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Circulating Brain Derived Neurotrophic factor (BDNF) and frequency of BDNF positive T cells in peripheral blood in human ischemic stroke: effect on outcome

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# Equal contribution as senior authors

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Key words: Acute Ischemic stroke, Brain derived neurotrophic factor, FoxP3, regulatory T cells, Flow cytometry, protective immunity, neuroprotection, stroke outcome

Abstract

The aim of this study was to measure the levels of circulating BDNF and the frequency of BDNF-producing T cells after acute ischaemic stroke. Serum BDNF levels were measured by ELISA. Flow cytometry was used to enumerate peripheral blood leukocytes that were labelled with antibodies against markers of T cells, T regulatory cells (Tregs), and intracellular BDNF. There was a slight increase in serum BDNF levels after stroke. There was no overall difference between stroke patients and controls in the frequency of $CD4^+$ and $CD8^+$ $BDNF^+$ cells, although a subgroup of stroke patients showed high frequencies of these cells. However, there was an increase in the percentage of $BDNF^+$ Treg cells in the $CD4^+$ population in stroke patients compared to controls. Patients with high percentages of $CD4^+$ $BDNF^+$ Treg cells had a better outcome at 6 months than those with lower levels. These groups did not differ in age, gender or initial stroke severity. Enhancement of BDNF production after stroke could be a useful means of improving neuroprotection and recovery after stroke.

1. Introduction

Ischemic stroke is caused by interruption of supply of blood to a part of the brain, creating an environment of hypoxia and glucose deprivation that leads to neuronal death and neurological deficits (Moustafa and Baron 2008). After stroke there is local inflammation at the site of injury (Gelderblom et al. 2009), which can be harmful in the early stages since activation of microglial cells and local inflammatory responses exacerbate injury to the brain (Jin et al. 2010). There is also systemic immune activation after stroke (Yan et al. 2009). We have previously reported that stroke subjects have increased levels of circulating anti-inflammatory cytokines (Yan et al. 2012a) and upregulation of $CD4^{+}CD25^{hi}$ $Foxp3^{+}$ T regulatory cells (Tregs) after stroke (Yan et al. 2012b). The increase in anti-inflammatory cytokines and the increased frequency of circulating Tregs could serve to reduce harmful inflammation. However, the overall effects of the systemic activation of the immune system after stroke are complex and may include beneficial effects (McCombe and Read 2008;Yilmaz et al. 2006).

Beneficial effects of the immune response after stroke could be mediated by protective immunity, which plays a role in recovery from injury and which is mediated through Tregs. Tregs have the functions of negative regulation of immune responses, self-tolerance and control of damage at the site of inflammation (Tang and Bluestone 2008;Wan 2010). In experimental stroke, the absence of Tregs in mice caused elevated expression of pro-inflammatory cytokines, indicating the role of Tregs in protection in acute ischemic stroke (Liesz et al. 2009). Adoptive transfer of Treg cells led to reduction of volume of ischaemia in experimental stroke (Brea et al. 2014). However, the role of Treg cells in recovery from stroke

may be different at different time points after stroke (Chen et al. 2013;Kleinschnitz et al. 2013;Xu et al. 2013).

Neurotrophins are molecules that promote the growth and survival of neurons (Huang and Reichardt 2001). One important neurotrophin is brain derived neurotrophic factor (BDNF) which improves neuronal survival and prevents neuronal death by activating intracellular protein kinases B and mitogen-activated protein kinases (MAPK/ERK) (Schabitz et al. 2000). Administration of BDNF in regional brain ischemia caused both a reduction in stroke volume and an improvement in functional outcome (Zhang and Pardridge 2006). There is only one study of circulating levels of BDNF after stroke, which found no increase in BDNF levels in 10 stroke subjects sampled at admission and daily for the following four days (Di Lazzaro et al. 2007).

However, even if circulating levels are unchanged, another possibility is that Tregs could deliver neurotrophins, such as BDNF, to the site of injury, and these could assist in recovery (Greenberg et al. 2009). It is known that activated human T cells, B cells and monocytes secrete BDNF *in vitro* (Kerschensteiner et al. 1999) and can be shown to produce BDNF by intracellular labelling with flow cytometry (Ziemssen et al. 2002). To investigate the possibility that Tregs produce BDNF after human stroke, we have measured circulating BDNF levels and production of BDNF by T cells after stroke. We also investigated whether the levels of $BDNF^+$ Tregs correlated with the clinical features after stroke.

2. Materials and Methods

Patients and controls

The study protocol was reviewed and approved by the Human Research Ethics Committee of the Royal Brisbane and Women's Hospital, the Wesley Hospital and the Medical Research Ethics Committee of the University of Queensland, Australia. All participants provided informed consent prior to study participation. The ischemic stroke patients were recruited from the Department of Neurology in the Royal Brisbane and Women's Hospital and the Wesley Hospital, Brisbane, Australia. Ischemic stroke was diagnosed on the clinical history, presence of focal neurological signs and symptoms and by Magnetic Resonance Imaging (MRI) or CT scans. Patients with subarachnoid haemorrhage, extradural or subdural haemorrhage, transient ischemic attack (TIA, defined using standard criteria) or a neurological deficit caused by trauma or neoplasm were excluded. We documented the clinical details, such as the presence of hypertension, through self-report by the patients or by questioning their relatives. Healthy controls were volunteers with no history of acute or chronic illness.

Table 1 shows the details of the subjects used for measurements of BDNF levels and for the flow cytometry study. The day of onset of stroke was taken as Day 0. For the flow cytometry study, blood was collected from stroke subjects up to three weeks after stroke. Six of the subjects were treated with thrombolysis in the emergency department on day 0. These subjects were enrolled in the present study on the next day, so that the initial clinical assessment was done the day after thrombolysis.

Classification of stroke and measurement of stroke severity

Patients were classified according to the Oxfordshire Stroke Classification (OSSC) (Bamford et al. 1991) into total anterior circulation infarcts (TACI), partial anterior circulation infarct (PACI), posterior circulation infarct (POCI) and lacunar infarcts (LACI). Stroke severity was assessed with the National Institutes of Health Stroke Scale (NIHSS) at day 1.Dependency was measured at baseline and 6 months using the modified Barthel index (mBI) (Mahoney and Barthel 1965;Sulter et al. 1999).

Isolation of PBMC and cell culture

Venous blood samples were collected within the first 3 weeks following stroke. For some patients, samples were collected at several times during this period; in those cases, only data from one time-point is reported in the current study. . Blood was collected on one occasion from controls. Serum was used for measuring BDNF levels. Peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation, as we have previously described (Yan et al. 2009) and cryopreserved. Cryopreserved PBMC samples were thawed and cultured in RPMI1640 supplemented with 10% FCS for 24 h before use to allow cells to rest and re-express cell surface molecules. We have previously reported results of Treg cells in freshly collected cells from subjects with stroke (Yan et al. 2012b), and six of those subjects were included in the present study.

Antibody labeling and flow cytometry

Prior to analysis on a Beckman Coulter Gallios flow cytometer with seven color acquisition, cells were stained with Aqua Live/Dead cell exclusion dye (Invitrogen) and surface stained with the following fluorochrome-conjugated antibodies: anti-CD25 fluorescein isothiocyanate (Becton Dickinson), anti-CD8 PerCP-Cy 5.5 (Becton Dickinson), anti-CD127 phycoerythrin-Cy7 (eBioscience) and anti-CD4 V450 (Becton Dickinson). Cells were fixed and permeabilized using a cytofix/cytoperm kit according to the manufacturer's instructions (Becton Dickinson), before intracellular staining with Anti-FoxP3 phycoerythrin (eBioscience) and anti-BDNF allophycocyanin (Neuromics) conjugated antibodies.

Single labelled tubes for each antibody, isotype-matched control antibodies, fluorescence-minus-one controls, dead cell exclusion and doublet discrimination were used to ensure accurate positive cut-off values and compensation matrices and to validate cell phenotype detection sensitivity and resolution.

Figure 1 shows the gating strategy used to identify BDNF+ve regulatory T cells. Flow cytometry data was analyzed using Kaluza 1.2 software (Beckman Coulter).

BDNF levels

BDNF concentrations in the serum were measured using BDNF Emax ImmunoAssay System sandwich ELISA kits (Promega, WI, USA) according to the manufacturer's instructions.

Briefly, 96-well flat-bottom plates (Corning Costar Corp., Cambridge, MA) were coated overnight at 4°C with anti-BDNF mAb. Next day, the plates were washed, blocked and then 100 µl of a 1:100 dilution of serum per well was added. Each sample was tested in triplicate. Serial dilutions of the BDNF standards were also tested on each plate, to allow the concentration of the BDNF in the serum samples to be determined. The plates were incubated for two hours at room temperature and then were washed and incubated with anti-human BDNF polyclonal antibody for two hours at room temperature. After washing, plates were then incubated with anti-IgY conjugated horseradish-peroxidase for one hour at room temperature. Finally, TMB (3,3,5,5-tetramethylbenzidine) substrate solution was added for 10 min, the reaction was stopped by addition of 1N HCl, and plates were read at 450 nm. The concentration of the BDNF was determined from the standard calibration curve. This ELISA kit detects a minimum of 15.6 pg/ml of BDNF, with less than 3% cross reactivity with other related neurotrophic factors at 100 ng/ml.

Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 5.03 (GraphPad Software Inc., San Diego, California, USA). The D'Agostino and Pearson omnibus normality test was used to determine whether distributions were normal. Depending on the distribution of the data, two-tailed t-tests or the Mann-Whitney U test were used to compare the healthy control and stroke populations. A *P* value of <0.05 was considered to be significant.

3. Results

3.1 Serum BDNF levels in stroke subjects and controls

The level of BDNF was measured in serum of 75 stoke patients and 56 healthy controls. The median serum BDNF concentration in the stroke subjects was slightly greater than that in the healthy controls (p=0.04). These results are shown in the left panel of Figure 2. The results for BDNF levels according to the time after stroke (Day 1, Week 1, Week 3) are also shown in Figure 2. The levels in these subgroups did not differ significantly from that of controls.

3.2 Frequency of BDNF-producing T cells after stroke

In 39 patients with stroke, the overall frequency of $CD4^{+}BDNF^{+}$ cells and $CD8^{+}$ $BDNF^{+}$ cells was not different from that of 15 healthy controls. However, a subgroup of 5 stroke subjects had very high levels of both of these groups of cells of CD4+ BDNF+ T cells (Figure 3). The CD4+ BDNF+ T cell percentages according to the time after stroke are also shown in Figure 3

We then examined the percentage of $CD4^{+}CD25^{hi}Foxp3^{+}CD127^{low/-}$ $BDNF^{+}$ cells in the $CD4^{+}$ population (Figure 4) The percentage of such $BDNF^{+}$ Tregs in the $CD4^{+}$ population was higher in stroke patients than in controls ($P<0.01$). Again, the 5 patients who had the high levels of $CD4^{+}$ and $CD8^{+}$ $BDNF^{+}$ T cells also had the highest levels of $BDNF^{+}$ Tregs. The percentage of $BDNF^{+}$ Tregs in the $CD4^{+}$ population at Day 1, Week 1 and Week 3 after stroke is also shown in Figure 4.

We also calculated the percentage of BDNF+ Tregs in the Treg population. This is shown in Figure 5. This did not vary between controls and stroke subjects, indicating that the increase in BDNF+ Tregs is part of the general increase in Treg cells.

3.3 Outcome at 6 months in subjects with high and low levels of $CD4^+BDNF^+$ Treg cells

The subgroup of stroke patients who had percentages of $BDNF^+$ Tregs in their blood that exceeded the 90% percentile of the healthy control group were classified as "hi BDNF". There was no difference between the two groups in the severity of the stroke as measured by NIHSS (dat not shown), or in age or gender. The hi BDNF group had slightly more dependency at onset, as measured by the mBI (Figure 5). However, their outcome following stroke, as measured by the mBI at 6 months, was significantly better than that of patients who had lower levels of $BDNF^+$ Tregs in their blood ("low BDNF" group; * $P<0.05$ using a 2-tailed Mann-Whitney U test) (Figure 6). Two patients in the low BDNF group had died before 6 months, and are given a mBI of 0 in the figure. Four other patients in the low BDNF group were not able to be contacted at the 6 month timepoint.

4. Discussion

In the present study we investigated the levels of circulating BDNF, levels of peripheral $BDNF^+$ Treg, and whether there was any association between $BDNF^+$ Treg levels and the outcome after stroke. There was a slight increase in circulating BDNF levels after stroke, a higher percentage of BDNF+ Tregs in the blood following stroke, and the subjects with high levels of $BDNF^+$ Tregs had a better outcome at 6 months. The levels of Treg cells are still low, and the increase in BDNF+ Treg cells is in proportion to the increase in Treg cells. However, these cells could play a role in recovery from stroke, because we also found that individuals with high levels had a better outcome at 6 months.

The role of nerve growth factors in recovery after stroke is a topic of interest, and administration of BDNF, which is thought to play a role in neuronal survival, differentiation, and neuroplasticity (Binder and Scharfman 2004;Greenberg et al. 2009), has been reported to improve outcome in experimental stroke in rats (Zhang and Pardridge 2006). In the current study, we found only a slight increase in circulating BDNF levels after stroke, and a previous small study found no increase in BDNF levels after stroke (Di Lazzaro et al. 2007). This would suggest that increases in circulating BDNF are unlikely to contribute to recovery after stroke.

The role of Tregs in recovery from stroke has been of great interest, because of the possibility that these cells could be beneficial, although there have been different opinions about this topic (Liesz et al. 2009;Chen et al. 2013). But results from experimental animal models of stroke suggest that Tregs can traffic to the site of the stroke. Our finding of the increased numbers of BDNF-producingTreg cells after stroke suggests the possibility that Treg cells may be able to supply BDNF to the site of injury to provide neuroprotection after the stroke. The stroke subjects showed variability in the levels of $BDNF^+$ Tregs after stroke. However, there was no difference between the hi BDNF group and the lo BDNF group in age, gender or severity of stroke. Therefore, the levels of these cells could be related to individual genetic factors, includingCD28 expression (Gogishvili et al. 2013) and a number of other genes (Ferraro et al. 2014). In those individuals with high levels of $BDNF^+$ Treg cells after stroke, the clinical outcome was better at 6 months. This suggests that high levels of these cells are beneficial.

The limitations of our study are that the sample size is relatively small, and that we do not have measurements of stroke volume for correlation with our results. We had complete data for the mBI as a measure of outcome, but did not have complete data for the modified Rankin scale, which is another common measure of stroke outcome (Sulter et al. 1999). Despite this, or data showed a significant effect of hi BDNF Treg cells on outcome.

We consider that the ability to produce high levels of BDNF Treg cells is most likely to be due to individual factors. For those subjects who do not produce high levels of these cells, we suggest that enhancing this response could beneficial, possibly as a means of delivering BDNF to the site of injury. Other studies that support the role of BDNF in recovery after stroke showed that BDNF-expressing neural stem cells were beneficial (Chang et al. 2013), as were mesenchymal stem cells (Jeong et al. 2014). Methods such as the use of histone deacetylase inhibitors (Kim et al. 2009) or a CD28 superagonist, as have already been shown to be beneficial in experimental stroke (Na et al. 2015), are possible strategies to increase the number of Treg cells. Given the lack of therapies to improve outcome after stroke, further studies of the role of BDNF-producing T reg cells would be useful.

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Table 1

	Ischemic stroke patients		Healthy controls	
	Serum ELISA	**FACS analysis**	**Serum ELISA**	**FACS analysis**
Number, n				
Total	75	39	56	15
Female	31	17	27	9
Male	44	22	29	6
Median age (range)	69 (27-89)	71 (32-88)	43 (20-95)	62 (27-78)
Hypertension (%)	70.7%	79.5%	19.6%	26.7%
Median NIHSS (IQR)*	3.5 (0-24)	3.0 (1-8)	N/A	N/A
Median mRS (IQR)*	3.0 (0-6)	3.0 (2-4)	N/A	N/A
OSCC, n (%)*				
LACI	21.3%	17.9%	N/A	N/A
PACI	41.3%	28.2%		
POCI	21.3%	35.9%		
TACI	12.0%	15.4%		
watershed	1.3%	2.6%		

*data not available for 1 patient in serum group

Figure legends:

Figure 1. Flow cytometric gating strategy.

Lymphocytes were identified on the basis of forward and side scatter (A). Viable lymphocytes were selected on the basis of Aqua viability dye exclusion (B), and, of these cells, CD4+ CD25+ T cells were selected (C). The portion of these cells that were negative for CD127 and positive for FoxP3 expression (D) were assessed for BDNF expression (E).

Figure 2. BDNF levels in serum

The left panel shows the BDNF levels in all samples taken from controls and stroke subjects. BDNF levels were slightly elevated in the serum of subjects with stroke (n=75) compared to healthy controls (n=56). The error bars shows the median and interquartile range.. *P*=0.03 using two-tailed Mann-Whitney U test. The right panel shows the levels of BDNF at day 1, week 1 and week 3 after stroke. When these subgroups are compared with the control group there was no significant difference.

Figure 3. Percentage of BDNF+ cells in CD4+ population

The left panel shows the percentages of $BDNF^+$ cells in the $CD4^+$ population. This did not differ significantly between subjects with stroke (n=39) and healthy controls (n=15), although 5 stroke patients had highly elevated levels of $BDNF^+$ producing cells in both the $CD4^+$

populations. Error bars show the median and interquartile range. The right panel shows the values for the stroke subjects at day 1,

Figure 4. Percentages of $CD4^+CD25^{hi}Foxp3^+CD127^{low/-}$ $BDNF^+$ cells in the $CD4^+$ population

Percentages of $BDNF^+$ Tregs in the $CD4^+$ population were significantly higher in stroke patients (n=39) than in controls (n=15) ($P<0.01$ by two tailed Mann-Whitney U test). Again, the 5 patients who had the high levels of $CD4^+$ and $CD8^+$ $BDNF^+$ T cells also had the highest levels of $BDNF^+$ Tregs. Error bars show the median and interquartile range.

Figure 5 Percentage of BDNF+ Tregs in the Treg population

This shows the percentage of BDNF Tregs in the Treg population, in healthy controls and subjects at Day 1, Week 1 and Week 7 after stroke. There was no difference between the groups, indicating that the increase in BDNF+ Tregs was in proportion to the overall in crease in Tregs in stroke. Error bars show the median and interquartile range.

Figure 6. Effects of levels of Cd4+BDNF+ T reg cells on outcome at 6 months.

6 month follow-up of patients with high and low levels of $CD4^+BDNF^+$Treg cells. The subgroup of stroke patients who had percentages of $BDNF^+$ Tregs in their blood that exceeded the 90^{th} percentile of the healthy control group were classified as "hi BDNF" and the other patients were classified as "lo BDNF". At baseline there was no significant difference between the Barthel index score (mBI) in the 2 groups. Their outcome following stroke, as measured by the mBI at 6 months, was significantly better than that of patients who had lower levels of $BDNF^+$ Tregs in their blood ("low BDNF" group; $P<0.05$ using a 2-tailed Mann-Whitney U test). Two patients in the low BDNF group had died before 6 months, and are given a mBI of 0 in the figure. Four other patients in the low BDNF group were not able to be contacted at the 6 month timepoint.

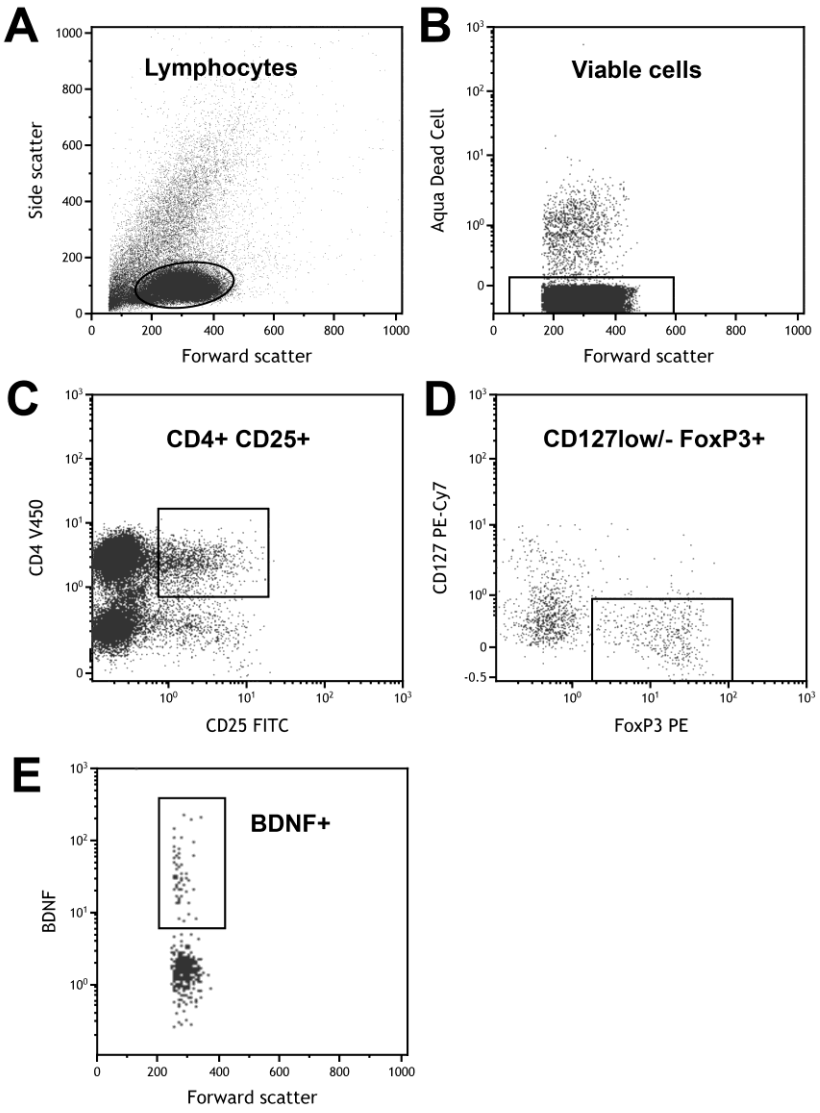

Fig. 1

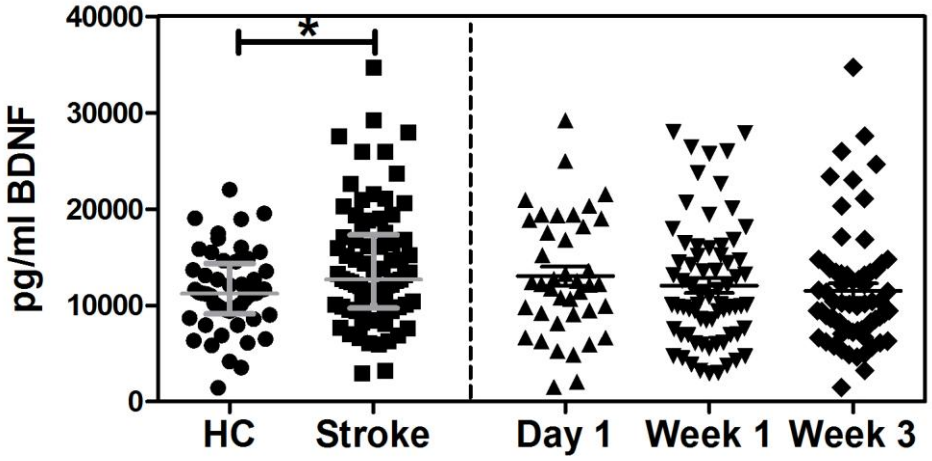

Fig. 2

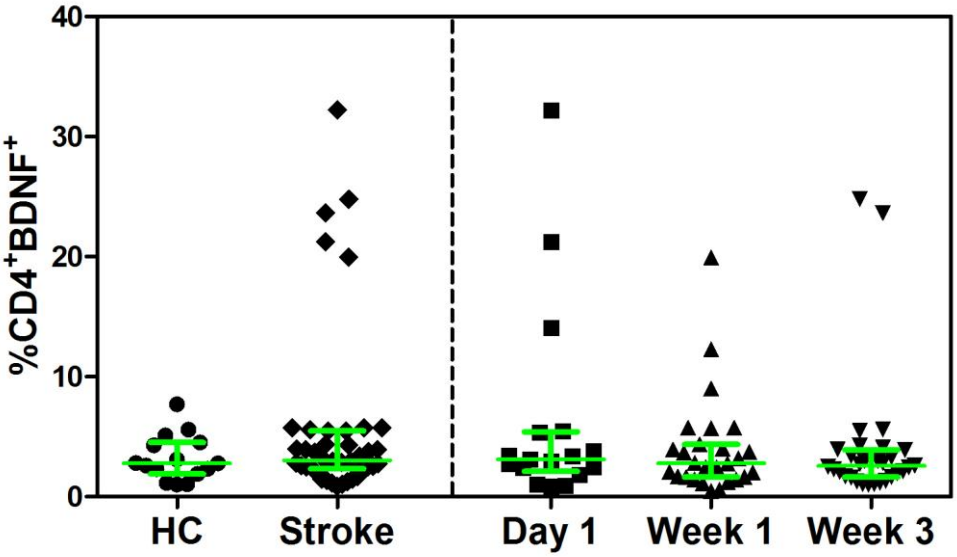

Fig. 3

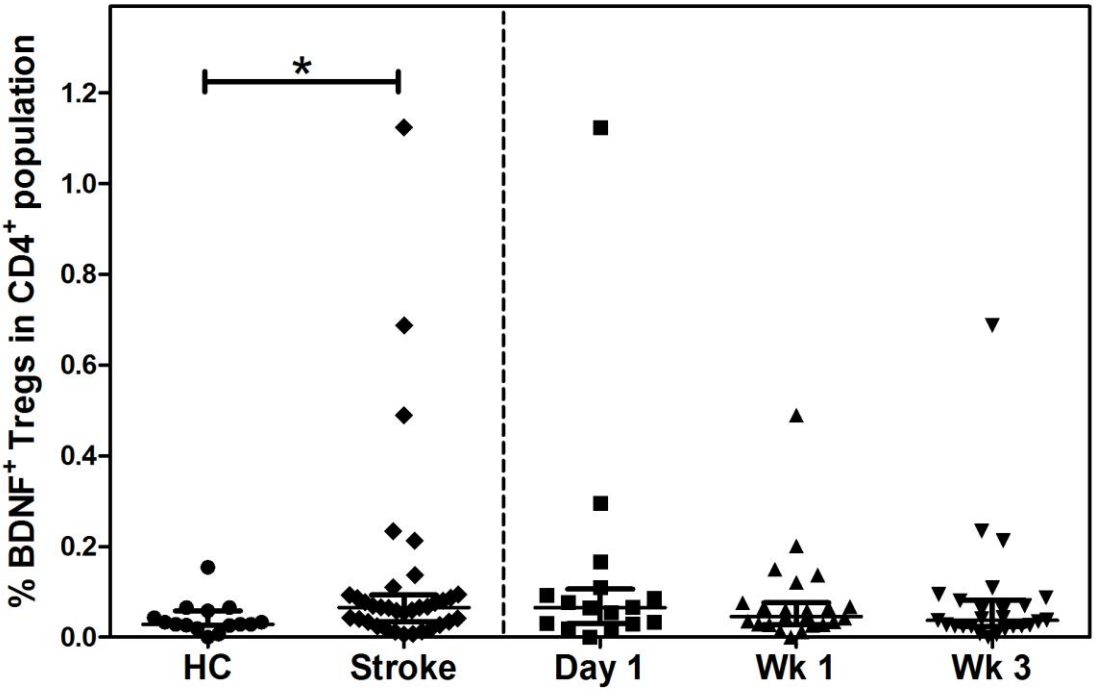

Fig. 4

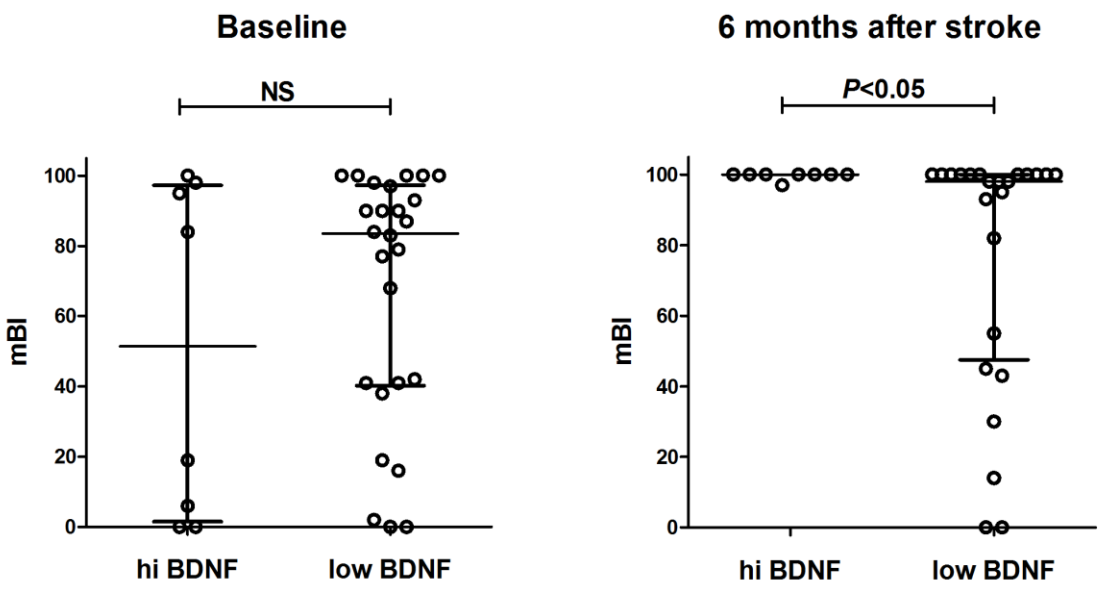

Fig. 5

Highlights

- We measured Treg cells and BDNF positive Treg cells in subjects with acute ischaemic stroke
- The frequency of Treg and BDNF positive Treg was increased in subjects after stroke
- Patients with high levels of BDNF positive Treg cells had a better outcome than other patients
- There was a slight increase in circulating levels of BDNF
- We propose that PDNF positive Treg cells could deliver BDNF to the site of stroke and could be beneficial