ONLINE SUPPLEMENT

Programmed death-1 pathway limits CNS inflammation and neurologic deficits in murine experimental stroke

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 2×10^7 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 (2×10^5/well) alone or co-cultured with B-cells (6×10^5/well) in 96-well plates were incubated in the presence or absence of plate-coated anti-CD3/CD28 (2μg/ml) for 24h. ^{3}H-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)

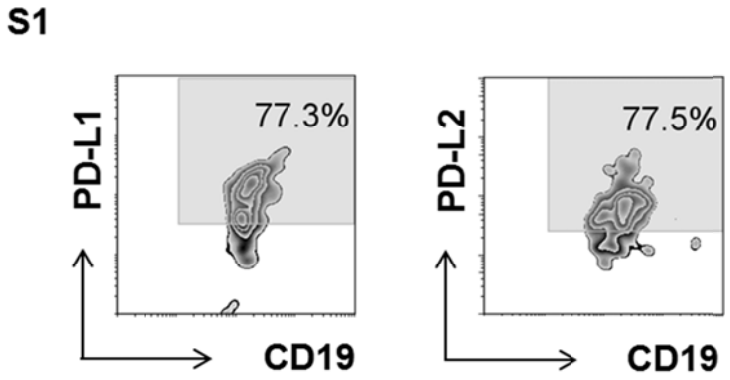
Co-culture assay of B-cells with macrophages

Harvested Mac (0.5×10^5/well) alone or co-cultured with B-cells (5×10^5/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)

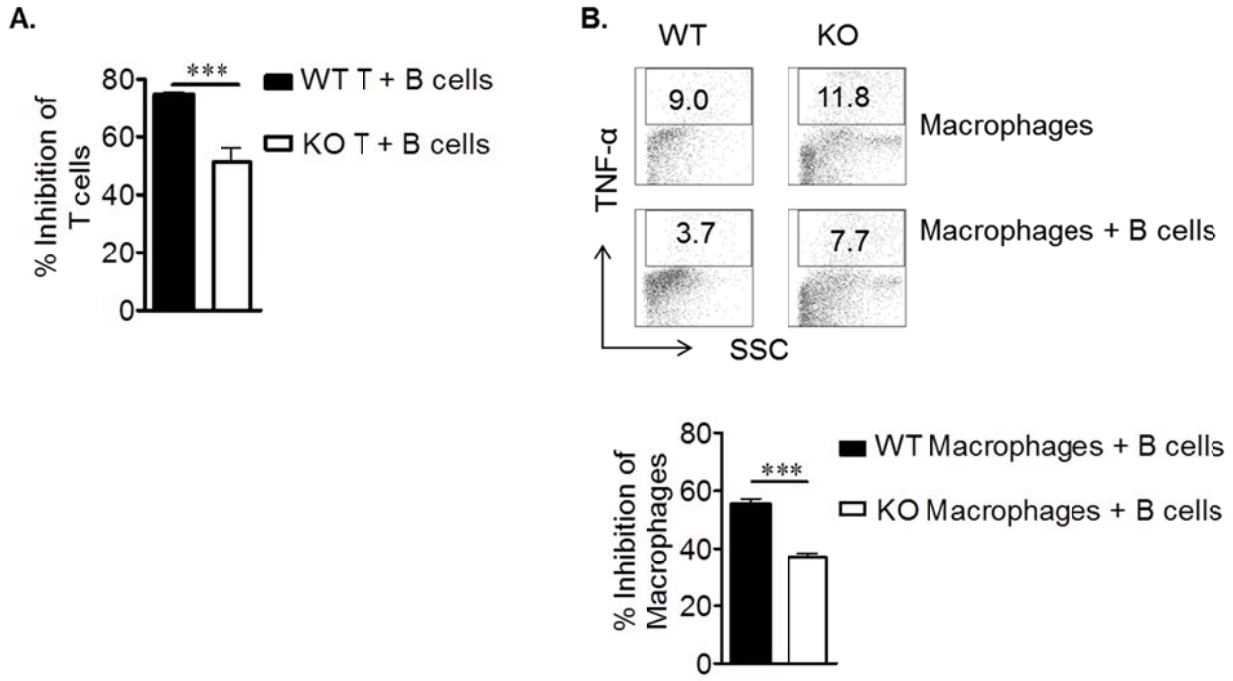
Statistics

Student's t-test was used to compare two groups. Data represent mean ± SEM.



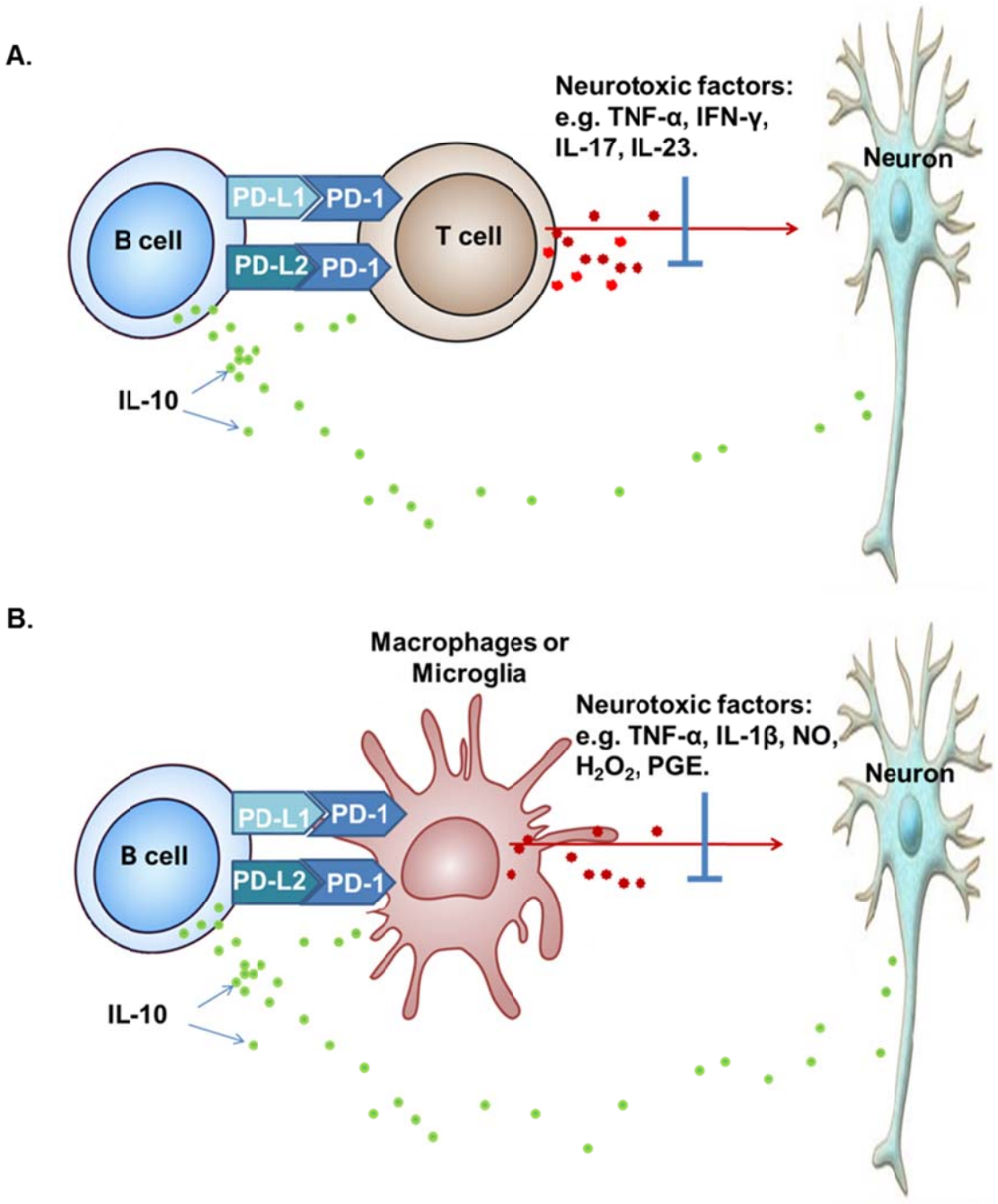
Supplemental Figure 1. Identification of PD-L1$^+$ and PD-L2$^+$ B-cells in the ischemic hemisphere of WT mice with MCAO. CD19$^+$ B-cells in the ischemic hemispheres were evaluated for the expression of PD-L1 and PD-L2 after 60min MCAO treatment and 48h reperfusion in WT mice. Inflammatory cells from 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.

S2



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. ^{3}H-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.

S3



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.

Variables	WT (n=4)			KO (n=3)		
	Baseline	Mid-MCAO	End-MCAO	Baseline	Mid-MCAO	End-MCAO
Arterial blood pH	7.38±0.06	7.40±0.08	7.4±0.06	7.46±0.08	7.41±0.06	7.40±0.10
P_aCO_2 (mm Hg)*	32±4	32±7	34±8	33±6	35±5	39±8
P_aO_2 (mm Hg)†	141±19	137±19	141±11	130±5	131±15	122±12
MABP (mm Hg)‡	83±5	83±5	75±7	73±10	74±17	63±15
Temperature (℃)	36.6±0.6	36.6±0.4	36.3±0.3	37.2±0.4	36.8±0	36.6±0.2
rCBF, %§	100±0	13±7	18±9	100±0	8±1	7±0

*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

1. Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, Bottazzi B, Doni A, Vincenzo B, Pasqualini F, Vago L, Nebuloni M, Mantovani A, Sica A. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappa B and enhanced IRF-3/STAT1 activation). Blood. 2006;107:2112-2122.
2. Ren X, Ye F, Jiang Z, Chu Y, Xiong S, Wang Y. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. Cell Death Differ. 2007;14:2076-2084.
3. Bodhankar S, Wang C, Vandenbark AA, Offner H. Estrogen-induced protection against experimental encephalomyelitis is abrogated in the absence of B-cells. Eur J Immunol. 2011. In press.