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A subpopulation of endothelial progenitor cells with low aldehyde dehydrogenase activity attenuates acute ischemic brain injury in rats

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Abstract

Previous studies have examined the therapeutic effect of endothelial progenitor cells (EPCs) during the chronic phase of cerebral infarction in rats; however, few studies have investigated the effects of EPCs during the acute phase of infarction. In this study, we evaluated the therapeutic effect of EPCs with low aldehyde dehydrogenase activity (Alde-Low EPCs) in rats with acute cerebral infarction, and our results provide insight that may help to identify a therapeutic mechanism of EPCs for acute cerebral infarction. The administration of Alde-Low EPCs into rats with acute cerebral infarction results in the accumulation and migration of the Alde-Low EPCs into the infarct area and the subsequent decrease of infarct volume. Moreover, we found that the stromal cell-derived factor-1 (SDF-1) and CXC chemokine receptor 4 (CXCR4) signaling pathway may regulate the accumulation of Alde-Low EPCs. The transplantation of Alde-Low EPCs may represent a potential treatment strategy for acute cerebral infarction.

Keywords: Endothelial progenitor cells; Aldehyde dehydrogenase activity; Cell transplantation therapy; SDF-1; CXCR4

1. Introduction

Due to recent developments in regenerative medicine and cell technology, cell therapy has shown great promise as a new treatment for ischemic stroke. In fact, a variety of cell types are currently being investigated as potential therapeutic agents: neural stem/progenitor cells, mesenchymal stem cells, endothelial progenitor cells (EPCs), embryonic stem (ES) cells, and induced pluripotent stem (iPS) cells [1,2,3,4].

Asahara and colleagues first identified EPCs as a subpopulation of the leukocyte fraction of human peripheral blood [5]. Recent studies have suggested that EPCs contribute to tissue vascularization during both physiological and pathological processes, e.g., wound healing and cancer, respectively [6,7]. In particular, there has been a growing interest in EPCs as potential therapeutic agents for ischemic disorders because of their ability to promote recovery after ischemic events, such as hind limb ischemia and myocardial infarction [6]. In stroke, however, EPC studies have been limited [8].

We have recently identified a population of EPCs derived from umbilical cord blood (UCB) that is suitable for the treatment of ischemic tissue [9]. We divided EPCs into two subpopulations based on the level of their aldehyde dehydrogenase (ALDH) activity and consequently found that EPCs with low ALDH activity (Alde-Low EPCs) possessed a

greater ability to proliferate and migrate compared with EPCs with high ALDH activity (Alde-High EPCs). In addition, previous studies have shown that the introduction of Alde-Low EPCs significantly reduced tissue damage in the ischemic condition of a mouse skin flap model [9].

Here, we confirmed the therapeutic effect of Alde-Low EPCs for acute cerebral infarction using a rat ischemia/reperfusion model.

2. Materials and Methods

2.1. Preparation of cells for administration

Alde-Low EPCs, Alde-High EPCs, and human umbilical vein endothelial cells (HUVECs) were prepared as previously described [9] and as briefly summarized below.

Human full-term UCB samples were collected from umbilical cord veins with permission from the local ethics committee of the University of Tsukuba. Mononuclear cells were separated from the UCB by density gradient centrifugation and cultured in medium consisting of Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen, Carlsbad, CA) with fetal bovine serum (FBS; Hyclone, South Logan, UT), L-glutamine (Invitrogen), and human basic fibroblast growth factor (hb-FGF; Peprotech, London, UK) in a flask without a specific surface coating. The adherent cells were analyzed for their incorporation of the PE-conjugated, low-density lipoprotein from the human plasma acetylated Dil complex (DiI-Ac-LDL; Molecular Probes, Eugene, OR); this marker is observed in both endothelial cells and macrophages. After the Dil-Ac-LDL assay, the cells were sorted into $CD45^-/CD31^+/Dil\text{-}Ac\text{-}LDL^+$ subpopulations by fluorescence-activated cell sorting (FACS) to exclude macrophages and monocytes. The sorted cells were then expanded for further

experiments. When grown on a Matrigel substrate, these cells formed capillary tube-like structures that resembled the morphology of endothelial cells. Because these cells exhibited responses and properties that were similar to those of EPCs, we concluded that they were EPCs. The UCB-derived EPCs were further separated according to ALDH activity into Alde-High and Alde-Low cells. The ALDH activity was analyzed using an Aldefluor reagent (StemCell Technologies, Vancouver, BC) [10], and the cells were sorted by FACS. The HUVECs were purchased from Cambrex (Walkersville, MD) and maintained in endothelial cell basal medium 2 (EBM-2; Cambrex).

The cultured HUVECs, Alde-High EPCs, and Alde-Low EPCs were transfected with green fluorescent protein (GFP) as described previously [11], and the GFP-labeled HUVECs and EPCs were purified using FACSVantage (BD Biosciences, San Jose, CA).

2.2. Transient middle cerebral artery occlusion (MCAo) and transarterial cell transplantation

All of the animal experiments were performed in a humane manner following approval from the Animal Experiment Committee of the University of Tsukuba and in accordance with the Regulation for Animal Experiments Committee of the University of Tsukuba.

Transient middle cerebral artery occlusion (MCAo) model was selected for the similarity with clinical condition during acute phase of ischemia, because reperfusion occured in 20% of patients within 24 hours after MCAo and 80% of patient within seven days [12,13].

Male Sprague-Dawley rats weighing 280-320 g were purchased from Charles River Japan Inc. (Yokohama, Japan). The induction of MCAo is an established technique in our laboratory and has been previously described [14]. In brief, after ligation of both the right common carotid artery (CCA) and the external carotid artery, a 4-0 nylon suture (Alfresa Pharma, Osaka, Japan) with silicon coating (Dow Corning Corporation, Midland, MI) on the tip was introduced from the CCA via the internal carotid artery (ICA) into the anterior cerebral artery (ACA) until the origin of middle cerebral artery (MCA) was occluded. Reperfusion was achieved by withdrawal of the suture following a transient occlusion of the MCA for 60 minutes. A neurological deficit, consisting of a left-sided hemiparesis and Horner's syndrome on the right side, was evaluated at 30 minutes and 24 hours after reperfusion using the modified Neurological Examination Grading System [15], and the regional cerebral cortical blood flow (rCoBF) was simultaneously measured trans-cranially using a Laser Doppler flowmeter (ADVANCE, Kyoto, Japan). The neurological deficit and the rCoBF were used as the criteria for a successful ischemic insult.

Thirty minutes after reperfusion of the MCA, the rats received an injection of HUVECs, Alde-High EPCs, or Alde-Low EPCs (5×10^5 cells) with 0.5 ml of phosphate-buffered saline (PBS) into the CCA via the same route through which the occluded suture was withdrawn. Immunosuppression was induced using an intraperitoneal injection of cyclophosphamide (cyclosporine-A; Wako, Osaka, Japan) at 10 mg/kg body weight 24 hours before and immediately after the transplantation.

2.3. Measurement of infarct volume

Twenty-four hours after transplantation, the rats were anesthetized with a lethal dose of intraperitoneal pentobarbital and perfused with PBS via the ascending aorta. The brains were carefully removed and cut into seven serial coronal slices (each 2 mm in thickness) from the frontal pole. These sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 minutes and imaged to calculate the volume of the infarct areas using SCION Image (SCION Corporation, Frederick, MD). We calculated the ratio of the total infarct area of the seven coronal sections to the contralateral hemisphere (% infarct volume) as the total infarct volume to reduce the distortion of the infarct area by brain edema.

2.4. Immunohistochemical and quantitative analysis of GFP-positive cells in the cerebral hemisphere

Twenty-four hours after cell transplantation, the perfused brains were removed as previously described. The brains were cut into 2-mm-thick coronal slices at the level of the caudate-putamen (6-8 mm caudal from the frontal pole); these slices included the MCA perfusion area. The sections were perfused with 4% phosphate-buffered paraformaldehyde (PBPA) for 24 hours and cryoprotected with 20% sucrose in PBS for an additional 24 hours. The fixed brain slices were then sliced into 10-μm-thick coronal sections. The sections were incubated with the following primary antibodies: a monoclonal mouse antibody against α-smooth muscle actin (α-SMA; DAKO, Glostrup, Denmark) and a rabbit polyclonal antibody against SDF-1 (Santa Cruz Biotechnology, Inc. Santa Crus, CA). Following incubation, the sections were then incubated with the following secondary antibodies: Alexa Fluor 555 goat anti-mouse (Invitrogen) and Alexa Fluor 555 goat anti-rabbit. The sections were counterstained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostics-Applied Science, Mannheim, Germany). The entire hemispheric area was observed by fluorescence microscopy (Biozero, BZ8000,

KEYENCE Inc., Osaka, Japan) for the detection and quantitative analysis of GFP-positive cells within designated rectangular regions (400 μm × 300 μm) for each rat in a consistent manner.

2.5. Analysis of the involvement of the SDF-1/CXCR4 signaling pathway in the accumulation of Alde-Low EPCs

To assess the involvement of the SDF-1/CXCR4 pathway in the accumulation of Alde-Low EPCs, we transplanted Alde-Low EPCs with CXCR4 down-regulation using small interfering RNAs (siRNAs) in MCAo rats. Stable down-regulation of CXCR4 was achieved by transduction of a lentiviral vector with the expression of short hairpin RNA for CXCR4 (CXCR4 shRNA), generated in the MISSION TRC-Hs1.0 (Sigma-Aldrich, Steinheim, Germany; the clone number is TRCN0000004053, and the sequence inserted to the vector was 5’-CCGGCATCATCTTCTTAACTGGCATCTCGAGATGCCAGTTAAGAAGATGATGT TTTT-3’). After incubating the Alde-Low EPCs for 24 hours in a 24-well plate (until the cells reached 80% confluency), 200 μl media and hexadimethrine bromide (Sigma-Aldrich; final concentration 8 μg/ml) were added to each well to enhance the

transduction efficiency. After the addition of 20 μl of the CXCR4 shRNA (Sigma-Aldrich) to the appropriate wells and an additional 48 hours of incubation, the Alde-Low EPCs that had been transduced with CXCR4 shRNA were selected using puromycin (Sigma-Aldrich; final concentration 2 μg/ml). Reverse-transcription–polymerase chain reaction (RT-PCR) revealed that CXCR4 gene expression had decreased to 14%. The CXCR4 primers used were 5'-CTGTGACCGCTTCTACCCCAATGACTT-3' and 5'-CTTGGGGTAGGAGATACGAAAGGAACC-3'.

The Alde-Low EPCs that had been down-regulated by the CXCR4 shRNA (Alde-Low EPCs CXCR4si) were also transfected with GFP. We evaluated the infarct volume and the accumulation of Alde-Low EPCs CXCR4si 24 hours after cell transplantation, as described above.

2.6. Statistical analysis

The values obtained in this study were statistically evaluated using Student's *t*-test for per-comparison analyses. All of the values are presented as the means ± SD.

3. Results

3.1. Reduction of the infarct volume

Twenty-four hours after the transplantation of EPCs, we examined the infarct lesions, which could be identified as unstained areas by TTC-staining (Fig. 1A-D). Interestingly, the infarct volume was significantly reduced in the rats of the Alde-Low EPC transplanted group (Fig. 1D) compared with the PBS, HUVECs, or Alde-High EPC transplanted group (Fig. 1A-C). The infarct volumes (% infarct volume) in each group were as follows: PBS, 77.0% ± 16.9%; HUVECs, 72.4% ± 18.2%; Alde-High EPCs, 63.1% ± 22.0%; and Alde-Low EPCs, 37.1% ± 10.4% (PBS or HUVECs vs. Alde-Low, $p < 0.0005$; Alde-High vs. Alde-Low, $p < 0.005$, n = 10) (Fig. 1E).

3.2. Distribution and accumulation of GFP-positive cells

In the Alde-Low EPC transplantation group, a larger number of GFP-positive cells had accumulated on the ischemic side compared with the control side (Fig. 2A, 2B), despite the lack of laterality in the distribution of GFP-positive cells in the HUVECs and Alde-High

EPC transplanted groups. The GFP-positive cells were detected morphologically as spindle-shaped cells with cytoplasmic GFP fluorescence and nuclear DAPI fluorescence or a round nuclear defect (Fig. 2C-E). In the Alde-Low EPC transplanted group, we observed scattered groups of GFP-positive cells that were localized within the cerebral parenchyma of the striatum and lateral cortex of the infarct side (Fig. 2F), indicating migration of the Alde-Low EPCs. Quantitative analysis of GFP-positive cells per hemisphere revealed that significantly greater numbers of GFP-positive cells were detected on the infarct side of the Alde-Low EPCs transplanted group (Fig.3). Consequently, the Alde-Low EPCs specifically accumulated on the infarct side even under the acute experimental conditions. The number of GFP-positive cells per hemisphere was as follows: HUVECs on the infarct side, 88.7 ± 9.5 cells per hemisphere; Alde-High on the infarct side, 118.3 ± 18.8; Alde-Low on the control side, 132.3 ± 48.0; Alde-Low on the infarct side, 318.7 ± 89.8 (HUVECs or Alde-High vs. Alde-Low on the infarct side, $p < 0.05$; Alde-Low on the control side vs. the infarct side, $p < 0.05$, n = 3) (Fig. 3).

3.3. Involvement of the SDF-1/CXCR4 pathway in the accumulation of Alde-Low EPCs

Fluorescent analysis revealed the expression of SDF-1 in the endothelial cells of capillaries in all the groups, and we found that the GFP-positive cells had accumulated in the capillaries (Fig. 4A). In addition, in the Alde-Low EPC CXCR4si transplanted group, the number of GFP-positive cells on the infarct side was reduced and the infarct volume had increased compared with the Alde-Low EPC transplanted group (Fig. 4B). Consequently, down-regulation of CXCR4 in the Alde-Low EPCs decreased their accumulation in the ischemic area. The number of GFP-positive cells in the Alde-Low EPCs CXCR4si transplanted group was 127.0 ± 6.1 cells per hemisphere, compared with 318.7 ± 89.0 cells per hemisphere observed in the Alde-Low EPC group ($p < 0.05$, n = 3). Moreover, the infarct volumes were 53.0% ± 11.8% in the Alde-Low EPC CXCR4si transplanted group and 37.1 ± 10.4% in the Alde-Low EPCs group ($p < 0.05$, n = 3) (Fig. 4B).

4. Discussion

EPC transplantation therapy shows great promise as a therapeutic option for ischemic disorders because of the ability of EPCs to promote recovery after ischemic events [6,16]. However, there have been limited EPC studies investigating the acute phase of stroke [8,17]. Moreover, the identification and isolation of EPCs have been controversial issues within the research community [18]. EPCs were originally characterized by their cell surface expression of CD34, CD133 and VEGFR-2 [5]; therefore, EPCs have been predominantly defined based on the expression of cell surface markers. EPCs can be isolated from a variety of sources, such as peripheral blood [19], bone marrow [17], spleen [20], adipose tissue [21,22], ES cells [2,23], and the UCB [9,24]. Because of their diverse origins, it has been unclear which type of EPC is the most suitable for cell therapy for stroke and other ischemic disorders.

Some studies examined the therapeutic time window of EPC transplantation during the chronic phase in a model of cerebral ischemia [1]. Another study of autologous mononuclear cell transplantation for cerebral ischemia in rats at 3-72 hours after induction of MCAo reported that the earlier administration of cells was more effective at decreasing the lesion size and increasing the functional outcome [25]. However, few studies have

reported on the beneficial effect of EPC transplantation during the acute phase, which is considered to be within 24 hours of transplantation [17,26].

In this study, we confirmed that transplantation of Alde-Low EPCs specifically attenuated the infarct volume and that EPCs accumulated in the ischemic region 24 hours after induction of MCAo in rats. These results are consistent with our previous study of Alde-Low EPC transplantation using the mouse skin flap model. However, during the acute phase of ischemia, the therapeutic mechanisms of EPCs remain unclear. During acute phase, trophic factors may also contribute to the therapeutic effects for cerebral ischemia through several different mechanisms, including free radical scavenging, anti-apoptotic activity, and anti-inflammatory activity, thereby reducing secondary brain damage after cerebral infarction [17,25,27,28].

We found the specific accumulation of Alde-Low EPCs at the ischemic site during the acute phase to be particularly interesting. To investigate the therapeutic mechanism of Alde-Low EPCs, we investigated the ability of the SDF-1/CXCR4 signaling pathway to mediate the accumulation of Alde-Low EPCs. CXCR4, a 7-transmembrane spanning G-protein-coupled receptor, is expressed on different types of stem cells, progenitor cells, and mature endothelial cells. The chemokine SDF-1 is a proinflammatory cytokine involved in cell trafficking and adhesion and binds exclusively to its cognate receptor,

CXCR4 [29,30,31]. Many studies have shown that the SDF-1/CXCR4 signaling pathway regulates angiogenesis and vasculogenesis during development via the mobilization, migration and differentiation of EPCs [30,32,33]. In addition, SDF-1 is expressed in the ischemic area even during the acute phase [34]. We have previously demonstrated that CXCR4 expression increased significantly in the Alde-Low EPCs compared with the Alde-High or control cells under hypoxic conditions and that Alde-Low EPCs have a higher migratory activity in vitro compared with Alde-High EPCs [9]. Moreover, we used CXCR4 siRNA to specifically block SDF-1/CXCR4 signaling [35,36]. We confirmed that Alde-Low EPCs CXCR4si exhibited a reduced ability to accumulate in vivo, strongly suggesting that SDF-1/CXCR4 signaling plays an important role in the accumulation of Alde-Low EPCs during the acute phase of ischemia.

In future studies, we intend to investigate the functional properties of Alde-Low EPCs and investigate the therapeutic mechanism in the sub-acute and chronic phases of infarction in addition to the acute phase.

We demonstrated that a specific subpopulation of EPCs, characterized by low ALDH activity, exerted a therapeutic effect and specifically accumulated in the ischemic area during the acute phase of stroke. We therefore concluded that transplantation of Alde-Low EPCs might be a promising strategy for the treatment of acute cerebral infarction. However,

further studies are essential to determine the safety, effectiveness and efficiency of EPC therapy for clinical application.

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Figure Legends

Fig. 1. Alde-Low EPCs reduced infarct volume at 24 hours after transplantation. (A-D) Representative coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride from the PBS injection group (A), HUVECs group (B), Alde-High EPC transplanted group (C), and Alde-Low EPC transplanted group (D). The infarct area (white) was significantly reduced in the Alde-Low EPC transplanted group (D). (E) The infarct volumes (% infarct volume) in the 4 groups of rats (n = 10) were measured. It should be noted that the smallest infarct volume was observed in the Alde-Low EPCs transplanted group (*$p < 0.005$, **$p < 0.0005$).

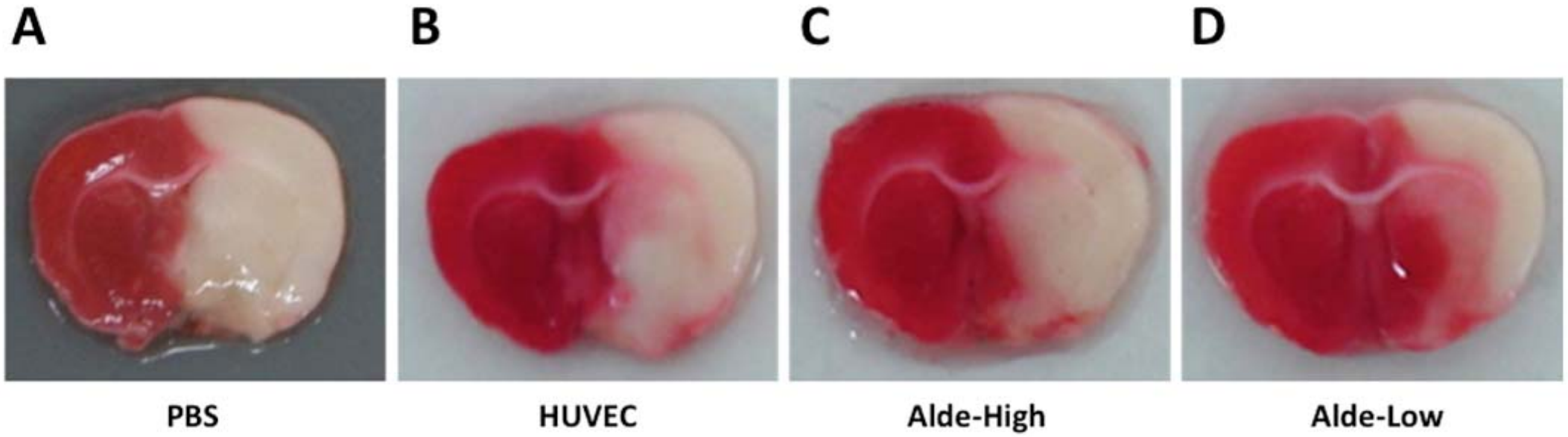
Fig. 2. The distribution and accumulation of GFP-positive cells in the Alde-Low transplanted group 24 hours after transplantation. Fluorescent immunohistochemical analyses were performed (A-F). (A) and (D) show the control side and (B), (C), (E) and (F) show the ischemic side. A larger number of GFP-positive cells (green) accumulated to the ischemic side (B) compared with the control side (A) in the caudate-putamen. (C) The GFP-positive cells were identified morphologically as spindle-shaped cells with cytoplasmic GFP fluorescence and nuclear DAPI fluorescence or as a round nuclear defect (arrow) localized predominantly on the capillaries stained with α-SMA (red; arrowheads).

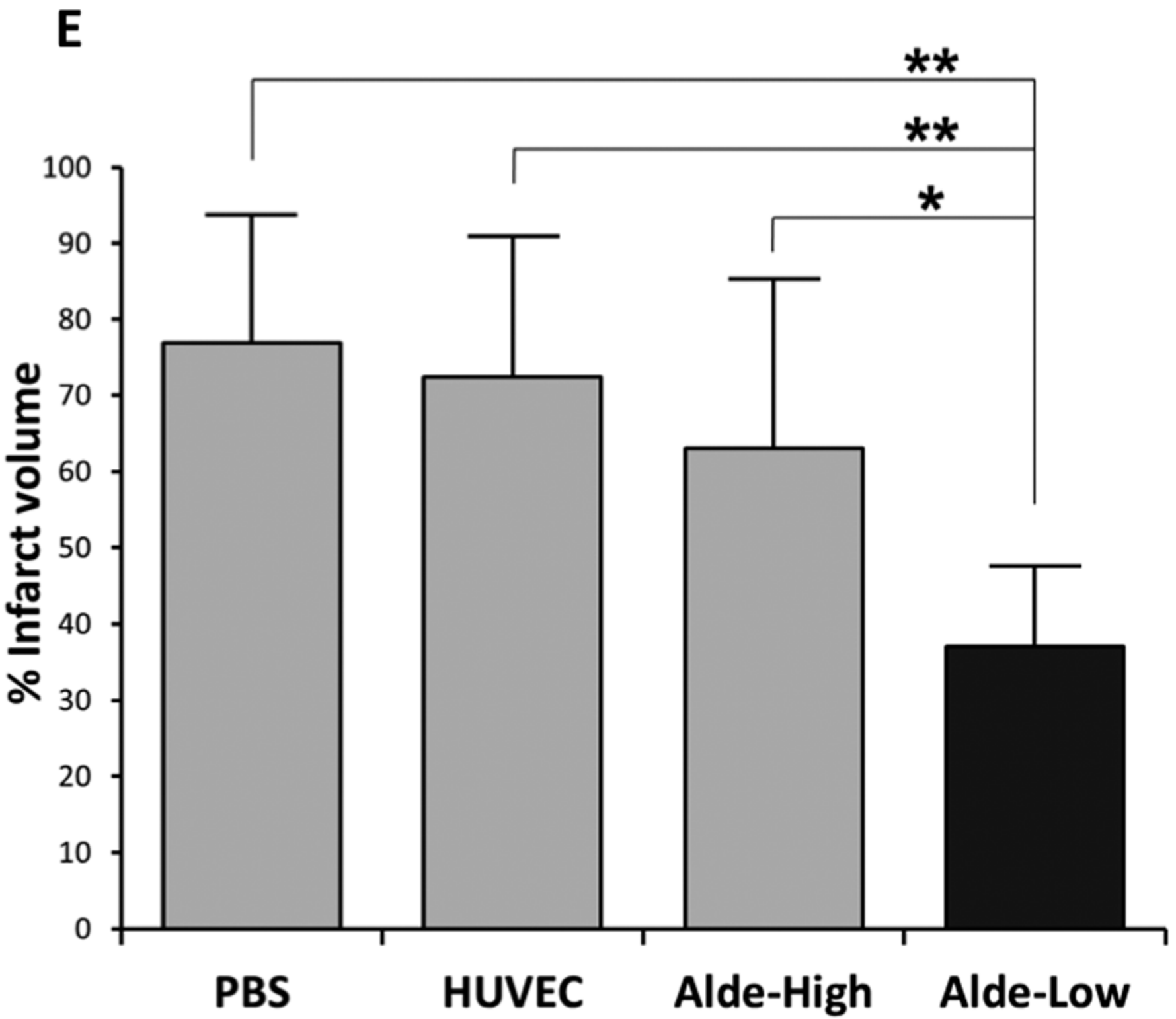
We quantified the GFP-positive cells, which were mainly found on the capillaries (D, E). Specifically in the Alde-Low EPC transplanted group, the migration of GFP-positive cells was observed to be localized within the cerebral parenchyma of the striatum and lateral cortex on the infarct side (F) as well as on the capillaries (E). Scale bars = 50 μm (A, B), 100 μm (C-F).

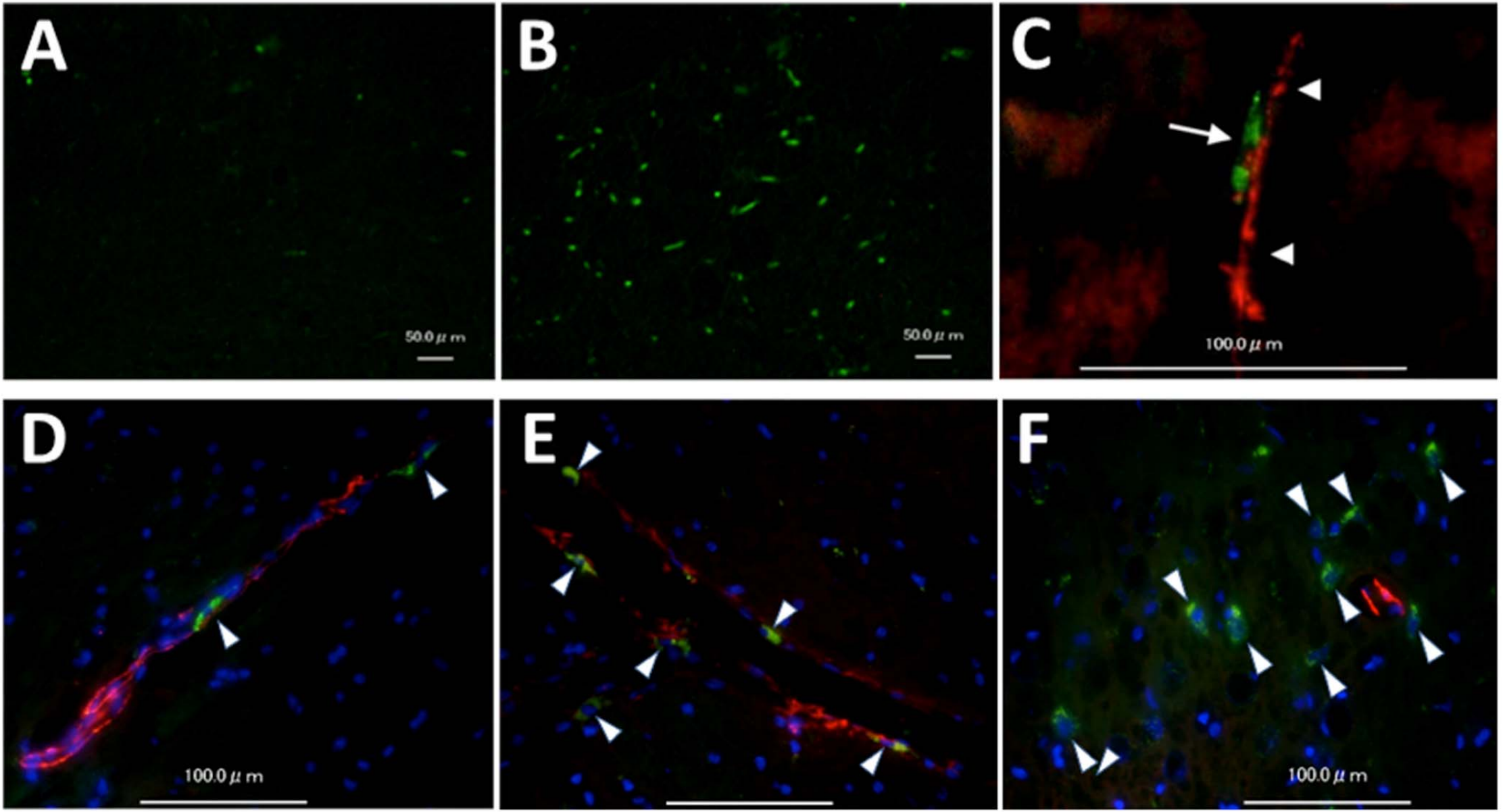
Fig. 3. The quantitative analysis of GFP-positive cells in each group that distributed to the control or ischemic hemisphere. It should be noted that the Alde-Low EPCs specifically accumulated on the ischemic side compared with the ischemic side of the control, the HUVEC and Alde-High EPC transplanted groups (*$p < 0.05$).

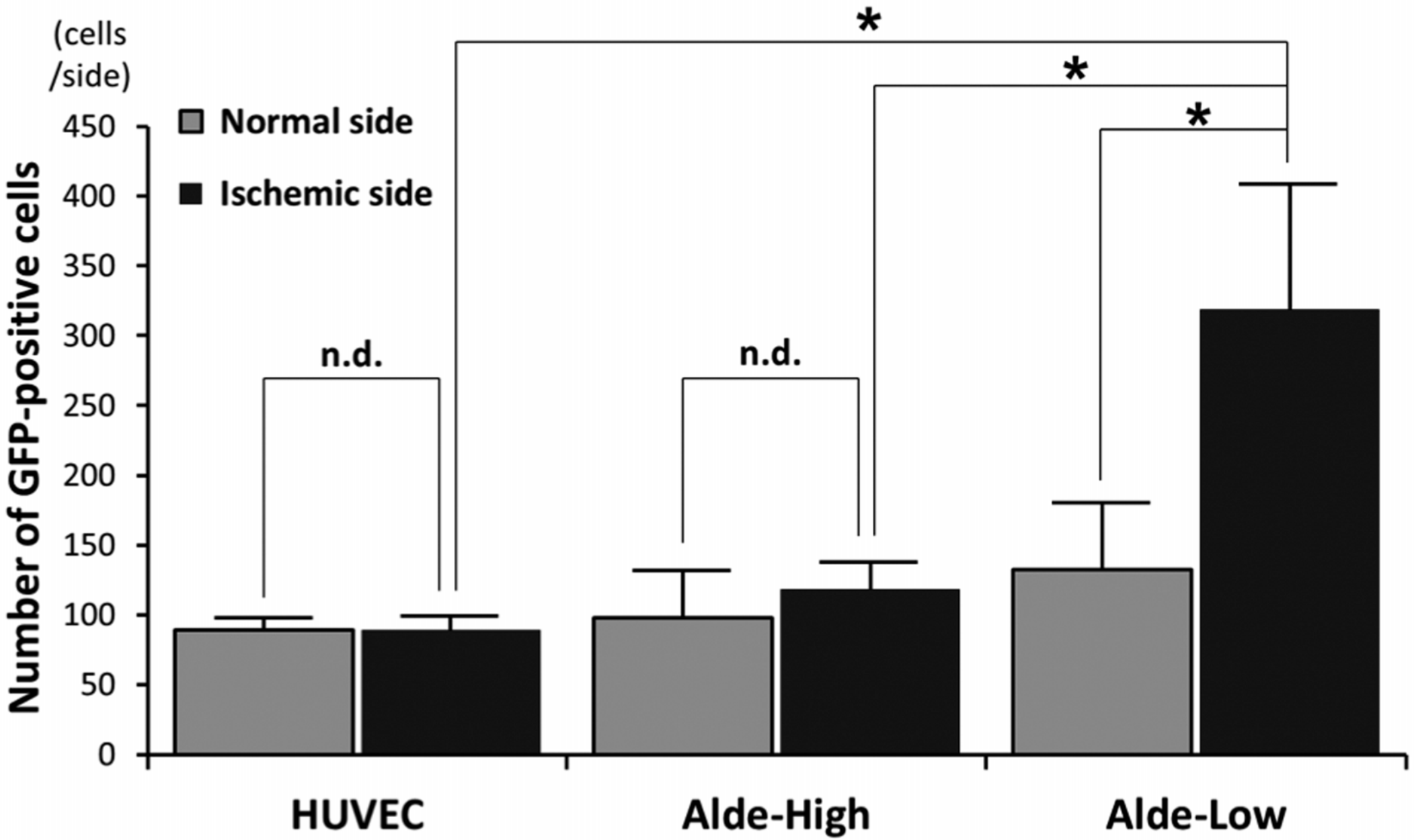
Fig. 4. Involvement of the SDF-1/CXCR4 signaling pathway. We observed the expression of SDF-1 in the endothelial cells of capillaries within the ischemic area 24 hours after MCAo (A). The GFP-positive cells (green) had accumulated toward the SDF-1-expressing endothelial cells (red) (A). In the Alde-Low EPC CXCR4si transplanted group, the number of GFP-positive cells on the infarct side was reduced and the infarct volume had increased compared with the Alde-Low EPC transplanted group (B). Consequently, down-regulation

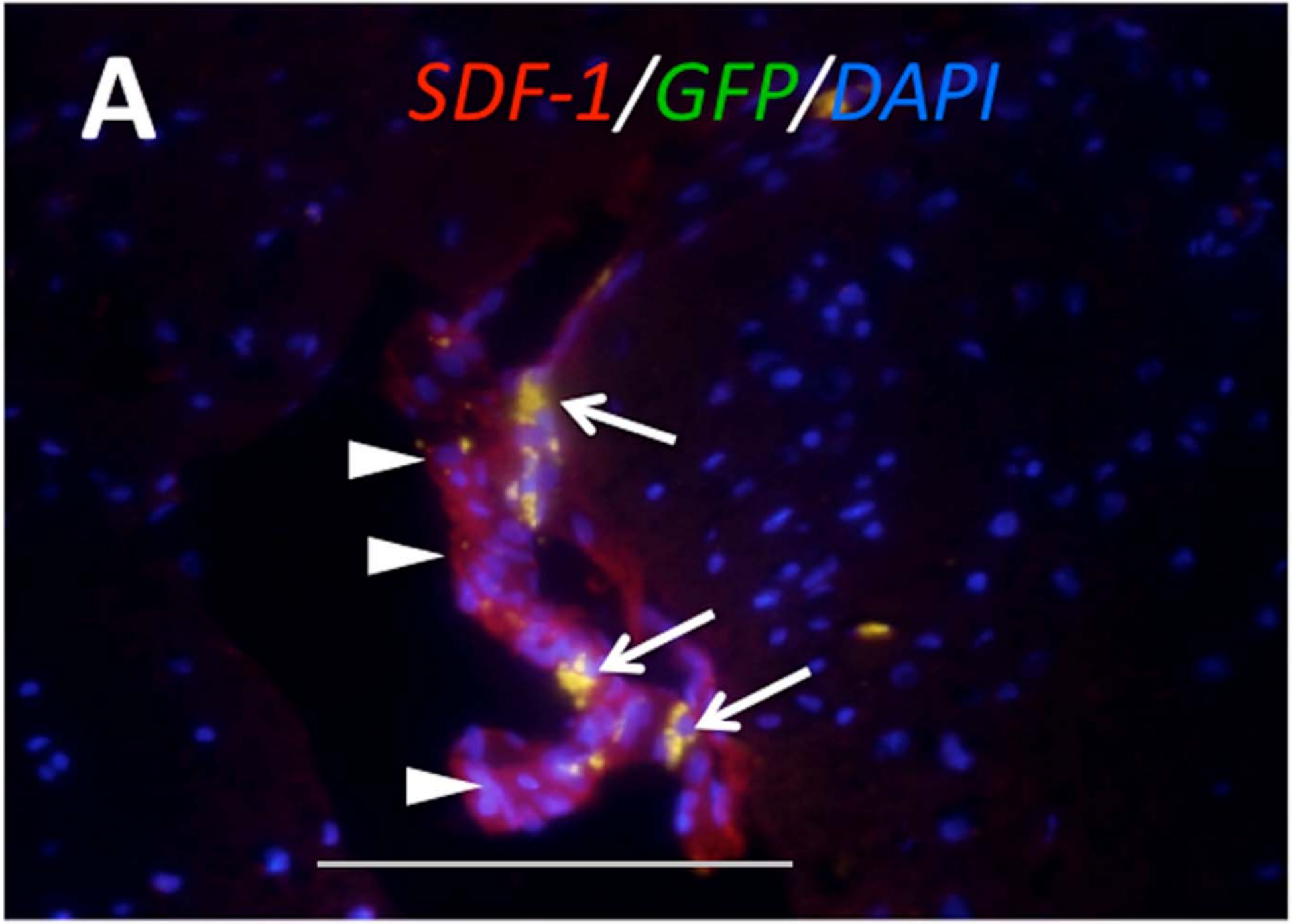
of CXCR4 in the Alde-Low EPCs decreased their accumulation within the ischemic area (*$p < 0.05$, **$p < 0.005$). Scale bars = 100 μm.

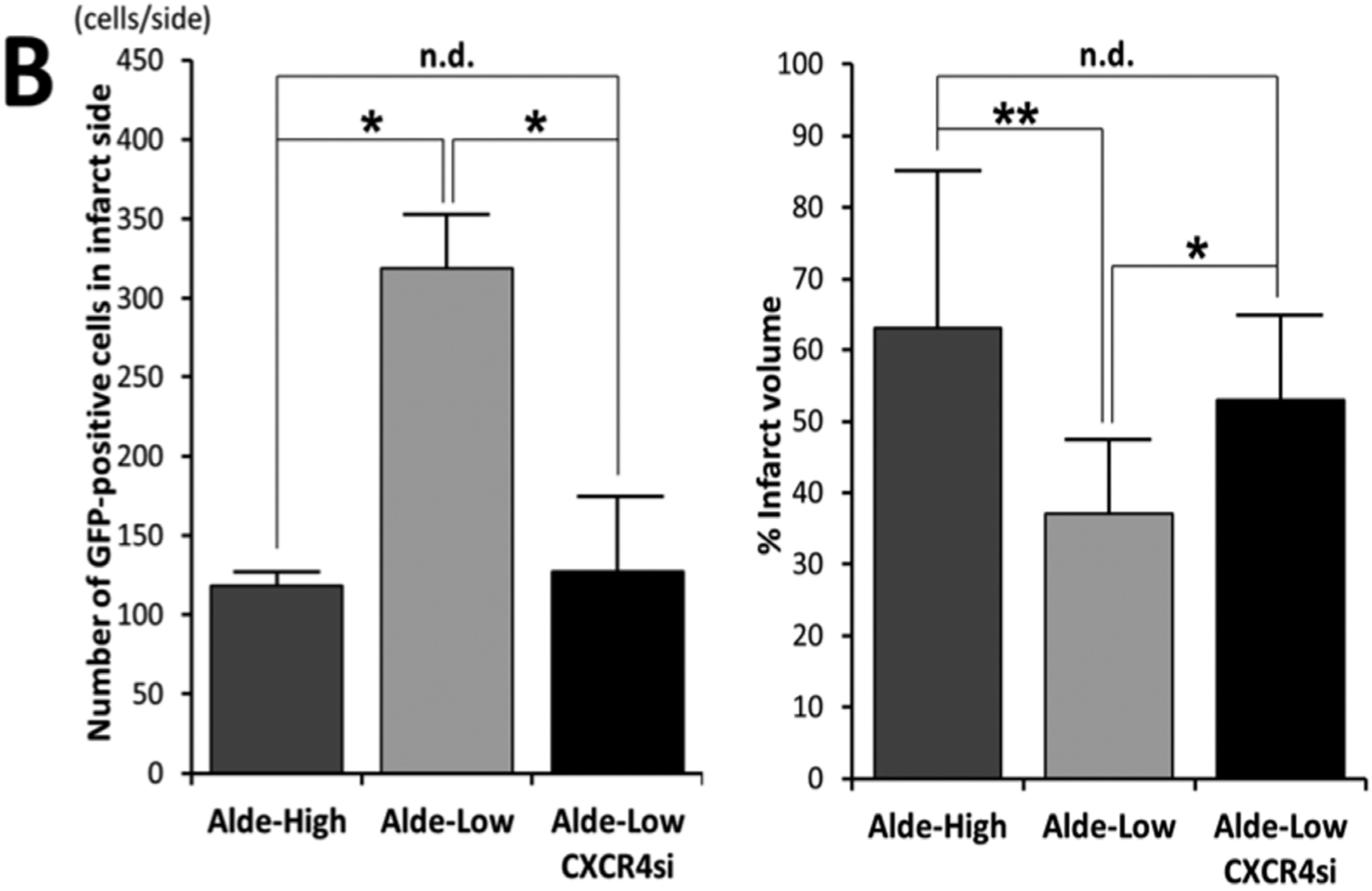

A
B
C
D
PBS
HUVEC
Alde-High
Alde-Low


E
**
**
*
100
90
80
70
60
50
40
30
20
10
0
% Infarct volume
PBS
HUVEC
Alde-High
Alde-Low


A
50.0 μm
B
50.0 μm
C
100.0 μm
D
100.0 μm
E
F
100.0 μm


(cells
/side)
Normal side
Ischemic side
Number of GFP-positive cells
450
400
350
300
250
200
150
100
50
0
n.d.
n.d.
*
*
*
HUVEC
Alde-High
Alde-Low


A
SDF-1/GFP/DAPI


B
(cells/side)
Number of GFP-positive cells in infarct side
450
400
350
300
250
200
150
100
50
0
n.d.
*
*
Alde-High
Alde-Low
Alde-Low CXCR4si
% Infarct volume
100
90
80
70
60
50
40
30
20
10
0
n.d.
**
*
Alde-High
Alde-Low
Alde-Low CXCR4si