Supplemental material

Regulatory T cells ameliorate tissue plasminogen activator-induced brain hemorrhage after stroke

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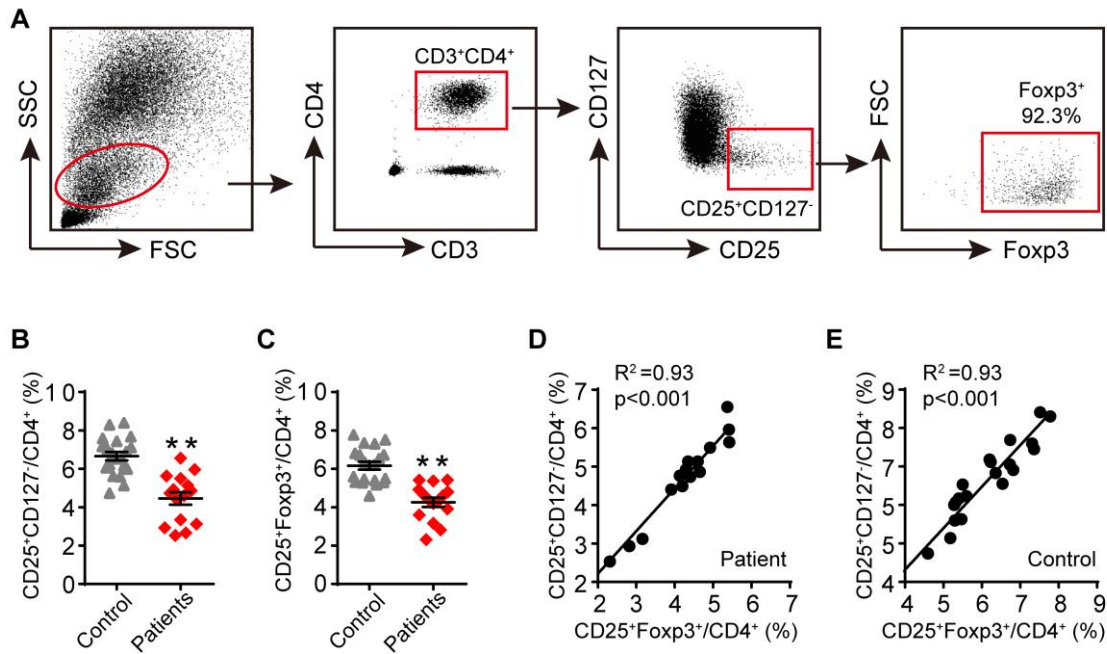
Supplemental Fig 1. In a group of blood samples from 15 stroke patients and 20 age and gender-matched healthy volunteers, we used $CD4^+CD25^+CD127^-$ and $CD4^+CD25^+Foxp3^+$ as selective markers for Tregs. (**A**) Representative flow cytometry plot showing that more than 92% of $CD3^+CD4^+CD25^+CD127^-$ cells were $Foxp3^+$. (**B-C**) Both sets of markers confirmed a significant decrease in Tregs after the onset of ischemic stroke, compared to healthy control subjects. Each dot represents an individual value. **$p<0.01$. (**D-E**) There was a highly significant correlation between the percentages of $CD25^+CD127^-$ and $CD25^+Foxp3^+$ cells within $CD3^+CD4^+$ populations in both patients ($r^2 = 0.93$; $p \leq 0.001$) and controls ($r^2 = 0.93$; $p \leq 0.001$).
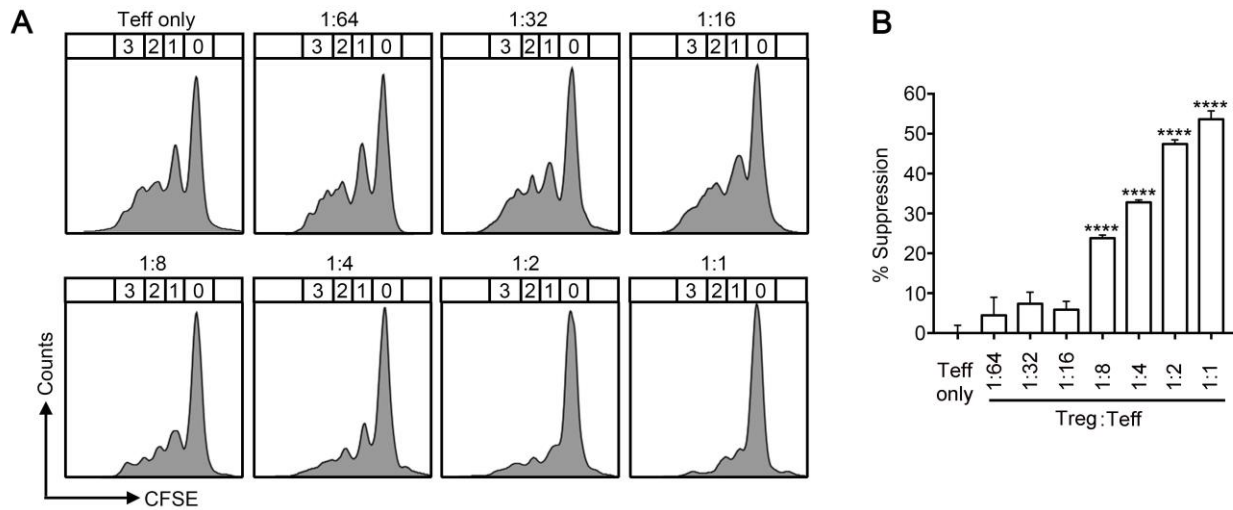
Supplemental Fig 2. Suppressive function of isolated Tregs *in vitro*. T effector cells (Teffs) were labeled with CFSE (1 μM, 37°C, 10 min) and then plated at 2×10^5 per well in a U bottom 96-well plate in the presence of CD3/CD28 activation beads (Miltenyi) to stimulate their proliferation. Tregs were added at a ratio of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, or 1:64 to the number of Teffs. Cells were incubated for three days. Suppression of Teff proliferation was determined by CFSE dilution on a flow cytometer. (**A**) Representative plots of suppression assay using CFSE-labeled CD4$^+$CD25$^-$ Teffs incubated with CD4$^+$CD25$^+$ Tregs at various ratios. (**B**) The bar graph indicates CFSE dilution in CD4$^+$CD25$^-$ gated Teffs. The percentage of suppression was calculated with the following formula: 100 − (% divided with Tregs present / % divided without Tregs present) $\times$ 100. Data are means ± SE of three independent experiments at each ratio of Tregs to Teffs. ****p<0.001 *vs* Teffs alone.
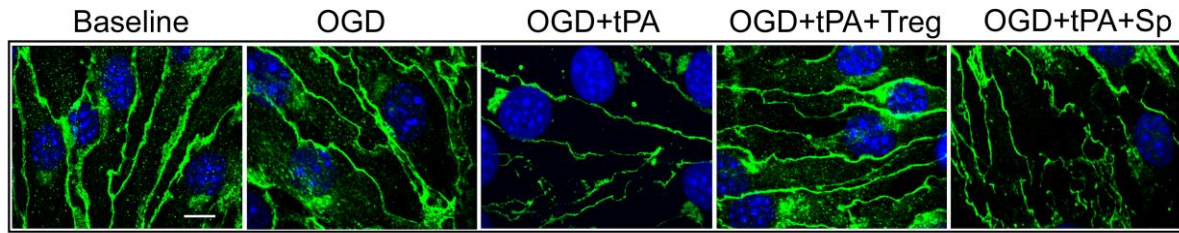
Supplemental Fig 3. Tregs inhibits OGD+tPA-induced degradation of AJ protein VE-cadherin in mouse endothelial cell cultures. Mouse primary endothelial cells were exposed to 4 h OGD, with or without tPA (500 ng/ml), and then co-cultured with Tregs or non-selected splenocytes at a 2:1 ratio. Immunostaining for VE-Cadherin was performed at 4h after OGD. Green: VE-cadherin; Blue: DAPI. Tregs protected endothelial cells against OGD+tPA-induced morphological disruption of VE-cadherin. Images are representative of slices from four experiments. Scale bar: 5 μM.

A

CCL2ShRNA Lenti
Two-way RM ANOVA   **Matching by cols**

Source of Variation	P	Significant?
Interaction	***	Yes
Time	***	Yes
Group	***	Yes

Bonferroni posttests

Baseline vs OGD				Baseline vs OGD+tPA				Baseline vs OGD+tPA+Treg			Baseline vs OGD+tPA+Sp		
Time	t	P value		Time	t	P value		Time	t	P value	Time	t	P value
1h	1.5	P > 0.05		1h	0.63	P > 0.05		1h	0.47	P > 0.05	1h	1.3	P > 0.05
2h	2.9	P < 0.05		2h	2.8	P < 0.05		2h	1.9	P > 0.05	2h	2.6	P > 0.05
3h	6.1	P<0.001	*	3h	7.5	P<0.001	*	3h	3.6	P<0.01	3h	5.9	P<0.001 *
4h	8.4	P<0.001	*	4h	7.9	P<0.001	*	4h	5.6	P<0.001 *	4h	7.5	P<0.001 *
6h	13	P<0.001	*	6h	16	P<0.001	*	6h	14	P<0.001 *	6h	15	P<0.001 *

OGD vs OGD+tPA				OGD vs OGD+tPA+Treg				OGD vs OGD+tPA+Sp		
Time	t	P value		Time	t	P value		Time	t	P value
1h	0.87	P > 0.05		1h	1	P > 0.05		1h	0.24	P > 0.05 ns
2h	0.12	P > 0.05		2h	0.97	P > 0.05		2h	0.23	P > 0.05 ns
3h	1.4	P > 0.05		3h	2.5	P > 0.05		3h	0.2	P > 0.05 ns
4h	0.49	P > 0.05		4h	2.8	P < 0.05		4h	0.82	P > 0.05 ns
6h	2.4	P > 0.05		6h	0.17	P > 0.05		6h	2.1	P > 0.05 ns

OGD+tPA vs OGD+tPA+Treg			OGD+tPA vs OGD+tPA+Sp		
Time	t	P value	Time	t	P value
1h	0.15	P > 0.05	1h	0.63	P > 0.05
2h	0.85	P > 0.05	2h	0.11	P > 0.05
3h	3.9	P<0.01	3h	1.6	P > 0.05
4h	2.3	P > 0.05	4h	0.33	P > 0.05
6h	2.2	P > 0.05	6h	0.32	P > 0.05

OGD+tPA+Treg vs OGD+tPA+Sp		
Time	t	P value
1h	0.79	P > 0.05
2h	0.74	P > 0.05
3h	2.3	P > 0.05
4h	2	P > 0.05
6h	1.9	P > 0.05
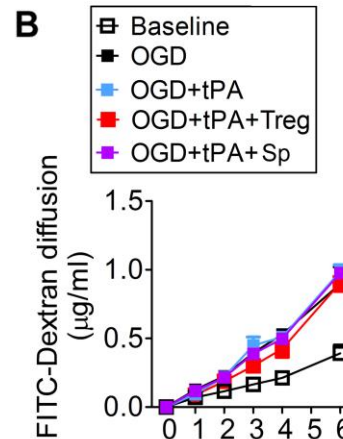
Supplemental Fig 4. CCL2 is critical for Treg-afforded BBB protection against OGD and tPA.
Mouse primary endothelial cells (ECs) were transfected with lenti-CCL2 shRNA for 3d, and then exposed to 4 hrs OGD, with or without tPA (500 ng/ml). The pretreated ECs were then co-cultured with Tregs or non-selected splenocytes at a 2:1 ratio. The diffusion of FITC-dextran from the luminal to abluminal chamber was measured over time. (**A**) Differences between groups at various timepoints were analyzed using a two-way repeated measures ANOVA followed by Bonferroni *post hoc* test. (n=3/group). Data are mean $\pm$ SE. Lentiviral-mediated knockdown of CCL2 in ECs provided BBB protection against OGD+tPA treatment. Tregs were unable to provide greater protection of the BBB than CCL2 knockdown. (**B**) Quantification of FITC-dextran leakage into the luminal chamber over time.

A Scramble Lenti

Two-way RM ANOVA		**Matching by cols**	
Source of Variation		P	Significant?
Interaction		***	Yes
Time		***	Yes
Group		***	Yes

Bonferroni posttests

Baseline vs OGD				Baseline vs OGD+tPA				Baseline vs OGD+tPA+Treg				Baseline vs OGD+tPA+Sp			
Time	t	P value		Time	t	P value		Time	t	P value		Time	t	P value	
1h	0.77	P > 0.05		1h	2.11	P > 0.05		1h	0.56	P > 0.05		1h	2.08	P > 0.05	
2h	2.41	P > 0.05		2h	2.79	P < 0.05		2h	2.07	P > 0.05		2h	3.11	P < 0.05	
3h	4.07	P<0.01		3h	7.98	P<0.001	*	3h	3.22	P < 0.05		3h	5.61	P<0.001	*
4h	9.08	P<0.001	*	4h	19.5	P<0.001	*	4h	11	P<0.001	*	4h	19.3	P<0.001	*
6h	17.1	P<0.001	*	6h	29.5	P<0.001	*	6h	16.6	P<0.001	*	6h	25.2	P<0.001	*

OGD vs OGD+tPA				OGD vs OGD+tPA+Treg				OGD vs OGD+tPA+Sp			
Time	t	P value		Time	t	P value		Time	t	P value	
1h	1.34	P > 0.05		1h	0.21	P > 0.05		1h	1.31	P > 0.05	
2h	0.38	P > 0.05		2h	0.34	P > 0.05		2h	0.71	P > 0.05	
3h	3.91	P<0.01		3h	0.85	P > 0.05		3h	1.54	P > 0.05	
4h	10.4	P<0.001	*	4h	1.93	P > 0.05		4h	10.2	P<0.001	*
6h	12.4	P<0.001	*	6h	0.54	P > 0.05		6h	8.13	P<0.001	*

OGD+tPA vs OGD+tPA+Treg				OGD+tPA vs OGD+tPA+Sp			
Time	t	P value		Time	t	P value	
1h	1.55	P > 0.05		1h	0.03	P > 0.05	
2h	0.72	P > 0.05		2h	0.32	P > 0.05	
3h	4.76	P<0.001	*	3h	2.37	P > 0.05	
4h	8.51	P<0.001	*	4h	0.23	P > 0.05	
6h	12.9	P<0.001	*	6h	4.24	P<0.001	*

OGD+tPA+Treg vs OGD+tPA+Sp			
Time	t	P value	
1h	1.52	P > 0.05	
2h	1.05	P > 0.05	
3h	2.39	P > 0.05	
4h	8.27	P<0.001	*
6h	8.67	P<0.001	*

B
- Baseline
- OGD
- OGD+tPA
- OGD+tPA+Treg
- OGD+tPA+Sp

FITC-Dextran diffusion (µg/ml)
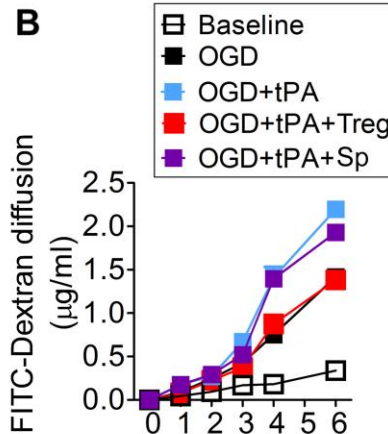
Supplemental Fig 5. Transfection of mouse endothelial cells (ECs) with lentivirus carrying scrambled shRNA sequences did not significantly influence the protective effects of Tregs on BBB integrity. Mouse primary endothelial cells were transfected with lenti-scramble for 3d, and then exposed to 4 h OGD, with or without tPA (500 ng/ml). The pretreated ECs were then co-cultured with Tregs or non-selected splenocytes at a 2:1 ratio. The diffusion of FITC-dextran from the luminal to abluminal chamber was measured over time. (**A**) Differences across groups at specific timepoints were analyzed using a two-way repeated measures ANOVA followed by the Bonferroni *post hoc* test. (**B**) The quantification of FITC-dextran leakage into the luminal chamber over time. (n=3/group). Data are mean ± SE.
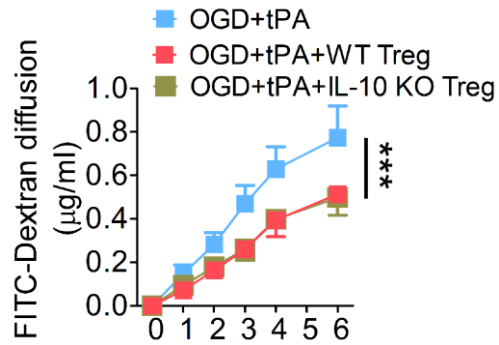
Supplemental Fig 6. IL-10 is not critical for Treg-afforded BBB protection. Primary mouse endothelial cells in cell culture inserts were exposed to 4h of oxygen-glucose deprivation (OGD) followed by tPA (500 ng/ml). WT or IL-10 KO Tregs were then added (endothelial cell:Treg= 2:1). The diffusion of FITC-dextran from the luminal to abluminal chamber was measured over time. (n=5/group). Data are mean $\pm$ SE. ***p≤0.001 OGD+tPA *vs* OGD+tPA+WT Treg; OGD+tPA *vs* OGD+tPA+IL-10 KO Treg.

Supplemental Table 1. Clinical characteristics of stroke patients

Treatment	Non-tPA treated (n=42)	tPA treated (n=23)	p
Age (years)	62.40±12.04	62.13±13.02	0.93
Gender (M:F)	29:13	15:8	0.787
Diabetes, n, (%)	N=10, (23.81%)	N=6, (26.09%)	0.57
Hypertension, n, (%)	N=34, (80.95%)	N=18, (78.26%)	0.54
Hyperlipidemia, n, (%)	N=18, (42.86%)	N=12, (52.17%)	0.31