Amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice

Supplemental Methods

Experimental stroke models

In most experiments, experimental brain ischemia was induced by permanent transcranial coagulation of the middle cerebral artery (MCA) distal from the lenticulostrial arteries as described previously\(^1\). Briefly, mice were anesthetized with 1.0–2.0% isoflurane in O\(_2\)/N\(_2\)O. After a 1 cm long skin incision between the left eye and ear, a burr hole was drilled, the dura mater was removed and the MCA was occluded permanently (pMCAO) using a bipolar electrocoagulation forceps (ERBOTOM, ERBE). For transient focal ischemia, mice were anesthetized with 1.0–2.0% isoflurane in O\(_2\)/N\(_2\)O. After neck dissection an incision was made into the external carotid artery between 2 ligations, and a silicon-covered 8–0 nylon monofilament (Doccol Corporation) was advanced via the internal carotid artery until a resistance was felt. Successful occlusion was verified by laser doppler flowmetry. Only animals with a reduction of relative cerebral blood flow to < 25% of baseline were included in the study. 60 minutes after filament insertion, mice were anesthetized again and the filament was removed.

Allocation of animals to treatment groups was randomized. Surgeons were blinded to treatment assignment. We excluded animals in which surgery lasted for more than 25 min, or in which mice lost more than 25% of their body weight during the 7d observation period.

Measurement of Infarct Volume
Mice were anesthetized with an i.p. injection of Ketamin/Xylazin (100mg/kg and 10mg/kg, respectively) and transcardially perfused with saline. Brains were removed, immediately frozen and 20µm thick coronal cryosections were cut every 400µm. Stained sections were scanned at 300dpi and the infarct area was analyzed using a public domain image analysis program (ImageJ) program. The total infarct volume was obtained by multiplying measured areas and distance between sections. Correction for brain edema was applied by subtraction of the ipsilateral minus contralateral hemisphere volume from the directly measured infarct volume. Evaluators were blinded to treatment assignment.

**Functional outcome test**

Mice were placed between two boards each with a dimension of 30 x 20 x 1cm set at a 30° angle with a small opening along the joint to encourage the mouse entering the corner. Left and right turns with rearing movement were counted. We scored 12 turns for each test and calculated the ratio of right turns of all turns and normalized on the performance prior surgery of each individual mouse. For the cylinder test, mice were placed in a glass cylinder (8 cm in diameter) and recorded for 10 min. We scored contact of the cylinder wall with one forelimb during a full rear and landing with one forelimb at the cylinder bottom. At least 20 independent contacts were counted for one forelimb.

**Flow cytometry analysis**

Cells were stained for FACS using the following antibodies: CD4-PerCP-Cy5.5, CD4 Alexa 647, MHCII-FITC, CD45.2-APC, CD11c-PE, CD3-PE-Cy7, CD8a-APC, CD69-PerCP-Cy5.5, IL-10-PE, CD11b-APC-Cy7, Ly6G-FITC (BD Biosciences) and Foxp3-PE or FITC, IFN-γ-PE, TNF-α-PE, Ki-67 Alexa 700 from eBioscience. For intracellular staining, cells were restimulated with 100ng/mL phorbol myristate acetate (Sigma-Aldrich) and 600ng/mL ionomycin (Sigma-Aldrich) in the presence of protein transport inhibitor (BD GolgiStop or
GolgiPlug, BD Biosciences) in RPMI containing 10% fetal calf serum for 4 hours and stained with CD3, CD45, CD4, IL-10, CD11b, IFN-γ-PE, TNF-α and Foxp3 according to the manufacturer’s instructions (eBioscience). Data were collected on a LSRII flow cytometer (Becton Dickinson, Heidelberg, Germany) and analyzed by FlowJo software (Tree Star, Ashland, OR).

Reference