Transient decrease in circulating dendritic cell precursors after acute stroke – potential recruitment into the brain

Atilla Yilmaz, Tanja Fuchs, Barbara Dietel, Regina Altendorf, Iwona Cicha, Christian Stumpf, Peter Schellinger, Ingmar Blümcke, Stefan Schwab, Werner G. Daniel, et al.

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

Transient decrease in circulating dendritic cell precursors after acute stroke – Potential recruitment into the brain

Atilla YILMAZ*[1], MD; Tanja FUCHS[2]; Barbara DIETEL[2], PhD; Regina ALTENDORF[2]; Iwona CICHA[2], PhD; Christian STUMPF[2], MD; Peter D. SCHELLINGER[3], MD; Ingmar BLÜMCKE[4], MD; Stefan SCHWAB[3], MD; Werner G. DANIEL[2], MD; Christoph D. GARLICHS[2], MD; Rainer KOLLMAR*[3], MD

[1]*Clinic of Internal Medicine I, Department of Cardiology, University Hospital Jena, Jena, Germany*

[2]*Medical Clinic II, University Hospital Erlangen, Erlangen, Germany*

[3]*Department of Neurology, University Hospital Erlangen, Erlangen, Germany*

[4]*Department of Neuropathology, University Hospital Erlangen, Erlangen, Germany*

*Corresponding authors:

- Atilla Yilmaz, M.D.; Clinic of Internal Medicine I, University Hospital Jena; Erlanger Allee 101, 07747 Jena, Germany; Phone: +49 3641 9 324101; Fax : +49 3641 9 324102; E-mail: A.Yilmaz@web.de
- Rainer Kollmar, M.D.; Department of Neurology; University Hospital Erlangen; Schwabachanlage 6; 91054 Erlangen, Germany. Phone: +49 9131 8534563; Fax: +49 9131 8536597. E-mail: rainer.kollmar@uk-erlangen.de

Running headline: Stroke - Circulating dendritic cell precursors

Keywords: Stroke, ischemic brain injury, inflammatory reaction, leukocyte infiltration, T cell activation

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

ABSTRACT

The role of dendritic cells (DC) as potent mediators of inflammation has not been sufficiently investigated in stroke. Therefore, circulating myeloid (mDCP), plasmacytoid (pDCP), and total DCP (tDCP) were flow cytometrically analyzed in (1) healthy controls (n=29), patients with (2) asymptomatic A. carotis interna stenosis (*ACI-S*, n=46), (3) transient ischemic attack (*TIA*, n=39), (4) acute ischemic stroke (*AIS*, n=73), and (5) acute hemorrhagic stroke (*AHS*, n=31). The National Institutes of Health Stroke Scale (NIHSS) and the infarction size in CT scan were evaluated after stroke. In a patient subgroup, postmortem immunohistochemical brain analyses were performed to detect mDC (CD209), pDC (CD123), T cells (CD3), and HLA-DR. In *AIS* and *AHS*, circulating mDCP ($p< 0.005$), pDCP ($p<0.005$), and tDCP ($p<0.001$) were significantly reduced. A significant inverse correlation was found between the NIHSS and circulating DCP ($p<0.02$), as well as between hsCRP and circulating DCP ($p<0.001$). Patients with large stroke size in CT scan had significantly lower mDCP ($p=0.007$), pDCP ($p=0.05$), and tDCP ($p=0.01$) than those with smaller stroke. Follow-up analysis showed a significant recovery of circulating DCP in the first days after stroke. In the infarcted brain, a dense infiltration of mDC colocalized with T cells, single pDC, and high HLA-DR expression were observed. In conclusion, acute stroke leads to a decrease in circulating DCP. Potentially, circulating DCP are recruited from the blood into the infarcted brain, and probably trigger cerebral immune reactions there.

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

INTRODUCTION

Inflammation is a major secondary pathophysiological factor after stroke [1, 2]. The local cerebral inflammation is involved in focal brain edema, hemorrhage, and tissue necrosis. These events are associated with further impairment of neurological outcome. Moreover, recent studies described the phenomenon of the so called CNS injury-induced immunodepression (CIDS), which is a result of a systemic reduction of circulating lymphocytes and impaired T- and natural killer cell activity, and frequently leads to severe systemic infections with an increase in mortality [3, 4]. Systemic infections are important in the context of stroke. On the one hand, it is well known that preceding infections are a significant risk factor for cerebral infarction [5]. On the other hand, it was shown that stroke-associated infections (SAI) are a predictor of a poor functional outcome [6]. However, the fact that SAI are not independently associated with the neurological outcome after stroke suggests that they do not contribute to cerebral damage, but are a simple marker of stroke severity [6]. This fact is reflected in great clinical trials showing the inefficacy of antibiotic prophylaxis to improve neurological outcome after stroke [7].

Under normal conditions the immune system, particularly T cells, are anergic to cerebral antigens. Following stroke, cerebral inflammation may lead to an autoimmune response directed against neuroepitopes, which can in turn result in further brain injury. Supporting this hypothesis, recent studies showed that administration of lipopolysaccharide (LPS) in a rat model of stroke induced a Th1 immune response toward myelin basic protein (MBP) resulting in persisting severe postischemic brain injury [8]. On the other hand, induction of tolerance by nasal vaccination to MBP or myelin oligodendrocyte glycoprotein led to reduction of infarct size and neurological improvement [9, 10]. Thus, antigen-presenting cells may play a crucial role for the neurological damage after stroke.

Dendritic cells (DC) are effective antigen-presenting cells with the unique ability to stimulate memory and naïve T cells. This provides them with the ability to induce an im-

mune response against antigen which were so far not recognized by the immune system [11]. Thus, DC might be the best candidates for the supposed primary immune response against neuroepitopes after stroke. Two lineages of DC can be differentiated: Myeloid DC which respond to bacteria and fungi, releasing IL-12, as well as plasmacytoid DC which release interferon-α upon viral infection [12]. Both lineages can be detected as *DC precursors* (DCP) in blood, patrolling through the circulation, invading the tissue in response to a local infection or other inflammatory situation. After migration into the tissue, as *immature DC* they are enabled to take up antigens, and upon subsequent maturation acquire the ability to stimulate T cells against those antigens (*mature DC)*. In recent times, the role of myeloid (mDC) or plasmacytoid (pDC) has been implicated in several pro-inflammatory diseases, e.g. atherosclerosis [13, 14]. Furthermore, in multiple sclerosis it was shown that mDC invade the human brain, subsequently triggering cerebral inflammation [15].

The aim of the present study was to investigate if circulating DCP are transiently reduced in patients with acute cerebral ischemia due to their recruitment into the infarcted brain, and if there is evidence for a DC-triggered cerebral (auto)immune response.

MATERIALS AND METHODS

Patients and controls

Between February 2006 and July 2007, 39 patients with *TIA*, 73 patients with acute ischemic stroke (*AIS)*, and 31 patients with acute hemorrhagic stroke (*AHS)* were enrolled, who were admitted within 24 hours after symptom onset to the Department of Neurology of the University Hospital Erlangen. Standard diagnostic clinical analyses on admission included cranial computer tomography (CT), repeated after 24 to 48 h, if the initial CT scan did not show pathological changes, to evaluate regions with cerebral ischemia or hemorrhage. Moreover, doppler sonography of extra- and intracranial carotid arteries, electrocardiography, and echocardiography were routinely performed. Pathological findings (ischemia, hemor-

rhage) were analyzed in the CT scan by a radiologist, and their maximum diameter was assessed. According to the maximum diameter, acute cerebral ischemia were radiologically classified as (1) small stroke (<10 mm), (2) medium stroke (10-30 mm), and (3) large stroke (>30 mm). Furthermore, the etiology of stroke was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [16]. The clinical severity of the stroke was evaluated at admission according to the National Institute of Health Stroke Scale (NIHSS) [17]. Patients with *AIS* admitted within 3 hours after onset of symptoms and 4 to 22 points according to NIHSS underwent rt-PA thrombolysis according to the National Institute of Neurological Disorders and Stroke criteria and AHA guidelines (n=18). The control group consisted of 29 sex- and age-matched cerebrovascular healthy subjects who presented themselves for a healthy checkup. Additionally, 46 patients were analyzed with significant ACI stenosis (> 70%) but without acute cerebral ischemia which were planned for routine carotid endarterectomy. Exclusion criteria were acute or chronic infections, malignancies, autoimmune diseases, hyperthyroidism, acute coronary syndromes, and immunosuppressive medication. Routine blood analyses were performed according to clinic standard. Standardized blood sampling for flow cytometrical analysis was carried out in the first hours after admission and in case of an acute cerebral ischemia at follow-up after 2 days (TIA) or 4 days (stroke). Immediately after blood withdrawal, samples were flow cytometrically analyzed. Informed consent was obtained from all patients. The study was approved by institutional ethics committee of the University Hospital Erlangen.

Serum analysis of pro-inflammatory markers

Serum concentration of high-sensitivity CRP (hsCRP) was measured using an immunonephelometric assay on a BN II analyzer according to the manufacturer's instructions (Dade Behring, Marburg, Germany).

Identification of DC precursors by Fluorescence Activated Cell Sorting (FACS)

Fresh blood samples collected on EDTA were analyzed using the *Blood Dendritic Cell Enumeration kit*^{TM} (Miltenyi Biotec, Bergisch Gladbach, Germany). Three color-staining and FACS analysis were performed as previously described [18]. FACS analysis was performed using the FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). Since circulating DCP comprise only 0.1 to 1% of white blood cells (WBC), a special gating strategy (Figure 1) was used to analyze mDCP, pDCP, and tDCP accurately, as described [18]. The relative cell numbers of circulating DCP were assessed as a percentage of WBC (% WBC). The absolute cell numbers (cells/µL) were calculated using relative cell numbers multiplied by white blood cell count.

Histological characterization of infarcted brain after stroke

Serial slides of brain tissue of patients deceased after stroke and control slides were kindly provided from Prof. I. Blümcke (Neuropathology, University Hospital Erlangen). Brain morphology was analyzed by an experienced neuropathologist using haematoxylin/eosin staining. No major autolytic changes have been observed after a thorough microcopical inspection. Minor changes were present in all specimens and included an increased extracellular space. Subsequently, specimens were classified as acute ischemic (n=17), acute hemorrhagic (n=12), or past stroke (*PS* > 4 weeks, n=12), and healthy tissue (n=11).

Immunohistochemical detection of DC and T cells in infarcted human brain

For immunohistochemical staining, the following monoclonal antibodies were used: anti-CD209 for mDC (Becton Dickinson, Heidelberg, Germany) and anti-CD123 for pDC (BioLegend, San Diego, U.S.A.), anti-CD3 for T cells (Dako, Hamburg, Germany), and anti-HLA-DR (Dako). For immunohistochemical staining of CD209, CD123, and HLA-DR the *Catalyzed Signal Amplification kit* was used according to manufacturer's instruc-

tions (*CSA System*™, Dako). For immunohistochemical analysis of CD3 the *EnVision G/2 Detection System*™ (Dako) was used. Double immunohistochemical stainings were performed with the EnVisionTM Doublestain System (Dako). Negative controls were treated with irrelevant isotype-matched antibodies.

Stained cells were identified with a CCD-camera (Nikon DXM 1200, Düsseldorf, Germany) at a magnification of 150x in each six representative sections (each 0.25 μm^2) in perivascular or interstitial stroke area. For controls, corresponding areas were analyzed. For each patient, the mean cell number of mDC, pDC, T cells, and HLA-DR^+ cells was calculated. Perivascular and interstitial cells are separately shown.

Statistical analysis

All values are reported as median. $P < 0.05$ was considered statistically significant. Clinical data (Table 1) were statistically compared between patients and controls by *one-way ANOVA* test. The correlation of mDCP, pDCP, and tDCP with different parameters was analyzed by the non-parametric *Spearman Rank Order* test (Table 2). Unless stated otherwise, all other comparisons and statistical analyses were done using the non-parametric *Mann-Whitney Rank Sum* test.

RESULTS

Baseline characteristics

The baseline characteristics are reported in Table 1. No significant differences were observed for age, gender, medical history, atherogenic risk factors, and current medication. Most of the serum parameters did not differ between the study groups. Patients with acute cerebral ischemia had a trend for higher serum glucose. Regarding inflammation, significantly higher levels of leukocytes and hsCRP were detected in patients with AIS and AHS than controls (Table 1). According to TOAST criteria the cause of stroke was large-artery

atherosclerosis in 20%, cardioembolism in 25%, small-artery occlusion in 23%, other determined etiology in 2%, and undetermined etiology in 30%.

Decrease in circulating DCP in patients with acute ischemic and hemorrhagic stroke

Compared to healthy controls, in patients with TIA, AIS, and AHS a significant decrease in relative and absolute levels of circulating mDCP and tDCP was observed (Figure 2). Regarding pDCP, a statistical significant reduction of their relative and absolute levels was observed in patients with AIS and AHS, whereas in patients with TIA, the values were slightly lower than in controls. Since both the relative and, more importantly, the absolute values of circulating DCP were reduced, we were able to exclude that their decrease might be caused by a secondary dilution phenomenon due to an increase in another WBC population. In follow-up analysis of patients with acute cerebral ischemia, a significant reconstitution of circulating DCP was observed after 2 days (TIA) or 4 days (AIS, AHS), suggesting that their reduction is a transient phenomenon in stroke (data not shown). In contrast, patients with asymptomatic ACI stenosis did not show any significant alterations of circulating DCP compared to the healthy controls (Figure 2).

Subgroup/correlation analysis of circulating DCP regarding various clinical factors

Regarding TOAST criteria, the decrease in circulating DCP in patients with acute cerebral ischemia was independent of their etiology (data not shown). In healthy controls, the values of circulating DCP did not differ significantly between the sexes. In contrast, females with acute cerebral ischemia had significantly lower mDCP (9.4 vs. 10.5 cells/µL, p=0.04), pDCP (4.9 vs. 6.2 cells/µL, p=0.01), and tDCP (15.0 vs. 18.4 cells/µL, p=0.02) compared to corresponding male patients. In diabetic patients, significantly lower circulating tDCP were observed (Table 2). Patients under current medication with clopidogrel had signifi-

cantly higher values of circulating mDCP and tDCP. Moreover, circulating pDCP were significantly elevated in patients under AT1-antagonist treatment.

To analyze whether the decrease in circulating DCP might be directly associated with other clinical factors, correlation analysis was performed (Table 2). The age of the patients inversely correlated with circulating DCP. Regarding the clinical severity of stroke, a significant inverse correlation was observed between all subtypes of circulating DCP and the NIHSS (Table 2, Figure 3). A significant inverse correlation between circulating DCP and leukocytes or hsCRP was observed (Table 2, Figure 3). Blood glucose or HbA1c levels were significantly inversely correlated with circulating DCP (Table 2).

Additionally we investigated whether the decrease in circulating DCP might be associated with the radiologically evaluated stroke size (Figure 4). For this reason, patients with ischemic and hemorrhagic stroke were subdivided according to the CT scan into three subgroups with small, medium, and large infarction diameter. In concordance with the results for the NIHSS, we observed significantly lower values of circulating mDCP, pDCP, and tDCP in patients with larger stroke. Separate analysis of patients with ischemic and hemorrhagic stroke provided the same results but without statistical significance due to the limited number of patients in the divided groups (data not shown). Altogether, these results suggest that the reduction of DCP in stroke can be caused by their rapid recruitment from blood into the infarcted brain.

Emergence of mDC colocalized with T cells in the infarcted brain

To investigate whether circulating DCP are recruited into the infarcted brain, we analyzed human autopsy samples for the presence of DC and T cells, and their immunostimulatory capacity. Compared to control tissue, in the area affected by acute ischemic or hemorrhagic stroke numerous mDC and T cells, and to a lower extent pDC, were observed (Figure 5A). Regarding the cell distribution, mDC and T cells were often located around intracerebral

vessels. In contrast, single pDC were located in a distance from the vasculature. Surprisingly, in patients with past stroke persisting high numbers of mDC and T cells were observed. In the stroke area a strong HLA-DR expression was detected. The number of HLA-DR expressing cells by far exceeded the number of mDC, pDC, and T cells, so that it is very likely that another, probably residential cell type might be contributing to HLA-DR expression. Indeed, many of HLA-DR-expressing cells had a very similar morphology to astrocytes. In past stroke, a persisting high expression of HLA-DR was detected in the infarcted brain, suggesting a long-lasting cerebral immune reaction.

In double immunohistochemical stainings, a colocalization of mDC and T cells and a high expression of HLA-DR close to mDC was observed, indicating that mDC are mature and able to activate T cells in the infarcted brain (Figure 5B).

DISCUSSION

Recent studies revealed that immunological mechanisms play an important role for the neurological and overall outcome after stroke. The breakdown of blood brain barrier contributes to an invasion of immune cells into the infarcted brain after stroke. These cells upon encountering novel brain antigens, initiate an autoimmune response which leads to further destruction of neurological tissue [19]. On the other hand, a systemic immunodepression syndrome emerging after stroke is the reason for frequent systemic infections [3, 4]. The aim of our present study was to investigate if local and systemic alterations of DC contribute to the local immune response, or to the systemic immunodepression after stroke.

In our present study, we show that circulating myeloid, plasmacytoid, and total DCP are significantly reduced in patients with TIA, acute ischemic, or hemorrhagic stroke. The extent of their decrease significantly correlated with the clinical stage and the radiological size of stroke. Regarding the TOAST criteria, no significant differences in circulating DCP were observed betweens groups with different stroke etiology, suggesting that it is

not a specific pre-existing situation, but rather the common consequences of stroke, that lead to the decrease in circulating DCP. In short-term follow-up analysis, a rapid recovery of circulating DCP was observed, suggesting that their transient decrease might be a result of an enhanced emigration from blood into the infarcted brain. Supporting this hypothesis, it is known from other inflammatory diseases that a decrease in circulating DCP is mostly caused by their recruitment into areas of inflammation [20].

Unexpectedly, no significant changes in the levels of circulating DCP were observed in patients with asymptomatic ACI stenosis compared to controls, although it is known that circulating DCP emigrate into atherosclerotic lesions [13, 21]. In contrast, in patients with stable coronary artery disease a significant decrease in circulating DCP was described which is dependent of the extent of coronary atherosclerotic burden [22]. However, limited number of patients in the ACI stenosis group may be the reason that we were unable to detect any alterations.

Regarding inflammation, a strong inverse correlation was found between circulating DCP and hsCRP. This is not surprising, since it is known that CRP is able to directly induce an upregulation of adhesion molecules on endothelial cells [23], as well as to induce the release of the chemokines CCL2, CCL3, and CCL4 from monocytes [24]. Thus, elevated CRP levels are probably a major contributing factor for the emigration of circulating DCP to the sites of inflammation.

As it is possible that the transient decrease in circulating DCP in acute cerebral ischemia is caused by their rapid recruitment into the infarcted brain, human cerebral specimens were immunohistochemically analyzed. We were able to show that in patients with acute ischemic or hemorrhagic stroke numerous mDC and T cells were located in the infarcted area. Those results are in concordance with the study of Kostulas et al., who described mDC in the rodent infarcted brain [25]. We observed a distribution of mDC, colocalized with T cells, around the cerebral vessels in the stroke area which is a strategic location

between the immune system and the CNS. Thus, it seems likely that mDC are initiating an antigenspecific immune response through T cell activation there. HLA-DR expressing cells by far exceeded the number of mDC in the stroke area. HLA-DR expression seemed to be associated with residential cells such as astrocytes. However, it is questionable whether those CNS-resident cells are able to sufficiently induce an immune response comparable to professional antigen-presenting cells. It is possible that the observed expression of HLA-DR on residential cells is only an epiphenomenon induced by invaded immune cells, as described for EAE [26]. An important finding was that patients with older stroke showed persisting mDC and T cells, and high HLA-DR expression in the infarcted area, suggesting a long-lasting immune response after stroke. In concordance, long-term persistence of macrophages was previously shown in the infarcted area after stroke by PK11195-PET [27].

Recent data suggest that the immune pathomechanisms of ischemic and hemorrhagic stroke are very similar [28]. Our present study confirms this opinion, showing that circulating DCP are reduced in both ischemic and hemorrhagic stroke, and are recruited into the affected brain under both conditions. According to the general clinical observation of higher severity of intracranial hemorrhage, our results show that the reduction in circulating DCP is more pronounced in hemorrhagic than in ischemic stroke.

Several limitations of our study have to be acknowledged. Systemic infections often occur after acute stroke. However, in our study we had to exclude patients with obvious systemic infections, since it is well known that circulating DCP are recruited to the site of infection. Thus, the exclusion of patient with systemic infections was in this case necessary to be able to investigate whether circulating DCP are reduced in acute stroke due to their recruitment into the infarcted brain.

Unexpectedly, no significant changes in the levels of circulating DCP were observed in patients with asymptomatic ACI stenosis compared to controls, although it is known that circulating DCP emigrate into atherosclerotic lesions [13, 21]. In contrast, in patients

with stable coronary artery disease a significant decrease in circulating DCP was described which is dependent of the extent of coronary atherosclerotic burden [22]. However, limited number of patients with ACI stenosis in the present study may be the reason that we were unable to detect any alterations.

As described above, the most likely reason for the decrease in circulating DCP in patients after stroke seems to be their recruitment into the infarcted brain. However, other theoretically possible reasons have to be taken into account: (1) reduced production of DCP in the bone marrow or (2) increased apoptosis of circulating DCP. Those other reasons could not be definitely excluded in our present study. To the best of our knowledge, there exists no published data reporting a selective depression of the production of circulating DCP in the bone marrow without any impairment of the production of other blood leukocytes. In the case of lymphocytes, an increased apoptosis was described as reason for their transient reduction after stroke [29]. However, apoptotic cells show a decreased size in flow cytometry. In our analysis, circulating mDCP and pDCP had a very constant size, so that apoptosis seems to be unlikely reason for their decrease in circulation. On the other hand, it is possible that not all myeloid DC which were found in the infarcted brain were recruited from the circulation, but might have developed from local cerebral cells [25].

In conclusion, we show for the first time a significant transient decrease in circulating DCP in patients with stroke. The reason for this decrease is probably an enhanced recruitment of DCP from blood into the infarcted brain. Less circulating DCP might be leading to immunodepression, resulting in frequent systemic infections and worse outcome in stroke patients. On the other hand, mDC invaded into the brain after stroke may activate T cells against newly-recognized neuroepitopes, inducing a long-lasting immune response, which leads to further neurological damage. Thus, novel strategies based on modulation of DC migration or function might be a promising therapeutic approach to prevent systemic infections or further neurological damage in stroke patients.

FUNDING

This work was supported by the *ELAN Fonds and* the *Interdisciplinary Center for Clinical Research* of the University Hospital Erlangen.

DISCLOSURES

None

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

FIGURE LEGENDS

Figure 1 **Gating strategy for the identification of circulating DC precursors.** Upper panel: DCP analysis, Lower panel: isotype control In region **R1** white blood cells (WBC) were separated from debris and platelets according to their typical forward (FSC) and side scatter (SSC). In region **R2** following cells were excluded: granulocytes by side scatter, B lymphocytes by CD19-staining, monocytes by CD14-staining, and dead cells by propidium iodide-staining. In region **R3** and **R4** circulating myeloid (mDCP) and plasmacytoid DC precursors (pDCP) were detected according to their specific BDCA-1- and BDCA-2-staining, respectively. The number of circulating total DC precursors (tDCP) represents the sum of cells detected in **R3** plus **R4.**

Figure 2 **Decrease in circulating DCP in patients with acute cerebral ischemia. (A)** Relative numbers of circulating mDCP, pDCP, and tDCP expressed as percentage of white blood cells (% WBC). **(B)** Absolute numbers of circulating DCP as cells per microliter (cells / µL). Healthy controls (**Control**), patients with ACI stenosis (**ACI-S**), with transient ischemic attack (**TIA**), acute ischemic stroke (**AIS**), or acute hemorrhagic stroke (**AHS**) were compared. ns = not significant compared to control.

Figure 3 **Correlation between circulating DCP and stroke severity or inflammation.** **(A)** Significant inverse correlation of mDCP, pDCP, and tDCP (% WBC) with the *National Institute of Health Stroke Scale* (NIHSS). **(B)** Significant inverse correlation of mDCP, pDCP, and tDCP (% WBC) with serum hsCRP (mg/L).

Figure 4 Association of the circulating DCP reduction with radiological stroke size. **(A)** Relative (% WBC) and **(B)** absolute numbers (cells / µL) of circulating DCP in patients with small (< 10 mm), medium (10-30 mm), and large stroke (> 30 mm). Stroke size was evaluated in the initial CT scan.

Figure 5 Cerebral immune response after stroke. **(A)** Immunohistochemical analysis of myeloid DC (CD209, brown), plasmacytoid DC (CD123, brown), T cells (CD 3, red), and HLA-DR expression (brown) in the area of ischemic, hemorrhagic, or past stroke (> 4 weeks). **Left:** Examples of immunohistochemical staining (each 150x). **Right:** Corresponding histograph showing the mean value and the standard error of mean (SEM). * $p < 0.001$. Percentage of cells located around intracerebral vessels as **diagonal stripes** (grey and white) and percentage of diffuse infiltrating cells as homogenous **grey bars**. Due to the high numbers of HLA-DR-positive cells, their grouping as perivascular or diffuse was not possible (grey bars). **(B) Left:** Colocalization of mDC and T cells was shown in double immunohistochemical staining using CD209 and CD3 antibodies (300x, 400x), **Right:** Double immunohistochemical analysis of the expression of HLA-DR on mDC (CD209-positive cells).

REFERENCES

1 Nilupul Perera, M., Ma, H. K., Arakawa, S., Howells, D. W., Markus, R., Rowe, C. C. and Donnan, G. A. (2006) Inflammation following stroke. J. Clin. Neurosci. **13**, 1-8

2 Huang, J., Upadhyay, U. M. and Tamargo, R. J. (2006) Inflammation in stroke and focal cerebral ischemia. Surg. Neurol. **66**, 232-245

3 Meisel, C., Schwab, J. M., Prass, K., Meisel, A. and Dirnagl, U. (2005) Central nervous system injury-induced immune deficiency syndrome. Nat. Rev. Neurosci. **6**, 775-786

4 Chamorro, A., Urra, X. and Planas, A. M. (2007) Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. Stroke **38**, 1097-1103

5 Emsley, H. C. and Hopkins, S. J. (2008) Acute ischaemic stroke and infection: recent and emerging concepts. Lancet Neurol. **7**, 341-353

6 Vargas, M., Horcajada, J. P., Obach, V., Revilla, M., Cervera, A., Torres, F., Planas, A. M., Mensa, J. and Chamorro, A. (2006) Clinical consequences of infection in patients with acute stroke: is it prime time for further antibiotic trials? Stroke **37**, 461-465

7 Chamorro, A., Horcajada, J. P., Obach, V., Vargas, M., Revilla, M., Torres, F., Cervera, A., Planas, A. M. and Mensa, J. (2005) The Early Systemic Prophylaxis of Infection After Stroke study: a randomized clinical trial. Stroke **36**, 1495-1500

8 Becker, K. J., Kindrick, D. L., Lester, M. P., Shea, C. and Ye, Z. C. (2005) Sensitization to brain antigens after stroke is augmented by lipopolysaccharide. J. Cereb. Blood Flow Metab. **25**, 1634-1644

9 Gee, J. M., Kalil, A., Thullbery, M. and Becker, K. J. (2008) Induction of immunologic tolerance to myelin basic protein prevents central nervous system autoimmunity and improves outcome after stroke. Stroke **39**, 1575-1582

10 Frenkel, D., Huang, Z., Maron, R., Koldzic, D. N., Hancock, W. W., Moskowitz, M. A. and Weiner, H. L. (2003) Nasal vaccination with myelin oligodendrocyte glycoprotein reduces stroke size by inducing IL-10-producing CD4+ T cells. J. Immunol. **171**, 6549-6555

11 Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K. (2000) Immunobiology of dendritic cells. Annu. Rev. Immunol. **18**, 767-811

12 Liu, Y. J. (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell **106**, 259-262

13 Yilmaz, A., Lochno, M., Traeg, F., Cicha, I., Reiss, C., Stumpf, C., Raaz, D., Anger, T., Amann, K., Probst, T., Ludwig, J., Daniel, W. G. and Garlichs, C. D. (2004) Emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques. Atherosclerosis **176**, 101-110

14 Niessner, A., Sato, K., Chaikof, E. L., Colmegna, I., Goronzy, J. J. and Weyand, C. M. (2006) Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha. Circulation **114**, 2482-2489

15 Greter, M., Heppner, F. L., Lemos, M. P., Odermatt, B. M., Goebels, N., Laufer, T., Noelle, R. J. and Becher, B. (2005) Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nat. Med. **11**, 328-334

16 Kolominsky-Rabas, P. L., Weber, M., Gefeller, O., Neundoerfer, B. and Heuschmann, P. U. (2001) Epidemiology of ischemic stroke subtypes according to TOAST criteria: incidence, recurrence, and long-term survival in ischemic stroke subtypes: a population-based study. Stroke **32**, 2735-2740

17 Muir, K. W., Weir, C. J., Murray, G. D., Povey, C. and Lees, K. R. (1996) Comparison of neurological scales and scoring systems for acute stroke prognosis. Stroke **27**, 1817-1820

18 Yilmaz, A., Weber, J., Cicha, I., Stumpf, C., Klein, M., Raithel, D., Daniel, W. G. and Garlichs, C. D. (2006) Decrease in circulating myeloid dendritic cell precursors in coronary artery disease. J. Am. Coll. Cardiol. **48**, 70-80

19 Schroeter, M., Jander, S., Witte, O. W. and Stoll, G. (1994) Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. J. Neuroimmunol. **55**, 195-203

20 Cravens, P. D. and Lipsky, P. E. (2002) Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. Immunol.Cell Biol. **80**, 497-505

21 Erbel, C., Sato, K., Meyer, F. B., Kopecky, S. L., Frye, R. L., Goronzy, J. J. and Weyand, C. M. (2007) Functional profile of activated dendritic cells in unstable atherosclerotic plaque. Basic Res. Cardiol. **102**, 123-132

22 Yilmaz, A., Schaller, T., Cicha, I., Altendorf, R., Stumpf, C., Klinghammer, L., Ludwig, J., Daniel, W. G. and Garlichs, C. D. (2009) Predictive value of the decrease in circulating dendritic cell precursors in stable coronary artery disease. Clin. Sci. (Lond) **116**, 353-363

23 Pasceri, V., Cheng, J. S., Willerson, J. T., Yeh, E. T. and Chang, J. (2001) Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. Circulation **103**, 2531-2534

24 Montecucco, F., Steffens, S., Burger, F., Pelli, G., Monaco, C. and Mach, F. (2008) C-reactive protein (CRP) induces chemokine secretion via CD11b/ICAM-1 interaction in human adherent monocytes. J. Leukoc. Biol. **84**, 1109-1119

25 Kostulas, N., Li, H. L., Xiao, B. G., Huang, Y. M., Kostulas, V. and Link, H. (2002) Dendritic cells are present in ischemic brain after permanent middle cerebral artery occlusion in the rat. Stroke **33**, 1129-1134

26 Youssef, S., Stuve, O., Patarroyo, J. C., Ruiz, P. J., Radosevich, J. L., Hur, E. M., Bravo, M., Mitchell, D. J., Sobel, R. A., Steinman, L. and Zamvil, S. S. (2002) The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. Nature **420**, 78-84

27 Pappata, S., Levasseur, M., Gunn, R. N., Myers, R., Crouzel, C., Syrota, A., Jones, T., Kreutzberg, G. W. and Banati, R. B. (2000) Thalamic microglial activation in ischemic stroke detected in vivo by PET and [11C]PK1195. Neurology **55**, 1052-1054

28 Rodriguez-Yanez, M. and Castillo, J. (2008) Role of inflammatory markers in brain ischemia. Curr. Opin. Neurol. **21**, 353-357

29 Urra, X., Cervera, A., Villamor, N., Planas, A. M. and Chamorro, A. (2009) Harms and benefits of lymphocyte subpopulations in patients with acute stroke. Neuroscience **158**, 1174-1183

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

Table 1 Baseline characteristics of controls and patients with asymptomatic A. carotis interna stenosis or acute cerebral ischemia

Score	Control	ACI-S	TIA	AIS	AHS	P
No. of patients	29	46	39	73	31	
Age (years)	68	69	70	70.5	70	NS
Male gender (%)	76	74	54	56	65	NS
Atrial fibrillation (%)	37	0	18	23	19	/
Carotid stenosis (%)	0	100	18	29	21	/
Medical history (%)						
Coronary artery disease	30	56	14	23	19	NS
Past acute myocardial infarction	9	22	9	11	15	NS
Past stroke	6	10	18	24	11	NS
Atherogenic risk factors (%)						
Diabetes mellitus	21	22	23	43	15	NS
Hypertension	76	95	57	91	78	NS
Smoking	15	42	13	20	12	NS
Hyperlipidemia	76	78	48	63	22	NS
Current Medication (%)						
Aspirin	10	63	33	47	19	NS
Statins	34	46	31	47	4	NS
Clopidogrel	0	11	5	8	7	NS
β Blockers	65	41	18	49	13	NS
ACE inhibitors	21	37	26	44	19	NS
AT1 antagonists	21	2	5	9	6	NS
Calcium channel blockers	3	20	10	14	16	NS
Serum parameters						
Total Cholesterol (mg/dL)	214	195	195	214	220	NS
LDL cholesterol (mg/dL)	138	116	129	141	146	NS
HDL cholesterol (mg/dL)	49	45.5	50	48	52.5	NS
Serum glucose (mg/dL)	109	109	110	115	134	NS
Creatinine (mg/dL)	1.03	1.13	1.04	1.10	0.95	NS
Leukocytes (cells/µL)	6.5	7.3	6.7	7.9	8.3	0.001
hsCRP (mg/L)	1.35	1.99	3.12	5.53	12.4	<0,001
Circulating DCP (% / cells/µL)						
mDCP	0.25 / 15.4	0.24 / 16.2	0.18 / 12.9	0.13 / 10.4	0.09 / 8.5	<0,001
pDCP	0.11 / 7.1	0.10 / 7.2	0.10 / 6.3	0.07 / 5.6	0.05 / 4.6	<0,001
tDCP	0.37 / 23.5	0.35 / 24.3	0.31 / 21.0	0.22 / 16.3	0.16 / 14.5	<0,001

Clinical data were compared between controls and patients with asymptomatic stenosis of the A. carotis interna (ACI-S), transient ischemic attack (TIA), acute ischemic stroke (AIS), and acute hemorrhagic stroke (AHS). Values are reported as median value or %.

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

Table 2 Comparative analysis of DCP levels with clinical data, medication, or other serum parameters

	mDCP			pDCP			tDCP		
	-	+	P	-	+	P	-	+	P
1. Subgroup Analysis									
Male gender	**11.5**	**12.5**	**0.04**	**5.3**	**6.4**	**0.02**	**17.0**	**20.6**	**0.003**
Atrial fibrillation	12.7	12.7	NS	5.9	6.8	NS	19.5	20.7	NS
Carotid stenosis	12.3	12.9	NS	6.1	6.3	NS	19.5	20.7	NS
Atherogenic risk factors (%)									
Diabetes mellitus	12.9	11.0	NS	6.2	5.6	NS	**20.0**	**15.8**	**0.05**
Hypertension	11.8	12.7	NS	6.2	5.9	NS	17.8	19.9	NS
Smoking	12.2	12.8	NS	5.8	6.4	NS	19.4	20.0	NS
Hyperlipidemia	10.7	11.0	NS	5.9	6.1	NS	19.6	20.3	NS
Family history of CVD	12.5	12.5	NS	5.9	5.6	NS	19.6	16.8	NS
Current Medication (%)									
Aspirin	12.2	12.9	NS	6.2	5.9	NS	19.6	20.0	NS
Clopidogrel	**12.3**	**16.8**	**0.05**	5.9	6.2	NS	**19.4**	**24.3**	**0.02**
Statins	12.3	12.7	NS	6.0	6.2	NS	19.4	20.8	NS
β Blocker	12.1	13.4	NS	6.0	6.0	NS	19.4	20.3	NS
Calcium channel blockers	12.2	13.6	NS	6.3	4.6	NS	19.9	18.4	NS
ACE inhibitors	12.5	12.7	NS	6.2	5.6	NS	20.0	19.5	NS
AT1-antagonists	12.4	13.5	NS	**5.8**	**8.2**	**0.01**	19.6	20.3	NS
2. Correlation Analysis	**R**	**P**		**R**	**P**		**R**	**P**	
Age	**- 0.16**	**0.02**		**- 0.18**	**0.007**		**- 0.2**	**0.003**	
Stroke Score (NIHSS)	**- 0.28**	**0.01**		**- 0.28**	**0.02**		**- 0.35**	**0.004**	
White blood cells	**- 0.36**	**< 0.001**		**- 0.33**	**< 0.001**		**- 0.39**	**< 0.001**	
hsCRP	**- 0.49**	**< 0.001**		**- 0.3**	**< 0.001**		**- 0.45**	**< 0.001**	
Platelets	0.03	NS		0.004	NS		0.03	NS	
Glucose	**- 0.17**	**0.03**		**- 0.25**	**0.001**		**- 0.22**	**0.004**	
HbA1c	- 0.18	0.1		**- 0.22**	**0.04**		**- 0.23**	**0.03**	
Creatinine	0.1	NS		- 0.004	NS		0.06	NS	
Total cholesterol	0.11	NS		0.08	NS		0.11	NS	
LDL-cholesterol	0.05	NS		0.08	NS		0.06	NS	
HDL-cholesterol	0.04	NS		0.09	NS		0.04	NS	
Triglycerides	- 0.04	NS		- 0.03	NS		- 0.06	NS	

1. **Subgroup analysis**: Absolute numbers of mDCP, pDCP, and tDCP (cells / µL) are calculated for the presence (+) or absence (-) of a clinical feature or medication. Values are reported as median.
2. **Correlation analysis**: Different parameters were correlated with the relative values of circulating mDCP, pDCP, and tDCP.

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

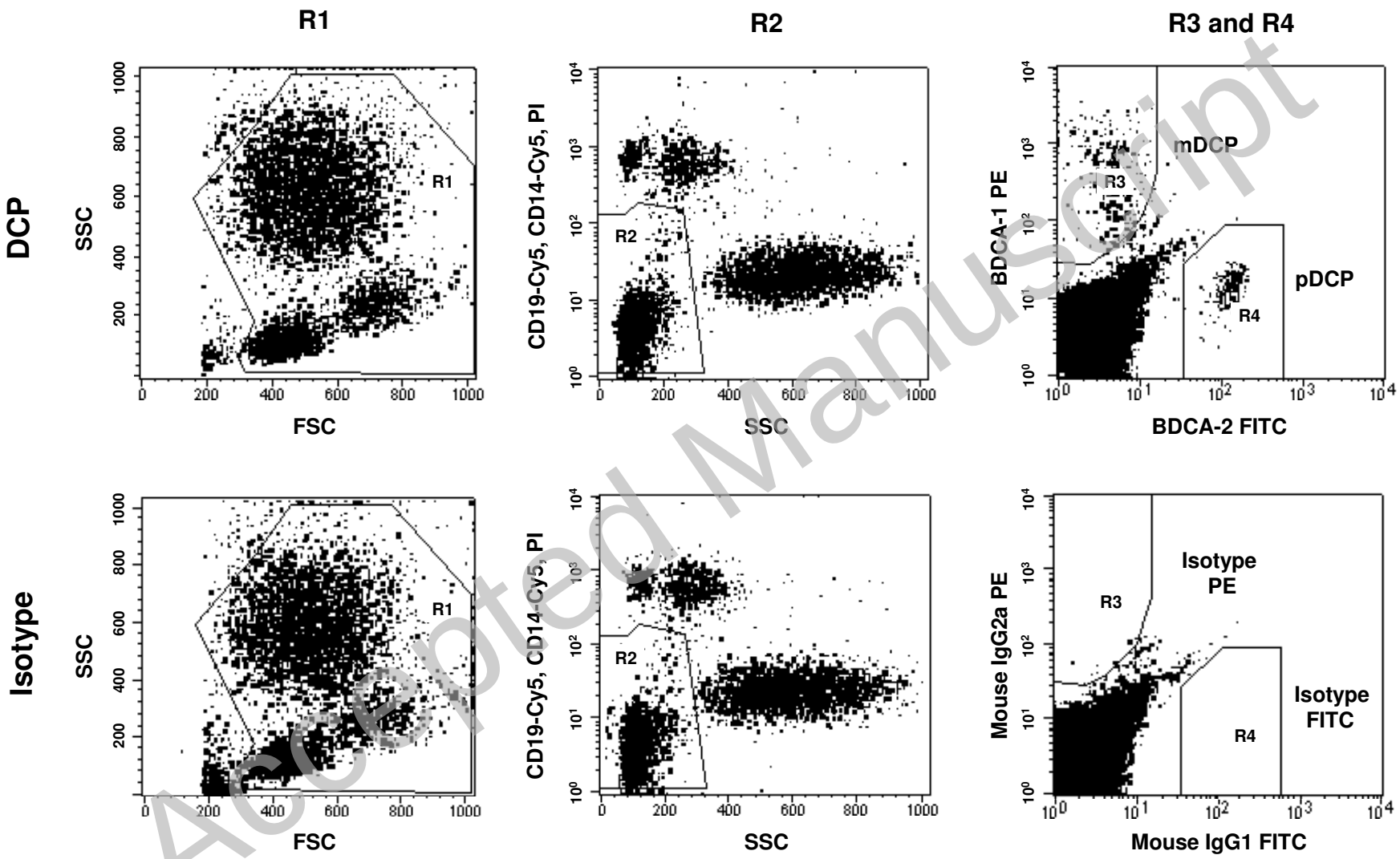


Figure 1

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

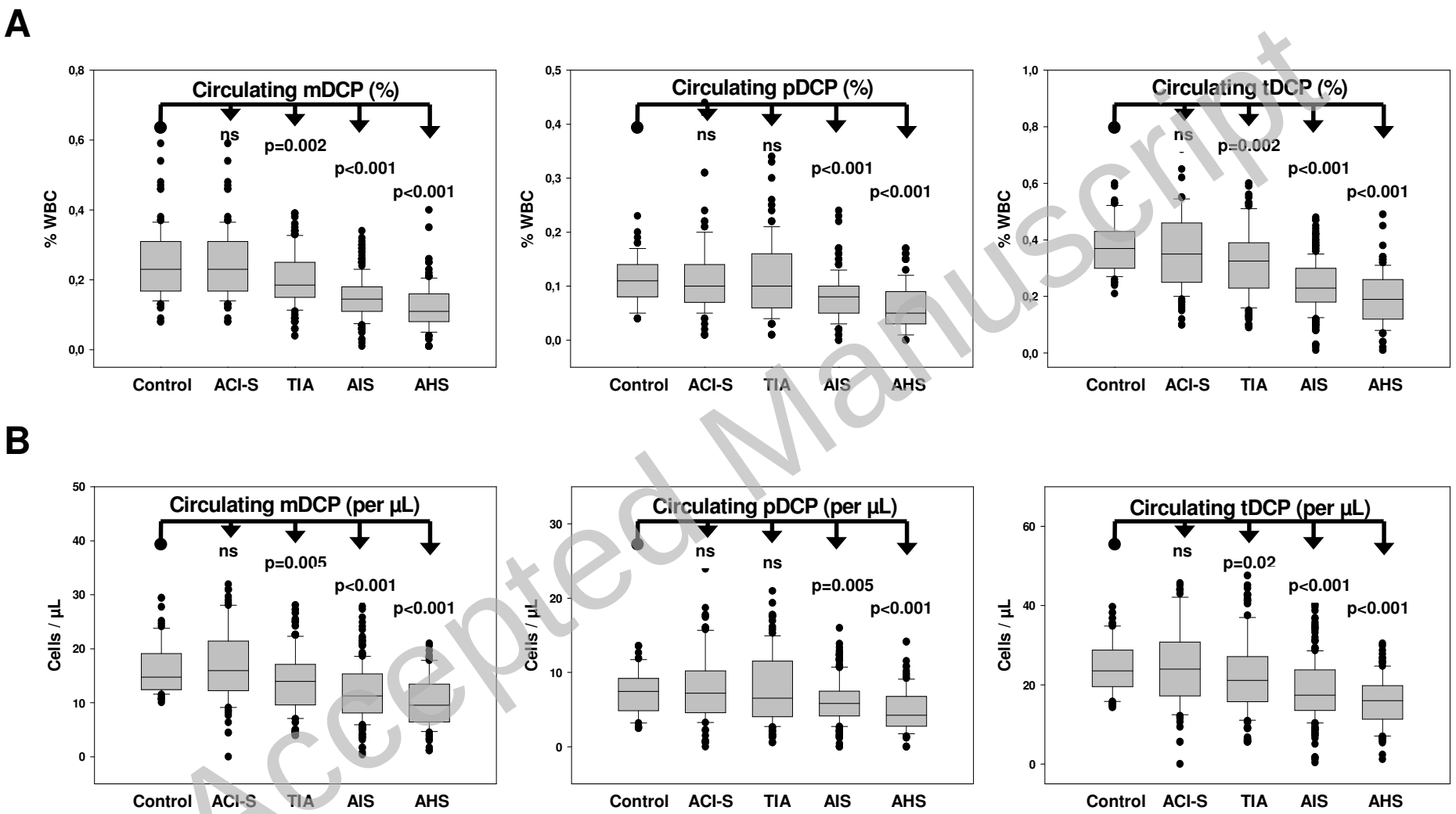
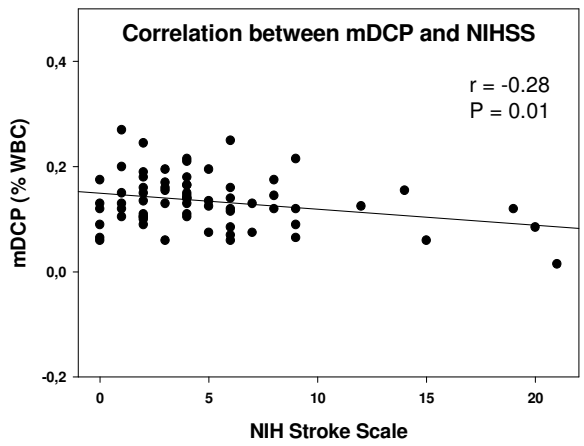
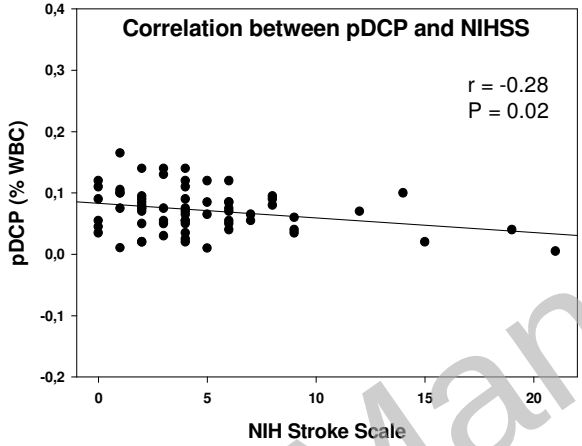


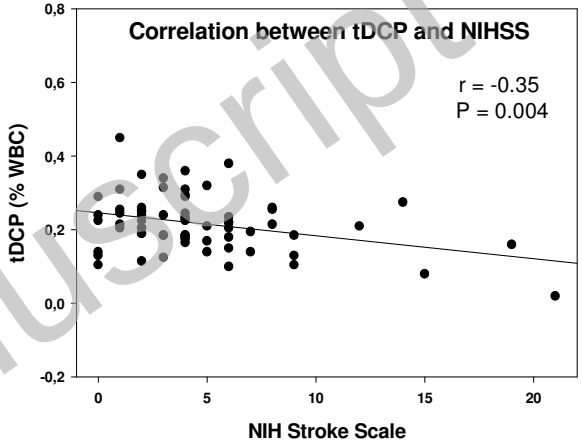
Figure 2

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

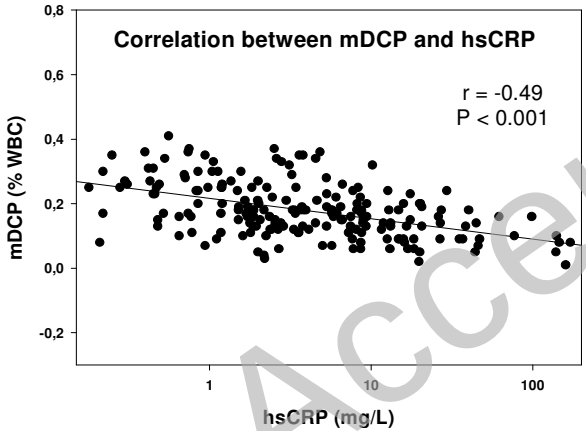
A

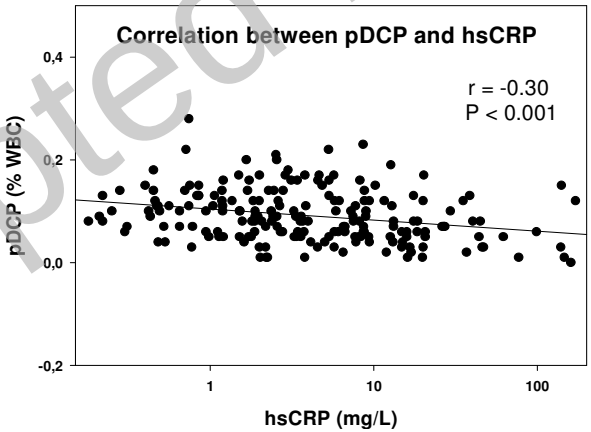






B





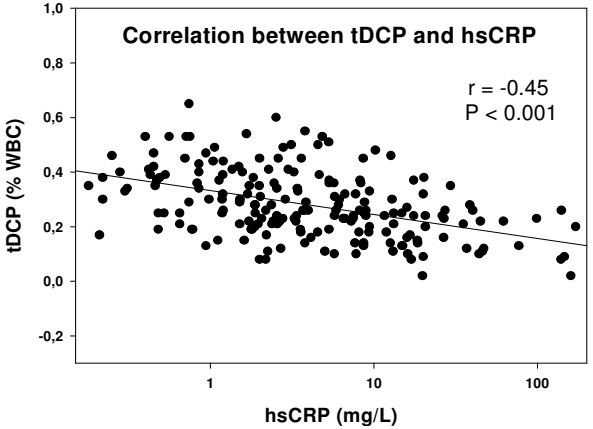


Figure 3

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

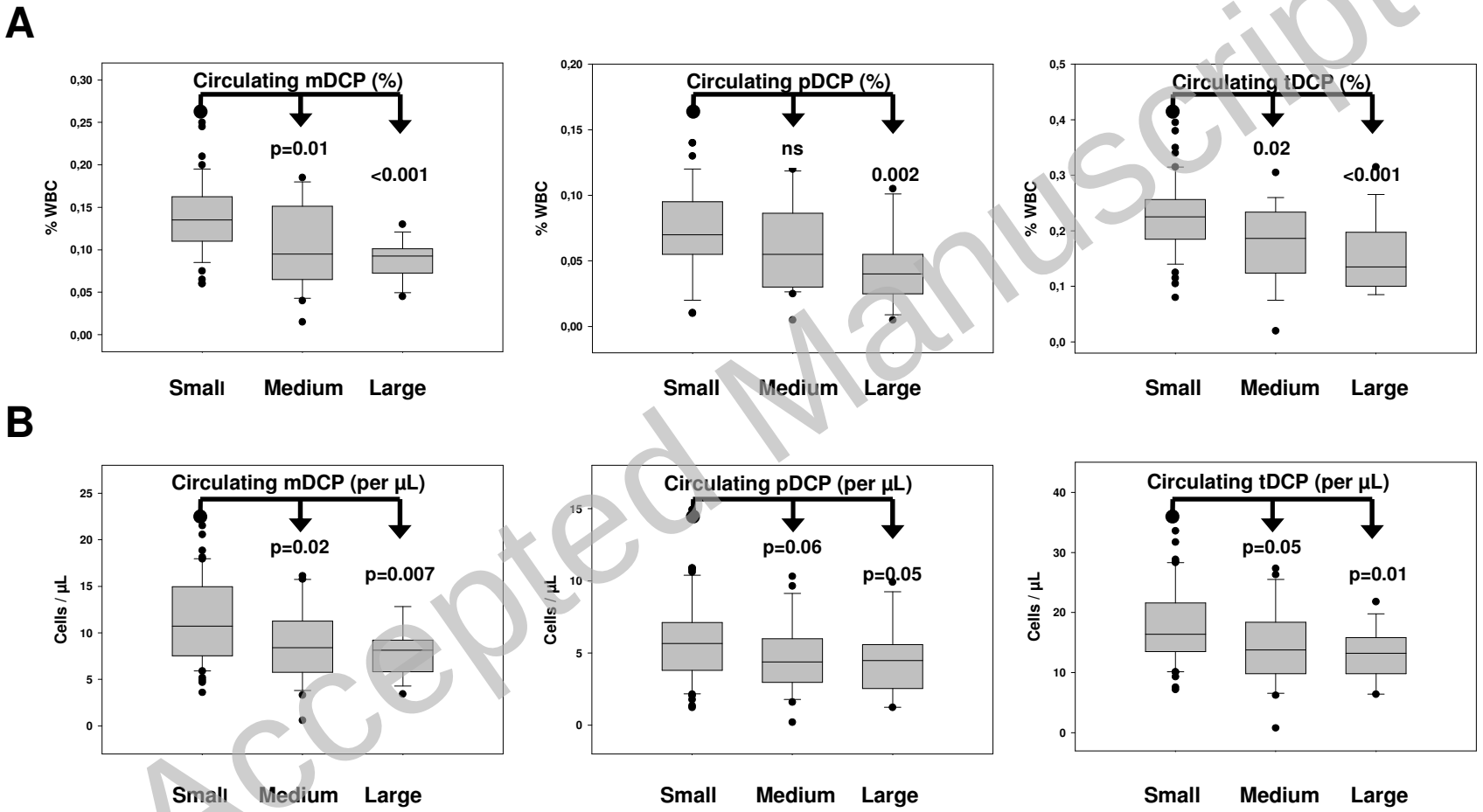


Figure 4

Clinical Science Immediate Publication. Published on 10 Jun 2009 as manuscript CS20090154

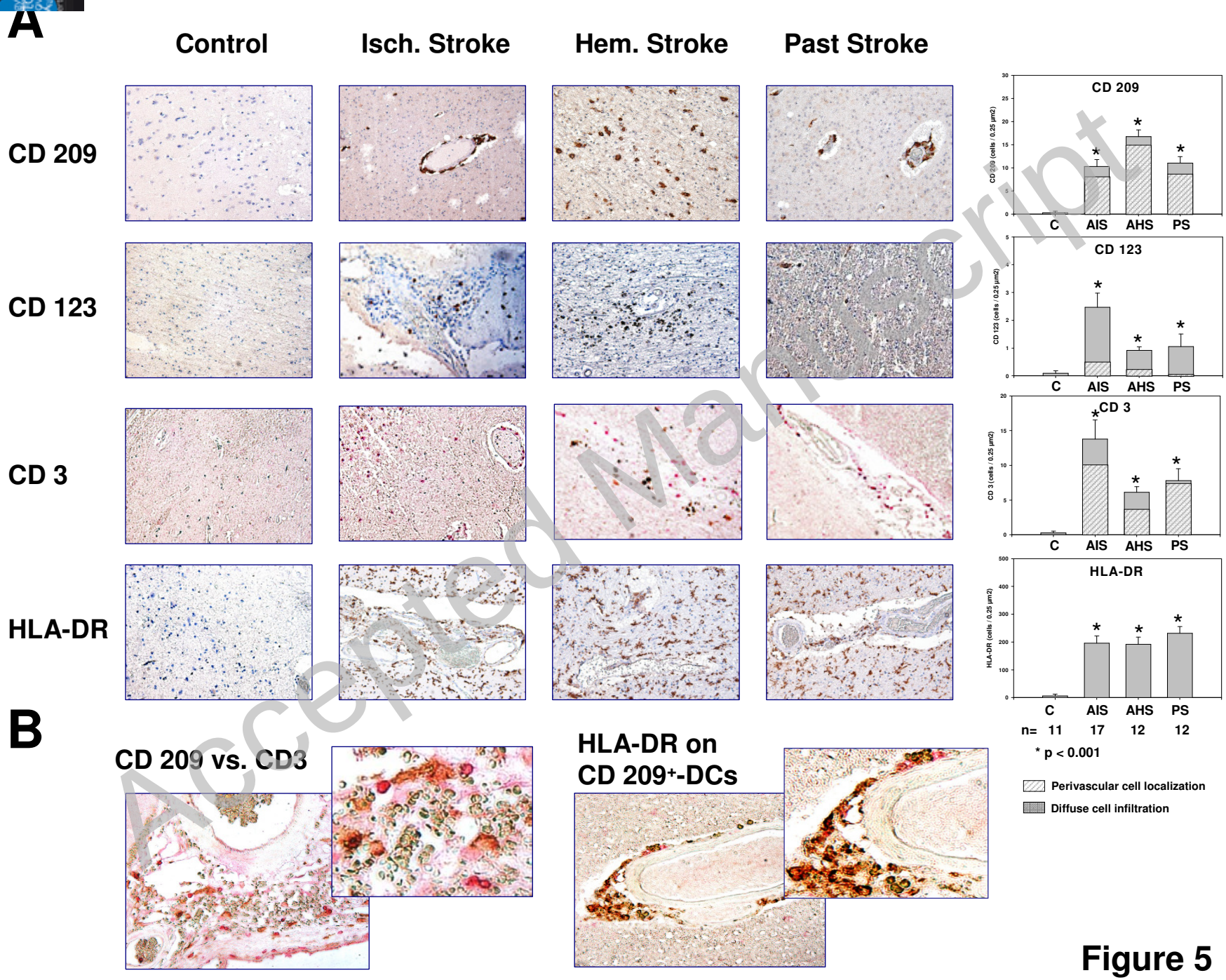


Figure 5