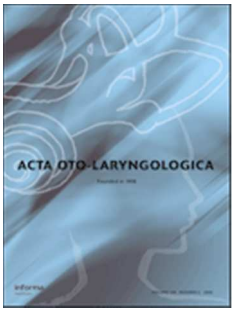

The engraftment and differentiation of transplanted bone marrow-derived cells in the olfactory bulb after methimazole administration.

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Abstract

Conclusion. Bone marrow-derived cells can be engrafted in the olfactory bulb and a few cells can differentiate into mitral/tufted cells in the olfactory bulb. *Objectives.* To investigate whether bone marrow derived-cells can be engrafted into the olfactory bulb and differentiate into neurons and glial cells after methimazole administration. *Method.* Bone marrow of GFP (green fluorescence protein) mice was transplanted into lethally irradiated recipient mice. Furthermore immunostaining was performed to confirm the cell types of bone marrow derived-cells expressing GFP. *Results.* GFP-positive cells were observed in the olfactory bulb at 2 days after methimazole administration. The number of dendritic GFP-positive cells increased up to 30 days after methimazole administration and then decreased. Double immunostaining for GFP and Iba1 or TBX21 showed that a large population of the GFP-positive cells had characteristics of microglia/macrophage and a few cells had characteristics of mitral/tufted cells.

Keywords: *transplantation, regeneration, olfaction, mitral cells, microglia*

Running title, bone marrow-derived cells in the olfactory bulb

Introduction

We have reported studies [1, 2] using bone marrow-derived cells for olfactory tissue regeneration. These studies have demonstrated that a part of donor bone marrow-derived cells engrafted in the olfactory epithelium of radiation-induced immunotolerant recipient mice had characteristics of olfactory receptor neurons [1] and that the early administration of G-CSF (granulocyte-colony stimulating factor) after the administration of methimazole (anti-thyroid function and toxicity specific to olfactory receptor neurons) was more effective to increase the engraftment rate of bone marrow-derived cells into the regenerated olfactory epithelium [2]. We have also found quite a few donor-marker cells in the olfactory bulb after methimazole administration.

The olfactory bulb is one of the relay sites of the olfactory pathway from the nose to the olfactory cortex and consists of five layers (glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer, and granule cell layer).

Mitral/tufted cells in the olfactory bulb, whose dendrites innervate the axon of olfactory receptor neurons at the glomeruli in the glomerular layer, relay olfactory information to the olfactory cortex. Apoptosis of olfactory receptor neurons after methimazole administration [3] induces the denervation of mitral/tufted cells and subsequently a fraction of these cells are fated to fall into cell death.

Mesenchymal stem cells are becoming universally accepted to be capable of differentiating not only into non-epithelial lineages such as chondrocytes, adipocytes, osteocytes, myocytes, but also into epithelial lineages such as hepatocytes and neuron-like cells [4-6] although it remains controversial if the plasticity of adult stem cells including mesenchymal stem cells is true or seems to be true [7, 8]. In addition mesenchymal stem cells have been suggested to be engrafted in damaged tissue as tissue-specific stem cells because of their plasticity and circulating in the body [9]. However, a wave of studies focusing on the possibility of mesenchymal stem cells for regenerative medicine have

elucidated that their roles are not only transdifferentiation into tissue-specific cells, but also secretion of growth and differentiation factors, induction of immune cell-mediated immuno-moderation, and secretion of anti-apoptotic factors [10, 11].

We transplanted bone marrow including mesenchymal stem cells into radiation-induced immunotolerant recipient mice and investigated the cell types of donor-marker positive bone marrow-derived cells in the olfactory bulb after methimazole administration.

Materials and methods

1. Bone marrow transplantation, drug administration, and tissue preparation

After eight-week-old female C57BL/6 recipient mice had undergone 10 Gy of lethal whole-body-irradiation split into two doses separated by 6.5 h, they were transplanted with bone marrow harvested from the femurs of age- and sex-matched GFP

transgenic mice (C57BL/6-Tg (CAG-EGFP)). Donor bone marrow was resuspended in Hank's balanced salt solution and 1×10^6 bone marrow cells were injected into the tail vein of the recipient mice. The recipient mice were maintained in a specific pathogen-free environment and received normal chow and hyper-chlorinated drinking water for the first 4 weeks after bone marrow transplantation.

One month after bone marrow transplantation, a dose of methimazole (50 mg/kg) (Wako, Richmond, VA, USA) was peritoneally administered to damage the olfactory epithelium of the recipient mice. These mice were sacrificed under diethyl-ether anesthesia at 2 days (n=5, 2-day group), 5 days (n=5, 5-day group), 10 days (n=5, 10-day group), 30 days (n=5, 30-day group), and 60 days (n=5, 60-day group) after methimazole administration.

Control mice (n=5, administration of 0.9% saline solution instead of methimazole, control group) were treated in the same manner and sacrificed at 30 days after the administration of saline solution. The anterior part of the head including the olfactory

bulb and epithelium of each mouse was then removed. After overnight fixation in 4% paraformaldehyde and decalcification for 14 days in 10% EDTA (ethylenediamine-tetraacetic acid), the tissue samples were embedded in paraffin and 4 μm thick consecutive coronal cross-sections were prepared.

All experimental protocols complied with the guidelines of the Okayama University's Committee on the Use and Care of Animals. This research was approved (OKU-2011135) by the Animal Experiment Control Committee of the Okayama University Graduate School of Medicine and Dentistry.

2. Immunohistochemistry for OMP (olfactory marker protein) or GFP

After blocking endogenous peroxidase, the sections were then incubated in 0.1% trypsin (Difco Laboratories, Detroit, MI, UAS) for 5 min at 37℃. After blocking of non-specific binding sites by incubation in 10% normal goat serum containing 1% BSA for 15 min, the sections were incubated overnight at 4℃ in an anti-OMP

goat polyclonal antibody (1:2000) (Wako Chemicals) or an anti-GFP rabbit polyclonal antibody (1:1000) (MBL, Nagoya, Japan).

The sections were then rinsed in TBS followed by treatment with a rabbit anti-goat immunoglobulin antibody conjugated-HRP (Santa Cruz Biotechnology, LA, USA) to OMP immunostaining or a goat anti-rabbit immunoglobulin antibody conjugated-HRP (Dako, Copenhagen, Denmark) to GFP immunostaining for 30 min. After rinsing in TBS, the sections were stained with DAB for 15 min.

Sections from the olfactory epithelium of the donor mice were used as positive controls, while negative control sections were processed in an identical way with the exception that the primary antibodies were excluded.

3. Statistical analysis of bone marrow-derived cells in the olfactory bulb

The engraftment rates of donor bone marrow-derived cells into

the olfactory bulb were calculated by counting the number of dendritic GFP-positive cells of the olfactory bulb per 1 mm^2 in a representative slice of the coronal sections. Measurements were carried out microscopically at high magnification (X400) in 2-day, 5-day, 10-day, 30-day, 60-day, and control groups. Data were expressed as medians, interquartile ranges at each group. Significant differences between groups were analyzed Kruskal-Wallis with multiple comparison test (Scheffe type that is based on the Kruskal-Wallis).

4. Double immunostaining for GFP and Iba1 or TBX21

Double immunostaining for GFP and Iba1 or TBX21 was performed in order to confirm the cell types expressing GFP. Iba1 and TBX21 stain the cytoplasm of microglia/macrophages [12] and the nuclei of mitral/tufted cells [13], respectively.

After incubation of the sections in the anti-GFP rabbit polyclonal antibody (1:50) or an anti-GFP goat polyclonal antibody (Abcam, Cambridge, MA, USA) (1:100), an anti-Iba1 goat polyclonal

antibody (1:50) (Abcam, Cambridge, MA, USA) or an anti-TBX21 rabbit polyclonal antibody (1:50) (Santa Cruz Biotechnology) was added. An anti-goat donkey antibody conjugated Alexa Fluor 568 (1:250) (Invitrogen, Carlsbad, CA, USA) for Iba1 and GFP and an anti–rabbit chicken antibody conjugated Alexa Fluor 488 (1:200) (Invitrogen, Carlsbad, CA, USA) for TBX21 and GFP were used as the secondary antibodies.

Results

1. Temporal expression of OMP in the olfactory epithelium and bulb after methimazole administration

The olfactory epithelium

At 2 days after methimazole administration, OMP expression was found only in the olfactory axons of the submucosal layer, because the olfactory epithelium exfoliated from the basal layer. At 5 days after methimazole administration, OMP expression and histological findings were similar to those of 2 days after

methimazole administration. At 10 days after methimazole administration, the regeneration of the olfactory epithelium was found, but OMP expression was weaker than that of the control. The olfactory epithelium was completely reproduced at 30 days after methimazole administration and OMP expression was recovered up to the control level. The findings of 60 days after methimazole administration were similar to those of 30 days after methimazole administration.

The olfactory bulb

Unlike the olfactory epithelium, the initial change of OMP expression was observed at 10 days after methimazole administration (Fig. 1A). Condensed OMP expression was limited to the atrophic glomerular layer. The histological structure and OMP expression of the olfactory bulb at 30 days after methimazole administration (Fig. 1B) were recovered up to those of the control. The changes of OMP expression in the olfactory bulb were delayed behind that of the olfactory epithelium.

A few round GFP-positive cells were found in the glomerular layer at 2 and 5 days after methimazole administration. Round GFP-positive cells increased after 10 days after methimazole administration, while a few dendritic GFP-positive cells were found in the glomerular layer and the external granular layer. At 30 days after methimazole administration, a quite few dendritic GFP-positive cells were present in the glomerular layer and the external granular layer (Fig. 2), while round GFP-positive cells decreased. At 60 days after methimazole administration, dendritic GFP-positive cells also decreased.

2. Statistical analysis of bone marrow-derived cells in the olfactory bulb

The means and standard deviations of dendritic-form GFP-positive cells/mm^2 of all groups were 1.32±1.34 (2-day group), 1.46±0.07 (5-day group), 10.72±4.12 (10-day group), 29.42±15.12 (30-day group), 15.62±9.70 (60-day group), and 2.04±1.62 (control group), respectively. The number of dendritic-form GFP-positive

cells increased up to 30 days after methimazole administration and afterwards decreased. Kruskal-Wallis with multiple comparison showed a significant difference between mean values by groups (P=0.0003). The Scheffe test, a post hoc test suitable for multiple comparison, showed significant differences between 30-day group and 2-day group (P=0.0230) or 5-day group (P=0.0418). Any difference between the other pairs failed to reach statistical significance (P<0.05) (Figure 3).

3. Double-immunostaining for GFP and Iba1 or TBX21

Double-immunostaining for GFP and Iba1 or TBX21 (Fig. 4) showed that numerous GFP^+ $Iba1^+$ cells were distributed in all layers of the olfactory bulb, while a few GFP^+ $TBX21^+$ cells were discovered mainly in the mitral cell layer. The ratio of GFP^+ $TBX21^+$ cells to GFP^+ $Iba1^+$ cells was about 1 percent.

Discussion

Stem cell transplantation has opened the possibility of

regeneration medicine for intractable diseases and mesenchymal stem cells residing in the bone marrow or adipose tissue are thought to be one of the most promising stem cells because of the easy harvest. Blau, *et al.* [14] have proposed the theory that stem cells circulate in the body and are engrafted into a damaged organ as tissue-specific stem cells inherent to the damaged organ. Their theory has been reinforced by numerous experimental and clinical studies. We have also reported that the insult of the olfactory epithelium by methimazole increased the engraftment rate of bone marrow-derived cells into the regenerated olfactory epithelium. The present study has demonstrated that the influences of methimazole to the olfactory bulb occurred behind those to the olfactory epithelium. In other words, methimazole acts on olfactory receptor neurons directly and induces them to apoptosis, while it does not act on the olfactory bulb directly [15]. The morphological changes of the olfactory bulb might be caused mainly by the degeneration of olfactory nerve axons and partly by the cell death of mitral/tufted cells by subsequent denervation. As

a similar secondary influence for the olfactory bulb, the increase of GFAP in the olfactory bulb has been shown to be caused by the primary damage to the olfactory mucosa of methimazole- and dichlobenil-treated mice [15].

Double immunostaining carried out to confirm the cell types expressing GFP demonstrated that all almost GFP-positive cells were microglia/macrophages and a few GFP-positive cells were mitral/tufted cells. This low frequency of transdifferentiation might be explained partly because only a fraction of mitral/tufted cells were denervated by the degeneration of olfactory axons and were induced to cell death.

From our temporal observation GFP-positive cells emerged in the glomerular layer of the olfactory bulb at 10 days after methimazole administration, increased significantly at 30 days after methimazole administration, and decreased down to the initial level at 60 days after methimazole administration. This time dependent change seemed to demonstrate that most GFP-positive cells were macrophages which entered the olfactory

bulb to phagocytose debris of damaged olfactory axons through blood brain barriers damaged by the secondary influences to the glomerular layer of the olfactory bulb by methimazole and macrophages were cleaned up after the histological restoration of the olfactory bulb, while a fraction of these cells differentiated into microglia as seen in a model of ischemic retinopathy [16].

We transplanted bone marrow including mesenchymal stem cells and reported that transplanted bone marrow-derived cells differentiated into olfactory receptor neurons. The present study also demonstrated that bone marrow-derived cells differentiated into microglia and rarely differentiated into mitral/tufted cells.

These results may be the first step to regeneration therapy using bone marrow transplantation for the secondary central dysosmia at the olfactory bulb although transdifferentiation to mitral/tufted cells is rare.

Conflict of interest

There is no conflict of interest for this manuscript, including financial, consultant, and other relations.

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Legends

Figure 1. OMP expression in the olfactory bulb.

(A) 10 days after methimazole administration, (B) 30 days after methimazole administration. Scale bar = 50μm.

Figure 2. GFP-positive cells in the olfactory bulb at 30 days after methimazole administration.

GFP-positive dendritic or round cells are mixed in the olfactory bulb, but the ratio of dendritic cells is superior. The inlet magnifies the dendritic cell indicated by the arrow.

Scale bar = 100μm.

Figure 3. The number of dendritic GFP-positive cells at each time point after methimazole administration.

Data were expressed as medians , interquartile ranges at each stage AMA. Significant differences between both stages were analyzed using Kruskal-Wallis with multiple comparison.

* $P<0.05$, *** $P<0.001$

Figure 4. Double immunostaining for GFP and Iba1 or TBX21 in the olfactory bulb on 30 days after methimazole administration. (A, D) GFP staining. (B) Iba1 staining. (C) Superimposing of GFP (green) and Iba1 (red) shows co-localization (orange). Superimposed image shows that a part of the donor bone marrow-derived cells became microglia/macrophages in the olfactory bulb. DAPI staining. (blue). (E) TBX21 staining. (F) Superimposing of GFP (red) and TBX21（green） shows co-localization (orange). Superimposed image shows that a fraction of the donor bone marrow-derived cells became mitral/tufted cells in the olfactory bulb. DAPI staining (blue). Scale bar = 20μm.

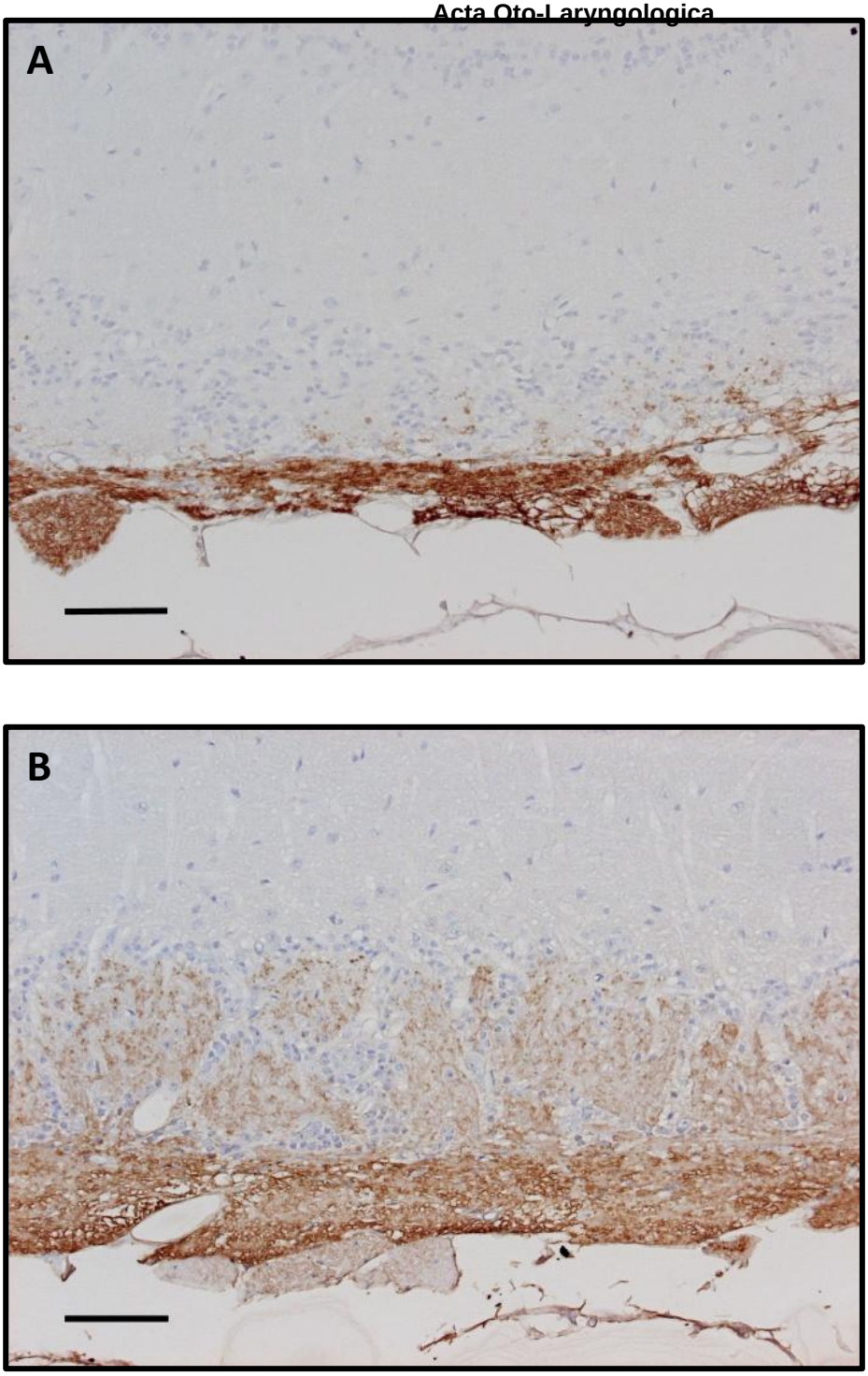

Fig. 1

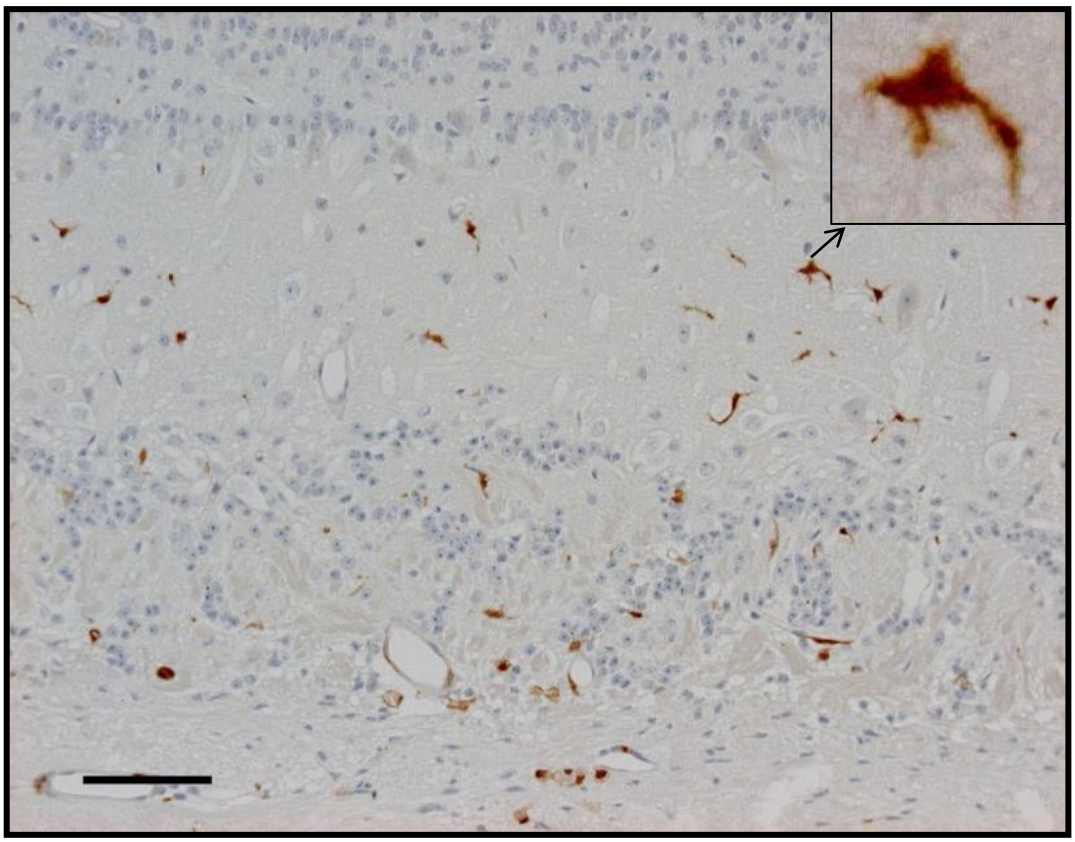

Fig. 2

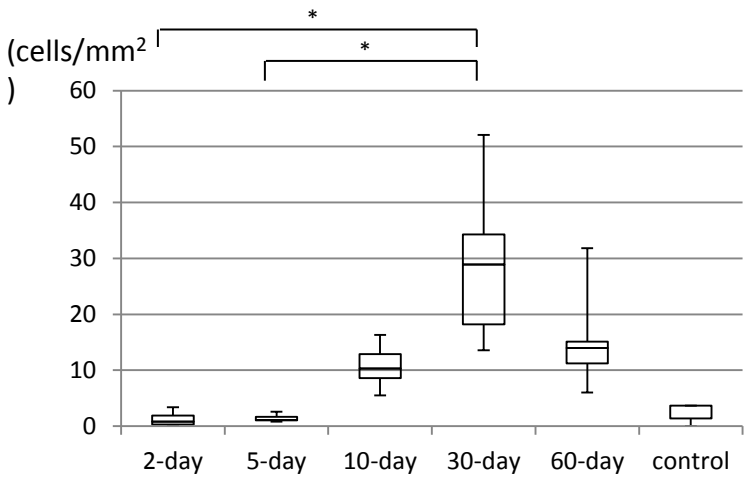
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Fig. 3

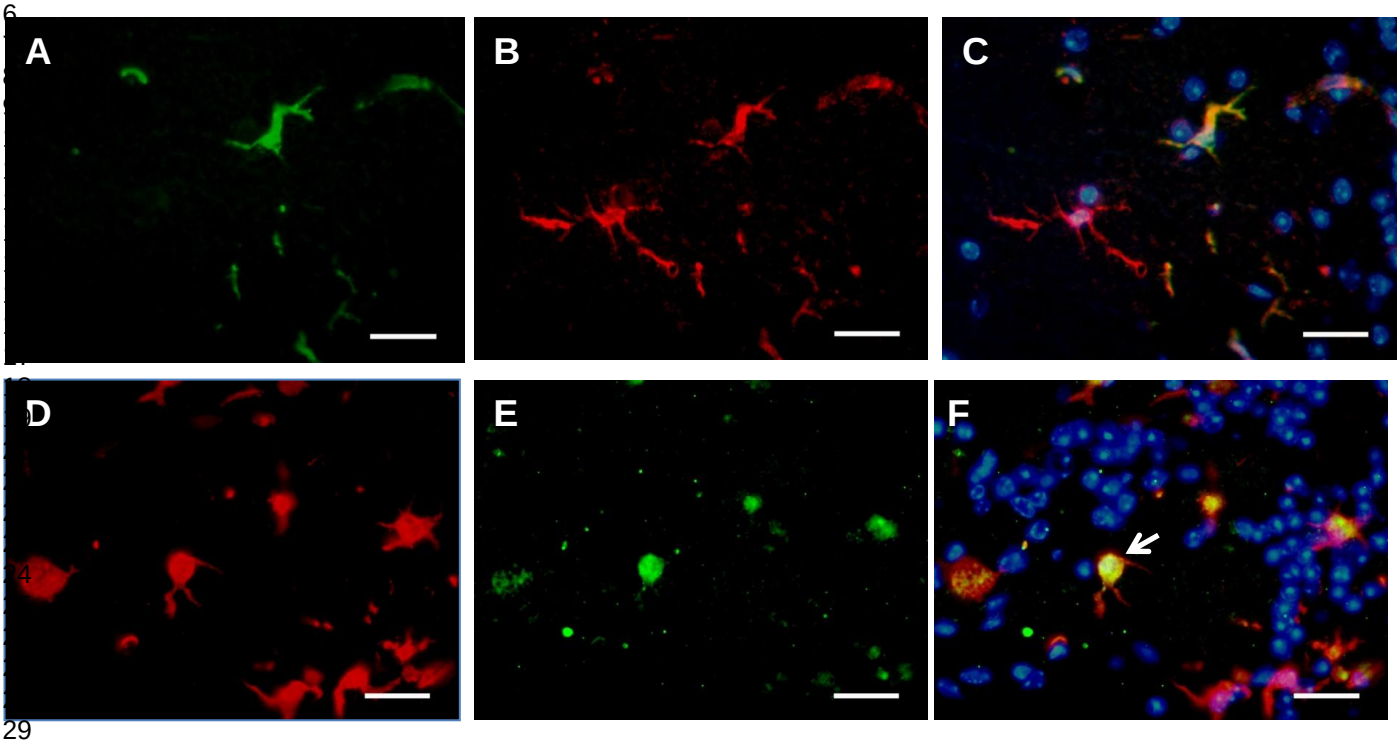

Fig. 4