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Research Article

The impact of fingolimod on Treg function in brain ischaemia

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Fingolimod has generally shown neuroprotective effects in stroke models. Here, we tested the hypothesis that fingolimod modulates T-cell cytokine production towards a regulatory phenotype. Second, we investigated how fingolimod altered the Treg suppressive function and the sensitivity of effector T cells to regulation. Mice that had undergone the permanent electrocoagulation of the left middle cerebral artery received saline or fingolimod (0.5 mg/kg) daily for 10-days post-ischaemia. Fingolimod improved neurobehavioural recovery compared to saline control and increased Treg frequency in the periphery and brain. Tregs from fingolimod-treated animals had a higher expression of CCR8. Fingolimod increased the frequencies of CD4⁺IL-10⁺, CD4⁺IFN- γ ⁺ and CD4⁺IL-10⁺IFN- γ ⁺ cells in spleen and blood, and CD4⁺IL-17⁺ cells in the spleen, with only minor effects on CD8⁺ T-cell cytokine production. Treg from post-ischaemic mice had reduced suppressive function compared to Treg from non-ischaemic mice. Fingolimod treatment rescued this function against saline-treated but not fingolimod-treated CD4⁺ effector T cells. In conclusion, fingolimod seems to improve the suppressive function of Treg post-stroke while also increasing the resistance of CD4⁺ effector cells to this suppression. Fingolimod's capacity to increase both effector and regulatory functions may explain the lack of consistent improvement in functional recovery in experimental brain ischaemia.

Keywords: Fingolimod · FTY720 · Ischaemia · Regulatory T cells · Treg · Stroke



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Fingolimod (FTY720) was introduced as an FDA-approved drug for the treatment of multiple sclerosis (MS) in 2010 under the brand name Gilenya [1]. The principal mechanism of action of fingolimod is thought to involve its action as a functional antagonist of the S1P1 receptor. This function prevents the movement of

potentially neurotoxic T cells from the lymph nodes to the peripheral circulation and to the central nervous system [2]. Due to the negative role of T cells in the evolution of acute brain ischaemia, and other commonalities between MS and stroke pathophysiology, fingolimod has been trialled in both pre-clinical and clinical ischaemic stroke [3]. The drug has been reported to reduce infarct size and ameliorate neurological deficits in transient and permanent models of experimental cerebral ischemia in both mice and rats [4, 5]. A meta-analysis concluded infarct volume was reduced by 30% and behavioural outcome improved by 34% [4]. Pilot

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ischaemic stroke clinical studies demonstrated fingolimod to be safe and effective, alone or when combined with thrombolysis [6–8]. Mechanistically, the lymphopenia associated with fingolimod treatment in MS may also play a major role in the neuroprotection observed in stroke [9]. However, effects unrelated to recirculating lymphocytes are also likely to be involved [10–17].

We previously demonstrated that fingolimod increased a subpopulation of CD4⁺T cells termed regulatory T cells (or ‘Tregs’; defined as being CD4⁺CD25⁺Foxp3⁺ cells) in blood and secondary lymphoid organs at 7-day post-permanent middle cerebral artery occlusion (pMCAO) and enhanced the number of brain infiltrating FoxP3⁺ cells in young, aged and hyperlipidaemic mice [18]. We also determined that the frequency of Tregs positively correlated with behavioural recovery to ischaemic stroke in saline-treated mice, suggesting a potential neuroprotective effect of this T-cell population [19]. Indeed, previous research has shown Tregs improve disease outcomes in stroke, through mechanisms that include IL-10 or TGF- β secretion, with the only detrimental effects thought to be a role in secondary microthrombosis [20].

Building on our previous evidence of fingolimod’s effect on Treg frequency post-ischaemia with the known beneficial effect of this drug in pre-clinical models of stroke, the primary aim of this study was to test the hypothesis that fingolimod shifts T cells towards a functionally suppressive phenotype. We determined the impact of fingolimod on Treg suppressive capacity and the sensitivity of conventional T cells to such suppression. Second, we focussed on determining fingolimod’s in vivo effects on CD4⁺ and CD8⁺ T-cell cytokine production and chemokine receptor expression, at 10-days post-pMCAO. Recent reports suggest Tregs that accumulate in brain in the weeks following ischaemic stroke highly express CCR6 and CCR8 chemokine receptors [21]. CCR5, which recognizes CCL3, CCL4 and CCL5, is critical for Treg recruitment to the ischaemic site and enhances Treg suppression [22]. Increased Treg expression of CCR6, which only binds CCL20, has been associated with more severe disease in an experimental model of MS [experimental autoimmune encephalomyelitis, (EAE)] [23]. A critical role of CCL1, and its receptor CCR8, on Treg in suppressing autoimmunity has been demonstrated in EAE. Thus, we wished to determine if these receptors may be involved in the fingolimod-mediated recruitment of Tregs to the infarct site.

A 10-day regimen of fingolimod (0.5 mg/kg) was chosen as our previous research demonstrated this would significantly enhance the Treg phenotype in spleen as well as maximize the number of Tregs that we could retrieve from infarcted brain tissue [18]. A secondary aim of this study was to confirm whether the 10-day course of fingolimod would improve neurobehavioural recovery post-ischaemia.

Results

Fingolimod attenuates neurobehavioural deficits post-pMCAO

The impact of fingolimod (0.5 mg/kg) treatment on functional recovery, at 3- and 10-days post-stroke, was assessed by quan-

tifying the effect of the drug on ischaemia-induced changes in cylinder test performance as compared to saline control. Before stroke induction (‘pre-behaviour’ baseline), no significant difference was observed between saline- and fingolimod-treated mice ($p = 0.8857$). An increased deficit score, reflecting poorer performance, in saline-treated mice was confirmed at days 3 ($p = 0.0003$) and 10 ($p = 0.0017$) post-brain ischaemia compared to pre-stroke baseline (Fig. 1). In contrast, fingolimod-treated mice did not show different performances at baseline, day 3 and day 10 ($p > 0.9999$). No significant differences were noted between saline- and fingolimod-treated mice in terms of mouse weight ($p = 0.9872$) or checklist neuroscore ($p = 0.7483$). As expected, the time since ischaemia positively impacted on recovery for both metrics (checklist score: $p < 0.0001$, mouse weight $p < 0.0001$). In contrast, fingolimod did not affect lesion size ($p = 0.6647$), ipsilateral/contralateral ratio ($p = 0.6433$) or tissue loss ($p = 0.5321$) versus saline control (Supporting Information Fig. 1).

Fingolimod increases the frequency of CCR8⁺ Tregs in multiple organs post-pMCAO

To determine if the modulation of chemokine receptor expression on Tregs is associated with fingolimod’s effect on previously observed increased Treg numbers in the brain [18], we determined the surface expression of CCR5, CCR6 and CCR8 in both the brain and periphery of mice treated or untreated with fingolimod post-stroke, compared to mice who did not have stroke (naïve). As fingolimod differentially affects the sequestration of Treg and effector T cells [24], we examined the frequency of Treg in multiple tissues. Intestinal IL-17⁺ $\gamma\delta$ T cells have also been shown to migrate to the meninges post-ischaemia [25].

We first confirmed the fingolimod-mediated increase in the Treg proportion of CD4⁺ T cells, observed in previous studies (Supporting Information Fig. 2) [18]. This was observed in the periphery, except for Peyer’s patches, and in the post-ischaemic brain (spleen: $p = 0.0258$, draining lymph nodes: $p = 0.033$, inguinal lymph nodes: $p = 0.0013$, blood: $p = 0.0044$, brain: $p = 0.0408$, compared to saline-treated animals). Fingolimod reversed the number of T cells that were found in the ischemic brain (Supporting Information Fig. 3).

The frequency of CD4⁺ Tregs that expressed CCR5 or CCR6 was not significantly altered by brain ischaemia or by fingolimod treatment at 10-days post-ischaemia in any organ (Fig. 2). CCR8 expression on Tregs, however, was significantly affected by stroke and by fingolimod treatment in most tissues. Brain ischaemia significantly reduced the frequency of CCR8⁺Tregs in spleen ($p = 0.0156$), non-draining lymph nodes ($p = 0.0235$) and draining lymph nodes ($p = 0.0489$). Ischaemia caused a significant increase in CCR8⁺ Tregs in blood. Fingolimod significantly increased CCR8⁺ Treg frequency in spleen ($p = 0.0007$), draining lymph nodes ($p = 0.0015$), non-draining lymph nodes ($p = 0.0067$), blood ($p = 0.0105$) and brain ($p = 0.0248$) compared to saline-treated animals, with levels restored to (spleen,

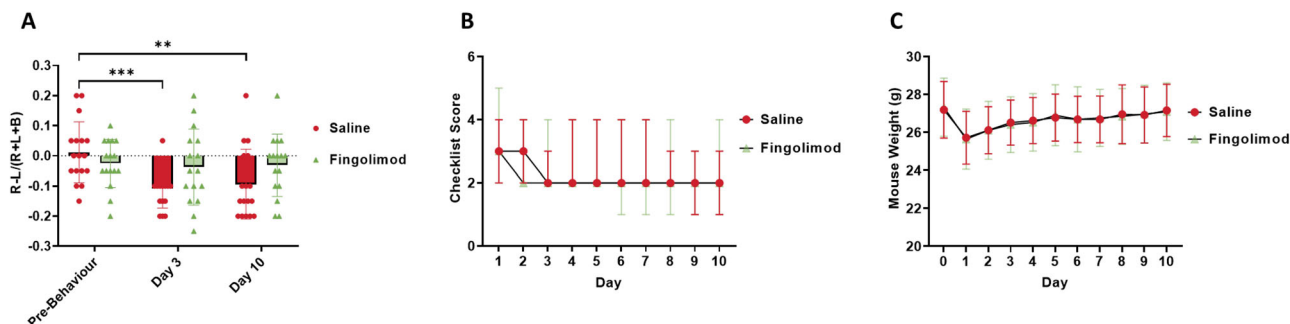


Figure 1. Neurobehavioural and clinical outcome post-stroke: (A) neurological deficit score as assessed by the cylinder test; (B) total checklist neuroscore; (C) body weight in saline- and fingolimod-treated mice. Two-way repeated measures ANOVA followed by post hoc Tukey's multiple comparisons was used to investigate differences between saline- and fingolimod-treated groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to saline). Plots A and C depict mean $\pm$ SD. Plot B depicts median (with range). $N = 16$ – 17 per group, pooled from eight individual experiments.

draining lymph nodes, non-draining lymph nodes) or exceeding (blood) those observed in naïve, non-pMCAO controls. No ischaemia- or fingolimod-induced changes were observed in CCR8⁺ Tregs in Peyer's patches. Overall, these findings demonstrate that fingolimod can restore or increase CCR8 expression on Treg in ischemic mice. Given the proposed role of CCR8 in Treg trafficking to the post-ischaemic brain [26], this trafficking may contribute to the enhanced frequency of Tregs observed in brains of fingolimod-treated mice (Supporting Information Fig. 2).

Fingolimod enhances secretion of IFN- γ , IL-17 and IL-10 by CD4⁺-T cells post-pMCAO

Next, we investigated how fingolimod modulates post-stroke cytokine expression by all T cells from mice post-stroke. The expression of IFN- γ , IL-17 and IL-10, subsequent to in vitro stimulation with PMA and ionomycin, were used as markers of Th1, Th17 and Treg cells, respectively, for CD4⁺ T cells. We also examined the effect of fingolimod on CD8⁺ T-cell cytokine expression.

Brain ischaemia alone did not influence IL-10, IFN- γ or IL-17 expression in CD4⁺ cells in any tissue. Fingolimod significantly increased the frequency of IL-10 and IFN- γ expressing CD4⁺ T cells in the spleen ($p = 0.0254$, $p = 0.0006$ for IL10 and IFN- γ , respectively) and blood ($p = 0.0027$ for IL-10, and $p = 0.0232$ for IFN- γ) compared to saline-treated animals (Fig. 3). Elevated frequencies of CD4⁺IL-17⁺ cells were noted in fingolimod-treated spleen ($p = 0.0168$). Expression of IL-10, IFN- γ or IL-17 by CD4⁺ T cells from lymph nodes, including Peyer's patches, were not significantly different in fingolimod-treated mice compared to saline-treated mice. Fewer changes were detected in CD8⁺ T cells. Brain ischaemia alone increased the frequency of CD8⁺IFN- γ ⁺ cells ($p = 0.0487$ compared to naïve) in blood; this was not further affected by fingolimod treatment (Fig. 4). Fingolimod increased the frequency of CD8⁺IL-10⁺ cells in non-draining lymph nodes ($p = 0.0035$). We investigated whether brain ischaemia and/or fingolimod-induced polyfunctional CD4⁺ and CD8⁺ T cells in blood or whether they caused selective expansion of polarized

Th1, Th17 or Treg cells (Fig. 5). Fingolimod treatment resulted in significantly increased frequencies of single-positive CD4⁺IFN- γ ⁺ ($p = 0.0378$), single-positive CD4⁺IL-10⁺ ($p = 0.0035$) T cells and double-positive CD4⁺IFN- γ ⁺IL-10⁺ cells ($p = 0.0428$). No effects of either brain ischaemia or fingolimod treatment on CD8⁺ T cells were observed. These results demonstrate that fingolimod promotes the secretion of one cytokine among unpolarized cells or causes the expansion of polarized IFN- γ secreting CD4⁺ Th1 cells or IL-10 secreting regulatory T cells. In contrast, brain ischaemia significantly increased the proportion of single-positive CD4⁺IL-17⁺Th17 cells; fingolimod reduced this population to frequencies observed in untreated animals.

Fingolimod rescues Treg function against saline-treated but not fingolimod-treated Tconv cells

Based on the effect on CD4⁺ cytokine production, we hypothesized that fingolimod's lack of effect on clinical outcome may be due to its dual effect on both regulatory and effector functions. To test this hypothesis, splenic Tregs (CD4⁺ CD25⁺) were isolated and pooled ($n = 3$ per pool) from post-ischaemic mice treated for 10 days with either saline (S-Treg) or fingolimod (0.5 mg/kg) (F-Treg) or from naïve controls (N-Treg) ($n = 2$ per pool). The ability of these Tregs to suppress the proliferation of conventional CD4⁺ T cells from each treatment group (N-Tconv, S-Tconv and F-Tconv), in the presence of plate-bound anti-mouse CD3 and soluble anti-mouse CD28, was assessed.

When Tconv and Treg were both derived from naïve mice, a linear increase in Treg suppression was observed as the number of Treg increased in the culture (Fig. 6A, black line). Tregs isolated from saline-treated post-ischaemic mice (S-Treg) were significantly less suppressive than N-Treg against N-Tconv at a 1:1 ratio ($p = 0.0062$) (Fig. 6A, red line) but had equivalent suppressive capacities at lower Treg:Tconv ratios. S-Tregs were also significantly less suppressive compared to N-Treg when co-cultured with S-Tconv ($p = 0.0039$ at 1:1 ratio) (Fig. 6B). No difference in efficacy was noted when N-Treg and S-Treg were co-cultured with F-Tconv (Fig. 6C). There was a trend for higher suppression by

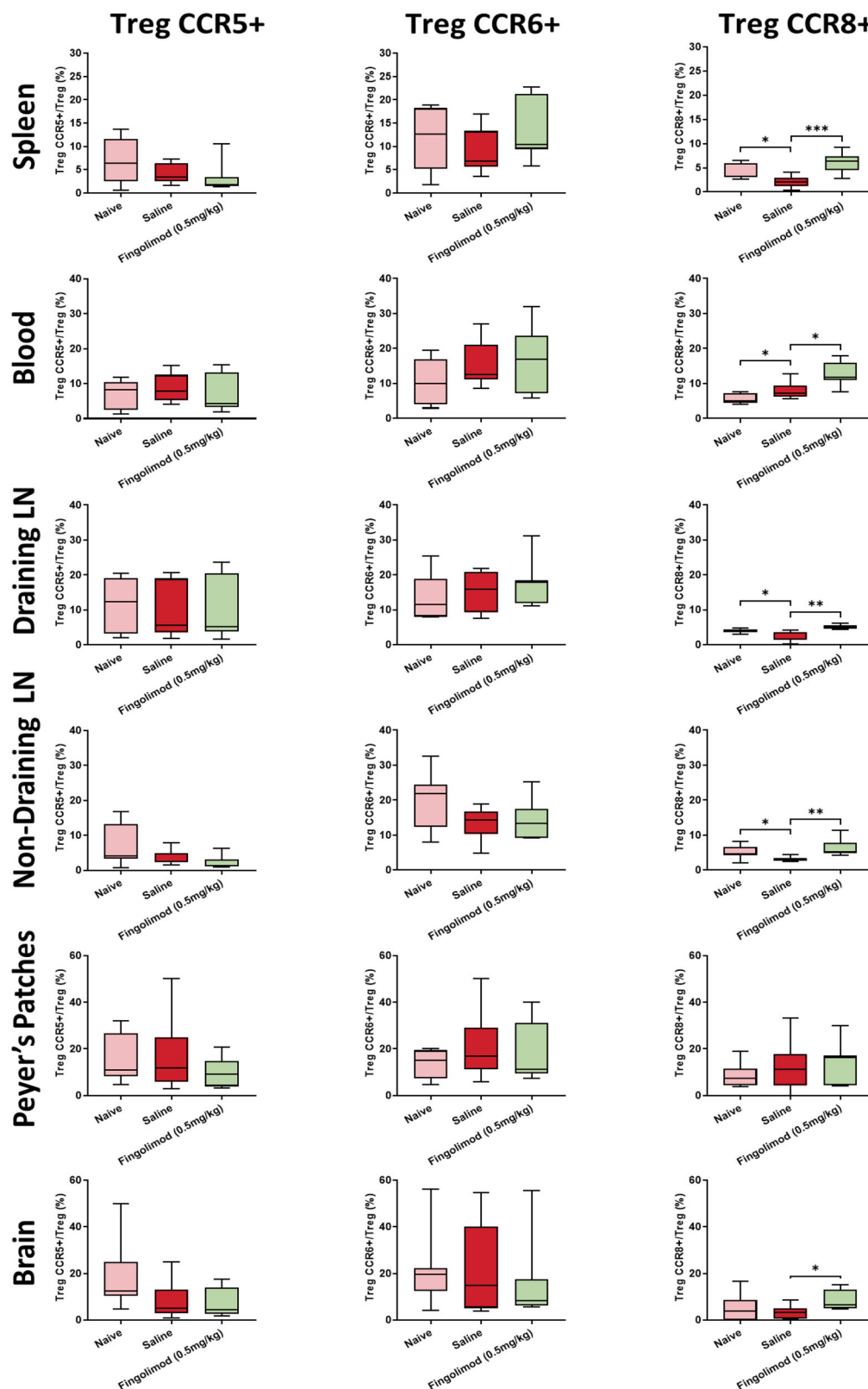


Figure 2. Expression of CCR5, CCR6 and CCR8 on Treg, post-brain ischaemia with or without fingolimod treatment: frequency of CCR5⁺, CCR6⁺ and CCR8⁺ CD4⁺CD25⁺Foxp3⁺ Treg cells, analysed by flow cytometry, in blood, brain and secondary lymphoid tissue in response to saline or fingolimod (0.5 mg/kg) treatment post-brain ischaemia in mice (t = 10 days) (n = 7 per group, pooled from five individual experiments). Two-sided, independent-sample t-tests investigated differences between two groups (*p < 0.05, **p < 0.01, ***p < 0.001 as compared to saline). Box and whisker plots display the 90/10 percentile at the whiskers, the 75/25 percentiles at the boxes and the median in the centre line.

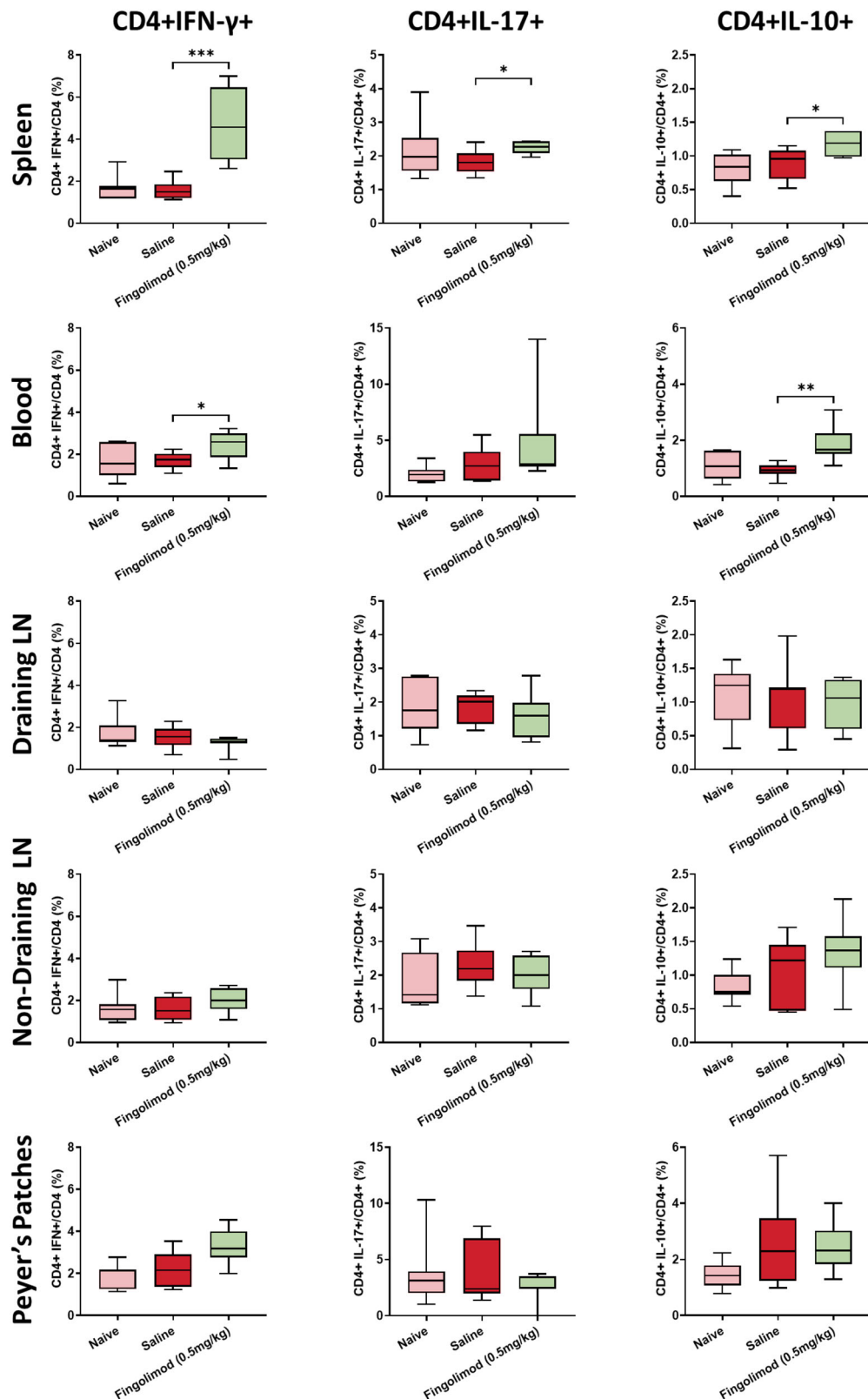


Figure 3. CD4⁺ T-cell cytokine production, post-brain ischaemia with or without fingolimod treatment: frequency of CD4⁺IFN- γ ⁺, CD4⁺IL-17⁺ and CD4⁺IL-10⁺ T cells, analysed by flow cytometry, in blood and secondary lymphoid tissue in mice treated with saline or fingolimod (0.5 mg/kg) post-brain ischaemia in mice (t = 10 days) or in untreated naïve mice (n = 7 mice per group, pooled from five individual experiments). Two-sided, independent-sample t-tests investigated differences between two groups (*p < 0.05, **p < 0.01, ***p < 0.001 as compared to saline). Box and whisker plots display the 90/10 percentile at the whiskers, the 75/25 percentiles at the boxes and the median in the centre line.

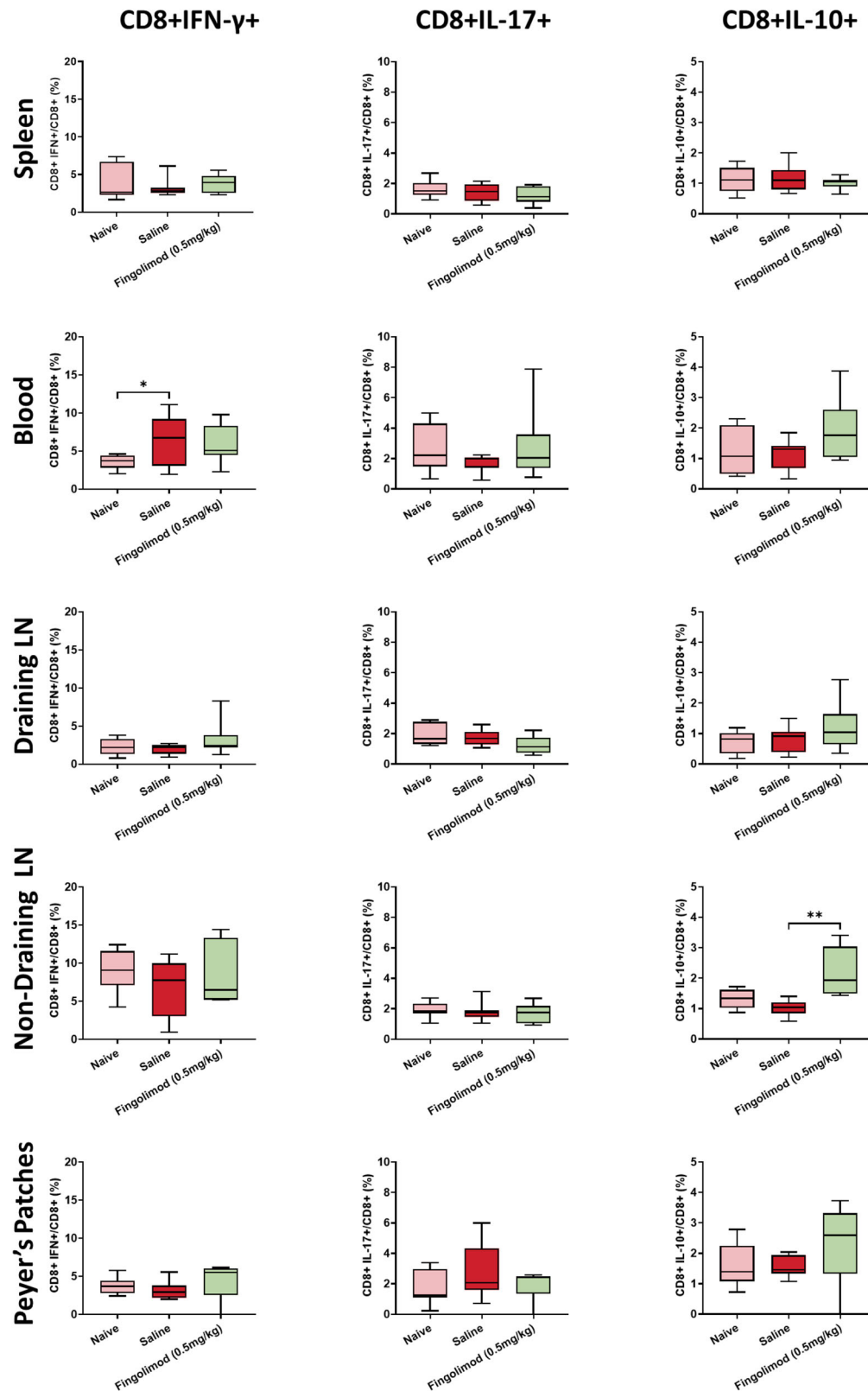


Figure 4. CD8⁺ T-cell cytokine production, post-brain ischaemia with or without fingolimod treatment: frequency of CD8⁺ IFN- γ ⁺, CD8⁺ IL-17⁺ and CD8⁺ IL-10⁺ cells analysed by flow cytometry, in blood and secondary lymphoid tissue in mice treated with saline or fingolimod (0.5 mg/kg) post-brain ischaemia in mice (t = 10 days) or in untreated naïve mice (n = 7 mice per group, pooled from five individual experiments). Two-sided, independent-sample t-tests investigated differences between two groups (*p < 0.05, **p < 0.01, ***p < 0.001 as compared to saline). Box and whisker plots display the 90/10 percentile at the whiskers, the 75/25 percentiles at the boxes and the median in the centre line.

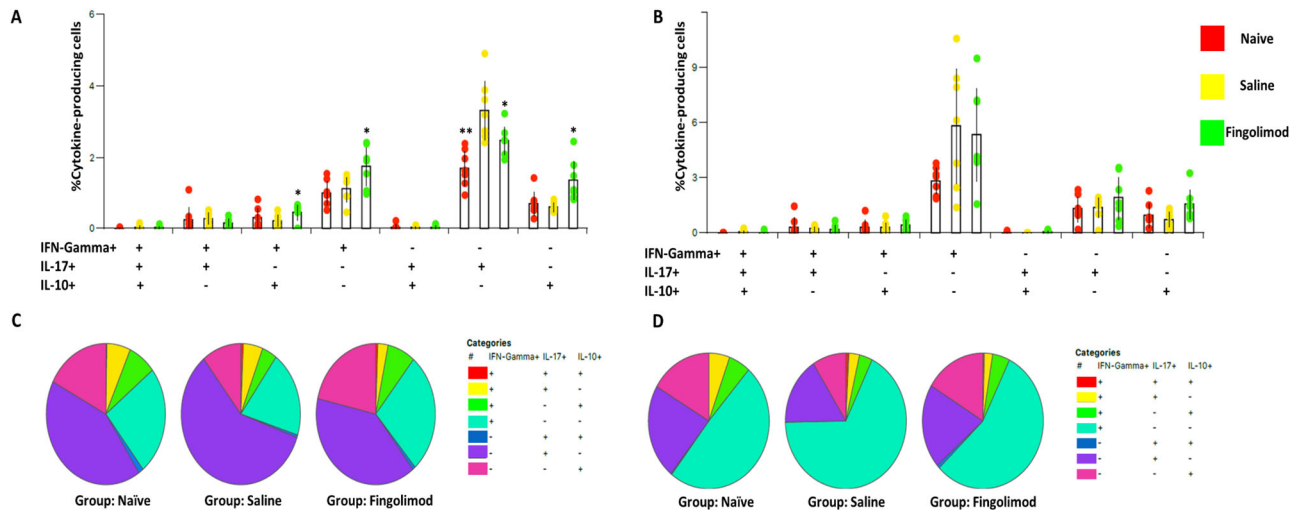


Figure 5. Polyfunctionality of CD4⁺ and CD8⁺ T-cell cytokine production, post-brain ischaemia with or without fingolimod treatment: the percentage of IFN- γ , IL-17- and/or IL-10-producing CD4⁺ and CD8⁺ T cells, analysed by flow cytometry, in blood of naïve, saline-treated and fingolimod-treated mice. Bar graphs display the percentage of single, double and triple cytokine-positive CD4⁺ (A) and CD8⁺ (B) cells in naïve (red), saline-treated (yellow) or fingolimod-treated (green) mice ($n = 7$, pooled from five individual experiments). Each dot represents one mouse. Pie charts (C/D) display relative percentages of CD4⁺ CD8⁺ T cells that are single, double or triple cytokine producers. Graphs were generated after performing a Boolean analysis in FlowJo and data analysis in SPICE software. Two-sided, independent-sample t-tests investigated differences between two groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with saline-treated mice).

Treg from fingolimod-treated mice (F-Treg) of N-Tconv, compared to S-Treg at low ratios. F-Tregs were also significantly more suppressive compared to S-Treg in suppressing S-Tconv ($p = 0.0378$ at 1:1 ratio), demonstrating that the drug enhances Treg suppressive capacity after brain ischaemia. However, no difference was seen between the suppressive capacity of Treg from saline- (S-Treg) and fingolimod (F-Treg)-treated mice when F-Tconv were the targets of suppression ($p = 0.1681$ at 1:1 ratio) (Fig. 6C). Furthermore, F-Tregs were significantly more capable of inhibiting N-Tconv ($p = 0.0364$ at 1:1 ratio) and S-Tconv ($p = 0.0034$ at 1:1 ratio) proliferation than F-Tconv. These results support our hypothesis that fingolimod is having a dual effect on suppressive function and on resistance to suppression.

Discussion

This study primarily investigated the effect of fingolimod on modulating T-cell function to better understand the disparity between the drug's effect on T cells and the variable effect on outcome in experimental stroke studies. Fingolimod-treated mice displayed no neurological deficit at days 3 and 10 post-pMCAO, in contrast to saline-treated post-ischaemic mice. Fingolimod increased the frequency of CCR8⁺ Tregs and increased single-positive CD4⁺ T cells expressing IL-10 or IFN- γ and dual-positive CD4⁺ IL-10⁺ IFN- γ ⁺. Fingolimod treatment restored Treg suppressive function that was reduced in brain ischaemia, but the drug also increased the resistance of effector T cells to suppression. These findings

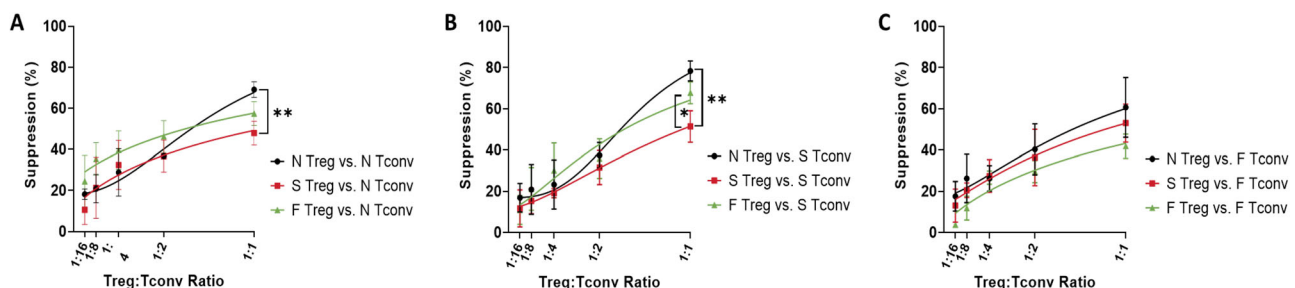


Figure 6. Treg suppressive capacity post-brain ischaemia with or without fingolimod treatment. Suppressive capacity of Tregs and susceptibility of Tconv to suppression, with cells isolated from naïve, saline-treated and fingolimod-treated mice: (A) naïve (black), saline-treated (red) and fingolimod-treated (green) Treg suppressive capacity versus naïve CD4⁺ cells at various ratios; (B) naïve (black), saline-treated (red) and fingolimod-treated (green) Treg suppressive capacity versus saline-treated post-ischaemic CD4⁺ cells at various ratios; (C) naïve (black), saline-treated (red) and fingolimod-treated (green) Treg suppressive capacity versus fingolimod-treated post-ischaemic CD4⁺ cells at various ratios. One-way ANOVA tests with post hoc Tukey's multiple comparisons were performed to investigate differences between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to saline). Curves depict mean $\pm$ SD for $n = 3$ replicate experiments. Each sample in each experiment is a pool of samples from $n = 2$ (naïve) or 3 (saline/fingolimod) mice.

indicate that fingolimod broadly enhances the function of both regulatory and effector CD4⁺ T cells with a lesser effect on CD8⁺ T cells. This dual effect likely underlies the lack of consistent efficacy of fingolimod in experimental stroke.^f

Our finding that fingolimod treatment improved neurobehavioural recovery post-ischaemia is in line with a recent pre-clinical meta-analysis [4]. However, most studies included in the analysis that recorded a positive treatment effect used a transient model of MCAO as opposed to the pMCAO employed here [4, 27]. Of the three studies wherein fingolimod failed to improve experimental stroke outcomes, one employed the same surgical model described above [28]. We previously observed in this pMCAO model that a 10-day course of once daily fingolimod treatment had no effect on functional outcome in young mice [29], and fingolimod only improved functional recovery in aged mice, despite increased CD4⁺ Treg frequency in young, aged and ApoE^{-/-} cohorts [19]. Pooling data from all fingolimod-treated mice under study, we observed Treg frequency negatively correlated with 7-day improvement. We hypothesize that nuisance factors (e.g. operator, animal husbandry and microbiome) may affect the balance between the pro-inflammatory and anti-inflammatory effects of fingolimod and, hence, its benefit in stroke.

To date, both pre-clinical and clinical stroke studies have focused on temporal shifts in circulating levels of chemokines, such as CCL5, CCL20 and CCL1, in acute ischaemic stroke (AIS) [30]. The corresponding chemokine receptors (CCR5, CCR6 and CCR8) also play a role in the neuroprotective activity of Tregs [31]. CCR5 signalling enhanced the immunosuppressive function of adoptively transferred Tregs via the induction of PD-1, CTLA-4 and CD39 [22]. However, chemokine receptor expression on Tregs may also negatively influence neuroprotection, as shown in a study in which human Tregs positively correlated with better functional outcome at 3 months, whereas CCR5⁺ Tregs showed a negative association [32]. In the current study, brain ischaemia or fingolimod had no effect on the frequency of Tregs expressing CCR5 or CCR6 at day 10. Our finding is in contrast with those found in a transient MCAO study in the subacute (14 days) stage [22]. It is possible a larger lesion, coupled with the inflammatory changes seen during reperfusion, is required to stimulate acute changes in chemokine receptor expression [33]. Ischaemia-induced changes in CCR8⁺ Tregs were observed in this study, suggesting focal injuries of the cortex can still produce peripheral immunomodulation in the absence of reperfusion. Notably, the absence of changes within Peyer's patches implies that ischaemia-induced changes in gut-derived T cells may not be as pronounced in mild stroke compared to larger infarct models [34]. The time since ischaemia onset may also affect CCR expression on Tregs [35]. Another study demonstrated that CCL1–CCR8 actions increase CCR8⁺ Tregs in the injured brain and improve neurological function [26]. Overall, it is likely that chemokine receptors such as CCR8 play a role in Treg-afforded neuroprotection, and this mechanism may underlie the improved recovery observed in fingolimod-treated mice in this study. Recent research in lung carcinomas shows S1PR1 levels positively correlate with CCR8 expression on Tregs, and it is possible that S1PR1 agonism by fin-

golimod leads to an increase in the frequency of CCR8⁺ Treg post-ischaemia [36]. Fingolimod may also polarize microglia towards an M2 phenotype which, through the secretion of CCL1, then stimulates CCR8 expression on Tregs [3, 37]. A greater understanding of the changes in the CCL1–CCR8 axis post-ischaemia may allow researchers to pinpoint the optimum time at which to intervene with fingolimod to exploit this mechanism.

In MS, myasthenia gravis and colitis, fingolimod administration was associated with reduced levels of IFN- γ and IL-17 coupled with enhanced production of IL-10 [38–40]. Here, fingolimod expanded single-positive IFN- γ -producing Th1 and IL-10-producing Treg cells but decreased the frequency of Th17 cells in blood. In agreement with previous studies, no major impact of fingolimod treatment on cytokine-producing CD8⁺ cells was observed [41, 42]. Long-term fingolimod treatment may be required to reduce pro-inflammatory CD8⁺ function [43]. In MS, fingolimod inhibited the plasticity of Treg [44]. The time since treatment initiation may be critical. A transient enhancing effect of fingolimod on IL-17 and IFN- γ has been shown, emerging in the first few weeks or months of treatment and fading thereafter [41, 45]. Mechanistically, this increase in IL-17/IFN- γ ⁺ cells may be explained by a relative expansion of effector memory T cells (which contain Th1/Th17 cells) upon fingolimod treatment in this autoimmune disease [46]. Overall, fingolimod treatment in our study promoted both pro- and anti-inflammatory single cytokine-producing CD4⁺ cells in the post-ischaemic mouse at a single measurement timepoint. This dual, pro- and anti-inflammatory effect of fingolimod on CD4⁺ T cells has potential consequences for clinical trials or treatment regimens that would assess short-term fingolimod treatment.

Understanding the balance of T-cell cytokines may also aid in understanding the impact fingolimod had on Treg suppressive function in this study. In line with clinical research on stroke, we demonstrated that the induction of brain ischaemia reduced Treg suppressive function [47, 48]. The administration of fingolimod improved Treg suppressive function compared to saline-treated post-pMCAO mice. This observation is in line with published MS results [49, 50]. However, in our study, fingolimod-treated Tregs were less capable of inhibiting the proliferation of fingolimod-treated CD4⁺ cells as compared to naïve/saline-treated effectors. These observations suggest an effect of fingolimod on both Treg and on CD4⁺ effector T cells. Previous studies of MS in humans and SIV infection in macaques demonstrate fingolimod is capable of rapidly increasing the frequency of proliferating CD4⁺ T cells [40, 51]. The enhanced secretion of pro-inflammatory cytokines by CD4⁺ cells (noted above) may also produce T-cell phenotypes with reduced suppressive capacity. It is possible an alternative suppression assay employing dendritic cells (DCs) may have more accurately captured an in vivo situation, as prior work suggests it is through DCs that fingolimod modulates effector T-cell proliferation (as opposed to any direct effect of the drug on T cells themselves) [52]. However, the assay we used demonstrates that fingolimod did impact on the capacity of a Treg to directly suppress T effector cell proliferation. The use of in vitro systems wherein only the Treg portion was pre-incubated with fingolimod may also

have overestimated its effect on suppressive capacity [49]. Overall, our study reveals that although fingolimod does enhance the suppressive capacity of Tregs, it also promotes resistance among CD4⁺ effector T cells to regulation. This may limit the usefulness of fingolimod as a Treg-expanding therapy.

The effect of fingolimod on cellular function was assessed at a single timepoint and additional, and perhaps opposing, effects in either the acute or recovery stage of ischaemic stroke cannot be ruled out. Drug dose and treatment duration could be refined to optimize the balance between Treg and effector T-cell functions. In an MS clinical study, 3 months of fingolimod treatment improved Treg suppressive capacity and reduced effector T-cell proliferation [50]. Given this enhanced suppression remained at 6 months, it is possible chronic treatment may be required to produce the same outcomes in the post-ischaemic patient.

Despite recent conflicting pre-clinical results, fingolimod continues to be assessed as a potential stroke therapy. This study shows that fingolimod also mediates an increase in CCR8⁺ expression on Tregs trafficking to the infarcted brain and upregulates Treg suppressive function. An increased production of IL-10 may be responsible, but other mechanisms are possible, such as the enhanced conversion of naïve/central Treg cells to an effector phenotype, or fingolimod-induced upregulation of surface molecules such as CD39 and CTLA-4 [38, 53–55]. The fact that this enhanced suppressive function was not seen when fingolimod-treated Tregs were co-cultured with fingolimod-treated effector T cells demonstrates the drug also promotes resistance to Treg suppression. This finding may explain why fingolimod failed to improve functional outcomes in recent experimental stroke studies. Further studies should ascertain whether the administration of fingolimod over a longer time window maximizes its Treg-mediated effects. However, the testing of drugs which can specifically enhance Treg suppressive function without upregulating inflammatory subsets should be prioritized.

Methods

Materials

Fingolimod hydrochloride was obtained from Novartis Institutes for Biomedical Research, Basel. All reagents and materials were obtained from Sigma-Aldrich or Miltenyi Biotec unless otherwise stated. Complete medium consisted of RPMI-1640 (with 2 mM L-glutamine and 1 mM sodium pyruvate), 10% FCS, 10 mM HEPES, 0.01 mM mercaptoethanol and 100 U/mL penicillin/streptomycin. All flow cytometry antibodies were obtained from eBioscience or Miltenyi Biotec.

Mice

Animal experiments were carried out in accordance with the European Directive 2010/63/EU, following approval by the Animal Experimentation Ethics Committee of University College Cork

and under authorization issued by the Health Products Regulatory Authority Ireland (license number AE19130/P075). The study was conducted, and its results were reported according to the ARRIVE guidelines. Male C57BL/6JOLA^{Hsd} mice (7-week old) (Envigo) were acclimatized for at least 1 week before any procedure took place. Mice were group-housed in individually ventilated cages in a specific-pathogen-free facility. Mice were exposed to a 12-h light/12-h dark cycle and kept at a temperature of 20–24°C and a relative humidity of 45–65%. Mice were provided with environmental enrichment and ad libitum access to both food and water. In total, 46 mice were used in this study. All surgeries and drug administration were conducted in the morning.

Ischaemic model

A pMCAO was employed, as previously described [18]. Briefly, mice were anaesthetized, using vaporised isoflurane (3–4% for induction; 1–2% for maintenance) in O₂/N₂ (30/70%). The skin between the left ear and eye was incised, and the temporal muscle retracted to expose the temporal and parietal bones. In sham animals, only the skin incision and exposure of the skull were performed. A small craniotomy was performed, and the meninges were perforated to expose the MCA. The distal portions, including the branches and the main artery below the bifurcation, were occluded using a small vessel bipolar electrocoagulation forceps (Bovie Bantam PRO electrosurgical generator (#A952)/McPherson 3 1/2" straight forceps (#A842) (Symmetry Surgical Inc., USA)). The MCA was cut to confirm successful occlusion, after which the incision was sutured, and anaesthesia was discontinued. Mice were allowed to recover in a heated chamber (32°C) for 30 min before being returned to their home cage. Experimental mice were monitored daily using a scoresheet which graded weight loss (from 0 to 3), appearance changes (from 0 to 3), behaviour (from 0 to 3) and neurological score (0: no observable deficit, 1: forelimb flexion, 2: decreased resistance to lateral push and forelimb flexion without circling, 3: same as 2, with circling) [56]. Naïve mice did not undergo anaesthesia or pMCAO as described above.

Fingolimod treatment

Once daily for 10 days post-ischaemia, mice received either normal saline or 0.5 mg/kg fingolimod via intraperitoneal (i.p.) injection. A researcher not associated with the surgery prepared treatment solutions (pH 7.0) for volumes no greater than 250 µL per injection.

Neurological deficit evaluation

Mice underwent neurological evaluation at 3- and 10-days post-stroke using the cylinder test. Each mouse was placed in a clear Perspex cylinder (12.5 cm diameter × 23.5 cm height) for 5 min.

The sessions were videotaped (Canon Legria HF R706). The number of times the mouse used each paw for weight support against the wall of the cylinder was counted over a 5-min period (a minimum of 20 contacts were recorded). Specifically, the independent use of the left (L) or right (R) forepaw, or simultaneous use of both (B) forepaws was noted. The three numbers were then expressed as a percentage of all paw contacts with the wall to obtain a neurological deficit score $(R - L)/(R + L + B)$.

Tissue collection and processing

At 10-days post-ischaemia, mice were culled by anaesthetic overdose (200 mg/mL i.p.; Euthatal; Merial, France). Blood was collected from the descending aorta and transferred into EDTA (approximately 500 µL). Mice were then perfused transcardially with 20 mL cold phosphate-buffered saline (PBS). The brain, spleen, Peyer's patches and cervical/inguinal lymph nodes were harvested. Spleen and lymphoid tissue were processed as previously reported [18]. Where relevant, red blood cells were lysed using a lysis buffer (eBioscience). After washing, samples were re-suspended in PBS and counted using Trypan blue to determine total cell number and viability. Brains were divided into ipsilateral and contralateral hemispheres. After mechanical dissociation, leukocytes were isolated using discontinuous 30/70% Percoll gradients, as previously described [18]. Post-centrifugation, the cell fraction was collected, washed and then counted.

Flow cytometric analysis

For the analysis of intracellular cytokines, 2×10^6 cells per well of a 96-well V-bottom plate were re-suspended in 200 µL complete medium and stimulated for 4 h at 37°C in a CO₂ incubator with PMA plus ionomycin, in the presence of brefeldin A and monensin (Cell Stimulation Cocktail eBioscience). Post-incubation, Fc receptors were blocked by incubation for 5 min with 50 µL of anti-mouse CD16/CD32 (Clone 93, 1:100) (eBioscience, #14016182). Cells were then stained for 30 min at 2–8°C in the dark with the antibodies outlined in Supporting Information Table 1. Samples were then washed, fixed, permeabilized and stained for 30 min at room temperature with intracellular antibodies as per Supporting Information Table 1. Samples were washed with permeabilization buffer and then re-suspended in PBS. Flow cytometric analysis was performed with an LSRII flow cytometer (Becton Dickinson). Compensation control was set using BD CompBeads Anti-Rat/Anti-Hamster Particles Set (BD, #552845). Data were analysed using FlowJo (v10) according to the following initial gating strategy: live cells (as determined by live/dead stain), lymphocytes (as determined by FSC/SSC), T lymphocytes (defined as CD3⁺), CD4⁺ and CD8⁺ and regulatory T cells (CD4⁺ cells which co-express high levels of CD25 and FoxP3). Gates were set according to unstained samples and fluorescent-minus-one controls (Supporting Information Fig. 4). Absolute cell counts for all tissues were calculated according to instructions provided with

the CountBright Absolute Counting Beads (Molecular Probes, #C36950). All results were reported according to the Minimum Information about a Flow Cytometry Experiment (MIFlowCyt).

Isolation of Tregs from post-ischaemic spleen

Treg cells were isolated using a mouse CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec, #130091041). Briefly, spleens were harvested from culled naïve (N), saline-treated (S) and fingolimod-treated (F) mice. For ischaemic mice, $n = 3$ spleens were pooled. For naïve controls, $n = 2$ spleens were pooled. CD4⁺ T cells were first negatively selected by magnetically labelling non-CD4⁺ T cells (CD8⁺, CD11b⁺, CD45R⁺, CD49b⁺, Ter-119⁺ cells). CD25⁺ (termed Treg) and CD25[−] (termed Tconv) T cells were then separated by magnetically labelling CD25⁺ cells. To achieve higher purity, the CD4⁺ CD25⁺ fraction was re-applied to a second MS column and processed as before. Flow cytometry was used to determine the purity of the isolated Treg and Tconv populations for each condition (Supporting Information Fig. 5). The mean percentages of CD25[−] cells were 96.77 ± 2.1 , 96.80 ± 1.67 and 93.97 ± 3.36 for naïve, saline and fingolimod conditions, respectively ($n = 3$ replicates). The mean percentages of Treg (CD4⁺ CD25⁺) among CD4⁺ cells were 98.3 ± 0.4 , 97.9 ± 0.7 and 96.3 ± 1.5 for cells from naïve, saline and fingolimod-treated mice, respectively, for the same samples.

Treg suppression assay

Wells of a 96-well U-bottomed plate were coated with anti-mouse CD3 (145-2C11) (eBioscience, #14003182) (50 µg) for 2 h at 37°C and then washed twice with PBS. Edge wells of the plates were filled with 200 µL PBS. Isolated Tconv were first re-suspended at 1×10^6 cells/mL and Tregs at 2×10^6 cells/mL. Anti-mouse CD28 (eBioscience, #14028182) was added at a final concentration of 1 µg/mL. For each condition, 5×10^4 Tconv were incubated with Tregs, the latter in decreasing number to create Treg:Tconv ratios of 1:1, 1:2, 1:4, 1:8, 1:16 and 1:0. Each condition was plated in triplicate and incubated at 37°C/5% CO₂ for 72 h. At hour 68, thiazolyl blue tetrazolium bromide (MTT) labelling reagent was added to each well at a final concentration of 0.5 mg/mL. After 4 h, plates were centrifuged at 1500 rpm for 5 min. The supernatant was carefully removed. The cell pellet was then dissolved in 200 µL DMSO/ammonium hydroxide (800 mM) and absorbance at 570 nm was read. For each condition, Treg suppression was calculated as a proportion of Tconv-cell proliferation in the absence of Treg.

Statistics

The Kolmogorov–Smirnov test was used to ascertain that all data were normally distributed. Flow cytometric data are

displayed as 10–90 percentile box-and-whisker plots. The ROUT method ($Q = 1\%$) was used to identify possible outliers, which were then screened for exclusion. Two-sided, independent-sample t -tests were employed to investigate differences between two groups. One-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparisons were used to investigate differences between three or more groups. One-way repeated measures ANOVA with post hoc Tukey's multiple comparisons was used to investigate neurobehavioural results. Two-way repeated measures ANOVA followed by post hoc Tukey's multiple comparisons was used to investigate differences between groups when the effects of two independent variables were studied. A p -value <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 8.0. All investigators remained blinded to treatment groups throughout mouse husbandry, data acquisition and analysis.

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Abbreviation: pMCAO: permanent middle cerebral artery occlusion

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