In vivo Positron Emission Tomography Imaging Demonstrates Diminished Microglial Activation after Fingolimod Treatment in an Animal Model of Multiple Sclerosis

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Running Title: Fingolimod-treatment reduces TSPO expression
ABSTRACT (318/350 words)

There is a great need for the monitoring of microglial activation surrounding multiple sclerosis lesions as this is thought to be driving the widespread neuronal damage. Recently, ‘second generation’ positron emission tomography (PET) radioligands have been developed which can reveal the extent of microglial activation by quantifying the increased expression of the 18 kDa translocator (TSPO) protein. Here, we investigate whether PET imaging can be used to demonstrate the reduction in microglial activation surrounding a chronic focal multiple sclerosis (MS)-like lesion following treatment with fingolimod, an established MS therapy.

Methods: Chronic focal experimental autoimmune encephalitis (EAE)-like lesions were induced in Lewis rats (n=24) via stereotaxic intrastriatal injection of heat-killed bacillus Calmette-Guérin (BCG) and subsequent activation using an intradermal injection of BCG in complete Freund’s adjuvant. This results in a delayed type hypersensitivity (DTH)-like EAE-lesion. The extent of neuroinflammation surrounding the lesion was measured using $^{18}$F-GE180 as a PET radioligand. The imaging was performed before and after treatment with fingolimod (0.3 mg/kg/day po, 28 days) or vehicle as a control. In addition to this, autoradiography and immunohistochemistry experiments were performed to verify the in vivo results.

Results: The chronic DTH-EAE lesion led to increased ligand binding in the ipsilateral compared to contralateral hemisphere when PET imaging was performed with the TSPO-binding radioligand $^{18}$F-GE180. Treatment with fingolimod led to highly significant reduction in the binding potential, which could be demonstrated using both in vivo and ex
**vivo** imaging (fingolimod vs. vehicle treatment, p<0.0001). The area of increased $^{18}$F-GE180 signal mapped closely to the area of activated microglial cells detected by immunohistochemistry.

**Conclusions:** PET-imaging, unlike magnetic resonance imaging (MRI), can be used to visualise the microglial activation surrounding a chronic DTH-EAE lesion. Importantly, treatment effect of fingolimod can be monitored *in vivo* by measuring the degree of microglial activation surrounding the chronic DTH-EAE lesion. This work gives promise for introduction of new outcome measures applicable in treatment studies of progressive MS.

**Key Words:** Multiple sclerosis, PET imaging, TSPO, fingolimod, neuroinflammation
INTRODUCTION

Endpoints for development of new therapies for multiple sclerosis (MS) have conventionally been suppression of relapses, reduction in the number of gadolinium-enhancing or new or enlarging T2 lesions in magnetic resonance imaging (MRI), and evaluation of disability using the expanded disability status scale (EDSS). However, it is increasingly clear that evaluation of the diffuse inflammation associated with progressive MS, and follow-up of the development of neurodegeneration need more accurate and sensitive imaging methods than can be achieved using MRI. Recent work suggests that neuronal damage in MS brain is associated with microglial activation, and is more widespread than previously envisaged. In particular, axonal damage occurs not only in MS lesions, but also within the normal appearing white matter, and spatially correlates to regions of activated microglia. In addition, it is clear that current MRI techniques are insensitive to the detection of pathologies associated with progression of MS. There is thus urgent need to develop novel imaging methods for the detection and monitoring of these processes. Methods that can accurately report on extra-lesional pathological processes may then be used to establish what therapies, if any, are able to modify this more widespread, extralesional damage.

It has been long recognised that microglial activation is associated with the increased expression of the 18kDa translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor. Thus an increase in expression of TSPO has been proposed as a surrogate marker for neuroinflammation. The increase in expression of TSPO can be visualised using positron emission tomography (PET) and an appropriate radiotracer such as $^{11}$C-PK11195. Recent years have seen the development of so-called...
second-generation TSPO-radioligands, which have better signal-to-noise ratio, and other more favourable physicochemical characteristics compared to the $^{11}$C-PK11195 ligand (10). One such radioligand in development is $^{18}$F-GE180 (11, 12).

Fingolimod (FTY720, Gilenya®) was the first oral therapy to be approved for treatment of MS (13). Fingolimod is phosphorylated \textit{in vivo} to fingolimod-phosphate (fingolimod-P), which is a broad Sphingosine 1-Phosphate receptor (S1PR) antagonist and binds to all S1PR apart from the S1P2R (14). The primary mechanism of action of fingolimod in MS therapy is thought to be that by modulating the S1P1 receptor expression on T cells, it blocks the T-cells from exiting lymph nodes and thus reduces the T-cell mediated autoimmune reaction in the central nervous system (CNS) (15). However, fingolimod also readily enters the CNS where it can bind to neurons, oligodendrocyte progenitor cells, astrocytes and microglia (13, 16). It has been demonstrated \textit{in vitro} that fingolimod can suppress the activation of microglia (17). The primary aim of this study was to evaluate whether fingolimod reduces microglial activation in an MS animal model when administered to the animals, and whether $^{18}$F-GE180 can be used to monitor the impact of treatment on microglial activation \textit{in vivo}. To study this, we used a delayed type hypersensitivity (DTH) model of a pattern I type MS lesion characterized by a T-cell and macrophage-dominated lesion (18, 19). Using this animal model we aimed to investigate the microglial activation within the lesion area, and in the area surrounding the lesion. The formation of stratified lesions in terms of spatial distribution and kinetics distinguishes this model from other types of experimental autoimmune encephalomyelitis (EAE), where lesion presentation is random and variable and hence not quantifiable (20). Regarding lesion pathology, the DTH-lesion closely resembles lesions seen in MS,
including blood-brain-barrier (BBB) breakdown at the height of the disease, demyelination, T-cell and macrophage infiltration, and widespread microglial activation (18).

MATERIALS AND METHODS

Animals

All animal experiments were carried out with the required ethical approval from the Southern Finland ethics board, application number ESAVI/6360/04.10.03/2011. Male Lewis rats (50-100g, n=18) were obtained from Charles River (Germany) and allowed to acclimatise for one week prior to the start of any experiments. All animals were housed in accordance with the Amsterdam protocol for animal experiments (21), food and water was available ad libitum.

Intrastriatal Injection of Bacillus Calmette-Guérin (BCG)

Prior to stereotaxic surgery the animals were anaesthetised using isoflurane (Baxter Medical AB, 4%) in air. Anaesthesia was maintained with 2.5% isoflurane and body temperature was maintained using a heating blanket. The rats were placed onto a stereotaxic frame (Kopf Instruments, US) and the skull was exposed. The brain of the animal was exposed via a hole which was drilled though the skull. Heat killed BCG (1x10^5 cells in 2 μL of PBS) was injected into the left striatum using a Hamilton syringe (10 μL, Sigma Aldrich). The acute inflammatory response to the intracranial injection of BCG resolves rapidly. The BCG remains sequestered behind the BBB and will escape recognition by the immune system (22).
Activation of the Lesion

28 days following the intracerebral BCG injection the lesions were activated by an intradermal injection of BCG (1.5 mg, Difco) in an emulsion of complete Freud’s adjuvant (100 μL, Sigma Aldrich, USA) mixed with phosphate-buffered saline (PBS; 100 μL). This peripheral sensitization of the immune system provokes an immune cell-mediated attack towards the intracranial BCG deposit, resulting in the formation of focal DTH lesions and bystander damage in the CNS (22).

Experimental Design

After the peripheral activation of the lesion, the DTH-EAE lesions were allowed to develop until day 127 to generate large chronic lesions and the animals were then treated for 28 days with either fingolimod (n=7) or vehicle (n=7; Figure 1). The fingolimod treatment was given daily (0.3 mg/kg in 0.5mL of water). Control group was given water (0.5 mL) as a vehicle control. The animals were dosed via oral gavage to ensure accurate dosing. In this model, the acute inflammation subsides after day 20 of activation of the lesion, and the BBB damage subsides (23). Thus, at day 127 the lesion clearly represents a well-developed chronic MS-lesion. PET imaging was performed immediately before initiation of treatment and at the end of the treatment period.

Radiotracer Production

18F-GE180 was synthesised using a previously published method (12). Briefly, [18F]F- was generated by proton irradiation of 18O-enriched H2O (97% enrichment). After suitable workup the [18F]F-anion was then reacted with the precursor molecule (GE Healthcare, Oslo, Norway) resulting in the formation of 18F-GE180. Finally, the tracer
was purified with semi-preparative high performance liquid chromatography and formulated for injection (12).

**In-vivo Imaging**

**In vivo** imaging was performed using an Inveon multimodality PET/computer tomography (CT; Siemens Medical Solutions, USA) small animal scanner with 1.2 mm³ resolution. The device generates images with 159 transaxial slices with a field of view with a 10 cm transaxial field of view and a 12.7 cm axial field of view. A CT scan was performed in order to correct for signal attenuation in the PET scan and provide anatomical references. Emission scans (40 min) were acquired for these studies. The data was acquired in list mode with an emission window from 350 to 650 MeV. The scans were initiated immediately after the intravenous injection of ¹⁸F-GE180. For the baseline scan, the amount of radioactivity given to animals in the control group was 49.0±3.1 MBq (mean±SD), and in the fingolimod-treated group 46.5±6.3 MBq. In the second scan, the amounts were 46.6±5.2 and 45.7±8.6 MBq, respectively.

**Digital Autoradiography**

Rats were killed for autoradiography analysis (n = 3) following the 40 minute **in vivo** PET imaging. The animals were killed 45 minutes post tracer injection, the anaesthetic was increased to a terminal level (5% in air), and the thoracic cavity was opened. Blood was collected via a cardiac puncture and the animals were transcardially perfused with heparinised saline (20 mL). The brains were removed, frozen and sectioned (20 μm) in a cryostat (Leica, Germany). Coronal sections were taken from different brain regions (olfactory bulbs, striatum, cerebellum) and exposed to a phosphor imaging plate (Fuji
Imaging Plate BAS-TR2025, Fuji Photo Film Co., Ltd., Japan) for a period of two half-lives of the radiotracer (220 min). The plates were then read in a phosphor imaging plate reader (BAS-5000, Fuji, Japan; 25 mm² resolution). Finally, the sections were frozen.

Regions of interest (ROI) were drawn in the left and right striatal and cortical areas on the images obtained from the autoradiography. The digital autoradiography images were analyzed for count densities (photo-stimulated luminescence per unit area, PSL/mm²) with a computerized image analysis program (Aida 2D densitometry program, Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany). ROI were drawn over the left and right hemisphere at the level of the striatum, and cerebellum. In order to quantify the binding potential of the radioligand the following calculation was performed:

\[ B_{nd} = \frac{ROI_{\text{Lesion}} - ROI_{\text{Contralateral}}}{ROI_{\text{Contralateral}}} \]

This was then averaged across the whole striatum to get an overall binding ratio for each injected animal. This binding potential was then normalised to the total lesion volume estimated from the cresyl staining (see below).

The 3D surface plots were generated in Image j, using the interactive 3D surface plot add-in.

In addition to the sections used in the autoradiography some sections were directly cut from the striatum and frozen for immunohistochemistry analysis. The fresh sections were post fixed with periodate-lysine-paraformaldehyde as previously described (24). Endogenous peroxide activity was quenched by placing the sections in a 1% H2O2 solution in MeOH for 20 minutes. The sections were then incubated with horse serum for one hour prior to the addition of the primary antibody (polyclonal goat anti-IBA-1,
Abcam (Cambridge, UK)) for overnight at 4 °C. The sections were then incubated with a biotinylated secondary antibody (Vector labs, CA, USA). The signal was amplified using avidin biotin system, (ABC kit, Vector Labs, CA, USA) and then developed using 3,3-diaminobenzidine. The sections were finally counterstained using cresyl violet. Serial sections were stained with the Bielschowsky silver impregnation method to demonstrate neuronal processes according to the standard Bancroft and Stevens protocol.

The sections which had been used for autoradiography were only stained with cresyl violet as this tissue was not suitable for immunohistochemistry. This allowed quantification of the lesion volume for the autoradiography analysis.

**In vivo Data Analysis**

The data was reconstructed using the ordered-subsets expectation maximization algorithm in three dimensions (OSEM3D) plus fastMAP iterative reconstruction protocols in the InveonTM acquisition software (Siemens Medical Solutions, USA). All data sets were aligned to a standard rat template (Sciffer, Pmod) using the rigid matching tool within Pmod v3.4 (PMOD Technologies Ltd., Switzerland). The images were then evaluated by drawing a spherical ROI in the injected striatum. This ROI was then mirrored in the contralateral hemisphere to act as a reference region. ROI analysis and segmentation was performed in Pmod. The time activity curve (TAC) from the VOI drawn in the lesioned hemisphere was subtracted from the TAC from the contralateral hemisphere to examine the point of maximal binding (25-50 minutes) during the imaging timeframe. The bound to free ratio was then calculated over the period of maximal binding, as described by Farde *et al.* (25). Briefly, the area under the curve was calculated
by integrating between the defined time limits during the maximal binding period for both the lesioned TAC and the contralateral TAC. In order to get the bound to free ratio the following calculation was performed:

$$B_{nd} = \frac{(TAC_{(Lesion)} - TAC_{(Contralateral)})}{TAC_{(Contralateral)}}$$

**Pixelwise Modelling of in-vivo Images**

The images were reconstructed into 3D using the in-built tool within Pmod and then automated segmentation of the lesions was performed using the threshold tool and placing a seed point in the lesion area.

**Statistical Analysis**

All statistical analysis was performed using Prism (5.01, Graph Pad software inc, USA). A result was considered significant if $p < 0.05$. Images were co-registered using Photoshop (CS4, Adobe, USA) to resize and overlay the images.

**RESULTS**

**Fingolimod Reduced Microglial Activation and ex-vivo $^{18}$F-GE180 Binding in a Chronic DTH-EAE Lesion**

The chronic focal EAE-lesion produced by intrastriatal injection of BCG and subsequent peripheral activation caused a large inflammatory lesion in the ipsilateral hemisphere, which was well visualized after 127 days of lesion induction. This late lesion had a wide area of microglial activation depicted by increased uptake of $^{18}$F-GE180 in the ipsilateral hemisphere (Fig. 2A), and a hypercellular core visualized by cresyl violet staining (Fig 2B). No inflammation was observed in the contralateral hemisphere (Fig. 2A and 2B).
The increased ipsilateral microglial activation was not solely limited to the area of hypercellularity but continued into the normal appearing brain tissue surrounding the lesion, and was demonstrated by an increase in perilesional $^{18}$F-GE180 binding (Figure 2A and 2B). Treatment of the animals with fingolimod for 28 days resulted with clear reduction in the binding of $^{18}$F-GE180 when compared to vehicle-treated animals and evaluated by ex vivo autoradiography (Fig. 3A). Quantification of the binding of the radiotracer revealed a significant reduction in the binding potential of $^{18}$F-GE180 ($p < 0.0001$) following treatment with fingolimod (Figure 4A).

**Evaluation of Microglial Activation and Axonal Integrity in Chronic Focal EAE Using Immunohistochemistry**

The area of increased uptake of the radiotracer corresponded to the area of activated microglia observed by immunohistochemistry (Figure 3A-C). Fingolimod-treatment prevented lesion expansion, demonstrated by diminished area of microglial activation surrounding the lesion in immunohistochemical staining (Figure 4B; for fingolimod-treatment; 7.22 arbitrary units (arb. unit) ± 3.40 vs. vehicle-treatment; 15.35 arb. unit ± 2.18, $p = 0.0264$). Closer examination of the appearance of the IBA-1-stained microglia revealed that the cellular processes were more elongated in the fingolimod-treated animals at equivalent extralesional sites compared to the vehicle-treated animals (3C). Silver staining revealed the presence of dystrophic axons surrounding the lesions. At equivalent sites, the silver stain demonstrated axon sparing in the fingolimod-treated animals compared to vehicle-treated animals (3D).
Effect of Fingolimod on Microglial Activation in Chronic Focal EAE Evaluated using in vivo TSPO-PET Imaging

From the in vivo PET images it was possible to observe binding of the $^{18}$F-GE180 radioligand in the ipsilateral but not contralateral striatum (Figure 5). When comparing a pre-treatment scan to a post-treatment scan a decrease in the size of the volume of $^{18}$F-GE180 binding following treatment with fingolimod was observed (Figure 5). A similar reduction was not observed in the vehicle-treated group, in fact the lesion actually increased in size (Figure 5B). Quantification of the PET data revealed a significant reduction in the $B_{nd}$ in the fingolimod-treated group ($-0.634 \pm 0.141$ arb. unit) compared to the vehicle-treated group ($0.266 \pm 0.281$ arb. unit), $p = 0.021$ (Figure 4C).

DISCUSSION

In this study, we have demonstrated for the first time that in vivo PET imaging using a TSPO-binding radioligand can be used to quantify microglial activation in a chronic focal EAE model in rat. Importantly, fingolimod treatment of the animals resulted with diminished microglial activation, demonstrable as a reduction both in lesion volume and in binding potential in in vivo PET. Furthermore, this reduction in TSPO binding was observed in autoradiography and could be attributed to reduction in the activation of the microglia, confirmed by anti-IBA-1 immunostaining. Importantly, earlier extensive work has validated the expression of TSPO to be localized in activated microglial cells and macrophages, and to some degree also in astrocytes in abnormal brain both in humans and in rodents (6, 26).
Fingolimod efficacy in the treatment of MS has been well demonstrated (27). The impact of fingolimod on the immune system is immunomodulatory, and dependent on the retention of lymphocytes within the lymph nodes (28). Due to its lipophilicity, fingolimod crosses the BBB, and pharmacologically relevant concentrations are found in the brain and spinal cord following oral administration (16). There is good evidence from MRI that fingolimod reduces brain atrophy development compared to placebo and interferon-beta therapy, an observation suggestive of neuroprotective action of this drug (27). S1PR are expressed on all CNS cell types, and binding of fingolimod-P to these results with intracellular signaling and potentially beneficial outcome effects in terms of preventing progression of MS (29).

Microglial activation is critical in MS pathogenesis. In secondary progressive MS, there is increased microglial activation and accumulating neurodegeneration throughout the white matter and gray matter (30). The observed correlation between diffuse inflammation and axonal injury suggests that inflammation likely contributes to neurodegeneration and disease progression, and thus therapeutic targeting of microglial activation might also reduce the axonal injury (30-32). The present work using the chronic focal EAE model mimicking progressive MS, and our earlier human PET imaging study on secondary progressive MS patients (9) together demonstrate that evaluation of microglial activation using TSPO-binding PET-ligands allows assessment of the diffuse brain inflammation and perilesional activity in progressive MS and animal models of the progressive disease in vivo. Importantly, our present work also demonstrates the close correlation between axonal damage and microglial activation.
MS is traditionally seen as an inflammatory autoimmune disease, but also neurodegeneration is present from early on. A plethora of medications has emerged in recent years for treatment of relapsing-remitting MS, but there is still no single treatment dedicated for progressive MS. Finding a treatment that would result with diminished microglial cell activity in progressive MS would be an attractive way towards drug development for the progressive forms of the disease. MRI imaging has been the ‘gold standard’ for imaging MS-related changes, but conventional MRI has a low detection threshold for detecting diffuse white matter changes typical for progressive disease. Hence, other functional imaging methods, such as advanced MRI imaging methods and PET imaging using TSPO-radioligands, are needed to complement conventional MRI.

Our work supports the notion that TSPO-PET-imaging, together with conventional and non-conventional MRI could potentially be used as an imaging biomarker e.g. in proof-of-concept treatment studies of progressive MS, where reduced radioligand binding could be interpreted as a quantifiable sign of reduced microglial activation. Interestingly, an \textit{in vivo} $^{11}$C-PK11195-study has already shown that glatiramer acetate-treatment leads to reduced microglial activation in relapsing remitting MS (33). Future studies will have to elucidate the prognostic significance of increased microglial activation in the normal appearing white matter, and the implications of being able to therapeutically reduce microglial activation.

The utility of focal DTH-EAE model to demonstrate both novel diagnostic techniques and treatment effects has been demonstrated in earlier studies (23, 34). The advantage of using a focal model over the more commonly used disseminated models, is that it is possible to monitor a specific lesion without the interference from new or neighbouring
lesions. This is especially important in micro-PET experiments due to the inherent low spatial resolution of the resultant image (35). The previous studies focused on the prevention of new lesion formation within the CNS as the treatment was already started simultaneously when the lesion was activated peripherally (23). In the present study we examined the effects of fingolimod on a well-established DTH-EAE lesion, which is a more clinically relevant paradigm, also in terms of progressive MS. The reduction of microglial activation surrounding the lesion occurred relatively quickly, after only 28 days of treatment, highlighting the efficacy of fingolimod in this model. This is probably due the direct effects of fingolimod within the CNS rather than the depletion of T-cells at this short time point.

Conclusions

This study demonstrates the effectiveness of $^{18}$F-GE180 as a ‘second generation’ TSPO-ligand in detecting the treatment effect of fingolimod in reducing microglial activation in a chronic focal rodent model of MS. The ability to visualise the reduction of microglia in vivo using PET imaging potentially provides a key tool in following this surrogate marker for diffuse inflammation in progressive MS.

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DISCLOSURE

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FIGURE 1. Experimental design showing the timeline of the procedures
FIGURE 2. An example of a digital autoradiography image of a coronal slice from the striatum at the beginning of the treatment period. White arrow marks the site of injection. Black arrows indicate the radioligand uptake in the ventricles. The area of high binding of $^{18}$F-GE180 is indicated in yellow (lesion hemisphere) and low binding of $^{18}$F-GE180 is indicated in purple (control hemisphere). B) Corresponding section stained with cresyl violet demonstrating the extent of the lesion in the left striatum (white arrow). $^{18}$F-GE180 not only binds to the lesion core, but to the surrounding inflamed area as well.
FIGURE 3. Reduced $^{18}$F-GE180 <i>ex vivo</i> binding following fingolimod-treatment is associated with reduced microglial activation. (A) Representative coronal striatal autoradiography images demonstrating the area of $^{18}$F-GE180 binding after treatment with fingolimod (left) or vehicle (right). (B) Representative whole brain photomicrographs demonstrating immunohistochemical staining of activated microglia
(anti-IBA-1) in a fingolimod-treated (left) and vehicle treated animals (right). (C) Highpowered photo micrographs taken from the border of the lesion (marked with * in B). (D). In fingolimod-treated animals, silver-staining shows normal-appearing axons at the site of * in B (left), whereas in vehicle-treated animals the axons are dystrophic and reduced in density at the corresponding site (right). High power scale bar = 20 μm.
FIGURE 4. Microglial activation surrounding the chronic focal EAE lesion is reduced following fingolimod treatment. (A) Autoradiography using $^{18}$F-GE180. Quantification of radioligand binding following fingolimod-treatment (fingolimod, n = 3, vehicle n = 3), Key: ***p<0.0001. (B) Immunohistochemical staining using anti-IBA-1 mAb. Quantification of the area of microglial activation, normalized to lesion area. Key: * p<0.05. (C) In vivo PET using $^{18}$F-GE180. Quantification of the change in binding potential following treatment (fingolimod: n = 5, white bar; Vehicle: n = 3, grey bar). Key: *p = 0.0206.
FIGURE 5. *In vivo* imaging demonstrates a reduction in $^{18}$F-GE180 binding following treatment with fingolimod. (A) Representative coronal *in vivo* PET images from a fingolimod-treated animal (left panel) or vehicle-treated animal (right panel). Top panels show images obtained from the animal before treatment and bottom panels show images after treatment. (B) Average 3D reconstructions of the PET images before (top panel) and after (bottom panel) treatment with either fingolimod (left panel, n = 5) or vehicle (right panel, n = 3). The red areas indicate the increased radiotracer uptake in the region of the lesion. The blue areas represent the ventricles.
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