheless, data from these studies have led to interesting discoveries. *E.g.,* a SNP associated with MS was found to express a modified tumor necrosis factor α (TNF-α) receptor 1, which blocks TNF-α instead of being activated by it. This explains why TNF blockers – that had been tried as MS drugs – exacerbate the disease course by acting similarly to these TNF-α receptor variants (Gregory et al., 2012), thereby mimicking or exacerbating a pathological process in MS.

Although they may not have revealed the etiologic origins, genome-wide association studies provide an excellent starting point for the development of treatment strategies, by at least suggesting possible leverage points, especially with regard to tyrosine kinase inhibitors (TKIs; see I. 1. 4. 2. b; also in Kingsmore et al., 2008; Tam et al., 2019).

I. 1. 2. Pathophysiology

In the past, the treatment of MS was largely guided by observed outcomes rather than a deep understanding of the pathophysiology of the disease. However, as outlined in the previous sections, our comprehension of MS has evolved. We are now able to take a more mechanistic approach to its management, thanks in part to the aforementioned SNPs, which have provided starting points for studying specific areas of the disease's pathological cascade. These SNPs have implicated both innate and adaptive immune cells in the causal pathway of MS (Gresle et al., 2020).
On the contrary, observational evidence for the success of B cell depletion therapies (see I. 2. 1. 1. b) has changed the perspective on a disease that was previously thought to be primarily T cell driven. In general, knowledge of the pathophysiology of MS has increased dramatically, *e.g*. with the discovery of immune cell (sub-)subpopulations and their cell polarizations (Attfield et al., 2022). An imbalance in these subpopulations is believed to form a milieu of pathogenic capacity that leads to the hallmark pathologies of perivenular inflammation and demyelinating plaques or lesions (Thompson et al., 2018b; Dobson and Giovannoni, 2019). Such lesions can be subdivided according to activity level and disease state (see Figure 3; Stadelmann et al., 2019).

I. 1. 2. 1. Initiation of autoimmunity

Strong evidence points to a misdirected autoreactive adaptive immune response as the primary event in MS, leading to the initiation of inflammatory demyelination. However, exactly how the initiation of immune cells against CNS components is induced is still unknown (Thompson et al., 2018b). Two hypotheses are discussed as most likely:
i) Initial intrinsic activation of APCs against autoantigens occurs in the CNS compartment. Their drainage to the peripheral immune system leads to autoreactivity with ensuing activation of B and T cells. The subsequent pathological processes are analogous to what is described herein:
ii) Dendritic presentation of extrinsic antigens in lymph nodes activates B and T cells. These are then erroneously rehomed to the CNS – possibly due to disturbances in the BBB (Hemmer et al., 2015; Thompson et al., 2018b).
Given the enormous amount of research efforts made, it is surprising that it remains

unclear whether MS is antigen-dependent, or autoimmune (or a combination), and which cells causally drive the disease's pathological cascade (Pachner, 2021; Attfield et al., 2022). Logically however, simply eliminating the autoreactive, pathogenic immune cells could be a compelling treatment strategy – especially since there is currently no alternative such as preventing their activation. However, their identification is obscured by the high degree of redundancy in the immune system (Attfield et al., 2022).
Classically, autoreactive monoclonal $CD8^+$ T cells are thought to be immanently involved in MS pathophysiology (see I. 1. 2. 2). They are activated by both B cells and monocytes. However, $CD8^+$ T cell subpopulations also have beneficial functions – as evidenced by the fact that the induction of such subpopulations is most likely the mechanism by which the MS drug glatiramer acetate can alleviate the disease course (see I. 1. 4. 2. a).

Activated mononuclear cells, *i.e.* macrophages and microglia, are another hallmark of MS lesions, but their role is not fully understood (see also I. 2. 1. 1. c). Furthermore, given the notion of insidious hidden neuronal damage that often goes undetected (see I. 1. 3 and Figure 5), there may be another pathological process that remains elusive. In support of this, neuronal damage has been reported prior to any detectable demyelination (Evangelou et al., 2000; DeLuca et al., 2006; Calabrese et al., 2015). It may involve $CD4^+$ T cells directed against β-synuclein, which are selectively located in the grey matter and predominantly found in patients with progressive MS (Lodygin et al., 2019). β-synuclein may be a particularly potent antigen due to its high expression, location in structures with high turnover, and proximity to APCs. Furthermore, since it is known to form aggregates and be post-translationally modified, it fits the pathophysiological notion outlined above (see I. 1. 1. 2; Hayashi and Carver, 2022). Nevertheless, a plethora of autoantigens appear to be present in MS patients, some of which have been identified, but they vary and may be evidenced by the presence of oligoclonal bands in MS patients (an alternate explanation is outlined in I. 2. 1. 1. b).
This leaves room for debate as to how such a variety of autoantigens can be generated and coexist in MS patients – one explanation is the physiological presence of myelin-directed T cells in healthy individuals that are pathologically proliferated and activated in MS patients (Pette et al., 1990; Elong Ngono et al., 2012; Lodygin et al., 2019).

I. 1. 2. 2. Lesion histopathology

The implications of the immune attack on the CNS outlined above are substantial, including inflammatory demyelination, axonal and neuronal loss, and astrocytic gliosis (Stadelmann, 2011). The actual pathological processes in the white and grey matter are complex and the subject of ongoing research. Regarding the cellular participation, $CD8^+$ T cells are the most abundant immune cells in MS lesions followed by macrophages/monocytes and $CD4^+$ T cells (Fransen et al., 2020; Attfield et al., 2022). In the earliest stages of the lesion, typically in the white matter of the brain, activated microglia and initially condensed-nuclei, later apoptotic oligodendrocytes dominate. Ensuing, macrophages scavenge myelin debris. They are accompanied by $CD8^+$ T cells, $CD4^+$ T cells, monocytes, and plasma cells. In late lesion states, remyelination often occurs in association with lymphocyte-rich perivascular cuffs. Chronic inactive lesions consist of a sparsely populated lesion center surrounded by a lesion rim abundant in microglia, $CD8^+$ T cells, and dendritic cells (see also Figure 3). Loss of axons and neurons is common in the early and later stages of the disease. It is the primary cause of disability and brain atrophy. Throughout, smoldering inflammation can persist and become self-sustaining behind the BBB, even in later stages, in spite of a restructured, impenetrable BBB for newly activated immune cells (see also I. 1. 4. 1).

As indicated above, lesions can also be classified according to their mode of expansion or state of activity, which would be interpreted from a more radiological (expansion characteristic) or histological (activity characteristic) point of view (see Figure 4).

FIGURE 3: IMMUNE CELLS IN MS

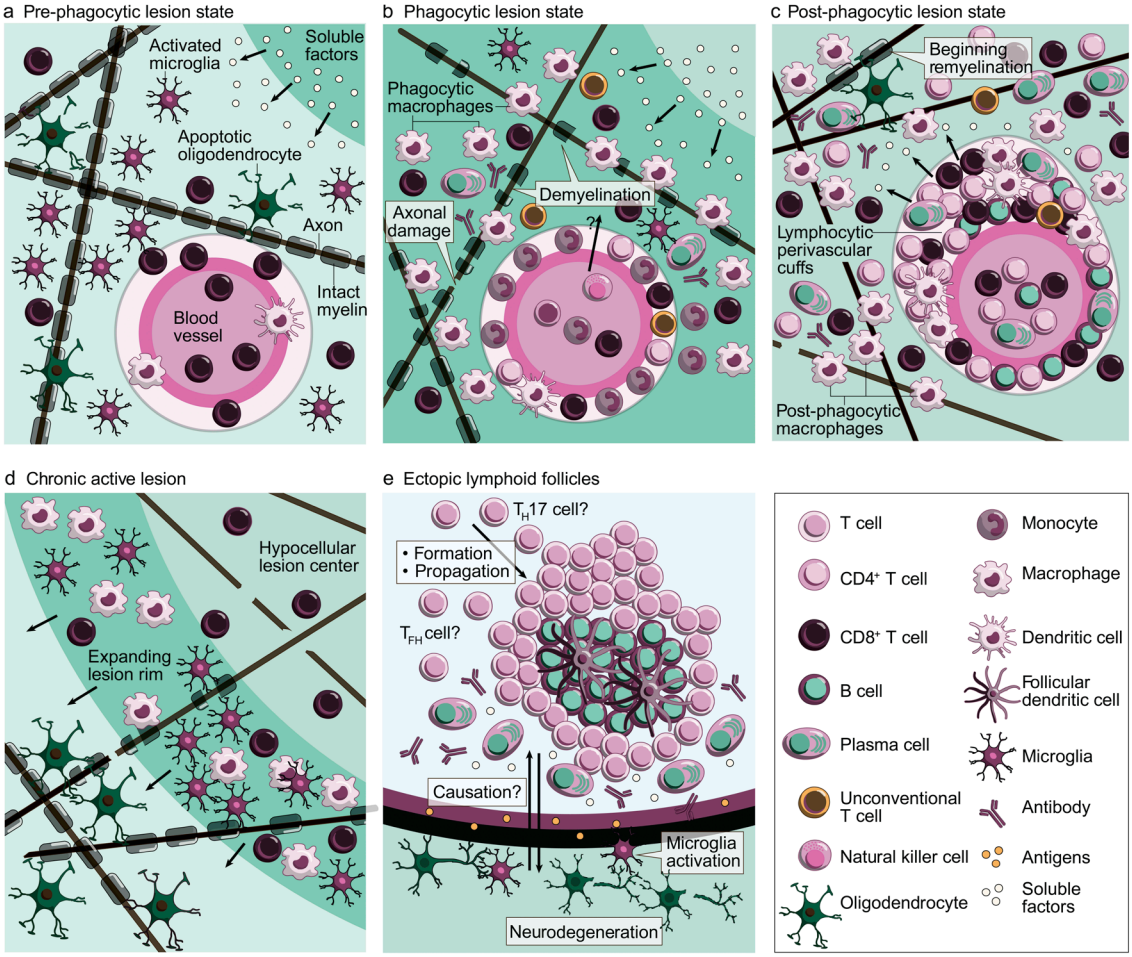


Figure 3 shows aspects of immune cell involvement in MS:
"***Multifaceted roles of immune cells in brain pathology in multiple sclerosis. a–c*** | *The pre-phagocytic lesion state is characterized by activated microglia and oligodendrocyte apoptosis, with few immune cells and little demyelination* […] *(part* ***a****). The phagocytic lesion state is characterized by* […] *macrophages, pronounced demyelination but limited axonal damage.* […] *(part* ***b****). In the post-phagocytic lesion stage* […] *demyelination is complete and macrophage digestion of myelin components has advanced. Peripheral immune cells are abundant in the parenchyma and prominent perivascular cuffs.* […] *(part* ***c****).* ***d*** | […] [C]*hronic active lesions* […] *are characterized by a rim of activated microglia and macrophages with a slowly expanding demyelinating front.* ***e*** | *Ectopic lymphoid follicles are localized predominantly in meninges near the brain surface. They* […] [could] *promote the B cell axis of adaptive immune responses* […]."
Source: Attfield KE, Jensen LT, Kaufmann M, Friese MA, Fugger L (2022): The immunology of multiple sclerosis. Nature Reviews Immunology; 22, page 745. (Attfield et al., 2022)
Modified and reproduced with kind permission from Springer Nature (RightsLink-License: 5452380397793)

FIGURE 4: MS LESION TYPES AND THEIR HISTOPATHOLOGY

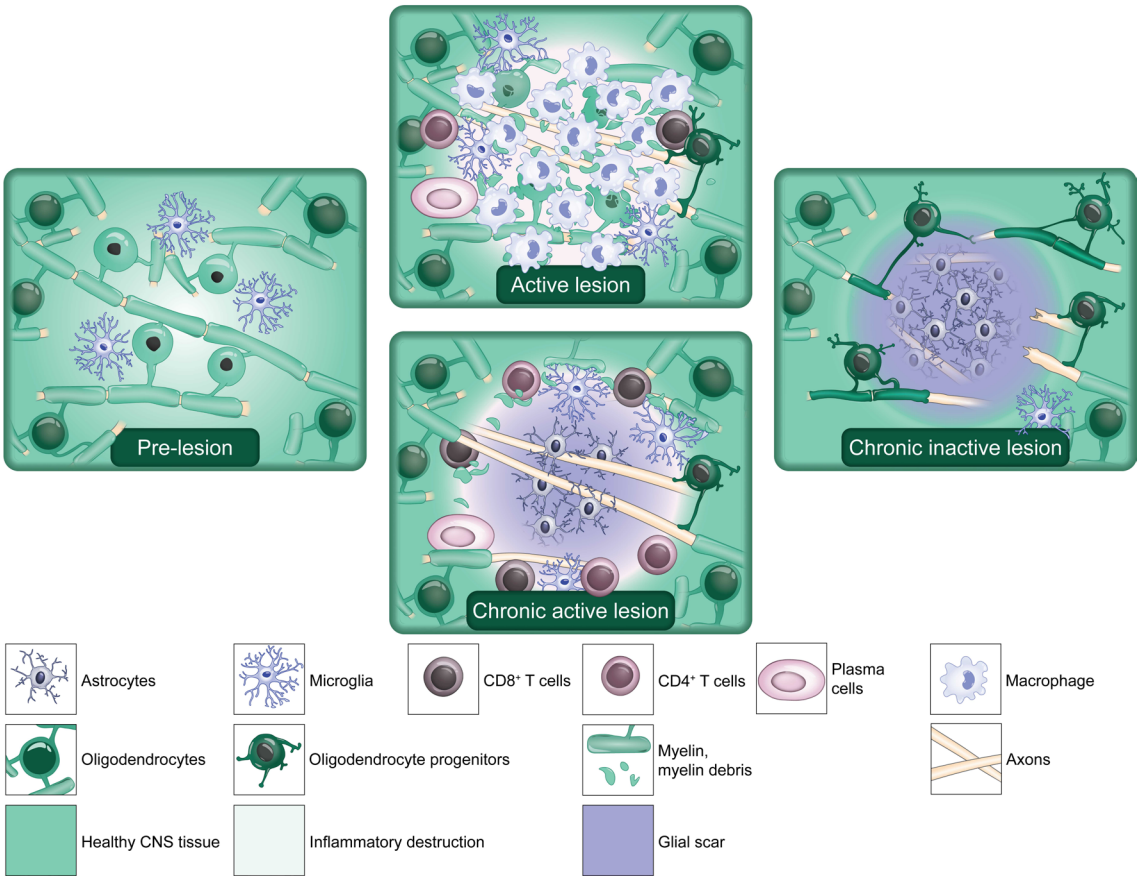


Figure 4 shows the lesions representing a continuum between inflammation and demyelination and axonal loss, which can correlate with the current phase of the disease (see I. 1. 3).

A pre-phagocytic lesion state has been proposed to precede the actual demyelinating inflammatory damage. It is unclear whether this corresponds to radiologic prelesions, which have been described to develop into typical gadolinium-enhancing lesions, or whether it is interindividual pathologic aspects that define these condensed nuclei of oligodendrocytes and microglia infiltration. Inflamed and actively demyelinating lesions (hereafter referred to as active lesions) are characterized by (foamy) phagocytes, myelin debris, perivascular lymphocyte infiltration with few B cells, and low levels of oligodendroglial apoptosis and normal density. Chronic active lesions have a more inert core, essentially a glial scar, and a smoldering rim of ongoing inflammation. This rim has features of activated T cells, BBB breakdown, phagocytes, and myelin debris. Remyelination is rarely observed in these lesions. Chronic inactive lesions represent a demyelinated, hypocellular core composed mainly of astrocytes in which axonal loss is often observed. The rim of these lesions often exhibits remyelination (thinner myelin sheaths). Lesion progression is not linear, and lesion states can merge into each other, except for the end-stage chronic inactive lesion core.

Lesion locations are typically the optic nerves, brainstem, juxtacortical, cortical and periventricular, and spinal cord (Filippi et al., 2019). However, in principle, any part of the human CNS can be a focus of lesion generation. This is consistent with the ‘chameleonic’ clinical presentation. An interesting site of demyelination is the cortical gray matter, where MS-specific bands of demyelination can occur. Scientifically important, cortical lesions are often extensively remyelinated. In contrast to these more easily detectable lesions, a more subtle neuroaxonal degenerative process may be ongoing, laying the foundation for prodromal disease activity and long-term progression (see also in Evangelou et al., 2000; DeLuca et al., 2006).

I. 1. 3. Clinical presentation

In congruence to the pathological diversity alluded to in Figure 4, the clinical presentation of MS can be equally diverse. Not only does the syndrome depend on the location of the lesions, but the course of the disease can be unpredictable. Although the brainstem, optic nerve, and spinal cord are the most common lesion sites, there are many different presentations (Thompson et al., 2018a). Furthermore, a latent phase of asymptomatic lesion development or even a separate pathological process – consisting of subtle neuroaxonal damage whose characteristics remain largely elusive – probably precedes the diagnosis. Thus, an MS prodrome has been proposed (Makhani and Tremlett, 2021).

Typically, a clinically isolated syndrome is the ‘first relapse’, a single episode of symptomatic disease activity, synonymous with attack or exacerbation. Classic symptoms include unilateral loss of vision in case of optic nerve involvement, sensory and motor deficits in focal brainstem, focal supratentorial, or cerebellar syndromes, but there are also several atypical manifestations (Miller et al., 2012; Thompson et al., 2018a; Thompson et al., 2018b; Makhani and Tremlett, 2021). These relapses are often associated with relapse-associated worsening (RAW), and disability results mainly from exacerbations, especially when historical data is considered. But even in the absence of attacks, the course of the disease is uncertain, with long-term progression to disability seen in 15-30% of cases (Cree et al., 2016; Thompson et al., 2018b). The clinical presentation of some patients begins with a primary progressive course (primary progressive MS, PPMS), while others show a progressive phase secondary to the relapsing-remitting onset of the symptomatic period (SPMS or RRMS, respectively). However, it may be more appropriate to regard MS pathophysiology as a spectrum with an inflammatory pole (immune infiltration and lesion formation; relapse) and a neurodegenerative pole (neuroaxonal damage; progression), as shown in Figure 5 (Kappos et al., 2020; Schmierer and Giovannoni, 2021; Kuhlmann et al., 2023).

FIGURE 5: MS DISEASE CONTINUUM AND PHASES OF MS

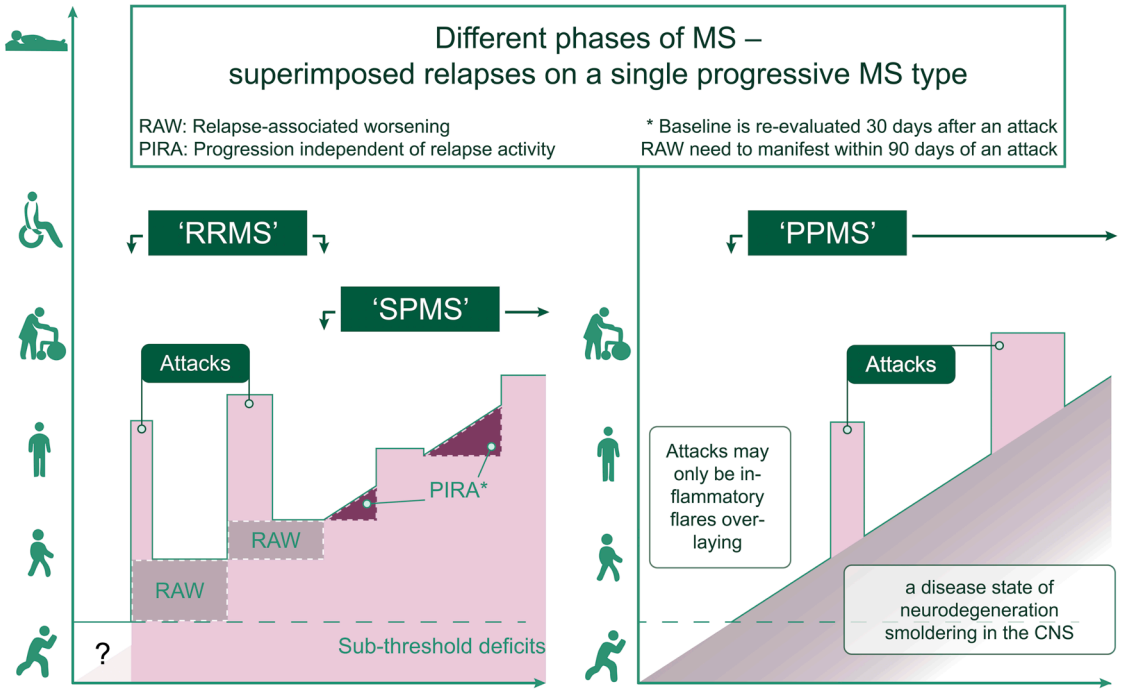


Figure 5 shows an interpretation of current findings (Hauser and Cree, 2020; Kappos et al., 2020; Pachner, 2021; Schmierer and Giovannoni, 2021) regarding the scarcity of RAW events in PPMS but also in RRMS (not shown), and a putative, underlying neuroaxonal damage process in all previously proposed 'types' of MS.

I. 1. 4. Diagnostics and current treatment options

I. 1. 4. 1. Diagnosis

Advances in the understanding of MS have been accompanied by improvements in diagnostic tools and sensitivity. The revised McDonald criteria of 2017 represent the latest step towards diagnosing increasingly mild presentations as MS (Thompson et al., 2018a; Pachner, 2021). The standards have changed dramatically since their introduction in 2001, but especially in comparison to the previous criteria. This is something to keep in mind when interpreting the success of current treatment regimens, as they may be confounded by overall fewer clinical symptoms due to more permissive diagnoses (Sormani et al., 2008; Pachner, 2021). In essence, diagnosis requires multitemporality as well as spatial independence of typical lesions or symptoms. Clinical, radiographic, and laboratory findings are used to objectify this as shown in more detail in Table 1.

I. 1. 4. 2. Treatment

The focus is on three strategies: Managing acute attacks, relieving symptoms, and reducing inflammatory activity (Hauser and Cree, 2020; Rajendran et al., 2021b). Acute attacks are usually treated with intravenous methylprednisolone or dexamethasone, followed by oral corticosteroids. Symptomatic treatment is multidisciplinary and includes lifestyle modification, mental health, exercise, and physical therapy. Given the multitude of studies suggesting beneficial effects of vitamin D with a very low risk of adverse effects, supplementation can be a reasonable option for those with vitamin D deficiency or low serum concentrations - although there may not be a direct link between vitamin D levels and MS (Laursen et al., 2016; Jagannath et al., 2018; Dörr et al., 2020).

TABLE 1: SIMPLIFIED MCDONALD CRITERIA

<table>
<tr><th>CLINICAL ATTACKS</th><th>LESIONS WITH OBJECTIVE EVIDENCE*</th><th>ADDITIONAL DATA NEEDED</th></tr>
<tr><td rowspan="2">≥ 2</td><td>≥ 2</td><td>None:
Dissemination in space (DIS) and Dissemination in time (DIT) criterium already met. Should conduct MRI if not already performed.
(If there is additional convincing historical evidence of a prior attack in another distinct anatomical location without documented objective evidence, one lesions is sufficient)</td></tr>
<tr><td>1</td><td>+ DIS by:
- Further clinical attack with objective evidence* OR
- ≥1 typical MRI lesions in ≥ 2/4 typical regions #</td></tr>
<tr><td rowspan="2">1</td><td>≥ 2</td><td>+ DIT by:
- Further clinical attack with objective evidence* OR
- Typical MRI lesions of different ages § OR
- Oligoclonal bands in the cerebrospinal fluid (CSF)</td></tr>
<tr><td>1</td><td>+ DIS (as above) AND
+ DIT (as above)</td></tr>
<tr><td colspan="2">Progression from onset</td><td>+ One year of progressive disability AND
+ ≥2 of these 3 criteria:
- ≥1 typical MRI brain lesions #
- ≥2 typical MRI spinal cord lesions #
- Oligoclonal bands in the CSF</td></tr>
</table>

*Anatomically corresponding findings from neurological examination, neurophysiological tests or imaging
#T2-hyperintense lesions in typical regions (periventricular, (juxta-)cortical, infratentorial and, spinal cord)
§Coexisting Gd-enhancing and -non-enhancing or new T2-hyperintense or Gd-enhancing lesions

Table 1 summarizes the 2017 McDonald criteria. For details and further description of symptoms and their specificity please refer to the original publication (Thompson et al., 2018a).

I. 1. 4. 2. a. Anti-inflammatory therapy

Given the great advances in relapse suppression with disease-modifying therapies (DMTs), the goals of treatment can now be defined as absence of disease activity in the form of relapses and no new evidence of MRI activity (new or enlarging lesions; Hauser and Cree, 2020; McGinley et al., 2021). Key milestones in the leaps in MS drug development, some of which are based on the underlying pathological processes in MS, are outlined in Figure 6.

FIGURE 6: HISTORICAL OVERVIEW OF MS THERAPIES

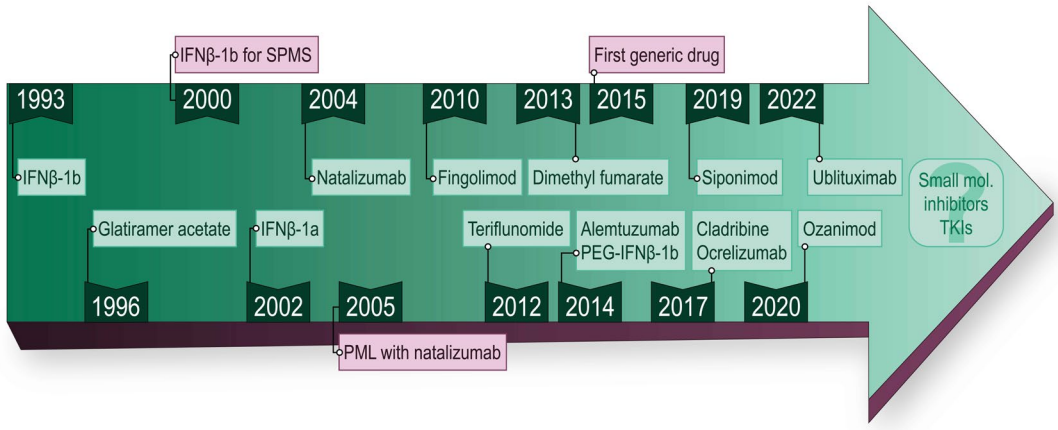


Figure 6 shows hallmarks of MS drug development.
Interferons and glatiramer acetate established the first DMTs while offering a favorable side effect profile. Since then, more effective drugs have been developed, enabling clinicians to substantially decrease relapses. Expectedly, these drugs also carry a greater risk of serious side effects – most famously with the resurgence of progressive multifocal leukoencephalopathy (PML) in 2005: Patients receiving immunosuppressive therapy with natalizumab may develop severe John Cunningham virus (JCV) induced encephalitis. Serious infectious complications in anti-CD20-treated B cell-depleted patients followed.
The possibility of high efficacy with tolerable side effects may be offered by small molecules that cross the BBB and are tailored to target specific pathways or tyrosine kinases involved in the pathophysiology. This presupposes a good knowledge of the pathological processes in order to identify potential target mechanisms (see I. 1. 4. 2. b).

Managing side effects rather than preventing relapses may be one of the greatest challenges facing clinicians today, as the stated goal of freedom from attacks is often already achieved. Various treatment regimens can be used, but there is no consensus as to which is the most appropriate:
Often a top-down approach is chosen, starting with highly effective drugs and then de-escalating the therapy. However, as the disease progresses, treatment options become more limited, less effective, and adverse effects increase (Hemmer et al., 2021).

An illustration of the insights gained from therapeutic interventions is glatiramer acetate, an MS drug with observed efficacy. The subsequent study of its mode of action has furthered our understanding of the pathophysiology of the disease. It led to the discovery that $CD8^+$ T cells also exert beneficial functions in MS. While it was previously thought that glatiramer acetate acts *via* APCs by diverting antigen presentation, glatiramer acetate has been shown to act on multiple cell types, including APCs, helper, killer and regulatory T cells, and plasma cells. Even though its exact mechanism of action still remains unclear, $CD8^+$ T cells have been shown to be essential for the therapeutic effects of glatiramer acetate (Tyler et al., 2013). It remains to be seen, whether this effect depends on certain $CD8^+$ T cell subpopulations themselves or whether interactions of

these cells with other parts of the immune system are decisive (Karandikar et al., 2002; Lalive et al., 2011b; Tyler et al., 2013). Another example of lessons learned from therapeutic interventions is the profound clinical impact of B cell depletion therapies. Previously, MS was thought to be T cell driven but the efficacy of B cell depletion has compellingly demonstrated the involvement of these immune cells (see I. 2. 1. 1. b).

FIGURE 7: TREATMENT OPTIONS IN DIFFERENT MS PHASES

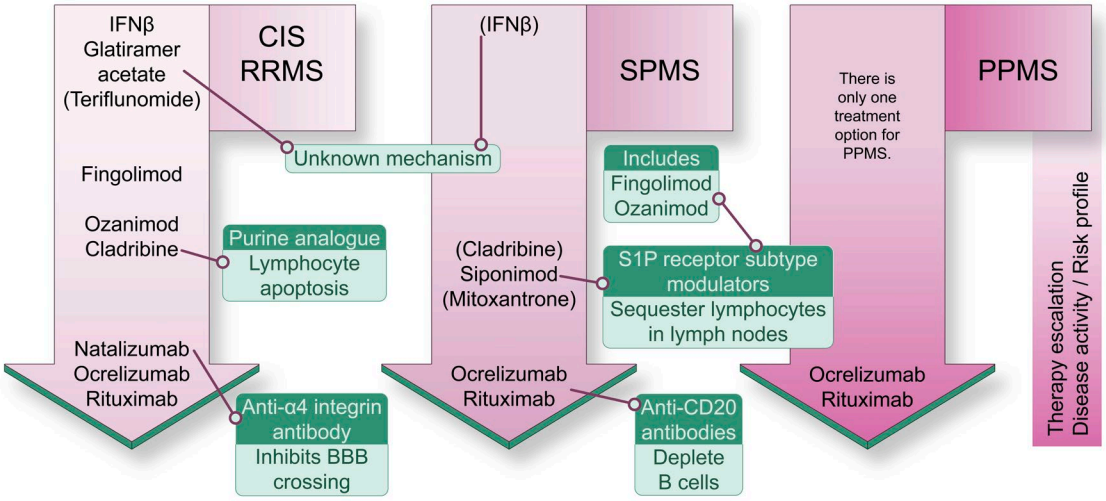


Figure 7 shows a possible treatment approach for different stages of the disease.
If the disease is in later or more severe stages, the options and their efficacy decrease dramatically. A high-risk profile includes high attack frequency, severe disability after acute attack treatment, poor remission after attacks, numerous or spinal cord lesions, young age, polysymptomatic onset, high intrathecal immunoglobulin. In RRMS, teriflunomide may be less effective (Kalincik et al., 2019); for patients with SPMS, cladribine is insufficiently studied (Hemmer et al., 2021), mitoxantrone has unfavorable side effects (Hauser and Cree, 2020; McGinley et al., 2021), interferons are at best marginally effective in SPMS patients with relapses. Rituximab is used off-label in Germany (Hemmer et al., 2021). Specific patient populations such as pregnant women and children require differentiated therapy selection.

While current treatment strategies appear to postpone and slow progression and greatly reduce the frequency of relapses, they cannot completely prevent them (Hemmer et al., 2021; McGinley et al., 2021; Rajendran et al., 2021b). In addition, even patients without relapses eventually develop disability (see I. 1. 3). It will thus be interesting to see if future long-term data can show a reduction in disability severity in those with infrequent attacks.

I. 1. 4. 2. b. Outlook

As described, addressing progression and causal pathology with novel treatment strategies is needed to help those who are at risk for disability. Given the inability to find a common autoantigen and the multitude of genetic variants as risk factors (Attfield et al., 2022), targeting subpopulations individually may be required to accurately address differing causal drivers of the autoreactive immune system.

The physiological remyelination mechanisms that occur in the disease state also hold great potential, especially for patients who are already affected or disabled. However, remyelination often fails and augmentation of the repair mechanism could offer rehabilitation from disability.

Antecedent in the causal chain, neuroprotective therapies could prevent the disabling neurodegeneration, *e.g.*, by modulating mitochondrial dysfunction (Dutta et al., 2006; Stadelmann, 2011; Angelova and Abramov, 2018; Lassmann, 2018; Tobore, 2020).
Therapeutics targeting molecules with unprecedented specificity already exist and are beginning to be implemented or are on the verge of widespread clinical use: TKIs – small molecules tailored to inhibit a small array of receptor tyrosine kinase (RTKs) or even a singular receptor type – have been intensively studied in recent decades, especially in oncology (Bedard et al., 2020), but also in inflammatory diseases (Decout et al., 2021; Ringheim et al., 2021; Gonzalez Lopez de Turiso and Guckian, 2022; Shalabi et al., 2022; Steinmaurer et al., 2022). They have already been approved by the major drug administrations and are expected to have far fewer side effects due to their specificity. Modulating only those pathways that are genuinely involved in immune system autoreactivity, or those that drive the specific inflammatory response and cytokine microenvironment may render more invasive techniques such as autologous hematopoietic stem cell transplantation (NCT04047628; Burt et al., 2019) avoidable.

Targeted therapies

One of these highly specific therapeutic targets is the Bruton's tyrosine kinase. It has gained much attention in the field of dermatologic autoimmune diseases (Ringheim et al., 2021) but also for MS (Steinmaurer et al., 2022). Tyrosine kinase 2 inhibitors and other TKIs (Gonzalez Lopez de Turiso and Guckian, 2022; Shalabi et al., 2022) may offer other pinpoint approaches. However, a large high-quality phase 3 trial failed to show superiority of Bruton's tyrosine kinase inhibitor evobrutinib over teriflunomide (Merck, 2023; Barboza, 2024). As a result, other pathways hypothesized to be the most crucial in MS, like the fibroblast growth factor (FGF) signaling cascade enter the spotlight. Given their variety and broad functions (see I. 3) inhibition of specific FGF receptors (FGFRs; Dai et al., 2019) offers great potential for precision-guided therapeutic modulation (see I. 1. 4. 2. b, and I. 3. 4).

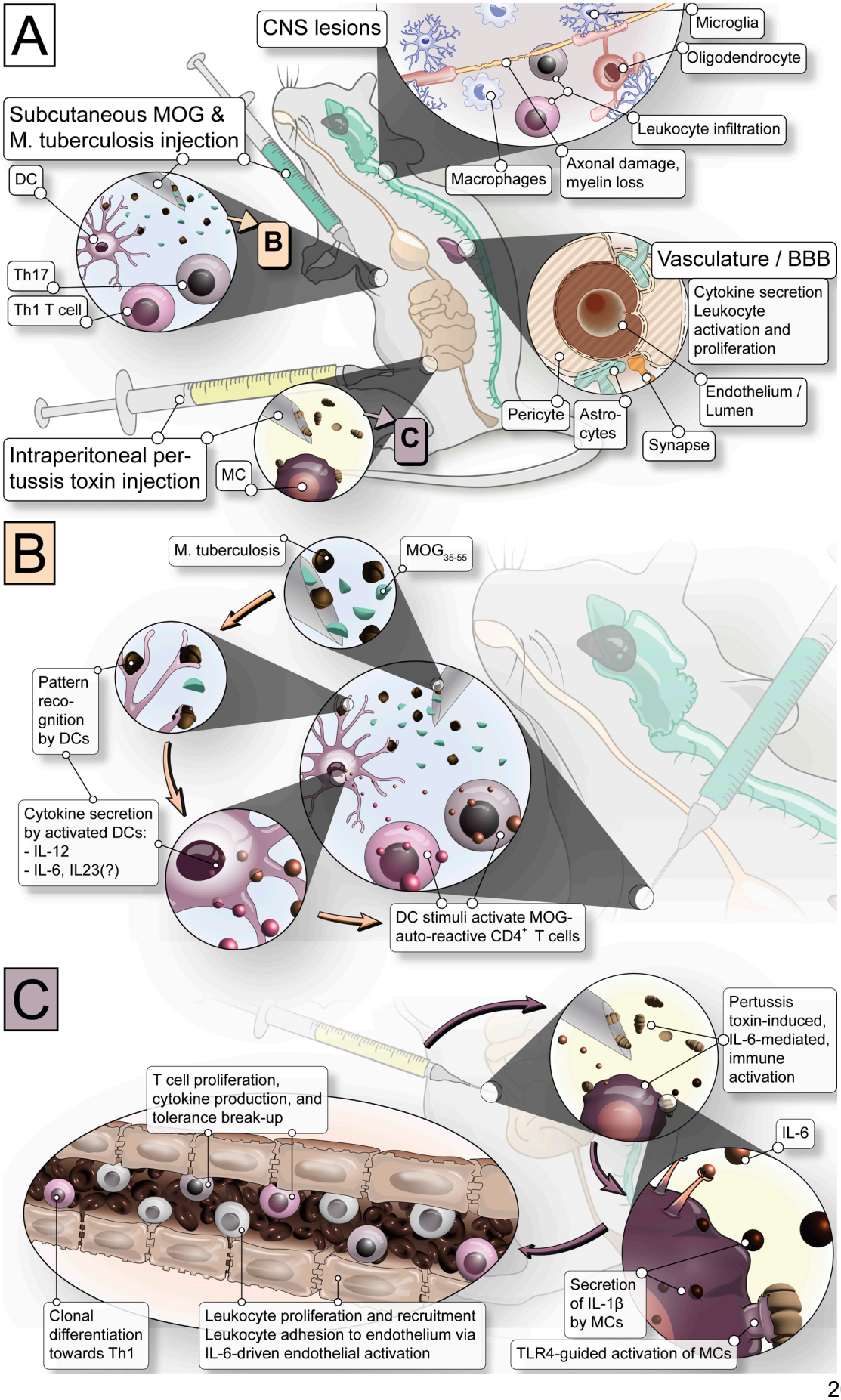
I. 2. EXPERIMENTAL AUTOIMMUNE ENCEPHALITIS

As repeatedly stated, a deeper understanding of MS is needed to improve the insufficient therapeutic tools available today. To this end, animal models remain indispensable for elucidating pathomechanisms and developing new drug targets. EAE is the most established and frequently used mouse model to study MS (Constantinescu et al., 2011; Robinson et al., 2014). It was initially developed in primates and then widely used in guinea pigs and rats. Most notably, the MS drugs IFN-β and glatiramer acetate have been developed in this model, validating it. Today, murine species are the most common application. In the case of this study, Black 6 (C57BL/6) mice were immunized subcutaneously against a component of the murine myelin sheath. Myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) has been used to induce a chronic, progressive clinical course that can enter a stable or recovery phase (hereafter, this specific form of EAE is referred to by using the term EAE; Robinson et al., 2014). In this model, *Mycobacterium tuberculosis* and *Bordetella pertussis* toxin are co-injected to generate an augmented (auto)immune response and weaken the BBB. This achieves the initiation of EAE (see Figure 8 for details) – a mechanism similar to that which may be responsible for infection-associated clinical worsening in MS (Dumas et al., 2014). During the course of the disease, animals present with ascending tetraparalysis emanating from the tail and other neurological symptoms, often followed by a partial recovery.

Figure 8 appears on the next page. Figure caption:

Figure 8 shows the main immunological venues of EAE. Two injections facilitate EAE: The antigen (MOG_{35-55}) is administered subcutaneously along with inactivated *M. tuberculosis* and intraperitoneal *B. pertussis* toxin. MOG_{35-55} and *M. tuberculosis* induce Th1 and Th17 T cells through DC activation, whereas *B. pertussis* elicits an augmented immune response including the release of interleukin 6 (IL-6) that activates – *inter alia* – MCs. It also leads to the proliferation and activation of leukocytes in the vasculature and culminates in the formation of CNS lesions where axonal damage and myelin loss are associated with a high presence of immune cells. (A).
M. tuberculosis is incorporated *via* pattern recognition receptors (PRRs). DCs subsequently secrete interleukins, including IL-6, -12 and -23. These stimulate physiologically existing MOG_{35-55} autoreactive T cells in the presence of the antigen to activate them and induce their ensuing proliferation (B).
In parallel, *B. pertussis* toxin has led to a global inflammatory response that is also driven by IL-6 secretion. This interleukin plays a role in the activation of MCs, which by exposition to the toxin secrete the highly pathological interleukin 1 beta (IL-1β). These cytokines lead to several pathological effects in the vasculature: T cells proliferate, partly towards Th1 phenotypes, release cytokines themselves, adhere to the inflamed endothelium and their tolerance is broken (C).
DC: Dendritic cells; MC: Myeloid cell; CNS: Central nervous system
BBB: Blood-brain barrier; IL: Interleukins; TLR4: Toll-like receptor 4

FIGURE 8: IMMUNOLOGICAL ASPECTS OF EAE



I. 2. 1. Background and limitations

EAE can be configured and tailored to highlight different aspects of the disease. The outcome and pathological processes of the model (*e.g.* chronicity, inflammation, degeneration) will vary depending on these configurations. The mouse strain, the immunogen applied – even if the only difference is the species of origin (Oliver et al., 2003) – and many other factors greatly influence the outcome (a detailed review can be found in Gold et al., 2006; Levy et al., 2010; Constantinescu et al., 2011; also reviewed in-depth in Robinson et al., 2014; Van Kaer et al., 2019). As an example of these many adaptations, adoptive-transfer EAE is mentioned here because of its importance in the study of T cell-induced autoimmunity and in understanding of the immunology inherent to this EAE model. It demonstrated that myelin-specific T cells (propagated *in vitro* and then transferred to the animals) suffice to generate an autoimmune response against the CNS that resembles an MS relapse (Robinson et al., 2014). However, a caveat in most EAE models regardless of their adaptation is the absence or limited occurrence of ataxia, spasticity, loss of motor coordination, and brain lesions highlighting their clinical difference from MS.

I. 2. 1. 1. Immunology of EAE vs. MS

Naturally, animal models cannot completely represent the disease for which they were developed. This is especially true for the complex immunology of an MS disease model. However, such models can yield profound insights into inflammatory demyelination and CNS immunity. Some important aspects of (in)congruence are summarized in Table 2.

I. 2. 1. 1. a. T cells

$CD4^+$ T cells include several subpopulations, which vary in cytokine profile, scope of duties and route of activation. The dichotomy of $CD4^+$ T helper 1 cells (Th1 cells), which are polarized by IL-12, secreting IL-2, -12, TNF-α and interferon gamma (IFN-γ), and $CD4^+$ T helper 2 cells (Th2 cells), which are polarized by IL-4 and -2, secreting IL-4, -5, -9, -10, -13 and -25, observed in mice is less pronounced in humans where these subpopulations tend to overlap (Robinson et al., 2014). In addition to Th1 cells, $CD4^+$ T helper 17 cells (Th17 cells) – which secrete IL-17, -21, and -22 and differentiate in the presence of IL-23 – have been identified as central to T cell-mediated autoimmunity in both EAE and MS (Attfield et al., 2022).

It is noteworthy that these cell polarizations are defined by specific cytokine compositions – a fact that may be the pivotal perspective when examining the aberrant immunity and the dysregulated microenvironment in the MS brain (see IV. 4). Moreover, while the

involvement of $CD8^+$ T cells in MS is well established, classical EAE models are based on $CD4^+$ T cell-driven autoimmunity, in which $CD8^+$ T cells may have a more regulatory, even beneficial role (York et al., 2010; Van Kaer et al., 2019; Attfield et al., 2022). However, both T cell subtypes are important for MS, as underscored by the aforementioned and mechanistically convincing study by Lanz *et al.*, which demonstrated high-affinity molecular mimicry of EBNA1 with post-translationally modified hepaCAM. Such autoreactive antibodies have been shown to worsen clinical course, induce activation of both $CD4^+$ and $CD8^+$ T cells, and exacerbate demyelination (Lanz et al., 2022).

I. 2. 1. 1. b. B cells

Oligoclonal bands secreted by CNS-resident plasma cells are a hallmark of MS, and their role is still a matter of discussion. Although causative antibodies have been discovered, oligoclonal bands may represent responses to neoantigens in the course of demyelination rather than evidence of the causal autoreactivity (Pryce and Baker, 2018). Counterintuitively, in MS, B cell depletion therapy does not seem to function by removing autoreactive antibodies (Cross et al., 2006; Piccio et al., 2010; Myhr et al., 2019). Rather, the overall reduction of B cell quantity, and their reduced interaction with other immune cells appears to be reason for the efficacy of B cell depletion. In contrast, in EAE, the antibody response is highly dependent on the immunogen and the animal background. Although debated, demyelinating antibody responses are the exception rather than the rule in the various EAE models (Lalive et al., 2011a; Hemmer et al., 2015). Notably, B cells can leverage the immune response *via* antigen presentation and cytokine-driven stimulation of T cells (Van Kaer et al., 2019). Although important in MS and EAE, they are not central to the pathology and certainly not in a classical role. Yet each of them has a unique part, especially since antibodies are generally absent in EAE and not the pivotal component of B cell pathology for MS either (Oliver et al., 2003; Gold et al., 2006).

I. 2. 1. 1. c. Innate immune cells

Overall, monocytes are centrally involved in antigen presentation, inflammation, and repair of tissue damage. Macrophages/monocytes from the periphery and CNS are abundant in both EAE and MS lesions but may act as bystanders rather than perpetrators. Importantly, myelin debris scavenging is a prerequisite for CNS repair mechanisms and possibly abatement of inflammation. Thus, myelin clearance putatively precedes remyelination and recovery (Lampron et al., 2015; Grajchen et al., 2020). However, cytokine secretion by macrophages/monocytes can also fuel inflammation and

immune cell activation. Disease severity correlates with their quantity. There is much debate as to whether these cells are required for clearance of myelin debris or whether they contribute to the pathology *via* oxidative stress or even initiate demyelination (Rajendran et al., 2021b).

Given the evidence for both a deleterious and reparative concept, one explanation may be that blood-derived monocytes, which comprise a distinct population of phagocytes from CNS-resident microglia, assume an imbalance in the diseased state. Thus, the functional diversity would be caused mainly by two distinct cell populations – one derived from the peripheral compartment where they were initiated, the other resident in the CNS where they control tissue homeostasis (Hemmer et al., 2015). Furthermore, microglia and macrophages are characterized by strong plasticity and changes in their transcriptome. They can be polarized towards anti- and pro-inflammatory M1/M2 phenotypes (Vergadi et al., 2017; Chu et al., 2018; Matsui and Mori, 2018), but this dichotomy is gradually resolved towards a functional continuum.

In EAE in particular, macrophages and microglia are responsible for axonal damage. This includes mitochondrial and oxidative excitotoxicity such as reactive oxygen species (ROS) – a characteristic of microglia known from other neurological diseases (Gold et al., 2006; Iovino et al., 2020). Concordantly, the majoritarian ablation of both microglia/macrophages reduced symptoms and demyelination in EAE (Nissen et al., 2018). However, depletion of microglia in a secondary progressive form of EAE has clear deleterious consequences, as seen after colony stimulating factor 1 receptor (CSF1R) inhibition, which markedly exacerbated disease progression (Tanabe et al., 2019). Vice versa, CSF1R stimulation had beneficial effects *via* the generation of $CD11^{+}$ microglia (Wlodarczyk et al., 2018). Overall, in both EAE and MS macrophages/monocytes and microglia are indispensable players in both cytokine homeostasis and inflammation and may be considered part of the culminating phase of the disease rather than the causal drivers. However, understanding their pathogenicity is immanent to MS pathology, and in a mechanistic parallel to FGF/FGFR signaling, microglia may be beneficial in the immediate management of an active lesion, but their continued activation in an imbalanced microenvironment may also contribute to neuroaxonal damage.

Innate-like lymphoid cells

Reflecting the complexity of immunology, even the dichotomy of innate vs. adaptive immune cells is increasingly being replaced by the notion of a functional continuum. Appropriately, immune cell populations such as natural killer T cells, mucosal-associated invariant T cells, marginal zone B cells, and other innate-like B cells are part of a limbo

of this continuum, combining both innate and adaptive properties. Given the diversity of these cell populations, the conflicting results from different models, their functional non-homology in Muridae and Hominidae, and the incomplete study of these cells in MS make it difficult to integrate their importance. However, several studies have shown their involvement in both EAE and MS, and further investigation is needed.

TABLE 2: DIFFERENCES AND SIMILARITIES OF EAE AND MS

	EAE	MS
Antigen(s)	MOG_{35-55}	Unknown, possibly cross-reactivity to EBNA, not MOG_{35-55}
Immunization	Direct immunization against antigen	Most likely indirect (Drainage from CNS or cross-reactive to external antigen)
T cell involvement	(Th1) $CD4^+$ T cell dominant Central role of Th17 $CD4^+$ T cell Conduciveness of $CD8^+$ T cells	$CD8^+$ T cell dominant Central role of Th17 $CD4^+$ T cell
B cell involvement	Most likely stimulatory rather than executive	
Antibodies	Most likely not assuming a central role	
Lesion location	Most notable in the spinal cord	Mainly in the brain, but also in the spinal cord
Clinical aspects	Different clinical presentation to MS Various phases and disease courses No model for long-term progression	Different clinical presentation to EAE Different disease phases Presumably smoldering chronic progression
BBB	Induced breakdown	Presumably functional disturbance on an unknown background
Trigger	Freud's Adjuvant Putatively confounding inflammation against adjuvants	EBV infection (Epi-)Genetic vulnerability Possibly further unknown triggers

Table 2 summarizes important differences and similarities of EAE in comparison to MS.

Given the obvious differences between the models and the human disease and the multitude of antigens in EAE and possibly MS listed here, it may come across surprising that so many accomplishments have accrued from this model. Many mechanisms, signaling pathways and cell types have been identified in EAE, emerged as important for EAE, presented differently in MS but turned out to be important in MS but by a different mechanism. Thus, major advances in understanding MS pathophysiology by understanding neuroinflammation and neurodegeneration *via* EAE have been achieved and may be derived from these models in the future.

Furthermore, the translation of new developments from other fields of research into this model allows the study of effects in an ecosystem that has been extensively explained. One of those developments – stemming from tumor pharmacology – are TKIs and in particular the FGF pathway.

I. 3. THE FIBROBLAST GROWTH FACTOR PATHWAY

Half a century after the first scientific emergence of FGFs (Armelin, 1973) and their cloning in the 1980s (Abraham et al., 1986), we are now witnessing a renaissance of interest and clinical application. Their historical name has remained, despite being somewhat imprecise given their functional fundamentality, ubiquity, and the absence of some of them on fibroblasts (Li et al., 2016). Their binding partners, the FGFRs, offer somewhat less variance. But even though there are only four different receptors, isoforms in the form of splice variants add to the diversity of FGF/FGFR signaling. The FGF/FGFR signaling pathway is fundamental to vertebrates and is involved in a plethora of developmental processes, disease states and regeneration.

I. 3. 1. Fibroblast growth factors

FGFs comprise 23 members in vertebrates (including rodent FGF15 and human FGF19 orthologs) of which three main categories can be distinguished (see Table 3; Ornitz and Itoh, 2015; Li et al., 2016; Ornitz and Itoh, 2022). FGFs are critical for embryogenesis, tissue homoeostasis, and regeneration and repair (Ornitz and Itoh, 2015). Their diversity and interconnection with several fundamental pathways, including metabolic regulation *via* mechanistic target of rapamycin (mTOR) and endocrine FGFs, complicates a general understanding of their function (see also Table 3). In the case of the canonical FGFs (auto-/paracrine FGFs, that signal classically), they can be described as partially redundant and relatively conserved vertebrate cell switches (Itoh and Ornitz, 2004).
They are fundamental in controlling cell programs such as survival, differentiation, and proliferation (Ornitz and Itoh, 2015). Therefore, it is not surprising that these factors are involved in tissue genesis and repair as well as in disease, and necessitate strict regulation (Ornitz and Itoh, 2015; Li et al., 2016). This is implemented – *inter alia* – through their affinity to heparan sulfate. Heparan sulfate is also a required cofactor for FGFR binding, its abundance in the extracellular matrix stabilizes and restricts canonical FGFs to a para-/autocrine scope (see I. 3. 2; Ornitz and Itoh, 2022). In keeping with their diversity, knockout of FGF genes varies from little to no apparent phenotype, to early embryonic lethality.

TABLE 3: SUMMARY OF FGF FAMILIES, FUNCTIONAL ASPECTS

FIBROBLAST GROWTH FACTOR FAMILIES	
PARA- AND AUTOCRINE	
FGF1 Subfamily	
FGF1 FGF2	▪ Activates all FGFRs ▪ Non-canonically secreted, which reduces angio- and oncogenic potential (Forough et al., 1993; Ornitz and Itoh, 2022) ▪ Tissue development, homeostasis, repair (heart, lung, bone) ▪ Inflammation: Up-regulation in inflammatory cells (macrophages, lymphocytes); both attenuating as well as aggravating effects
FGF4 Subfamily	
FGF4 FGF5 FGF6	▪ Activate FGFR1-3 IIIc splice variants ▪ Skeleton development and disorders; vascular and skeletal muscle formation ▪ Breast cancer (FGF5); germ cell and urinary tumors (FGF4)
FGF7 Subfamily	
FGF3 FGF7 FGF10 FGF22	▪ Activate FGFR1-2 IIIb splice variants ▪ Ontogenesis: ▫ FGF3: Neural, skeleton development ▫ FGF7, 10: Lung, urinary tract development, lung disease and repair, angiogenesis, mucosa regeneration ▪ Tissue-dependent inflammatory regulation
FGF8 Subfamily	
FGF8 FGF17 FGF18	▪ Activate FGFR1-3 IIIc splice variants and FGFR4 ▪ Ontogenesis: ▫ FGF8: Highly important for skeleton development ▫ FGF17/18: Bone, cartilage, urinary system development and repair ▪ Cancer: Prostate, hepatocellular
FGF9 Subfamily	
FGF9 FGF16 FGF20	▪ Activate FGFR3 IIIb splice variant ▪ Activate FGFR1-3 IIIc splice variants and FGFR4 ▪ Ontogenesis: ▫ Skeleton development and repair ▫ Lung, heart, urinary system development ▪ Cancer: Lung, ovarian
ENDOCRINE	
FGF15/19 Subfamily	
FGF15/19 FGF21 FGF23	▪ Do not relevantly bind to heparan sulfate ▪ Co-factors αKlotho, βKlotho and Klotho-LPH related protein ▪ Metabolic functions ▫ Energy homeostasis, glucose-lowering, beneficial for lipid and hepatic metabolism ▫ FGF21 crosses BBB, increases insulin sensitivity ▪ FGF23: Mineral metabolism, prostate cancer
INTRACELLULAR	
FGF11 Subfamily	
FGF11 FGF12 FGF13 FGF14	▪ Not conventionally secreted, may still activate FGFRs (Sochacka et al., 2020) ▪ Modify excitability of neurons and other cells ▪ Aggravating effect in some cancers

(Xie et al., 2020)

Table 3 lists all known FGFs and summarizes important known functions and characteristics.

I. 3. 2. Fibroblast growth factor receptors

The appropriate receptors to the FGFs were also revealed in the late 80's, and shortly afterwards, all four FGFR (FGFR1-4) were cloned (Coughlin et al., 1988; Pasquale, 1990; Stark et al., 1991). They belong to the group of RTKs that signal intracellularly by

transferring phosphates from adenosine triphosphate (ATP) to tyrosine residues. Besides these four main receptor variants there is one closely related, FGFR-like 1 (FGFRL1), sometimes referred to as FGFR5. Furthermore, *FGFR1-3* exist in a variety of splice variants (potentially up to 256 per gene; Hou et al., 1991). Functionally important, FGFR1-3 are expressed in two isoforms as FGFR1-3b/c, respectively (see Figure 9). Beneath these spliced immunoglobulin (Ig)-like domains lie an acid box, a transmembrane domain and two tyrosine kinase domains that initiate the complex signaling cascade (see Figure 10).

FIGURE 9: STRUCTURE AND ISOFORMS OF FGFR

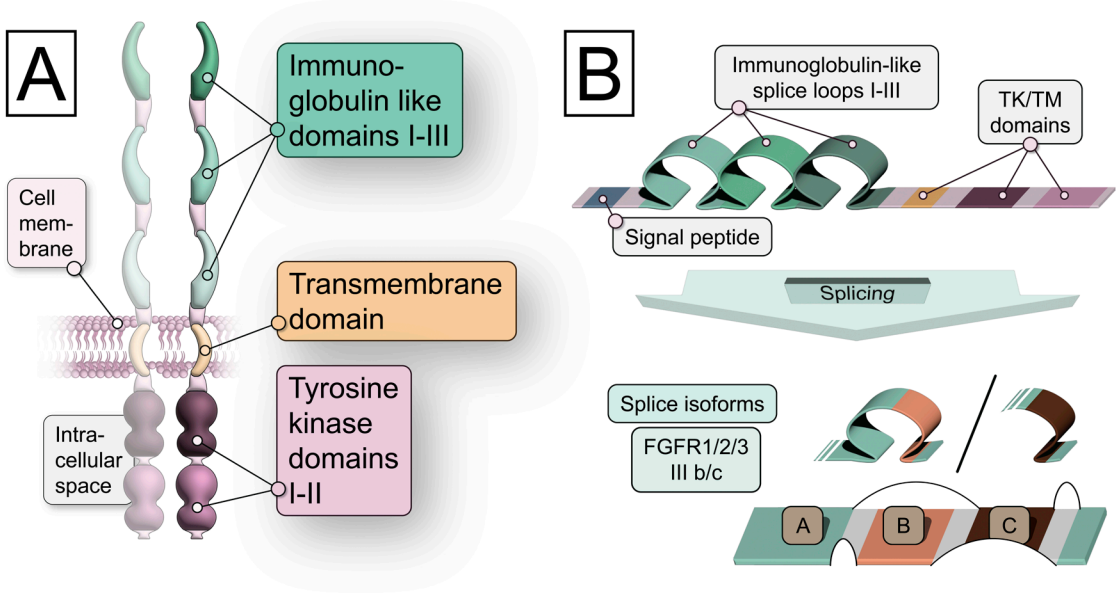


Figure 9 shows the structure of the FGFR molecule, which consists of 3 functional domains: 3 Ig-like binding sites for FGF, a transmembrane domain (TM) and 2 tyrosine kinase domains (A). Splicing at the third Ig-like domain allows for the generation of FGFR1-3 b/c isoforms (B). FGFR4 is not spliced into isoforms.

Upon activation of the receptor, its dimerization and trans-/autophosphorylation recruits and phosphorylates downstream molecules of three main effector pathways, i) mitogen-activated protein kinase (MAPK), ii) Akt (named after AKR mouse strain thymoma), and iii) signal transducer and activator of transcription (STAT). All FGFRs induce these signaling pathways, but with different intensities, *e.g.*, FGFR1 and FGFR2 emphasize on the MAPK extracellular signal-regulated kinase (ERK), and on phospholipase C gamma (PLCγ), which is the main inducer of protein kinase C (PKC; Eswarakumar et al., 2005; Brewer et al., 2016). The simplified signaling pathway of the FGFs with FGFR substrate 2 (FRS2) as an obviously central component is depicted in Figure 10.

Furthermore, several interactions of these effector molecules underscore their redundancy and reciprocity. For example, the phosphoinositide 3-kinase (PI3K) / Akt

pathway interacts with both the ERK and mTOR pathways to influence basal cellular metabolism and cycling (Mendoza et al., 2011; Okkenhaug et al., 2014; Ishii et al., 2019). STAT is another fundamental signaling pathway involved in cell transformation and presumably polarization (Hart et al., 2000). PKC is known to activate ERK (Mendoza et al., 2011; Fearon and Grose, 2014) and all STATs, Akt, ERK and PKC induce multiple genetic switches themselves. Not surprisingly, FGFR1/2 are essential for embryonic development. Knockout of these genes renders embryos inviable within the first two weeks (Eswarakumar et al., 2005).

FIGURE 10: EXCERPT OF THE FGFR SIGNALING CASCADE

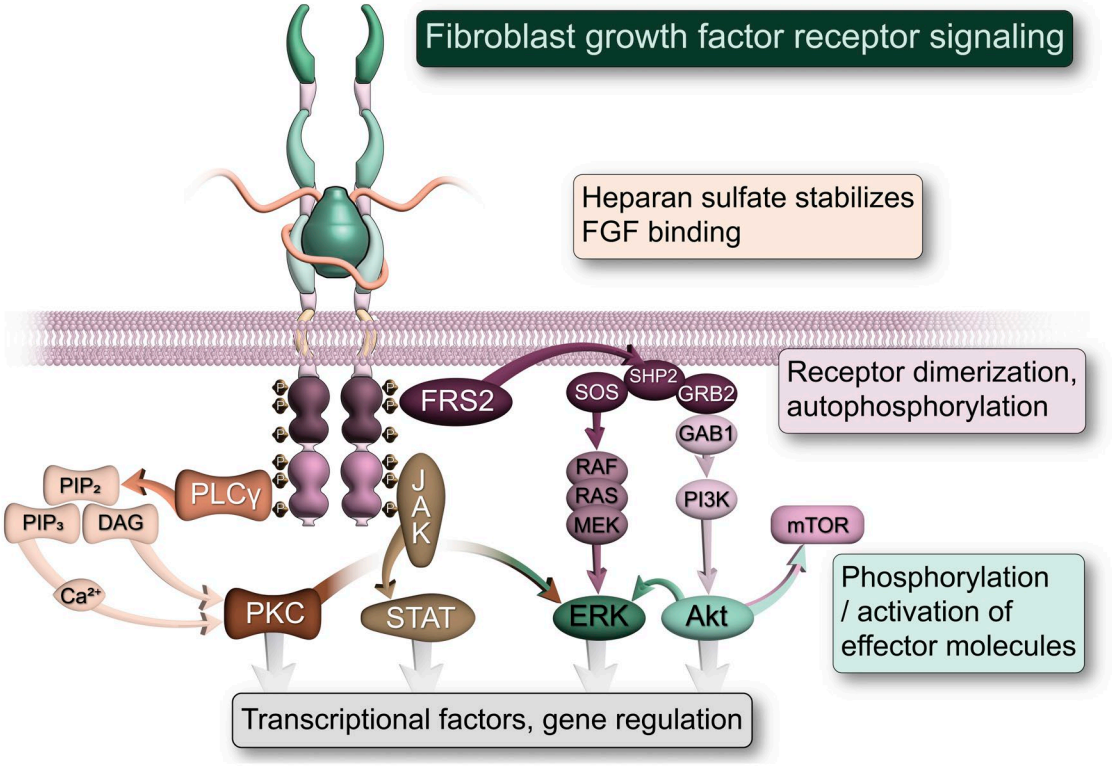


Figure 10 shows the main segment of FGFR signaling for the canonical (para/autocrine) pathway. For details on the FGFRs also see Figure 9.
There is strong regulation of signaling: Next to lack of heparan sulfate, ubiquitination, and degradation of the receptor, the following signaling inhibitors exist:
The most upstream intracellular inhibitor is Casitas B-lineage lymphoma (Cbl), which induces the degradation of FRS2. Through this ubiquitin ligase, FGF/FGFR signaling appears to be tightly regulated and self-limiting: Upon activation of FRS2 by FGFs, Cbl signals for FGFR ubiquitination, internalization and trafficking to lysosomes (Wong et al., 2002; Haugsten et al., 2008).
Sprouty is another protein that can block FGF/FGFR signaling through its antagonistic effect on RAS-MAPK signaling (Zheng et al., 2022).
FGFRs are known to interact with other critical pathways as well: *E.g.*, the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Wang et al., 2018; Yusuf et al., 2021) and – particularly in development – GSK3β which interacts with Wnt, establishing Wnt as an indirect target of FGF/FGFR signaling (see I. 3. 2. 1. a and I. 3. 3. 1. c; also in Wright et al., 2015).

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There are other factors that strongly influence FGFR signaling: Cell adhesion molecules modulate its signaling. N-cadherin stabilizes FGFR to prevent ubiquitination (Kon et al., 2019). Integrins act as co-ligands to amplify downstream signaling (Xie et al., 2020), neural cell adhesion molecule (NCAM) even acts as a ligand and promotes the appearance of FGFRs on the cell surface (Francavilla et al., 2009), heparan sulfates are highly important for stabilization with ligands and phosphorylation activity.
Cell adhesion molecules bind to an acidic box between the Ig-like domains I and II, heparan sulfates bind to FGFs themselves and to Ig-like domain II (Ornitz and Itoh, 2015).
Going further, FGFR1 also has intranuclear functions, it is translocated from the Golgi to the nucleus. There, it acts as a signaling hub for gene regulation, particularly important for development (Klimaschewski and Claus, 2021). *E.g.* it binds to cAMP response element-binding protein binding protein (CREB).

I. 3. 2. 1. FGFRs in immune cells and inflammation

FGFRs live up to their name by being implicated in proliferation in a wide variety of cell types. However, they are functionally diverse and have been indirectly associated with inflammation through their downstream targets. Further studies are needed to elucidate the immune cell-specific effects, as these are not well understood. This would help to define their modulatory actions on inflammation.
In general, FGFRs are putatively ubiquitous but specifically, they have been shown to be expressed – *inter alia* – on T cells, B cells, microglia, and macrophages (Liu et al., 1998; Byrd et al., 1999; Lee et al., 2011; Noda et al., 2014; Kuang et al., 2020). Since their discovery they have been known to be involved in immune responses (Blotnick et al., 1994; Zhao et al., 1995; Byrd et al., 1999). On macrophages, FGFRs facilitate angiogenesis and endothelial cell proliferation as well as inflammatory atherosclerotic events (Liu et al., 1998; Pakala et al., 2002; Raj et al., 2006). Similar mitogenic effects of FGF/FGFR signaling are also known from wound healing, FGFs attract macrophages and monocytes (Markan and Potthoff, 2016). In metabolic and cardiovascular diseases, some FGFs are linked to proliferative and inflammatory responses (Xiao et al., 2016; Zhang et al., 2018).

There is also an interesting association with another autoimmune disease: In autoimmune arthritis, FGF2 (*via* FGFR1) and IL-17 (relevant to MS, see also I. 2. 1. 1. a and I. 3. 2. 1) act in concert to induce inflammation (Shao et al., 2017). Several recent studies have implicated FGF2 in the exacerbation of inflammation in various disease states, so it is conceivable that future findings will solidify the pro-inflammatory role of FGF2/FGFR signaling (see also I. 3. 3. 1. c; also Shao et al., 2017; Liang et al., 2018; Zhang et al., 2018; Tan et al., 2022). Nevertheless, in certain settings (*in vitro* treatment of human umbilical vein endothelial cells), pro-inflammatory cytokines can also reduce FGFR1 abundance (interestingly, with the exception of Interleukin 6 (IL-6; Chen et al., 2012a), again pointing to the situational ambiguity of FGF/FGFR signaling responses.

These ambiguous effects will be addressed in the following sections with a focus on cell specificity.

I. 3. 2. 1. a. FGFR signaling in T cells and microglia

Since the present work examined FGFR inhibition in T cells and microglia *in vitro*, and due to their prominence in EAE and MS, it is appropriate to summarize what is known about FGFR signaling in these cells.

Microglia

The aforementioned plasticity of microglia gives rise to pro-inflammatory M1 and anti-inflammatory M2 phenotypes with corresponding cytokine and transcriptional profiles. How FGFs influence these polarizations remains to be seen, the evidence is conflicting, and integration is needed – yet another example of cytokine composition shaping immunology (see also discussed in IV. 4)
FGF1 treatment of primary microglia promotes M2 switching while decreasing Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (Dordoe et al., 2022). NF-κB is not a typical downstream target of para/autocrine FGFs, yet it can also be modulated by the MAPK pathway. Similarly, FGF10 attenuated inflammation and reduced NF-κB expression in microglia after spinal cord injury (Chen et al., 2017). Other cells responded differently to FGFs. In hepatic stellate cells, NF-κB is reduced after FGFR inhibition (Wang et al., 2020a). In addition, FGF21 treatment of primary microglia promoted a switch to M2. However, as an endocrine FGF, FGF21 has a different signaling cascade than canonical FGFs, but endocrine FGFs can also act through FGFRs (Ornitz and Itoh, 2022).

In a model of inflammatory depression induced by intraventricular lipopolysaccharides (LPS), intraventricularly applied FGF2 was anti-inflammatory and its inhibition was pro-inflammatory (Tang et al., 2018). In contradiction, high concentrations of FGF2 injected into the fourth ventricle led to microglial activation and proliferation (Goddard et al., 2002). To make it even more complex, FGF2 did not induce proliferation or activate microglia in neuron-microglia cocultures. It did, however, promote migration toward FGF2 through Wnt signaling. FGF2 also enhanced the phagocytosis of neuronal debris (Noda et al., 2014).

Microglia themselves are a source of FGF2 and – due to its para-/autocrine properties – a constant target of these factors. The amount of secreted FGF2 correlates with the extent of demyelination induced by cuprizone treatment (see also I. 3. 3. 1. b; Voss et

al., 2012). Again, it is difficult to dissect the various implications of FGF/FGFR signaling on microglia. To date, studies on this topic have mainly focused on FGF2. To the best of the author's knowledge, there are no studies investigating signaling blockage at the receptor level of this pathway specifically in microglia.

Conflicting results are common in emerging fields, especially when effects are as model-dependent as immune interactions. Furthermore, it should not be overlooked that these cells act in close coordination with their microenvironment and that any external stimuli will profoundly influence the CNS equilibrium. It must be recognized that FGFs can have both pro- and anti-inflammatory effects depending on the circumstances. Important factors are the particular FGF observed, the duration of its presence (Noda et al., 2014), the site of its application within the CNS and its concentration (Goddard et al., 2002), and the cause of inflammation/immune activation (Chen et al., 2017; Tang et al., 2018).

T cells

FGF/FGFR signaling in T cells has been investigated in context of T cell lymphoma and also in other carcinoma regarding immune cell migration and intrinsic anti-tumor activity. *E.g.*, FGFR1 on gastric tumors may represent a chemoattractant marker for T cells (Yang et al., 2022). Interestingly, the T cell receptor (TCR) is known to induce FGFR co-localization on the plasma membrane (Byrd et al., 2003). Counterintuitively, $CD4^+$ T cells are stimulated by FGFR inhibition in cancer models (Kono et al., 2022). Similar effects have been found for macrophages and $CD8^+$ T cells (Kato et al., 2019; Palakurthi et al., 2019; Im et al., 2020; Yi et al., 2021; Adachi et al., 2022; Ruan et al., 2023; Suzuki et al., 2023). Another example of T cell vigilance induced by FGFR inhibitors can be derived from breast cancer studies: FGFR inhibition induced downregulation of MAPK/ERK pathway and subsequently broke the physical and chemical resistance posed by fibroblasts. This coincided with reduced secretion of vascular cell adhesion molecule 1 (VCAM1; Wu et al., 2022) – a factor implicated in microglial pathology in MS (Peterson et al., 2002). Mouse tumor models revealed pro-inflammatory effects of FGFR inhibition for $CD8^+$ T cells, which increased their secretion of IFN-γ and granzyme B and reduced tumor-associated macrophages (Kato et al., 2019).

However, these findings may be tumor-specific: T cells *per se* are presumably activated by FGFs. Stimulation of T cells with FGF1 leads to induction of NFκB. However, in addition to FGF1/FGFR stimulation, co-stimulatory signals may be necessary for the activation of $CD4^+$ T cells. (Zhao et al., 1995; Byrd et al., 1999). T cells themselves are a source of FGF2 (Blotnick et al., 1994). FGF2 upregulation can worsen myocardial damage by infiltration of $FGFR1^+$ T cells (Meij et al., 2002). On a T cell line, neural cell

adhesion molecule (NCAM) can stimulate the production of IL-2 when co-stimulated by TCR-activation (Kos and Chin, 2002). Recent studies have uncovered that this stimulation is FGFR1-dependent and that NCAM/FGFR1 signaling is distinct from FGF2/FGFR signaling (Kos and Chin, 2002; Wong et al., 2002; Haugsten et al., 2008; Francavilla et al., 2009). NCAM inversely correlates with MS (Axelsson et al., 2019; Ziliotto et al., 2019).

As described for microglia, T cells respond differentially to FGF/FGFR signaling. Therefore, caution is warranted when translating findings from cancer models to neuroinflammation. The literature review indicated a modulatory, rather accessory role of FGF/FGFR signaling in T cells, which is primarily contingent on the activation status of the cells.

I. 3. 3. FGF/FGFRs in neurological disease

In addition to its indispensable role in neuronal development, the adult CNS is a complex scene when it comes to FGF/FGFR signaling. FGFRs are universally expressed in the brain and spinal cord: While FGFR1 is more abundant in neurons and astrocytes, oligodendrocytes and microglia tend to synthesize more FGFR2 and FGFR3 than FGFR1 (Gonzalez et al., 1995; Bansal and Pfeiffer, 1997a; Lee et al., 2011).
Overall, FGF/FGFR signaling represents a main regulator of CNS homeostasis (Klimaschewski and Claus, 2021). For neurogenesis, protective effects of FGF signaling and negative effects of induced FGFR deletion have been shown (for reviews on older studies see also Reuss and von Bohlen und Halbach, 2003; Eckenstein et al., 2006; Zhang et al., 2013). Against this, in animal models of spinal cord injury, FGF1 and FGF2 are one of the few substances that can improve axonal growth and overall recovery (Li et al., 2018b; Zhou et al., 2018). FGF1 application promotes M2 polarization of macrophages, induces BDNF secretion, reduces glial scar formation, and ameliorates oxidative stress in peripheral nervous system (PNS) cells (Zhou et al., 2018; Li et al., 2021). Reduction of astrocytic scarring may also be beneficial in inflammatory lesions (Klimaschewski and Claus, 2021). However, the mechanisms responsible, including those for remyelination, differ from those in inflammatory states (see next section).

There are further interdependencies as, *e.g.*, FGFs also bind to other receptors such as Nogo receptor 1 (Lee et al., 2008), and up-regulation of tight junctions can result from FGF/FGFR signaling (Huang et al., 2012) – interesting in context of BBB disruption in MS (see also in IV. 3. 3. 3). Thus, implications in the PNS diverge from those in the CNS neural tissue. Beneficial effects for FGFs in spinal cord, the PNS and hemorrhagic injury are mainly due to their pleiotropic effects. At least in the CNS, increased FGFR1

signaling alone cannot mimic these beneficial effects, and other confounding factors such as duration and inflammatory microenvironment are also important.

I. 3. 3. 1. FGF/FGFR signaling in demyelinating pathologies

The situational ambiguity of FGF/FGFR signaling is also present in the context of myelination, and there is no answer to its conductivity in its entirety. On the contrary, it is important to strictly separate findings from inflammatory models (such as EAE) from those from non-inflammatory models (such as cuprizone-induced demyelination, see I. 3. 3. 1. b). Furthermore, when considering inflammatory demyelination, the phase of the disease is critical, as lesions change dramatically over time in both EAE and MS (see also I. 1. 2. 2). First, it may be helpful to consider observations of FGF/FGFR signaling and its absence on myelin *in vitro,* in knockouts and on myelination physiology independent of disease or pathology.

I. 3. 3. 1. a. Physiological functions of FGFRs and its knock-outs on myelination

In adult mice, where remyelination still spontaneously follows demyelination, differentiation and proliferation of oligodendrocyte progenitor cells (OPCs) as well as oligodendrocytes is controlled by FGF signaling (Bansal et al., 1996; Bansal and Pfeiffer, 1997a, 1997b; Murtie et al., 2005).
Conditional oligodendrocyte-specific FGFR1/FGFR2 knock-out decreases myelination associated with a reduction in ERK1/2-MAPK signaling (Furusho et al., 2012; Ishii et al., 2012; Ishii et al., 2014; Furusho et al., 2015). In absence of disease or injury, FGFR2 appears to be more important than FGFR1 for myelination. Downstream, Akt, mTOR and ERK pathways contribute to myelin gene expression (Furusho et al., 2017; Kang et al., 2019). FGF/FGFR signaling promotes oligodendrocyte proliferation similar to what has been observed in spinal cord injury models (see below; also in Clemente et al., 2011). However, it also inhibits oligodendrocyte differentiation and decreases the expression of myelin proteins (Rajendran et al., 2021a). FGF2 administered into a ventricle in the CNS of healthy rats can even induce the loss of myelin, which coincides with a regression of oligodendrocytes to an immature state (Goddard et al., 2002; Butt and Dinsdale, 2005). Treatment of primary oligodendrocytes with FGF1 reduced differentiation and promoted myelination in tissue culture. It also induced chemoattractant expression in astrocytes (Leukemia inhibitory factor CXCL8; Mohan et al., 2014). FGF2 and FGF9 inhibited differentiation and slightly stimulated proliferation of oligodendroglia in organotypic slice cultures. As an example of the contextual dependency of FGFs, FGF9 applied to monocultured OPCs has the opposite effect (Fortin, 2005). Thus, FGF/FGFR signaling

may be a migratory and proliferative signal for oligodendroglia, whereas prolonged activation may impede differentiation and myelin expression.

I. 3. 3. 1. b. Non-inflammatory demyelination

Several studies of chronic cuprizone-mediated demyelination have reported enhanced remyelination in the absence of FGFR1 and/or FGF2 (Tobin et al., 2011; Zhou et al., 2012a; Mierzwa et al., 2013). Cuprizone experimental demyelination is dependent on the ingestion of the copper-chelating agent cuprizone that induces mitochondrial dysfunction leading to oligodendroglia loss and astrogliosis (Toomey et al., 2021). The Armstrong research group has consistently reported that proliferation of OPCs is not halted by induced FGFR1 deletion in the cuprizone model, while in spinal cord injury models FGF1 was responsible for improved neuronal survival, reduction of ROS and proliferation of oligodendrocytes (Zhang et al., 2013; Li et al., 2018b). While these findings are inconsistent with the notion that FGF/FGFR signaling is conducive to OPC proliferation and migration *in vitro* and in traumatic nerve injury, a conception resolving this contradiction could be that FGF/FGFR should signal proliferation and migration prior to remyelination *via* differentiated mature oligodendrocytes. It is noteworthy, that by Armstrong *et al.*, knock-out was induced by tamoxifen while the animals were already demyelinated – and only in oligodendroglia (Zhou et al., 2012a; Zhou et al., 2012b; Mierzwa et al., 2013). Thus, another interpretation may be that FGFR1 was present long enough before – or may still be present after – tamoxifen administration to provide sufficient proliferative stimuli, but below a threshold that prevents differentiation.

I. 3. 3. 1. c. Inflammatory demyelination

Findings in animal models in which demyelination results from an inflammatory autoimmune attack on the CNS place FGFRs in a different position in the framework – possibly with FGF/FGFR signaling itself driving inflammation.
However, there are two EAE studies, by Ruffini *et al*., and by Rottländer *et al*., that report beneficial effects of FGF2. The first used a viral vector to knock-up *FGF2* in cells in contact with the cerebrospinal fluid. Effects – *inter alia* reduced immune cell infiltration and lowered clinical scores – were still seen even in the chronic phase of EAE. These benefits had resulted from a single application at the beginning of the clinical onset. Yet when they repeated these injections, the effect was reversed (Ruffini et al., 2001). This is interesting as it alludes to the versatility of FGF/FGFR signaling duration and intensity, and even more so in light of the fact that chronically high FGF2 expression is representative of at least some MS lesions.

The second study by Rottländer *et al*., used EAE in *Fgf2*$^{-/-}$ mice to show that the systemic absence of FGF2 in all cells leads to elevated monocyte and CD8^{+} T cell infiltration but no changes in overall inflammatory signals (Rottlaender et al., 2011). Other studies on FGF2 in EAE (Liu et al., 1998) and other inflammatory demyelination-remyelination models (intracranial injection of murine hepatitis virus) have reported conflicting results, suggesting FGF2 as an astrocytic catalyzer for remyelination (Messersmith et al., 2000; Albrecht et al., 2003). It is important to note that the hepatitis virus model exhibits extensive spontaneous remyelination independent of any treatment. Inflammation does not play a significant role after lesion formation in this model. Nevertheless, the virus persists, including in oligodendrocytes, and there is immune cell infiltration (Donati, 2020). Despite the immunological background of this model, FGF2 as a pro-inflammatory cytokine may remain below a threshold that, when exceeded (as in inflammatory models), would switch it to an inhibitor of differentiation and a promoter of proliferative cell programs.

Thus, the ligands have ambiguous roles that vary depending on their abundance and the duration and composition of the tissue to which they are exposed. Therefore, it may be more appropriate to target one or more of their receptors. The research group that hosted this thesis had conducted oligodendrocyte-specific knock-out studies on both *FGFR1* and *FGFR2* to generate *Fgfr1*$^{ind\ -/-}$ and *Fgfr2*$^{ind\ -/-}$ mice in which EAE was then induced. These deletions in oligodendrocytes resulted in an improvement of all clinical outcomes, axonal loss and demyelination (Rajendran et al., 2018; Kamali et al., 2021; Rajendran et al., 2021c; Rajendran et al., 2022). As a precursor to the current studies, it was also shown that the number of infiltrating immune cells in the spinal cord was greatly reduced for monocytes as well as T and B cells – which was also replicated with pharmacological FGFR inhibition in EAE (Rajendran et al., 2023; Gurski et al., 2024).

Downstream signaling effects varied between the two receptor deletions:
While oligodendroglial loss of *FGFR1* resulted in what has been proposed as neuroprotective autoimmunity – a concept of heightened immune vigilance that proposes the necessity of generating autoreactive immune cells, including their secretion of neuroprotective factors (Hohlfeld et al., 2000; Kipnis et al., 2002; Jones et al., 2010; Stroet et al., 2013), oligodendroglial loss of *FGFR2* resulted in decreased remyelination inhibitory factors such as FGF2, transforming growth factor β (TGF-β) and SEMA3A, while mainly inducing Akt signaling through mechanisms that remain indeterminate.
In conclusion, two settings must be distinguished: While FGF/FGFR signaling reduces nerve damage and promotes inflammatory homeostasis in models of traumatic nerve injury, nerve damage and demyelination by autoimmune attack or in an inflammatory state is not a setting in which FGF/FGFR signaling appears to be favorable, possibly owing to its overabundance.

Multiple sclerosis

The limited data directly derived from patients available to the author at the time of writing have established that FGFs and FGFRs are involved in MS. To date, no definitive answer can be given as to their exact role. The studies presented often make comparisons within the same patients and their normal-appearing white matter. Given that even normal-appearing CNS matter in MS patients differs from that of healthy individuals, this limits the inferences that can be drawn (Allen et al., 2001; Moll et al., 2011; Gallego-Delgado et al., 2020). Overall, FGF2 expression is higher in MS tissue than in healthy controls (Clemente et al., 2011). Again, as seen in the various models, this FGF2 may attract $FGFR1^+$ oligodendroglia to migrate to these sites of de-/remyelination. FGF2 is presumably secreted by $FGF2^+$ microglia/macrophages. $FGFR^+$ oligodendroglia are detectable in areas where remyelination is possible (Clemente et al., 2011). $FGF2^+$ microglia/macrophages were preferentially located in the active lesions and in the rim area of both active and inactive chronic lesions; astrocytes were also labeled $FGF2^+$ especially in perivascular location. Thus, $FGF2^+$ cells collocate with ongoing inflammation and myelin pathology. Interestingly, in the context of the smoldering neurological decline that may be present in MS, FGF2 has also been found to be upregulated in normal-appearing gray matter of MS patients, FGF9 also seems to be associated with neuronal damage (Thümmler et al., 2023). In support of Liu *et al*., Ruffini *et al*. and Rottländer *et al*., Clemente *et al.* propose FGF2 as a factor favoring remyelination. However, a recent study found FGF2 to be upregulated in astrocytes and to a lesser extent in microglia and OPCs also within lesions. Therefore, Thümmler *et al*.

propose that FGF2 has mitogenic functions *via* FGFR1 and myelination-inhibitory functions *via* FGFR2. While inhibition of myelination could execute downstream of FGFR2 *via* Wnt signaling and be excluded by inhibition of Wnt specifically, it is proposed that FGFR1 mainly induces immunomodulatory effects on leukocytes and BBB integrity (Thümmler et al., 2019). However, these findings have not been reported in other studies, most of which have focused on specific ligands (Thümmler et al., 2023).

FGF1 was found to be elevated in remyelinated lesions, lower in active lesions, and even lower in demyelinated lesions. It was also found in astrocytes in remyelinated areas but not in the active rim (Mohan et al., 2014). However, the high interindividual variance and small sample size must be considered when interpreting these findings. Furthermore, in active lesions, some microglia/macrophages were $FGF1^+$ (Mohan et al., 2014).
FGF9 was found to be elevated mainly in oligodendrocytes and to a lesser extent in astrocytes and OPCs in active lesions. This difference was also observed in the rims of chronic active lesions – albeit less pronounced (Fortin, 2005). In very early lesion states FGF9 was variably expressed in “immunoreactive glia” (astrocytes, oligodendrocytes; Lindner et al., 2015).

Studies examining serum and cerebrospinal fluid (CSF) from MS patients have shown that FGF2 is elevated in both serum (Su et al., 2006; Sarchielli et al., 2008; Harirchian et al., 2012) and CSF (Sarchielli et al., 2008) compared to healthy individuals in each disease state. FGF2 abundance in CSF was found to correlate with lesion burden. Increased CSF FGF2 expression coincided with relapses in PPMS (Sarchielli et al., 2008). In the serum of patients with disability progression in secondary progressive phases or relapses in PPMS, the expression of FGF2 and vascular endothelial growth factor (VEGF) corresponded to the clinical course – although these changes in serum expression were less pronounced than in CSF (Su et al., 2006; Sarchielli et al., 2008). FGF2 expression seems to be generally very low or undetectable in CSF (Harirchian et al., 2012).

In summary, in MS, FGFs are linked to lesions and especially to active demyelination. Overall, they correlate with disease activity. This correlation alone allows for some, but rather little mechanistic explanation given that remyelination almost always takes place simultaneously with demyelination. There is evidence that FGF/FGFR signaling can induce both the necessary proliferative effects and the deleterious anti-differentiation effects. This supports the notion that either a delicate balance or a two-phase action is required for successful remyelination. Nevertheless, MS most likely displays an imbalance of this pathway, making its modulation an interesting leverage point.

I. 3. 4. Pharmacological inhibition of FGFRs

As mentioned above, FGF/FGFR signaling is involved in tumor growth and inflammatory diseases. Its blockade has therefore been extensively investigated as a potential therapeutic leverage point in the context of targeted therapies for various malignancies (Huynh et al., 2012; Katoh and Nakagama, 2014; Zhang et al., 2014; Kang et al., 2015; Porta et al., 2017; Seckl et al., 2017; Katoh, 2019; Das et al., 2020; Lam et al., 2020; Kommalapati et al., 2021). To date, there are a large number of different FGFR inhibitors that differ in potency and selectivity. There is increasing evidence that these agents are BBB-penetrating and hold great therapeutic potential beyond cancer treatment. Fexagratinib (formerly AZD4547 WHO, 2023; Shan et al., 2024), infigratinib (formerly BGJ398), and dovitinib (formerly TKI-258 / CHIR-258) were selected due to their characteristics and progress in clinical application (see I. 3. 4. 1).

Unfortunately, the promising results of the early phase cancer trials of FGFR inhibitors and the great hopes attached to these substances have not materialized in later phase clinical cancer trials due to various compensatory mechanisms induced in these tumors. Thus, FGFR alteration is not (anymore) a predictive biomarker to select cancer patients for treatment with FGFR inhibitors (Katoh and Nakagama, 2014). However, some promising clinical trials have led to accelerated approval status and actual FDA approval of several FGFR inhibitors. In addition, the pharmacological differentiation of FGFR inhibition has progressed considerably, with a wide range of more or less selective FGFR inhibitors now available. These can be divided into FGFR1/2/3, pan-FGFR and multikinase FGFR inhibitors. A selection of current and clinically available FGFR inhibitors is shown in Table 4.

I. 3. 4. 1. FGFR inhibitors dovitinib, fexagratinib, infigratinib

Fexagratinib was chosen because of its high selectivity as a FGFR1/2/3 inhibitor including FGFR4, VEGFR2 and CSF1R (Katoh, 2016). As is common with FGFR inhibitors, other RTKs, such as the Anexelekto RTK (AXL) in the case of fexagratinib, are also inhibited. Infigratinib is also highly selective and clinically tested but does not inhibit CSF1R, VEGFR and inhibits FGFR2 less efficiently than fexagratinib. Dovitinib is a multikinase inhibitor that has off-target effects that may contribute to its efficacy but also to its adverse effects.

Currently, there are no phase 3 or higher clinical trials for fexagratinib (one completed; NCT02965378) or dovitinib (two completed; NCT01223027; NCT02116803). There is only one active clinical trial beyond phase 2 for infigratinib (NCT03773302), one of which

was terminated. Infigratinib is also being evaluated in clinical trials for achondroplasia (NCT04265651; NCT05145010). There are several phase 1 and 2 trials with fexagratinib, infigratinib or dovitinib that are active or not yet recruiting.

Apart from differences in potency, blocking mechanism and selectivity (see Table 5), there are no fundamental differences between these inhibitors in their mode of action. Until 2016, there were no data on BBB penetration for these agents (Heffron, 2016). Dasatinib and ponatinib have since been shown to cross the BBB but are subject to efflux pumps. BBB disruption is known to facilitate BBB penetration for these substances (Ravi et al., 2021). For derazantinib, however, low CNS penetration has been suggested (McSheehy et al., 2022). The authors of a study demonstrating efficacy of infigratinib in brain glioblastoma argue that there is preliminary evidence suggesting that infigratinib crosses the BBB while announcing further studies to clarify this (Lassman et al., 2022). Dovitinib is propagated to be BBB-penetrable (Schäfer et al., 2016), but no direct evidence has been provided. Of course, there are many ways to introduce BBB permeability for these drugs in general (Brar et al., 2022). Cross-over to the CNS has been demonstrated for both fexagratinib and infigratinib, especially in areas where the BBB is disrupted (Knight et al., 2022). Overall, it is plausible to assume CNS penetration for the three inhibitors used in the present work.

Several observations have been made regarding the modulation of downstream signaling by fexagratinib: While RAS-MAPK is suppressed, PI3K is differentially modulated, PI3K gene products are mostly slightly up-regulated (Delpuech et al., 2016). In another cancer study (Phanhthilath et al., 2020), fexagratinib also inhibited downstream ERK, but also Akt and S6 pathways. An increase in STAT3 was only seen in the cell lines used in the aforementioned study that were sensitive to fexagratinib (Phanhthilath et al., 2020). In some tumor cells, much higher IC_{50} values were required to inhibit cell growth for these inhibitors (above 20 µM for fexagratinib, 10 µM for infigratinib and 3 µM for dovitinib; Phanhthilath et al., 2020). The availability of these FGFR inhibitors offers the opportunity to study the implications of inhibiting these pivotal pathways in MS with the prospect of clinical application due to their selectivity and favorable side effect profile.

TABLE 4: EXCERPT OF CLINICALLY IMPORTANT FGFR INHIBITORS

DRUG NAME	SELECTIVITY	CHARACTERISTICS	
Fexagratinib * AZD4547	FGFR1/2/3 inhibitor	Has been deployed in a phase 3 clinical cancer trial (NCT02965378)	2nd generation FGFR inhibitors ATP-competitive inhibition type I (reversible; (Katoh, 2016, 2019) Common adverse effect is hyperphosphatemia (compensatory FGF23 upregulation by FGFR blockade leads to renal phosphate reabsorption; (Katoh, 2019)
Infigratinib * # BGJ398	FGFR1/2/3 inhibitor	Is currently deployed in a phase 3 clinical cancer trial (NCT03773302) Might have stronger vascular endothelial growth factor receptor (VEGFR) 2 inhibition *in vivo* than previously thought (Konecny et al., 2013)	
Dovitinib * TKI 258 / CHIR258	Multikinase inhibitor	1st generation FGFR inhibitor ATP-competitive inhibition type I (reversible; (Katoh, 2016, 2019) Reduces FRS2 and ERK phosphorylation *in vitro* and *in vivo* (Lee et al., 2005). Suppresses PI3K/Akt pathway (Das et al., 2020) Low potency for FGFR2 inhibition (Byron et al., 2013) Risk of hypertension and cardiovascular effects as well as liver toxicities *via* inhibition of VEGF is a common adverse effect in multikinase inhibitors (Katoh, 2019)	
Derazantinib ARQ 087	Multikinase inhibitor	2nd generation FGFR inhibitor ATP-competitive inhibition type I and II (Hall et al., 2016) No phase 3 clinical trials, several in phase 2 Risk of hypertension and cardiovascular effects (see above)	
Erdafitinib JNJ-42756493 FDA-approved drug	Pan-FGFR inhibitor	ATP-competitive inhibition type I (reversible; (Katoh, 2016, 2019) Is currently deployed in a phase 3 clinical cancer trial (NCT03390504) FDA accelerated approval status (Facchinetti et al., 2020)	2nd generation FGFR inhibitors Hyperphosphatemia, liver and cardiac toxicity (see above)
Futibatinib TAS-120 FDA-approved drug	Pan-FGFR inhibitor	ATP-competitive inhibition type II (irreversible: (Facchinetti et al., 2020) Is currently deployed in phase 3 clinical cancer trials (NCT05615818, NCT03784014, NCT04093362)	
Lucitanib AL3810	Multikinase inhibitor	1st generation FGFR inhibitor No phase 3 clinical trials Risk of hypertension and cardiovascular effects (see above)	
LY2874455	Pan-FGFR inhibitor High-potency VEGFR inhibition	2nd generation FGFR inhibitor No phase 3 clinical trials, two phase 1 Hyperphosphatemia, liver and cardiac toxicity (see above)	
Pemigatinib INCB054828 FDA-approved drug	FGFR1/2/3 inhibitor	2nd generation FGFR inhibitor Is currently deployed in a phase 3 clinical cancer trial (NCT03656536) Hyperphosphatemia (see above)	
Ponatinib AP24534	Multikinase inhibitor	1st generation FGFR inhibitor ATP-competitive inhibition type II (irreversible) Several phase 3 clinical cancer trials, including leukemia, severe adverse effects have been reported there (Katoh, 2016) Risk of hypertension and cardiovascular effects (see above)	
Rogaratinib BAY 1163877	Pan-FGFR inhibitor	2nd generation FGFR inhibitor ATP-competitive inhibition type I (reversible) Has been deployed in a phase 3 clinical cancer trial (NCT03410693) Hyperphosphatemia, liver and cardiac toxicity (see above)	
Zoligratinib Debio 1347	FGFR1/2/3 inhibitor	2nd generation FGFR inhibitor ATP-competitive inhibition type II (irreversible; (Goyal et al., 2019) No phase 3 clinical cancer trial, one phase 2 completed Hyperphosphatemia (see above)	

* Applied *in vitro* in the present work

\# Applied in EAE in the present work

Table 4 shows the most studied and used FGFR inhibitors currently available. The inhibitors used in this study are highlighted.

TABLE 5: SELECTIVITY AND POTENCY OF DEPLOYED FGFR INHIBITORS

FEXAGRATINIB (GAVINE ET AL., 2012; KWAK ET AL., 2015; KATOH, 2016)					
KINASE	**ENZYMATIC IC_{50}**	**CELLULAR IC_{50} ***	**KINASE**	**ENZYMATIC IC_{50}**	**CELLULAR IC_{50} ***
FGFR1	0.2 / <1 / 2 nM	12 nM	VEGFR2	24 nM	258 nM
FGFR2	2.5 / 3 / 1 nM	2 nM	Insuline-like growth factor 1 receptor (IGF1R)	581 nM	828 nM
FGFR3	1.8 / 2 / 7 nM	40 nM	C-KIT	24 nM	Others reported no change in activity at 50 nM (Gudernova et al., 2016)
FGFR4	165 / 56 nM	142 nM	AXL	>2.000 nM	2 nM
CSF1R	Not reported	9.7 nM	Fms like tyrosine kinase 3 (FLT3)	9% activity at 1 µM	85 nM
BGJ398 (GUAGNANO ET AL., 2011; KONECNY ET AL., 2013)					
KINASE	**ENZYMATIC IC_{50}**	**CELLULAR IC_{50} ***	**KINASE**	**ENZYMATIC IC_{50}**	**CELLULAR IC_{50} ***
FGFR1	1 nM	3 nM	VEGFR2	180 nM (Konecny et al., 2013) reported strong in vivo effects on VEGFR2.	1449 nM
FGFR2	1 nM	2 nM Konecny *et al.* reported values in cancer cells of up to 10.000 nM	IGF1R	No change in activity at 20 nM (Gudernova et al., 2016)	
FGFR3	1 nM	2 nM	C-KIT	750 nM	2501 nM
FGFR4	60 nM	168 nM	Lyn	581 nM	3019 nM
DOVITINIB (LEE ET AL., 2005; TRUDEL ET AL., 2005)					
KINASE	**ENZYMATIC IC_{50}**	**CELLULAR IC_{50} ***	**KINASE**	**ENZYMATIC IC_{50}**	**CELLULAR IC_{50} ***
FGFR1	8 nM	90 - 1000 nM (Chase et al., 2007)	VEGFR2	13 nM	40 - 1260 nM (Chen et al., 2012b)
FGFR2	40 nM (Katoh, 2016)	420 - 4751 nM (Byron et al., 2013; Konecny et al., 2013)	IGF1R	>10.000 nM	Not reported
FGFR3	9 nM	Not reported	C-KIT	2 nM	Not reported
FGFR4	Not reported		CSF1R	36 nM	(Yan et al., 2017) report no inhibition of phosphorylation at 900 nM
VEGFR1	10 nM	<50 nM	VEGFR3	8 nM	Not reported
PDGFRα	210 nM	524 - 1940 nM (Huang et al., 2010)	PDGFRβ	27 nM	<50 nM
FLT3	1 nM	185 - 795 nM (Cao et al., 2021)	InsR	2.000 nM	Not reported
EGFR1	2.000 nM	Not reported			

Values are rounded to nM (nmol/L)

* It has to be considered, that cellular IC_{50} values depend on the deployed cell line and incubation conditions. Thus, they can only provide some general orientation.

Tyrosine-protein kinase Lyn is of the Src family (named after Ick/yes-related novel tyrosine kinase (LYN) gene

Table 5 shows the cellular and molecular specificities of the inhibitors used in this study.

I. 4. AIMS AND HYPOTHESIS

The historical hypothesis originating from M. Giraldo-Velasquez of the experimental neuroimmunology research group of Prof. Dr. med. Martin Berghoff at the Justus-Liebig-University Giessen, which later hosted this thesis, was that FGFR knockouts are detrimental for myelin expression, myelination and presumably remyelination. It soon became evident that the opposite was true and that neuroprotective pathways could be induced by blockade of FGF/FGFR signaling (namely the BDNF/TrkB pathway).
Next, the EAE model of inflammatory demyelination was established, demonstrating that the absence of FGF/FGFR signaling promotes remyelination. Conditional oligodendrocyte-specific *FGFR1* and *FGFR2* deletions resulted in significantly reduced clinical symptoms and histopathologic evidence of reduced neuroglial damage.
This was followed by pharmacological studies to translate these findings into clinical application. At this time, strong anti-inflammatory effects were observed in the infigratinib-treated animals.

Thus, we hypothesized that immunomodulatory effects were occurring very early in the disease and treatment period, presumably in the peripheral immune system, that remained elusive at that time. Given the importance of FGF/FGFR signaling for myelin pathology and the immune system, and the preliminary immunomodulatory effects described above, insights into the effects of inhibition of FGF/FGFR signaling on immune cells are of great relevance in advancing our comprehension of MS pathophysiology.

To this end, I applied *in vivo* and *in vitro* approaches to evaluate i) the effects of infigratinib on immune cell populations in EAE *in vivo* and ii) the effects of dovitinib, fexagratinib, and infigratinib on SIM-A9 microglia and Jurkat T cells *in vitro*.
Thus, the objectives of the present work were i) to establish quantitative differences in immune cell populations in the spleen and blood of mice treated with infigratinib compared to vehicle and ii) to evaluate whether the observed effects can be replicated with respect to cytotoxicity and proliferation of microglia and T cell lines *in vitro* and iii) to differentiate downstream effects of receptor inhibition in dependence of cell type and respective inhibitor.

I aimed to characterize the immunomodulatory effects of FGFR inhibition *in vivo* and *in vitro* to deepen the understanding of FGF/FGFR signaling in the context of inflammatory demyelination and on immune cell populations in general, and to discuss and translate these findings to the context of MS.

II. Material and Methods

II. 1. Materials

II. 1. 1. Animals

Female mice (*Mus musculus*) of the C57BL/6J strain were purchased at 7 weeks of age from Charles River Laboratories, Sulzfeld, Germany.

The mice were fed standard chow (recipe 1320 from Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) in pellet form (Cat. No. 1324) and given *ad libitum* access to autoclaved water. DietGel Boost® and HydroGel® from ClearH2O® (Westbrook, Maine, USA) were added to the diet during the experiments. For living conditions see methods section (II. 2. 1. 2).

II. 1. 2. Cell lines

Jurkat cells (Jurkat E6.1, European Collection of Authenticated Cell Cultures (ECACC), Art. No. 88042803) were purchased from Merck KGaA, Darmstadt, Germany (Art. No. 88042803-1VL) at passage 5. Jurkat cells are derived from a human leukemic T cell lymphoblast. They were initially isolated from a 14-year-old human male with acute lymphoblastic leukemia and designated 'JM' (Schwenk and Schneider, 1975). The cell line has since been named 'Jurkat' (Schneider et al., 1977), and has been modified and frequently applied to study T cells. The subclone employed here is an IL-2 producing cell line derived from Jurkat FHCRC cells (Weiss et al., 1984).

SIM-A9 cells (SIM-A9, American Type Culture Collection (ATCC), Art. No. CRL3265™) were obtained from ATCC Manassas, Virginia, USA. SIM-A9 cells are derived from a microglial clone of a day 1 postnatal mouse cortex that spontaneously attained immortality (Nagamoto-Combs et al., 2014).
They resemble mouse microglia and respond differentially to immune stimuli (Gill et al., 2018; Dave et al., 2020; Jayakumar et al., 2021).

II. 1. 3. Chemicals

II. 1. 3. 1. Solutes, solvents, solutions

Table 6: Solutes, solvents, solutions

Compound name	Manufacturer
2-Mercaptoethanol Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Ammonium persulfate (APS)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany

COMPOUND NAME	MANUFACTURER
Fexagratinib (AZD4547)	Selleckchem, Houston, Texas, USA
Bromophenol Blue	Neolab, Heidelberg, Germany
Bovine Serum Albumin (BSA)	Capricorn Scientific GmbH, Ebsdorfergrund, Germany
Carboxymethylcellulose Sodium Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Collagenase D	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
Complete Freund´s adjuvant Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Complete Freund's adjuvant Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Dako fluorescence mounting medium	Agilent Technologies, Inc., Santa Clara, California, USA
4',6-Diamidino-2-phenylindol dihydrochloride (DAPI)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Dipotassium phosphate (K_2HPO4)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Disodium phosphate (Na_2HPO_4)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Dulbecco's Modified Eagle Medium (DMEM)/F-12 Gibco™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Dimethyl sulfoxide (DMSO)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
DNAse I Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
DNase Phosphate buffered saline (PBS)	Qiagen, Venlo, Netherlands
Dovitinib (TKI-258)	Selleckchem, Houston, Texas, USA
Dulbecco's phosphate buffered saline (DPBS) ROTI®Cell 10X	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
DPBS ROTI®Cell DPBS CELLPURE®	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Eosin	Merck KGaA, Darmstadt, Germany
Ethanol 100% Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Fetal bovine serum (FBS) Gibco™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Fragment crystallizable receptor (FcR) Blocking Reagent, mouse	Miltenyi Biotec, Bergisch Gladbach, Germany
FGF2 (Fibroblast growth factor 2) Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein R&D Systems®	Bio-Techne, Minneapolis, Minnesota, USA
Glucose	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Glutaraldehyde	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Glycerol	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Hematoxylin	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Horse serum, New Zealand Gibco™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Hydrochloric acid 37% Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Hydrogen peroxide 3%	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Infigratinib (BGJ398)	Selleckchem, Houston, Texas, USA
Isoflurane 100%	Ecuphar, Greifswald, Germany
Ketamine 10%	bela-pharm GmbH, Vechta, Germany
Laemmli Sample Buffer	Bio-Rad Laboratories, Inc., Hercules, California, USA
Luxol-Fast-Blue Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Methanol Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany

COMPOUND NAME	MANUFACTURER
MOG_{35-55}, peptide sequence: MEVGWYRSPFSRVVHLYRNGK	Institute for Medical Immunology, Charité University Hospital, Berlin, Germany
Mycobacterium tuberculosis H37Ra BD Difco™ Adjuvants	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Non-fat dry milk	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA
Nonidet-P40	United States Biological, Swampscott, Massachusetts, USA
Nuclease free water Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
PBS for flow cytometry (FC)	PAA Laboratories GmbH, Cölbe, Germany
Penicillin/Streptomycin Gibco™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Pertussis toxin	Merck KGaA, Darmstadt, Germany
Paraformaldehyde (PFA) Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Phosphatase inhibitor cocktail 2 Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Poly-L-lysine solution (0.1%) Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Potassium chloride (KCl)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Protease inhibitor cocktail	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
RBC Lysis Solution	Qiagen, Venlo, Netherlands
ROTI®Fair HBS	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
ROTIPHORESE® Gel 30% acrylamide mix	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
ROTIPHORESE® Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis PAGE 10X	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
RPMI 1640 Medium Gibco™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Sodium dodecyl sulfate (SDS)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Sodium azide (NaN3) Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Sodium carboxymethyl cellulose Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Sodium chloride (NaCl)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Sodium hydroxide (NaOH) Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Tetramethyl ethylenediamine (TEMED)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Trishydroxymethyl aminomethan (Tris)	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Tris-HCl	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Triton™ X-100 (10%) Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Trypan blue	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Trypsin (2.5 g / l) Gibco™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Tween® 20 Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Xylazine 2%	CP-Pharma GmbH, Burgdorf, Germany

II. 1. 3. 2. Buffers

TABLE 7: BUFFERS

BUFFER NAME	INGREDIENTS	AMOUNT
WESTERN BLOT		
APS solution	H_2O APS	10 ml 1 g
Blocking Buffer	Tris buffered saline (TBS)-Tween (TBST) BSA	300 ml 15 g
Lysis buffer	Glycerol NP40 NaCl Tris EDTA NaN3	25 ml 2.5 ml 2.19 g 0.61 g 0.07 g 0.025g
Milk blocking solution	TBST Non-fat dry milk	100 ml 5 g
PBS 10X	H_2O NaCl Na_2HPO_4 KH_2PO_4 KCl	1000 ml 80 g 14.4 g 2.4 g 2 g
Running Buffer	H_2O SDS-PAGE 10X	1800 ml 200 ml
SDS solution	H_2O SDS	10 ml 1 g
TBS	H2O NaCl Tris	1000 ml 87.7 g 24.2 g
TBST	Tris buffered saline (TBS) 1X Tween® 20	1000 ml 1 ml
Transfer Buffer	H_2O Methanol SDS-PAGE 10X	1400 ml 400 ml 200 ml
CELL CULTURE AND IMMUNOCYTOCHEMISTRY		
Blocking Buffer	DBPS BSA Triton™ X-100 (10%)	110 ml 5.5 g 3.3 ml
Detachment DPBS (DDPBS)	DPBS Glucose EGTA EDTA	500 ml 0.5 g 0.19 g 0.146 g
Incubation buffer	DPBS Triton™ X-100 (10%) BSA	100 ml 3 ml 1 g
Permeabilization / wash buffer	DBPS Triton™ X-100	495 ml 5 ml
Polylysine solution	H2O Poly-L-lysine	99 ml 1 ml
FLOW CYTOMETRY		
Digestion buffer	RPMI 1640 Collagenase DNAse	12 ml 3 ml 15 µl
FC Buffer	DBPS FBS EDTA	50 ml 0.25 g 73 mg

II. 1. 3. 3. Cell culture media

TABLE 8: CELL CULTURE MEDIA

NAME	INGREDIENTS	AMOUNT
Jurkat freezing medium	Jurkat growth medium DMSO	9 ml 1 ml
Jurkat growth medium	RPMI 1640 FBS Penicillin/Streptomycin	500 ml 50 ml 5 ml
SIM-A9 freezing medium	SIM-A9 growth medium DMSO	9 ml 1 ml
SIM-A9 growth medium	DMEM/F12 FBS Horse serum Penicillin/Streptomycin	500 ml 50 ml 25 ml 750 µl

II. 1. 4. Antibodies

II. 1. 4. 1. Primary antibodies

TABLE 9: PRIMARY ANTIBODIES

TARGET	COMPANY	HOST SPECIES	ART. NO.
WESTERN BLOT			
FGFR1	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	9740
FGFR2	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	23328
GAPDH	Santa Cruz Biotechnology, Inc., Dallas, Texas, USA	Mouse	sc-365062
IL1β	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Mouse	12242
pAkt (Ser473)	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	4060s
pERK p-44/42	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	4370s
pP38	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	9215
pSAPK/JNK p-46/54	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	9251
pSTAT3	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	9131
TNF-α	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	11948
IMMUNOCYTOCHEMISTRY (ICC)			
FGFR1	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	9740s
FGFR2	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Rabbit	23328s

II. 1. 4. 2. Secondary antibodies

TABLE 10: SECONDARY ANTIBODIES

TARGET	COMPANY	HOST SPECIES	ART. NO.
ICC			
Rabbit	Invitrogen Thermo Fisher Scientific, Waltham, Massachusetts, USA	Goat	A-11008
WESTERN BLOT			
Rabbit – Horseradish peroxidase (HRP)	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Goat	7074
Mouse – HRP	Cell Signaling Technology, Inc., Danvers, Massachusetts, USA	Horse	7076

II. 1. 4. 3. Flow cytometry antibodies

TABLE 11: FC ANTIBODIES

TARGET	FLUOROPHORE	COMPANY	ART. NO.
	DESCRIPTION		
CD11b	PerCP/Cyanine5.5	BioLegend, San Diego, California, USA	101228
	Integrin alpha M (ITGAM): Monocytes/macrophages, granulocytes, natural killer (NK) cells marker		
CD183	PerCP/Cyanine5.5	BioLegend, San Diego, California, USA	126514
	CXC chemokine receptor 3 (CXCR3): Effector T cells, T cell trafficking and T cell function		
CD19	APC	BioLegend, San Diego, California, USA	115520
	B-lymphocyte antigen: B cell marker during all stages of B cell lymphopoiesis		
CD3	Pacific Blue	BioLegend, San Diego, California, USA	100214
	T cell coreceptor: T cell marker of both $CD4^+$ and $CD8^+$ cells, forms TCR complex with CD3ζ and TCR		
CD4	Brilliant Violet 510™	BioLegend, San Diego, California, USA	116025
	TCR coreceptor: $CD4^+CD3^+$ cells: T helper cells		
CD8a	Alexa Fluor® 488	BioLegend, San Diego, California, USA	100723
	TCR coreceptor: $CD8^+CD3^+$ cells: Cytotoxic T cells		
CD11c	APC/Cyanine7	BioLegend, San Diego, California, USA	117324
	Integrin, alpha X: Primarily dendritic cells		
CD45	FITC	BioLegend, San Diego, California, USA	103108
	Protein tyrosine phosphatase, receptor type, C (PTPRC): Nucleated hematopoietic cells		
CD45	PE	BioLegend, San Diego, California, USA	12-0451-82
	PTPRC: Nucleated hematopoietic cells		
CD64	PE/Cyanine7	BioLegend, San Diego, California, USA	139314
	Fc gamma receptor 1 (FCγR1): Primarily macrophages/monocytes, activated granulocytes and dendritic cells		
EMR1	PE	BioLegend, San Diego, California, USA	123110
	F4/80: Macrophages/monocytes		
Ly6G	Brilliant Violet 510™	BioLegend, San Diego, California, USA	108438
	Part of granulocyte receptor-1 antigen: Myeloid cells, especially differentiated granulocytes (neutrophils)		
MHC II	Allophycocyanin (APC)	BioLegend, San Diego, California, USA	116418
	Professional antigen-presenting cells		

EMR1: EGF-like module-containing mucin-like hormone receptor-like 1
MHC: Major histocompatibility complex Ly6G: Lymphocyte antigen 6G

II. 1. 5. Western blot ladder

TABLE 12: WESTERN BLOT LADDERS

NAME	MANUFACTURER
PageRuler™ Prestained Protein Ladder No. 26616	Thermo Fisher Scientific, Waltham, Massachusetts, USA

II. 1. 6. Primers

Sequences for forward primers (FPs) and reverse primers (RPs) were adapted from the available literature or used as established by the research group. All primers for human

genes were then ordered from and manufactured by Eurofins Genomics, Ebersberg, Germany.

II. 1. 6. 1. Human primers

Primers used for Jurkat cells are listed in Table 13:

TABLE 13: HUMAN PRIMERS

GENE NAME	TARGET PROTEIN	SEQUENCE 5' → 3'
CCL2	Monocyte chemoattractant protein 1 (MCP 1) / C-C motif chemokine ligand 2	FP GCA ATC AAT GCC CCA GTC AC RP GAC ACT TGC TGC TGG TCA TTC
CX3CL1	Fractalkine / C-X3-C motif chemokine ligand 1	FP GAC CCC TAA GGC TGA GGA AC RP CTC TCC TGC CAT CTT TCG AG
*CX3CL1**	Fractalkine / C-X3-C motif chemokine ligand 1	FP ACC ACG GTG TGA CGA AAT G RP TGT TTG ATA GTG GAT GAG CAA GC
*CX3CL1**	Fractalkine / C-X3-C motif chemokine ligand 1	FP GAC CCC TAA GGC TGA GGA AC RP CTC TCC TGC CAT CTT TCG AG
FGFR1	Fibroblast growth factor receptor 1	FP CCA AAG ACG GTC GTT TAG TGG RP ACA GCC AAA GTA AAG TCA AGG TT
FGFR2	Fibroblast growth factor receptor 2	FP ACA GTT TCG GCT GAG TCC AG RP GGT GTC TGC CGT TGA AGA GA
*FGFR2**	Fibroblast growth factor receptor 2	FP GGT GGC TGA AAA ACG GGA AG RP AGA TGG GAC CAC ACT TTC CAT A
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	FP ACA ACT TTG GTA TCG TGG AAG G RP GCC ATC ACG CCA ACA GTT TC
IFNG	Interferon gamma (IFN-γ)	FP TCG GTA ACT GAC TTG AAT GTC CA RP TCG CTT CCC TGT TTT AGC TGC
IL12A	Interleukin 12A (IL-12A)	FP CCT TGC ACT TCT GAA GAG ATT GA RP ACA GGG CCA TCA TAA AAG AGG T
IL1B	Interleukin 1 beta (IL-1β)	FP ATG ATG GCT TAT TAC AGT GGC AA RP GTC GGA GAT TCG TAG CTG GA
IL21	Interleukin 21 (IL-21)	FP GGC AAG ACC AGT ATG AAG AGC RP TGA XAX TGA AAA TGT CGT CGG
IL6	Interleukin 6 (IL-6)	FP TGA ACT CCT TCT CCA CAA GCG RP TCT GAA GAG GTG AGT GGC TGT C
*IL6**	IL-6	FP ACT CAC CTC TTC AGA ACG AAT TG RP CCA TCT TTG GAA GGT TCA GGT TG
TNF	TNF-α	FP GAG ACA GAT GTG GGG TGT GAG RP AGC TGT CAT ATT TCC CGC TC
** did not accomplish amplification*		

II. 1. 6. 2. Murine primers

Primers used for SIM-A9 cells are listed in Table 14:

TABLE 14: MURINE PRIMERS

GENE NAME	GENE PRODUCT	SEQUENCE 5' → 3'
Ccl2	MCP1 / C-C motif chemokine ligand 2	FP CAC TCA CCT GCT CGT ACT CA RP GCT TGG TGA CAA AAA CTA CAG C
*Ccl2**	MCP1 / C-C motif chemokine ligand 2	FP GCA ATC AAT GCC CCA GTC AC RP GAC ACT TGC TGC TGG TGA TTC
Cx3cl1	Fractalkine / C-X3-C motif chemokine ligand 1	FP ACC ACG GTG TGA CGA AAT G RP TGT TGA TAG TGG ATG AGC AAA GC
Fgfr1	FGFR1	FP GCC CTG GAA GAG AGA CCA GC RP GAA CCC CAG AGT TCA TGG ATG C

GENE NAME	GENE PRODUCT	SEQUENCE 5' → 3'	
Fgfr2	FGFR2	FP RP	CGG AGA CAG GTA ACA GTT TCG GC CTA TTC CCG GAG GTT GCC TTT C
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	FP RP	TGG CAA AGT GGA GAT TGT TGC C AAG ATG GTG ATG GGC TTC CCG
Ifng	IFN-γ	FP RP	TGG CAT AGA TGT GGA AGA AAA GAG TGC AGG ATT TTC ATG TCA CCA T
*Ifng**	IFN-γ	FP RP	TCA AGT GGC ATA GAT GTG GAA GAA TGG CTC TGC AGG ATT TTC ATG
*Il12a**	IL-12A	FP RP	TGG CTA CTA GAG AGA CTT CTT CCA CAA GCA CAG GGT CAT CAT CAA AGA C
Il1b	IL-1β	FP RP	CTG TGT CTT TCC CGT GGA CC CAG CTC ATA TGG GTC CGA CA
Il6	IL-6	FP RP	ACT CAC CTC TTC AGA ACG AAT TG CCA TCT TTG GAA GGT TXA GGT TG
Tnf	TNF-α	FP RP	GAG ACA GAT GTG GGG TGT GAG AGC TRG TCA TAT TTC CGC GTG

** did not accomplish amplification*

II. 1. 7. Kits and assays

TABLE 15: ASSAYS AND KITS

KIT NAME	MANUFACTURER
Cell Proliferation Reagent water soluble tetrazolium salt (WST-1) Cell proliferation assay	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
Cytotoxicity Detection Kit (Lactate dehydrogenase (LDH) Cytotoxicity detection assay	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
Enzyme-linked immunosorbent assay (ELISA Kits): Human IL-6 ELISA Human VEGF ELISA	Biomedica Medizinprodukte GmbH, Vienna, Austria
iTaq Universal SYBR Green Supermix	Bio-Rad Laboratories, Inc., Hercules, California, USA
Luminex® Assays Human Premixed Multi-Analyte Kit Mouse Premixed Multi-Analyte Kit Mouse Luminex Discovery Assay Human Luminex Discovery Assay R&D Systems®	Bio-Techne, Minneapolis, Minnesota, USA
peqGOLD Total ribonucleic acid (RNA) Kit RNA isolation kit PEQLAB - Life Science	Avantor, Inc., Radnor, Pennsylvania, USA
Pierce™ BCA Protein Assay Kit Protein quantification assay	Thermo Fisher Scientific, Waltham, Massachusetts, USA
QuantiTect® Reverse Transcription Kit	Qiagen, Venlo, Netherlands
Quick Start™ Bradford Protein Assay Protein quantification assay	Bio-Rad Laboratories, Inc., Hercules, California, USA
RNeasy Plus Mini Kit RNA isolation kit	Qiagen, Venlo, Netherlands
SuperSignal™ West Pico PLUS Chemiluminescent Substrate HRP detection for Western blot	Thermo Fisher Scientific, Waltham, Massachusetts, USA

TABLE 16: ANALYTES FOR LUMINEX® KIT

TARGET (AS PER MANUFACTURER)		MANUFACTURER
FGF-2	IL-6	R&D Systems® Bio-Techne, Minneapolis, Minnesota, USA
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	IL-10	
IFN-gamma	IL-12p70	
IL-1beta	IL-17A	
IL-2	TNF-α	
IL-4	VEGF	

II. 1. 8. Instruments

TABLE 17: LABORATORY INSTRUMENTS

INSTRUMENT	MANUFACTURER
Arpege 75 Liquid nitrogen tank	Air Liquide Medical GmbH, Düsseldorf, Germany
Axio Scan Z1 Microscope	Carl Zeiss Microscopy GmbH, Oberkochen, Germany
Biological safety cabinet	NuAire, Plymouth, Minnesota, USA
Biometra TPersonal thermocycler	Analytik Jena GmbH, Jena, Germany
Enhanced chemoluminescence (ECL) ChemoCam Imager	INTAS Science Imaging Instruments GmbH, Göttingen, Germany
EV 231 power supply PEQLAB – Life Science®	Avantor, Inc., Radnor, Pennsylvania, USA
Feeding gavage, plastic, FTP-22-25	Instech Laboratories, Inc., Plymouth Meeting, Pennsylvania, USA
Hanna edge® pH meter	Hanna Instruments, Woonsocket, Rhode Island, USA
Hettich Universal 320R Refrigerated Centrifuge	Hettich GmbH, Kirchlengen, Germany
IKA Magnetrührer RET basic Magnetic stirrer	IKA®-Werke GmbH & CO. KG, Staufen, Deutschland
Integra pipet boy 2	INTEGRA Biosciences GmbH, Biebertal Germany
Inverted microscope for cell culture plates	A.KRÜSS Optronic GmbH, Hamburg, Germany
Luminex® MAGPIX® CCD Imager	Luminex Corporation - A DiaSorin Company, Austin, Texas, USA
MACSQuant Analyzer 10 Flow cytometer	Miltenyi Biotec, Bergisch Gladbach, Germany
Mikro 120 Tabletop centrifuge	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Multiskan EX Microplate photometer	Thermo Fisher Scientific, Waltham, Massachusetts, USA
MyCycler™ Thermal Cycler System	Bio-Rad Laboratories, Inc., Hercules, California, USA
NanoPhotometer® P330	Implen GmbH, München, Germany
Neubauer improved counting chamber	Glaswarenfabrik Karl Hecht GmbH & Co KG
Peristaltic pump	Carl Roth® GmbH + Co. KG, Karlsruhe, Germany
Pipettes, Multi-Dispenser pipettes	Various companies
QuantStudio™ 3 Real-Time PCR System, 96-well	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Refrigerators and freezers	Various companies
Roller RM5 Assistent 348	Glaswarenfabrik Karl Hecht GmbH & Co KG
Sanyo MCO-20AIC CO_2 Incubator	Sanyo Denki K.K., Moriguchi, Osaka, Japan
Scientific Industries SI™ Vortex-Genie™ 2	Thermo Fisher Scientific, Waltham, Massachusetts, USA
StepOne® Real-Time PCR system Applied Biosystems™	Thermo Fisher Scientific, Waltham, Massachusetts, USA

INSTRUMENT	MANUFACTURER
Surgical instruments	Various companies
Thermomixer comfort	Eppendorf SE, Hamburg, Germany
TissueRuptor	Qiagen, Venlo, Netherlands
Trans-Blot® SD Semi-dry transblotting cell	Bio-Rad Laboratories, Inc., Hercules, California, USA
WB 22 water bath	Memmert GmbH + Co. KG, Schwabach, Germany
Weighing balances	Various companies
Western blotting system Casting frames Glass plates Combs Roller Mini-PROTEAN® Tetra Cell for SDSPAGE	Bio-Rad Laboratories, Inc., Hercules, California, USA

II. 1. 9. Consumables

TABLE 18: LABORATORY CONSUMABLES

ITEM	MANUFACTURER
Amersham Hybond ECL Nitrocellulose Membrane	GE HealthCare, Chicago, Illinois, USA
Cannula 26G, sterile, disposable	B. Braun SE, Melsungen, Germany
Cell culture dish 60 x 15 mm	Sarstedt AG & Co. KG, Nümbrecht, Germany
Cell scraper	Greiner Bio-One GmbH, Frickenhausen, Germany
Cellstar® 6 well plate 24 well plate Flat bottom lid TC-Plate 96 well sterile U-shape lid TC-Plate 96 well sterile	Greiner Bio-One GmbH, Frickenhausen, Germany
Cellstar® cell culture flasks: Suspension culture flask 50 ml Suspension culture flask 250 ml Cell culture flask 25 cm^2 Cell culture flask 75 cm^2	Greiner Bio-One GmbH, Frickenhausen, Germany
Cryoboxes	Ratiolab GmbH, Dreieich, Germany
Cryogenic vials	Various companies
Eppendorf tubes: Standard tubes, 1.5 ml, 2 ml PCR tubes (pyrogen and DNase free) 0.2 ml, 0.5 ml, 1.5 ml, 2 ml	Various companies
Falcon tubes 15 ml; 50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Falcon® 70 µm Cell Strainer	Corning, Inc., Corning, New York, USA
Glass Pasteur pipettes 150 mm	BRAND GmbH & Co. KG, Wertheim, Germany
Glassware: Erlenmeyer flasks, Volumetric flasks, Beakers, Bottles Measuring cylinders, Burettes, Microscope slides	Various companies
Gloves, nitrile	Various companies
MicroAmp™ PCR disposables: Optical 96-Well Reaction Plate 8-Cap Strip, 8-Tube Strip Applied Biosystems™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Microscope cover slips	Glaswarenfabrik Karl Hecht GmbH & Co KG
Minisart® Syringe Filters (0.2 µm)	Sartorius AG, Göttingen, Germany

ITEM	MANUFACTURER
Mr. Frosty™ freezing container	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Parafilm® Sigma-Aldrich®	Merck KGaA, Darmstadt, Germany
Pipette tips (pyrogen and DNase free) 10 µl; 20 µl; 100µl; 200 µl; 1000 µl	nerbe plus GmbH & Co. KG, Winsen, Germany
Pipette tips standard 10 µl; 20 µl; 100µl; 200 µl; 1000 µl	Sarstedt AG & Co. KG, Nümbrecht, Germany
Plastic feeding tubes 22ga 25mm, sterile	Instech Laboratories, Inc., Plymouth, Pennsylvania, USA
Plastic pipettes: 5 ml; 10 ml; 25 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Syringes	B. Braun SE, Melsungen, Germany
Western blot disposables: Blot paper 19x18.5 cm	BioRad, München, Germany

II. 1. 9. 1. Preparation of coated adhesive cover slips

The coverslips were sterilized in pure ethanol overnight at room temperature, then washed with sterile dH_2O, dried, and transferred to a 6-well plate. The coverslips were coated with poly-L-lysine solution and incubated for at least 5 hours at room temperature. Excess poly-L-lysine solution was then removed, and the coverslips were placed in a fresh 6-well plate and stored at 8 °C until further use.

II. 1. 10. Software

TABLE 19: SOFTWARE

ITEM	MANUFACTURER
Adobe CC Adobe Illustrator 24.1.3 Adobe Photoshop 21.1.3 Adobe InDesign 15.0.3	Adobe Inc., San Jose, California, USA
ChemoStar Imager 0.4.18.0	INTAS Science Imaging Instruments GmbH, Göttingen, Germany
Design & Analysis Software 2.6.0	Thermo Fisher Scientific, Waltham, Massachusetts, USA
FlowJo software 10.2.0	Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA
GraphPad Prism 9.1.2.226	Dotmatics, Boston, Massachusetts, USA
ImageJ 1.53s	Public Domain, BSD-2
StepOne™ Software 2.3 Life Technologies™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
ZEN (blue edition) 3.4.91.0	Carl Zeiss Microscopy GmbH, Oberkochen, Germany

II. 2. METHODS

The experiments were either carried out in Biomedizinisches Forschungszentrum Seltersberg (BFS) of the Justus-Liebig-Universität (JLU), Schubertstraße 81, Medizinisches Forschungszentrum Seltersberg (ForMED) of the JLU, Aulweg 128, or

Institut für Anatomie und Zellbiologie of the JLU, Aulweg 123, Gießen, Germany. Housing and animal experiments were performed in the animal facility of the BFS. For manufacturer information on materials referenced to in this section please refer to II. 1.

II. 2. 1. *In vivo* experiments

Animal handling and EAE experiments were conducted by Dr. rer. nat. Ranjithkumar Rajendran, Dr. rer. nat. Vinothkumar Rajendran and Kian Shirvanchi on the basis of to their qualification to oversee animals as per the Federation of European Laboratory Animal Science Associations (FELASA).

II. 2. 1. 1. Ethics Statement

Prior to all animal experiments, approval was obtained from the regional council of Hesse, Giessen, Germany (Regierungspräsidium Gießen, reference number: GI 20/18 Nr. G3-2018) according to the German animal welfare law (Tierschutzgesetz, TSchG, BGBl. I S. 1826) and the European legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). The procedures were performed in conformity with the above-mentioned laws and under the supervision of the animal care officer of the Justus-Liebig-University (registration number: 891_GP). The BFS facility where the experiments were carried out was licensed for this purpose (Az: IV44-53r 30.03 UGI118.11.03) and the method of euthanasia was in accordance with the American Veterinary Medical Association (AMVA) Guidelines for the Euthanasia of Animals.

II. 2. 1. 2. Acclimatization and EAE induction

C57BL/6J mice were maintained in temperature- and light-controlled surroundings at the animal facility and were utilized in the experiments at 8-12 weeks of age. At 8 weeks of age, mice were anesthetized by isoflurane inhalation and immunized against 300 µg MOG_{35-55}, which was emulsified in Complete Freund's adjuvant (including 10 mg/ml *Mycobacterium tuberculosis* H37Ra). Subcutaneous injections were placed ventrally into the four flanks. Additionally, 300 ng *Bordetella pertussis* toxin in 100 µl PBS was injected intraperitoneally at 0 hours and 48 hours post-induction. This mixture was used to boost immune response and weaken the BBB, thereby inducing EAE. EAE then progressed without further intervention. Weight and neurological disability were assessed in a blinded fashion until the animals were sacrificed. The EAE score was assessed as follows:

TABLE 20: EAE SCORING PATTERN

SCORE	SYMPTOMS	SCORE	SYMPTOMS
0	Inapparent	0.5	Distal tail weakness
1	Complete tail weakness	1.5	Mild hindlimb weakness
2	Ascending hindlimb weakness	2.5	Severe hindlimb weakness
3	Hindlimb paralysis	3.5	Hindlimb paralysis and forelimb weakness
4	Tetraparesis	4.5	Tetraplegia
5	Moribund or death		

The sample size for evaluation was pre-specified by biomathematical power analysis as $n \geq 8$ in three independent experiments. Animals were excluded from the experiment if they exceeded a symptom burden defined as a humane endpoint approved by the local animal welfare authorities. Therefore, a total score was calculated from the EAE score (Table 20) and other behavioral and physiological measures. If this score surpassed a predetermined sum, animals were excluded.

II. 2. 1. 3. Experimental design of the EAE study

The experimental design depicted in Figure 11 was chosen to reveal differences in the effect of infigratinib depending on the temporal availability of the drug during the course of EAE. After induction of EAE (see II. 2. 1. 2) infigratinib was administered orally to the treatment group by gavage at a dose of 30 mg/kg body weight. Infigratinib was dissolved in 100 µl 1% sodium carboxymethylcellulose in PBS for treated mice, while vehicle group mice received vehicle only (*i.e.*, 100 µl 1% sodium carboxymethylcellulose). Mice were fed either solution in a blinded fashion. The dosing was consistent with study doses that have shown efficacy at this concentration (Guagnano et al., 2011; Konecny et al., 2013; Fumarola et al., 2017).

Two treatment approaches were selected. Infigratinib was administered

i) preventively, injected once daily for 10 days from day 0 post injection (pi) to day 9 pi;

ii) or suppressively, administered from day 10 pi to day 19 pi.

Sample collection times for the prevention experiment for these approaches were day 17 pi in the acute phase and day 41 pi in the chronic phase. Sample collection times for the suppression experiment were day 20 pi and 42 pi, respectively. Blood/tissue of interest was collected after euthanasia with CO_2.

FIGURE 11: EXPERIMENTAL DESIGN OF THE EAE STUDY

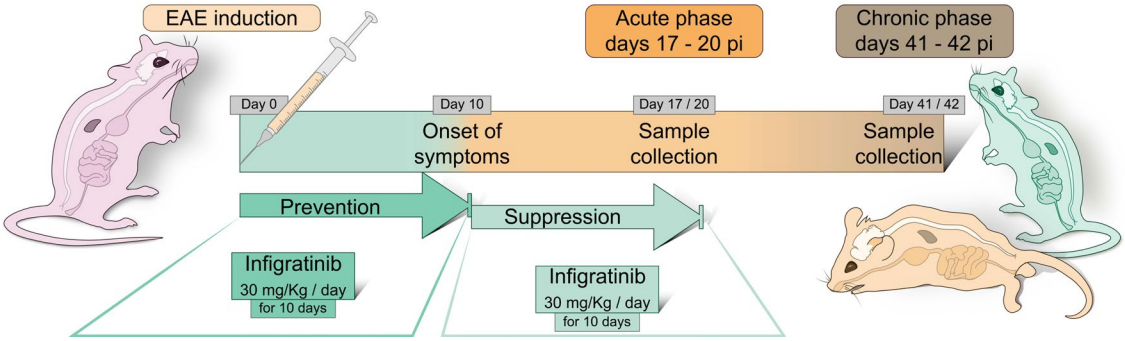

Figure 11 shows important timings of EAE induction, treatment, and sample collection.

II. 2. 1. 4. Flow cytometric analysis of animal tissue

Blood was immediately collected in EDTA-coated tubes and lysed with RBC lysis solution for 2 minutes to remove erythrocytes. To remove clots, the blood was passed through a Falcon® 70 µm cell strainer. The spleens were also immediately collected and stored in ice-cold PBS. The spleen was then minced with scissors and incubated in digestion buffer at 37 °C for 30 minutes. To obtain a single-cell suspension, the minced organ was passed repeatedly through 21G cannulas and a Falcon® 70 µm cell strainer with a syringe plunger, the tissue solution was incubated with RBC lysis solution for 2 minutes with PBS. Both blood and tissue solution were then centrifuged for 7 minutes at 4 °C, 300 × *g*. The supernatant was discarded. The resulting single cell suspension was incubated with FcR blocking reagent for 15 minutes at 4 °C in 1.5 ml tubes. After blocking, antibody cocktails were added, and cells were incubated for 30 minutes at 4 °C in the dark. The cells were then washed and centrifuged as described above and resuspended in FC buffer. This cell solution was then used for analysis. The cell populations of interest were separated using a gating strategy. In a first step, live cells were identified by plotting side scatter-area (SSC-A) against forward scatter-area (FSC-A) to estimate size and granularity (P1 gate). Second, the CD45-positive population was selected (P2 gate). Third, doublets were excluded by plotting SSC-A against side scatter-height (SSC-H) and FSC-A against forward scatter-height (FSC-H, P3 gate). This hematopoietic single cell population was then probed with the antibodies listed in Table 11 except CD45 (for an overview see Figure 12). FC was performed using a MACSQuant Analyzer 10 flow cytometer. The resulting data were analyzed using FlowJo software.

FIGURE 12: CELL IDENTIFICATION IN FC

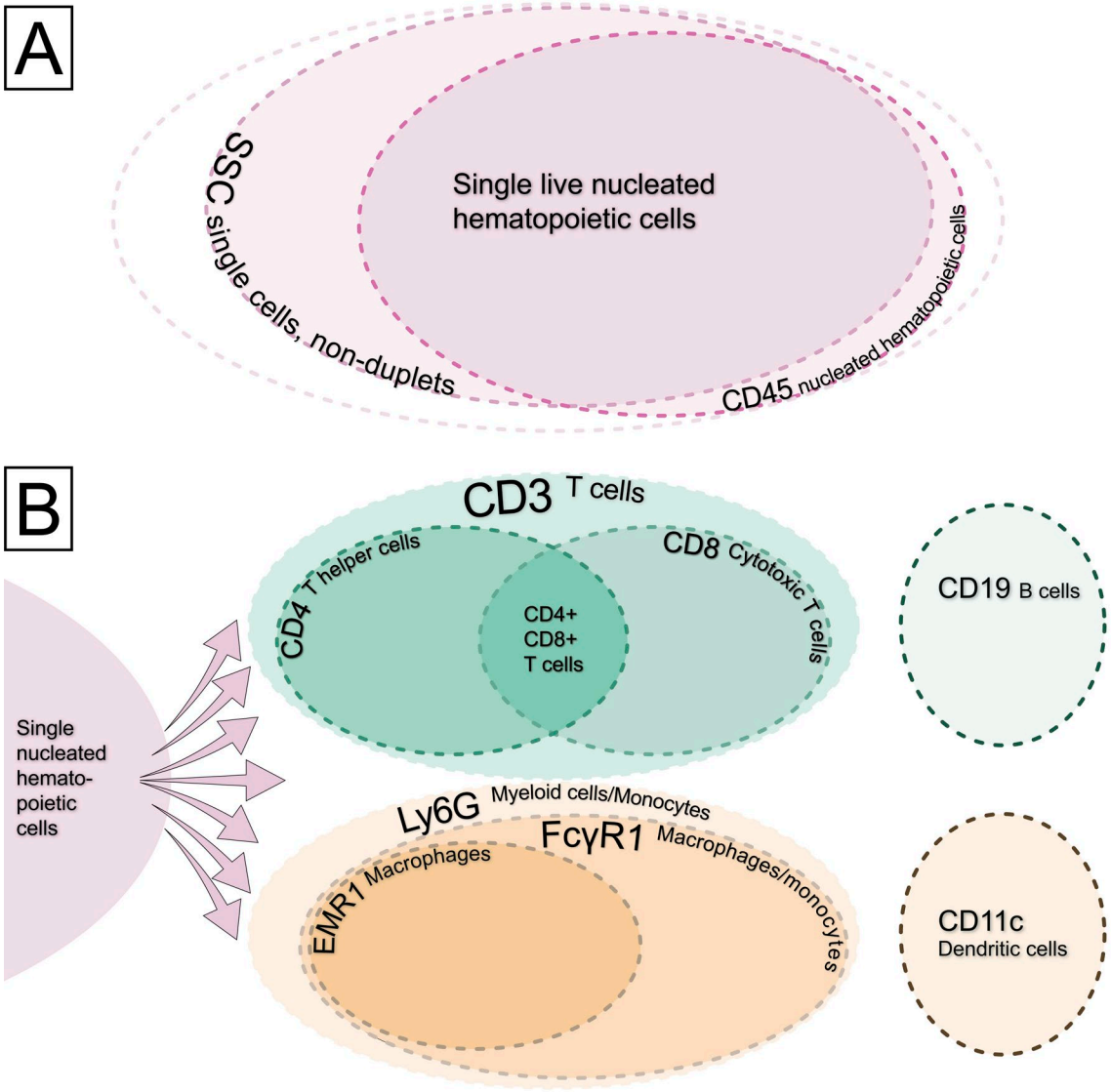

Figure 12: Antibodies used, and subpopulations identified in the FC.

II. 2. 2. *In vitro* experiments

All cell culture experiments were conducted in the ForMED, in a biological safety cabinet under sterile conditions. Cells were revived, brought to exponential growth, and then maintained in this phase by splitting on an average of every other day. All experiments were performed in the exponential growth phase. Cell concentration was measured using an improved Neubauer counting chamber. Trypan blue was used to identify dead cells (Strober, 2015), which can only penetrate the cytosol if cell membrane integrity is compromised (as defines cell death).

II. 2. 2. 1. Cell cultivation

All cells were maintained under the following standard growth conditions:
Incubation was performed in the Sanyo incubator under a constant controlled environment of 37 °C, 5% CO_2 and 95% humidity. When live cells are described as having been centrifuged, the following settings were used: 130 × *g* for 4 minutes at room temperature. If material is described as heated or warm, it has been warmed to 37 °C in a water bath. For a description of the cell lines, see II. 1. 2.

II. 2. 2. 1. a. Jurkat cells

Revival

Jurkat cells were rapidly thawed in a warm water bath by gentle agitation in the manufacturer's frozen cryotube. The cell solution was added to 10 ml of CO_2 pre-acclimatized pre-warmed Jurkat growth medium in a 15 ml Falcon® tube to rapidly dilute the cryoprotectant DMSO. This tube was then centrifuged, the supernatant discarded, and the resulting cell pellet resuspended in 20 ml of warm Jurkat growth medium in a 50 ml Falcon® tube. Depending on the number of cells counted, cells were then seeded into an appropriate number of flasks to achieve a final density of 1×10^5 cells / ml.

Passage

Once Jurkat cells reached a density of 1×10^6 cells / ml, or close to it, were divided. Cell culture flasks were therefore emptied into one or more 50 ml Falcon® tubes, centrifuged, the supernatant discarded, and the pellet resuspended in an appropriate volume of growth medium. After counting, the cell suspensions were plated at 1×10^5 cells/ml in fresh cell culture flasks and labeled as one passage higher.

Conservation

Jurkat cells were frozen periodically to maintain passage stocks. Therefore, cells were centrifuged and resuspended in Jurkat freezing medium at a density of 1×10^6 cells / ml in a cryovial. This vial was then placed in a freezing container filled with isopropanol at room temperature and then transferred to a -80 °C freezer to slowly cool the cells. The cells were then resuspended as described above.

II. 2. 2. 1. b. SIM-A9 cells

Revival

SIM-A9 cells were revived analogously to Jurkat cells as described above with the use of SIM-A9 growth medium. Cells were placed into adhesive cell culture flasks and the desired initial confluency was between 20% and 30%.

Passage

Cells were passaged when they reached 80% - 90% confluency. Generally, no change of media was required prior to that. To split the cells, pre-acclimatized pre-warmed adhesive cell culture flasks were prepared, the supernatant of the existing vegetated flasks was transferred to a 50 ml Falcon® tube and the flasks were gently washed with 2 ml 1x DPBS, which was then added to the same tube. Next, 5 ml DDPBS was added to the cell growth inside the flask and incubated for 10 minutes in warmth. When the cells dissociated, the (D)DPBS-cell-solution was also moved to the tube. The cells could now be centrifuged and reseeded as described above for the Jurkat cells.

Conservation

Cells were detached as described under passaging. They were then slowly frozen as described above for the Jurkat cells but using SIM-A9 freezing medium.

II. 2. 2. 2. Experimental design of the cell culture experiment

Both cell lines were treated analogously except for the use of their respective growth media. Only cells in exponential growth were used if there was no evidence of contamination. Between 1 × 10^4 cells and 1 × 10^6 cells were used for each group, depending on the intended material for analysis. Treatment and control groups were defined as follows:

TABLE 21: TREATMENT SCHEME AND CONTROLS

GROUP NAME	TREATMENT	CODE/COLOR
Control	None	C (Control)
Fexagratinib	1 µM fexagratinib	A (AZD4547)
Infigratinib	1 µM infigratinib	B (BGJ398)
Dovitinib	1 µM dovitinib	D (Dovitinib)
FGF2	25 pg / L	F (FGF2)
Vehicle control	1 µM	V (Vehicle)

Table 21: Cell culture treatment groups. FGFR inhibitors were dissolved in DMSO, FGF2 was dissolved in 1% BSA in DPBS

The experimental design is shown in Figure 13. Every experiment was done in biological triplicates. Some methods were additionally performed in technical replicates.

FIGURE 13: EXPERIMENTAL DESIGN OF THE CELL CULTURE EXPERIMENT

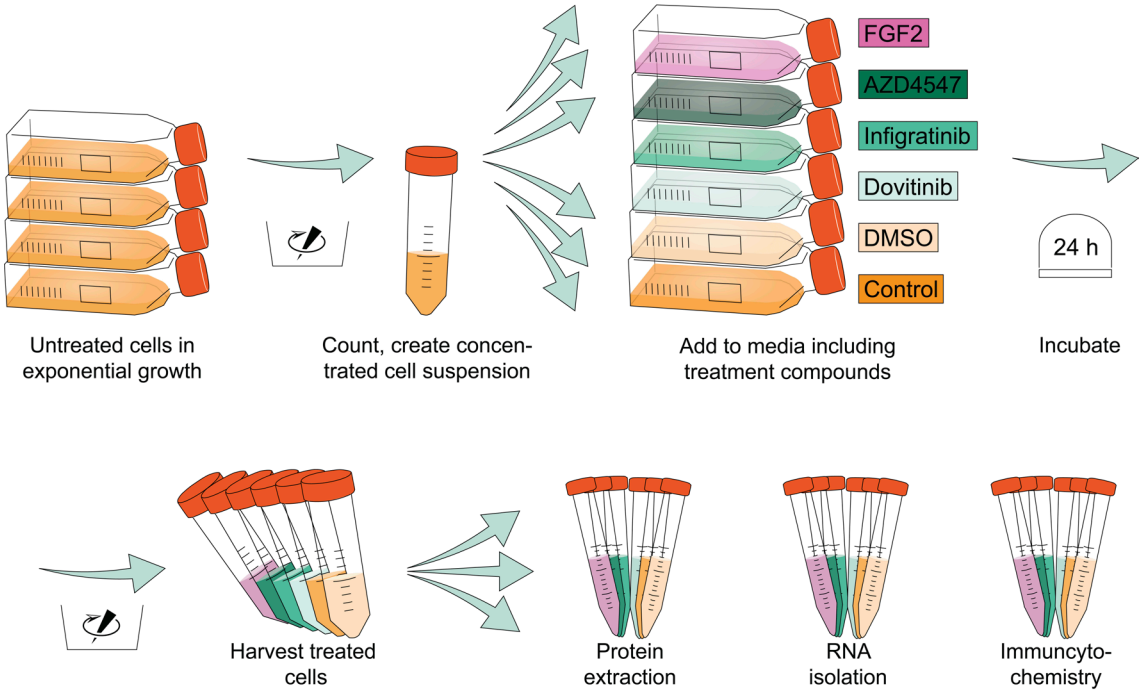

Figure 13 shows the procedure of the cell culture experiment: Exponentially growing cells were centrifuged, the supernatant discarded, and then diluted to a predetermined concentration. This cell suspension was then added to pre-prepared growth media flasks containing the treatment compounds at a higher than desired concentration. By diluting this compound-containing medium with the cell suspension, the desired target concentration was achieved. The cells were then incubated for 24 hours under standard growth conditions to allow the compounds to affect the cells. After this period, the cells were harvested and processed. While protein, RNA and whole cells were obtained by treatment in cell culture flasks, the treatment of cells for WST-1 and lactate dehydrogenase (LDH) assays were performed in 96-well plates.

II. 2. 3. Molecular biological analyses

For materials and instruments mentioned in this section, see II. 1. Unless otherwise noted, standard laboratory conditions were maintained.

II. 2. 3. 1. Cytotoxicity measurement

To exclude possible cytotoxic effects from the mode of action of the inhibitors, cytotoxicity screening was performed. Cell death is conventionally defined as the loss of plasma membrane integrity (Kroemer et al., 2009). The release of lactate dehydrogenase (LDH), which is an enzyme found inside cells, thus correlates with cell death and has since long been used to measure cytotoxicity (Decker and Lohmann-Matthes, 1988). It is suitable due to its properties of offering a high sensitivity and specificity, with the exception of an

overestimation of cytotoxicity when cell growth inhibition is strong (Smith et al., 2011). Therefore, the cell proliferation was measured independently (see II. 2. 3. 2).

II. 2. 3. 1. a. Principle

In the Cytotoxicity Detection Kit used, a catalyst, NAD^+, the tetrazolium salt dye 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) and sodium lactate are added to the supernatant. LDH catalyzes the turnover of lactate to pyruvate and vice versa. When lactate is oxidized to pyruvate, the LDH enzyme reduces NAD^+ to $NADH^+/H^+$. Thus, two protons are transferred on INT which is converted by diaphorase to a red dye. This reaction directly correlates to the amount of LDH present. This applies only to a limited period of time; optimal timing was established beforehand.
Contrary to INT, the red dye absorbs at 500 nm (Roche, 2016). Color intensity was measured (492 nm filter), background was excluded (600 nm) after shaking.

II. 2. 3. 1. b. Cell preparation and incubation

Jurkat or SIM-A9 cells were treated as described in II. 2. 2. 2. They were seeded in U-shape TC-Plates at a density of 1×10^5 cells / ml (counted in a Neubauer chamber). A volume of 100 µl and a quantity of 1×10^4 cells per well was used. After incubation, plates were centrifuged (250 × g, 10 minutes). Under strict conservation of the cell debris, only the supernatant was transferred to a flat bottom TC-Plate. An equal amount of reaction mixture of the kit was added, the plate was incubated in darkness for 30 minutes (Roche, 2016). It was then read in the photometer.

II. 2. 3. 2. Proliferation measurement

To account for potential overestimation of cytotoxicity and to reveal effects of the inhibitors to the growth rate of Jurkat and SIM-A9 cells, their proliferation was measured. For standardization and simultaneity an Enzyme-linked immunosorbent assay (ELISA) was used.

II. 2. 3. 2. a. Principle

Tetrazolium salts have long been used as a measuring tool for cell proliferation (Mosmann, 1983). 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1; Ishiyama et al., 1993) is suitable due to its water solubility and stability (Ishiyama et al., 1997; Berridge et al., 2005). In the utilized kit, WST-1 is added to the sample and reduced to a red formazan salt most likely through transmembrane transport of electrons from $NADH^+/H^+$ to WST-1 through an intermediate

electron acceptor (Berridge et al., 2005). This turnover is directly proportional to the number of viable cells. Discriminatory power was optimal after 4 hours incubation. Contrary to the substrate, the resulting formazan salt absorbs at approximately 440 nm (Roche, 2021). Color intensity was measured with a 450 nm filter, which is in the range of absorption maximum. The background absorption was excluded with a 620 nm filter. Measurements were taken after shaking.

II. 2. 3. 2. b. Cell preparation and incubation

Jurkat or SIM-A9 cells were treated as described in II. 2. 2. 2. They were seeded in flat bottom TC-Plates at a density of 1×10^5 cells / ml, after counting them in a Neubauer chamber, to result in a volume of 100 µl and a quantity of 1×10^4 cells per well. After incubation, 30 µl reaction mixture, consisting of 13 µl WST-1 solution and 17 µl RPMI 1640 or DMEM/F12, were added to each well and the plate was incubated in standard growth conditions for 4 hours. It was then read in the photometer.

II. 2. 3. 3. Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed in a two-step protocol.
Resulting cycle threshold (C_T) values were used with the $2^{-\Delta\Delta C_T}$ or Livak method to calculate the relative x-fold changes in gene expression levels (Livak and Schmittgen, 2001). RT-qPCR was either performed with the StepOne or Quant Studio 3.

II. 2. 3. 3. a. RNA isolation for RT-qPCR

Jurkat or SIM-A9 cells were treated as described in II. 2. 2. 2. They were seeded in T-25 flasks of 2.2×10^5 cells / ml to result in a volume of 9 ml and a quantity of at least 0.8×10^6 cells per flask to achieve sufficient RNA yield. After incubation, cells were washed with DPBS and centrifuged at 250 × *g* for 4 minutes. Supernatant was stored for further analysis. The cell pellet was either immediately stored in -80 °C for later use or was joined with 400 µl RNA lysis buffer T included in the total RNA kit to release the RNA to the solution.

II. 2. 3. 3. b. Principle of two-step RT-qPCR

Since the changes at the messenger ribonucleic acid (mRNA) level were investigated using a two-step approach, the production of complementary deoxyribonucleic acid (cDNA) to the mRNA of the cell lysate was necessary. Thus, the first step of the RT-qPCR is the cDNA synthesis shown in Figure 14. Total RNA Kit was used according to

the manufacturer's manual (Avantor, n.d.). The polymerase chain reaction was followed by the use of SYBR green, which is displayed in Figure 15. This reaction was done in either of the two PCR machines with a short or long protocol, each cycle was done following a protocol displayed in Figure 16. As soon as fluorescence passes a predetermined threshold, the current cycle is read out. This C_T-value corresponds to the relative number of mRNA molecules of the region of interest in the respective sample calculated with the $2^{-\Delta\Delta C_T}$ method.

FIGURE 14: FIRST STEP OF TWO-STEP-RT-QPCR

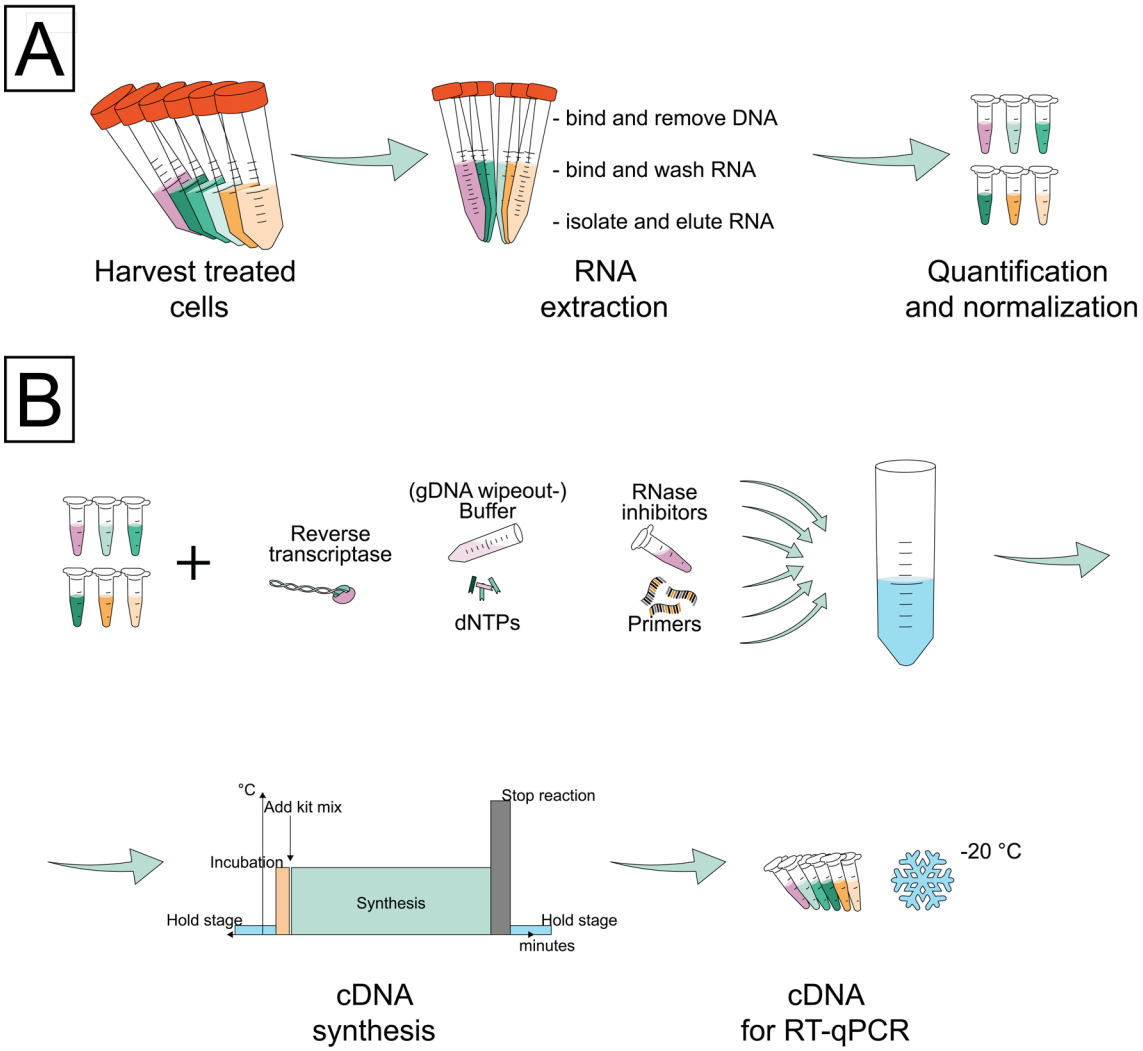

Figure 14 shows the first step of the two-step RT-qPCR.
DNA was removed with the help of DNA binding columns, subsequently RNA was bound to an RNA binding column, twice washed with included wash buffers, and then eluted in 70 µl of nuclease free dH_2O. This elution was then quantified in the NanoPhotometer for its amount of RNA and normalized with nuclease free dH_2O to 1 µg per 20 µl (A).
For the following steps, the Reverse Transcription Kit was used. The isolated RNA was first joined with the genomic DNA (gDNA) wipeout-buffer and incubated for 2 minutes at 42 °C. The Mastermix consisting of reverse transcriptase, deoxyribonucleotide triphosphates (dNTPs), random primers, and RNase inhibitors was then added to the RNA solution (Qiagen, 2016). Synthesis was done for 30 minutes at 42 °C and stopped by heating to 95 °C for 3 minutes. Samples were then cooled to 4 °C and stored at -20 °C (B).

FIGURE 15: PRINCIPLE OF RT-QPCR

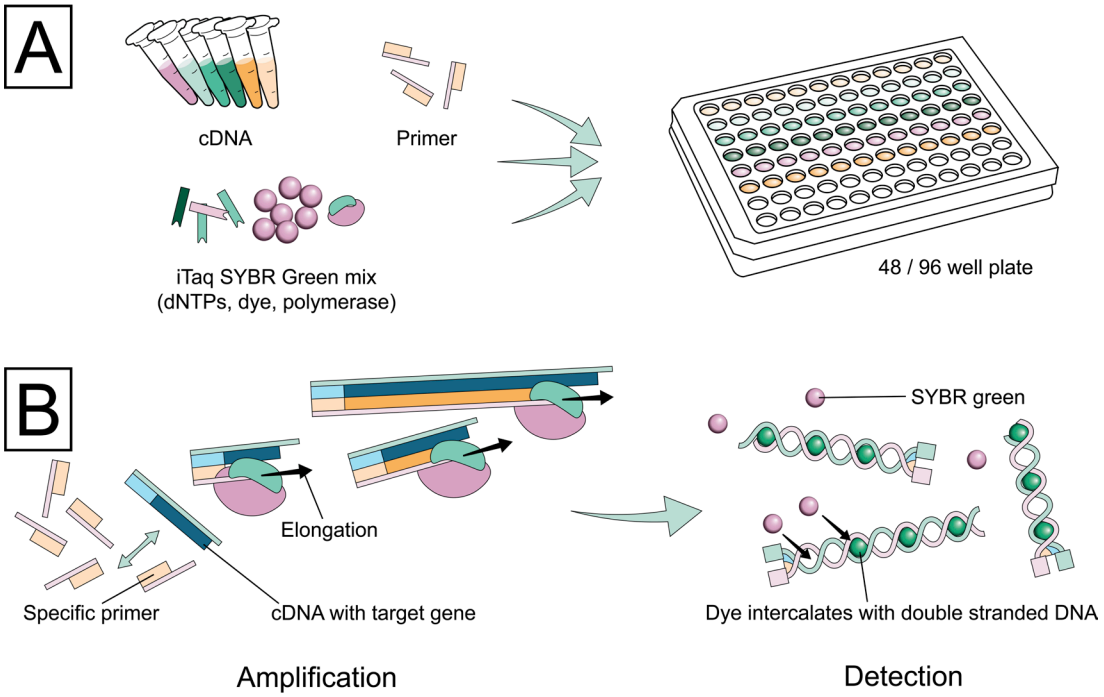

Figure 15 shows the principle of RT-qPCR: 1 µl sample, 1 µl each of specific primer (FP and RP) for the gene of interest and 7 µl of the kit mix are combined in a multiwell plate (A). When the polymerase replicates primer sequences bound to a cDNA, primer-specific double stranded DNA is produced. SYBR green binds non-specifically to the double-stranded DNA and emits green light, which thus correlates to the cDNA abundance of the initial mRNA of interest (B).

FIGURE 16: RT-QPCR PROTOCOLS

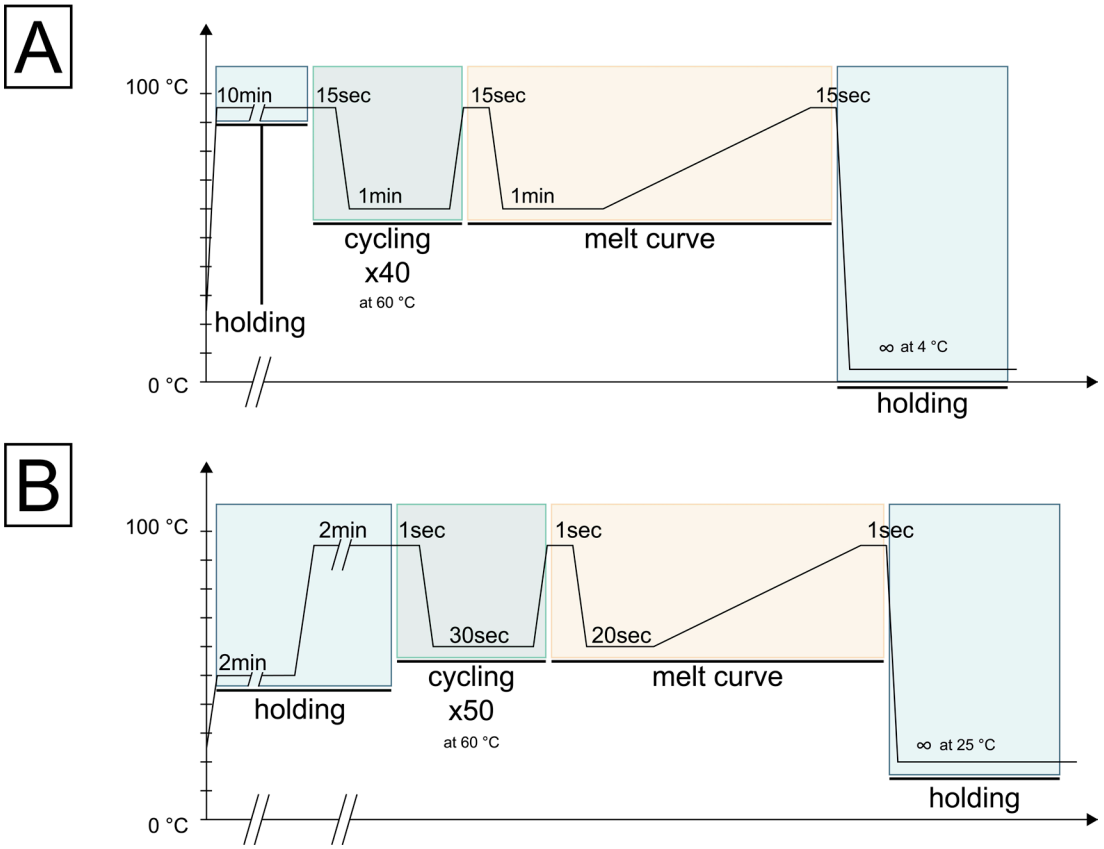

Figure 16 shows the long (A) and short (B) protocol of the RT-qPCR: Cycles were repeated 40 or 50 times, respectively, after which RT-qPCR products were analyzed (see next section).

II. 2. 3. 3. c. RT-qPCR analysis

After cycling, the RT-qPCR products were analyzed on the basis of their melt curve to exclude unspecific amplicons. Livak and Schmittgen demonstrated that, for efficient RT-qPCRs, the amount of target mRNA is equal to their C_T-values when compared to a reference gene and a calibrator control. This results in the equation:

$$\text{amount of target} = 2^{-\Delta\Delta C_T}$$

$$\text{for } -\Delta\Delta C_T = -(\Delta C_{T,q} - \Delta C_{T,cb})$$

(Livak and Schmittgen, 2001)

ΔC_T is calculated as the difference of C_T-values of the target gene and the reference gene (Livak and Schmittgen, 2001). After that, C_T-values of the treatment groups ($C_{T,q}$-values) are subtracted from C_T-values of a calibrator ($C_{T,cb}$-values). Accordingly, C_T-values of amplicons with a consistent melt curve were used with the $2^{-\Delta\Delta C_T}$ method using GAPDH as a reference gene and the control group as a calibrator. The control group was normalized to 1.0.

II. 2. 3. 4. Western blot

The protein immunoblotting was done with SDS-PAGE and Enhanced chemoluminescence (ECL) HRP substrate (see Figure 18). Due to low occurrence of the proteins of interest and difficult detection conditions, optimization of the Western blot protocol was carried out for each protein of interest.

II. 2. 3. 4. a. Protein normalization

Jurkat or SIM-A9 cells were treated as described in II. 2. 2. 2. The normalization of the protein samples is shown in Figure 17.

II. 2. 3. 4. b. Principle of SDS-PAGE and HRP ECL immunoblotting

After lysis, normalized protein samples were linearized and electrophoretically separated by SDS-PAGE according to their molecular weight. The distributed peptides were then transferred to a nitrocellulose membrane. When this membrane was incubated with antibodies and ECL solution, the signal of specific proteins was detectable with an ECL imager (for details please refer to the figure caption of Figure 18).

FIGURE 17: PROTEIN ISOLATION AND NORMALIZATION

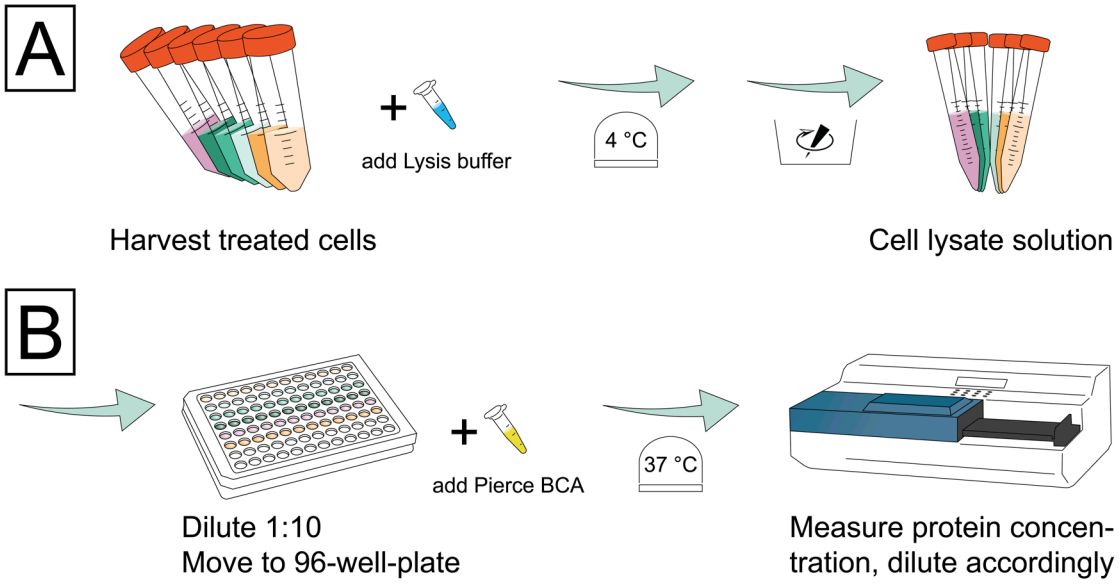

Figure 17 shows the process of protein normalization: Cells at 2.2 × 10^5 cells / ml in a volume of 15 ml and a quantity of at least 3 × 10^6 cells per flask were incubated, plates were centrifuged at 250 × *g* for 4 minutes. After treatment, the cells were joined with 400 µl Lysis buffer, incubated on ice and regularly vortexed for 30 minutes. The cell lysate was centrifuged at 12000 rotations per minute (rpm) for 30 minutes and supernatant collected (A).
1 µl of each sample was diluted 1:10 and transferred to a 96-well plate in triplicates. 200 µl working reagent of the Protein Assay Kit was added, incubated at 37 °C for 30 minutes and measured in the ELISA reader at 562 nm. Samples were normalized to 2 µg / µl accordingly (B).

FIGURE 18: WESTERN BLOT OVERVIEW

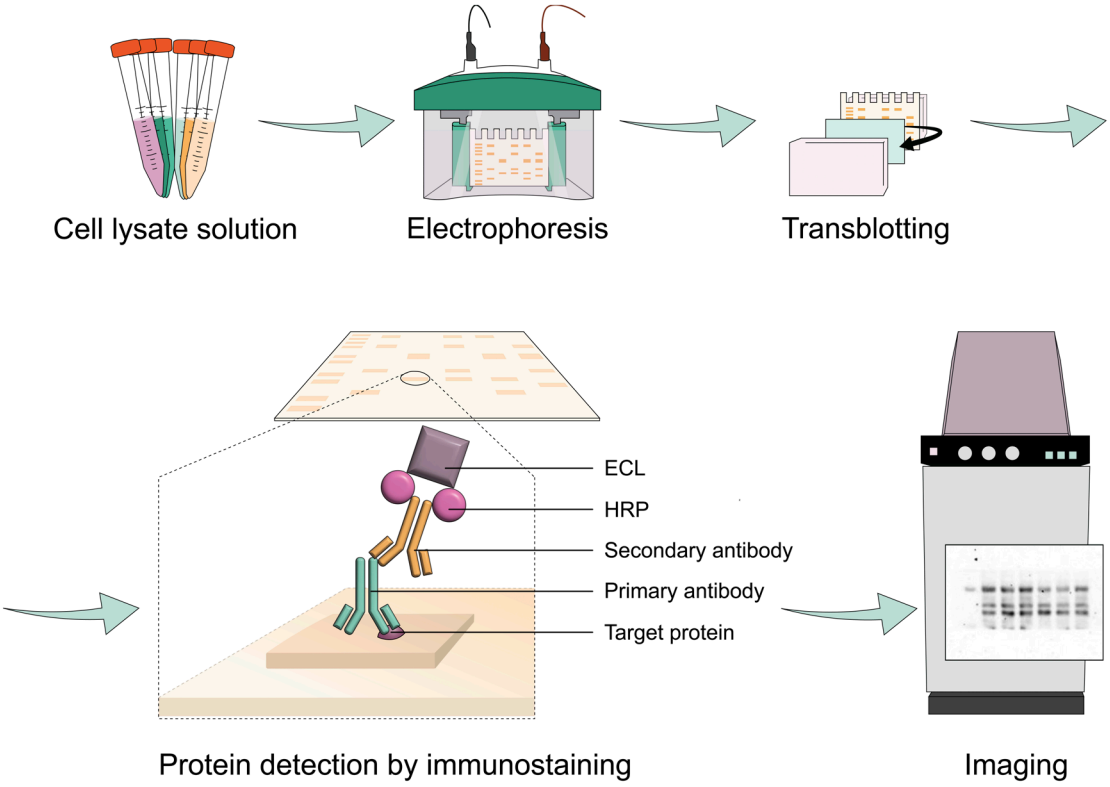

Figure caption of Figure 18 appears on the next page.

Figure 18 shows the procedure of SDS PAGE and ECL HRP immunoblotting.
SDS-PAGE was used to electrophoretically distribute the proteins according to their molecular weight.
1. Linearization, neutralization:
SDS was added to the protein sample in excess (up to 60 µg) along with a loading dye and additives such as 2-mercaptoethanol (to cleave disulfide bonds). By adding this Laemmli buffer and boiling the solution for 5 minutes, secondary and tertiary protein structures were degraded. In addition, SDS bound to amino acids neutralized charges and added negatives in proportion to the number of amino acids (Janson and Tischler, 2012). The result was a negatively charged linear peptide chain that allowed proteins to be separated regardless of their structure and intrinsic charge.
2. Two-Step Electrophoresis:
a) 90 V and 300 mA for 30 minutes and b) 120 V and 1000 mA for 150 minutes. This current applied to the buffer in the chamber system resulted in an electric field into which a 10% SDS gel was placed. It consisted of two parts, a stacking and a resolving gel. These gels differed in pH and molecular mesh granularity.
By loading the proteins on wells, their flow through the first part of the gel resulted in a stacking effect that condensed the bands before they passed to the resolving gel. The current was cut off as the attached ladder approached the bottom of the gel.
Depending on the granularity of the resolving gel molecular network (mainly proportional to the acrylamide fraction), the transit speed of the proteins to the anode was modifiable, as smaller proteins moved faster through the pores of the gel (Janson and Tischler, 2012). This allowed the resolution range to be optimized according to the molecular weight of the protein of interest.
3. Transblotting, blocking, incubation :
An electric field (25 V and 354 mA for 70 minutes) perpendicular to the gel transferred the peptides to the surface of a nitrocellulose membrane, making them accessible for binding. Nitrocellulose was chosen for its high retention and affinity for peptides (Mahmood and Yang, 2012). Membranes were then blocked with nonfat dry milk to mask nonspecific binding sites (1 hour at 4 °C). The following washing steps were performed to maintain sensitivity and reduce background signal (Mahmood and Yang, 2012). Antibodies specific to the proteins of interest were incubated with the membrane on a rolling machine (overnight at 4 °C, for antibodies see Table 9). After another wash, an enzyme-conjugated secondary antibody was added (1:1000 in nonfat dry milk). This antibody, directed against the host species of the primary antibody, bound to the primary antibody (Janson and Tischler, 2012).
4. Imaging:
The membrane was then washed and continuously covered with chemiluminescent substrate for approximately 30 seconds. Luminol was oxidized by peroxide buffer, and this HRP-catalyzed conversion emitted blue light. The addition of enhancers allowed for longer duration and higher intensity (due to an excited state product) of light emission. This occurs only during the enzyme/substrate reaction in the vicinity of the enzyme. Thus, the amount of light emitted is proportional to the amount of protein bound to the membrane.
The light emittance was captured by a CCD camera and the resulting *tiff* - images were analyzed using the ImageJ software.
5. Image evaluation:
All bands of the molecular weight of interest were selected to confirm that the bands belonged to the protein of interest. While plotting the area under the curve of signal intensity, each signal was separated from its neighboring signals and assigned to the treatment group. The area under the curve value was analyzed and compared to the control group (which was normalized to 100%).
Modifications and optimization:
Depending on the protein, various optimizations were applied to the basic protocol described above: SDS gel concentration was adjusted between 10% and 12%, protein lysate was added at a level of up to 60 µg per group (30 µl protein (on ice) combined with 10 µl loading dye).
Duration of electrophoresis was adapted (proportional to molecular weight).
All subsequent blocking and washing steps were shortened or lengthened, or the incubation temperature was adjusted (room temperature vs. 4 °C).
Primary antibodies were diluted between 1:100 and 1:1000 (in blocking buffer). The incubation time of the chemiluminescent substrate was adjusted according to the expected amount of protein available and the sensitivity of the antibody, as well as the exposure times on the CCD imager to obtain optimal grayscale depth.

II. 2. 3. 5. Immunocytochemistry

Jurkat or SIM-A9 cells were treated as described in II. 2. 2. 2. They were seeded in T-25 flasks of 1.6 × 10^5 cells / ml, after counting them in a Neubauer chamber, to result in a volume of 12.5 ml and a quantity of 2 × 10^6 cells per flask. After incubation, cell solutions were centrifuged at 12000 rotations per minute (rpm) for 4 minutes.

A summary of the ICC protocol is shown in Figure 19 and described in the figure caption.

FIGURE 19: ICC SUMMARY

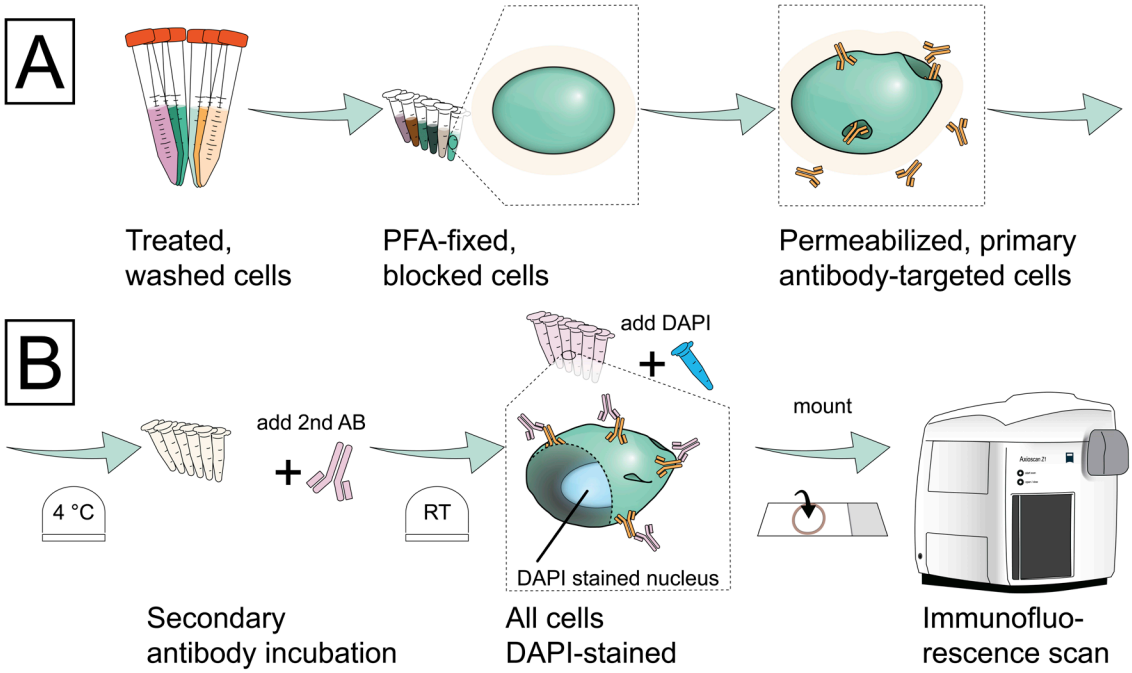

Figure 19 shows the principal steps needed for immunofluorescent labelling of cells for a specific antigen.
The treated cells were first washed with DPBS, centrifuged and the supernatant discarded. They were then transferred to 2 ml Eppendorf tubes, fixed with 4% PFA for 15 minutes at room temperature, washed again and permeabilized with 0.1% Triton™ X-100 for 15 minutes at room temperature. After another round of washing, non-specific binding sites were blocked with blocking buffer for 1 hour at room temperature. After one further wash, the primary antibody was diluted with incubation buffer at a ratio of 1:200 and 1:250 (FGFR1 and FGFR2, respectively). It was incubated overnight at 4 °C (A).
The cells were then washed again and incubated with secondary antibody (1:1000 in incubation buffer) for 1 hour at room temperature. After removal of the secondary antibody solution, the nuclei of all cells were stained with DAPI (2 µg/ml) to measure the ratio of negative to positive cells. They were then transferred to coated adhesive coverslips (see II. 1. 9. 1), upended to mounting slides prepared with mounting medium, sealed with nail polish, and scanned with the Axio Scan microscope.
A filter for Alexa Fluor 488 (450 - 488 nm) and for DAPI (370 - 400 nm) was used to detect signals with the Zen software. A positivity threshold was defined after gamma and histogram adjustment for both filters to achieve maximum contrast and high specificity for positivity.
Those cells with adequate signal location and strong signal were counted as positive, while all other cells were counted as negative. This was done analogously for 5 fields of view at an objective-magnification of × 20 for each group. Thereby, semi-quantitative data was gained, allowing for a proportional comparison of FGFR 1 or FGFR2 positivity to the control group, which was normalized to 100% (B).

II. 2. 3. 6. Luminex® assay for cytokine secretion

In the cell culture experiments to obtain cell lysate for RT-qPCR and Western blot, the cell supernatants containing substances excreted by the cells, were collected after centrifugation and stored for further analysis. These supernatants were analyzed with Luminex® assay kits by Biomedica Medizinprodukte GmbH, Vienna, Austria.

II. 2. 3. 6. a. Principle and protocol of magnetic bead based multiplex assay

Multiplex assays are based on magnetic microparticles that have been coated with specific antibodies for the cytokine of interest and can be detected by their light emittance. For a summary see caption of Figure 20:

FIGURE 20: PRINCIPLE OF LUMINEX MAGNETIC BEAD ASSAY

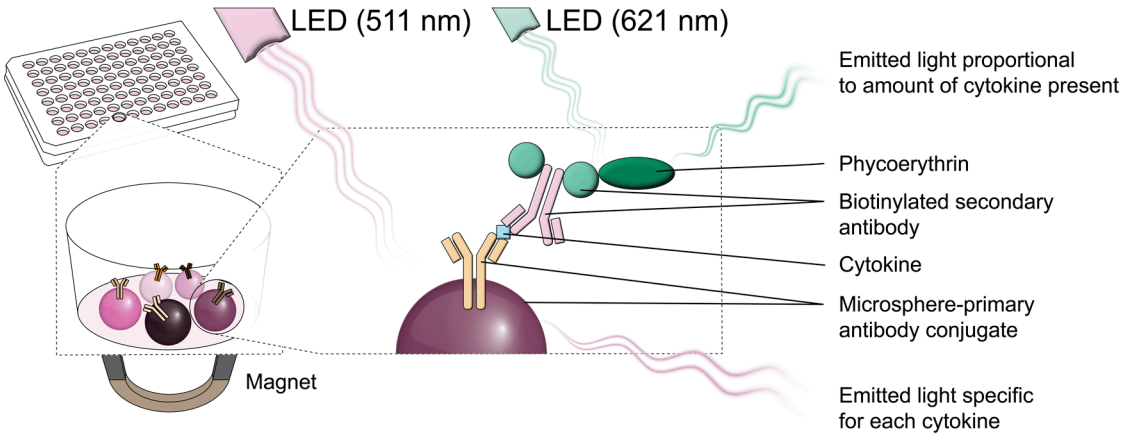

Figure 20 shows the mechanism applied in a Luminex® magnetic bead assay.
The microparticles are produced as microspheres containing a specific dye in their polystyrene core (red to infrared fluorophores) and a magnetite layer (Reslova et al., 2017). Specific antibodies were conjugated to the microspheres. The microspheres were combined with the supernatant and incubated in a sealed microplate for 2 hours at room temperature, including standards.
After incubation, the plate was placed on a magnetic plate to hold the beads at the bottom of the plate wells. Washings were performed to remove unbound material. Samples were then incubated with biotinylated secondary antibodies for 1 hour at room temperature. Washing was performed as described above. A streptavidin-phycoerythrin solution was then added and incubated for 30 minutes at room temperature.
A magnet aligns them in a monolayer and the plates can now be imaged. Two different measurements were made: First, the beads were illuminated by a light-emitting diode at 621 nm to identify them by their specific dye.
Second, another light-emitting diode (511 nm) determined the amount of bound cytokine, which was detected according to the amount of phycoerythrin present. Repeated measurements were made for each target cytokine and the amount of light emitted by phycoerythrin in a Luminex® CCD imager.
The antibody/bead conjugates included in the Premixed Multi-Analyte Kit are listed in Table 16.

II. 2. 3. 7. ELISA

Supernatant collected as described in II. 2. 3. 6 was used for analysis in ELISA kits by Biomedica Medizinprodukte GmbH, Vienna, Austria.

II. 2. 3. 7. a. Principle and protocol of ELISA

ELISA sandwich enzyme immunoassays are based on pre-coated well plates with specific antibodies for the cytokine of interest and can be detected by their light emittance similar to the mechanism of the Luminex® assays (see II. 2. 3. 6). The supernatant was applied to these pre-coated microplate wells and incubated. After incubation, the plate wells were washed so that only bound antigen-antibody-conjugates reside and unbound material is removed. Next, samples were incubated with secondary HRP-Streptavidin conjugated antibodies. Washing followed as described above; a tetramethylbenzidine solution was then added and the color change of tetramethylbenzidine is detectable in a microplate reader at 450 nm (analogous to II. 2. 3. 1).

II. 2. 4. Statistics and graphing

GraphPad (see Table 19) was used for statistical analysis. Graphs were prepared using GraphPad and processed in Adobe Illustrator. Some Western blot bands were scaled to better fit their respective frames, however, no manipulations in histogram or contrast/brightness were applied. If not declared otherwise, relative values are shown (in comparison to the control group, which was normalized to 1.0 or 100%).

II. 2. 4. 1. Cell culture

Each cell culture method experiment (cytotoxicity, proliferation, RNA extraction, protein extraction, ICC) was biologically replicated three times. Each method was technically replicated three to four times. First, ANOVA was performed with a post-hoc Tukey test. For non-normally distributed data (according to the Shapiro-Wilk test), a Kruskal-Wallis test with a post-hoc Dunn's test was used. *P*-values are marked as shown in Table 22.

II. 2. 4. 2. Animal experiments

Treatment and analysis were performed in a blinded fashion, and all mice and samples were included in the analysis. There were 2 to 4 mice in each group. A Student's *t*-test was used to calculate significant findings for the animal experiment as it consisted of only one treatment and one vehicle group. *P*-values are marked as shown in Table 22.

TABLE 22: STATISTICAL ANNOTATIONS

ASTERISK	*P*-VALUE	ASTERISK	*P*-VALUE	ASTERISK	*P*-VALUE
***T*-TEST (ANIMAL EXPERIMENTS) / TUKEY (CELL CULTURE)**					
*	$p < 0.05$	**	$p < 0.01$	***	$p < 0.001$
KRUSKAL-WALLIS/DUNN'S TEST (CELL CULTURE)					
#	$p < 0.05$	##	$p < 0.01$	###	$p < 0.001$

All values shown as mean. Error bars are displaying the **95%-confidence** interval, if calculable.

Table 22 shows the labels/asterisks displayed in this work marking statistically significant results.

III. RESULTS

III. 1. *IN VITRO* DATA

The results of the cell culture experiment in this section are coded according to the key shown in Table 21.
Data and results from methods (or parts of methods) below the lower sensitivity threshold are not depicted (e.g. Luminex® assays). Unless clearly stated otherwise, the implicit comparison is always to the control group.

III. 1. 1. Jurkat and SIM-A9 cells excreted few cytokines

Sensitive Luminex® assays were deployed to compare the excretion of cytokines and growth factors following treatment with FGFR inhibitors or FGF2. However, the levels of secreted cytokines (see Table 16) stayed below the sensitivity of the employed kits, thus rendering them undetectable, with the exception of those shown in Figure 21.

FIGURE 21: JURKAT CELLS FGF2 AND SIM-A9 CELLS TNF-α SECRETION

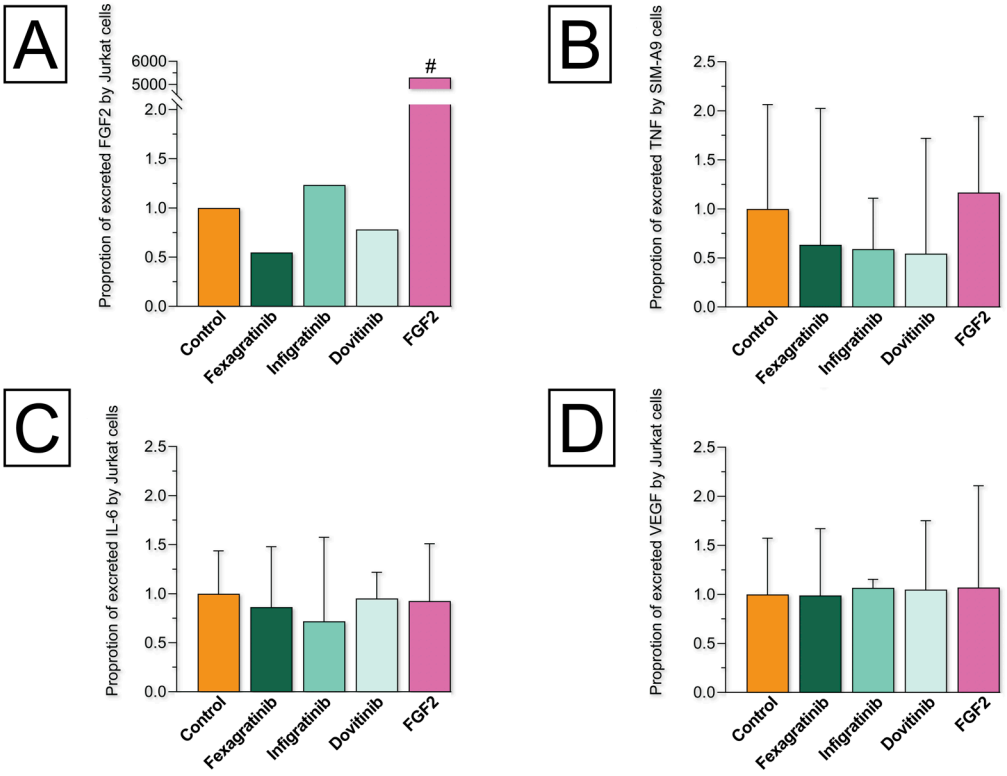

Figure 21: There were no significant changes in the secretion of FGF2 by Jurkat cells following any of the treatments. Due to the low prevalence only one biological replicate returned measurable FGF2 levels. #) FGF2 levels in the FGF2 treated group originated from artificially applied compound (A).
There is no significant effect to TNF-α secretion by treating SIM-A9 cells with any of the compounds (B).
Measurements for IL-6 and VEGF also did not expose differences in cytokine secretion (C-D).
Statistical annotations can be found in Table 22.

III. 1. 2. FGFR inhibitors marginally affected mRNA levels

Semi-quantitative assessment of mRNA levels of key cytokines showed no significant changes after treatment of either cell line with FGFR inhibitors or FGF2 except for *IL21* levels in Jurkat cells (see Figure 22). Fexagratinib resulted in a trend toward decreased *IL21* levels (fexagratinib p = 0.08, fexagratinib vs. dovitinib: p = 0.02). Results are presented in Figure 22 and Figure 23. A general trend toward reduced cytokine levels after treatment with all FGFR inhibitors, but particularly fexagratinib and infigratinib, was observed in Jurkat cells.

FIGURE 22: JURKAT CELL RT-QPCR DATA

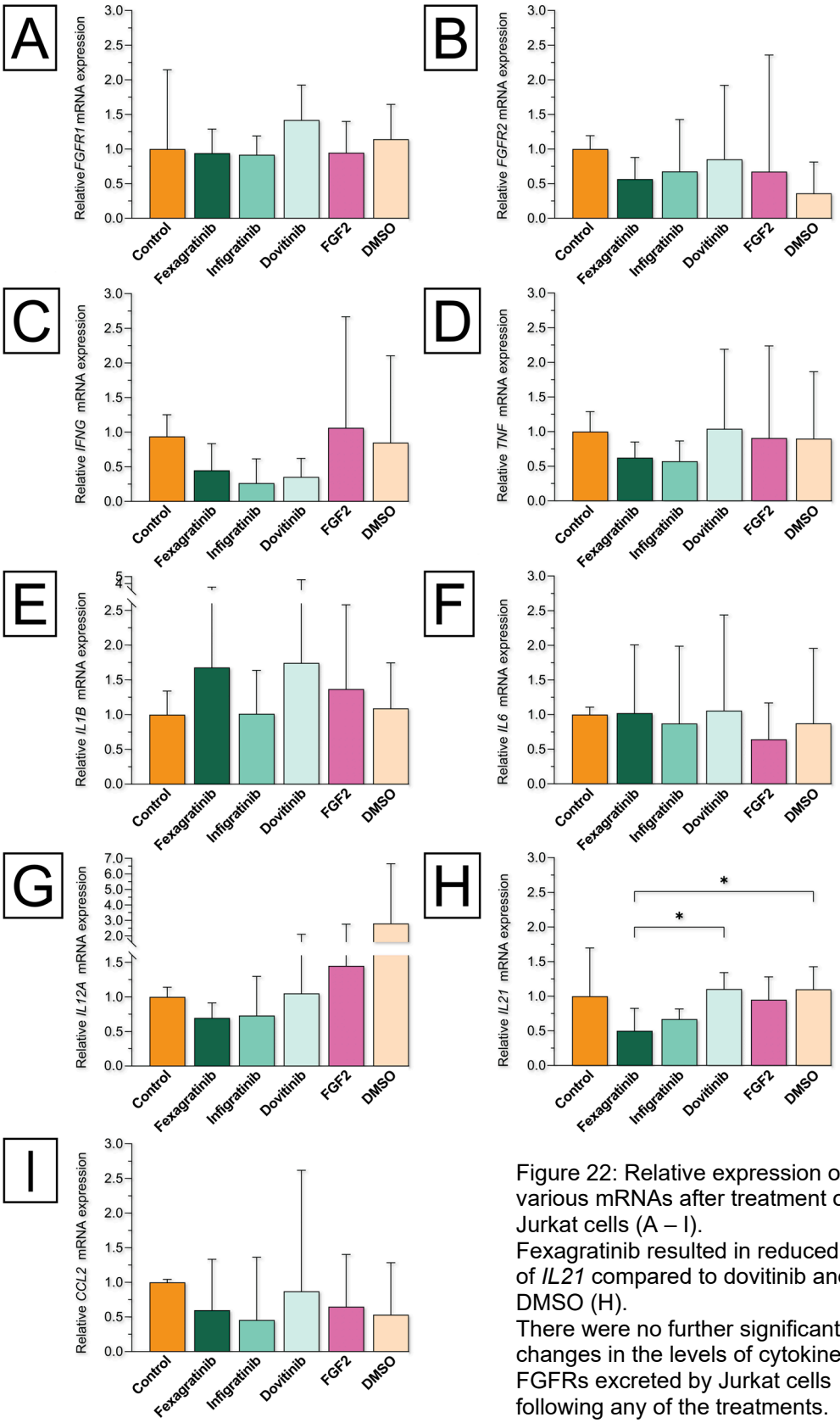

Figure 22: Relative expression of various mRNAs after treatment of Jurkat cells (A – I).
Fexagratinib resulted in reduced levels of *IL21* compared to dovitinib and DMSO (H).
There were no further significant changes in the levels of cytokines or FGFRs excreted by Jurkat cells following any of the treatments.
Statistical annotations can be found in Table 20.

FIGURE 23: SIM-A9 CELL RT-QPCR DATA

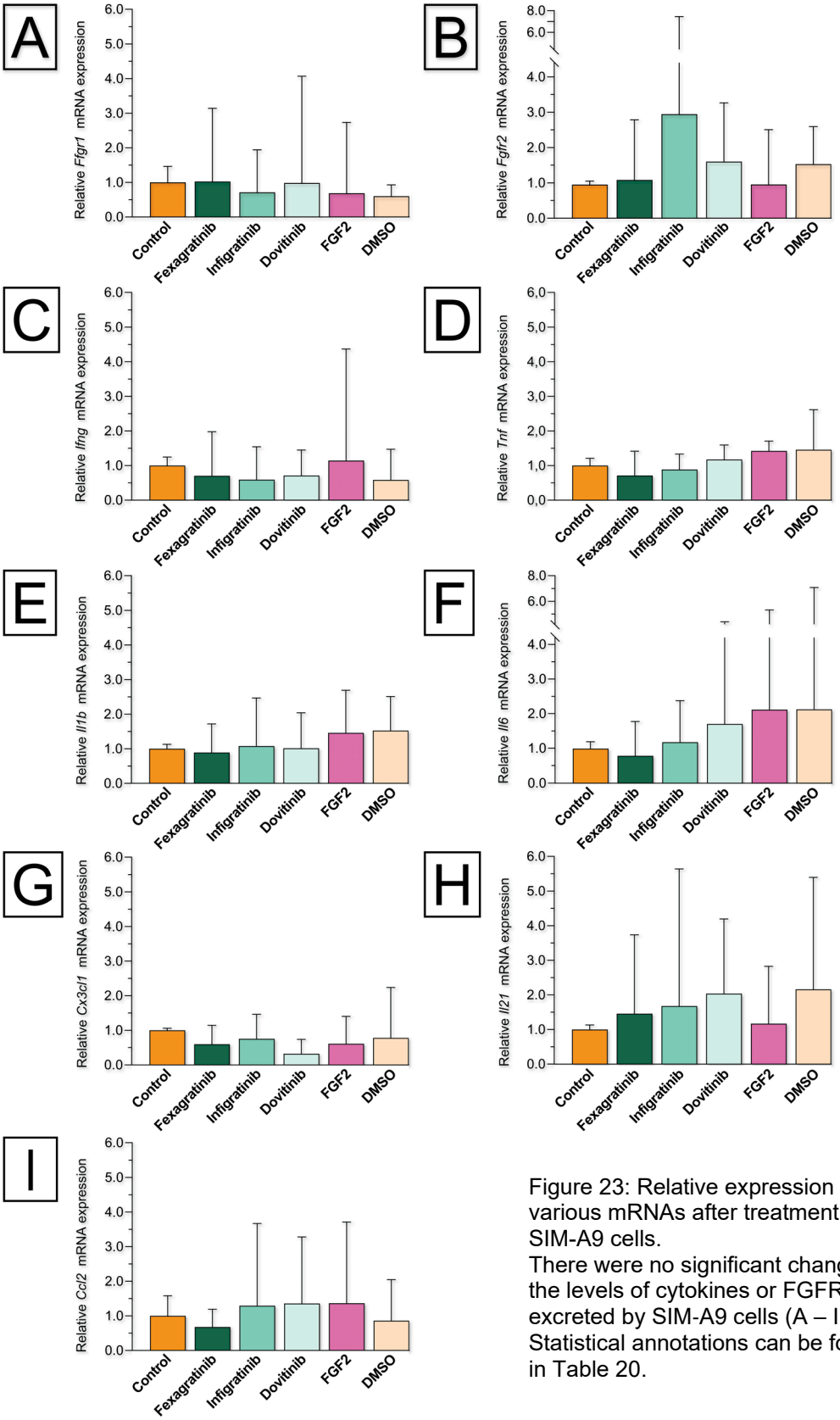

Figure 23: Relative expression of various mRNAs after treatment of SIM-A9 cells.
There were no significant changes in the levels of cytokines or FGFRs excreted by SIM-A9 cells (A – I). Statistical annotations can be found in Table 20.

III. 1. 3. Utilized inhibitors had anti-proliferative but not cytotoxic effects

Figure 24 summarizes the proliferation and cytotoxicity measurements after treatment.

In Jurkat cells, treatment with all inhibitors resulted in reduced proliferation (fexagratinib: $p = 0.005$; infigratinib: $p < 0.001$; dovitinib: $p < 0.001$). The strongest effect was exhibited by dovitinib, followed by infigratinib and fexagratinib. Similarly, a comparison with FGF2 revealed anti-proliferative effects of the inhibitors. However, FGF2 itself did not induce proliferation in Jurkat cells.

In SIM-A9 cells, the picture was more ambiguous. Both fexagratinib and dovitinib reduced proliferation (fexagratinib: $p = 0.04$; dovitinib: $p = 0.01$). Dovitinib showed the strongest effect, followed by fexagratinib. However, infigratinib did not reach significancy (infigratinib: $p = 0.12$). By contrast, in comparison to FGF2, cells treated with infigratinib showed reduced proliferation (infigratinib vs. FGF2: $p < 0.001$). Similar to the Jurkat cells, comparison to FGF2 revealed anti-proliferative effects of the inhibitors. FGF2 itself did not induce proliferation in SIM-A9 cells.

None of the inhibitors, DMSO or FGF2 had cytotoxic effects on either SIM-A9 cells or Jurkat cells.

FIGURE 24: PROLIFERATIVE AND CYTOTOXIC EFFECTS OF THE TREATMENTS

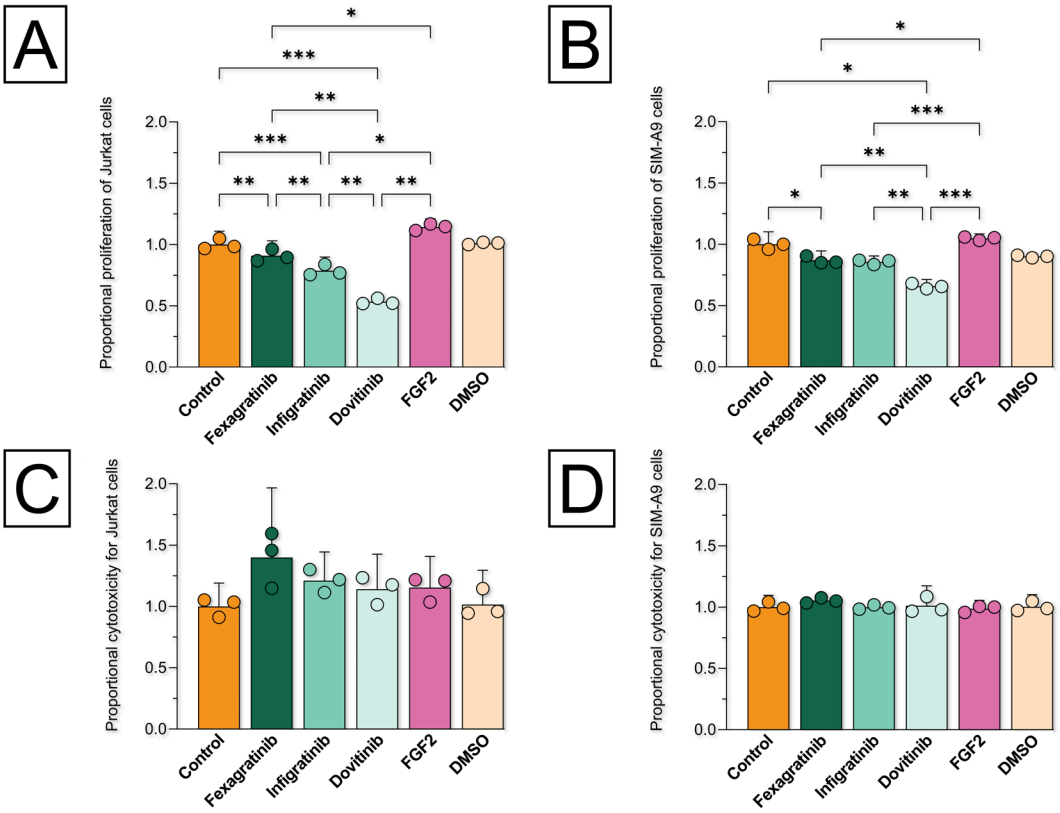

Figure caption of Figure 24 appears on the next page.

Figure 24: All three FGFR inhibitors reduced Jurkat cell proliferation. This applies for both the comparison to the control and FGF2 groups. FGF2 did not induce proliferation (A).
Anti-proliferative effects were observed with fexagratinib and dovitinib in SIM-A9 cells. In addition, infigratinib reduced the proliferation of Jurkat cells compared to FGF2-treated cells (B).
None of the inhibitors or FGF2 had cytotoxic effects on any of the cell lines (C-D).
Pairwise comparisons (asterisks) with DMSO are removed for a more comprehensive view. Statistical notes can be found in Table 22.

III. 1. 4. Expression of FGFRs was distinctly affected by their inhibition

FGFR expression was measured by Western blot of whole cell lysate and by ICC. The expression of FGFRs varied depending on cell type and inhibitor.

Generally, FGFR1 tends to be downregulated by infigratinib in Jurkat cells. In SIM-A9 cells, FGFR2 tends to be downregulated by fexagratinib, with similar trends for infigratinib and dovitinib.

III. 1. 4. 1. FGFR1

Fewer FGFR1$^+$ Jurkat cells were detectable *via* ICC in the infigratinib group (infigratinib: $p = 0.02$). In contrast, Western blot analysis did not reveal a change of FGFR1 in the lysate. Data are presented in Figure 25.

SIM-A9 cells' FGFR1 expression in the ICC was not affected by treatment with any of the inhibitors or FGF2 (DMSO: $p = 0.25$; infigratinib vs. DMSO: $p = 0.01$). Data are presented in Figure 26.

III. 1. 4. 2. FGFR2

In contrast to FGFR1, the number of FGFR2$^+$ Jurkat cells was not lower in the infigratinib group (infigratinib: $p = 0.22$). In addition, Western blot analysis showed that infigratinib increased the expression of FGFR2 in these cells (infigratinib: $p = 0.002$). However, DMSO may have affected FGFR2 expression in Jurkat cells (DMSO: $p = 0.01$).
No significant influence of FGF2 on FGFR2 expression was measurable. Data are presented in Figure 27.

FGFR2$^+$ SIM-A9 cell numbers were reduced by treatment with fexagratinib. A strong trend towards similar levels was noticed after treatment with infigratinib or dovitinib (infigratinib: $p = 0.05$; dovitinib: $p = 0.06$). Again, this was not reflected on protein expression measured by Western blot. Data are presented in Figure 28.

FIGURE 25: JURKAT CELLS FGFR1 EXPRESSION

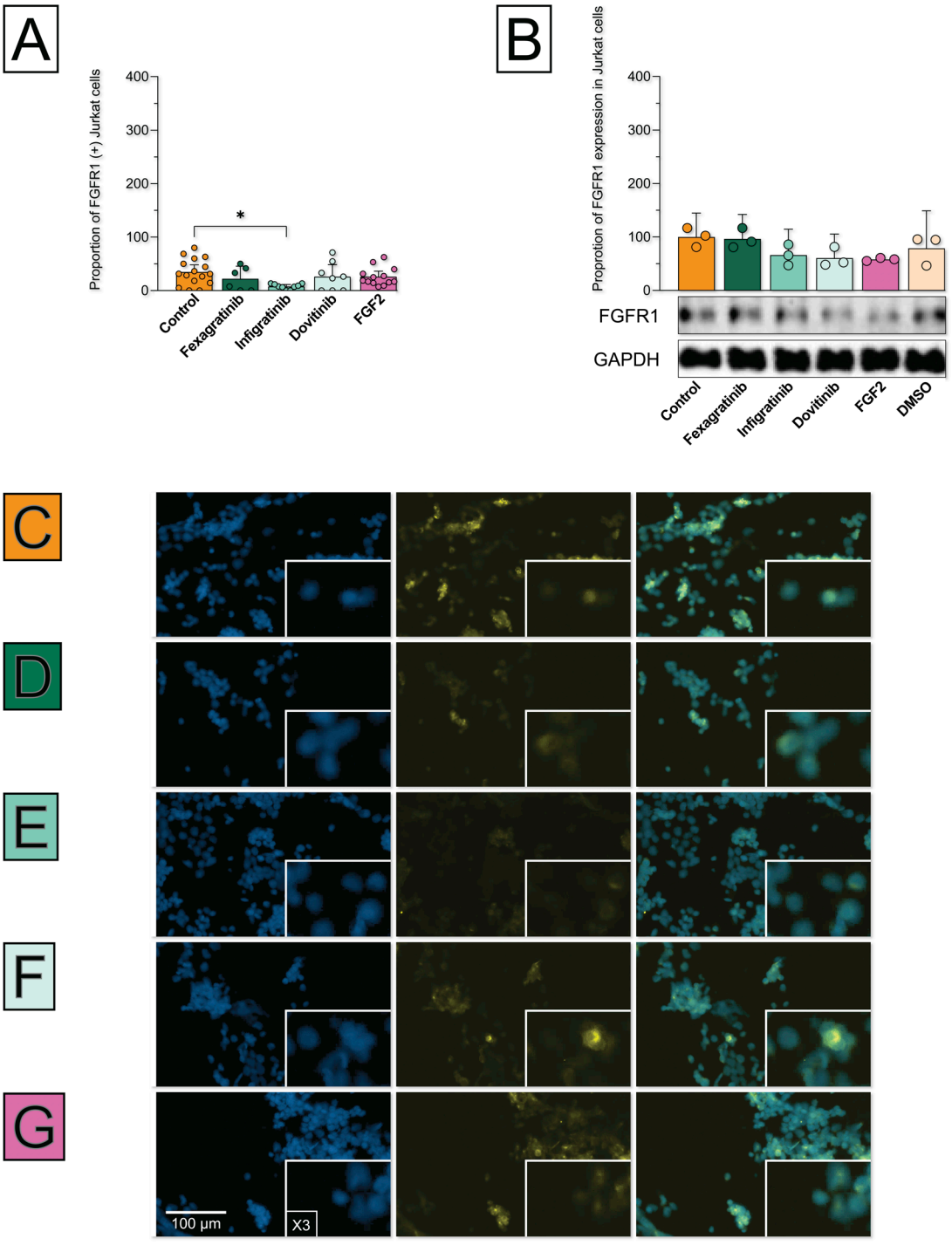

Figure 25: ICC stainings revealed a reduction in FGFR1⁺ cell numbers after treating with infigratinib (A).
Western blot data showed no difference between groups. Representative bands are shown (B).
Representative ICC images are displayed in (C-G), exemplary cell bodies are shown enlarged (inserts, × 3).
Statistical annotations can be found in Table 22.

FIGURE 26: SIM-A9 CELLS FGFR1 EXPRESSION

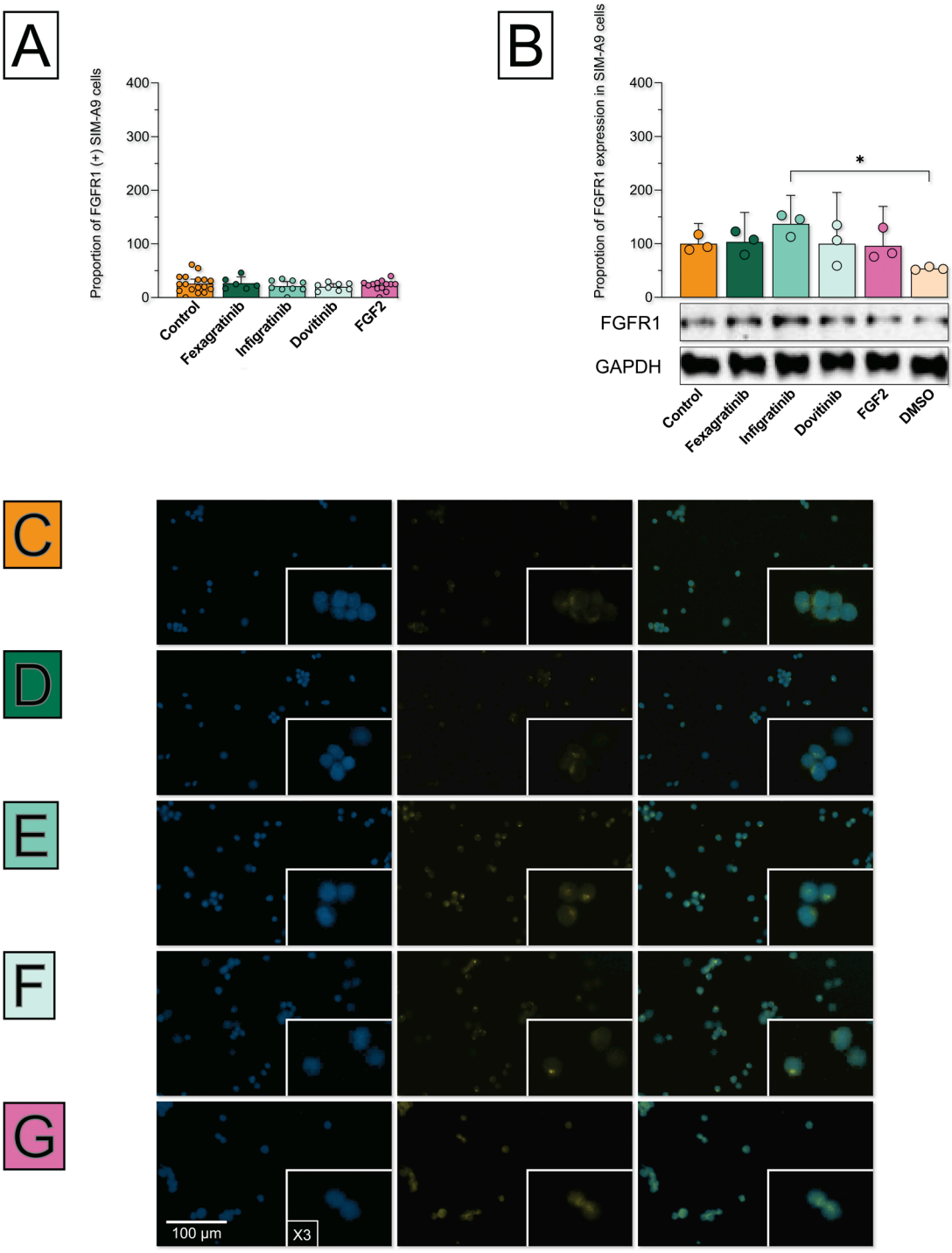

Figure 26: No differences were detected in the ICC staining of FGFR1 in SIM-A9 cells for any treatment (A).
Infigratinib showed a trend towards higher expression of FGFR1 (B).
Representative ICC images are displayed in (C-G), exemplary cell bodies are shown enlarged (inserts, × 3).
Statistical annotations can be found in Table 22.

FIGURE 27: JURKAT CELLS FGFR2 EXPRESSION

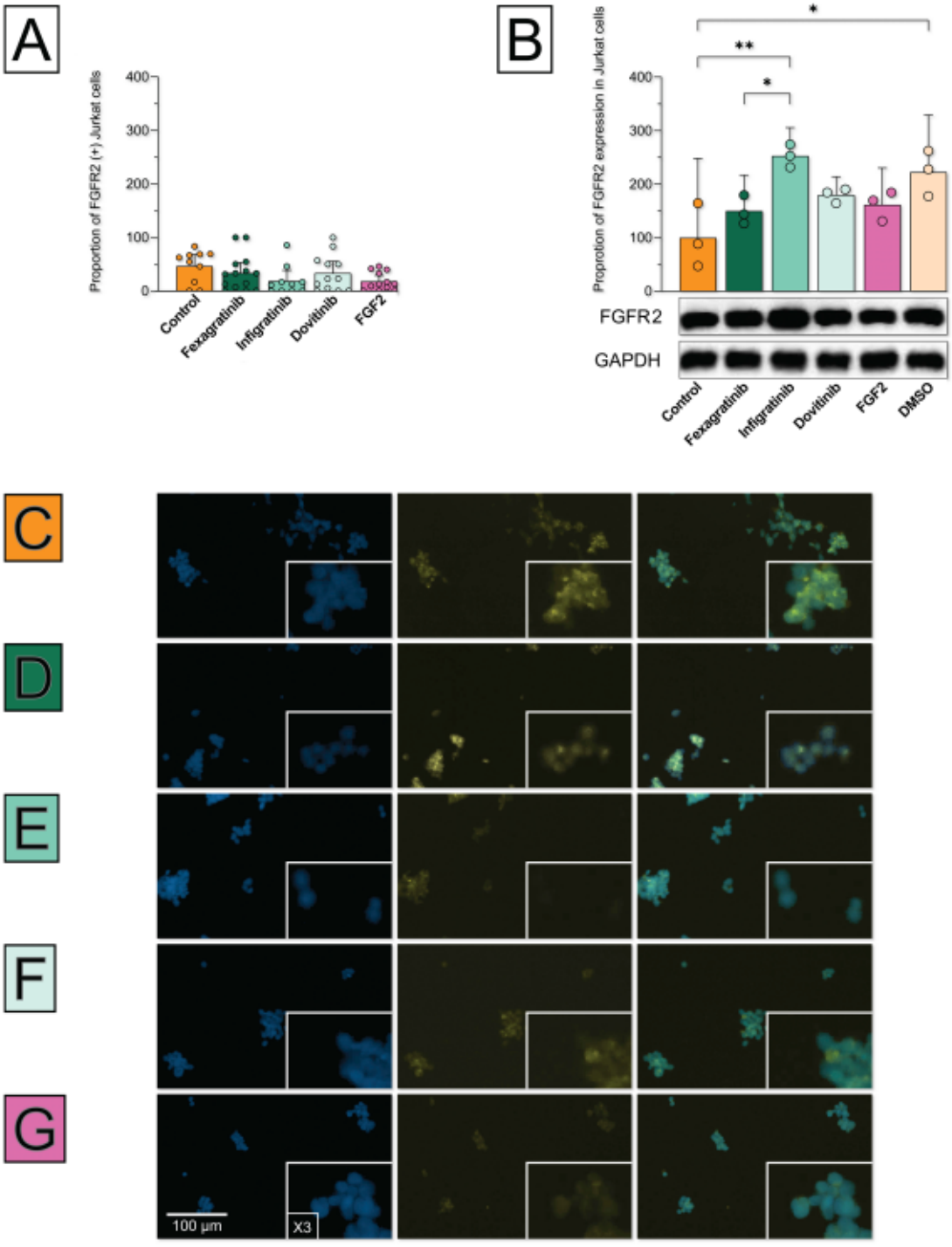

Figure 27: No differences were detected in the ICC staining of FGFR2 in Jurkat cells for any treatment (A).
DMSO affected FGFR2 expression in Jurkat cells. Infigratinib increased FGFR2 expression (B).
Representative ICC images are displayed in (C-G), exemplary cell bodies are shown enlarged (inserts, x 3).
Statistical annotations can be found in Table 22.

FIGURE 28: SIM-A9 CELLS FGFR2 EXPRESSION
ex

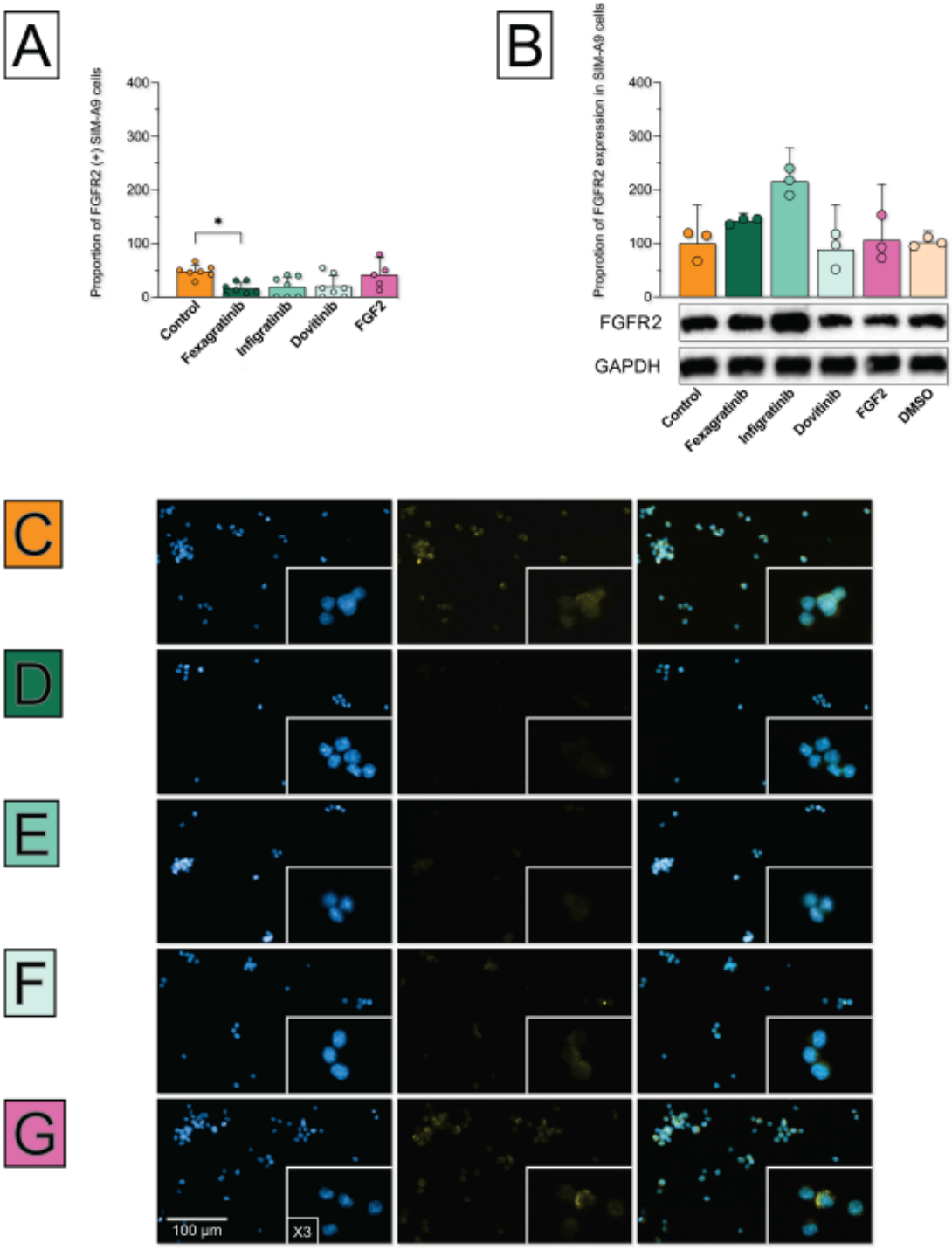

Figure 28: Fexagratinib reduced the number of FGFR2⁺ SIM-A9 cells (A).
No differences could be detected in Western blot analysis of FGFR2 expression (B).
Representative ICC images are displayed in (C-G), exemplary cell bodies are shown enlarged (inserts, x 3).
Statistical annotations can be found in Table 22.

III. 1. 5. Downstream signaling effects depended on specific setting

To evaluate the influence of FGFR inhibitors on downstream singaling molecules of the receptor, the phosphorylation status of ERK and Akt were measured by Western blot. FGFR inhibitors had differential effects depending on the cell line and the inhbitiors. Jurkat cells treated with dovitinib tended to have lower phosphorylation levels of both main downstream targets of FGFR1 and FGFR2. In SIM-A9 cells, however, both dovitinib and FGF2 reduced the phosphorylation of Akt.

III. 1. 5. 1. Dovitinib resulted in trends of reduced phosphorylation of ERK and Akt in Jurkat cells

Jurkat cells showed little response to inhibition or stimulation of FGFR in their downstream molecules. Only dovitinib exerted a significant impact on ERK (dovitinib: p = 0.05; dovitinib vs. fexagratinib: p = 0.01) and Akt phosphorylation (dovitinib vs. infigratinib: p = 0.09; dovitinib vs. FGF2: p = 0.02). However, there was no effect of either FGFR inhibitors, nor did FGF2 induce a significant increase in downstream signaling (trend to increase, FGF2: p = 0.27). Data are presented in Figure 29:

FIGURE 29: JURKAT CELLS' DOWNSTREAM SIGNALING

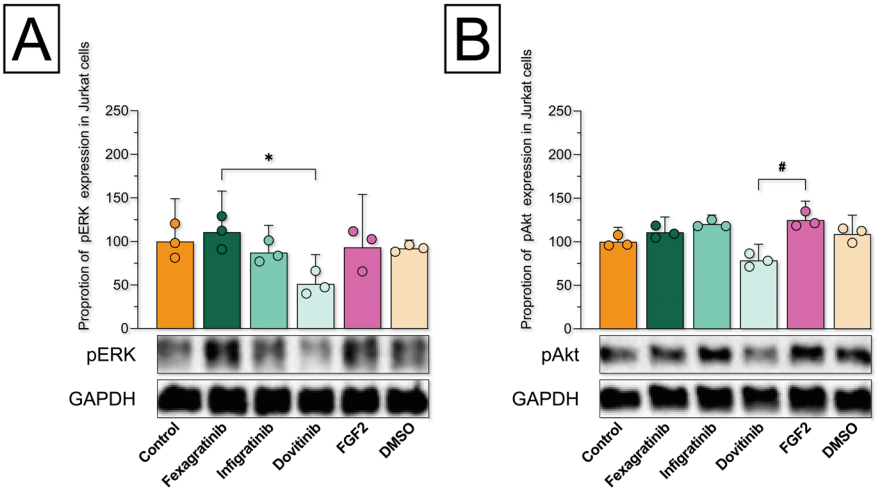

Figure 29: Western blot analysis of pERK and pAkt in Jurkat cells. Of all FGFR inhibitors, only dovitinib compared to fexagratinib or FGF2 resulted in decreased downstream signaling of ERK or Akt. (A, B, respectively) .
Statistical annotations can be found in Table 22.

III. 1. 5. 2. Dovitinib and FGF2 reduced phosphorylation of Akt in SIM-A9 cells

pERK expression was not affected by inhibition or activation of FGFRs in SIM-A9 cells. Dovitinib strongly reduced the expression of phosphorylated Akt in SIM-A9 cells. Surprisingly, FGF2 had a similar effect on SIM-A9 cells:

FIGURE 30: SIM-A9 CELLS' DOWNSTREAM SIGNALING

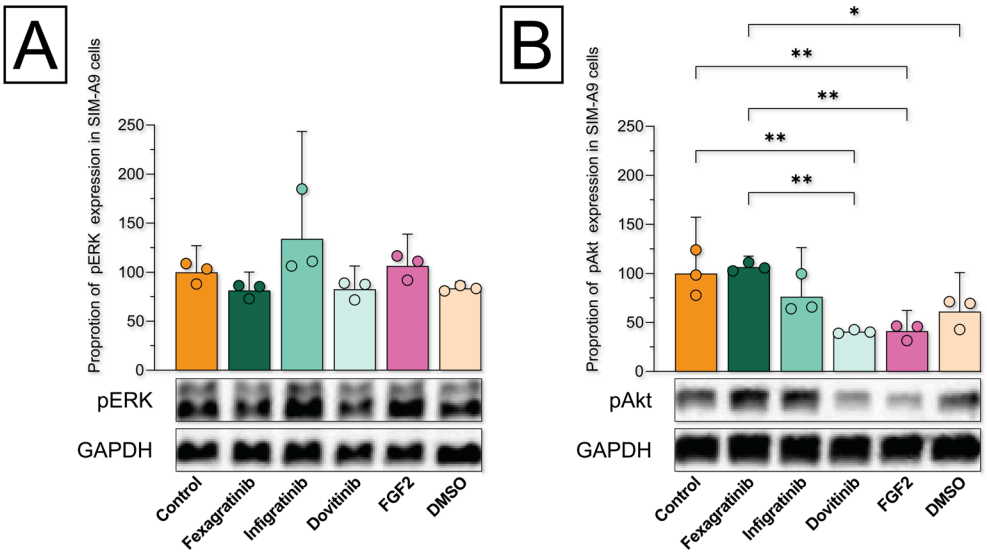

Figure 30: Western blot analysis of pERK and pAkt in SIM-A9 cells.
Phosphorylation of ERK were not affected by any of the treatments in SIM-A9 cells (A).
Akt on the contrary, was activated less after treatment of SIM-A9 cells with dovitinib. The same applied to the FGF2 treatment group (B).
Statistical annotations can be found in Table 22.

III. 1. 6. Cytokine expression varied only marginally after FGFR inhibition

To assess the activation of Jurkat or SIM-A9 cells, cytokines and/or their phosphorylation pattern were measured by Western blot.

In general, few effects were observed in SIM-A9 cells, except for infigratinib, which led to an increase of IL-1β and pP38 compared to some groups. In Jurkat cells, there was no influence on the expression or phosphorylation of the cytokines analyzed, except for pP38, which was up-regulated by the application of dovitinib.

III. 1. 6. 1. Treatment of Jurkat cells with dovitinib mildly induced IL-1β, pP38

With respect to IL-1β, Jurkat cells showed no significant effect of FGFR inhibition or stimulation (dovitinib: $p = 0.19$; dovitinib vs. fexagratinib: $p = 0.24$). pP38 expression was induced by dovitinib (dovitinib: $p = 0.02$; infigratinib: $p = 0.14$).

c-Jun N-terminal kinase (JNK) phosphorylation was not affected by any treatment in Jurkat cells, which was also true for pSTAT3. Data are shown in Figure 31:

FIGURE 31: JURKAT CELLS' CYTOKINE EXPRESSION

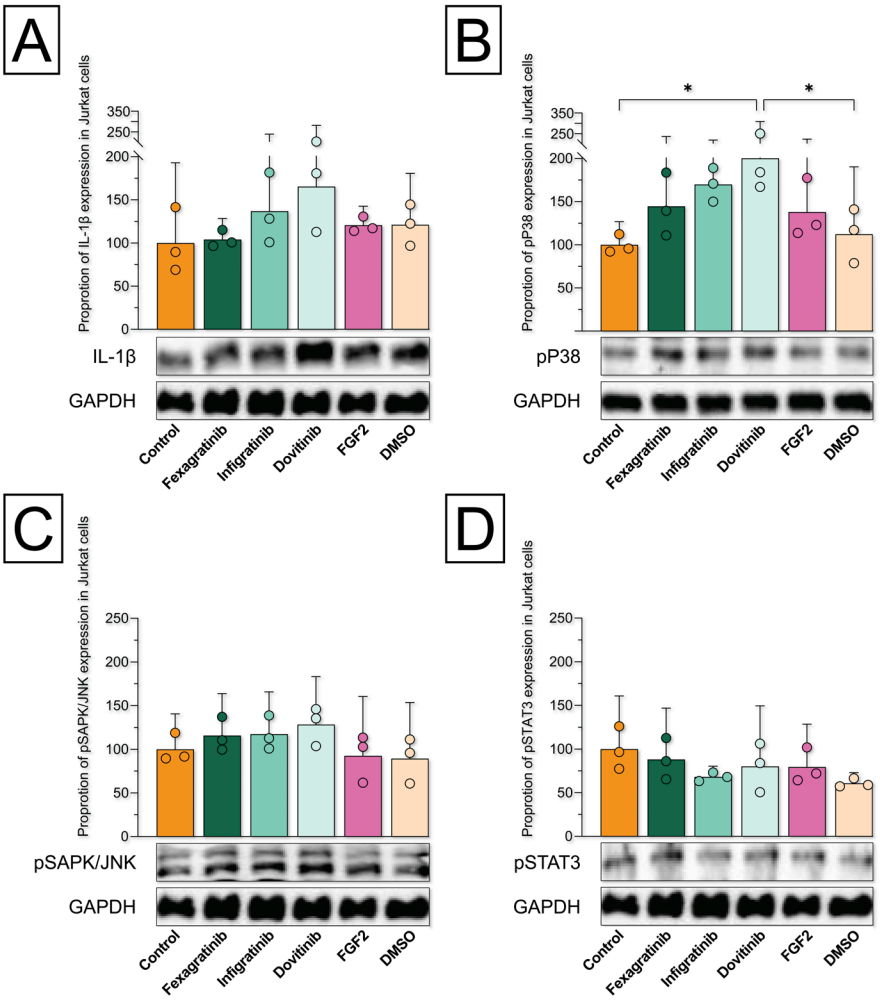

Figure 31: Protein expression of various cytokines in Jurkat cells. There was no effect of treatment to the IL-1β expression (A).
Phosphorylated P38 was more strongly expressed after treating Jurkat cells with dovitinib, the other inhibitors trended towards increased pP38 expression (B).
pSAP and pSTAT3 expression remained unchanged after treating the cells (C-D).
No effect was seen by FGF2 on Jurkat cells regarding their cytokine expression (A-D).
Statistical annotations can be found in Table 22.

III. 1. 6. 2. Infigratinib showed trends to induce IL-1β, pP38, pSTAT3 in SIM-A9 cells

SIM-A9 cells treated with infigratinib expressed more IL-1β than cells treated with the solvent DMSO alone (infigratinib vs DMSO: p = 0.009). However, no significance was found when compared to the control. Consistently, pP38 expression was induced by infigratinib compared to FGF2 (infigratinib: p = 0.40; infigratinib vs FGF2: p = 0.03). JNK phosphorylation was not significantly reduced by fexagratinib compared to the cells treated with FGF2 (fexagratinib vs. FGF2: p = 0.17). For pSTAT3, no significant effects

were observed with any of the compounds (infigratinib vs. dovitinib: $p = 0.12$; infigratinib vs. DMSO: $p = 0.20$; dovitinib vs. FGF2: $p = 0.16$). Neither FGFR inhibition nor activation influenced TNF-α expression in SIM-A9 cells. Data are presented in Figure 32:

FIGURE 32: SIM-A9 CELLS' CYTOKINE EXPRESSION

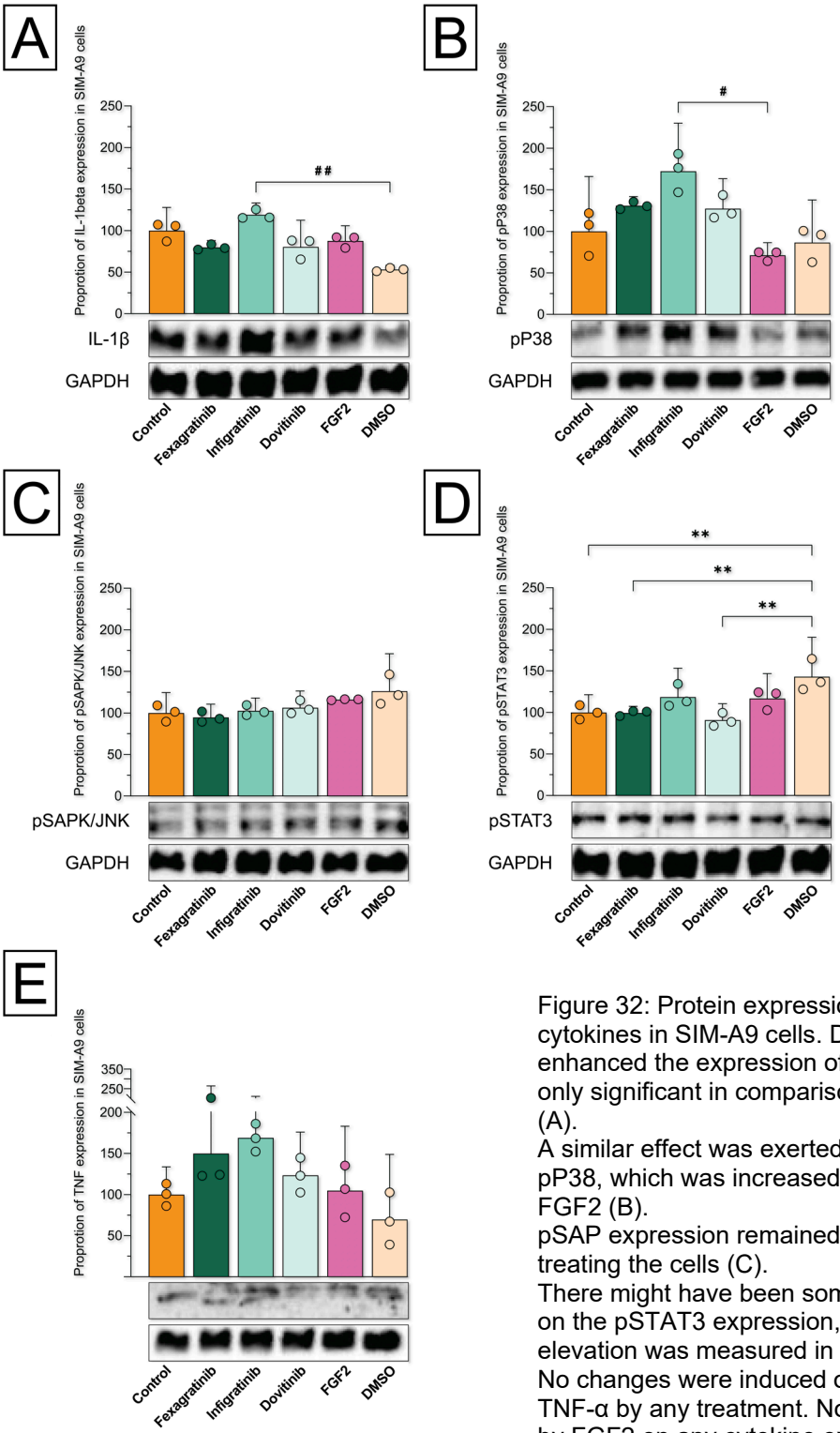

Figure 32: Protein expression of various cytokines in SIM-A9 cells. Dovitinib may have enhanced the expression of IL-1β, which is only significant in comparison to DMSO control (A).
A similar effect was exerted by dovitinib on pP38, which was increased in comparison to FGF2 (B).
pSAP expression remained unchanged after treating the cells (C).
There might have been some effect of DMSO on the pSTAT3 expression, of which an elevation was measured in Western blot (D).
No changes were induced on the expression of TNF-α by any treatment. No effect was seen by FGF2 on any cytokine expression (A-E).
Statistical annotations can be found in Table 22.

III. 2. FLOW CYTOMETRY DATA

Overall, the peripheral immune system induction initiated by EAE was ameliorated by treating with infigratinib. However, profound effects were only found in the acute phase.

III. 2. 1. Acute phase of EAE

The consequences of infigratinib treatment in the acute phase of EAE differed depending on the treatment approach. In summary, the preventive administration of infigratinib strongly reduced the number of T and B cells, but increased the number of dendritic cells and macrophages/monocytes. When the drug was administered after the onset of symptoms, *i.e.* suppressively, these effects did not occur.

III. 2. 1. 1. Preventive treatment ameliorated immune induction

The effects further varied depending on the compartment of interest. In the spleen of mice treated with infigratinib, immune cells, including T helper and cytotoxic T cells, and B cells were greatly reduced ($CD3^+$: $p < 0.001$; $CD4^+$: $p = 0.034$; $CD8^+$: $p < 0.001$; $CD19^+$: $p = 0.002$). Double positive (DP) T cells did not differ in numbers. In contrast, macrophages/monocytes and dendritic cells were more abundant in the treated mice (monocytes: $p < 0.001$; macrophages: $p < 0.001$; dendritic cells: $p < 0.001$). Figure 33 shows respective data.

In blood of EAE-affected mice of the infigratinib treatment group, both T helper cells and cytotoxic T cells were reduced in numbers ($CD4^+$: $p = 0.001$; $CD8^+$: $p < 0.001$). A trend towards less abundant DP T cells was visible ($CD4^+$&$CD8^+$: $p = 0.076$). Overall T and B cell numbers were not distinguishable. Contrarily, monocytes and dendritic cells were represented in larger numbers, proportionally (monocytes: $p < 0.001$; dendritic cells: $p = 0.011$). Figure 34 shows respective data.

III. 2. 1. 2. Suppressive treatment had few effects immune cell populations

In the spleen of mice that were suppressively treated with infigratinib, only dendritic cell numbers were slightly lower (dendritic cells: $p < 0.001$). The trends observed in the adaptive immune system at this time point were an overall decrease in T cells and a decrease in cytotoxic T cells ($CD3^+$: $p = 0.063$; $CD8^+$: $p = 0.063$). Figure 35 shows respective data.

In blood of mice after suppressive infigratinib treatment, no significant changes manifested, but both cytotoxic T cells and dendritic cells trended towards increased numbers ($CD8^+$: $p = 0.063$; dendritic cells: $p < 0.070$). Figure 36 shows respective data.

FIGURE 33: IMMUNE CELLS IN ACUTE EAE – PREVENTION – SPLEEN

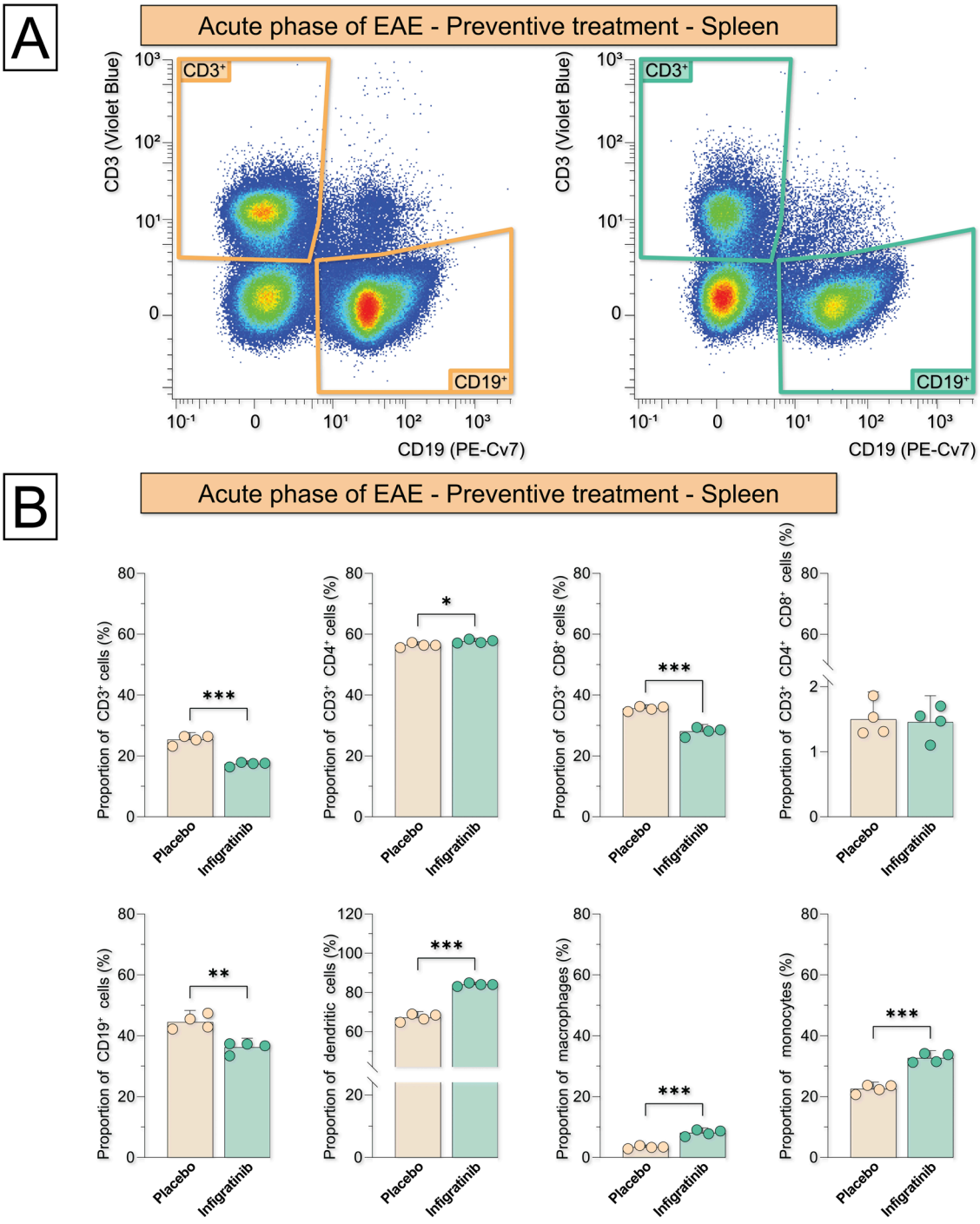

Figure 33: FC data of the spleen for the acute phase of EAE, preventive treatment. Representative plots for $CD3^+$ and $CD19^+$ cells (A).
B cell and T cell (helper and cytotoxic) numbers were proportionally lower in infigratinib-treated mice. Macrophages/monocytes and dendritic cells were proportionally induced (B). Statistical annotations can be found in Table 22.

FIGURE 34: IMMUNE CELLS IN ACUTE EAE – PREVENTION – BLOOD

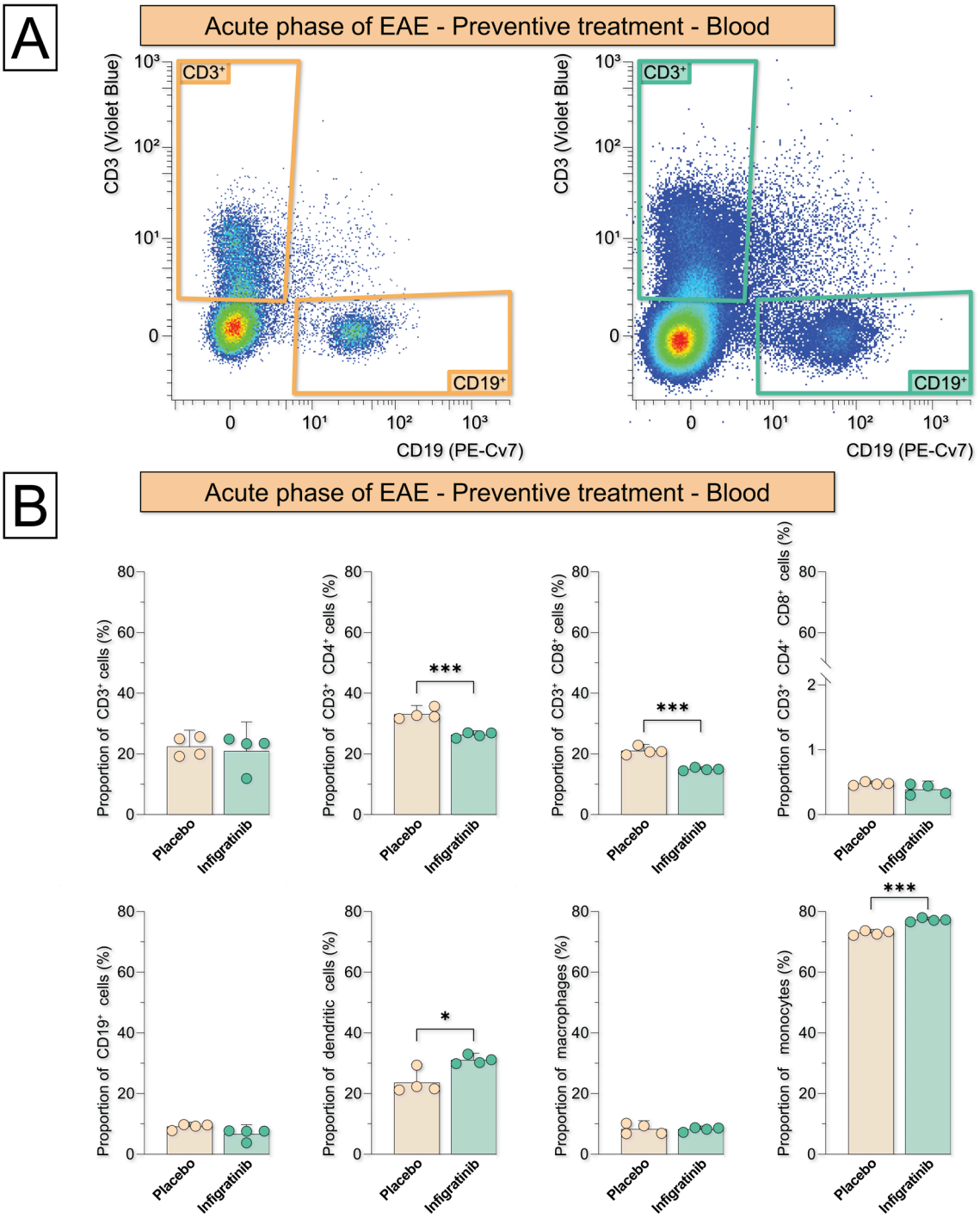

Figure 34: FC data of blood for the acute phase of EAE, preventive treatment. Representative plots for $CD3^+$ and $CD19^+$ cells (A).
Proportions of T helper cells and cytotoxic T cells were reduced in the infigratinib compared to the vehicle group. Monocytes and dendritic cells were proportionally induced by treatment with infigratinib (B).
Statistical annotations can be found in Table 22.

FIGURE 35: IMMUNE CELLS IN ACUTE EAE – SUPPRESSION – SPLEEN

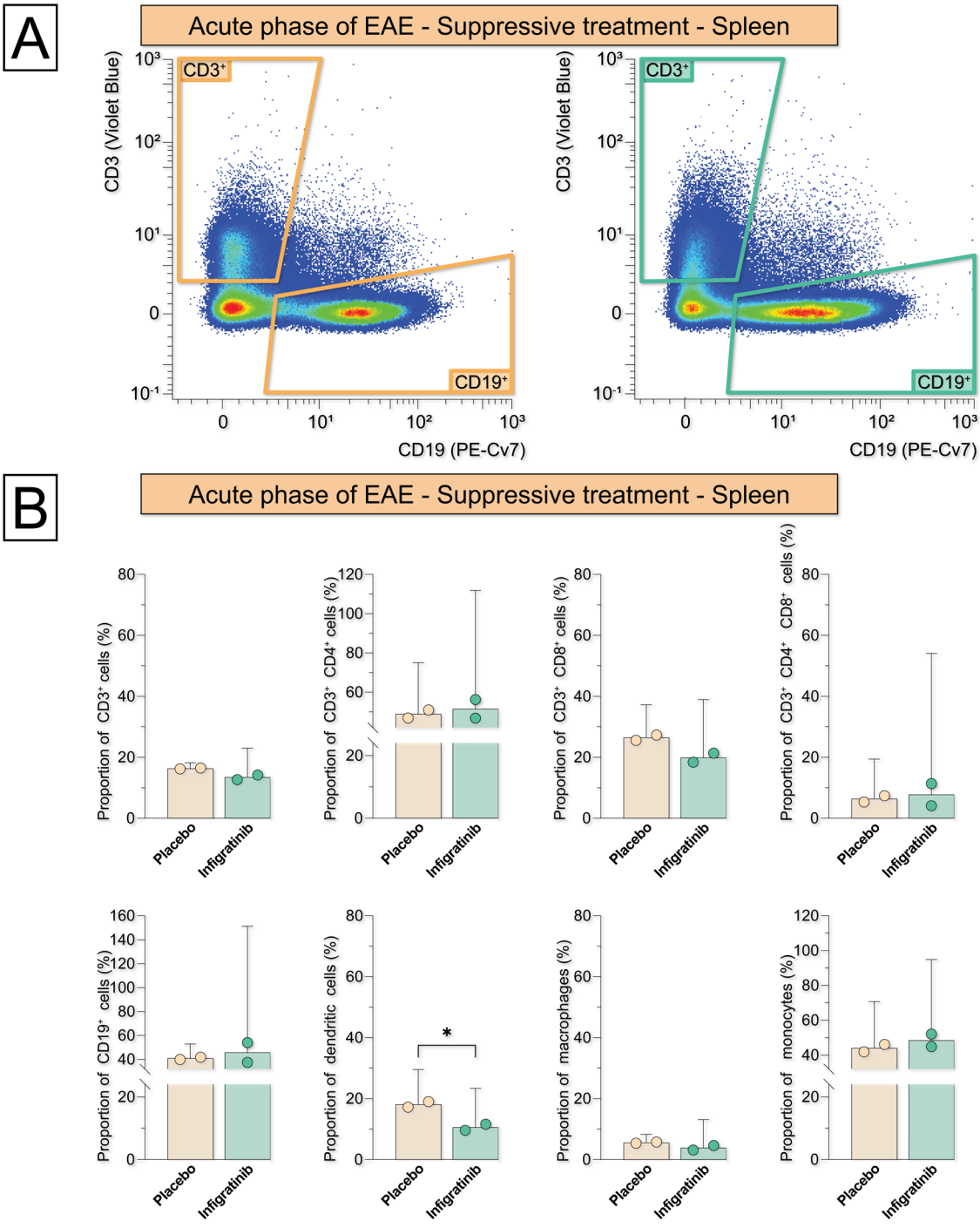

Figure 35: FC data of the spleen for the acute phase of EAE, suppressive treatment. Representative plots for $CD3^+$ and $CD19^+$ cells (A).
Dendritic cells were reduced by treating mice with infigratinib (B).
Statistical annotations can be found in Table 22.

FIGURE 36: IMMUNE CELLS IN ACUTE EAE – SUPPRESSION – BLOOD

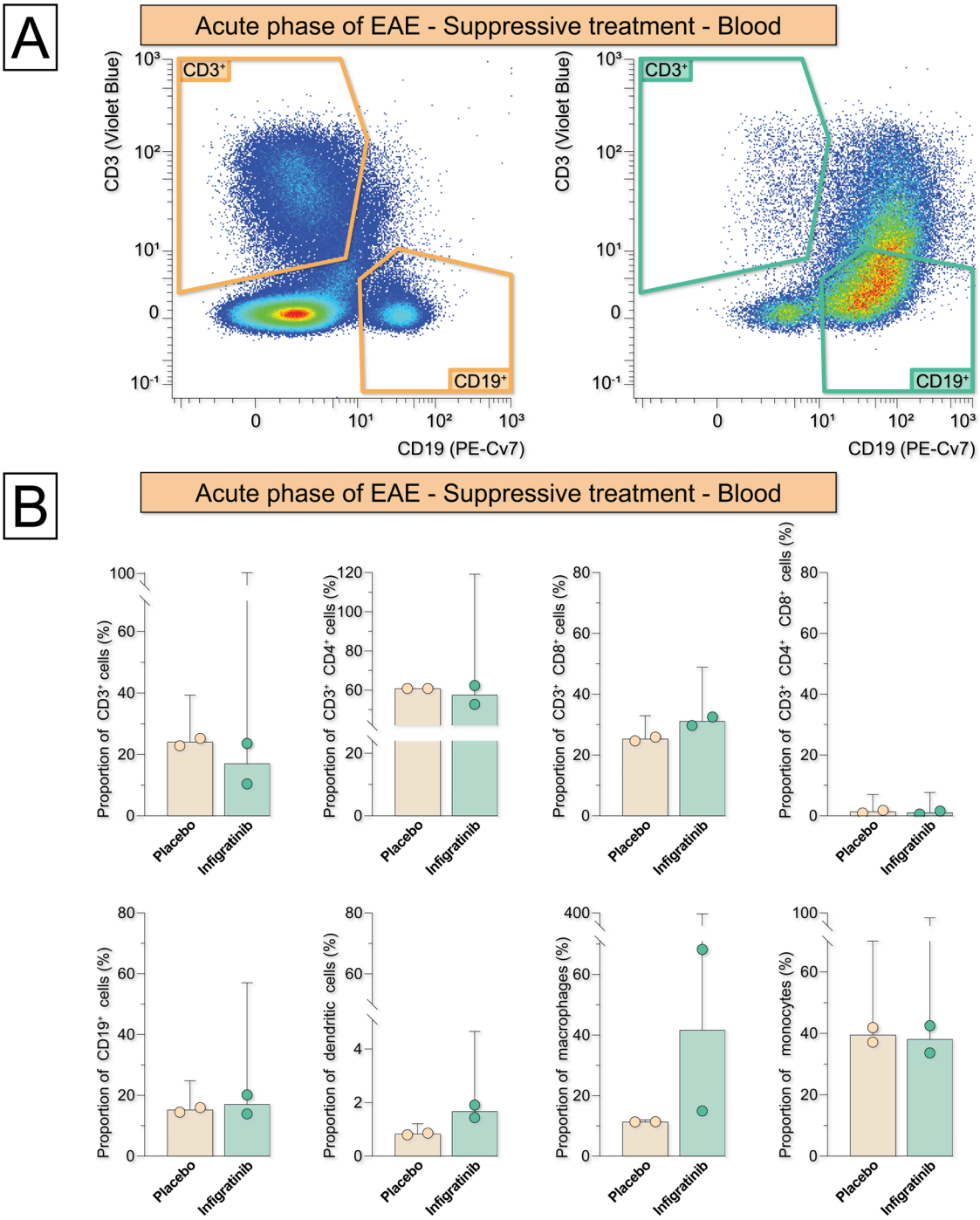

Figure 36: FC data of blood for the acute phase of EAE, suppressive treatment. Representative plots for CD3⁺ and CD19⁺ cells (A).
No proportional differences in cell numbers were exhibited in infigratinib-treated mice compared to the vehicle group in any of the B, T cell, and cells of the innate immune system populations (B).
Statistical annotations can be found in Table 22.

III. 2. 2. Chronic phase of EAE

Contrastingly to the acute phase of EAE, none of the treatment approaches appeared to have an effect on immune cell populations at the later time point in the chronic phase of the disease.

III. 2. 2. 1. Preventive treatment effects were not observable long-term

Figure 37 shows respective data of preventively treated EAE-affected mice with infigratinib in the chronic phase. There were no significant changes in the proportions of any of the cell populations analyzed in the spleen, but trends toward fewer total T cells and DP-T cells were observed ($CD3^+$: $p = 0.052$; $CD4^+\&CD8^+$: $p = 0.065$). Similarly, in blood, as illustrated in Figure 38, no effects were detectable in the proportions of any of the immune cell populations in this disease state.

III. 2. 2. 2. Lasting immunomodulatory effects from suppressive treatment

Consistent with data for preventive treatment in the chronic phase, Figure 39 shows that suppressive infigratinib treatment had no effect in the spleen. A trend was seen in the monocyte population, which was proportionally lower (monocytes: $p = 0.068$).

Unlike the spleen compartment, there were a few exceptions in the blood compartment: As shown in Figure 40, the number of $CD3^+$ cells was increased compared to the vehicle group in EAE-mice in the chronic phase that were suppressively treated with infigratinib ($CD3^+$: $p = 0.015$). Also, macrophages were proportionally induced in the treatment group (macrophages: $p = 0.027$). As in the spleen, monocytes tended to be lower and DP T cells were slightly reduced ($CD4^+\&CD8^+$: $p = 0.099$; monocytes: $p = 0.068$)

FIGURE 37: IMMUNE CELLS IN CHRONIC EAE – PREVENTION – SPLEEN

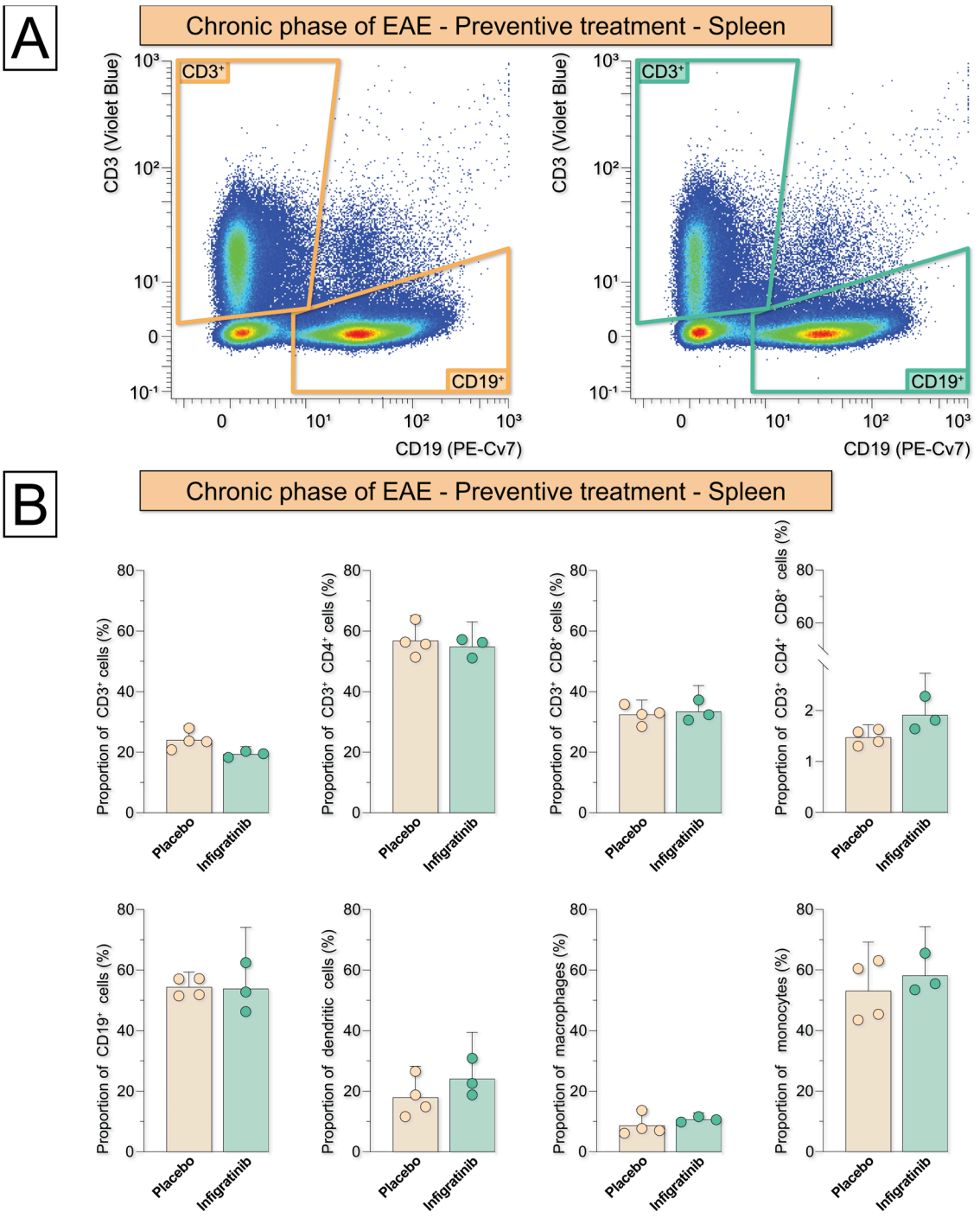

Figure 37: FC data of the spleen for the chronic phase of EAE, preventive treatment. Representative plots for $CD3^+$ and $CD19^+$ cells (A).
No proportional differences in cell numbers were exhibited in infigratinib-treated mice compared to the vehicle group in any of the B, T cell, and cells of the innate immune system populations (B).
Statistical annotations can be found in Table 22.

FIGURE 38: IMMUNE CELLS IN CHRONIC EAE – PREVENTION – BLOOD

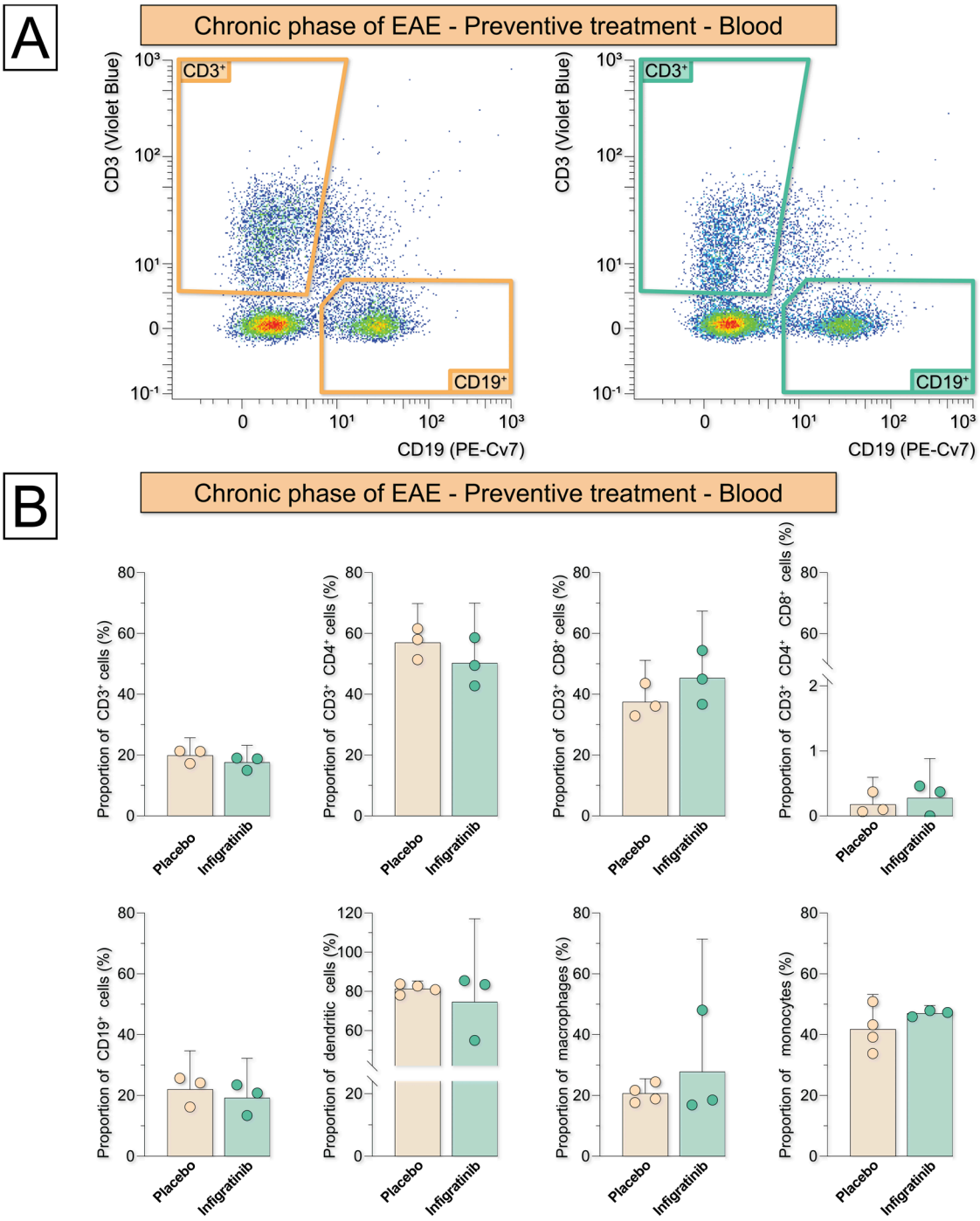

Figure 38: FC data of blood for the chronic phase of EAE, preventive treatment. Representative plots for $CD3^+$ and $CD19^+$ cells (A).
No proportional differences in cell numbers were exhibited in infigratinib-treated mice compared to the vehicle group in any of the B, T cell, and cells of the innate immune system populations (B).
Statistical annotations can be found in Table 22.

FIGURE 39: IMMUNE CELLS IN CHRONIC EAE – SUPPRESSION – SPLEEN

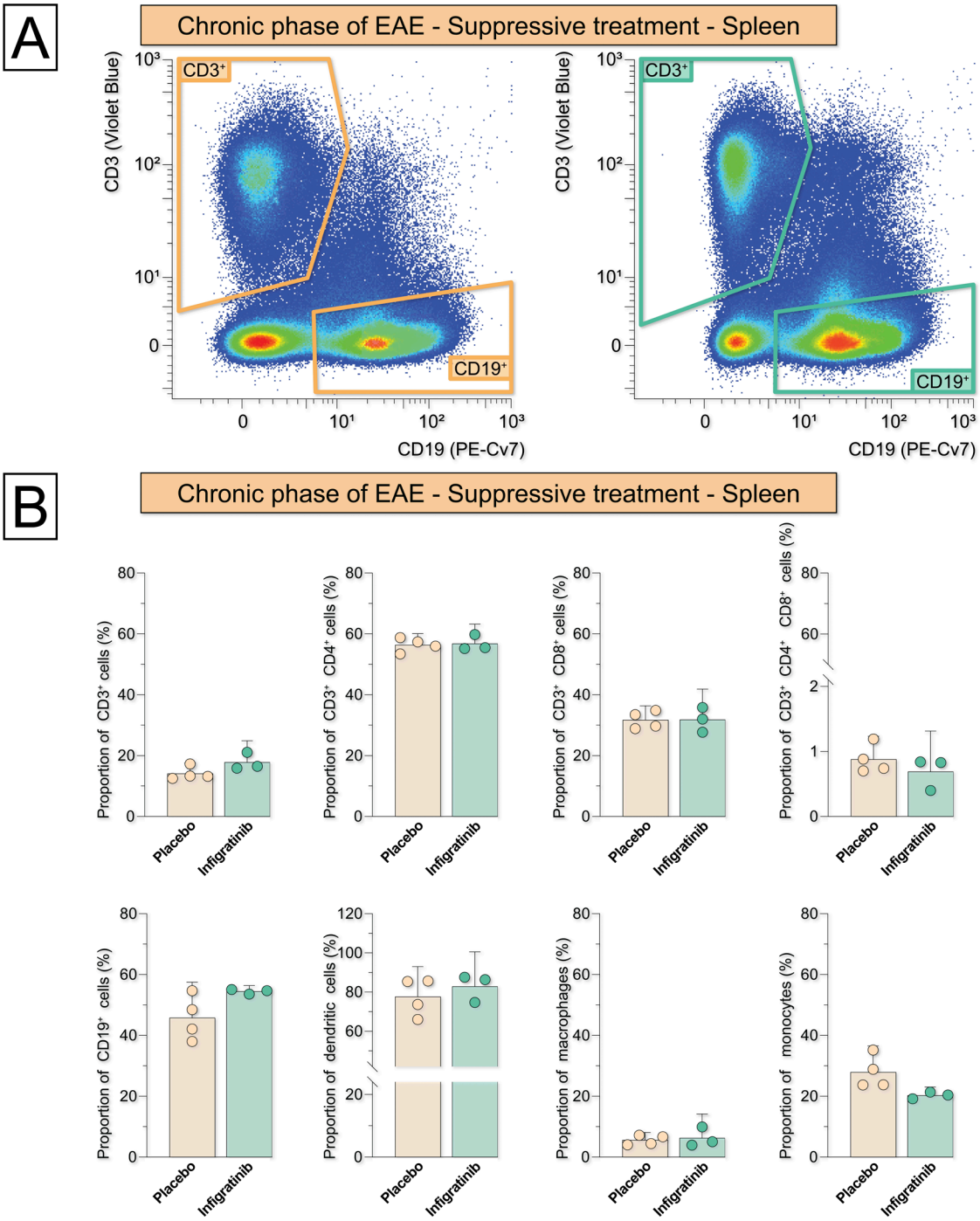

Figure 39: FC data of the spleen for the chronic phase of EAE, suppressive treatment. Representative plots for $CD3^+$ and $CD19^+$ cells (A) .
No proportional differences in cell numbers were exhibited in infigratinib-treated mice compared to the vehicle group in any of the B, T cell, and cells of the innate immune system populations (B).
Statistical annotations can be found in Table 22.

FIGURE 40: IMMUNE CELLS IN CHRONIC EAE – SUPPRESSION – BLOOD

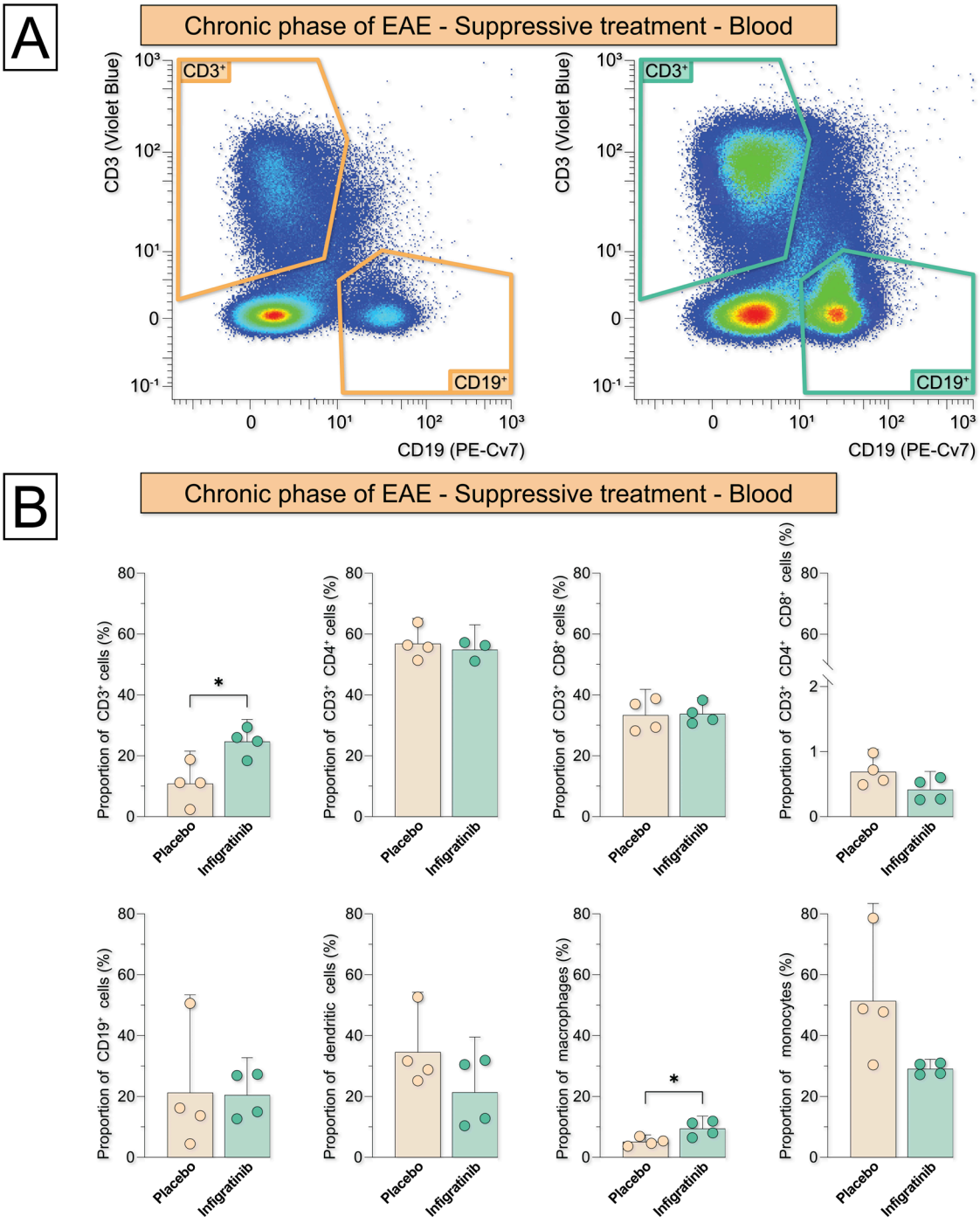

Figure 40: FC data of blood for the chronic phase of EAE, suppressive treatment. Representative plots for $CD3^+$ and $CD19^+$ cells (A).
Overall, T cell numbers were proportionally increased in mice treated with infigratinib compared to those given the vehicle. The same applies to the macrophage populations of the treatment group. No further effects on other cell populations including T cell subpopulations were observable (B).
Statistical annotations can be found in Table 22.

IV. DISCUSSION

IV. 1. SUMMARIZED FINDINGS

The present work investigated the effects of three TKIs on two cell lines and the effect of one of these inhibitors, infigratinib, *in vivo* in an experimental MS model. Thus, a universal summary of the effects of these FGFR inhibitors is arguably not feasible. However, to encapsulate the findings of the experiments described above, it can be said that FGFR inhibition

i) acts anti-proliferative on immune cells (Figures 24, 33, 34),
ii) results in trends to differentially influence cytokine excretion and expression (Figures 22, 23, 31, 32)
iii) lowers FGFR expression only on cell surface (Figures 25, 26, 27, 28),
iv) has cell-specific consequences regarding downstream signal conduction, *e.g.*,
    dovitinib decreases Akt signaling,
    dovitinib reduces ERK signaling in Jurkat but not SIM-A9 cells,
v) acts *via* or influences pP38, particularly in Jurkat cells (Figures 31, 32),
vi) has differential anti-inflammatory effects in EAE regarding immune cell proliferation especially when administered preventively / in the acute phase (Figures 33 - 40).

Taken together, the present work provides a strong rationale for immunomodulatory effects of FGFR inhibition. Furthermore, the data presented here show that FGFR inhibition exerts an anti-inflammatory effect by strongly inhibiting proliferation in the context of an MS-like immune response, and indicate that this translates into anti-inflammatory and modulatory signaling changes in both microglia and T cells. These downstream pathways involve ERK, Akt, and pP38 signaling, depending on the compound and cell type.
In accordance with the hypothesis, the effects of FGFR inhibition were highly dependent on the model, inhibitor, and cell type. Therefore, the diverse specific impacts are described, reflected, and integrated in the following sections.

IV. 2. *IN VITRO* DATA

In an effort to study cell biology and cell-specific pathways, we chose an *in vitro* methodology focused on microglia and T cells. While acknowledging the inherent limitations of this simplification, such as differences in subpopulations, not to mention the

complexity of the lesion microenvironment, it nevertheless allowed for specific insights into singular cell populations and analyses of their response as a cell identity.

IV. 2. 1. Summarized effects of FGFR inhibition in Jurkat cells

Immunological effects on T cells are one of the most significant ones to consider regarding MS and EAE due to their executive role and abundance in lesions (see I. 1. 2 and I. 2. 1. 1. a, also in Fransen et al., 2020; Attfield et al., 2022; Krishnarajah and Becher, 2022). Jurkat cells are $CD4^+$ T cell-derived lymphoblasts and are commonly used to study effects in T cells.

Most importantly, Jurkat cell proliferation was significantly reduced after treatment with each of the three inhibitors. A trend towards a cytotoxic effect on this cell line was observed following the application of both fexagratinib and dovitinib, which coincided with increased pP38 expression for dovitinib but not for fexagratinib – rather a trend towards lower levels was observed with fexagratinib compared to dovitinib. FGF2 induced a weak trend towards proliferation.

Thus, in agreement with our hypothesis, the T cells employed in this study were restrained in their cell growth after application of each of the three inhibitors. Principally, Jurkat cells exhibit exponential growth (Schwenk and Schneider, 1975; Weiss et al., 1984), and, utilization of a growth factor inhibitor can be expected to reduce cell proliferation. However, given the overall tolerance of Jurkat cells to growth-suppressive signals and their non-mutation on *FGFR*, the effects observed here point towards anti-proliferative effects of FGFR inhibition on T cells. In addition, cell surface expression of FGFR1 was significantly reduced upon treatment with infigratinib, with similar trends observed for the other inhibitors as well as for FGFR2. However, total protein expression was not affected – on the contrary, a trend towards higher FGFR2 expression was observed in the Western blots (see IV. 2. 4. 3).

Downstream of FGFR, canonical signal transducers were reduced by dovitinib. This included pERK as well as pAkt when compared to FGF2, which itself induced non-significantly higher pAkt expression. As mentioned above, dovitinib induced increased pP38, a convergent trend was promoted subsequent to employing infigratinib, with the opposite being the case for fexagratinib. Thus, the multikinase inhibitor dovitinib induces the pP38 pathway. Inhibition of this pathway was shown to inhibit apoptosis and, in concert with FGF1 signaling, might aid tissue repair processes in injured tissue (Cuevas et al., 1997; Baines and Molkentin, 2005; Engel et al., 2005; Xie et al., 2020). Together with decreased pERK and pAkt, dovitinib has an anti-proliferative and

potentially differentiating effect, as these cell programs are often mutually exclusive. However, pERK is a cell signaling pathway with unspecific effects, which is embedded in fundamental cell cycle programs (Pende et al., 2004; Meloche and Pouysségur, 2007). Thus, integration with other signals and the situational microenvironment will determine specific cell responses. Interestingly, pP38 phosphorylation levels were unchanged following fexagratinib treatment. However, like with dovitinib, employment of fexagratinib trended towards cytotoxicity albeit without activating the P38 pathway. This could mean that other implications of pP38 have to be considered (see IV. 2. 4. 2. b).

Regarding the effects of the FGFR inhibitors on cytokines, only mRNA levels of *IL21* were significantly affected. *IL21* was down-regulated by application of fexagratinib compared to dovitinib with aligned trends against the control group exhibited after fexagratinib and infigratinib treatment. Apart from that, weak trends towards lower *IFNG*, *TNF* and higher IL-1β and pJNK were seen post-dovitinib-treatment. These might be attributable to cellular stress induced by growth factor deprivation *via* dovitinib-mediated multikinase inhibition. Cytokines like TNF-α, IFN-γ, IL-1β, IL-6, IL-21 were not altered by treatment with FGF2. Other factors such as TCR activation, NCAM stimulation or IKKβ/NF-κB signaling might be more influential than FGF/FGFR signaling for these cells (Kos and Chin, 2002; Wong et al., 2002; Byrd et al., 2003; Haugsten et al., 2008; Francavilla et al., 2009). It is known that $CD8^+$ T cells respond to FGFR inhibition in a pro-inflammatory manner, but also that FGF2 can be chemoattractive to these cells (Meij et al., 2002; Kato et al., 2019), which means that both the microenvironment as well as a possible beneficial role of $CD8^+$ T cells that could be enhanced by FGFs need to be considered. Not surprisingly, there may have been broader effects following multikinase inhibitor treatment: Trends toward decreased canonical pro-inflammatory *IFNG* and *TNF* but conversely increased IL-1β and pJNK following dovitinib treatment suggested ambiguity of these signals in T cells (see IV. 2. 4. 4).

IV. 2. 2. Summarized effects of FGFR inhibition in SIM-A9 cells

Like T cells, microglia are centrally involved in the composition of lesions, but beyond that, their plasticity and ambivalence with respect to pathogenicity (Hemmer et al., 2015; Vergadi et al., 2017; Chu et al., 2018; Matsui and Mori, 2018) characterize them as promising candidates for immunomodulation. To allow for analogies to the histopathological data of our EAE experiments, we chose SIM-A9 cells, which relatively closely resemble CNS resident murine microglia (Nagamoto-Combs et al., 2014; Gill et al., 2018; Dave et al., 2020; Jayakumar et al., 2021).

Applying the FGFR inhibitors to SIM-A9 cells produced similar effects to those described for the Jurkat cells above. Their proliferation was strongly reduced by all substances except infigratinib, where only a trend towards lower proliferation was measurable. Interestingly, this particular group treated with infigratinib was also the only one to show significantly higher pP38, suggesting other downstream mechanisms following FGFR inhibition in this cell line as in Jurkat cells (see IV. 2. 4. 1). Underlining these differences, cytotoxicity could only be suspected in the fexagratinib group where a trend toward cytotoxic effects was observed. Other than the weak trend in the T cells, FGF2 did not induce any change in cell growth.
Taken together, in agreement with our hypothesis, FGFR inhibition reduces microglial cell growth, while the most selective substance, infigratinib, showed the weakest effect that stayed below the significant threshold.

Again, cell surface expression of FGFRs was affected by their inhibition, but to a much lesser extent than in Jurkat cells. In the case of SIM-A9 cells, only FGFR2 was visibly reduced after the use of each of the three inhibitors, but only the cells treated with fexagratinib reached significantly lower amounts of FGFR2. Analogous to Jurkat cells, no significant changes in the total cellular amounts of FGFR1 and FGFR2 were measurable in Western blots, while trends for their respective mRNAs showed a reduction by FGF2 and induction by inhibitor application, respectively (see IV. 2. 4. 3). Downstream, a different picture emerged: Akt phosphorylation levels were reduced only by dovitinib and FGF2. Utilization of infigratinib exhibited a trend towards slightly lower levels. pERK expression remained unchanged in SIM-A9 cells.
Taken together, the growth inhibition in SIM-A9 cells is completely independent of both pERK and pAkt. Furthermore, pP38 has a different role, as infigratinib was the only substance that increased its phosphorylation levels while not displaying any cytotoxicity and being the weakest growth inhibitor of the deployed substances (see IV. 2. 4. 2).
Interestingly, infigratinib was also the only compound that showed trends to induce TNF-α and IL-1β expressions. Apart from that, only weak trends were observed with respect to other cytokines (or their mRNA) with one of the most robust ones being that of reduced *CX3CL1* after deploying dovitinib. Subsequent to the application of fexagratinib, *IL1B* and *TNF* and pJNK were weakly reduced – all not statistically significant.

IV. 2. 3. Limitations

As mentioned above, there are several caveats to the present study that are inherent to the methods employed and to the models and their implementation.
Given that Jurkat cells are significantly diverged from human $CD4^+$ T cells and they had not been activated *via* their TCR, conclusions are contextually limited. Nevertheless, in an attempt to initiate first insights and to generate generalized statements, the experimental design was chosen as described in II. 2. 2. 2. but is expandable as discussed in IV. 5. In light of the fact that peripheral macrophages/monocytes do not contribute to the CNS microglial pool, we considered microglia to be more important to the CNS lesion microenvironment (Ajami et al., 2011) than macrophages/monocytes. Again, these cells were not actively immunostimulated per se, which is a caveat of these experiments, but allows for a more generic description of FGFR inhibition in microglia.

The mRNA detection using RT-qPCR yielded normally distributed data with a very high variance, which was not log-transformed. The effectiveness of gene amplification was often low, resulting in repeated measurements and high C_T values. However, the method was well-established in the research group that hosted this thesis, and gene amplification product confirmation, for example *via* Northern blot, was not considered necessary. Furthermore, not only the mRNA levels, but also the secretion of cytokines was very low, rendering them undetectable by ELISA and often even by Western blot. This indicates an overall low expression of cytokines. Protein concentration, such as precipitation of the supernatant, may therefore be necessary for future experiments (see IV. 5).

An important reason for these low cytokine levels is certainly the lack of immunological activation of the cells by LPS (microglia) or TCR stimulation (T cells). The following considerations were made: The pathway of both microglial and T cell activation and their sustained engagement in MS and EAE is unknown. Hypothetically, these frameworks of prolonged activity centrally determine cellular responses to external stimuli. Therefore, it was considered more relevant to determine the physiological cellular response to FGFR inhibition rather than an artificially stimulated response that may not resemble the actual pathological state. Furthermore, given the biological distance of the cell lines employed from their primary counterparts, the experiments were designed as an entry point for further studies (such as with primary cultures, see IV. 5). However, due to the low expression of cytokines, inferences are limited both by sensitivity issues and mechanistically by the different activation status that will be found in actual lesions or in the immune cells responsible for inflammatory damage. Of course, the effects of FGFR

inhibition on relatively quiescent microglia and T cells can be evaluated in the context of possible side effects.

In addition to the absence of direct immune stimuli, indirect ones, as postulated for chronic lesions and focal inflammation in the CNS of patients with MS, could not be observed due to the monoculture required for the *in vitro* approach. This is particularly unsatisfactory with respect to the postulated microenvironmental feedback mechanisms that are believed to be present. Such cross-reactions are encouraging for future research efforts and could be addressed using tissue culture or mixed primary cell cultures.
Obviously, the *in vitro* setup is *per se* limited in terms of temporal changes and organ-wide or even effects based on organism-dependent changes such as in the course of EAE or in chronic vs. active and smoldering vs. acute pathologies, which is something that has been and must be addressed in other studies *in vivo* (see IV. 5; also in Rajendran et al., 2018; Kamali et al., 2021; Rajendran et al., 2021c; Rajendran et al., 2022).

Restrictions are also rooted in the limited response to FGF2 application in both cell lines, in particular lack of up-regulation of FRS2 downstream proteins phosphorylation ERK and Akt. FRS2 serves as the major signaling mediator, yet its modulation varies depending on the route of receptor activation and the specific site of phosphorylation (either threonine or tyrosine residues; Lax et al., 2002). Though this may convey a notion of autoregulation in response to prolonged exposition to high levels of FGF2 *via* ERK and P38 mediated FRS2 phosphorylation (Zakrzewska et al., 2019), it might inadequately capture the pathological state in inflammatory demyelinated CNS tissue. However, given the evident consequences of FGFR inhibition and the demonstrated efficacy, such as the growth inhibition, the diminished pAkt levels in microglia might indicate the involvement of other downstream pathways that hold greater significance for these immune cells.

Nevertheless, apart from that, only weak trends were seen as a result of FGF2 treatment: The more robust ones are lowered FGFR2 surface expression on and growth induction in T cells. Notably, the use of FGF2 had no effect on cytokine secretion, although the aforementioned limitations for cytokine secretion must be kept in mind.

IV. 2. 4. Integration of the *in vitro* findings

IV. 2. 4. 1. Proliferation

Consistent with our hypothesis, proliferation of both cell lines was strongly reduced by all inhibitors except infigratinib in SIM-A9 cells. Nevertheless, there was a strong congruent trend for cell growth inhibition in SIM-A9 cells by infigratinib. As previously discussed, T cell pathology in the MS-like CNS involves drainage, aberrant priming, rehoming, activation, and proliferation (see I. 1. 2 and I. 2. 1. 1, also in Korn and Kallies, 2017; Ruder et al., 2022). The abrogation of these processes could potentially offer a curative treatment. However, it is known from autologous hematopoietic stem cell transplantation studies that $CD8^+$ T cells reconstitute relatively quickly, and $CD4^+$ T cells follow soon after (Ruder et al., 2022). Thus, the T cell repertoire in the CNS is highly variable and undergoes constant renewal but also exhibits some persistent features. In other tissues than the CNS, self-renewing resident T cell populations are known to form after viral infection. These could also exist in a pathologically affected CNS environment (Mackay et al., 2012; Korn and Kallies, 2017) but conceivably also in the form of a beneficial regulatory resident T cell population. Given the *in vitro* impact of FGFR inhibition on T cell proliferation observed in this study, it is plausible that this anti-proliferative effect would be particularly pronounced in those T cells that are pathologically altered in MS due to their active and proliferative status. This is particularly compelling given the robust reduction in inflammation and immune cell infiltration, including T cells, observed in both *FGFR* knockout studies and the infigratinib and fexagratinib treatment studies in EAE (Rajendran et al., 2018; Kamali et al., 2021; Rajendran et al., 2021c; Rajendran et al., 2022; Rajendran et al., 2023; Gurski et al., 2024).

The presence of microglia in the earliest lesions and their coincidence with pre-apoptotic oligodendrocytes, in addition to their functional implication in MS lesion pathology, and their quantitative correlation with CNS tissue damage, consolidates their association with functional damage. However, as described in I. 2. 1. 1. c, microglia also align themselves with coinciding repair mechanisms. Ultimately, the precise nature of their role remains to be elucidated. However, in the context of EAE, there is a growing body of evidence indicating that these cells may exert detrimental effects (Gold et al., 2006; Lampron et al., 2015; Grajchen et al., 2020; Iovino et al., 2020). Analogous to T cells, our previous studies demonstrated a significant reduction in microglia abundance, concomitant with a reduction in demyelination, axonal loss, and an improved clinical outcome (Rajendran et

al., 2018; Kamali et al., 2021; Rajendran et al., 2021c; Rajendran et al., 2022; Rajendran et al., 2023; Gurski et al., 2024). Consequently, it could be inferred that pharmacological FGFR inhibition induces an anti-proliferative effect on microglia, irrespective of the reduced lesion formation or decreased inflammatory stimuli that may be attributable to other effects of the inhibition.

IV. 2. 4. 2. Downstream signaling

The inhibition of T cell and microglial growth following FGFR inhibition is evident, but the pathway ultimately responsible remains elusive. This is due to the fact that downstream signal alterations varied between substances, and in particular, pERK and pAkt were not found to be responsible, as they remained unchanged after treatment with infigratinib and fexagratinib despite growth inhibition by these agents.

IV. 2. 4. 2. a. ERK and Akt

This is an interesting finding, as above all, both pAkt and pERK were expected to be influenced by the inhibition of FGFRs because they are the most important downstream signals of FRS2. Moreover, alterations in downstream signaling following treatment with dovitinib have been previously described in other cancer cell lines (Lee et al., 2005; Langdon et al., 2015; Das et al., 2020). Notably, dovitinib was the only substance to reduce both pAkt and pERK (in Jurkat cells) but did not differ significantly from the other substances in terms of its anti-proliferative effect. The changes in ERK and Akt phosphorylation are presumably based on the multikinase activity of dovitinib. Given the potent inhibition of VEGFRs and other FLTs as well as PDGFRβ and C-Kit, which all signal through PI3K/Akt and/or MAPK/ERK (Rönnstrand, 2004; Heldin, 2013; Shibuya, 2014; Kazi and Rönnstrand, 2019), dovitinib has broader effects than all other inhibitors.

It is generally accepted that both of these pathways, particularly the MAPK/ERK pathway, are centrally involved in cell cycle regulation and proliferative stimulus. This does not appear to be the case for the cell lines investigated here. However, there is evidence that abatement of the MAPK/ERK pathway (Wu et al., 2022), especially in conjunction with microglia, can be beneficial (Peterson et al., 2002), *e.g.* by reducing the secretion of VCAM1. This reduction in VCAM1 centrally involves the IKKβ/NF-κB pathway (Blotnick et al., 1994; Zhao et al., 1995; Byrd et al., 1999; Wu et al., 2022). It is noteworthy that the impact on direct downstream targets differs in SIM-A9 cells compared to the Jurkat cells. While pAkt was reduced by dovitinib, it was also lowered by FGF2, exhibiting a comparable trend after infigratinib treatment. It was not expected

that FGF2 would enhance the rate of cell growth *in vitro*, but rather that it would increase the expression of both pAkt and pERK (Goddard et al., 2002; Noda et al., 2014). This absence of direct downstream upregulation in response to receptor activation could be explained by FGFR autoregulation in SIM-A9 cells (see IV. 2. 4. 3). Overall, the marginal response of both cell lines to FGF2 exposition mitigates the deductibility of these experiments, and furthermore, both predominant downstream signals ERK and Akt are not significantly activated by exposure to the FGFR ligand.

IV. 2. 4. 2. b. Other downstream pathways

Nevertheless, the reduction in growth observed established the efficacy of the inhibitors. Further analysis of the FGF/FGFR signaling pathway components may present the opportunity to unravel the more pivotal components in microglia and T cells (see IV. 5).

pP38

It is therefore reasonable to presume that other signaling pathways are more crucial for the anti-proliferative and potentially anti-inflammatory effects observed in both Jurkat and SIM-A9 cells. One such pathway could be P38, which was differentially affected by the inhibitors. However, the incongruence between the phosphorylation levels of P38 and the respective proliferation data precludes the possibility that pP38 is *per se* responsible for cell growth inhibition.

Nonetheless, the P38 pathway is auspicious in the context of CNS injury, as it is connected to FGF/FGFR signaling, including indirect feedback through phosphorylation of FRS2 (Zakrzewska et al., 2019; Ornitz and Itoh, 2022). It is also expressed in T cells at high levels in MS and EAE (Shin et al., 2003). Its inhibition has several protective effects in cardiac tissue damage, including prevention of cardiomyocyte apoptosis (Cuevas et al., 1997; Baines and Molkentin, 2005), improvement of cardiomyocyte proliferation and cardiac regeneration (Engel et al., 2005; Xie et al., 2020). These effects involve FGF/FGFR signaling, which inhibits the pP38 pathway thereby promoting proliferative responses. When applied to the microenvironment of MS lesions, the superabundance of FGF and sustained receptor activation may promote apoptotic pathways, as proliferative and apoptotic pathways are always intertwined (Rönnstrand, 2004; Ashwell, 2006; Heldin, 2013; Shibuya, 2014; Kazi and Rönnstrand, 2019). Conversely, FGFR inhibition in this context could promote differentiation (*e.g.*, as known from OPC differentiation to oligodendrocytes; Rajendran et al., 2021a) and induce immune cell apoptosis. Further links between tissue damage and P38 involve ROS. *In vitro*, neuronal cells may protect themselves against ROS through the inhibition of pP38,

pERK and pJNK – which are all upregulated in EAE lesions in various cells including astrocytes and glia cells (Shin et al., 2003). Interestingly, instead, the activation of the PI3K-AKT pathways is promoted (Hu et al., 2015). In response to GM-CSF stimulation, inflammatory microglia up-regulate both the ERK and P38 pathways thereby increasing their inflammatory potency (Parajuli et al., 2012).

Notably, pP38 expression varied depending on the inhibitor and to some extent in a cell-specific manner. While there was a discernible tendency towards increased expression of pP38 in Jurkat cells in response to each of the three inhibitors, only dovitinib achieved statistical significance. Multikinase inhibition in SIM-A9 cells did not result in an increase in pP38 in contrast to the effect observed with FGFR1/2/3 inhibition by infigratinib. Trends towards cytotoxicity did not coincide with changes in pP38. Therefore, it can be concluded that more specific FGFR inhibition in microglia leads to upregulation of pP38, whereas in T cells other RTKs such as PDGFRβ or VEGFR play a more important role in inducing pP38 signaling.
Due to the caveats of this study, it is challenging to assess the consequences of this pP38 signaling alteration regarding the inflammatory impetus in EAE or even MS (see IV. 2. 3).

If the results of this work are rendered on known implications of pP38, it can be postulated that the induction of pP38 in microglia and T cells could promote inflammation. However, the consequences of pP38 induction are complex and include growth inhibition. Therefore, if a microenvironment with an excess of pro-inflammatory and proliferative signals is postulated for MS lesions, the induction of pP38 particularly in overly active and detrimental cell types might represent a counterbalance towards restoring an equilibrium in which remyelination and regression of immune infiltration can be achieved. Thus, it would be of interest to ascertain the impact of pP38 in the context of a chronic inflammatory milieu (see IV. 5).

IV. 2. 4. 3. Receptor autoregulation

After having reviewed the downstream targets and their counter-intuitive response to FGFR inhibition, it may be informative to take a step back and consider the regulatory mechanisms of FGFRs. Receptor autoregulation, membrane expression, and feedback mechanisms are critical for a pathway that is so centrally involved in potentially deleterious cell programs (see I. 3. 1). Ligands are tightly regulated (see I. 3. 1), but the receptor itself is subject to several regulatory mechanisms that are still incompletely

understood. Glycosylation of the receptor influences membrane association and on T cells is dependent on TCR activation (Byrd et al., 2003). Endocytosis is one of the most important negative feedback mechanisms and is mediated by both clathrin-dependent and clathrin-independent mechanisms. Furthermore, dimerization or oligomerization increases uptake efficiency even for inactive receptors (Pozniak et al., 2020). FGF mimics, such as galectins, have been demonstrated to both facilitate and impede FGFR clustering and constitutive receptor endocytosis (Opaliński et al., 2018; Kucińska et al., 2019). Consequently, internalization and subsequent degradation is both receptor- and host-cell-type-specific (Szybowska et al., 2021; Ornitz and Itoh, 2022).

This is particularly interesting in the context of alternative ligands such as NCAM. NCAM exhibits a negative correlation with MS and serves to stabilize FGFR against ubiquitination and degradation (Axelsson et al., 2019; Kon et al., 2019; Ziliotto et al., 2019). NCAM is also active in T cells, stimulating IL-2 secretion (Kos and Chin, 2002) and promoting FGFR cell surface expression. This occurs *via* a two-step process: First, internalization is induced, followed by trafficking to endosomes *via* Proto-oncogene tyrosine-protein kinase Src. In contrast to the usual internalization route of FGF-mediated FGFR stimulation *via* lysosomes, endosomes are targeted here instead (Wong et al., 2002; Haugsten et al., 2008; Francavilla et al., 2009).
This underscores the flexibility of FGFR processing and points towards a heavy dependency on ligand-receptor interactions, which comprises cell surface expression and thus the availability of the receptors for prolonged signaling, such as in environments where FGFs are superfluous. Furthermore, signal transduction is also regulated by molecular autoinhibition, preventing ligand-independent autophosphorylation. Mutations in this receptor region constitute potential malignant transformations (Chen et al., 2007).

In light of these regulatory mechanisms, it is not surprising that T cell surface expression of FGFR1 was significantly reduced upon treatment with infigratinib, with the same trends noted for the other inhibitors and for FGFR2. Furthermore, microglial FGFR cell surface expression was differentially affected. There, only FGFR2 was reduced only on the cell surface, whereas total protein and their respective mRNAs either showed trends towards upregulation or remained unchanged after FGFR inhibition. Thus, internalization is the most likely mechanism to explain these findings, and the absent changes in downstream signaling could be due to or confounded by decreased availability of the receptor.
In conclusion, it would be unwise to focus solely on the downstream consequences of FGFR inhibition, as the regulation of FGFR is inherently complex. Interestingly, non-canonical ligands such as NCAM may primarily modulate the cascade *via* this regulatory

mechanism. If one accepts the hypothesis that FGF is excessively abundant in MS lesions and that receptor autoregulation is perturbed by a pro-inflammatory MS-typical microenvironment, it can be concluded that impaired internalization and degradation of the receptors is imperative to maintain the pathologically overactive signaling cascade that is otherwise tightly regulated. Only then would lesion extension be possible and physiologic regeneration efforts be prevented.

IV. 2. 4. 4. Cytokine microenvironment

This hypothetical microenvironment is characterized by a specific cytokine composition. However, in both cell lines, treatment with FGF2 and FGFR inhibitors did not strongly affect cytokine secretion or expression. Overall, cytokine secretion remained below detectable levels (see Figure 21).

IV. 2. 4. 4. a. *IL21*

However, *IL21* was slightly down-regulated after FGFR inhibition in T cells. As $CD4^+$ T cells-derived IL-21 guides differentiation and function of lymphoid and myeloid cells it is involved in autoimmunity and inflammation not least by $CD4^+$ T cells' modulation of antibody production. Most importantly, IL-21 plays an extremely important role for chronic viral infections, in which it helps to recover $CD8^+$ T cells function (Asao, 2021; Ren et al., 2021). It remains to be established whether IL-21 is as pivotal for EAE or MS but the evidence available points towards a detrimental albeit context-dependent role of the cytokine (Ghalamfarsa et al., 2016; Edo et al., 2022). One potential mechanism by which FGFR inhibition may be beneficial in EAE is through the reduction of this cytokine, particularly during the acute phase of the disease.

IV. 2. 4. 4. b. IL-1β and CXC3L1

Moreover, and to a greater extent than IL-21, IL-1β is a pleiotropic cytokine that is considered to be markedly pro-inflammatory and involved in MS pathology. Monocytes, including microglia/macrophages, astrocytes, and endothelial cells, are the source of this cytokine. It facilitates the transmigration of activated immune cells across the BBB and activation and recruitment of CNS resident glia (Ferrari et al., 2004; Murphy et al., 2010; Maghzi and Minagar, 2014). In the present work, a trend towards lower *Il1b* levels in microglia was observable following infigratinib utilization.

A similar trend was observed for *Cx3cl1*, albeit after dovitinib treatment. The CX3CL1/CX3CR1 signaling pathway is associated with microglial CNS pathology in various disease states, yet it has proven difficult to integrate into the inflammatory

cascade as it is another example of profound context-sensitivity of the immune system. Nevertheless, it is involved in neurodegenerative states as well as inflammatory demyelination, where it has resulted in contrasting results in EAE. Genetic polymorphisms in MS include *CX3CR1* but their role is unclear (Pawelec et al., 2020; Subbarayan et al., 2022).

IV. 2. 4. 4. c. TNF-α

The role of TNF-α is much more clearly defined as a prototypical ubiquitous pro-inflammatory cytokine that is involved in immune cell cycle control as well as activation and apoptosis (Fresegna et al., 2020). Additionally, TNF-α is involved in the completion of T cell responses *via* both TNF receptor 1 and 2 (Twu et al., 2011). The differences in these receptors might constitute the basis for the pleiotropy of TNF signaling in MS. In this context, the pharmacological abrogation of this pathway has been associated with deleterious effects (see I. 1. 1. 2. d), suggesting that a nuanced approach targeting both receptors individually might be more adequate (Fresegna et al., 2020). In light of these considerations, the contrasting trends after fexagratinib and infigratinib treatment may not be very meaningful if only the ligand TNF-α is considered.
However, if microglia were indeed modulated toward reduced IL-1β and CX3CL1 secretion in conjunction with reduced IL-21 secretion by T cells, they could establish a microenvironment conducive to regenerative processes such as remyelination or, if present at earlier stages, even prevent the derailment of autoreactivity.

IV. 2. 4. 5. Conclusions from the *in vitro* findings

In conclusion, the anti-proliferative effects of FGFR inhibition in T cells and microglia are based on pathways other than ERK and Akt, such as P38 and IKKβ/NF-κB. FGFR autoregulation may also have played a role. Translationally, receptor surface expression, degradation, glycosylation and ligand specificity need consideration. Furthermore, non-canonical ligands such as NCAM in modulating FGFR signaling must be appreciated. *In vitro*, FGFR inhibition has minimal effects on cytokine secretion, but small changes in the mRNA levels of IL-21, IL-1β, and CX3CL1 may indicate the possibility of a shift in the pathologic microenvironment of the MS brain that could be achieved by FGFR inhibition. However, firstly, these cytokines may also be involved in the anti-inflammatory and regenerative effects of FGFR inhibition in MS, and secondly, in the disturbed CNS immune environment of MS, cell-cell and organ-wide interferences are to be expected, which is why consideration and integration of the *in vitro* data with the *in vivo* data is of great importance.

IV. 3. *IN VIVO* DATA

The peripheral immune system is considered to be the origin of autoimmunity in MS, or at the very least, immune cells from the periphery cross the BBB and contribute to CNS inflammation. Therefore, to complete our understanding of FGFR inhibition in EAE, and to evaluate the *in vitro* effects in an MS model, we analyzed immune cell populations in two peripheral compartments at two time points of EAE following either vehicle treatment, or preventive or suppressive infigratinib application (see II. 2. 1. 3).
Both innate and adaptive immune cells were considered, including identification of B cells, $CD4^+$ and $CD8^+$ T cells, dendritic cells, macrophages, and monocytes.

IV. 3. 1. Effects of FGFR inhibition on peripheral immune cells in EAE

Overall, *in vivo*, strong effects of infigratinib were seen only subsequent to the preventive treatment, and only in the acute phase of EAE. In this setting, a pronounced reduction in T cell abundance was measurable in the spleen, while interestingly there was an increase in $CD4^+$ T cells and a decrease in $CD8^+$ T cells. In addition, the quantity of all considered innate immune cells increased markedly (see Figure 33). Notably, in the same setting, both $CD4^+$ T cells and $CD8^+$ T cells were reduced in the blood, while the number of innate immune cells was increased to a similar extent as in the spleen, except macrophages, which remained unchanged (see Figure 34). The number of B cells was reduced in both compartments following employment of infigratinib, but this change did not reach significant levels in the blood.

There were two other experimental settings in which significant changes were identified: Suppressive infigratinib treatment reduced the number of dendritic cells in the spleen in the acute phase of EAE and, in the chronic phase, increased the number of T cells and macrophages in the blood (see Figures 35, 40).

In summary, the use of infigratinib in parallel with immunization prior to the onset of symptoms partially prevented the activation and proliferation of peripheral immune cells. Interestingly, CD4/8 ratios differed in blood and spleen, and there seemed to be a stimulatory effect on the innate immune system, as these cells were more abundant despite FGFR inhibition.

IV. 3. 2. Limitations

Limitations of this particular part of the study include the small number of animals in the acute EAE setting after suppressive treatment and in both chronic EAE settings in the infigratinib group (n = 2, n = 3, respectively).

Of course, as discussed previously, EAE does not fully represent MS (see I. 2. 1. 1) but is one of the most successful and appropriate models available to date to study inflammatory demyelination. With respect to immune cells, the results must be weighed against the differences in immunology between the two diseases. Given the importance, but distinct roles, of $CD8^+$ T cells in both conditions, insights into cell signaling may aid our understanding of both diseases. Similarly, microglia have a profound impact on both EAE and MS, and a better comprehension of their response to FGF/FGFR signaling may allow for targeted treatments.

Some methodological problems were posed by cell clumping and possible exclusion of lymphocytes in the process of red blood cells lysis and tissue homogenization.

Furthermore, cell numbers alone do not fully reveal the activation status and their distribution in the organism cannot be deduced from these experiments. In addition, the antibody panel employed in this study did not fully assess subpopulations. However, a sequential sorting method such as fluorescence-activated cell sorting (FACS) and a culture approach could provide comprehensive knowledge as a follow-up experiment to the data described here (see IV. 5).

IV. 3. 3. Integration of the *in vivo* data

Consistent with our hypothesis, and fitting the *in vitro* data, the relative quantities of both T cells and B cells were reduced by treatment of EAE with infigratinib. Surprisingly however, this was mainly the case in one setting, *i.e.*, preventive treatment at 17 days pi. Otherwise, only modest changes were observed, though we knew from previous studies that immune cell infiltration in the CNS is diminished regardless of the treatment approach or sampling time. Nevertheless, we have now demonstrated an immuno-modulatory peripheral effect of FGFR inhibition that may prevent persistent CNS damage without preventing overall disease development. The exact mechanism can only be speculated, but it would most likely involve peripheral T and B cell activation. As a result of reduced T and B cell activity, and possibly by promoting quiescence through FGFR inhibition itself, the number of activated T and B cells is decreased, thereby reducing the number of lymphocytes that are erroneously relocated into the CNS. The implications of FGFR inhibition for the different cell types, temporal changes, and by the substances are discussed in the following sections.

IV. 3. 3. 1. T cells

As described above, mononuclear and T cells are critical, albeit dichotomous in the pathophysiology of MS. T cells are presumably activated by autoreactive APCs or are directed against post-translationally generated molecular mimicry peptides themselves (see I. 1. 1. 2. a, I. 1. 2. 2., I. 1. 4. 1 and I. 2. 1. 1. c; also in Fransen et al., 2020; Attfield et al., 2022). Not all alterations in immune cells are quantifiable by the FC approach employed in the present work. Non-quantifiable changes may be most striking with respect to $CD8^+$ T cell subpopulations, which have been associated with various beneficial properties. Although a pathological role has been attributed to $CD8^+$ T cells in MS and sometimes EAE, an increase in $CD8^+$ T cells may be beneficial in certain situations as they are known to remove autoreactive $CD4^+$ T cells and contribute to regulatory anti-inflammatory responses. (Jiang et al., 1992; Cunnusamy et al., 2014; Saligrama et al., 2019). Based on these paradoxical findings, these T cells either form a plethora of activation states or polarizations that are yet to be determined, or they are part of several subpopulations that vary in number and activity and thus shape the recovering immune responses induced against autoinflammatory processes.
Within the scope of the present work, it was not possible to further investigate this complex research topic and stratify immune cell populations according to their molecular markers and/or activation status, as this would have required methodology not available to the author and additional (approval of) animal experiments.

One of the T cell populations that has recently been partitioned into several microenvironmentally induced subpopulations are $CD4^+$ T cells (Attfield et al., 2022). Regulatory T cells are $CD4^+$ (Treg cells), also express CD25 and forkhead box P3 (FOXP3), and are the major component of the regulatory immune system.
Th1 cells on the other hand, may be one of the main drivers of CNS infiltration and destruction that can be targeted by dimethyl fumarate – or with respect to CNS invasion by natalizumab (Attfield et al., 2022). Ongoing research is likely to provide an even finer grained profile of such cells, and it remains uncertain which of them provide regulatory impulses and which drive oligodendroglial apoptosis and neuronal destruction (e.g., β-synuclein-directed $CD4^+$ T cells, see I. 1. 2. 1; Zhao et al., 2017). Moreover, the transitions are fluid and the plasticity is extensive, so it is plausible that even more of these cells than we currently know are polarizable and can actually transform into each other (Piconese et al., 2020; Attfield et al., 2022).
Furthermore, $CD4^+$ T cells are pivotal for microglial activation and maturation, and their interplay can sustain and also abrogate an inflammatory response (Aloisi et al., 2000).

In EAE, the combined secretion of IFN-γ and IL-17 by $CD4^+$ T cells leads to microglial activation and IL-1β-, IL-6- and TNF-α-positive inflammation (Murphy et al., 2010).
For simplicity, if we look at the immune cells in the blood and spleen in just one setting, animals preventively treated with infigratinib, we have to argue for a dampening of the inflammatory cascade induced by FGFR inhibition. This would be evidenced by the reduction of $CD8^+$ T cells and total T cell counts. The increase of $CD4^+$ T cells, however, does not fit the picture and needs further interpretation. The increase in this particular subpopulation may consist mainly of regulatory T cells (Treg cells), which may indeed explain the success of both genetic and pharmacological silencing/suppression of the FGFR signaling cascade, leading to an improvement in clinical course, reduced immune cell invasion, reduced demyelination and remyelination inhibitors, enhanced axonal density, and increased myelin formation and mature oligodendrocytes (Rajendran et al., 2018; Kamali et al., 2021; Rajendran et al., 2021c; Rajendran et al., 2022; Rajendran et al., 2023; Gurski et al., 2024).

IV. 3. 3. 2. Innate immune cells

As mentioned above, innate immune cells are as important in EAE as adaptive immune cells. Central to its antigen presentation are dendritic cells, which can be divided into several subpopulations (Guilliams et al., 2014). One of them, conventional dendritic cells – that secrete *inter alia* TGF-β – are dependent on FLT3 (which interestingly is blocked by fexagratinib but not by infigratinib, see IV. 3. 3. 4) and are responsible for the differentiation of Treg cells (Roncarolo et al., 2001; Cheong et al., 2010; Kushwah and Hu, 2011; Yin et al., 2021). TGF-β is particularly relevant to CNS homeostasis of the innate immune system (Lund et al., 2018). Although monocytes promote Treg cells *via* TGF-β in an inflammatory steady state, they are essential for the maintenance of peripheral tolerance (Yin et al., 2021) and under FGFR inhibition they could shift their weight toward regulatory differentiation.

Similarly, monocytes are known to shape the cell differentiation of immune cells, especially in the blood, and are classically the main source of IL-12 (Yin et al., 2021). They have also been attributed a detrimental role in neuroinflammation. There is evidence that monocytes within the spleen are destined to differentiate into macrophages or dendritic cells (Gordon, 2007; Auffray et al., 2009), whereas tissue-resident macrophages are already established during embryonic development (Bronte and Pittet, 2013). Many monocytes reside in the spleen and can migrate to inflammatory sites where they irreversibly differentiate into dendritic cells or macrophages (Swirski et al., 2009;

Cheong et al., 2010; Grainger et al., 2013). Thus, these monocyte-derived cells exhibit invasive behavior and mobility within tissues and can contribute to local macrophage pools. They are a source of pro-inflammatory input and *de novo* macrophages in conditions of acute inflammation. Their turnover is high and half-life low, but unlike tissue-resident macrophages established before birth, they are more plastic and modulatory (Swirski et al., 2009; Cheong et al., 2010; Grainger et al., 2013).

In EAE and neuroinflammation, monocytes are thought to be centrally involved in demyelination following CNS invasion, but they may also be critical for peripheral EAE induction by secreting IL-23 (Shemer and Jung, 2015). But, as expected, monocytes cannot be so clearly blamed. Myeloid-derived suppressor cells exist that are important for generation of Treg cells and immunosuppressive functions (Owens et al., 2020), including autoimmune diseases (see below; Boros et al., 2016; Su et al., 2016).

IV. 3. 3. 3. Discordance between peripheral and central immunologic findings

Given the proven efficacy of infigratinib and fexagratinib in all treatment approaches and phases of EAE with respect to both CNS histology and clinical outcome (Rajendran et al., 2023; Gurski et al., 2024), but the contrasting unique setting of the present work (day 17 pi, preventive treatment) showing peripheral immunologic efficacy, the peripheral immune system as the sole site of activation and/or action must be disregarded. However, it remains conceivable that infigratinib may modulate immune cell activation without affecting proliferation and thus the overall population quantities of immune cells at later stages. This may be particularly relevant in the spleen, which is an important reservoir of both innate and adaptive immune cells, but also highly important for the generation of self-tolerance (Bronte and Pittet, 2013). Considering the clinical and histopathologic success of suppressive infigratinib treatment against the backdrop of the FC data generated in the suppressive arm of the present study, other mechanisms must be at play, as no far-reaching changes were observed in these FC data.
This would raise the question of why the effect in the aforementioned studies was seen across all clinical stages and treatment approaches. Several explanations are conceivable:

i) Induction of regulatory subpopulations:
   Hypothetically, in preventively treated mice with acute EAE, immunization and immune cell activation occurs predominantly in the peripheral immune system, including the spleen, based on the route of administration. Due to FGFR inhibition, Treg cells are upregulated and actively target autoreactive $CD8^+$ T cells. This would

also reflect the divergent picture seen in the blood. Here, $CD4^+$ T cells are reduced concordantly with their $CD8^+$ counterparts because hypothetically Treg cells have reduced the number of $CD8^+$ T cells in the activation compartment, *i.e.* the spleen, and thus the release of $CD8^+$ and Th1 cells is reduced. Later in the disease, the immune cell activation would shift to the CNS or become more distributed in the lymphoid tissues, rendering substantial changes in leukocyte populations in the blood or spleen undetectable (see IV. 3. 3. 5). Another possible or parallel explanation for the reduction in $CD8^+$ T cells could be the pP38 induction observed by FGFR inhibition (with the caveat that infigratinib only trended and only dovitinib achieved significant induction of pP38 in Jurkat cells – for a discussion of this see IV. 2. 4. 2. b). Even though not cytotoxic in our experiments, pP38 has been shown to induce apoptosis in $CD8^+$ T cells and may switch these cells towards an anti-proliferative state (Shin et al., 2003; Liu et al., 2016). Under this notion, however, it would remain elusive how different subsets of T cells respond differently to FGFR inhibition and proliferate instead of what was observed in our $CD4^+$ T cell-derived Jurkat cells. Yet the *in vivo* data demonstrate that T cells respond differentially to FGFR inhibition either due to direct effects or due to the immunological background it creates.

In extension of the concept presented above, the induction of macrophages observed in the spleen would be caused by increased cell-cell toxicity of Treg cells against $CD8^+$ and Th1 cells or by direct actions of FGFR inhibition. As a result, they cannot enter the circulation (as evidenced by the unchanged number of macrophages in the blood) and cannot invade the CNS to become activated destructive CNS macrophages.

An integration of the increased number of dendritic cells and monocytes in both compartments into this concept would be that these cells are also part of the regulatory arm of the immune system. Under this assertion, myeloid-derived suppressor cells described above are induced by FGFR inhibition and promote regulatory immune responses in the spleen and CNS.

Thus, according to this concept, the efficacy of infigratinib treatment of EAE would be based on the induction of immune cell populations that promote self-tolerance and regulation of inflammation.

ii) Low importance of peripheral immune system

It could be argued that, apart from initiation, the peripheral immune system does not play a major role in EAE, a CNS disease characterized by inflammation in the brain and spinal cord. This could be supported by the lack of peripheral effects in

the later stages of the disease and in the delayed treatment approach. Therefore, FGFR inhibition might mainly affect the CNS, where it causes the changes discussed above (see I. 3. 3. 1. c, IV. 2. 4. 1, IV. 3. 3. 1; Rajendran et al., 2023; Gurski et al., 2024), and the peripheral immunological effects are mostly incidental. Although FGF/FGFR signaling is considered mitogenic and has been shown to be so in many circumstances (see I. 3. 2. 1), its inhibition has also been shown to promote proliferation, primarily in tumor microenvironments (Tang et al., 2018; Kato et al., 2019; Palakurthi et al., 2019; Im et al., 2020; Yi et al., 2021; Adachi et al., 2022; Ruan et al., 2023; Suzuki et al., 2023). Vice versa, its activation has been shown to be non-proliferative (Goddard et al., 2002). This could be the explanation for the elevated leukocyte count observed here. In contrast, there are highly variable proliferative effects for each leukocyte population, underscoring the context-dependent nature of FGF/FGFR signaling, and *in vitro*, FGFR inhibition acts anti-proliferative. Thus, it seems more likely that possible effects in later stages are obscured by the methodological coarseness of the analysis.

iii) BBB protection

Another mechanism of action may be an as yet unidentified alteration in BBB breakdown that occurs after EAE induction. According to this idea, a largely intact BBB would be the reason for the reduced inflammation and immunological invasion of the CNS observed after FGFR inhibition, rather than a direct anti-proliferative or immunomodulatory effect on immune cells in the periphery. This is highly speculative, as we have no concrete evidence at this time - however, there are some data that the FGF/FGFR axis influences the BBB (Huang et al., 2012; Wu et al., 2017; Thümmler et al., 2019; Chen et al., 2020; Kriaučiūnaitė et al., 2023). It could be a mechanism that runs parallel to what is described in i). Consequently, in the chronic phase, (peripheral) immune induction is long past, and changes in the peripheral immune cell compartment are no longer detectable. Against this, in the CNS, microglia would have been inactivated, leading to the robust anti-inflammatory effects. However, given the strong effects of FGFR inhibition on T cells described here and by others, this seems unlikely.

iv) Antigen presentation is decisive

On account of what is described in i), FGFR inhibition may instead primarily affect the innate immune system. This is proposed to occur in two ways: first, by stimulating the proliferation of innate immune cells, and second, by preventing the activation of autoreactive T and B cells. Macrophages/monocytes may be able to

scavenge and eliminate (in the case of EAE, foreign) antigens and reduce their immunogenicity. In addition, FGFR inhibition may also generate myeloid-derived suppressor cells. Together, this would prevent the activation of autoreactive immune cells. Therefore, the changes in lymphocyte populations are not a direct effect of FGFR inhibition, but rather a consequence of the lack of innate immune system stimulation on the adaptive immune system.

IV. 3. 3. 4. Comparison to our fexagratinib EAE study

While the broad multikinase inhibition of dovitinib may explain the more pronounced changes downstream *in vitro* as well as on the receptor level, *in vivo,* the distinct specificities of fexagratinib and infigratinib may provide answers to the discrepancies in peripheral immune modulation observed in the present work. In contrast to the findings after infigratinib treatment presented here, in our other pharmacological EAE study (Gurski et al., 2024), fexagratinib did not affect the number of T cells in the blood or in the spleen, but increased the total number of $CD45^+$ cells in the spleen in the acute preventive setting. It would be too speculative to say which population of these hematopoietic $CD45^+$ cells, which are neither T nor B cells, may have increased in quantity and thus be responsible for the efficacy of fexagratinib – if this shift in cell numbers is playing a significant role at all. But apparently, fexagratinib is not able to significantly reduce overall proliferation of specifically $CD3^+$ and $CD19^+$ immune cells at the onset of EAE. Infigratinib however, impacts peripheral immune cells, particularly during preventive treatment, leading to reduced T and B cell numbers in the spleen. Additionally, it may modify the activation and polarization of $CD8^+$ T cells and Treg cells.

Conversely to our fexagratinib EAE study, in tumor models, fexagratinib induces $CD8^+$ T cells, while other FGFR1-4 inhibitors reduce macrophage infiltration by inhibiting metabolic pathways (Suzuki et al., 2023). Interestingly, in a tumor microenvironment, FGFR inhibition activates T cells and cells of the innate immune system leading to increased cell-cell cytotoxicity (Kato et al., 2019; Palakurthi et al., 2019; Im et al., 2020; Yi et al., 2021; Adachi et al., 2022; Ruan et al., 2023). As previously mentioned (see I. 3. 4. 1), fexagratinib has opposing effects on the MAPK/ERK and PI3K/Akt pathways, as well as STAT, depending on the cell type. Some cells exhibit greater resistance to its inhibition (Delpuech et al., 2016; Phanhthilath et al., 2020). Consequently, inhibition of FGFR leads to varied outcomes, which are influenced by the specific inhibitor, cell type, and immunological context.
It appears that $CD8^+$ T cells and microglia are independent of the PI3K/Akt pathway for

FGF/FGFR signaling with regard to their proliferation and cell cycle control, as indicated by the lack of change in pAkt levels in SIM-A9 and Jurkat cells. Nevertheless, the observed systemic changes *in vivo* diverge from the *in vitro* results, suggesting that interactions among immune cell subsets or within the immune microenvironment are likely involved. Therefore, FGFR inhibition, particularly with infigratinib, may primarily affect the regulatory components of both the adaptive and innate immune systems, facilitating the elimination of autoreactive T cells in EAE.

In the acute preventive setting, fexagratinib reduced spleen granulocytes, likely through CSF1R inhibition. This additional CSF1R blockade may also have masked a potential increase in macrophages, or, alternatively, fexagratinib does not influence macrophage proliferation. Another RTK blocked by fexagratinib but not infigratinib is FLT3. This receptor signals through various pathways that have been addressed here, including Src family kinases, Akt, STAT and ERK. It promotes continuous dendritic cell differentiation, preferentially in the spleen and is activated by hematopoietic sources of FLT3 ligands, particularly by $CD4^+$ T cells (Kazi and Rönnstrand, 2019; Wilson et al., 2021). This inhibition of FLT3 by fexagratinib could explain absence of innate immune cell effects in the fexagratinib-treated mice in contrast to infigratinib-treated mice, as dendritic cell activation may be dampened. Additionally, fexagratinib's inhibition of AXL, a RTK that attenuates the innate immune system's inflammatory response (Scutera et al., 2009), could explain the observed differences between inhibitors. By blocking AXL signaling, fexagratinib may facilitate the elimination of autoreactive T cells, particularly through the activation of dendritic cells.

Taken together, fexagratinib had a similar clinical and histopathologic outcome to infigratinib in our EAE experiments, and closer examination of the immune cell populations may reveal similar mechanisms. However, compared to the study presented here, the effects on immune cells were much less pronounced in the fexagratinib treatment experiments. While we saw that infigratinib strongly affected the peripheral immune cell compartment, but fexagratinib did not, the anti-inflammatory properties in the CNS still persisted. This may indicate that blocking FGFRs has a more profound effect within the CNS than in the peripheral immune compartments, or that the method of measurement, *i.e.* counting cells only, is not sensitive enough to detect actual effects on cell activation, polarizations and elevation of some subpopulations. In addition, systemic or local changes in cytokine composition may have modified the interaction of the innate and adaptive immune systems, inducing Treg and eliminating autoreactive T cells after FGFR inhibition, which would also not be reflected in the FC data as collected here.

IV. 3. 3. 5. Chronic phase

Furthermore, analogously to the acute preventive setting after infigratinib treatment, B cells in the blood were reduced in the suppressive treatment setting with fexagratinib. Except for a slight decrease in dendritic cells in the spleen after preventive treatment in the acute phase, only more $CD3^+$ T cells and macrophages persisted into the chronic phase in the spleen of mice receiving suppressive treatment. Thus, in line with our hypotheses, FGFR inhibition modulates the induction of EAE specifically and does not have a lasting impact on leukocyte levels, at least outside the central nervous system. This is relevant in terms of possible (long-term) adverse effects, but also allows some guesswork on the mechanism of action. Fexagratinib has off-target effects on FLT3, CSF1R, AXL and C-KIT that infigratinib does not share. Both inhibit VEGFR2 to some extent, though fexagratinib is more potent. Given the importance of these targets for immune cells, differential modulation of the immune system is to be anticipated. The fact that these receptors signal in part through common downstream targets demonstrates that there is a high degree of redundancy, but also that cross-talk is to be expected in these fundamental cellular pathways. This makes it challenging to draw clear conclusions, especially when looking at cell counts alone.

Despite the less receptor specific properties of fexagratinib, infigratinib still more pronouncedly reduced leukocyte proliferation *in vivo*. This suggests that FGF/FGFR signaling may be more relevant for leukocyte proliferation and turnover than previously thought. However, as discussed above, several systemic effects cannot be ruled out, such as changes in the immune status due to systemic FGFR inhibition, which in turn modulate the immune system and the proliferation of its cellular components. The impact of tissue-resident populations and the effects of invading immune cells on them during the chronic stage of the disease are challenging to study, and yet hard to unravel. However, it is conceivable that the altered infiltration generated in peripheral tissues under FGFR inhibition may exert a profound influence on the disease course. In addition, the elimination of autoreactive T cells by promoting regulatory T cells in the central nervous system may also contribute to the long-term effects observed in the chronic phase of EAE.

IV. 3. 3. 6. Conclusions from the *in vivo* findings

In summary, infigratinib has peripheral immunologic efficacy, particularly during preventive treatment, reducing splenic T and B cell counts. In comparison, fexagratinib had less pronounced effects on peripheral immune cells despite similar clinical and

histopathologic outcomes in different treatment approaches for EAE. Fexagratinib may primarily affect the immune microenvironment of the CNS and may not affect proliferation as much, despite being less specific for FGFRs. Thus, polarization and activation of the immune system may be more relevant to the beneficial effects of FGFR inhibition in EAE but it may also involve other mechanisms not captured by the measurements. Both inhibitors may modify the activation and polarization of $CD8^+$ T cells (possibly *via* pP38) and Treg cells, but the evidence is stronger for infigratinib. Speculatively, these effects may have contributed to fewer autoreactive lymphocytes entering the CNS to induce inflammation. Separately, the clinical and histopathologic success of suppressive infigratinib treatment suggests that additional mechanisms are at play. In particular, FGFR inhibition may have induced both myeloid-derived suppressor cells and Treg cells, as infigratinib – the more specific FGFR inhibitor, but not fexagratinib, increased the levels of macrophages/monocytes (possibly due to cell-to-cell cytotoxicity of Treg cells) and $CD4^+$ T cells in the periphery, which do not enter the circulation and cannot invade the CNS. These cells could exert immunosuppressive functions, promote regulatory immune responses and remove autoreactive $CD4^+$ T cells. However, the peripheral immune system is likely not the only site of activation, later stage immune cell modulation and/or changes in cytokine profiles may occur when immunologic action has shifted to the CNS or has become more distributed in lymphoid tissues.

Thus, a notion is proposed where direct immune cell modulation by FGFR inhibition induces regulatory anti-inflammatory responses, explaining the efficacy in EAE. The observed changes in immune cells populations *via* FC cannot fully explain the pharmacological efficiency which warrants further investigation.

IV. 4. CONCLUSIONS

The effects of FGFR inhibitors, specifically dovitinib, fexagratinib and infigratinib, on immune cells in the context of MS and EAE were reviewed here. The *in vitro* studies with human $CD8^+$ T cells (Jurkat cells) and murine microglia (SIM-A9 cells) revealed that all three TKIs were effective in suppressing the proliferation of both cell types. However, they exhibited distinct responses with respect to cytokine expression, FGFR surface expression, and intracellular signaling pathways. The *in vivo* studies using an EAE model as a surrogate for MS demonstrated that infigratinib, a selective FGFR1/2/3 inhibitor, exerted significant effects on peripheral immune cells when administered preventively during the acute phase of EAE. It altered the composition of T cells and B cells in the spleen, modulated the balance of $CD4^+$ and $CD8^+$ T cells, and increased the number of innate immune cells such as dendritic cells, macrophages, and monocytes. However, these effects alone cannot fully explain the efficacy of FGFR inhibition. Because of the dissociation between histopathologic CNS inflammation and peripheral immune activation measured by FC, there must be effects that remain elusive.

Nevertheless, the present studies provide a strong rationale for the immunomodulatory effects of FGFR inhibition in MS. FGFR inhibition appears to act as an anti-inflammatory agent by inhibiting the proliferation of immune cells, particularly T cells and microglia, which are implicated in MS pathology. However, cell numbers alone are unlikely to reflect the nuanced changes that occur; instead, modulation of the cytokine expression profile, activation status, and polarization of lymphocytes is proposed. In addition, FGFR inhibition appears to modulate the ERK, Akt, and P38 pathways in a cell- and substance-specific manner, potentially affecting inflammation and tissue repair processes. Given the complex ramifications of FGFR inhibition at both the cellular and immunological scales, integrating the effects on the microenvironmental background is paramount. Just as there are no single cytokines, antigens or cell types that can be isolated and blamed for MS, the immunological state of the MS brain is multifactorial, redundant and robust. Since FGFR signaling is equally multidimensional, its modulation of this microenvironment may be exactly what this dysregulated state demands, rather than blaming or eliminating specific cell subtypes (Gor et al., 2003). However, more research is needed to better understand this and possibly find more appropriate or additional modulators.

Under this notion, MS may be interpreted as the culmination of several fateful predispositions: Genetic risk factors and cells that are inherently autoreactive and reactive to EBV form the backdrop. Under the influence of epigenetic modifications and a shifted cytokine profile, these cells contribute to a sub-inflammatory milieu and pathological microenvironment likely within the CNS but possibly within the peripheral immune compartments. Under these preconditions an immune system that lacks sufficient regulatory oversight (possibly due to a transformation in plasticity away from Treg cells) initiates a focal cascade that manifests as characteristic MS pathology.
This is where FGFR inhibitors come into play and show great promise in modulating immune responses in MS, both *in vitro* and *in vivo*. They may exert their effects through a variety of mechanisms, including regulation of FGFRs, modulation of ligand-receptor interactions, cross-influencing downstream pathways, profound interference with immune cell populations, and subsequent alteration of cytokine composition.

The insights gained from tumor medicine, where FGFR inhibition was initially employed as an anti-proliferative tool, have led to the realization that FGFR inhibition has immunostimulatory effects and can restore immune vigilance to the tumor microenvironment. Similarly, the MS microenvironment may be imbalanced, and when exposed to FGFR inhibition, it could be reset towards an immune response that is directed against autoreactive cells instead of autoantigens. This would integrate how *in vivo* some immune cells are actually stimulated to proliferate with what was originally hypothesized and what is contrary to what was shown here *in vitro*. Thus, FGFR inhibition would not constitute an anti-inflammatory treatment but a corrective measure that prompts the immune system to consider its attack from within itself instead of attacking its host (CNS).
In addition, there is evidence for FGFR inhibitions' beneficial role in reparative processes, either indirectly or directly. However, additional research is needed to fully elucidate these mechanisms and their implications for MS treatment.

IV. 5. OUTLOOK

The exploration of FGFR inhibition is comparable to solving a complex puzzle, with each study representing a single piece. Each discovery, while illuminating, often raises at least three new questions, prompting us to identify contradictions while trying to focus on relevant and consistent facts. As suggested in the beginning, the approach is reminiscent of speleology, where side passages might lead to dead ends or remain inaccessible, but they could also open up to a new cave of insight. The fundamental concept that must be grasped is (inter)cellular signaling, as it holds the key to unlocking the mysteries of FGFR inhibition and its potential therapeutic implications in MS. Consequently, each piece of the puzzle contributes to the formation of a more comprehensive picture, even as it reveals the vast expanse of what remains to be discovered.
In the context of a smoldering progressive neurodegenerative process that may be underway before the clinical threshold of MS is exceeded, we are faced with the challenge of identifying individuals with clinically silent MS. We are struggling to understand what we are looking for and where to look, with the most promising avenue being the detection of pathological changes within the blood. However, our knowledge is advancing rapidly such as in immune biology, which is now based on cell subpopulations and characterizes interindividual differences. While this study was rather broad in scope, it does attempt to take a step toward individualized therapies through understanding cell signaling. However, given the large amount of redundancy in the immune system and our incomplete understanding, individual targeting of selected pathological cells may not (yet) be the ultimate solution.

Such a tailored, individualized medicine would be ideal in light of the possibility that multiple individual or non-classical antigens may mask the discovery of a single causal targetable antigen in MS (which we already suspect may not exist). However, the notion of a tailored approach to eliminating the culpable immune cell subpopulation may not fully reflect the unorthodox mechanisms that may be at play in MS. In addition, the redundancy of the immune system, particularly with respect to T cells (Attfield et al., 2022), makes this idea less convincing. In addition, it may be overstated to consider it highly specific in the face of unknown side effects of those so-called targeted manipulations. Given our limited understanding of immunology, possible knock-on effects must be carefully and repeatedly evaluated. Perhaps the ‘coarser’ tool of using a growth inhibitory effect that may target particularly those cells involved in the pathology (*i.e.* by having a high turnover) or that are sensitive to the inhibition because it triggers their regulatory features would be sufficient. Given the fact that there is also an excess

of FGF in the lesions themselves and the good favorable side effect profile (maybe with the exception of dovitinib), FGFR inhibition may simply be the most suitable tool currently available for development.

As acknowledged, there are several limitations of the present work's methodology and models that could be remedied in future follow-up studies. In particular, the lack of immunological activation of the cell lines and the low expression and secretion of cytokines should be considered.

One such possibility for further studies would include protein precipitation, or other more sensitive methodology, but most likely, addressing the absent stimuli would be a more useful first step. Immunostimulation of the cell cultures with TCR activation or application of LPS would be a facile solution that had been considered initially, but the disadvantage of this approach would be that such cells would not reflect the activation route in EAE/MS.

The absence of cross-reactions between different cell types in the monocultured *in vitro* setting also warrants an extension of the study. This could be achieved in primary cultures of FACS-acquired cells from EAE-affected mice, which as such were already primed by their immune microenvironment. Further studies could then explore the effects of FGFR inhibition on different subpopulations that were obtained through FACS and their activation states and polarizations. Should such molecular markers prove effective, one potential avenue for translating this into medical appliance would be the harvesting of such marked pathogenic T cell subpopulations from the blood of MS patients. At the very least, this could address differences in immunology between EAE and MS. Even more significantly, it could further elucidate the interplay of immune cells in MS or help identify early stages of MS.

The limited response to FGF2 application of the cell lines utilized in the present study was also perplexing. Further investigations on this topic could employ tissue culture or mixed primary cell cultures, or again co-stimulation with cytokines. It is possible that the FGFR ligands may exert their effects only in such a multicellular microenvironment.

A more comprehensive *in vivo* analysis of the FGF/FGFR downstream signaling components in an MS-like brain, such as in EAE, would also be of great interest. The use of histopathologic multiplex assays or confocal laser scanning microscopy could facilitate the elucidation of the origin of the FGF/FGFR signaling components, their interactions, and even the cytokine responses of subpopulations. As described, FGFRs are highly regulated at the receptor level by dimerization, cell adhesion molecules, and even by other RTKs (Latko et al., 2019). Given the unexpected and unconventional effects, such as receptor downregulation upon inhibition, this may be an important future research objective for understanding inhibition of FGFRs and RTKs in general.

Although not covered here, ROS and glutamate-induced excitotoxicity are also contributing factors in MS (Lau and Tymianski, 2010; Thompson et al., 2018b; Stadelmann et al., 2019; Chávez and Tse, 2021; López-Muguruza and Matute, 2023). P38 represents a link to this pathological aspect of MS because it is involved in excitotoxicity and in the deleterious ROS cascade (Kim et al., 2014). In contrast to the lack of effects on cytokines but the effects on pP38, Kim *et al.* showed that P38 is increased in microglia after treatment with a thiazole derivative that regulates glutamate transmission. This resulted in reduced secretion of pro-inflammatory cytokines such as prostaglandin E2, TNF-α, IL-1β and ROS (Kim et al., 2014). In light of the distinct responses observed, such as these alterations in P38 phosphorylation, further insight into the FGF/FGFR signaling axis in the chronic inflammatory milieu may yield tailored approaches within the downstream cascade that go beyond addressing individual receptors in specificity. Instead, specific downstream molecules could be targeted.

Regarding the marginal effects on the canonical downstream targets pERK and pAkt, the IKK/NF-κB pathway may be worth reassessing in this regard. Interestingly, in hepatic stellate cells, for example, FGFR inhibition reduces inflammation, whereas FGF21 reduces neuroinflammation in stroke models, importantly and non-canonically by reducing NF-κB (Wang et al., 2020a; Wang et al., 2020b). FGF/FGFR signaling also results in NF-κB activation in T cells (Byrd et al., 2003) but NF-κB is not a typical downstream target of para-/autocrine FGFs and was therefore not focused on here. However, it can be modulated by the MAPK pathway. Next to IKK/NF-κB, CD200, which can also act as a FGFR ligand in neurons and microglia would be one of those downstream targets of interest. It is important for self-tolerance and can induce regulatory immune responses (Pankratova et al., 2016; Kotwica-Mojzych et al., 2021; Choe and Choi, 2023). To better categorize these effects on cell subtypes and their activation status, gene expression profiling would be another valuable tool to identify those cells that shape the proposed pathological microenvironment.

A further interesting approach currently being tested is the use of syngeneic splenocytes or erythrocytes coupled to host-specific peptides or proteins. These induce self-tolerance *via* clearance of apoptotic cells and erythrocytes by splenic phagocytes, and induction of Treg cells (Ravishankar and McGaha, 2013). Although the role of the spleen is still unclear, the treatment may be effective for patients with multiple sclerosis and has been tested in humans where it reduced T cell responses to these myelin-related antigens (Bronte and Pittet, 2013; Lutterotti et al., 2013). Similarly, antigen-loaded dendritic cells or, more recently polymers, induce T-cell apoptosis (Lutterotti et al., 2013; Li et al.,

2018a; Pearson et al., 2019; Zubizarreta et al., 2019). Analogously, after FGFR inhibition, stimulatory effects even on $CD4^+$ T cells that are not FGFR-reactive have been observed in tumor models (Kono et al., 2022) as well as in $CD8^+$ T cells (Kato et al., 2019) alluding to the paradoxical pro-inflammatory mechanism of action of FGFR inhibition proposed in the previous sections. Thus, in an interesting parallel to the mechanism of action of FGFR inhibition proposed here, polymer-loaded dendritic cells may open up a new therapeutic approach, especially in the face of a better understanding of pathogenic antigens (such as EBV-related ones that could be used as such polymers). At the very least, it points to our findings here that the peripheral immune system and the spleen may be worth a renewed focus to therapeutically promote regulatory immune responses.

Taken together, several future research directions are conceivable, including the use of more sophisticated *in vitro* cultures, more detailed methodology to dissect intercellular interplay, and a more comprehensive analysis of the FGF/FGFR signaling components, such as the IKK/NF-κB and pP38 pathway. They may disregard or reinforce the notion presented here. Meanwhile, the potential of FGFR inhibition to induce the immune system is a very exciting proposition. This inductive property may allow it to emerge as a motor of regulatory immunologic impetus, resolving the imbalanced microenvironment that proposedly is the pivotal factor in the development of the autoreactive immune system, thereby unraveling the pathology of the debilitating condition, the MS brain.

ABSTRACT

The fibroblast growth factor (FGF) signaling pathway, fundamental to vertebrates, is involved in embryogenesis, a plethora of developmental processes and disease states. Beyond this, the pathway is also a major regulator of tissue homeostasis and is essential for the integrity, regeneration, and repair of the central nervous system (CNS). Its importance in pathological conditions is underscored by accumulating evidence indicating that it plays a critical role in the pathogenesis of both multiple sclerosis (MS) and experimental autoimmune encephalitis (EAE). Interestingly, FGF receptor (FGFR) signaling in T cells and microglia is primarily modulatory and contingent upon the activation status of these cells. This is particularly relevant in diseases such as EAE and MS, where the microenvironment and disease phase are crucial to the drastic temporal change of lesions.

Based on our recent studies, we hypothesized that FGFR inhibition would lead to a reduction in inflammation and immune cell activation and proliferation.

To investigate this, we used an *in vitro* and *in vivo* approach. *In vitro*, we utilized two cell types, human $CD4^+$ T cells (Jurkat cells) and mouse microglia (SIM-A9 cells), and three drugs: the multi-kinase inhibitor dovitinib, the non-specific FGFR1/2/3 inhibitor fexagratinib, and the selective FGFR1/2/3 inhibitor infigratinib. The study examined *in vivo* how infigratinib affected the immune cells both in the blood and in the spleen of mice with EAE.

The study showed that *in vitro,* all three drugs reduce the proliferation of both cell types. However, they had varying impacts on cytokine release, FGFR surface expression, and intracellular signaling pathways.

In vivo, infigratinib demonstrated significant effects on immune cells particularly during the acute phase of EAE, when administered preventively. Infigratinib reduced the proportion of T and B cells in the spleen, altered the balance of $CD4^+$ and $CD8^+$ T cells, and increased the number of innate immune cells.

The data also suggested that FGFR inhibition affects the ERK, Akt, and pP38 pathways in a cell- and substance-specific manner, which itself may have differential context-specific effects on inflammation and tissue repair.

The present work discusses the potential role of FGFR autoregulation, ligand-receptor interactions and cytokine composition in the modulation of the immune response in MS. It proposes the notion of immunostimulatory modulation by FGFR inhibition, which may inhibit the activation and proliferation of peripheral adaptive immune cells and promote cells of the regulatory parts of the adaptive immune system as well as regulatory cells of the innate immune system. In doing so, it could actually reduce inflammation and aid tissue repair in MS by reducing pathological and increasing beneficial immune cells. It is thus postulated, that FGFR inhibition has both anti-inflammatory but also pro-inflammatory properties, stimulating regulatory parts of the immune system.

In conclusion, the microenvironment in inflammatory or autoimmune states plays a pivotal role in modulating FGFR signaling in immune cells, including their proliferation and/or activation. For the understanding of this modulation as well as for the understanding of the immunologic pathophysiology of demyelinating diseases, the downstream pathways of FGFR are highly important.

However, further research is needed to overcome the limitations of the present study and to explore the specific implications of FGFR inhibition on different immune cells and their modulatory effects on inflammation and the CNS environment in MS.

ZUSAMMENFASSUNG

Der Fibroblasten-Wachstumsfaktoren (FGF)-Signalweg ist fundamental für Wirbeltiere, an deren Embryogenese, diversen Entwicklungsprozessen und Krankheiten beteiligt. Darüber hinaus ist er ein wichtiger Regulator der Gewebehomöostase und essenziell für Integrität, Regeneration und Reparatur im zentralen Nervensystem (ZNS). Seine pathologische Bedeutung wird durch zunehmende Evidenz für seine entscheidende Rolle in der Pathogenese der Multiplen Sklerose (MS) und der experimentellen Autoimmunenzephalitis (EAE) unterstrichen. Interessanterweise ist der FGF-Rezeptor (FGFR)-Signalweg in T-Zellen und Mikroglia in erster Linie modulatorisch und hängt von deren Aktivierungszustand ab. Dies ist besonders relevant für Krankheiten wie EAE und MS, bei denen das Mikromilieu und die Krankheitsphase die drastischen zeitlichen Veränderungen der Läsionen bestimmen.
Aufgrund unserer jüngsten Studien war unsere Hypothese, dass eine FGFR-Inhibition Entzündung, die Aktivierung und die Vermehrung von Immunzellen reduziert.
Um dies zu untersuchen, nutzten wir einen *in vitro*- und *in vivo*-Ansatz. *In vitro* wurden zwei Zelltypen verwendet, menschliche $CD4^+$ T-Zellen (Jurkat-Zellen) und murine Mikroglia (SIM-A9-Zellen), sowie drei Medikamente: den Multi-Kinase-Inhibitor Dovitinib, den unspezifischen FGFR1/2/3-Inhibitor Fexagratinib und den selektiven FGFR1/2/3-Inhibitor Infigratinib. *In vivo* wurde in der Studie untersucht, wie Infigratinib die Immunzellen im Blut und in der Milz von Mäusen mit EAE beeinflusst.
Die Studie konnte zeigen, dass alle drei Medikamente *in vitro* die Proliferation beider Zelltypen reduzieren. Die Effekte auf die Zytokinfreisetzung, die FGFR-Oberflächenexpression und die intrazellulären Signalwege variierten jedoch.
In vivo zeigte Infigratinib signifikante Effekte auf Immunzellen, insbesondere während der akuten Phase der EAE, wenn es präventiv verabreicht wurde. Infigratinib reduzierte den Anteil an T- und B-Zellen in der Milz, veränderte das Verhältnis von $CD4^+$-und $CD8^+$ T-Zellen und vermehrte die Zahl angeborener Immunzellen.
Die Daten legen zudem nahe, dass die FGFR-Inhibition die Signalwege ERK, Akt und pP38 zell- und substanzspezifisch beeinflusst, was ihrerseits spezifische Folgen für Entzündung und Gewebsreparatur haben könnte.
Die vorliegende Arbeit diskutiert die mögliche Rolle von FGFR-Autoregulation, Ligand-Rezeptor-Interaktionen und Zytokinzusammensetzung bei der Modulation der Immunantwort von MS. Es wurde die Vorstellung einer modulierenden Immunstimulation durch FGFR-Inhibition entwickelt, die die Aktivierung und Proliferation peripherer adaptiver Immunzellen hemmen und das Zellen der regulatorischen Komponente des adaptiven, sowie des angeborenen Immunsystems induzieren könnte. Durch die Unterdrückung schädlicher und die Förderung vorteilhafter Zellen könnte Inflammation reduziert und die Gewebsreparatur bei MS unterstützt werden. Es wird daher postuliert, dass durch die FGFR-Inhibition sowohl entzündungshemmende als auch -fördernde Eigenschaften *via* regulatorischer Anteile des Immunsystems propagiert werden.
Zusammenfassend spielt die Mikroumgebung bei entzündlichen oder autoimmunen Zuständen eine zentrale Rolle bei der Modulation der FGFR-Signaltransduktion in Immunzellen, einschließlich ihrer Proliferation und/oder Aktivierung. Für das Verständnis dieser und der Immun-Pathophysiologie demyelinisierender Erkrankungen sind die den FGFR nachgeschalteten Signalwege sehr bedeutend.
Weitere Forschungsarbeiten sind jedoch erforderlich, um die Einschränkungen der vorliegenden Studie zu umgehen und die spezifischen Auswirkungen der FGFR-Inhibition auf verschiedene Immunzellen und ihre modulierende Wirkung auf Entzündung und das MS-ZNS-Milieu zu untersuchen.

LIST OF ABBREVIATIONS

APC	Professional antigen presenting cell
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AXL	Anexelekto receptor tyrosine kinase
BBB	Blood-brain barrier
BSA	Bovine serum albumin
Cbl	Casitas B-lineage lymphoma
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
CSF1R	Colony stimulating factor 1 receptor
CT	Cycle threshold
CXCR	CXC chemokine receptor 3
DAPI	4’,6-Diamidino-2-phenylindol dihydrochloride
DDPBS	Detachment DPBS
DIS	Dissemination in space
DIT	Dissemination in time
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMT	Disease-modifying therapies
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DP	Double positive
DPBS	Dulbecco’s phosphate buffered saline
EAE	Experimental autoimmune encephalomyelitis, if not stated otherwise: Murine MOG35-55 induced EAE on C57BL/6J background
EBNA1	Epstein-Barr nuclear antigen 1
EBV	Epstein-Barr virus
ECACC	European Collection of Authenticated Cell Cultures
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMR	EGF-like module-containing mucin-like hormone receptor-like 1
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum

FC	Flow cytometry
FcR	Fragment crystallizable receptor
FCγR	Fc gamma receptor 1
FELASA	Federation of European Laboratory Animal Science Associations
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FGFRL1	FGFR-like 1 (or FGFR5)
FLT	Fms like tyrosine kinase 3
ForMED	Medizinisches Forschungszentrum Seltersberg
FOXP3	forkhead box P3
FP	Forward primer
FRS	Fibroblast growth factor receptor substrate 2
FSC-A	Forward scatter - Area
FSC-H	Forward scatter - Height
gDNA	Genomic DNA
GIT	Gastro-intestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hepaCAM	Hepatocyte cell adhesion molecule
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGF1R	Insuline-like growth factor 1 receptor
IL-12A	Interleukin 12A
IL-1β	Interleukin 1 beta
IL-21	Interleukin 21
IL-6	Interleukin 6
INT	2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride)
ITGAM	Integrin alpha M
JCV	John Cunningham virus
JLU	Justus-Liebig-Universität
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharides
LY6G	Lymphocyte antigen 6G
LYN	lck/yes-related novel tyrosine kinase
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MOG35-55	Myelin oligodendrocyte glycoprotein peptide 35-55
mRNA	Messenger ribonucleic acid

MS	Multiple sclerosis
MSIF	Multiple Sclerosis International Federation
mTOR	Mechanistic target of rapamycin
NCAM	Neural cell adhesion molecule
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
OPC	Oligodendrocyte progenitor cell
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pi	Post injection
PI3K	Phosphoinositide 3-kinase
PLCγ	Phospholipase C gamma
PKC	Protein kinase C
PML	Progressive multifocal leukoencephalopathy
PNS	Peripheral nervous system
PPMS	Primary progressive MS
PRR	Pattern recognition receptor
PTPRC	Protein tyrosine phosphatase, receptor type, C
RAW	Relapse-associated worsening
RNA	Ribonucleic acid
RP	Reverse primer
rpm	Rotations per minute
RRMS	Relapsing-remitting MS
RTK	Receptor tyrosine kinase
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SNPs	Single-nucleotide polymorphisms
SPMS	Secondary progressive MS
SSC-A	Side scatter - Area
SSC-H	Side scatter - Height
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TBST	TBS-Tween
TCR	T cell receptor
TEMED	Tetramethyl ethylenediamine
TGF-β	Transforming growth factor β
Th1 cell	$CD4^+$ T helper 1 cell
Th2 cell	$CD4^+$ T helper 2 cell
Th17 cell	$CD4^+$ T helper 17 cell
TKI	Tyrosine kinase inhibitor
TNF-α	Tumor necrosis factor α

Treg	Regulatory $CD4^+$ T cell
Tris	Trishydroxymethyl aminomethan
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule 1
VEGFR	Vascular endothelial growth factor receptorregulatory T cells (Treg cells)

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LITERATURE

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