Programmed Death-1 Pathway Limits Central Nervous System Inflammation and Neurologic Deficits in Murine Experimental Stroke

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Background and Purpose—Evaluation of infarct volumes and infiltrating immune cell populations in mice after middle cerebral artery occlusion strongly implicates a mixture of both pathogenic and regulatory immune cell subsets that affect stroke outcome. Our goal was to evaluate the contribution of the well-described coinhibitory pathway, programmed death (PD-1), to the development of middle cerebral artery occlusion.

Methods—Infarct volumes, functional outcomes, and effects on infiltrating immune cell populations were compared in wild-type C57BL/6 versus PD-1-deficient mice after 60 minutes middle cerebral artery occlusion and 96 hours reperfusion.

Results—The results clearly demonstrate a previously unrecognized activity of the PD-1 pathway to limit infarct volume, recruitment of inflammatory cells from the periphery, activation of macrophages and central nervous system microglia, and functional neurological deficits. These regulatory functions were associated with increased percentages of circulating PD-ligand-1 and PD-ligand-2 expressing CD19+ B-cells in blood, the spleen, and central nervous system with the capacity to inhibit activation of inflammatory T-cells and central nervous system macrophages and microglial cells through upregulated PD-1.

Conclusions—Our novel observations are the first to implicate PD-1 signaling as a major protective pathway for limiting central nervous system inflammation in middle cerebral artery occlusion. This inhibitory circuit would likely be pivotal in reducing stroke-associated Toll-like receptor-2- and Toll like receptor-4-mediated release of neurotoxic factors by activated central nervous system microglia.

Key Words: coinhibitory pathway inflammatory cells MCAO programmed death-1

Stroke is a devastating central nervous system (CNS) condition marked by death of brain cells, loss of cognitive and motor function, and systemic infections that often lead to death. Mouse models of focal cerebral ischemia incurred by middle cerebral artery occlusion (MCAO) support a biphasic effect of stroke on the peripheral immune system. The initial phase is characterized by early signaling from the ischemic brain to the spleen, resulting in massive production of inflammatory factors, transmigration of splenocytes to the circulation, and infiltration of stroke-damaged areas of the brain by inflammatory polymorphonuclear leukocytes, macrophages, T-cells, and B-cells. Early activation is followed by compensatory systemic immunosuppression that occurs within days of focal stroke by profound (90%) loss of immune T- and B-cells in the spleen and thymus and reduced T-cell activation. These changes were accompanied by an increase in transferase-mediated dUTP nick-end labeling, Annexin V+, and PI+ splenocytes committed to apoptosis that suggested involvement of the programmed death-1 (PD-1) coinhibitory pathway.

PD-1 (CD279) is an Ig-superfamily member containing an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif that are inductively expressed by activated T-cells, B-cells, natural killer cells, monocytes, and some dendritic cell subsets. Binding of PD-1 to either of 2 ligands, PD-L1 (B7-H1/CD274) or PD-L2 (B7-DC/CD273) with overlapping expression patterns, induces inhibitory signals that control induction and maintenance of peripheral T-cell tolerance and immune homeostasis. Much work has focused on regulation of effector T-cell responses due to the autoimmune phenotype of PD-1-deficient (knockout [KO]) mice and linkage of PD-1 genes...
with autoimmune disorders. Moreover, other inhibitory pathways involving PD-1/PD-ligand (L) have been described, including our studies on estrogen-mediated suppression through Treg-cell activation,

inhibition of encephalitogenic T-cell responses by PD-L-expressing myeloid APC,

"reverse signaling" through PD-L that induces suppressive dendritic cells, PD-L1 protection from CD8 T-cell lysis of virally infected target cells, and PD-1-dependent immunemediated damage of CNS oligodendroglial cells.

Currently, there are no reports that have implicated the PD-1/PD-L co-inhibitory pathway in stroke. In this report, we found increased expression of PD-1 on brain macrophages and microglial cells and PD-L on B-cells from the spleen, blood, and CNS in mice after induction of MCAO. We thus compared MCAO in PD-1 KO versus wild-type (WT) male mice after 96 hours reperfusion. Cortical, striatal, and total infarction volumes were significantly larger in PD-1 KO versus WT C57BL/6 mice with MCAO (P<0.01). This study clearly implicates the PD-1/PD-L pathway in limiting infarct lesion volume in MCAO and provides new insights into immune regulatory interactions.

**Methods**

**Animals**

PD-1 deficient (PD-1−/− or PD-1 KO) mice backcrossed with C57BL/6 mice were obtained from Dr Tasuku Honjo, Kyoto University, Japan. Age-matched 8- to 12-week-old WT C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used as controls. Animals were randomized to treatment groups. All experiments were performed under approved protocols in the Animal Resource Facilities at Oregon Health & Science University and the Veterans Affairs Medical Center, Portland, OR.

**MCAO Model**

The mice were subjected to MCAO as previously published by reversible right MCAO (60 minutes) under isoflurane anesthesia followed by 96 hours of reperfusion. Body and head temperatures were controlled at 37±0.5°C. Occlusion and reperfusion were verified in each animal by laser Doppler flowmetry (Moor Instruments).

**Quantification of Infarct**

Brains were collected at 96 hours for standard 2,3,5-triphenyltetrazolium chloride histology and digital image analysis of infarct volume as previously published. To control for edema, corrected infarct volume is expressed as a percentage of contralateral structure, that is, cortex, striatum, or total hemisphere.

**Neurological Deficit Score**

Neurological function was evaluated in a blinded fashion using a 0-5-point score at 24, 48, and 96 hours post-MCAO (0=normal neurological functions; 1=failure to extend left forelimb fully when lifted by tail; 2=circling to the contralateral side; 3=failing to the left; 4=no spontaneous walk or in a comatose state; 5=death).

**Cell Isolation**

Blood mononuclear cells were prepared by using red cell lysis buffer (eBioscience) following the manufacturer’s instructions. Splenocyte suspensions were prepared by mechanical disruption. For preparation of inflammatory cells from the brain, each mouse was perfused transcardially with 30 mL saline to exclude blood cells, the forebrain was dissected from the cerebellum and suspended in RPMI-1640 medium, and the suspension was digested with Type IV collagenase (1 mg/mL; Sigma-Aldrich) and Dnase I (50 micro grams/mL; Roche) at 37°C for 45 minutes in a shaker at 180 times/minute. Inflammatory cells were isolated by 37% to 70% Percoll density gradient centrifugation as described. The cells were washed twice with RPMI 1640, counted, and resuspended in stimulation medium containing 10% fetal bovine serum for phenotyping.

**Analysis of Cell Populations by Fluorescence-Activated Cell Sorter**

Antimouse antibodies CD19 (1D3; BD Pharmingen), CD45 (30-F11; Invitrogen), CD11b (M1/70; eBioscience), MHCII (2G9; BD Pharmingen), Gr1 (IA8; BD Horizon), CD3 (17A2; eBioscience), interferon-γ (XMG1.2; eBioscience), tumor necrosis factor-α (MP6-XT22; BD Pharmingen), PD-1 (RMP1-30; eBioscience), PD-L1 (MH5; eBioscience), and PD-L2 (TY25; eBioscience) were used for the study. Single-cell suspensions were washed with staining medium (phosphate-buffered saline containing 0.1% NaN3 and 2% fetal calf serum). After incubation with mAb and washing, cells were acquired with LSRII (BD Biosciences) and analyzed (Flowjo software; TreeStar) using isotype control antibodies to set quadrants before calculating the percentage of positive cells.

**Intracellular Staining**

Intracellular staining was visualized using a published immunofluorescence protocol. Briefly, 2×10⁶ cells/mL were resuspended in complete medium (RPMI 1640 containing 10% fetal calf serum, 1 mmol/L pyruvate, 200 μg/mL penicillin, 200U/mL streptomycin, 4 mmol/L L-glutamine, and 5×10⁻⁵ mol/L 2-β-ME) with PMA (50 ng/mL), ionomycin (500 ng/mL), and Brefeldin A (10 μg/mL; Sigma-Aldrich) for 5 hours. Fc receptors were blocked with anti-FcR mAb (2.3G2; BD Pharmingen) before cell-surface staining and fixed and permeabilized cells (eBioscience) were stained with antitrustor necrosis factor-α or anti-interferon-γ mAb or isotype-matched mAb.

**Statistical Analysis**

Data were reported as means±SEM. Student t test was used for comparison of infarct volumes; analysis of variance Kruskal-Wallis test followed by post hoc Dunn multiple comparison test was used for comparison of neurological deficits and Fisher exact test was used for comparing mortality rates. There was no difference in MCAO mortality between WT (5 of 26) and PD-1 KO (5 of 25) mice. For ≥3 groups, the analysis of variance followed by post hoc Tukey test was applied. For all tests, probability values <0.05 were considered statistically significant.

**Results**

**Increased Expression of PD-1 and PD-L After MCAO Suggests Involvement of the Coinhibitory Pathway**

We have recently shown that peripheral B-cells have strong regulatory activity in limiting MCAO severity. B-cells are known to express the PD-1 ligands, PD-L1 and -2, that are important in delivering inhibitory signals through the PD-1 receptor to control induction and maintenance of peripheral T-cell tolerance and immune homeostasis and to inhibit activation of other PD-1-expressing cells. We thus evaluated the possible involvement of this important coinhibitory pathway in limiting immune-mediated inflammation in MCAO. We found that B-cells predominantly expressed PD-L1 and PD-L2 (67% to 86% of total) in blood and the spleen (Figure 1A–B) of sham-operated mice and that their levels were strongly increased (2- to 8-fold) after MCAO (60 minutes occlusion, 96 hours reperfusion). Moreover, 77% to 78% of B-cells that are enriched in the ischemic hemisphere of MCAO mice expressed PD-L1 and PD-L2 (Supplemental Figure I; http://stroke.ahajournals.org). Conversely, activated resident microglia and infiltrating macro-
phages that are major inflammatory cell types that contribute to stroke damage in CNS had strongly enhanced levels of PD-1 expression in the ischemic hemisphere of MCAO mice (Figure 1C–D). The importance of these changes was further demonstrated by the impaired ability of purified B-cells to inhibit proliferation of T-cells and production of tumor necrosis factor-α by macrophages in PD-1-deficient mice (Supplemental Figure II). These results demonstrate the capacity of PD-L1 and PD-L2 B-cells to ligate and potentially to inhibit activation of PD-1 inflammatory cells during stroke.

PD-1 Deficiency Exacerbates Stroke Outcomes and Alters Cerebral Inflammatory Cell Infiltration

To assess the role of PD-1 in stroke development, infarct volume, neurological outcome, and infiltration of inflammatory cells into the brain were evaluated in cohorts of PD-1-deficient versus C57BL/6 WT mice treated with 60 minutes of focal cerebral ischemia and 96 hours of reperfusion. Loss of PD-1 resulted in significantly larger hemispheric infarct volumes in the cortex (P=0.01), striatum (P=0.01), and total hemisphere (P=0.0001) relative to WT mice (Figure 2A). Representative histological staining of injured brain is shown in Figure 2B. These data clearly implicate the role of PD-1 in limiting histological damage after MCAO. In a further cohort, there was significant recovery of neurological scores in WT mice that did not occur in PD-1 KO mice (Figure 2C). To confirm that the ischemic insult was equivalent among all animals, relevant physiological parameters were assessed before and during MCAO. As is shown in Supplementary Table I, rectal temperature, mean arterial blood pressure, arterial blood gases, and pH were comparable between groups. Similarly, intras ischemic cortical blood flow as estimated by laser Doppler flowmetry was not different between PD-1-deficient and WT mice.

Leukocytes are major effectors of inflammatory damage after experimental brain ischemia. To determine if the loss of PD-1 altered leukocyte composition in the brain after MCAO, numbers of infiltrating CD3+ T-cells, Gr1+ neutrophils,
CD11b^+CD45^{low} microglia, and CD11b^+CD45^{high} macrophages were evaluated by flow cytometry. After 96 hours reperfusion, accumulation of all of these leukocyte subtypes was significantly greater in the ischemic (right) versus nonischemic (left) hemisphere of MCAO-treated PD-1 KO mice as compared with MCAO-treated WT mice (Figure 3A–D).

**PD-1 Limits Inflammatory T-Cell Responses in the Periphery and CNS of MCAO Mice**

To further evaluate possible regulatory effects of PD-1 on T-cell cytokine production during MCAO, inflammatory factors were quantified in ex vivo activated cells from blood, the spleen, and CNS after 96 hours reperfusion in PD-1 KO versus WT mice. As is shown in Figure 4A–B, the percentages of CD3^+ T-cells secreting tumor necrosis factor-α and interferon-γ were significantly increased in both blood and the spleen in PD-1-deficient versus WT mice after MCAO. Loss of PD-1 further permitted significant 3- to 5-fold increases in the absolute numbers of interferon-γ- and tumor necrosis factor-α-secreting CD3^+ T-cells in the ischemic hemisphere of MCAO mice (Figure 4C–D).

**Increased PD-1 Expression Inhibits Activation of Microglia and Macrophages in the Ischemic Brain After MCAO**

As is shown (Figure 1C–D), PD-1 expression was strongly upregulated on both microglia and macrophages within the ischemic CNS lesion after MCAO in WT mice and obviously could not be expressed similarly in PD-1 KO mice. The effect of the loss of PD-1 was to permit significant 2- to 3-fold increases in the absolute numbers of Major Histocompatibility Complex Class II^+ and tumor necrosis factor-α-secreting microglia (Figure 5A–B) and macrophages (Figure 5C–D) in the ischemic hemisphere after activation ex vivo. Of note, there was approximately 2- to 3-fold more infiltrating macrophages present in the ischemic hemisphere than T-cells or microglia after MCAO. These results clearly demonstrate enhanced infiltration of inflammatory cells into the affected CNS in PD-1-deficient mice after MCAO.

**Discussion**

The results presented demonstrate 2 important and novel findings, which have potentially high impact in our understanding of immunologic mechanisms of ischemic brain injury. First, a previously unrecognized activity of the PD-1/
PD-L coinhibitory pathway contributes to limit infarct volume and functional neurological deficits as well as to inhibit activation and recruitment of inflammatory T-cells, granulocytes, macrophages, and microglia into the growing infarct. These regulatory activities were significantly decreased in MCAO-treated PD-1 deficient mice, thus implicating unequivocally the protective activity of this regulatory pathway. Second, PD-L1 and -2 expression was increased on peripheral and CNS B-cells and PD-1 expression was upregulated on CNS microglia and infiltrating macrophages within the lesioned brain hemisphere 96 hours after MCAO. Because peripheral B-cells, T-cells, and macrophages migrate across the blood–brain barrier to contribute to the ischemic injury, our novel results suggest a previously undescribed regulatory circuit in which PD-L1/2, interleukin-10-secreting B cells may directly inhibit T-cells and regulate activation and release of neurotoxic factors by PD-1+ microglia and macrophages. These putative interactions are illustrated in Supplemental Figure III. These findings implicate the PD-1/PD-L1 immunoregulatory pathway as a novel target for protection from CNS damage in experimental stroke.

It is of potential importance that PD-1 has been reported to be constitutively expressed on neurons, and it is conceivable that interaction of PD-L+ APC with PD-1 expressed by neurons could be directly neurotoxic. This event would contribute to the MCAO lesion only in WT but not PD-1 KO mice, thus producing larger lesions in WT mice (the opposite of what was observed in our study). In the absence of PD-1, there would be less neuronal death due to PD-L/PD-1 interactions but more neuronal damage from neurotoxic factors released by unregulated microglial cells and macrophages.

Although our results unequivocally implicate PD-1 as a contributor to CNS lesion development during 96 hours reperfusion after MCAO, a prior report did not observe a change in lesion volumes in PD-1-deficient mice 24 hours after MCAO. This result suggested a lack of involvement of PD-1 as a costimulator for adaptive T-cell responses in early stroke, and the 24-hour observation time in PD-1-deficient mice was apparently too early after occlusion to allow for the production of larger infarct volumes as was observed in our study. Congruent with this interpretation, our study implicates the PD-1 effect at a later time point involving reduced recruitment and cytokine release by activated T-cells, macrophages, and brain microglial cells.

As mentioned, the cytoplasmic domain of PD-1 contains an immunoreceptor tyrosine-based switch motif sequence, and it was later demonstrated that the tyrosine within the immunoreceptor tyrosine-based switch motif is essential for binding the protein tyrosine phosphatases SHP-1 and SHP-2, which mediate inhibitory PD-1 function. In T-cells, inhibition of activation requires coligation of the TCR. As mentioned, the cytoplasmic domain of PD-1 contains an immunoreceptor tyrosine-based switch motif sequence, and it was later demonstrated that the tyrosine within the immunoreceptor tyrosine-based switch motif is essential for binding the protein tyrosine phosphatases SHP-1 and SHP-2, which mediate inhibitory PD-1 function. In T-cells, inhibition of activation requires coligation of the TCR. In macrophages (and presumably microglia), PD-1 can be upregulated and cell activation inhibited by an interferon-sensitive response element. Toll-like receptor-2, Toll-like receptor-3, and Toll-like receptor-4 and other agents. This is of particular importance in stroke, where microglia activation and release of CNS neurotoxic factors is known to occur through ligation of Toll-like receptor-2 and Toll-like receptor-4 and other agents. Possibly by heat-shock protein 60 released from CNS cells undergoing necrotic or apoptotic cell death. Thus, ligation of PD-1 expressed on macrophages and microglia by PD-L1 expressed by regulatory B-cells could result in the inhibition of a key neurodestructive process in stroke.

In conclusion, our study provides new insights into the contribution of the PD-1/PD-L1 coinhibitory pathway that limits stroke-induced endogenous inflammatory responses.

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References


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Supplementary Methods

Purification and isolation of CD19+ B-cells and CD4+ T-cells
CD19+ B-cells or CD4+ T-cells from splenocytes of WT or PD-1-KO mice were purified by magnetic beads (MiltenyiBiotec, Auburn, CA) according to manufacturer’s instructions. The purity of each cell preparation was examined by flow cytometry. For better purity, untouched T-cells were purified twice by negative selection while B-cells were purified once. Cell preparations demonstrating >98% purity of each cell type were used for co-culture studies in Supplemental Figure 2.

Preparation of peritoneal macrophages (Mac)
Peritoneal exudate cells were harvested from WT and PD-1-KO mice and peritoneal Mac purified according to the protocol described earlier (1). A total of 2×10⁷ peritoneal cells were seeded in 14.0-cm Petri dishes in a final volume of 20ml incomplete medium and, after 1 hour of incubation, non-adherent cells were thoroughly washed off with jets of medium. Monolayers were assessed by labeling with anti-CD11b and analyzed by flow cytometry. Cell preparations demonstrating >95% purity of Mac were used for co-culture studies in Supplemental Figure 2.

T-cell proliferation assay
The proliferation assay was performed by modification of previously published protocols (2, 3). Purified CD4+ T-cells (2×10⁵/well) alone or co-cultured with B-cells (6×10⁵/well) in 96-well plates were incubated in the presence or absence of plate-coated anti-CD3/CD28 (2µg/ml) for 24h. ³H-thymidine was added during the last 8h of culture and the cells were harvest on glass fiber filters and counted by liquid scintillation. All experiments were performed in triplicate. Inhibition of T-cell proliferation in the presence of B-cells was calculated from the following formula:

Stimulation index (SI) = CPM (with anti-CD3/CD28)/CPM (without anti-CD3/CD28)

Inhibition of T cells [%] = [SI (without B cells) - SI (with B cells)]/SI (without B cells)

Co-culture assay of B-cells with macrophages
Harvested Mac (0.5×10⁵/well) alone or co-cultured with B-cells (5×10⁵/well) in 96-well plates were incubated in the presence of LPS (1ng/ml) for 24h. B-cells were then washed off and Mac were stained for intracellular TNF-α as described in Materials and Methods. All experiments were performed in triplicate. Inhibition of Mac activation in the presence of B-cells was calculated from the following formula:

Inhibition of Mac [%] = [%TNF-α⁺ (without B-cells) – %TNF-α⁺ (with B-cells)]/%TNF-α⁺ (without B-cells)

Statistics
Student’s t-test was used to compare two groups. Data represent mean ± SEM.
Supplemental Figure 1. Identification of PD-L1\(^+\) and PD-L2\(^+\) B-cells in the ischemic hemisphere of WT mice with MCAO. CD19\(^+\) B-cells in the ischemic hemispheres were evaluated for the expression of PD-L1 and PD-L2 after 60min MCAO treatment and 48h reperfusion in WT mice. Inflammatory cells from brain were prepared as described in Materials and Methods and stained with anti-CD19, anti-PD-L1, anti-PD-L2 mAbs and propidium iodide (PI). The B-cells were analyzed on the CD19\(^+\) PI\(^+\) gate (live B-cells). The expression of PD-L1 and PD-L2 on B cells was analyzed on CD19\(^+\) cells as shown. Results represent at least three independent experiments with three mice per group.
Supplemental Figure 2. T-cell proliferation and Mac activation were regulated by B-cells through the PD-1/PD-L co-inhibitory pathway. (A) B-cells significantly inhibited T-cell proliferation in vitro through the PD-1/PD-L pathway. WT or PD-1-KO T-cells were cultured alone or with B-cells (T:B-cells at a 1:3 ratio) with or without anti-CD3/CD28 (2µg/ml) stimulation for 24h. ³H-thymidine was added during the last 8h of culture and the cells were harvest on glass fiber filters and counted by liquid scintillation. Data represent inhibition rate relative to SI with or without B-cells. (B) B-cells significantly inhibited Mac activation in vitro through the PD-1/PD-L pathway. WT or PD-1-KO Mac were cultured alone or with B-cells (Mac:B-cells at a 1:10 ratio) in the presence of LPS (1ng/ml) for 24h. B-cells were then washed off and Mac were evaluate for expression of intracellular TNF-α. Data represent inhibition rate relative to TNF-α expression with or without B-cells. Error bars represent mean ± SEM. *** P<0.001 comparing WT vs. PD-1-KO mice. Results represent three independent experiments.
**Supplemental Figure 3.** B-cell regulation of peripheral and CNS inflammation may be mediated through the PD-1/PD-L co-inhibitory pathway after MCAO. MCAO causes enhanced expression of PD-L1 and PD-L2 by peripheral B-cells. B-cells may inhibit activation of T-cells (A) and microglia or macrophages (B) through the PD-1/PD-L co-inhibitory pathway and secretion of IL-10, thus inhibiting the release of neurotoxic factors.
Supplementary Table 1. Physiological parameters at baseline, mid-MCAO and end-MCAO in wild-type (WT) and PD-1 deficient (KO) mice.

<table>
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<th>Variables</th>
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<tr>
<td>rCBF, %§</td>
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</table>

$^*$Arterial CO$_2$ tension.

$^†$Arterial O$_2$ tension.

$^‡$MABP indicates mean arterial blood pressure.

$§$rCBF indicates regional cerebral blood flow. Other abbreviations are as defined in text.

There were no differences in physiological parameters determined between the genotypes.
Supplementary References

